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Optimisation of protocols for *ex vivo* expansion of limbal stem cells and their enrichment

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Abstract

The corneal epithelial cells are constantly replaced by the stem cells located at the limbus, the peripheral edge of the cornea, therefore known as limbal stem cells (LSCs). LSCs can be destroyed by numerous factors which results in the condition called limbal stem cell deficiency (LSCD).

Ex vivo expansion of LSCs is a well-established technique used successfully to cure patients with LSCD. Therapeutic use of LSCs must be performed in compliance with good manufacturing practice (GMP) as a quality assurance system. However, traditional culture media for e*x vivo* expansion of LSCs contains a number of ingredients derived from animal sources which may compromise its safety profile for human transplantation. The first aim of the study was to define new GMP grade medium for cultivation and maintenance of LSCs *in vitro*. Formulation of new GMP compliant media resulted in equal growth to non-GMP grade media.

Strick regulations for cell therapy promote centralization of culture units, therefore definition of reliable and practical transportation strategies is vitally important. The second aim of this study was to optimise the transport conditions for limbal biopsies (LBs) and cultured limbal epithelial cells (LECs). Transport of LBs at room temperature proved to be significantly superior to 4°C transport. We also showed that cultured LECs may be stored in serum-free media and transported up to 7 days at 23°C without any negative effect on cell number, viability, colony forming efficiency or gene expression profile.

Due to the absence of specific LSC markers, identification and isolation of putative LSCs is a complicated task. The third and final aim of this study was to identify novel cell surface markers for LSCs. We reported herein the identification of a new cell surface marker for LSCs (CD200) as well as a cell surface marker for proliferating progenitor cells (CD109).

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At the end, I would like to thank my family for their unconditional support and faith in me.

Declaration

I confirm that no part of the material offered has previously been submitted by me for a degree in this or any other University. Material generated through joint work has been acknowledged and appropriate publication cited. In all other cases, material from the work of others has been acknowledged, and quotations and paraphrases suitably indicated.

Signature:

Sanja Bojic

Date: 19th March 2020

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Abbreviations

LSC - Limbal Stem Cell

LSCD - Limbal Stem Cell Deficiency

GMP - Good Manufacturing Practice

LB - Limbal Biopsy

LEC - Limbal Epithelial Cell

TAC - Transient Amplifying Cell

PMC - Post Mitotic Cell

TDC - Terminally Differentiated Cell

EGF - Epidermal Growth Factor

EGFR - Epidermal Growth Factor Receptor

CK - Cytokeratin

NGF - Nerve Growth Factor

HGF - Hepatocyte Growth Factor

CLAU - Conjunctival Limbal Autograft

LR-CLAL - Living Related Conjunctival Allograft

KLAL - Cadaveric Keratolimbal Allograft

SLET - Simple Limbal Epithelial Transplantation

MSC - Mesenchymal Stem Cell

CLET - Cultivated Limbal Epithelial Transplantation

HAM - Human Amniotic Membrane

COMET - Cultivated Oral Mucosal Epithelial Transplantation

MSCT - Mesenchymal Stem Cell Transplantation

RM - Regenerative Medicine

ATMP - Advance Therapy Medicinal Product

SOP - Standard Operating Procedures

IPC - In Process Controls

HIV - Human Immunodeficiency Virus

HBV - Hepatitis B Virus

HCV - Hepatitis C Virus

HTLV - Human T- Lymphotropic Virus

NHS - National Health Service

NHSBT - NHS Blood and Transplant

DMEM - Dulbecco's Modified Eagle's Medium

FBS - Fetal Bovine Serum

T3 - Triiodothyronine

CT - Cholera toxin

AMP - Adenosine monophosphate

SEM - Standard Error of the Mean

PBS - Phosphate-Buffered Saline

CFE - Colony Forming Efficiency

CPC - Cell Processing Centre

MHRA - Medicines and Healthcare products Regulatory Agency

HBSS - Hank's Balanced Salt Solution

KCM - Keratinocyte Culture Medium

FACS - Fluorescence-Activated Cell Sorting

ECM - Extracellular matrix

Chapter 1. Introduction

1.1 Corneal structure and function

The cornea is a transparent dome-shaped window at the front of the eye with significant refractive and barrier functions (Eghrari *et al.*, 2015). Major function of the cornea is enabling the transmission and focussing of light, together with the crystalline lens, onto the retina at the back of the eye for visual perception. The cornea is also a major protective shield of the interior of the eye functioning as a barrier to debris and infection. A damage of the corneal tissue, if not promptly treated, could lead to scarring and consequential visual impairment and often blindness (Hertsenberg and Funderburgh, 2015). Millions of people worldwide suffer from blindness and visual impairment caused by corneal opacities. According to the World Health Organization data from 2002 corneal scaring and opacity are listed as the fourth most common cause of blindness globally, causing around 5.1% of all registered cases of blindness around the world (World Health Organization; Resnikoff *et al.*, 2008).

The anterior surface of the eye is composed of the cornea centrally and the sclera and conjunctiva peripherally. The human cornea measures approximately 11.5 mm horizontally and 10.5 mm vertically. Its transparency and anterior radius of curvature centrally of 7.8 mm makes the cornea responsible for three-fourths of the total refractive power of the human eye (Eghrari *et al.*, 2015). Therefore, preservation of its transparency is vital for this role. The central corneal thickness is approximately 520 μm and increases towards the periphery where it can reach up to 650 μm. The junction region where the cornea becomes continuous with the sclera, approximately 1.5–2 mm in width, is known as the limbus (**Figure 1.1**). The limbus is demarcated on the corneal side by the termination of the Bowman's layer. At the limbus, the corneal avascular epithelium continues into the vascular conjunctival epithelium, the corneal stroma into the sclera and the corneal endothelium continues into the anterior surface of the iris and the suprachoroidal space (Takacs *et al.*, 2009).

The presence of an intact limbal epithelium is essential for maintaining corneal transparency in two different ways. Firstly, the limbal epithelium acts as a barrier that prevents the conjunctival epithelium and its stromal blood vessels from encroaching onto the cornea, thereby impairing its clarity (Chen and Tseng, 1991). Secondly, the limbal epithelium also harbours the limbal stem cells which proliferate and differentiate to provide a lifetime source of corneal epithelial cells (Dua and Azuara-Blanco, 2000; Daniels et al., 2001; Sangwan et al., 2003).

Human cornea is composed of three cellular layers: the outer epithelial layer of ectodermal origin, the stroma and the endothelium, both of mesenchymal origin (Cvekl and Tamm, 2004; Takacs et al., 2009). Although the outermost epithelial layer is the most exposed to environmental damage it also has the best mechanism of a constant self-renewal assured by corneal epithelial stem cells. On the contrary, cell turnover in the corneal stroma is very slow (Doutch et al., 2012) and long believed non existing in corneal endothelial layer of the adult organism (Takacs et al., 2009). However, some recent findings indicate that progenitor cells may be found in these layers as reviewed by Hertsenberg and Funderburgh (Hertsenberg and Funderburgh, 2015).

Corneal development begins with primitive formation of epithelium and lens from the surface ectoderm, followed by waves of migration of neural crest cells between these two structures to create the stroma and endothelium. Descemet membrane is secreted by the endothelial layer and gradually thickens (Eghrari et al., 2015). The stream of neural crest cells migrates between the lens and presumptive corneal epithelium to form the corneal endothelium and stromal keratocytes (Lwigale, 2015). The corneal stroma and epithelium are abundantly innervated by the sensory nerves that originate from the neural crest- and ectodermal placode-derived trigeminal ganglion. Simultaneous with the corneal innervation is the formation of the limbal vascular plexus and the establishment of corneal avascularity (Lwigale, 2015). Unlike most tissues in the body, the cornea contains no blood vessels. Avascularity, together with a highly organized architecture, is essential for corneal transparency and normal vision. Instead, it receives all nutrients including glucose and oxygen essential for maintenance of normal metabolic functions from the limbal circulation, the aqueous humour and the tear film (Utheim, 2013). On the other hand, it is heavily innervated, with the density of nerve endings approximately 300-400 times greater than in the skin (Rozsa and Beuerman, 1982).
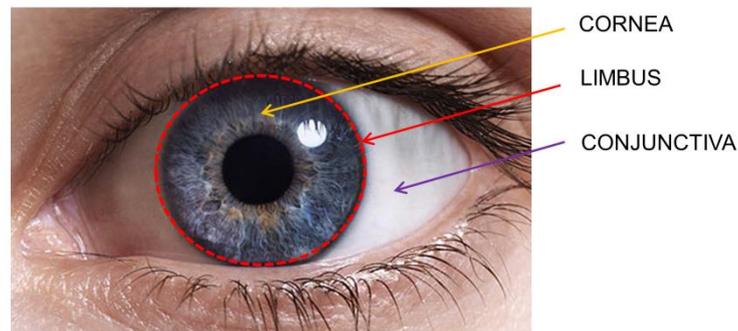
Figure 1.1 The location of the limbus on the ocular surface. The limbus represents the interface between the peripheral cornea and neighbouring conjunctiva.

In humans, the corneal tissue is arranged in five distinct layers (three cellular layers with two thinner layers or membranes between them), each having an important function (**Figure 1.2**):

1. **Corneal epithelium**

   The epithelium is the most anterior layer that serves as the principal barrier to fluid and foreign materials including pathogens and dust, a function performed through production of tight junctions and constant turnover of cells. It is supported posteriorly by basement membrane and Bowman's layer and assists in the maintenance of stromal dehydration. It also provides a smooth surface that absorbs oxygen and cell nutrients from tears, and then distributes these nutrients to the other layers of the cornea. The epithelium is filled with thousands of nerve endings that make the cornea extremely sensitive to pain. The corneal epithelium is composed of five to six layers (4-6) of non-keratinized, stratified squamous epithelial cells, comprising about 10% of the corneal thickness. In humans it's approximately 50 μm thick (Eghrari *et al.*, 2015). Its transparency is a result of the homogeneity of the refractive index of all its constituent cells (Dohlman, 1971). Superficial two to three layers are composed of flat and polygonal cells with apical microvilli and microplicae and covered by a charged glycolcalyx which maximizes surface area with the mucinous layer of the tear film (Eghrari *et al.*, 2015). The most superficial cells

of the corneal epithelium are attached by tight junctions that prevent the penetration of the tear film and its components and assist in the prevention of pathogenic organisms from entering the cornea. Directly posterior, two to three layers of cuboid "wing" or suprabasal cells demonstrate tight junction complexes while basal, columnar cell layer is anchored to the basal lamina via hemidesmosomes (Alison *et al.*, 2002; Eghrari *et al.*, 2015). In humans, the density of hemisdesmosomes is higher in the cornea compared to the limbus (Gipson, 1989). Similarly, the density of anchoring fibrils and adhesion plaques is also higher in the cornea compared to the limbus (Keene *et al.*, 1987; Gipson, 1989). The lower density of all these structures in the limbus may be compensated by the undulating nature of the limbal epithelial-stromal interface.

The outermost layer of the corneal epithelial cells is continuously shed and replenished by proliferation of wing and basal cells (Thoft and Friend, 1983). The lifespan of epithelial cells is 7-14 days on average (Cenedella and Fleschner, 1990; Eghrari *et al.*, 2015; Gonzalez *et al.*, 2018).

The limbal epithelium, the thickest of the ocular surface, is composed of 7-10 layers of stratified epithelial cells. Unlike the central corneal epithelium, the limbal epithelium contains also other cell types such as melanocytes and antigen-presenting Langerhans cells (Dziasko and Daniels, 2016).

2. Bowman's layer

Bowman's layer is a thin acellular condensation of the outer portion of the corneal stroma consisting mainly of collagen type I and type III fibres and proteoglycans. It's approximately 8-12 μm thick and decreases in thickness over time. Unlike the epithelial layer, it has no regenerative potential (Eghrari *et al.*, 2015). If injured, it can form a scar as it heals. If large and centrally located, these scars may cause vision loss.

3. Corneal stroma

The stroma composes the majority of corneal volume, around 90% of the total corneal thickness, measuring approximately 500 μm in humans. The corneal

stroma plays several pivotal roles within the eye - optically it is the main refracting lens and mechanically it has to be extremely tough to protect the inner contents of the eye. Both these functions are governed by its structure at all hierarchical levels (Meek and Knupp, 2015). Beside it provides support and clarity, it also assists in ocular immunity (Eghrari *et al.*, 2015).

The stroma contains densely packed, tough yet transparent connective tissue. The transparency of the stroma originates from its regularly ordered and equally spaced collagen bundles that are produced by the corneal fibrocytes known as keratocytes (Takacs *et al.*, 2009).

The stroma consists of an extracellular matrix made up of collagens (type I, V and VI) and proteoglycans (decorin associated with dermatan-sulfate and lumican associated with keratin-sulfate). The collagen fibrils are regularly arranged into bundles or lamellae in order to allow corneal transparency. There are about 300 lamellae of parallel collagen fibrils in the centre of the cornea reaching to nearly 500 lamellae at the limbus (Radner *et al.*, 1998). The orientation of the alternate lamellae differs with each other, but they are all parallel with the corneal surface. Keratocytes from which the collagen fibrils are produced during development are scattered throughout the stroma between the lamellae. Whilst the corneal stroma is avascular tissue, the limbal stroma is heavily vascularized (Goldberg and Bron, 1982).

4. Descemet's membrane

Descemet's membrane is the thick basement membrane of the corneal endothelium and measures approximately 3 μm in thickness in children, gradually thickening to 10 μm in adults. Similar to the stroma it contains laminin and fibronectin as well as keratin sulfate, heparin sulfate and dermatan sulfate. On the other hand, it contains collagen IV and VIII fibrils. In contrast to other basal membranes throughout the body in which collagen type IV is common, collagen type VIII is relatively specific to the Descemet's membrane (Eghrari *et al.*, 2015). Descemet's membrane repairs itself easily after injury.

5. Endothelium

The endothelium is the most posterior layer and it separates the cornea from the aqueous humour of the anterior chamber of the eye. It provides nutrients for stromal keratocytes and participates in the maintenance of the stromal transparency (Takacs *et al.*, 2009). The posterior cornea, composed of Descemet membrane and endothelium, is essential for stromal dehydration, maintained through tight junctions and endothelial ionic pumps located in basolateral plasma membranes. Beside tight junctions, gap junctions are also present in corneal endothelium and contribute to the electrical coupling of endothelial cells (Eghrari *et al.*, 2015). Insufficiency of the endothelial pump function results in corneal oedema, that leads to loss of corneal clarity and vision impairment (Takacs *et al.*, 2009).

The corneal endothelium is composed of a single layer of closely interdigitating, flat, polygonal cells which line the posterior surface of the cornea. Unlike corneal epithelial cells, corneal endothelial cells do not proliferate *in vivo* and cell replacement does not occur through mitosis but through cell migration. Therefore, central loss of cells results in centripetal migration of adjacent endothelial cells with subsequent formation of tight junctions and restoration of pump function (Eghrari *et al.*, 2015).

5. 4.   3.   2. 1.

1. Epihelium (50μm)
2. Bowman's membrane (12μm)
5. Endothelium (5μm)
3. Stroma (480-500μm)
4. Descemet's membrane (8-10μm)
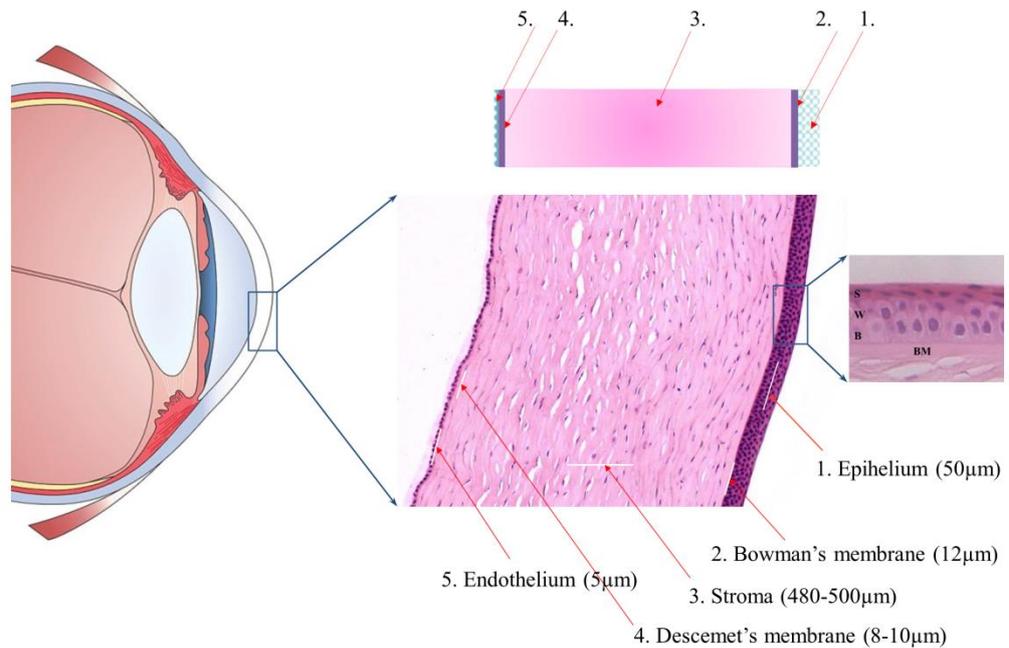
Figure 1.2 Schematic diagram of a sagittal section of the anterior portion of the human eye (left) and corneal structure (right). The cornea is a transparent dome-shaped window at the front of the eye that covers the iris, pupil, and anterior chamber. It is composed of five distinct layers, three cellular layers with two thinner membranes between them. From the outside to inside the layers of the human cornea are the corneal epithelium, Bowman's membrane, corneal stroma, Descemet's membrane and corneal endothelium.

In conclusion, corneal transparency essential for normal vision, is determined by:

- a smooth epithelium with no invasion of conjunctival cells (Puangsricharern and Tseng, 1995);
- the absence of vasculature (Utheim, 2013);
- a highly organized stromal architecture (Fini and Stramer, 2005);
- a functional endothelium that regulates corneal hydration (Meek *et al.*, 2003);
- a production of crystalline proteins by keratocytes in the stroma (Jester *et al.*, 1999).

1.2 Corneal epithelial stem cells

1.2.1 Stem cells

Stem cell is an undifferentiated cell, which can self-renew to replicate itself as well as give rise to the specialized cells under appropriate conditions (Weissman, 2000).

Tissue-specific or adult stem cells are found in almost every adult tissue, with exception of the heart, and are able to maintain and regenerate the given tissue for a lifetime (Alison *et al.*, 2002; Takacs *et al.*, 2009) (**Figure 1.3**). Adult stem cells are either multipotent or unipotent (Wagers and Weissman, 2004). Multipotent stem cells can differentiate into many cell types but within a particular lineage while unipotent stem cells can differentiate towards only one cell type.
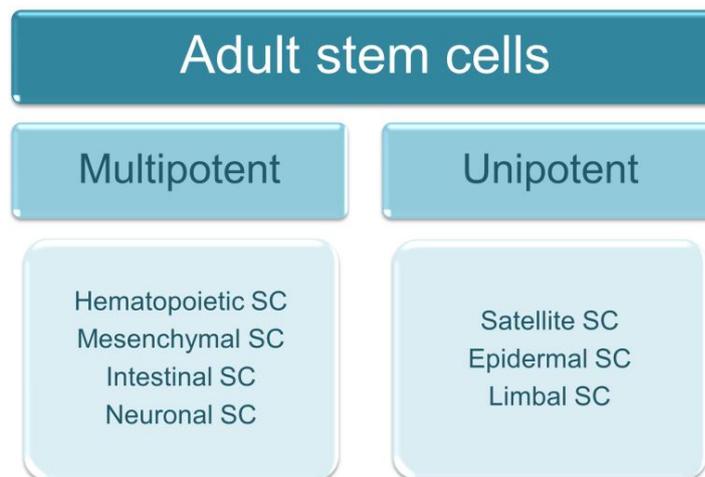
Figure 1.3 Adult stem cells, types and examples. Differentiation capacity of adult stem cells is ranging from multipotent to unipotent. Multipotent stem cells are capable to develop into multiple specialised cell types present in a specific tissue or organ whilst unipotent stem cells are capable to generate only one specialised cell type.

The adult stem cells form a very small percentage of the total cellularity. For example, in the mouse small intestine there are 4–5 stem cells found at the bottom of the crypt out of a total crypt population of about 250 cells (Bjerknes and Cheng, 1999). In skeletal muscle, similarly, satellite cells comprise about 5% of all nuclei while in the bone marrow the multipotent haematopoietic stem cell is even rarer, with

a frequency of only 1 in 10 000 or more amongst all blood cells (Alison *et al.*, 2002). Most of the time stem cells remain in growth arrested state but can enter the cell cycle on demand (e.g. tissue injury) and give a rise to a differentiating and highly proliferating progeny – transient amplifying cells. Transient amplifying cells (TACs) have a lower proliferative potential compared to stem cells, but still have some self-renewal capacity which contributes to cellular turnover and regeneration. These fast dividing progenitors represent the vast majority of the proliferative cells in the corneal/limbal epithelium (Schlotzer-Schrehardt and Kruse, 2005).

Stem cells are characterized by the following properties (Alison *et al.*, 2002; Takacs *et al.*, 2009):

1. Unlimited self-renewal and asymmetric cell division. For normal homeostasis and functioning of some tissue is vital that a constant pool of stem cells is maintained throughout the lifetime (Kolli *et al.*, 2009). That is achieved by so-called asymmetric cell division, giving rise to one daughter cell that remains stem cell and a second daughter cell that will enter differentiation and become TAC (**Figure 1.4**). Thanks to this process, stem cells have a long life and possess the ability to proliferate indefinitely during the lifetime of the organism in which they reside (Kolli *et al.*, 2009). The main purpose of TACs is to increase the number of cells resulting from each stem cell division (Kolli *et al.*, 2009) and protect stem cells of going through the cell cycle often and accumulate DNA damage over time. Both stem cells and TACs belong to the proliferative tissue compartment. TACs differentiate into post-mitotic cells (PMCs) which fall belong to the non-proliferative (differentiation) tissue compartment and are not capable of further divisions (Kolli *et al.*, 2009). PMCs are irretrievably committed to cellular differentiation and mature into terminally differentiated cells (TDCs) (Kolli *et al.*, 2009) which are carriers of the functional aspect of the tissue (**Figure 1.5**).
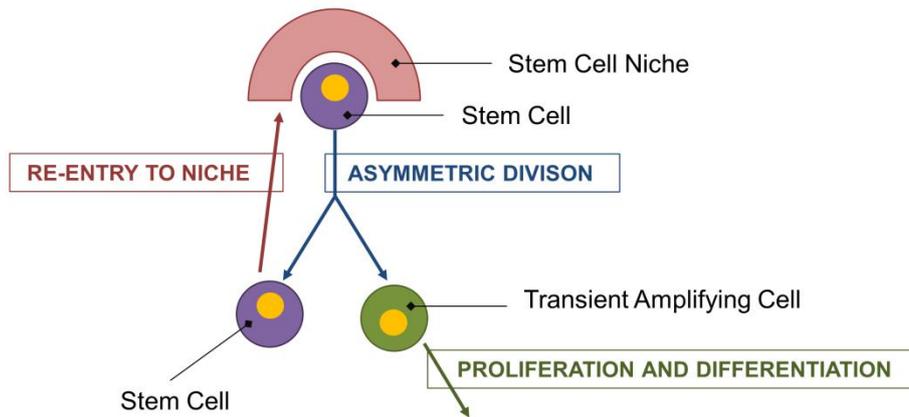
Figure 1.4 Asymmetric cell division. Through asymmetric cell division stem cell produces two daughter cells with different cellular fates, one daughter cell is identical to mother cell and will re-enter the niche to maintain the pool of stem cells whilst a second daughter is programmed to differentiate into a specific cell type and become TAC.
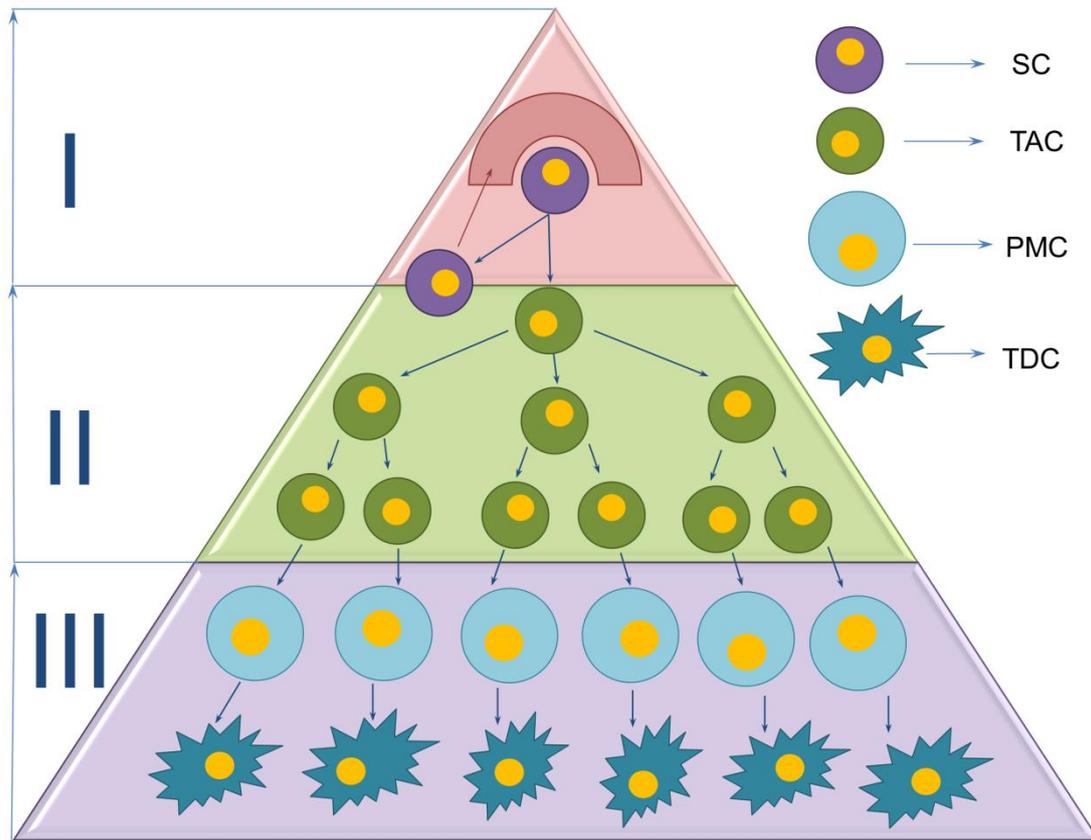
Figure 1.5 The hierarchy of cells in the adult stem cell system including the corneal epithelium. The corneal epithelium is composed of pyramid structure of cells, with only a few stem cells (SCs) at the top of the pyramid, greater but still relatively small number of TACs in the middle of the pyramid and the majority of PMCs that give rise to TDCs in the base of the pyramid. Therefore there are three different cell compartments present: the stem cell compartment (I), the proliferative compartment (II) and differentiation compartment (III).

2. Relatively undifferentiated state but high differentiation potential, implying that despite the stem cells do not have the functional specializations of the progeny they give rise to, they have the ability to differentiate into all cell types of their home tissue;

3. Slow cell cycle but high clonogenic potential, meaning that stem cells divide less frequently than transient-amplifying cells in order to lower accumulation of DNA errors because DNA synthesis can be error-prone. For that reason, stem cells divide less frequently than TACs. In hair follicles for example, the hair haft and its surrounding sheaths are produced by the hair matrix, which is

itself replenished by the bulge stem cells. The bulge cells divide less frequently, but are more clonogenic than the TACs of the hair matrix (Oshima *et al.*, 2001).

1.2.2 Limbal stem cells

The corneal epithelium is rapidly regenerating stratified squamous epithelium in a state of constant renewal as the superficial terminal cells are naturally continuously shed into the tear film. As one of the most rapidly regenerating mammalian tissues, it is estimated that the corneal epithelial cell layers turn over every 7-14 days (Cenedella and Fleschner, 1990; Eghrari *et al.*, 2015; Gonzalez *et al.*, 2018). *In vivo*, the corneal epithelium is renewed every 9–12 months (Utheim, 2013). The central corneal epithelium in humans has no capacity to renew (Ebato *et al.*, 1988). Instead, it is widely accepted that, during normal homeostasis, the corneal epithelium is maintained by the stem cells located at the peripheral edge of the cornea known as limbus that proliferates slowly unless stimulated by injury (Davanger and Evensen, 1971; Cotsarelis *et al.*, 1989). Due to their anatomical location these corneal epithelial stem cells are more commonly known as limbal stem cells (LSCs). LSCs give rise to fast-dividing TACs, which migrate centripetally in the basal layer of the corneal epithelium (Kinoshita *et al.*, 1981; Buck, 1985; Nagasaki and Zhao, 2003). Here they proliferate for a limited time before undergoing a final division, whereupon both daughter cells usually detach from the basement membrane, move vertically (apically) through the suprabasal layers, becoming terminally differentiated and are eventually shed from the most superficial layer (Beebe and Masters, 1996; Lehrer *et al.*, 1998).

1.2.3 Location of limbal stem cells

The existence of epithelial stem cells in the limbus has been proposed in 1971 by Davanger and Evensen (Davanger and Evensen, 1971). The limbus is a specialized region, the narrow band of tissue which encircles the cornea, which is "highly vascularized, innervated and protected from potential UV light damage by the presence of melanin pigmentation" (Schlotzer-Schrehardt and Kruse, 2005).

Over the years numerous experimental and clinical observations supported hypothesis that the basal layer of the limbal epithelium harbour the stem cells for the corneal epithelium:

1. **Pigment migration studies** – Davanger and Evensen were the first to propose the basal layer of limbal epithelium as a "generative organ for corneal epithelial cells". Their study with guinea pigs, which naturally have pigmented basal layer of the limbal epithelium, showed that the cornea was healed by the pigmented epithelium when the normally non-pigmented central corneal epithelium was removed (Davanger and Evensen, 1971).

2. **Cell culture studies** – Many studies suggested the presence of cells with high proliferative potential in the limbal basal epithelium. Ebato and co-workers showed that *in vitro* cultured human limbal epithelium has a higher proliferative potential compared to the corneal and conjunctival epithelium. Having in mind that proliferative potential is a vital property of stem cells, this finding provided further evidence of the limbal location of the corneal epithelial stem cells (Ebato *et al.*, 1988). Other studies showed that cells from the central cornea generated mostly paraclones (terminally differentiated epithelial colonies) that could not be passaged more than twice while cells from the limbal region could proliferate for many generations (between 80-100 doublings) and form holoclones (large colonies with regular borders) (Lavker *et al.*, 1991; Pellegrini *et al.*, 1999a).

3. **Radio labelled thymidine studies** – Stem cells have a slower cell cycle and can be detected experimentally as label retaining cells (Bickenbach, 1981) using pulse/chase experiments with a DNA precursor such as tritiated

thymidine (^{3}HT) or 5-bromo-2′-deoxyuridine (BrdU) (Rodriguez and Nguyen, 2018). Following a chase period of 4-8 weeks, rapidly dividing cells lose the label but slow dividing cells will remain labelled. Lavker and Cotsarelis were the first to show that label retaining cells were found exclusively in the basal layer of the limbal epithelium but were completely absent in the central cornea. They also showed using tritiated thymidine to label cells in S phase of the cell cycle in mouse cornea that the basal cells of the limbal epithelium are normally slow cycling cells but upon wounding of the central corneal epithelium can be stimulate to proliferate more rapidly (Cotsarelis *et al.*, 1989). Label retaining studies further showed that the percentage of stem cells represents less than 10% of the total limbal population (Lavker *et al.*, 1991). Recently, Sartaj and colleagues localized, purified, and characterized slow cycling cells in the cornea in an inducible transgenic "pulse-chase" murine model (Sartaj *et al.*, 2017).

4. **Animal studies** – Corneal wound healing studies in rabbits showed that surgical removal of the limbus resulted in insufficient re-epithelization and conjunctival invasion of the corneal surface (Kruse *et al.*, 1990; Huang and Tseng, 1991).

5. **Clinical studies** – There are several pieces of clinical evidence that confirm limbal location of corneal epithelial stem cells. Firstly, it was observed that larger peripheral corneal epithelial wounds heal significantly faster than smaller central wounds (Matsuda *et al.*, 1985). Secondly, clinical studies showed that limbal transplantation leads to long term restoration of the corneal surface in patient with limbal damage (Kenyon and Tseng, 1989; Holland and Schwartz, 1996). Finally, corneal tumours almost exclusively arise from the limbus (Waring *et al.*, 1984) that further corroborate the limbal location of corneal epithelial stem cells (Reya *et al.*, 2001).

6. **Lineage tracing experiments** – In 2015, Amitai-Lange *et al.* provided a direct evidence of limbal cell migration under both homeostasis or injury using a mouse model, *R26R-Confetti* mouse, that allows multicolour lineage tracing of limbal and corneal cytokeratin 14 positive cells (Amitai-Lange *et al.*, 2015). They reported that in homeostasis limbal epithelial cells undergo slow

centripetal migration of 4–5 months until reaching the corneal centre, while upon wounding, they observed significant corneal contribution to the healing process and rapid appearance of limbal stripes (Amitai-Lange *et al.*, 2015). The same year, another two research groups reported very similar findings using lineage tracing mouse models, confirming that labelled clones appeared at the periphery of the cornea and extended centripetally as radial stripes (Di Girolamo *et al.*, 2015; Dora *et al.*, 2015).

Over the years lots of other evidences further supported the limbal location of the corneal epithelial cells such as a lack of the corneal epithelial differentiation associated cytokeratin pair CK3/12 in the limbal basal cells (Schermer *et al.*, 1986; Rodrigues *et al.*, 1987; Kasper *et al.*, 1988; Kurpakus *et al.*, 1994). Also, most epithelial tumours of the ocular surface originate from the limbal area. Likewise, the limbus is a common region for certain congenital abnormalities to occur, such as limbal dermoids with ectopic brain or bone tissue, which suggest the presence of undifferentiated cells (Emamy and Ahmadian, 1977; Weinstein *et al.*, 1979). This widely accepted LSC dogma is, however, challenged by several studies which reported that the murine cornea remained healthy following deletion of the entire limbal epithelium (Vauclair *et al.*, 2007; Majo *et al.*, 2008). Moreover, the central cornea of patients with a destroyed limbus remained transparent for over 60 months (Dua *et al.*, 2009). These contradictions were recently addressed by Nasser and colleagues who reported that "committed corneal cells possess plasticity to dedifferentiate, repopulate the stem cell pool, and correctly re-form the tissue boundary in the presence of intact stroma" (Nasser *et al.*, 2018). Furthermore, the lineage-tracing experiments clearly demonstrate that during normal homeostasis, cells labelled in adult mice generate clones of cells that emerge from the limbus and extend across the corneal radius toward the centre. The distributions of labelled clones are inconsistent with the "corneal epithelial stem cell hypothesis" and strongly support the conventional "limbal epithelial stem cell hypothesis" (Amitai-Lange *et al.*, 2015; Di Girolamo *et al.*, 2015; Dora *et al.*, 2015).

1.3 Limbal stem cell niche

Stem cells are dispersed and kept in a unique anatomic location of each self-renewing tissue called niche, where they maintain quiescence while performing self-renewal to replenish the stem cell population lost to progeny production. The concept that a stem cell niche provides a unique microenvironment to support self-renewal and multipotent activity was first proposed in the late 1970s by Scofield (Schofield, 1978). According to the stem cell niche hypothesis stemness is maintained by certain extrinsic factors in the niche microenvironment which regulate stem cell fate decisions and prevent differentiation (Watt and Hogan, 2000). Removal of stem cells from the niche leads to loss of their stem cell identity, self-renewal capacity, and consequential onset of differentiation (Voog and Jones, 2010).

Variety of the limbal niche components ensure preservation of LSC activity including the limbal extracellular matrix (ECM), vasculature and surrounding cells (Nowell and Radtke, 2017). The limbal stroma is heavily vascularised and contains different ECM components compared to the corneal stroma, in particular α1 and α2 collagen IV, β2 laminin and fibronectin (Dziasko and Daniels, 2016). The limbal niche is populated by various cell types which are in close contact with the corneal epithelial layer such as melanocytes, vascular cells, nerve cells, immune cells and mesenchymal stromal cells (Yazdanpanah *et al.*, 2019). The expression of specific biochemical factors by limbal stromal cells including Wnt ligands (Nakatsu *et al.*, 2011; Han *et al.*, 2014), various cytokines and chemokines has proven to be important for LSC preservation.

There are three different groups of cytokines in the limbus initially described by Li and Tseng (Li and Tseng, 1995), according to their interaction with surrounding cells:

1. **type I cytokines** released by the epithelium with their receptors found mainly in stromal cells: TGFβ, IL-1β, PDGFβ, NGF, GDNF (Li and Tseng, 1995; Qi *et al.*, 2007),
2. **type II cytokines** with receptors found both in stromal and epithelial cells: IGF1, TGFβ1, TGFβ2, βFGF, NT-3, NT-4 (Li and Tseng, 1995; Qi *et al.*, 2007) and
3. **type III cytokines** released by the stroma whereas their receptors are found in the epithelium: KGF, HGF, BDNF (Li and Tseng, 1995; Qi *et al.*, 2007).

Importantly, many of these cytokines, including KGF, HGF, NGF, TGFβ1, TGFβ2, βFGF, have been identified in human amniotic membrane (HAM), which can support *in vitro* growth and undifferentiated phenotype of limbal epithelial cells in the absence of feeder cells and therefore can serve as surrogate LSC niche (Grueterich *et al.*, 2003a).

Touhami and co-workers examined the expression of different members of the neurotrophin family of growth factors and their receptors in the human limbal epithelium and found the high expression of the TrkA receptor in limbal basal epithelial cells, suggesting that nerve growth factor (NGF) signalling is involved in the control of the LSC behaviour (Touhami *et al.*, 2002). Recently, Kolli *et al.* reported that NGF signalling is indeed a key promoter of LSC proliferation, colony-forming efficiency, and a maintainer of the LSC phenotype (Kolli *et al.*, 2019).

Another feature of the stem cell niche that has been shown to be important in preserving stem cell function is the mechanical properties of the surrounding tissue. Every stem cell niche is composed of different ECM components which results in different mechanical properties of the tissue. Factors, such as elasticity and topography, have been shown to influence how a cell responds to other microenvironmental cues. For example, the specific ECM composition of the limbal stroma may confer distinct mechanical properties compared to the central conea and favour stem cell maintenance at that specific location. Engler *et al.* were the first to demonstrate the influence of substrate stiffness on stem cell differentiation. They reported that mesenchymal stem cells show sensitivity to tissue-level elasticity and capability to "respond" by either muscle, bone, or neural cell differentiation depending on substrate elasticity (Engler *et al.*, 2006). Related to the corneal tissue in particular, Moers *et al.* reported that corneal epithelial cells grown on substrates of physiological stiffness maintained an early differentiation state while on the contrary growth on unphysiologically stiff substrates induced their differentiation (Moers *et al.*, 2013). Another study, using very soft uncompressed and compressed collagen gels, showed not only that corneal epithelial cells exhibited differentiation on the stiffer gels but also that the cellular layer exhibited better stratification on the stiffer gel (Jones *et al.*, 2012).

Apart from the functional aspects, the LSC niche has also anatomic dimensions. Anatomically, the LSC niche is located at the Palisades of Vogt, a series of radially

orientated fibro-vascular ridges at the limbus, proposed for the first time in 1971 as a location of the cells responsible for renewal of the corneal epithelium (**Figure 1.6**) (Davanger and Evensen, 1971). These papillae-like structures are located in the sub-epithelial connective tissue at the limbus and more prominent along the superior and inferior limbus, where the upper and lower eyelids provide protection to the LSCs (Davanger and Evensen, 1971). The region in between the palisades, interpalisade ridges, are occupied by the epithelial rete pegs which consist of 10 to 15 layers of limbal epithelial cells (Townsend, 1991). The Palisades of Vogt can be seen and observed clinically, using a slitlamp microscope on the surface of the limbus, giving it a corrugated appearance (Goldberg and Bron, 1982; Townsend, 1991). These structures are easily identified in darkly pigmented individuals due to a concentration of melanin- containing cells and limbal melanocytes lining the interpalisade ridges (Zheng and Xu, 2008; Lagali *et al.*, 2013).

Figure 1.6 The Palisades of Vogt. Magnified view of the limbal area demonstrating visible corrugations, projections of limbal stroma into the epithelium, the palisades of Vogt (PV), and the rich blood supply (blood vessels, BV).

In corneal tissue sections, putative stem cells have been detected at the bottom of the epithelial papillae between the palisades, which provide a protective environment for LSCs (Schlotzer-Schrehardt and Kruse, 2005). Not only this junction between the limbal epithelial and stroma shelters LSCs from stress but also the neighbouring blood vessels supply them with nutrients and growth factors (Boulton and Albon, 2004).

In addition to these visible undulations of the limbal basement membrane, some later

studies reported existence of deeper epithelial outgrowths into the limbal stroma. In 2005, Dua *et al.* described structures named "limbal epithelial crypts" as invaginations that were perpendicular to the surface epithelium and then extended parallel to the surface (Dua *et al.*, 2005). These invaginations provide even closer proximity and increased contact with the limbal microenvironment. Limbal epithelial crypts are therefore putatively similar to intestinal crypts where stem cells reside in the base of the crypts. In 2007 Shortt *et al.* revealed further ultrastructural details of the LSC niche using *in vivo* confocal microscopy and three-dimensional reconstruction. They identified two novel candidate niche structures, the first named as "limbal crypts" located between the palisades of Vogt, and the second named as "focal stromal projections" which project upwards at the open end (facing the cornea) of the crypts (Shortt *et al.*, 2007a). Limbal crypts are downward projections of the limbal epithelium that are open to the corneal surface and are in close association with the limbal vasculature. Focal stromal projections are, on the other side, finger-like projections of stroma containing a central blood vessel surrounded by small, tightly packed epithelial cells. The highest number of putative stem cells, p63α and ABCG2 positive cells, were observed in the basal epithelial layers of these two described structures (Shortt *et al.*, 2007a).

Dziasko *et al.* identified cells with the stem cell like morphology exclusively within the basal layer of the limbal crypts using high-resolution imaging and showed these cells are capable of holoclone formation *in vitro*. They also confirmed that these cells are in direct contact with cells in the underlying stroma and that the contact is facilitated by focal basement membrane interruptions (Dziasko *et al.*, 2014) (**Figure 1.7**).
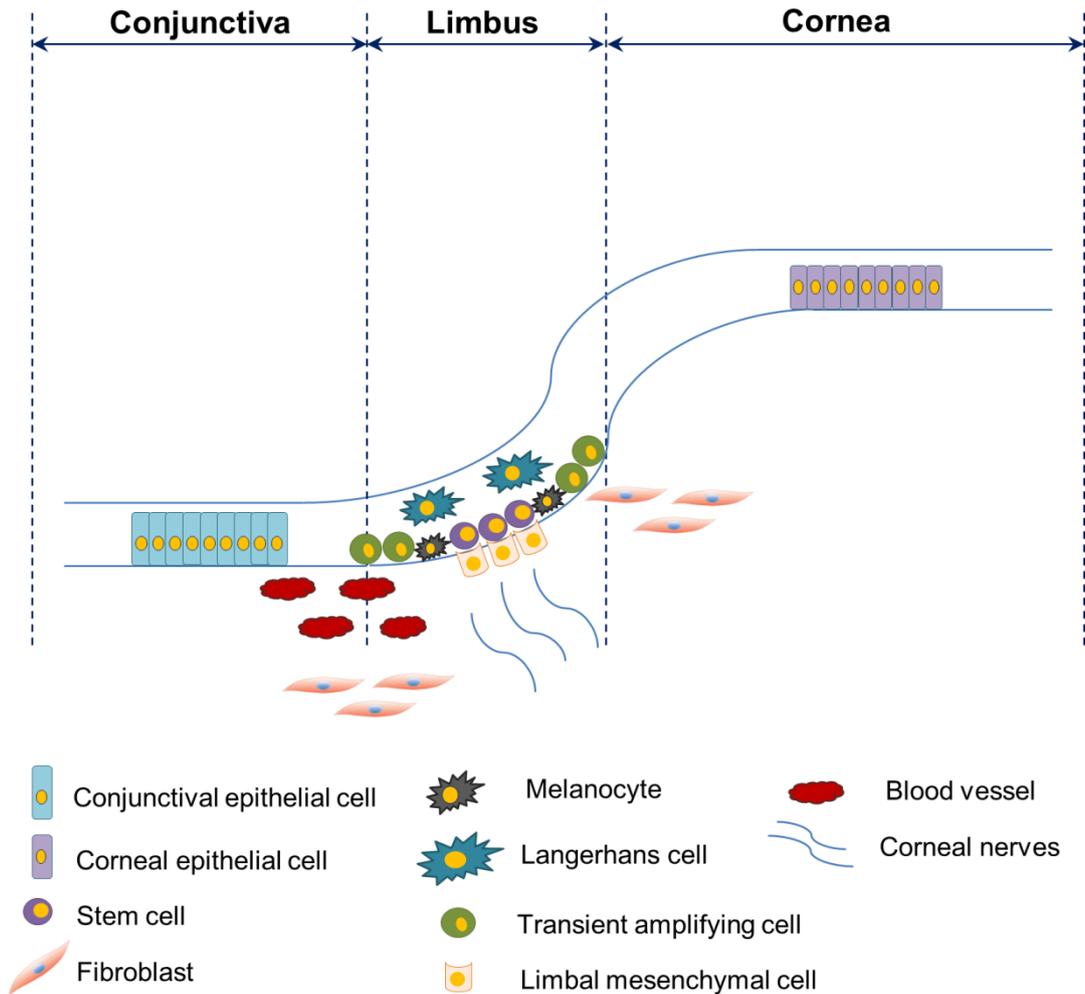
Figure 1.7 Simplified model of the LSC niche and niche cells. The LSCs that reside in the basal layer of the limbal epithelium are uniquely positioned to receive cellular signals from a variety of sources both by direct cell contact and diffusible cytokines. Daughter TACs divide and migrate centripetally towards central cornea where they terminally differentiate and eventually shed from the ocular surface. According to the recent findings, LSC are closely associated with limbal mesenchymal cells and limbal melanocytes. Besides these two cell types, limbal and corneal fibroblasts and Langerhans cells are also important components of the limbal stem cell niche.

The limbal stem cell niche is highly pigmented due to the presence of melanocytes (Davanger and Evensen, 1971; Higa *et al.*, 2005; Dziasko *et al.*, 2015) and is infiltrated with antigen-presenting Langerhans's cells (Baum, 1970; Vantrappen *et al.*, 1985) and suppressor T lymphocytes (Vantrappen *et al.*, 1985). The niche cells provide sheltering environment that protects stem cells from stimuli that may promote

differentiation or apoptosis, threatening stem cell reserves (Moore and Lemischka, 2006). For example, melanocytes, distributed in the limbal basal layer with their cellular projections extending to surrounding basal epithelial cells, produce and transport melanin pigments into epithelial cells. Melanin minimizes damage caused by ultraviolet irradiation together by the upper and lower eyelids that covers the superior and inferior limbus, where Palisades of Vogt are primarily located (Hertsenberg and Funderburgh, 2015). A three dimensional reconstruction of the LSC niche was proposed by Dziasco *et al.* in which the stem cell is closely associated and maintained by both dendritic pigmented limbal melanocytes and elongated limbal stromal cells (Dziasko *et al.*, 2014). Limbal epithelial cells co-cultured *in vitro* with mitotically active human limbal melanocytes were able to generate large epithelial colonies of small and compact cells with morphological stem cell characteristics, positive for the expression of the putative stem cell markers CK15, Bmi-1 and p63α and negative for the marker of terminal cell differentiation CK3 (Dziasko *et al.*, 2015). It is also generally accepted that mesenchymal cells from the limbal stroma play a role in the maintenance and support of LSCs and are considered as an important element of the stem cell niche (Chen *et al.*, 2011; Li *et al.*, 2014b; Nakatsu *et al.*, 2014).

1.4 Corneal epithelial cell turnover

The "X, Y, Z hypothesis of corneal epithelial maintenance" proposed by Thoft and Friend in 1983, prior to our knowledge of the existence of stem cells in the cornea, summarize the movement of cells in a healthy corneal epithelium (Thoft and Friend, 1983). Since there is a continual loss of the superficial epithelial cells through desquamation while the epithelial mass remains relatively constant, they hypothesized that the corneal epithelium required continual replacement of naturally lost epithelial cells. According to their hypothesis corneal epithelial maintenance can be defined by the equation: $X + Y = Z$, where X is the proliferation of the basal epithelial cells and their movement towards the corneal epithelial surface, Y is the movement of the peripheral cells to the central corneal epithelium and Z is the loss of cells from the corneal epithelial surface due to constant desquamation (**Figure 1.8**). This equation states that cell loss (Z) must be balanced by cell replacement (X+Y) to enable the normal maintenance of corneal epithelium.
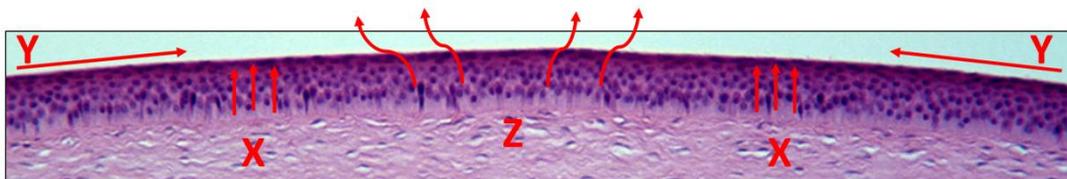
Figure 1.8 The X, Y, Z hypothesis of corneal epithelial maintenance. X represents the migration of epithelial cells from the basal layer of the corneal epithelium to the epithelial surface. Y represents the migration of epithelial cells from periphery to the centre of the cornea. Z represents the loss of epithelial from the corneal surface from which terminally differentiated cells continuously slough off. For maintaining the corneal homeostasis it's important that the sum of X and Y is equal to Z.

Although it was questioned, there is a plenty of evidence that supported the original observations of the hypothesis. Different radio-labelling studies have been proved the movement of epithelial cells from the basal layer of the corneal epithelium towards the epithelial surface (Hanna and O'Brien, 1960; Lavker *et al.*, 1991). The movement of epithelial cells from the periphery of the cornea is also confirmed by numerous studies (Buck, 1979; Kaye, 1980; Kinoshita *et al.*, 1981; Dua and Forrester, 1990). Clinical observations confirmed that the cell movement from the peripheral corneal epithelium ends in eventual loss of cells from the central corneal surface (Lemp and Mathers, 1989).

1.5 Characteristics of limbal stem cells

LSCs fulfil all the criteria which apply to other types of stem cells such as low level of differentiation with a primitive phenotype, slow cell cycle, high proliferative potential after activation by wounding or *in vitro* culture conditions and capacity for unlimited self-renewal (Schlotzer-Schrehardt and Kruse, 2005).

The capability to generate new cells is important characteristic of both stem cells and TACs. To be able to fulfil this function, they first need to replicate their deoxyribonucleic acid (DNA), through a process known as DNA replication which

occurs in the S phase of the cell cycle. Both tritiated thymidine and 5-bromo-2'-deoxyuridine (BrdU) have been used to label cells in the S phase (Bickenbach, 1981; deFazio *et al.*, 1987; Ahmad *et al.*, 2006). Whilst stem cells go through the cell cycle slowly and retain these S phase markers for longer periods of time (Morris *et al.*, 1985; Kruse and Tseng, 1992; Ahmad *et al.*, 2006), TACs continuously generate new cells and therefore cycle more rapidly. Consequently they retain S phase markers for shorter periods of time. The "label retaining property" has traditionally been used to distinguish stem cells from TACs. Using the proportion of label retaining cells present in the limbal region of the human cornea reported by Lavker and colleagues, it has been estimated that the percentage of stem cells may represent less than 10% of the total basal cell population (Lavker *et al.*, 1991).

Further characteristics that distinguish LSCs from TACs are morphological criteria and difference in cell size (Schlotzer-Schrehardt and Kruse, 2005). The smallest cells are located in the basal layer of the limbal epithelium as compared to the basal corneal epithelium (10.1 ± 0.8 vs. 17.1 ± 0.8 µm) (Romano *et al.*, 2003). Another striking characteristic of limbal basal cells is their pigmentation that is result of intrinsic melanogenesis and play role in the protection of these cells from solar damage (Davanger and Evensen, 1971; Wolosin *et al.*, 2000). Recently, Liu *et al.* showed association between pigmentation and differentiation, using a comparative transcriptomic analysis, with the p63[+] population being the most pigmented and immature of the progenitors compared to ABCB5^{+}, p63[+]ABCB5^{+} and p63[+]ABCB5^{+}CK3^{+} populations (Liu *et al.*, 2018). The limbal epithelial cells in the basal layer of the limbal epithelium show characteristics of immature cells including small cell size, high nuclear-cytoplasmic ratio, euhromatin-rich nuclei, hardly detectable nucleoli and cytoplasm rich in tonofilaments (Chen *et al.*, 2004). Schlotzer-Schrehardt and Kruse reported that the adult LSCs exist in close spatial relationship with their early progenitor cells (Schlotzer-Schrehardt and Kruse, 2005). Using transmission electron microscopy, they observed "groups of small, roundish, densely packed cells at the bottom of the epithelial papillae forming the palisades of Vogt", putative stem cell niche. Two different cell types could be observed within these clusters: one or few small, primitive appearing putative stem cells surrounded by larger melanin-containing early progenitor cells – putative TACs. The putative stem cells lay on a delicate basement membrane without forming cytoplasmic processes. Moreover, hemi-desmosomes and intercellular junctions are absent in this

region. On the other hand, the putative TACs possess cell processes which interdigitate with the matrix underneath and also have numerous hemi-desmosomes (Schlotzer-Schrehardt and Kruse, 2005). Additionally, the putative stem cells showed a high nuclear-cytoplasmic ratio, heterochromatin-rich nuclei without visible nucleoli, sparse cytoplasm with small melanin granules, few intermediate filaments, few mitochondria and ribosomes (Schlotzer-Schrehardt and Kruse, 2005). In contrast, the putative TACs have nuclei with increased euchromatin and distinct nucleoli, prominent melanin granules and tonofilament bundles (Schlotzer-Schrehardt and Kruse, 2005).

1.6 Molecular markers of limbal stem cells

Although a variety of putative LSC markers have been proposed up to date, identification of specific markers that would precisely identify LSC remains elusive and their role in identification of LSCs is still controversial. One of the possible reasons may be a very low number of LSCs present, probably as few as 100 in the mouse limbus (Collinson *et al.*, 2002). The major markers proposed for epithelial stem cells in ocular or non-ocular tissues can be categorized into at least three groups: A) nuclear proteins such as the transcription factors p63 and PAX6, and signalling protein WNT7A B) cell membrane or transmembrane proteins including integrins (integrin β1, α6, α9), receptors (epidermal growth factor receptor - EGFR, transferrin receptor CD71, nerve growth factor receptor TrKA etc.), and drug resistance transporters (ABCG-2, ABCB5), and C) cytoplasmic proteins such as cytokeratin 19 (CK19), nestin, and α-enolase (Chen *et al.*, 2004). In addition, a variety of differentiation markers have also been proposed to distinguish stem cells from differentiated cells. These include cytokeratin 3 and 12 (CK3 and CK12), involucrin, intercellular adhesive molecule E-cadherin, and gap junction protein Connexin 43, etc. (Chen *et al.*, 2004). Notable markers are summarized in **Table 1.1**.

Prominent candidate markers of LSC include transcription factor p63, C/EBPδ, Bmi1, ABCG2, and Notch-1 and recently proposed ABCB5 whilst most studied negative markers are cytokeratin dimer 3/12 and Connexin 43 (Pellegrini *et al.*, 2001; Chen *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005; Barbaro *et al.*, 2007; Notara *et al.*, 2010; Joe and Yeung, 2014; Ksander *et al.*, 2014). Many of the markers associated

with limbal basal cells in the past, such as α9 and β1 integrins, α-enolase, and Connexin 43, are widely believed to be associated with increased mitotic activity related to TACs, but not with true quiescent stem cells (Schlotzer-Schrehardt and Kruse, 2005; Notara *et al.*, 2010; Pellegrini *et al.*, 2011). For many years, this area remains a highly controversial with numerous conflicting reports.

Table 1.1 Putative LSC markers (with corresponding references).

Putative Markers	Presence (+) or absence (-) in basal limbal epithelium	Reference

I NUCLEAR PROTEINS

	Putative Markers	Presence (+) or absence (-)	Reference
Transcription factors and signalling proteins	p63	+	(Pellegrini et al., 2001)
	C/EBPδ	+	(Barbaro et al., 2007)
	Bmi1		(Barbaro et al., 2007)
	PAX6		(Ouyang et al., 2014)
	WNT7A		(Ouyang et al., 2014)

II CELL MEMBRANE OR TRANSMEMBRANE PROTEINS

	Putative Markers	Presence (+) or absence (-)	Reference
Receptors	EGFR	+	(Zieske and Wasson, 1993)
	TrKA	+	(Touhami et al., 2002)
	CD71	+	(Hayashi et al., 2008)
	RHAMM/HMMR	-	(Ahmad et al., 2008)
	Notch-1	+	(Thomas et al., 2007)
Transporters	ABCG2	+	(de Paiva et al., 2005)
	ABCB5	+	(Ksander et al., 2014)
Adhesion proteins	Integrin β1 & α9	+	(Chen et al., 2004)
	E-cadherin	-	
Gap junction proteins	Connexin 43	-	(Matic et al., 1997)
	Connexin 50	-	

III CYTOSOLIC AND CYTOSKELETON PROTEINS

	Putative Markers	Presence (+) or absence (-)	Reference
Cytokeratins	CK3	-	(Rodrigues et al., 1987; Kasper et al., 1988)
	CK12	-	
	CK13	-	(Merjava et al., 2011)
	CK14	+	(Bickenbach, 2005)
	CK15	+	(Ohyama et al., 2006)
	CK19	+	(Michel et al., 1996)
Intermediate filament proteins	Vimentin	+	(Kasper et al., 1988)
Structural proteins	Involucrin	-	(Chen et al., 2004)
	Cytochrome oxidase	+	(Hayashi and Kenyon, 1988)
	Nestin	-	(Schlotzer-Schrehardt and Kruse, 2005)
Metabolic enzymes	α-enolase	+	(Zieske et al., 1992)
	Na/K-ATPase	+	(Zieske, 1994)
	Carbonic anhydrase	+	(Steuhl and Thiel, 1987)
	Protein kinase C-γ	+	(Tseng and Li, 1996)
Cell-cycle proteins	Cyclin A, D & E	+	(Joyce et al., 1996)

1.6.1 p63

p63 is a transcription factor which belongs to a family of tumour suppressor proteins including also p53 and p73 (Benard *et al.*, 2003) and is involved in morphogenesis (Mills *et al.*, 1999).

TP63, the gene encoding p63, produces six isoforms by the use of two promoters, TA and ΔN, and alternative splicing at the 3'-terminus (Aberdam and Mantovani, 2009). All isoforms consist of a DNA-binding domain and a carboxyl-terminal oligomerization domain. Isoforms with an added amino-terminal transactivation domain are named - TAp63 and those without - ΔNp63 (also known as p40) (Ahmad *et al.*, 2006). In addition, three forms of the carboxyl-terminal oligomerization domain exist – α, β and γ (Ahmad *et al.*, 2006). This results in a total of six p63 isoforms, three with an added transactivation domain (TAp63 α, β and γ) and three without (ΔNp63 α, β and γ) (Ahmad *et al.*, 2006), of which ΔNp63α is most highly associated with LSCs (Di Iorio *et al.*, 2005). ΔNp63α is shown to be more specific to LSCs compared to the other isoforms (Di Iorio *et al.*, 2005), and its expression increases in limbal basal cells during wound healing (Barbaro *et al.*, 2007). Moreover, ΔNp63α is thought to be a marker of both resting (quiescent) and activated LSCs (Barbaro *et al.*, 2007). While some studies showed that ΔNp63α is expressed in holoclones (formed by stem cells) but not paraclones (formed by TACs) in quiescent cornea (Barbaro *et al.*, 2007), numerous studies, on the other hand, showed that ΔNp63α is also expressed by early TACs (Du *et al.*, 2003; Espana *et al.*, 2004; Harkin *et al.*, 2004; Joseph *et al.*, 2004).

However, it is important that higher ΔNp63α expression in limbal epithelial grafts is associated with greater success in clinical LSC transplantation, whereas grafts containing less than 3% of ΔNp63α - bright cells are at an increased risk for failure (Rama *et al.*, 2010).

Beta and gamma isoforms of ΔNp63 are expressed in the suprabasal layers of the limbal and corneal epithelia in response to wounding and are expressed in more differentiated cells (Daniels *et al.*, 2006a).

Biological processes known to require p63 include epidermal lineage commitment, epidermal differentiation, cell adhesion, and basement membrane formation (Koster,

2010). Aberdam and colleagues suggested a key role of p63 in BMP-4 induced epidermal commitment of embryonic stem cells. They showed that although ΔNp63 is not required for ectodermal fate it enhances embryonic stem cell-derived ectodermal cell proliferation and epidermal commitment (Aberdam *et al.*, 2007). ΔNp63 may be dispensable for some epithelial differentiation, but is necessary for the commitment of embryonic stem cells into CK5/CK14 positive squamous stratified epithelial cells (Medawar *et al.*, 2008).

In vivo studies, transgenic and knock-out mice have demonstrated that p63 plays an essential role in squamous epithelial development and regeneration (Mills *et al.*, 1999; Yang *et al.*, 1999; Rinne *et al.*, 2006; Aberdam *et al.*, 2007; Rinne *et al.*, 2007). The generation of mouse models lacking p63 led to severe changes in their phenotype: the mice die soon after birth with severe defects in limbs, craniofacial development, and absence of skin (Mills *et al.*, 1999; Yang *et al.*, 1999) . The fact that p63 knockout mice suffer from severe abnormalities in their epithelial development and lack of stratified epithelium suggests that p63 might be involved in maintaining the stem cell population (Celli *et al.*, 1999). It is thought to regulate the stem cell population by promoting cell senescence and genomic stability, thereby sustaining their proliferative potential (Su *et al.*, 2009).

p63 is consistently expressed in the basal cells of stratified epithelia and was previously identified as a stem cell marker for keratinocytes (Pellegrini *et al.*, 2001). Later studies showed that p63 was not an exclusive LSC marker and it's expressed not only in LSC, but also in some TACs, especially proliferative epithelial cells (Kim *et al.*, 2009). It is highly expressed in both epidermal and limbal holoclones (Mills *et al.*, 1999; Yang *et al.*, 1999; Pellegrini *et al.*, 2001). Studies on human corneas partially supported these findings, demonstrating predominant immuno-localization of p63 in the nuclei of the limbal epithelial basal layer scattered in between the patches of p63 negative cells (Chen *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005). Another study, on the other hand, reported positive p63 staining not only in the limbal region but also among most of the basal cells of the peripheral and central corneal epithelium (Dua *et al.*, 2003). Opposite to the observations in human corneas, studies on murine corneas showed p63 expression not only in the limbal region but throughout whole corneal epithelium (Moore *et al.*, 2002).

1.6.2 Cytokeratins

Cytokeratins or keratins are a group of cytoskeletal proteins that form intermediate filaments in epithelial cells and are expressed in distinct patterns during epithelial development and differentiation. They are expressed in different combinations according to the type of epithelium and its state of differentiation (Sun *et al.*, 1983; Schlotzer-Schrehardt and Kruse, 2005).

Cytokeratins have been intensively studied and represent either markers of differentiation (CK3, CK12) (Rodrigues *et al.*, 1987; Kasper *et al.*, 1988; Liu *et al.*, 1993), or unspecific markers of limbal basal cells (CK5, CK14, CK15, CK19). Some of these cytokeratins were also found in conjunctival epithelium (CK5, CK14, CK19) (Merjava *et al.*, 2011). CK14 has been proposed as a marker of proliferating keratinocytes in the skin (Bickenbach, 2005) as well as CK19 that was used to localize epidermal stem cells in hair follicles (Michel *et al.*, 1996). CK19 together with another structural protein, vimentin, have also been foundin the basal cells of both humane and murine limbal epithelium (Kasper *et al.*, 1988; Kasper, 1992; Lauweryns *et al.*, 1993; Schlotzer-Schrehardt and Kruse, 2005). Cells which co-express both of these proteins were found to be equivalent to label retaining cells (Kasper, 1992). CK15 is the one that deserves a particular attention as reported to be a marker of hair follicular bulge stem cells (putative epidermal stem cells) and also considered as a marker of LSC (Ohyama *et al.*, 2006; Yoshida *et al.*, 2006). However, CK15 is also widely expressed in limbal basal cells and has not been directly linked to LSC functions such as clonogenicity or self-renewal (Joe and Yeung, 2014). On the other hand, dimer CK3/12 identifies more differentiated cells in corneal epithelium (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). This dimer is absent in LSCs and early TACs and therefore represents an important negative marker for LSCs.

1.6.3 Connexin 43 and Connexin 50

Connexin 43 and connexion 50, the gap junction proteins, are other important negative markers of LSC. They are abundantly expressed in the corneal epithelium, Connexin 50 throughout all layers whilst Connexin 43 is mainly confined to the basal cell layer (Dong *et al.*, 1994; Matic *et al.*, 1997; Wolosin *et al.*, 2002). In contrast, they

are both absent from the basal layer of human, mouse, chicken and neonatal rabbit limbal epithelium where both LSCs and early TACs reside. It has been suggested that the absence of gap junction communication and consequential metabolic isolation from differentiating signals is an important characteristic of stem cells (Matic *et al.*, 1997).

1.6.4 C/EBPδ and Bmi1

Transcription factor CCAAT enhancer binding protein δ (C/EBPδ) is expressed in many epithelial cell types and is involved in cell cycle arrest (Barbaro *et al.*, 2007). Bmi1, a polycomb ringfinger protein, has also been detected in human limbus (Barbaro *et al.*, 2007) but was also reported in stem cells in a number of other organs (Molofsky *et al.*, 2003; Park *et al.*, 2003; Iwama *et al.*, 2004). Similarly to ΔNp63α, C/EBPδ and Bmi1 are expressed in holoclones but not paraclones (Pellegrini *et al.*, 2011). Coexpression of C/EBPδ, Bmi1 and ΔNp63α is thought to identify mitotically quiescent limbal cells. Both C/EBPδ and Bmi1 are involved in regulation of LSC quiescence, and the expression of both factors becomes downregulated upon corneal injury. LSC continue to express ΔNp63α during the initial proliferative phase, but lose this expression on terminal differentiation (Barbaro *et al.*, 2007). However, more recent findings demonstrated that despite the fact that Bmi1 positive cells did participate in tissue replenishment within the central cornea, they were unable to maintain homeostasis of the cornea for more than 3 months, suggesting their status as progenitor rather than stem cells (Kalha *et al.*, 2018).

1.6.5 ABCG2 and ABCB5

ABCG2, an ATP-binding cassette transporter protein, is expressed in stem cells in various tissues (Zhou *et al.*, 2001). In these cells ABCG2 is responsible for the efflux of different anticancer drugs but also for their capability to exclude Hoechst 33342 dye that can be detected by flow cytometry (Zhou *et al.*, 2001). The exclusion of Hoechst 33342 dye is a unique property of the so called side-population (SP) cells. This characteristic has been associated with stem and progenitor cells in many tissues (including blood and skeletal muscle) and potentially can be used for cell

purification (Goodell *et al.*, 1996; Chang *et al.*, 2011; Ergen *et al.*, 2013). However, this isolation strategy is not entirely specific on its own, and should be used only in combination with other positive or negative stem cell markers. ABCG2 is expressed by only a small subset of limbal basal cells (less than 1%) but interestingly its expression was not associated with extensive colony forming capacity (Umemoto *et al.*, 2006).

ABCB5, ATP-binding cassette, sub-family B, member 5, is firstly identified as a marker of skin progenitor cells (Frank *et al.*, 2003) and melanoma stem cells (Schatton *et al.*, 2008). It functions as a regulator of cellular differentiation (Frank *et al.*, 2003) and is required for LSCs maintenance, corneal development and repair (Ksander *et al.*, 2014). ABCB5 expression on label-retaining LSCs in mice and p63α positive LSCs in humans, along with reduced ABCB5 positive limbal cell frequency in LSC-deficient patients, suggest that ABCB5 preferentially marks LSCs (Ksander *et al.*, 2014). Ksander *at al.* also showed that isolated humane or murine ABCB5 positive cells possess the exclusive capacity to fully restore the cornea upon grafting to LSC-deficient mice (Ksander *et al.*, 2014). Moreover, they showed that depletion of quiescent LSCs due to enhanced proliferation and apoptosis in *Abcb5* knockout mice results in defective corneal differentiation and wound healing (Ksander *et al.*, 2014). Recently however, another research group reported that p63 is superior to ABCB5 as a marker for stemness, whilst ABCB5, either alone or in co-expression patterns with p63 and CK3, identifies more committed progenitor cells (Liu *et al.*, 2018).

1.6.6 PAX6 and WNT7A

In 2014, Quyang and colleagues reported that "the transcription factors p63 and PAX6 (paired box protein PAX6) act together to specify LSCs and WNT7A controls corneal epithelium differentiation through PAX6" (Ouyang *et al.*, 2014). Additionally, they showed that loss of either WNT7A or PAX6 induces change of LSCs into skin-like epithelium and furthermore that transduction of PAX6 in skin epithelial stem cells is sufficient to convert them to LSC-like cells. Importantly, these reprogrammed skin epithelial stem cells were able to repair damaged corneal surface and regenerate corneal epithelium upon transplantation in a rabbit corneal injury model (Ouyang *et*

al., 2014). Taken together, their findings suggested a central role of the WNT7A-PAX6 axis in corneal epithelial cell fate determination.

1.6.7 Notch-1

Notch-1 is a transmembrane receptor involved in maintaining cells in an undifferentiated state and has been proposed as a potential LSC marker by Tomas and co-workers. It has been found that Notch-1 expression was localised to a small number of cells in the limbal epithelial basal layer expressing also ABCG2. While all Notch-1 positive cells were ABCG2 positive, not all ABCG2 positive cells expressed Notch-1. It seems to be highly expressed in quiescent cells, supporting the notion that it could be an LSC marker (Thomas *et al.*, 2007).

1.6.8 RHAMM/HMMR

RHAMM/HMMR, the hyaluronan receptor, is located in all layers of corneal epithelium and in the suprabasal layers of the limbal epithelium but is completely absent from the basal layer of the limbus. Moreover, the absence of RHAMM/HMMR expression is correlated with properties associated with LSC. Namely, RHAAM/HMMR negative limbal epithelial cells are smaller in size, express negligible CK3 but higher levels of ΔNp63α and have higher colony forming efficiency compared to RHAAM/HMMR positive limbal epithelial cells. For all this reason, RHAMM/HMMR represents a putative negative marker of LSC (Ahmad *et al.*, 2008).

1.6.9 Periostin

Periostin is a non-structural matricellular protein originally found in murine osteoblasts (Takeshita *et al.*, 1993). Later studies have shown that periostin is produced not only by stromal tissues, but also by epithelial tissues including epithelial cells in prostatic, ovarian and oral tumours (Gillan *et al.*, 2002; Siriwardena *et al.*, 2006; Tsunoda *et al.*, 2009). Recently, periostin was found to be exclusively localized in the basal layer of human limbal epithelium and co-localized with p63 but not with

corneal differentiation marker CK3. In primary human limbal epithelial cells, periostin expression at both mRNA and protein levels was significantly higher in sub-confluent cultures at exponential growth stage than in confluent cultures at slow growth condition. Moreover, periostin expression increased significantly during epithelial regeneration in wound healing process, especially 16-24h at the wound edge, similar to activation and upregulation of p63 and integrin β1 (Qu *et al.*, 2015).

1.7 Limbal stem cell deficiency

According to the World Health Organization, as the 4th cause of blindness globally (5.1%), corneal blindness is one of the major causes of visual deficiency after cataract, glaucoma and age-related macular degeneration (World Health Organization). It has been estimated that corneal vascularisation and opacity cause blindness in 8 million people worldwide each year.

Corneal integrity and function depend on self-renewing properties of the corneal epithelium. The corneal surface is constantly renewed by the stem cells located at the peripheral edge of the cornea, in a region known as limbus. These cells can be destroyed by numerous factors, including chemical and thermal burns, infections or autoimmune diseases, which result in the condition called limbal stem cell deficiency (LSCD), very painful and debilitating eye disease (O'Callaghan and Daniels, 2011; Ahmad, 2012).

The cornea is surrounded by the conjunctiva which lays on fibrovascular connective tissue containing blood vessels and lymphatics (Utheim, 2013). Numerous cell types are interspersed within the conjunctival epithelium including lymphocytes, melanocytes, Langerhans cells and goblet cells. Goblet cells produce mucin, essential for maintenance of the tear film and ocular surface integrity, and are present exclusively in conjunctival but not in healthy corneal epithelium (Utheim, 2013). The limbus acts as a barrier separating the clear avascular corneal epithelium from the surrounding vascular conjunctival tissue.

LSCD is characterized by either a loss or dysfunction of the stem cells in the limbus (Ahmad, 2012). In LSCD both the stem cell function and the barrier function of the limbus fail (Ahmad *et al.*, 2010). Either loss or dysfunction of LSCs lead to failure of

corneal epithelial healing, therefore persistent corneal epithelial defects appear. These epithelial defects cause chronic ocular surface discomfort and pain. The failure of the barrier function, on the other side, will allow conjunctival epithelium along with its blood vessels to invade the corneal surface. This phenomenon is known as conjunctivalization of the cornea that leads to loss of corneal clarity with consequent visual impairment or blindness (Ahmad, 2012).

1.7.1 Etiology and classification of LSCD

The two pathologic mechanisms of LSCD are the direct destructive loss of LSCs and the loss of the limbal microenvironment/niche needed for LSC survival (Le *et al.*, 2018). Many acquired and congenital diseases may cause LSCD either by the direct loss of LSC pool, by the destruction of their niche or both (Li *et al.*, 2007; Le *et al.*, 2018).

There are many known causes of LSCD, if the cause is unknown these cases are classified as idiopathic (Espana *et al.*, 2002) (**Table 1.2**). Primary LSCD is a direct result of genetic mutations that lead to LSC dysfunction or destruction. Some hereditary diseases including the limbus such as aniridia, a developmental dysgenesis of the anterior segment of the eye (Nishida *et al.*, 1995; Ramaesh *et al.*, 2005; Skeens *et al.*, 2011), keratitis associated with multiple endocrine deficiencies (Puangsricharern and Tseng, 1995; Mohammadpour and Javadi, 2006), dyskeratosis congenita (Aslan and Akata, 2010; Aslan *et al.*, 2012), ectodermal dysplasia (Di Iorio *et al.*, 2005; Felipe *et al.*, 2012), Turner syndrome (Strungaru *et al.*, 2014), lacrimo-auriculo-dento-digital (LADD) syndrome (Cortes *et al.*, 2005), and xeroderma pigmentosum (Fernandes *et al.*, 2004) are connected with LSCD.

Secondary LSCD results from external factors that directly destroy LSCs, damage the stem cell niche or both. The most common acquired LSCD is caused by chemical or thermal injuries (Sangwan, 2001; Le *et al.*, 2010; Ahmad, 2012; Sejpal *et al.*, 2013), while other possible acquired causes are contact lens wear and ocular surface inflammatory diseases such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid, and chronic limbitis (Puangsricharern and Tseng, 1995; Dua and

Azuara-Blanco, 2000; Sridhar *et al.*, 2001; Bhatia *et al.*, 2009). Other inflammatory conditions including severe chronic vernal keratoconjunctivitis and microbial infection involving the limbus can directly destroy the stem cells too (Sangwan *et al.*, 2011). In contact lens wear, LSCD may be caused by either toxicity of the contact lens solution or chronic mechanical micro trauma, hypoxia and inflammation of the limbus as a result of contact lens friction (Utheim, 2013). Iatrogenic causes are also present: extensive cryotherapy, radiation, multiple ocular surgeries involving the limbal region, toxicity from topical medications such as mitomycin C and 5-FU can also directly destroy LSC and their niche (Pires *et al.*, 2000; Dudney and Malecha, 2004; Utheim, 2013). LSCD secondary to other ocular surface disorders has also been reported; these disorders include neurotrophic (neural and ischemic) keratopathy (Bonini *et al.*, 2003), bullous keratopathy (Paris Fdos *et al.*, 2010), and extensive ocular surface tumours (Gupta *et al.*, 2011). In most of the acquired cases of LSCD, probably both LSCs and their niche are affected (Secker and Daniels, 2008; Utheim, 2013).

Table 1.2 Causes of limbal stem cell deficiency.

I PRIMARY / HEREDITARY

- Aniridia
- Multiple endocrine deficiency
- Ectodermal dysplasia
  - Ectrodactyly–ectodermal dysplasia–cleft (EEC) syndrome
  - Keratitis-ichthyosis-deafness (KID) syndrome
- Dyskeratosis congenital
- Turner syndrome
- Xeroderma pigmentosum
- Lacrimo-auriculo-dento-digital (LADD) syndrome

II SECONDARY / AQUIRED

- Chemical or thermal injury
- Contact lens wear
- Ocular surface inflammatory diseases
  - Stevens-Johnson syndrome / toxic epidermal necrolysis
  - ocular cicatricial pemphigoid
  - chronic limbitis (vernal / atopic conjunctivitis)
- Iatrogenic causes
  - extensive cryotherapy
  - radiotherapy
  - multiple ocular surgeries involving the limbal region
  - cytotoxic agents (mitomycin C and 5-fluorouracil)
- Severe microbial infections involving limbus
- Mechanical trauma
- Pterygium
- Limbal tumours and extensive ocular surface tumours
- Neurotrophic keratopathy
- Bullous keratopathy

III IDIOPATHIC (unknown cause)

1.7.2 Presentation of LSCD

LSCD can affect both eyes (bilateral) and just one eye (unilateral). Depending on the extent of the disorder LSCD is classified as either partial or total (Kolli *et al.*, 2010).

Depending on the severity of the disease, LSCD can present with various symptoms and signs. Characteristic symptoms and signs can be easily understood by considering the main functions of limbus, the stem cell function and barrier function.

As a result of either loss or dysfunction of LSCs corneal epithelial defects start to appear and fail to heal normally (Utheim, 2013). Recurrent corneal erosion or persistent epithelial defects together with inflammation cause severe pain, irritation, redness, tearing, photophobia, epiphora, blepharospasm and reduced vision and may lead to scarring, corneal thinning and even perforation (Espana *et al.*, 2002). As the barrier function of limbus also fail conjunctival epithelium along with blood vessels encroach and invade the corneal surface. This process known as conjunctivalization of the cornea and represents the hallmark of LSCD. The conjunctivalization causes loss of corneal clarity and consequential visual impairment.

The direct loss of LSCs and the conjunctivalization of the corneal surface lead to a number of clinical signs (Dua *et al.*, 2000; Kolli *et al.*, 2009; Sejpal *et al.*, 2013):

Corneal epithelial haze. As a direct consequence of scarring and the conjunctivalization the corneal epithelium loses its transparency and becomes hazy (**Figure 1.9**). Normal corneal epithelial cells are attached by tight junctions but the cellular connections between conjunctival epithelial cells are much looser making the conjunctival epithelium more permeable for hydrophilic molecules such as fluorescein compared to corneal epithelium (Kolli *et al.*, 2009). This fact results in the late staining of conjunctivalized corneal epithelium with fluorescein usually seen in LSCD (Kolli *et al.*, 2009). Moreover, these loose connections between the conjunctival epithelial cells permit entry of leucocytes from the tear film which then contribute to the redness and the chronic inflammation seen in LSCD (Kolli *et al.*, 2009).
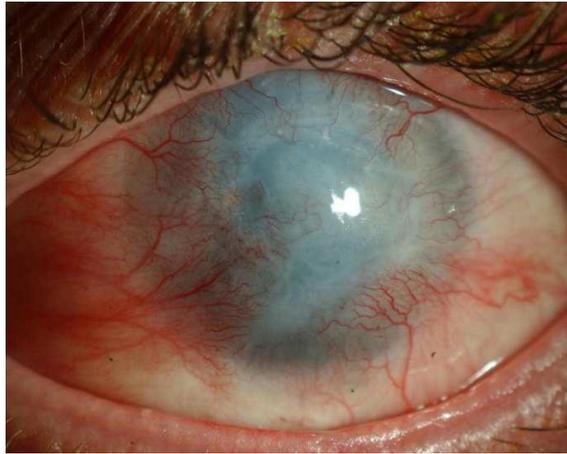
Figure 1.9 Clinical signs of limbal stem cell deficiency showing corneal haziness, conjunctivalization and neovascularisation of the corneal surface. The photo is taken from the paper "Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency" (Kolli *et al.*, 2010).

Persistent epithelial defects. Failure of corneal epithelial healing due to loss or dysfunction of LSCs lead to formation of persistent corneal epithelial defects. These persistent epithelial defects may lead to scarring, ulceration, stromal neovascularisation, corneal thinning, secondary stromal infiltration and melting or even perforation of the cornea (Kolli *et al.*, 2009).

Loss of limbal architecture. In LSCD, there is loss of the palisades of Vogt and characteristic late irregular and radial staining of the limbal epithelium with fluorescein due to conjunctival invasion (Kolli *et al.*, 2009). The absence of Palisades of Vogt alone, however, does not indicate LSCD (Zheng and Xu, 2008).

Corneal neovascularisation. Conjunctival cells unlike corneal cells are not capable of secretion of anti-angiogenic factors that leads to the development of both superficial in milder and deep corneal vascularization in more severe cases of LSCD (Kolli *et al.*, 2009). Neovascularisation is often seen but may not be present in some cases (**Figure 1.9**).

Corneal scarring. In case the epithelial defects do not progress to thinning and perforation, remodelling of the affected epithelial tissue occurs and forms a scar tissue resulting in further loss of vision (Kolli *et al.*, 2009).

Keratinization. If there is also a presence of tear deficiency, keratinisation may occur.

Clinical manifestation of LSCD varies depending on severity and extent of involvement (Dua *et al.*, 2000). In partial LSCD only one segment of the limbus is involved and clinical presentation is milder compared to total LSCD characterised by a complete absence of LSC population accompanied by conjunctivalization of the entire corneal surface (Sejpal *et al.*, 2013). Recurrent epithelial defects cause severe pain, photophobia and together with conjunctivalization, vascularisation and scarring will result in functional blindness.

1.7.3 Diagnosis of LSCD

Accurate diagnosis of LSCD is crucially important because appropriate treatment can prevent progression of the condition and further damage to the ocular surface. Although the diagnosis of LSCD is mainly made on clinical grounds (Ahmad *et al.*, 2010), laboratory tests are necessary to confirm the diagnosis and monitor the disease progression. Inherent limitations are associated with the interpretation of clinical signs (Dua *et al.*, 2009). For example, the presence of a fibrovascular pannus may be caused by previous infectious keratitis rather than by LSCD (Le *et al.*, 2018). Beside patient's medical history and slit-lamp examination, laboratory tests, such as corneal impression cytology and *in vivo* confocal microscopy, are useful diagnostic methods used to confirm the diagnosis of LSCD and monitor success of surgical interventions. Sophisticated testing is not required for the patients with condition known to cause LSDC, such as chemical injuries and Stevens-Johnson syndrome, with typical clinical signs (loss of the Palisades of Vogt, conjunctivalization and persistent epithelial defects) (Utheim, 2013). In patients with the less clear symptoms and signs, however, additional diagnostic tools including corneal impression cytology and *in vivo* confocal microscopy are of a great importance, especially if LSC transplantation is being considered as a therapy.

1.7.3.1 Clinical examination

The slit-lamp examination have been the main basis for the diagnosis and classification of LSCD (Le *et al.*, 2018). The slit-lamp examination reveals a dull and irregular reflex from the conjunctivalized corneal surface (Dua *et al.*, 2000). So-called "waterfall" or "whorled" epithelium is often present along with the loss of the Palisades of Vogt (Liang *et al.*, 2009). Corneal haze, recurrent erosions or persistent epithelial defects and neovascularisation are also seen during the examination together with abnormal fluorescein staining. In advanced cases of LSCD, fibrovascular pannus and corneal scaring predominate (Dua *et al.*, 2000).

Clinical findings under slit-lamp biomicroscopy were summarized by Le and colleagues (Le *et al.*, 2018):

- ➢ **Mild stage LSCD**
    - Dull/irregular corneal surface with loss of light reflex
    - Corneal epithelial opacity
    - Fluorescein epithelial staining
    - Loss of palisades of Vogt
- ➢ **Moderate stage LSCD**
    - Vortex keratopathy
    - Superficial vascularisation and peripheral pannus
- ➢ **Severe stage LSCD**
    - Recurrent/persistent epithelial defects
    - Stromal neovascularisation
    - Stromal scarring and opacity.

1.7.3.2 Corneal impression cytology

Impression cytology is the gold standard diagnostic test for LSCD (Puangsricharern and Tseng, 1995). A nitrocellulose acetate filter paper (Egbert *et al.*, 1977; Singh *et al.*, 2005) or a polytetrafluoroethylene membrane (Thiel *et al.*, 1997) may be pressed onto the corneal surface under the topical anesthesia to remove superficial cells

which can be then subjected to histological, immunohistological or molecular analysis. Thereafter the epithelial morphology and the presence of markers of conjunctivalization are evaluated. The presence of goblet cells on the cornea proves conjunctival epithelial invasion, since these cells are not normally present within the corneal epithelium. Besides the goblet cells other commonly used markers specific to conjunctival cells are CK7, CK13, mucin-1 and less specific CK19 (Barbaro *et al.*, 2010; Jirsova *et al.*, 2011; Ramirez-Miranda *et al.*, 2011). However, in some cases, such as those arising from severe chemical injury or Stevens-Johnson syndrome, goblet cells are often absent leading to false negative results (Liang *et al.*, 2009). Sangwan *et al.* showed that even one third of patients with clinical diagnosis of LSCD demonstrated the absence of goblet cells (Sangwan *et al.*, 2005). These patients received transplantation of *ex vivo* cultured LSCs which resulted in restoration of the ocular surface (Utheim, 2013).

1.7.3.3 In vivo confocal microscopy

Using *in vivo* confocal microscopy for examination in patients with LSCD the loss of normal limbal architecture, cystic changes in the epithelium and sub-epithelial fibrosis can be seen (Nubile *et al.*, 2013). The main advantages of *in vivo* confocal microscopy compared to impression cytology are the possibility of non-invasive examination that does not require removal of corneal epithelial cells and obtaining information about both the superficial and deeper zones of the limbal area (Deng *et al.*, 2012; Nubile *et al.*, 2013). Using this technique normal corneal epithelial cells with bright well-defined membranes can be observed in contrast to hyper-reflective conjunctival epithelial cells with ill-defined membranes (Dua *et al.*, 2009). Although the quality of images generated is outstanding, there are some disadvantages of using *in vivo* confocal microscopy such as a need of direct physical contact between the imaging probe and the epithelium examined and a small field of view (Utheim, 2013). Spectral domain optical coherence tomography represents an alternative technique allowing larger field of view in non-invasive manner but with significantly lower resolution (Bizheva *et al.*, 2011).

1.7.4 Management of LSCD

Successful reconstruction of the ocular surface in LSCD requires not only restoration of LSC number (by LSCs transplantation) but also restoration of the normal LSC niche environment (normal lid function and tear function, possible use of amniotic membrane and autologous serum etc.) (Kolli *et al.*, 2009). Optimization of the ocular surface health is the very first step in the management of both partial and total LSCD. In partial LSCD improvement of the ocular surface health provides a better environment for the remaining LSCs to survive whilst in total deficiency cases these measures provide the best chance for the transplanted tissue to survive.

LSCD treatment is usually complex and varies a lot among patients. The choice of treatment depends on severity (mild, moderate, severe LSCD) and extent of the disease (partial or total LSCD). For milder and moderate cases, treatment involve the control of the symptoms and causes. For patients with severe LSCD, the ocular surface reconstruction is required. Moreover, although for milder cases conservative management can be sufficient, if the pupillary area of the eye is covered by encroaching conjunctival tissue, surgical intervention is also required (Utheim, 2013). Various surgical procedures have been developed over the past 30 years to treat and reconstruct severely damaged or diseased ocular surface epithelia including amniotic membrane transplantation, conjunctival limbal grafting, simple limbal epithelial transplantation and cultivated limbal and oral mucosal epithelial transplantation (Yin and Jurkunas, 2018).

There are at least five main principles in the management of LSCD, as summarized by Fernandez-Buenaga and colleagues (Fernandez-Buenaga *et al.*, 2018):

1. Understanding and control of inflammation;
2. Correcting lid malposition and fornix adhesions;
3. Managing dry eye disease;
4. Partial versus total LSCD – since one of the main goals of ocular surface reconstruction is vision improvement, involvement of the central cornea is an important indication for surgical reconstruction;

5. Unilateral versus bilateral LSCD – knowing of LSCD aetiology is important in determining the surgical options, mainly in terms whether autograft or allograft procedures should be employed.

1.7.4.1 Partial LSCD treatment

Partial LSCD involves only a few sectors of the cornea and can be treated conservatively with frequent ocular lubrication and topical steroid therapy. Repeated mechanical scraping of the conjuntivalized epithelium, known as the sequential sector conjunctival epitheliectomy, may be performed to allow regrowth of normal corneal epithelium in its place providing there is some remaining healthy corneal tissue with functional stem cells left (Dua, 1998). Amniotic membrane transplantation and ipsilateral limbal translocation are also suggested as an early therapeutic option (Anderson *et al.*, 2001; Nishiwaki-Dantas *et al.*, 2001). Amniotic membrane transplantation helps to recreate the integrity of the ocular surface by promoting epithelialization, reducing angiogenesis and inflammation (Koizumi *et al.*, 2001) and could be used with a high success instead of limbal transplantation in the management of partial LSCD.

1.7.4.2 Complete LSCD treatment

In case of total LSCD, whether unilateral or bilateral, the treatment of choice is surgical. Conventional corneal transplantation replaces only the central cornea and cannot successfully treat the ocular surface with extensive and complete LSCD. Therefore, therapeutic strategies evolved to replace limbal epithelium with or without corneal transplantation surgery.

Jose Barraquer was the first to report managing "superficial burns" of one eye using "epithelial conjunctivocorneal limbus taken from the other eye" in 1964 (Holland,

2015). Thoft later reported "initial transplantation of conjunctiva, then lenticules of peripheral cornea, from cadaveric eyes" (Thoft, 1977).

In unilateral cases of LSCD, donor tissue is obtained from the healthy fellow eye, called **limbal autograft**; in bilateral cases of LSCD, donor tissue is obtained from an allogeneic source (cadaveric, living related and unrelated donors), called **limbal allograft**. Both procedures have the same purpose - a transplantation of a new source of epithelium after the removal of the patient's scarred and diseased epithelium (Thoft, 1977; Thoft, 1984). Allogeneic transplantation requires prolonged systemic immunosupression since the procedure carries the risk of rejection. It was also reported that long time survival of allografts is worse than autologous grafts (Miri *et al.*, 2010).

The corneal transplantation (penetrating keratoplasty) cannot restore sight to an eye blinded by LSCD before functional LSCs are restored (Dua *et al.*, 2000). Corneal transplantation can be performed either simultaneously with limbal grafting or after the limbal grafting procedure has proven to be successful, which is more common. Restoration of the tear film may be achieved with frequent lubrication and use of autologous serum drops (Liang *et al.*, 2009). In case the tear film is not efficiently restored, the resulting dryness contributes to the failure of traditional corneal transplantation. In that case keratoprosthesis is the only available terapeutic option available (Cauchi *et al.*, 2008). Keratoprosthesis can be used as an alternative to allogeneic transplantation to avoid immunosupression. Athough patient with keratoprosthesis show improved vision postoperatively they may develop glaucoma and retroprosthetic membranes (Kamyar *et al.*, 2012).

1.7.5 Surgical treatment of LSCD

1.7.5.1 Conjuntival limbal autograft (CLAU)

The pioneer work by Kenyon and Tseng in 1989 identified that the transplanted limbal tissue can rehabilitate the corneal surface. They showed that in cases of unilateral total LSCD a contralateral conjunctival limbal autograft harvested from the healthy fellow eye may be used to help recover dieased eye's corneal surface (Kenyon and Tseng, 1989), but the procedure is linked with the risk of iatrogenic

LSCD development in the donor eye as this technique requires the removal of relatively large free conjuntival lenticule (Kenyon, 1989; Kenyon and Tseng, 1989).

Iatrogenic LSCD in the healthy donor eye has been reported clinically (Jenkins *et al.*, 1993) and validated in experimental animal studies (Chen and Tseng, 1990; Chen and Tseng, 1991). The risk of iatrogenic LSCD is lower when fewer than four to six clock-hours of limbal tissue is transplanted (Dua *et al.*, 2000), but the success rate decreased in these smaller grafts and complications were seen (Liang *et al.*, 2009; Baradaran-Rafii *et al.*, 2012).

1.7.5.2 Allograft limbal transplant

In total bilateral LSCD, allograft limbal transplantation is one of the approaches to reconstruct the ocular surface (Tsai and Tseng, 1994). Allogeneic limbal transplantation can be carried out as as living-related conjunctival allograft (LR-CLAL), cadaveric keratolimbal allograft (KLAL), or the combination of LR-CLAL and KLAL (also referred to as the "Cincinnati Procedure") (Holland, 2015; Haagdorens *et al.*, 2016). Allograft limbal stem cell transplant can provide immediate postoperative epithelialization and rapid reconstruction of the ocular surface (Dong *et al.*, 2018). However, in order to avoid rejection of the allograft, systemic immunosuppression is necessary. Adverse effects related to long-term immunosuppression including anemia, hyperglycemia, elevated creatinine, and elevated levels of liver function markers are commonly seen (Holland *et al.*, 2012; Krakauer *et al.*, 2012).

Interestingly, a long-term study showed that eventually, only recipient DNAs were detectable in the regenerated epithelium of the majority of the successful cases. This finding suggests that the allografted limbal epithelium promotes regeneration of the corneal epithelium in patients with LSCD, at least in part, by activating residual stem cells and enhancing their self-renewal (Daya *et al.*, 2005). One of the possible explanations is that allografted LSCs secrete factors necessary for maintaining stem cell homeostasis. It is important to further elucidate what exactly these factors are and whether their direct application onto the ocular surface could possibly restore the corneal epithelium (Dong *et al.*, 2018).

1.7.5.3 Simple limbal epithelial transplantation (SLET)

In 2012, Sangwan and colleagues introduced a novel technique called "simple limbal epithelial transplantation", that incudes direct transplantation of the 2 x 2 mm piece of healthy limbal tissue cut into pieces and secured on amniotic membrane using fibrin glue without *ex vivo* cultivation (Sangwan *et al.*, 2012). Promising results are reported after a multicenter study on 68 eyes from patients who underwent SLET for unilateral LSCD (Basu *et al.*, 2012). Basu *et al.* reported that clinical success was achieved in 57 (84%) cases and the survival probability exceeded 80%, with a median follow-up of 12 months (Basu *et al.*, 2012). In 2016, the same autors reported long-term clinical outcomes of a large cohort of patients (125 cases) with unilateral LSCD occurring after ocular burns with median postoperative follow-up of 1.5 years. They showed that 76% patients maintained a successful outcome and in addition to the ocular surface restoration, most patients showed a significant improvement in visual acuity (Basu *et al.*, 2016).

Gupta and coworkers reported that SLET showed a similar success rate to the traditional autologous limbal transplantation with an important advantage - autologous SLET requires only a tiny amount of limbal tissue from the unaffected eye therefore carrying a minimal risk to the donor eye (Gupta *et al.*, 2018).

1.7.6 LSCD cell-based therapies

Cell therapy of LSCD involves tissue engineering techniques focused primarily on *ex vivo* expansion of the least amount of limbal tissue on different carrier scaffolds (substrates) and production of limbal epithelial sheets which will further be used to reconstruct severely damaged ocular surfaces by transplantation. Beside limbal epithelial cells, several studies focused on use of oral mucosal epithelial cells and mesenchymal stem cells (MSCs) in LSCD treatment.

1.7.6.1 Cultivated limbal epithelial transplantation (CLET)

A new chapter in the treatment of LSCD started with the laboratory expansion of limbal epithelial cells (LECs) when Pellegrini *et al.* first reported cultivating a small

limbal biopsy of the healthy eye of patients with severe alkali burn and successful restauration of the ocular surface by transplantation of the cultured cell sheet on the other eye (Pellegrini *et al.*, 1997). Many studies published later confirmed that a small piece of limbal tissue, usually 2 x 2 mm, may be taken from the healty eye and transplanted following *ex vivo* cultivation of LECs, achieving excellent long term clinical outcome (Pellegrini *et al.*, 1997; Kolli *et al.*, 2010; Rama *et al.*, 2010; Baylis *et al.*, 2011). The limbal tissue can be harvested from the healthy fellow eye in unilateral LSCD, or living related donors or cadaveric eyes in bilateral cases. Cells can be cultured in either an explant or cell suspension system using various culture media and substrates (Shortt *et al.*, 2007b; Ahmad *et al.*, 2010; Shortt *et al.*, 2010; Baylis *et al.*, 2011). In the suspension technique, cells are separated by enzyme digestion from the limbal niche for culture while in the explant culture LSCs along with the entire limbal niche are placed in a culture (Ramachandran *et al.*, 2014). In suspension culture, because cells are removed from their natural niche, additional support in the form of feeder cells is required to maintain stemness of LSC population (Ramachandran *et al.*, 2014).

The possibility to get enough cells for transplantation from a very small amount of starting material has made LSCD treatment simpler and safer. Also, it became possible to repeat CLET in cases in which the first CLET failed, without adversely affecting the health of the donor eye (Basu *et al.*, 2012). It has been reported that by repeating CLET 3-12 months after the first CLET, a stable ocular surface could be generated in more than 66% of the failed cases (Basu *et al.*, 2012).

Rama *et al.* reported "permanent restoration of a transparent, renewing corneal epithelium" in 76.6% of eyes after CLET using autologous LSCs cultivated on fibrin to treat 112 patients with corneal damage, most with burn-dependent LSCD (Rama *et al.*, 2010). Restored eyes remained stable over time, with up to 10 years of follow-up. Importantly, they also showed that successful outcome was associated with the percentage of p63-bright, holoclone-forming stem cells in culture. Namely, cultures with more than 3% of p63-bright cells were associated with successful transplantation in 78% of patients while, in contrast, cultures in which such cells made up 3% or less of the total number of cells were associated with successful transplantation in only 11% of patients (Rama *et al.*, 2010). Moreover, they showed that graft failure was also associated with the type of initial ocular damage and postoperative complications (Rama *et al.*, 2010). The next long-term multicentre

prospective study on 152 patients treated with autologous LSCs cultured on fibrin from the same research group, scored outcomes as full success, partial success and failure in 66.05%, 19.14% and 14.81% of eyes, respectively (Pellegrini *et al.*, 2013).

Although Pellegrini *et al.* initially recommended use of clinical-grade 3T3-J2 feeder cells as a substrate for LSCs cultivation, many research groups later tended to avoid the use of animal feeder cells in the process of cell product preparation for human administration. Kolli *et al.* for example, recommended use of HAM as a more suitable substrate for *ex vivo* LSCs expansion for clinical application (Kolli *et al.*, 2010). Having in mind that HAM mimics the natural stem cell niche and has the potential to enhance the self-renewal of LSCs (Nakamura *et al.*, 2006), it represents an ideal substrate for various transplantation procedures on the ocular surface (Sangwan *et al.*, 2006). In their study, Kolli *et al.* reported treating of eight eyes of eight consecutive patients with unilateral total LSCD with *ex vivo* expanded autologous LSC transplant on HAM. After transplantation, successful ocular surface reconstruction with a stable corneal epithelium was obtained in all eyes (100%) with a mean follow-up of 19 months (Kolli *et al.*, 2010).

Over the past 22 years since the first CLET was performed, various modifications of the culture and transplantation techniques emerged. Consequently, due to variation within the studies and between the studies, it is often difficult to make an objective assessment. Outcomes of limbal epithelial therapy were reviewed by different research groups (Shortt *et al.*, 2007b; Baylis *et al.*, 2011; Holland, 2015; Fernandez-Buenaga *et al.*, 2018). Pooling the data of 13 studies with long-term outcomes, Holland analysed the long-term overall success rate (Holland, 2015). Overall, 720 eyes were treated with either autologous (89%) or allogenic CLET (11%) and the single most common indication for the surgery was chemical/thermal injury (89%) (Holland, 2015). Using ocular surface reconstruction success and improvement in visual acuity as the main outcome measures, he reported the overall success rate of these 13 studies of 72% (Holland, 2015).

1.7.6.2 Cultivated oral mucosal epithelial transplantation (COMET)

As severe LSCD is mostly bilateral and allogeneic limbal transplant or CLET require intesive and prolonged postoperative immunosupressive therapy, scientists

worldwide become interested in an extraocular source of stem cells to avoid dependence on allogeneic LSCs. So far, oral mucosal epithelium and MSCs were used in regeneration of the corneal surface. It has been shown that COMET is a feasible substitute for allogenic CLET without the need for long-term systemic immunosuppression (Nakamura *et al.*, 2004; Nakamura and Kinoshita, 2011; Nakamura *et al.*, 2011; Eslani *et al.*, 2012; Sotozono *et al.*, 2013; Kolli *et al.*, 2014; Sotozono *et al.*, 2014; Prabhasawat *et al.*, 2016).

Gaddipati *et al.* reported that cultivated oral epithelial cells formed a stratified tissue and expressed proliferation and progenitor markers Ki67 and p63 in the basal layer of the cell sheets, suggesting that the epithelium had regenerative capacity (Gaddipati *et al.*, 2014). Furthermore, the transplanted epithelium also expressed CK3, CK19, p75, and the cornea-specific PAX6 and CK12 (Gaddipati *et al.*, 2014). This study confirms that the oral cells acquire some of the corneal epithelial-like characters at the ectopic site. However, although transplantation of *ex vivo* cultivated autologous oral mucosal epithelial cells in patients with bilateral total LSCD achieved success in stabilization of the ocular surface, the visual improvement was not as optimal (Nakamura and Kinoshita, 2003; Nakamura *et al.*, 2004; Kolli *et al.*, 2014). Moreover, compared with cultured LECs, oral epithelial cells have significantly higher angiogenic potential (Dong *et al.*, 2018; Duan *et al.*, 2019). Recently, Duan and colleagues suggested that this issue may be solved by using allogenic limbal niche cells instead of mouse-derived 3T3 cells as a feeder layer, as they reported that the cultivated oral mucosal epithelial cells obtained in this system are less likely to induce postsurgical neovascularization (Duan *et al.*, 2019).

Kolli *et al.* reported transplantation of autologous *ex vivo* expanded oral mucosa epithelium in two patients with bilateral total LSCD that resulted in successful reversal of LSCD in the treated eye up to 24 months (Kolli *et al.*, 2014). In another study with mean follow up of 25.5 months in 40 eyes, corneal surface stability underwent an early decline in transplanted COMET over the first 6 months, remaining stable thereafter (1 year: 64.8%, 2 years: 59%, 3 years: 53.1%) (Satake *et al.*, 2011). Fernandez-Buenaga *et al.* recently reported that COMET success rate vary between 50 and 70% at 3-4 years follow up (Fernandez-Buenaga *et al.*, 2018).

1.7.6.3 Mesenchymal stem cell transplantation (MSCT) in LSCD treatment

MSCs are relatively easy to obtain from a variety of tissues, including bone marrow, adipose tissue, umbilical cord, etc. Importantly, MSCs possess immunomodulatory and non-immunogenic properties (Aggarwal and Pittenger, 2005), thereby removing the need for immunosuppression. There is plenty of evidence from both *in vitro* and *in vivo* studies regarding the potential use of bone marrow derived MSCs for ocular surface regeneration. All studies reported promising results in animal models and demonstrated significant corneal regeneration, improved corneal transparency, and rapid healing associated with the restoration of vision (Holan and Javorkova, 2013; Yao and Bai, 2013; Harkin *et al.*, 2015). Another promising, easily accessible and abundant source of MSCs is adipose tissue (Strioga *et al.*, 2012). Adipose tissue derived MSCs also show the beneficial role in corneal epithelial regeneration. Under basic culture conditions they express corneal epithelial markers, suggesting that these cells may have some inherent properties to regenerate the corneal epithelium (Nieto-Miguel *et al.*, 2013). Moreover, several studies reported that in experimental models of LSCD, administration of adipose tissue derived MSCs either subconjunctivally (Lin *et al.*, 2013a), topically (Lin *et al.*, 2013b; Zeppieri *et al.*, 2013; Almaliotis *et al.*, 2015), or overlaid on scleral contact lenses or nanofiber scaffolds (Espandar *et al.*, 2014; Holan *et al.*, 2015) promoted regeneration of the corneal epithelium. A case report in which adipose tissue derived MSCs were applied topically to a patient with persistent sterile epithelial defects further supports the potential benefit of MSCs from this source (Agorogiannis *et al.*, 2012). Furthermore, Galindo *et al.* showed that adipose tissue derived MSCs improved corneal and limbal epithelial phenotypes in animal LSCD models (Galindo *et al.*, 2017). These cells transplanted in total LSCD models developed in rabbits were well tolerated, reduced inflammation, and restrained the evolution of corneal neovascularization and corneal opacity. The expression profile of the corneal epithelial cell markers CK3 and E-cadherin, and the limbal epithelial cell markers CK15 and p63 was lost in the LSCD models, but was partially recovered after human adipose tissue derived MSCT (Galindo *et al.*, 2017).

On the contrary, a study published by Holan and colleagues suggests superiority of bone marrow derived MSCs in comparison with adipose tissue derived MSCs (Holan *et al.*, 2015). Clinical characterization of the healing process, evaluation of corneal thickness, re-epithelialization, neovascularization, and the suppression of a local

inflammatory reaction, were comparable in the bone marrow derived MSC and LSC-treated eyes, but results were significantly better than in injured, untreated eyes or in eyes treated with adipose tissue derived MSCs (Holan *et al.*, 2015). Due to these contradictory results it is still not clear whether or not one of these two MSC sources represent superior option for LSCD treatment.

MSCs could also be used as a support to LSC transplantation. It's been shown that co-transfer of murine LSCs with MSCs, which have immunosuppressive properties, significantly inhibited local inflammatory reactions and supported the healing process (Zajicova *et al.*, 2010).

Mittal *et al.* showed, using an *in vivo* mouse model of ocular injury, that MSCs possess the capacity to restore corneal transparency by secreting high levels of hepatocyte growth factor (HGF) and moreover that HGF alone can restore corneal transparency (Mittal *et al.*, 2016).

Recently, the first reported proof-of-concept clinical trial provided an evidence that allogeneic bone marrow-derived MSCT to the ocular surface is as safe and as effective as allogeneic CLET at facilitating recovery of LSCD (Calonge *et al.*, 2019). They conducted a 6-12 month randomized, double-masked pilot trial to test whether allogeneic bone marrow-derived MSCT (performed in 17 patients with LSCD) was as safe and as equally efficient as allogeneic CLET (performed in 11 patients with LSCD), to improve corneal epithelial damage due to LSCD. Global success after 6-12 months was 72.7% - 77.8% for CLET cases and 76.5% - 85.7% for MSCT cases, without any significant differences found. Since there were no adverse events related to cell products reported, their study showed that MSCs used in MSCT can safely and effectively help treating corneal pathology due to LSCD (Calonge *et al.*, 2019).

Chapter 2. Cultivation of LSCs under good manufacturing practice (GMP) conditions

2.1 Introduction

2.1.1 Regenerative medicine and cell therapy

Regenerative medicine (RM) is an innovative, fast-moving field with the ultimate aim to return patient to full health. RM has been defined as the "process of replacing, engineering or regenerating human cells, tissue and organs to restore or establish normal function" (Mason and Dunnill, 2008). Stem cell research plays a central role in RM through translational research as stem cells can be used to repair or replace damaged or ageing cells in the human body by either promoting endogenous regenerative processes or directly replacing damaged tissues after cellular transplantation (Eming *et al.*, 2014).

There are a number of major medical conditions, such as heart failure, insulin-dependent diabetes, spinal cord injury, Parkinson's disease etc. which could be addressed via cell-based therapies. Stem cell derived β islet cells, for example, could potentially replace a patient's requirement for insulin injections (Phillips *et al.*, 2007).

Cell manufacturing for clinical applications is a unique form of biological manufacturing that depends on maintenance of strict work practices invented to ensure product consistency and prevent contamination by either microorganisms or by another patient's cells. For considerable period of time hematopoietic stem cell transplantation has been the principal cell therapy but since the adult stem cells were discovered cellular therapies evolved beyond. Limbal epithelial transplantation is a prime example of the cell-based therapy that has been used successfully in patients suffering from LSCD.

The cell therapy market is one of the most emerging sectors of the global biopharmaceutical market with approximately $100 billion in revenues and very

promising compound annual growth rate predicted to reach at least 20% in the near future (Abbasalizadeh and Baharvand, 2013).

In 2004, medicinal products for human use which are either based on gene therapy, somatic cell therapy (including stem cell therapy), tissue engineering or a combination of those are classified as "Advance Therapy Medicinal Products" (ATMPs) by European Commission (EC) (Hartmann-Fritsch *et al.*, 2016). Recently, ATMPs have been regulated by pharmaceutical rules applying to this new area of medicine. Regarding their manufacturing and testing, the ATMP regulation EC 1394/2007 defines the standard in Europe (Off J Eur Union, 2007). The regulation sets out tailor-made technical requirements and establishes new standards for clinical trials in the development of ATMPs. In enforcement of the European regulation 1394/2007, all ATMPs must be produced under conditions following the guidelines of Good Manufacturing Practice (GMP). The principles of GMP are described in the Commission Directive 2003/94/EC (Off J Eur Union, 2003). This directive is concerned with medicinal products of human use only.

GMP is a quality assurance system which ensures the highest quality and safety of medicinal product. It ensures that products are manufactured consistently and to defined standards as well as that the end product meets preset specifications. It requires traceability of raw materials and production that follows validated standard operating procedures (SOPs) (Arjmand *et al.*, 2012). The GMP regulations address also record keeping, personnel qualification, sanitation, cleanliness, equipment verification, the validation of the process and complaint handling (Skottman *et al.*, 2006).

The translation of a successful research process into a process following GMP guidelines is a challenging procedure. The common misconception is that clinical-grade cell lines may be produced only by transfer of current methodology into clean room facilities. These GMP facilities are indeed important in avoiding contamination of the product but equally important in implementing GMP standards is the development of validated SOPs for the entire process, from cell isolation to freezing and cell storage. Another important aspect is transfer of cell production to GMP grade standards is establishment of quality control methodology and release criteria of the final product (Unger *et al.*, 2008).

2.1.2 Hurdles to produce ATMPs under GMP conditions

The translation of a research process into a GMP process is a long and challenging procedure that requires many adaptations to be made at different levels (Hartmann-Fritsch *et al.*, 2016):

1. Risk management

A complex process such as ATMPs production should be divided into small achievable production steps, for example: starting materials, consumables, handling techniques, equipment used etc. Careful risk management ensures quality of the manufacturing process and ultimately safety of the patient (European Medicines Agency, 2015). General risk assessment includes assessment, control, communication and review of risks. Risk can affect any area of the process from:

- ✓ materials - not all material required is available in GMP grade;
- ✓ involved personnel - working with living tissues includes risk of transmitting pathogens to staff or cross-contamination with other batches/products;
- ✓ patient – if the batch manufacturing has to be stopped due to contamination etc.

2. Documentation

Documentation is of crucial importance to GMP (EudraLex, 2010). It ensures the traceability in case of batch-specific problems, reduces the risk of mistakes by defining the complete process, assures reproducibility, and confirms responsibilities by signatures. A precise documentation system has to be established, describing the whole manufacturing process in details (EudraLex, 2012). Besides manufacturing steps of the ATMP itself, this includes also preparation protocols for culture media, solutions and matrixes. In addition to these, all used materials including starting materials and consumables need to be described in details.

3. Starting materials

Although over the past years GMP grade materials become more and more
available or may be produced by some companies on customer request,
majority of starting material is made for "research use only". The use of these
materials under GMP conditions may only be approved by authorities under
special circumstances if no other option is available and on a risk-based
assessment.
Human tissue sample is a starting material of special interest. Before tissue
removal minimally required laboratory analyses have to be performed (HIV1,
HIV2, HBV, HCV and syphilis) together with additional tests depending on the
type of tissue or donor.

4. Consumables

Single-use single-pack consumables are the first choice for manufacturing
according to the GMP guidelines. For reusable materials efficient cleaning has
to be performed and validated.

5. In process controls (IPCs)

IPCs have to be established for the complete manufacturing process. These
controls are necessary in order to ensure the safety of the product and the
quality of the product (EudraLex, 2014).
IPCs of the first category ensure safety of the product, verifying for instance
that a certain cell population used for the production of a tissue engineered
product is free of endotoxins and mycoplasma. Sterility is of vital importance
for the safety of the product and is broadly and carefully checked. Cell
populations need to be tested on their identity and purity to assure that the
appropriate cells are present in the final product in the defined number.
Whenever possible objective controls with measurable outcome should be
chosen, for example cell morphology identification using only microscopic
assessment should be replaced by immunofluorescence analysis, using
specific markers for the cell type under investigation.

6. Release controls

The use of reference standards and potency assays in the manufacture of ATMPs is recommended to ensure product safety and potency prior to transplantation. It's crucial to ensure that final products are consistently manufactured and meet all necessary criteria in terms of viability, function, purity and sterility (Neofytou *et al.*, 2015).

At the end of the process, the end product needs to be analyzed for safety and quality by a qualified person. The strategy regarding sterility of the final product requires particular attention. Widely used sterilization methods such as autoclaving, gamma ray and ethylene oxide sterilization are not applicable for living tissues. Since no final sterilization can be performed sterility of the product has to be ensured during manufacturing itself, all the way from starting materials to packing materials (EudraLex, 2008). Living ATMPs has relatively short shelf life and often clinical application of the batch takes place before final sterility results are available (sterility results available after approximately 14 days), which leads to so called conditional release of ATMP for transplantation. Other mandatory release controls regarding product safety involve analysis for mycoplasma and endotoxins. As a part of quality control, analysis regarding functionality and performance of the final product should also be included (the amount of living cells, the concentration of secreted factors etc.).

For example, control measures and well defined release criteria should be employed for LSC cultures before they are used for human transplantation as for other stem cell products, to determine the quality of the final cell product (Daniels *et al.*, 2006b). To release the epithelial cell graft for ocular surface reconstruction, critical quality parameters such as adherent and healthy epithelial cells with cobblestone morphology, integrity of the fibrin gel and no contamination must be met (Sheth-Shah *et al.*, 2016). Pellegrini *et al.* proposed stringent quality criteria that should be adopted for LSC cultures in order to ensure that they contain a sufficient number of stem cells essential for long term epithelial survival (Pellegrini *et al.*, 2011). According to the same research group, quality control should also include rigorous clonal analysis or the evaluation of cell doublings generated during serial cultivation of LSCs (Pellegrini *et al.*, 2011). Another important analysis that has to be done before the LSC product is released for human

transplantation is evaluation of levels of contaminating murine fibroblasts in LSC cultures. It was previously estimated that more than 5% contamination was unsuitable for clinical use (Di Iorio *et al.*, 2010). Ideally, the use of murine 3T3 fibroblasts should be eliminated in the future and substrates more appropriate for clinical use such as HAM should be employed. Furthermore, mandatory microbial analyses of LSC cultures have to be conducted before these cells are applied to treat patients with LSDC proving that cultures are sterile and safe for human administration.

Once all the required specifications are confirmed, certificates of analysis could be generated for all product lots to certify them for clinical use (Neofytou *et al.*, 2015).

7. Training of staff

The vital point for implementing a successful GMP practice and process is the staff (EudraLex, 2013). The level and quality of implementation of all documentation, protocols and controls by qualified staff define the quality of the end product.

8. Approval by the Regulatory Authority

The final step after successful establishment and validation of the entire GMP process is approval of the manufacturing process by the regulatory authority. The basis for approval is the investigational medicinal product dossier which structure is set out in "Guideline on the Requirements to the Chemical and Pharmaceutical Quality Documentation Concerning Investigational Medicinal Products in Clinical Trials" (European Medicines Agency, 2016).

The use of defined cultured systems should be implemented with manufacturing clinical grade cell products. Using chemically undefined media or material of animal origin, such as fetal bovine serum and mouse embryonic fibroblasts, should be avoided if possible. The cultures should be animal substance free in order to exclude the risk of infections and immunogenity (Skottman *et al.*, 2006; de Lazaro *et al.*, 2014). The use of xeno-free media for cell generation, maintenance and differentiation in culture is for these reasons one of the clinical grade requirements. The safety of the exposure to variety of growth factors and culture conditions also

has to be thoroughly investigated prior to any clinical application (de Lazaro *et al.*, 2014).

2.1.3 Limbal stem cell therapy

Therapeutic use of LSCs must be performed in compliance with GMP as a quality assurance system to ensure highest quality and safety of cell product for transplantation in accordance with the European Union, Regulation (EC) No 1394/2007 of the European Parliament and of the Council, on ATMPs (Off J Eur Union, 2007).

Replacement of a pathologically altered cornea with healthy corneal tissue from a suitable donor is among the most common and successful transplantation procedures in medicine. With a success rate of 90%, the outcome of cornea transplantation is very favorable. However, one global survey from 2016, that included 95% of the world's population, quantified the considerable shortage of corneal graft tissue, with only 1 cornea available for 70 needed (Gain *et al.*, 2016). In 2017, NHS Blood and Transplant reveals that its eye banks are 21% below the level needed to supply hospitals (NHS Blood and Transplant, 2017). Having in mind the shortage of corneal tissue worldwide, innovative, cell-therapeutic approaches may open new, promising treatment perspectives.

The patients with severe total LSCD have been successfully treated with autologous limbal tissue transplants taken from the healthy contralateral eye (unilateral cases) or allogeneic transplants obtained from living related or cadaveric donors (unilateral and bilateral cases) (Kenyon and Tseng, 1989), but this technique is connected with the risk of iatrogenic LSCD development in the living donor eye. Importantly, improvements in the *ex vivo* expansion of LSCs obtained from the culture of small limbal biopsies and the successful reversal of LSCD upon their transplantation has revolutionized the field and has reduced the risk to the donor eye, making this a widely used technique for treatment of LSCD in humans (Pellegrini *et al.*, 1997). In 2015, the European Commission granted marketing authorization to the first stem cell-based treatment in the European Union. The product named Holoclar® is an

ATMP for the treatment of moderate to severe LSCD due to physical and chemical burns in adults (Pellegrini *et al.*, 2018).

2.1.3.1 Sources of the LSCs for transplantation

In clinical trials both autologous and allogeneic sources have been used. Autologous cells are however preferred, as their transplantation does not cause any immunoreactivity and therefore does not require systemic immunosuppression. However, this option is not possible in cases of bilateral disease when tissue donation from deceased person or living related or unrelated donor is the only option available (Behaegel *et al.*, 2017).

2.1.4 Basic concepts of epithelial cell culture

Rheinwald and Green pioneering work made possible the cultivation of human keratinocytes using a feeder layer of lethally irradiated 3T3-J2 mouse embryonic fibroblasts (Rheinwald and Green, 1975). The same principle has been used to cultivate human limbal and epidermal keratinocytes for more than 3 decades (Gallico *et al.*, 1984; Pellegrini *et al.*, 1997; Pellegrini *et al.*, 1999b; Ronfard *et al.*, 2000; Pellegrini *et al.*, 2013). *Ex vivo* expansion of limbal biopsies on HAM as a substrate is also a well-established technique which has been used successfully to cure patients with total LSCD (Kolli *et al.*, 2010; Yu *et al.*, 2016).

Epithelial cells on a substrate grow optimally at 37C° in a humidified atmosphere with 95% air and 5% carbon dioxide (Rheinwald, 1980; Kolli *et al.*, 2010; Yu *et al.*, 2016).

Additionally, there are a certain number of nutritional requirements for successful epithelial cell culture:

1. Dulbecco's modification of Eagle's medium (DMEM) – The basic nutritional requirements of epithelial cells in culture are fulfilled by DMEM (Rheinwald, 1980; Yu *et al.*, 2016).

2. HAM's nutrient mixture F12 – Addition of HAM's nutrient mixture F12 to DMEM at the ratio of 3:1 has been shown to improve epithelial cell culture (Allen-Hoffmann and Rheinwald, 1984).

3. Fetal bovine serum (FBS) – Addition of FBS is an essential requirement for successful epithelial cell culture (Maciag *et al.*, 1981). It contains a mixture of growth factors, hormones and cytokines (some of them unknown) important for successful cell culture. All the attempts to eliminate it from epithelial cell culture, particularly limbal epithelial cultures, led to poor maintenance of stem cells in cultures (Kruse and Tseng, 1991).

4. Hydrocortisone – Besides the presence of hydrocortisone in culture medium improves the growth and morphology of epithelial cells in culture (Rheinwald and Green, 1975), it also prevents deterioration of the 3T3 fibroblast feeder layer (Pera and Gorman, 1984).

5. Insulin – Insulin stimulates glucose transport into cultured cells and it has an important role in glycogen synthesis within the cells (Hayashi *et al.*, 1978). As it's one of the most important hormonal constituents of FBS, its addition to the culture media has shown to reduce the FBS requirements (Hayashi *et al.*, 1978; Maciag *et al.*, 1981).

6. Triiodothyronine (T3) – Similarly to insulin, T3 is a hormone involved in cellular metabolic processes, and its addition to the culture medium also lowers the requirement for FBS (Hayashi *et al.*, 1978). The addition of both insulin and T3 together lowers the requirement of FBS in epithelial culture medium from 20% to 10%.

7. Adenine – It was shown that addition of adenine to the culture medium improves the ability of epithelial cells to form colonies (Allen-Hoffmann and Rheinwald, 1984).

8. Cholera toxin – The addition of cholera toxin to the culture medium stimulates DNA synthesis in cells by increasing levels of cyclic AMP (adenosine monophosphate) therefore promoting epithelial cell proliferation (Green,

1978). Importantly, its addition also opposes terminal differentiation of the epithelial cells (Sun and Green, 1976).

9. Epidermal growth factor (EGF) – The addition of EGF has been shown to antagonize the differentiation of epithelial cells in culture, promoting undifferentiated state. It also promotes migration of growing epithelial cells therefore preventing crowding at the center of the colonies (Rheinwald and Green, 1977).

2.1.4.1 Types of epithelial clones

The proliferative compartment of human squamous epithelia contains three different types of clonogenic keratinocytes with different capacities for multiplication, named holoclones, meroclones and paraclones (Barrandon and Green, 1985; Barrandon and Green, 1987; Rochat *et al.*, 1994; Pellegrini *et al.*, 1999a; Pellegrini *et al.*, 2014). The stem cell of all squamous epithelia is the holoclone forming cell (De Luca *et al.*, 2006). Meroclone and paraclone forming cells, on the other hand, have properties of progenitor cells also known as transient amplifying cells (Pellegrini *et al.*, 2014). Holoclone forming cells have the greatest growth potential giving rise to meroclones, a clones of mixed composition, which further give rise to paraclones (Barrandon and Green, 1987). These transitions from holoclone to meroclone to paraclone are unidirectional and result in progressively restricted growth potential.

In vitro holoclones give rise to large colonies with smooth circular perimeter (**Figure 2.1**). Due to their greatest growth potential they can be sub-cultured for the longest time compared to meroclones and paraclones. When sub-cultured, holoclones give rise to only 0-5% of terminal looking colonies, described below as paraclones.
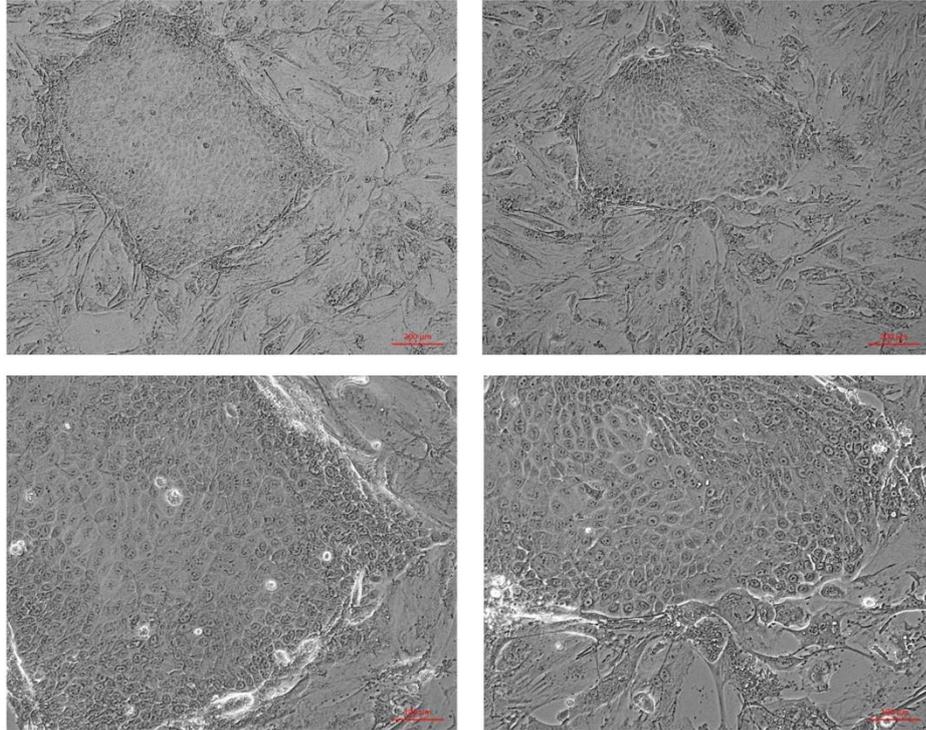
Figure 2.1 Phase contrast micrographs of typical holoclones showing closely packed, small cells that pile up around the edge of the colony.

Meroclones in culture give rise to intermediate sized colonies with slightly irregular or wrinkled perimeter (**Figure 2.2**). When sub-cultured, meroclones give rise to 5-95% of terminal differentiated colonies.
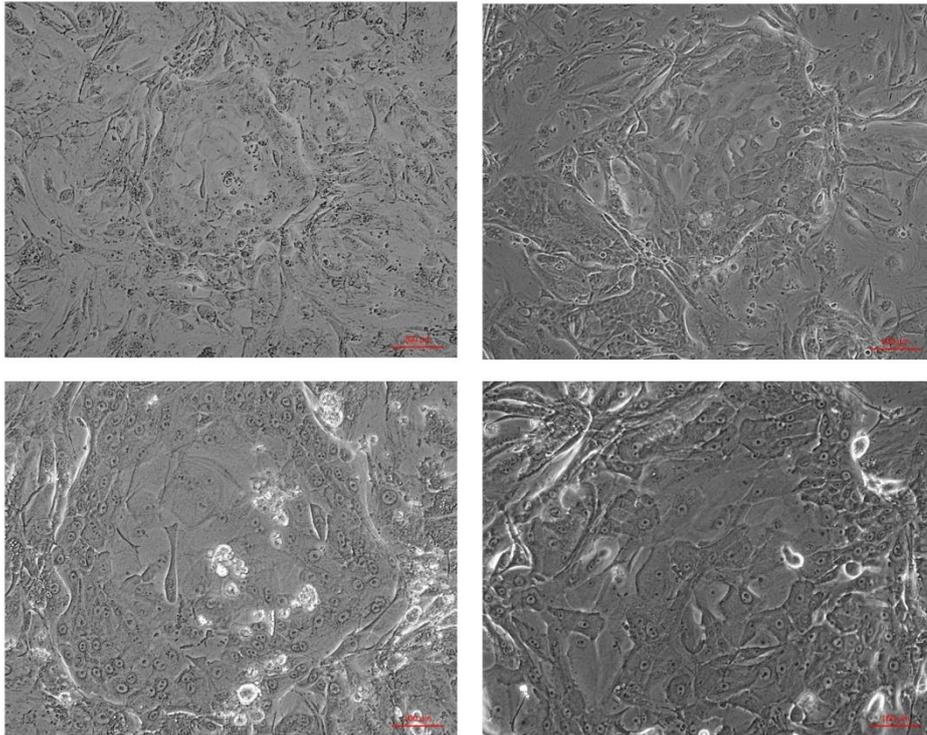
Figure 2.2 Phase contrast micrographs of typical meroclones with small cells around the edge of the colony and larger, flatter cells in the centre.

Paraclones give rise to small colonies with highly irregular perimeter referred as terminal colonies due to their terminal looking appearance (**Figure 2.3**). Terminal colonies generated by paraclones cannot be effectively sub-cultured since they form over 95% of terminal differentiated colonies.

The typical clonal hierarchy has also reported in limbal epithelium. Dziasko and co-workers showed that limbal epithelial cells isolated from the limbal crypts were able to generate the highest proportion of holoclones while this ability was dramatically decreased when cells were isolated from the non-crypt-rich limbus, confirming the limbal crypts as a stem cell reservoir (Dziasko *et al.*, 2014).
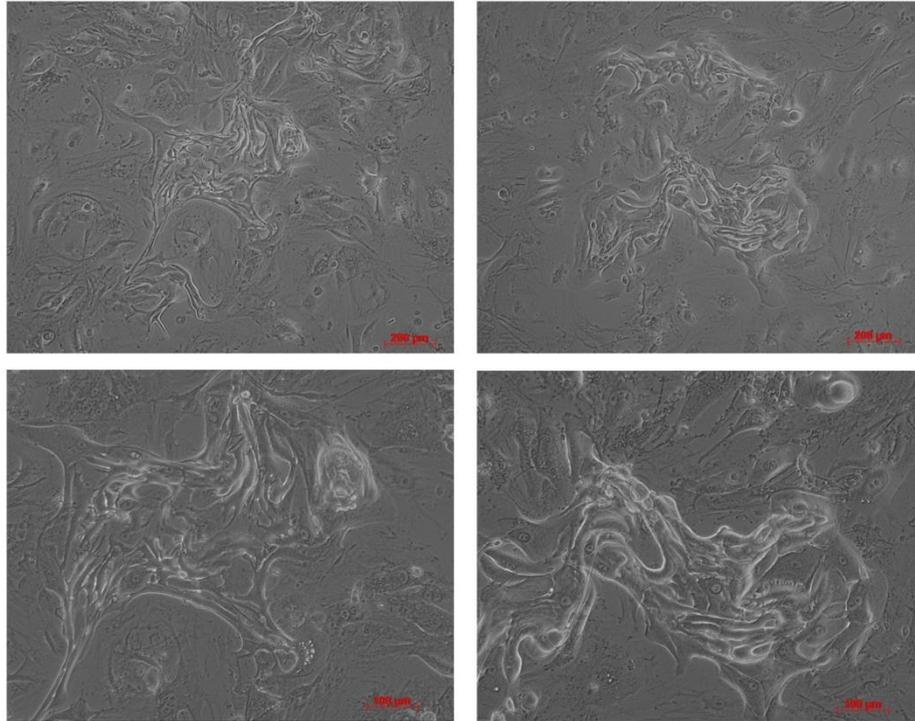
Figure 2.3 Phase contrast micrographs of typical paraclones, a small terminal-looking colony with irregular borders.

2.1.5 LSC culture techniques

LSCs culture can follow either the explant or cell suspension method (Utheim *et al.*, 2018c). In **the explant method**, cells grow out from a small biopsy attached to the base of a culture dish or a substrate (Tsai *et al.*, 2000; Kolli *et al.*, 2008; Kolli *et al.*, 2010). In **the cell suspension method** cells are first enzymatically released from the tissue and seeded as single cells (Ahmad *et al.*, 2007; Shortt *et al.*, 2008). Once attached to the bottom of a culture dish or substrate the single cells divide, grow and form colonies until they merge and form a confluent layer. Some culture methods include so called "air-lifting" to promote differentiation of the superficial layer (Schwab *et al.*, 2000; Nakamura *et al.*, 2006).

The use of irradiated or Mytomicin C growth arrested mouse embryonic fibroblasts was originally developed to enable culture of skin epidermal cells *in vivo*, now these

feeder cells are widely used for culture of all types of epithelial cells to supply cytokines and growth factors that promote their proliferation (Llames *et al.*, 2015). Puck and Marcus reported for the first time in 1955 the use of feeder cells in cell culture (Puck and Marcus, 1955). Feeder layer cells usually consist of adherent growth-arrested, but viable and bioactive, cells. Beside feeder cells, LSC growth can be supported by different native and synthetic scaffolds.

2.1.6 Scaffolds for LSC transplantation

In vitro, cells are maintained in culture much more readily if they are supported by substrate components most closely resembling the extracellular matrix (ECM) in which they occurred *in vivo* (Kruk and Auersperg, 1994; Baharvand *et al.*, 2005). The choice of culture substrate as the substitute for the *in vivo* niche is known to affect the phenotype of the cultured cells (Utheim *et al.*, 2018c). A wide range of biological and synthetic materials have been identified as substrates for cultivation of limbal epithelial cells so far, each of them with own advantages and limitations (Nguyen *et al.*, 2018). Among native scaffolds HAM is by far the best characterized. Seventy years after its first use in ophthalmology the HAM represents one of the major developments in ocular surface reconstruction (Eidet *et al.*, 2012). Tsai *et al.* were the first to introduce the use of HAM to culture limbal epithelial cells (Tsai *et al.*, 2000). HAM is especially suited for clinical use thank to its effects on promoting epithelialization, reducing pain and decreasing inflammation (Tseng, 2001). HAM harbors a variety of endogenous signaling molecules (growth factors and cytokines)(Hao *et al.*, 2000; Gicquel *et al.*, 2009), most of which are associated with various stem cell activities, rendering HAM an excellent surface for maintaining stemness before, during and after grafting (Riau *et al.*, 2010). Various culture techniques are used to culture LSCs on HAM which differ regarding the composition of HAM (with or without epithelium), air-lifting prior to transplantation and the use of an additional 3T3 feeder layer (Utheim *et al.*, 2018c). Many studies have shown that limbal epithelial cells cultured on an intact HAM maintain a more stem cell-like phenotype compared to limbal epithelial cells cultivated on denuded HAM (Grueterich *et al.*, 2002; Grueterich *et al.*, 2003b; Sudha *et al.*, 2008). The precise role of devitalized amniotic epithelium is not fully understood, however studies have shown that intact amniotic membrane (with the amniotic epithelium) exhibits higher levels of

growth factors compared with epithelially denuded HAM (Koizumi *et al.*, 2000). Previously it was shown that self-renewal of LSCs, their migration and differentiation can be mimicked by *ex vivo* culture system on HAM where the outgrowth zone nearer to explant contains the highest proportion of stem/progenitor cells and as they move away from the explant they differentiate to acquire features typical of corneal epithelial cells (Kolli *et al.*, 2008).

2.1.7 Cultivation of LSCs under GMP conditions

The therapeutic use of limbal epithelial cultures for the permanent regeneration of corneal epithelium in patients with LSCD has been reported in many studies. In accordance with GMP guidelines, strictly regulated procedures and stringent quality control tests are required to manipulate stem cells as "medicinal products" and make engraftment safer and eventually more successful. Accordingly, in order to be used for human transplantation, human limbal epithelial cells need to be propagated under GMP quality requirements in a validated GMP facility. However, the traditional culture media for *ex vivo* expansion of LSCs contains a number of ingredients derived from animal sources which may compromise its safety profile for human transplantation that ideally should be replaced with more safe alternatives (Yu *et al.*, 2016).

The *ex vivo* expansion of limbal epithelium prior to clinical transplantation is still a relatively new technique, and as such, optimization and constant evaluation of the culture medium components are required for minimizing any risk to patients (Yu *et al.*, 2016). The traditional culture media for the *ex vivo* expansion of limbal biopsies on HAM used by our and other groups includes hydrocortisone, triiodothyronine, adenine and cholera toxin (Pellegrini *et al.*, 1997; Tsai *et al.*, 2000; Meller *et al.*, 2002; Kolli *et al.*, 2008; Yu *et al.*, 2016). Hydrocortisone is shown to be important for the maintenance of distinct epithelial colonies as well as keratinocyte proliferation (Rheinwald and Green, 1975). Triiodothyronine is a thyroid hormone which was reported to reduce the requirement for fetal calf serum in epithelial cultures to minimal levels (Hayashi *et al.*, 1978). Cholera toxin (CT), a protein complex secreted by the bacterium *Vibrio cholera,* is responsible for the profuse, watery diarrhea characteristic of cholera infection. It has been reported that CT strongly stimulates colony growth from a small number of cultured human epidermal keratinocytes. The

effect of CT on proliferation of keratinocytes has been associated with increased intracellular cyclic AMP level (Okada *et al.*, 1982), whilst the addition of adenine to the culture media improves the colony forming of epithelial cells (Flaxman and Harper, 1975; Allen-Hoffmann and Rheinwald, 1984). Their individual contribution for the expansion and differentiation of LSCs in this culture system was examined in detail by Yu, Bojic and co-workers (Yu *et al.*, 2016).

2.1.8 Aim of the study

The aim of the study was a replacement of all research grade ingredients of traditional, research grade medium with GMP grade reagents wherever possible and consequent formulation of new GMP grade medium for cultivation and maintenance high percentages of LSCs *in vitro*. With this in mind Solu-Cortef® (hydrocortisone sodium succinate) was used as hydrocortisone replacement, Actrapid® (human insulin produced in Saccharomyces cerevisiae) as insulin replacement, Liothyronine (liothyronine sodium) as triiodothyronine replacement, Isoprenaline (L-isoproterenol) as cholera toxin replacement together with GMP grade EGF.

2.2 Material and Methods

2.2.1 Human donor tissue

Cadaveric adult human limbal tissue was obtained from the corneo-scleral rings remaining (3 males and 1 female, average age 62, SEM 2.72, range from 55 - 68 years), after removal of the central cornea for transplantation supplied by the NHS Blood and Transplant (NHSBT) Cornea Transplantation Service eye banks based in Manchester and Bristol, UK. Human amniotic membranes were provided as individual units of 3 x 3 cm^2 mounted on nitrocellulose paper by NHSBT, Tissue Services. Human tissue was handled according to the tenets of the Declaration of Helsinki and informed consent was obtained for research use of all human tissue

from the next of kin of all deceased donors. The study was approved by the NRES Committee North East - Newcastle & North Tyneside 1 (REC number: 11/NE/0236, protocol number 5466) on the 29th October 2013.

2.2.2 Cell culture

2.2.2.1 Traditional culture media and GMP grade media

"Traditional" complete epithelial medium was prepared according to a previously validated composition (Kolli *et al.*, 2010), containing low-glucose Dulbecco's modified Eagle's medium and HAM's nutrient F12 mixture in 3:1 ratio (both Gibco, UK). This composition was further supplemented with FBS, penicillin/streptomycin (both Gibco, UK), hydrocortisone, insulin, triiodothyronine, adenine, cholera toxin and EGF (all Sigma-Aldrich, UK) (**Table 2.1**).

Newly formulised, "GMP grade" complete medium contained DMEM and HAM's F12 medium in the same 3:1 ratio, supplemented with human serum instead of FBS (SigmaAldrich, UK), penicillin/streptomycin (Gibco, UK), Solu-Cortef® as hydrocortisone replacement (Pharmacia Limited, UK), Actrapid® as insulin replacement (Novo Nordisk, Denmark), Liothyronine as triiodothyronine replacement (Mercury Pharmaceuticals Ltd., UK), Isoprenaline (South Devon Healthcare, UK) as cholera toxin replacement, adenine (Sigma-Aldrich, UK) and GMP-grade EGF 10 ng/ml (Miltenyi Biotec, UK) (**Table 2.1**). Penicillin/streptomycin was removed from culture medium after the first 3 days of culture, as per our GMP protocol, in accordance with the Medicines and Healthcare products Regulatory Agency (MHRA). In addition to complete GMP grade media, four different complete media were prepared with replacement of just one ingredient in isolation (either Solu-Cortef®, Actrapid®, Liothyronine or Isoprenaline) to assess the impacts of individual replacements of GMP grade ingredients.

The medium was changed on the third day and then every other day thereafter. All cultures were placed in a tissue culture incubator at 37°C with a humidified atmosphere containing 5% CO_2.

Table 2.1 Composition of traditional and GMP grade medium.

Traditional medium		GMP grade medium	
REAGENT	**COMPOSITION**	**REAGENT**	**COMPOSITION**
DMEM : F12	3:1	DMEM : F12	3:1
FBS	10%	Human serum	10%
Pen/Strep	1%	Pen/Strep*	1%
Hydrocortisone	0.4 µg/ml	Solu-Cortef®	0.4 µg/ml
Insulin	5 µg/ml	Actrapid®	5 µg/ml
Triiodothyronine	1.4 ng/ml	Liothyronine	1.4 ng/ml
Cholera toxin	8.4 ng/ml	Isoprenaline	2 mg/ml
Adenine	24 mg/ml	Adenine	24 mg/ml
EGF	10 ng/ml	GMP-grade EGF	10 ng/ ml

*removed from culture medium after the first three days of culture

2.2.2.2 LSCs culture under GMP conditions

Limbal explant culture was performed as previously described (Kolli *et al.*, 2010). Briefly, the HAM was defrosted at room temperature in a class II laminar flow hood and washed twice with phosphate-buffered saline (PBS) (Gibco, UK) containing 1% penicillin/streptomycin, and once more with culture medium. After washing, HAM was trimmed and wrapped around a sterile 24 x 24 mm^2 glass coverslip with the epithelial side facing up and the overhanging part being folded over the edges of the coverslip. The HAM and its associated coverslip were placed on the top of a second sterile

glass coverslip to lock the HAM in place. Finally the whole construction was placed in a 9.6 cm^2 tissue culture well (well of a six well plate).

Two limbal explants were prepared from each of four corneo-scleral rings, which had been stored in organ culture (supplied for corneal transplantation). The limbal explants were expanded in the GMP facility either in previously described traditional, non GMP grade complete medium described above (n=4) or newly formulised GMP grade complete medium (n=4) using HAM as a substrate. The stromal tissue and deeper layers of the corneo-scleral rings were dissected away together with excess sclera leaving a ring containing approximately 2 mm of peripheral cornea and 2 mm of adjacent sclera, thereby including all the corneo-scleral limbus. Each ring was then divided into separate 4 mm^2 segments and one of each such segment was carefully placed at the centre of the prepared HAM with a slight pressure for a few second to facilitate adhesion. Complete epithelial medium were slowly added to ensure the explants were covered in medium without causing them to detach from the HAM (1.5ml of medium).

The medium was changed on the third day and then every other day thereafter. All cultures were placed in a tissue culture incubator at 37°C with a humidified atmosphere containing 5% CO_2.

2.2.2.3 Measuring of explant outgrowth

The expansion of each epithelial outgrowth was marked at every medium change for the full length of the culture to allow comparison of growth rates (**Figure 2.4**).
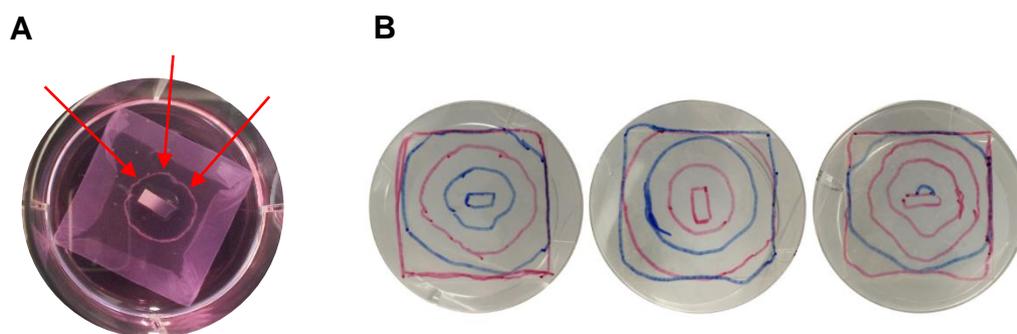
Figure 2.4 Examples of explant growth rate monitoring. (A) Macroscopic picture of limbal biopsy explant culture on HAM showing present outgrowth as the white ring pointed with red arrows (B) Every single line represents external border of a tissue outgrowth at the corresponding day of medium change.

2.2.2.4 Division of outgrowths into three zones

The cultures were terminated prior to the growth reaching the edge of the slides (at ~14 days), upon reaching 90% confluence. The epithelial outgrowth was divided into three equal zones depending on proximity to the explant (4.0 mm from the edge of the biopsy or the adjacent zone) as described by Kolli and co-workers (Kolli *et al.*, 2008): zone A indicating growth adjacent to the explant, zone B indicating intermediate outgrowth and zone C indicating the growth furthest away from the explant (**Figure 2.5**). At the end of the culture period, the epithelial cells from these zones were released and detached from the underlying HAM by 0.05% trypsin treatment for 10 min at 37°C. The cells from these three samples were further analysed for any differences in stem cell properties including colony-forming efficiencies and immunocytochemistry.
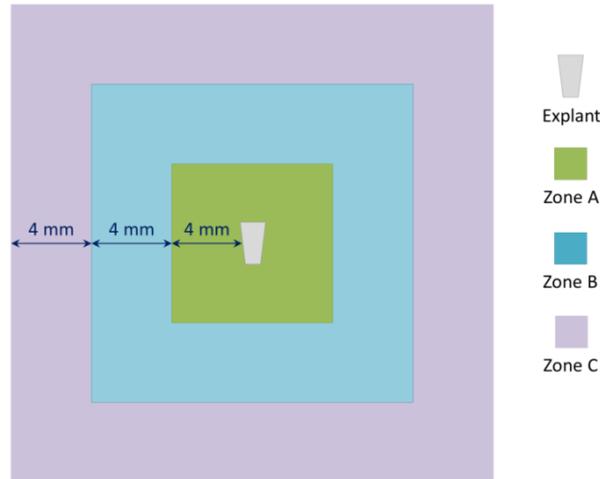
Figure 2.5 Schematic representation of an explant outgrowth and its different zones. The outgrowth zone nearest to explant contains the highest proportion of stem/progenitor cells (zone A) and the lowest proportion in the zone furthest from the explant (zone C). As cells grow and move further away from the explant they differentiate to acquire features typical of corneal epithelial cells (Kolli *et al.*, 2008).

2.2.2.5 Cell counting and viability

20 µL of cells were transferred to a 1.5-mL clear Eppendorf tube and incubated for 3 minutes at room temperature with an equal volume of 0.4% (w/v) trypan blue solution prepared in 0.81 % NaCl and 0.06 % (w/v) dibasic potassium phosphate. Cells were counted using a dual-chamber haemocytometer and a light microscope. Viable and nonviable cells were recorded and used for viability measurement.

2.2.3 Colony forming efficiency (CFE) assay

Colony-forming efficiency assay (CFE) assay is a method for determining the ability of the limbal epithelial progenitor cells to form colonies and assess their frequency. Mitotically inactivated 3T3-J2 mouse embryonic fibroblasts (Kerafast, USA) were suspended in complete medium containing: high-glucose DMEM (89%), FBS (10%) and penicillin/streptomycin (1%) and plated in a 9.6 cm^2 tissue culture well at a final

density of 2.4×10^4 cells per cm^2 and placed in a tissue culture incubator overnight to allow the establishment of a 3T3 feeder layer. The following day, 500 viable limbal cells from the limbal epithelial culture of interest were plated onto the prepared 3T3 feeder cells together with 2 ml of epithelial medium. The CFE culture was then placed in the tissue culture incubator and the epithelial medium was changed on the third day and then every second day thereafter with regular microscopic examination (Eclipse TS100, Nikon, Japan) for the presence of LSC colonies. The CFE was measured on the 12th day of the culture. This was performed by removal of the epithelial medium followed by two brief washes with PBS. The culture was then fixed with 3.7% formaldehyde (VWR International, UK) in PBS for 10 minutes at room temperature. Next, the formaldehyde solution was removed and the culture was irrigated with PBS. The colonies were then stained by incubation with 1% Rhodamine B (Sigma-Aldrich) in methanol for 10 minutes at room temperature. Following staining, the colonies were counted under dissecting microscope (SMZ645, Nikon, Japan). The CFE was calculated using the formula: number of colonies formed/number of cells plated ×100.

2.2.4 Immunofluorescence

2.2.4.1 Cytospin

Cells were prepared by cytospin using a cytocentrifuge obtained from Shandon Southern Instruments, Sewickley, USA, with centrifugation speed of $1000 \times g$ in order to provide a better cell distribution over the slides.

2.2.4.2 Immunofluorescent staining and microscopy

Immunocytochemistry was performed as previously described (Polak *et al.*, 1975). In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 (Sigma-Aldrich, UK), blocked with 5% BSA for 1 h, and incubated with primary antibodies including anti- ΔNp63 antibody, also known as p40 (NBP2-29467, Novus, USA), CK3 (08691431, MP Biomedicals, USA) and Connexin 43 (C6219, Sigma-Aldrich, UK) in recommended dilutions overnight at 4 °C. Next day, the slides were washed three times with PBS for 5 min and then incubated with secondary antibody

conjugated with FITC for 30 min in the dark at room temperature. An isotype control was used as a negative control where the primary antibody was omitted. Following this, cells were washed and then mounted in Vectashield anti-fading media containing Hoechst (Vector Laboratories, UK). Images were obtained with Zeiss Axio Imager (Carl Zeiss Microscopy, Germany). The images were analyzed with ImageJ by marking and counting the immunostained cells as well as total cells separately. A minimum of 300 cells per treatment were counted and the percentages of immunostained cells was calculated.

2.2.5 Statistical analysis

For comparison of two groups Student T-test analysis was used whilst the growth rates were compared using one-way ANOVA analysis. Results were considered significant if p value was less than 0.05.

2.3 Results

Growth rate, cell number and viability, colony-forming efficiency together with expression of putative stem cell marker ΔNp63 and markers of corneal differentiation (cytokeratin 3 and Connexin 43) at the transcriptional and protein level were used to fully analyze the potential impacts of the newly formulized GMP media.

2.3.1 Growth rate in limbal explant epithelial cells expanded in traditional and GMP grade medium

Successful cultures for explant outgrowths and CFE assay were obtained using both non GMP and GMP grade media. 90% confluence was reached at day 13 in both groups. Growth rates, however, differed significantly between the groups at day 6 (***$p < 0.001$) and day 13 (*$p < 0.05$) with limbal epithelial cultures showing earlier onset of cell growth and greater growth area at the end of culture for explants cultivated in GMP grade medium (**Figure 2.6**) (Yu *et al.*, 2016).
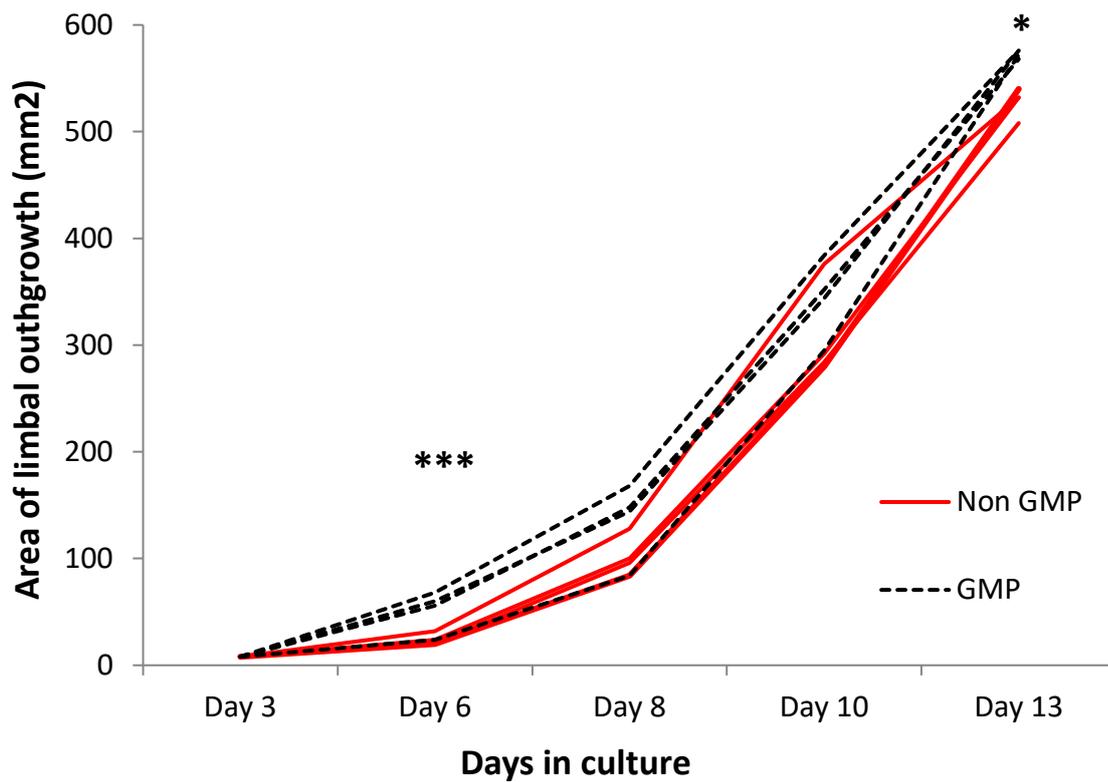
Figure 2.6 Schematic graph showing the area of explant outgrowth (mm^2) on different days of culture for the explants cultivated in non GMP and GMP grade medium (n=4).

2.3.2 Cell number and viability in limbal explant epithelial cells expanded in traditional and GMP grade medium

Cell number in zone A was not significantly different whilst cell numbers in zones B and in zone C were significantly higher in the explants cultivated in non GMP grade medium (**Figure 2.7**) (Yu *et al.*, 2016).
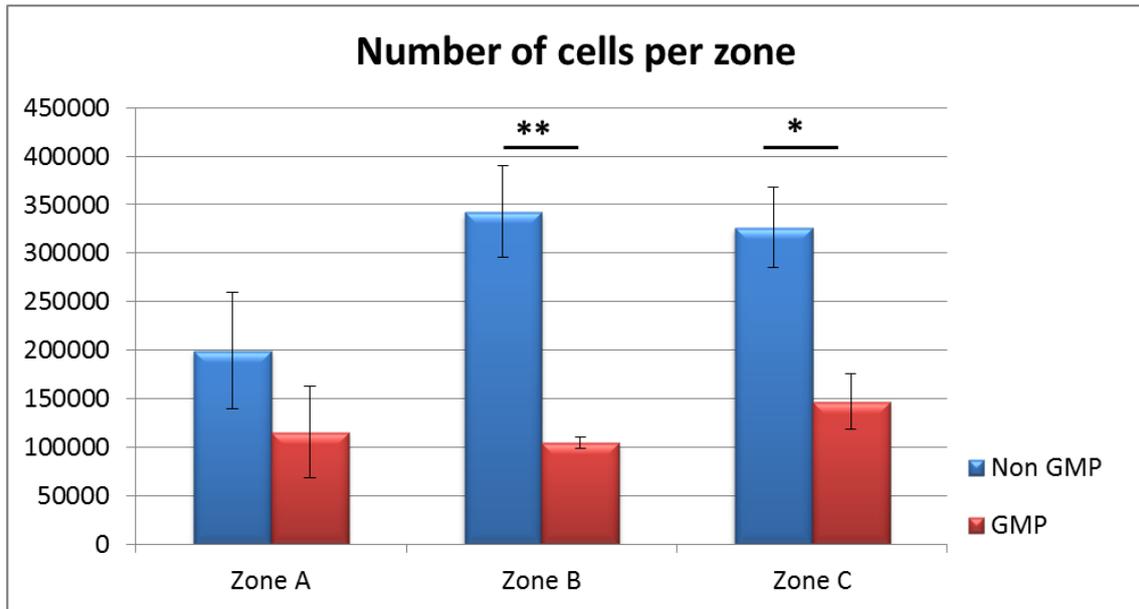
Figure 2.7 Cell numbers per zone of outgrowths cultivated in non GMP and GMP grade medium. Data are presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$.
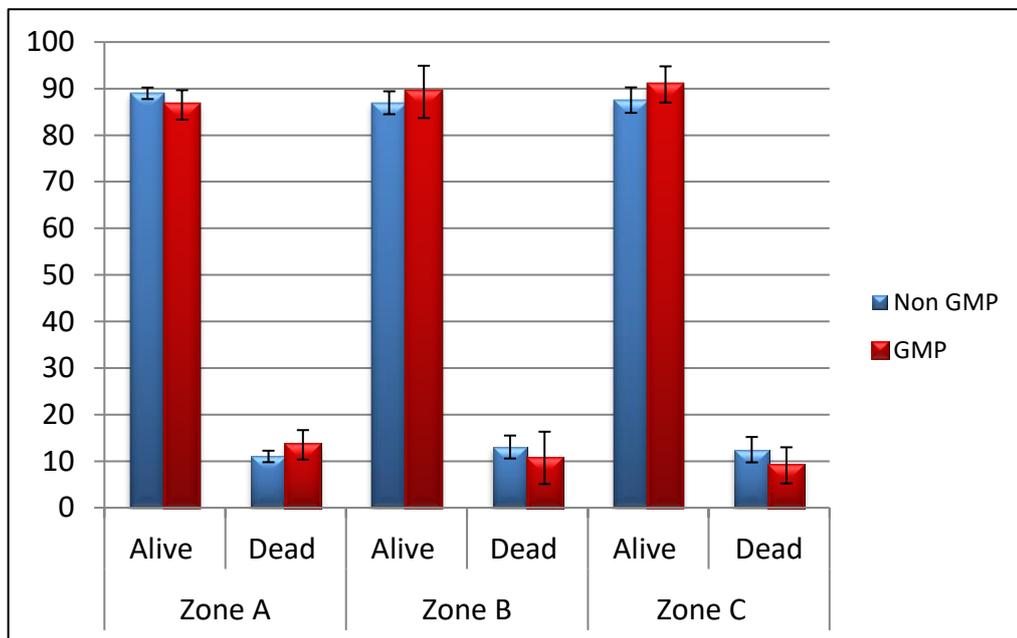
Figure 2.8 Cell viability per zone of outgrowths cultivated in non GMP and GMP grade medium. Data are presented as mean ± SEM.

Cell viability did not differ over different zones between traditional and newly formulized GMP grade medium (**Figure 2.8**).

2.3.3 Morphology of colonies derived by cells expanded in traditional and GMP grade medium

Cells cultivated in the GMP grade medium formed colonies with more differentiated appearance compared with cells cultivated in the non GMP grade medium (**Figure 2.9A-B**) (Yu *et al.*, 2016).
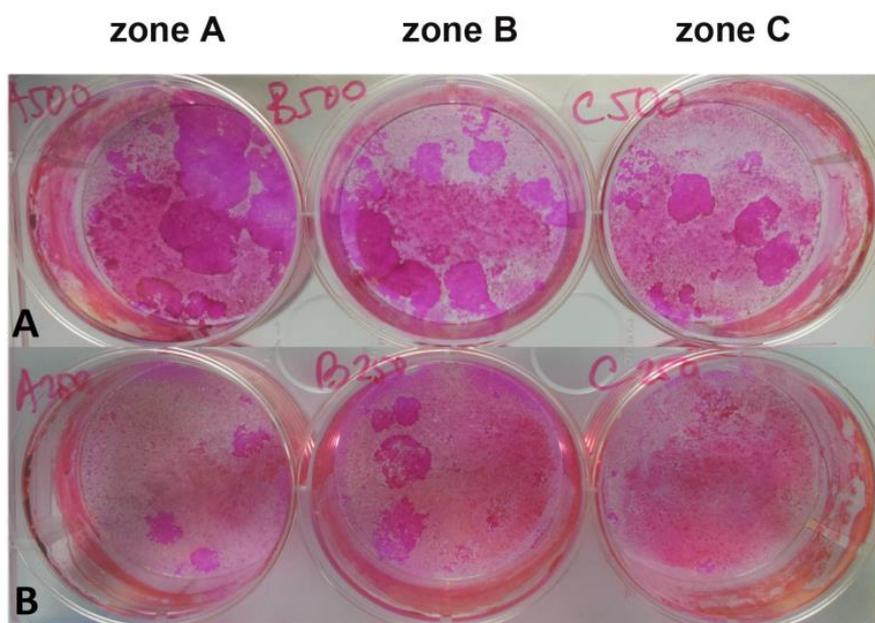
Figure 2.9 Appearance of colonies per zone of outgrowths cultivated in different media: (A) non GMP grade medium; (B) GMP grade medium.

2.3.4 CFE assay in limbal explant epithelial cells expanded in traditional and GMP grade medium

Analysis of CFE didn't reveal any significant difference between the zones of outgrowths cultivated in non GMP and GMP grade medium (**Figure 2.10**) (Yu *et al.*, 2016).
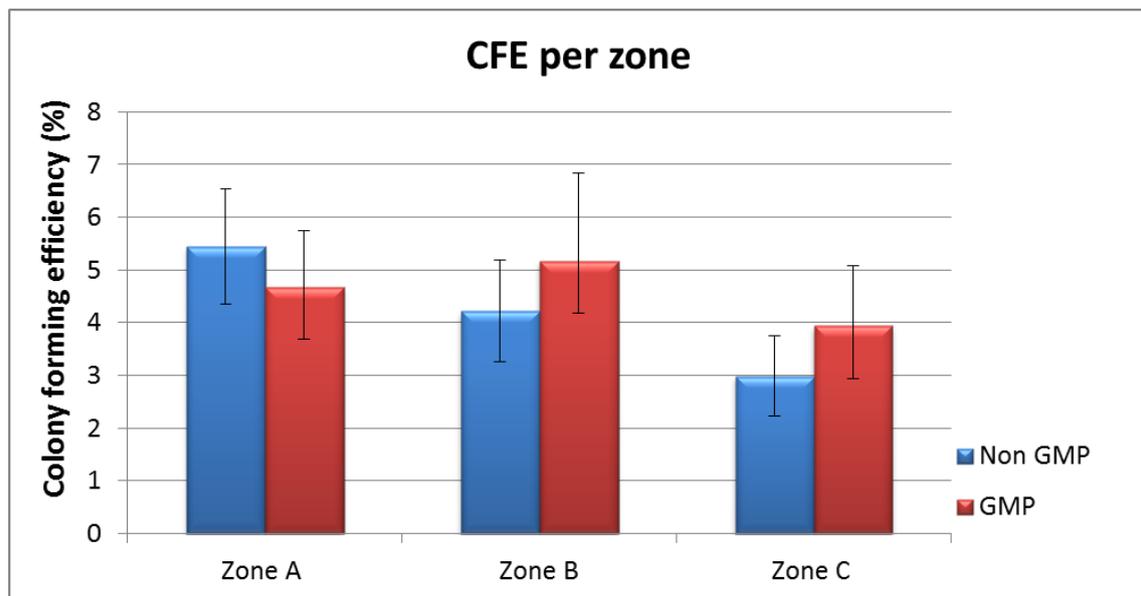
Figure 2.10 The colony forming efficiency of cells from different zones of outgrowths cultivated in non GMP or GMP grade medium. Data are presented as mean ± SEM.

2.3.5 Expression of stem cell related and differentiation markers in limbal explant epithelial cells expanded in traditional and GMP grade medium

The percentage of ΔNp63 positive cells (**Figure 2.11A**) didn't reveal any significant difference between the zones of outgrowths cultivated in non GMP and GMP grade medium (Yu *et al.*, 2016).

The percentage of cytokeratin 3 (**Figure 2.11B**) and Connexin 43 positive cells (**Figure 2.11C**) was significantly higher in zone A suggesting the presence of more

differentiated cells in zone A of outgrowths cultivated in GMP grade medium (Yu *et al.*, 2016).
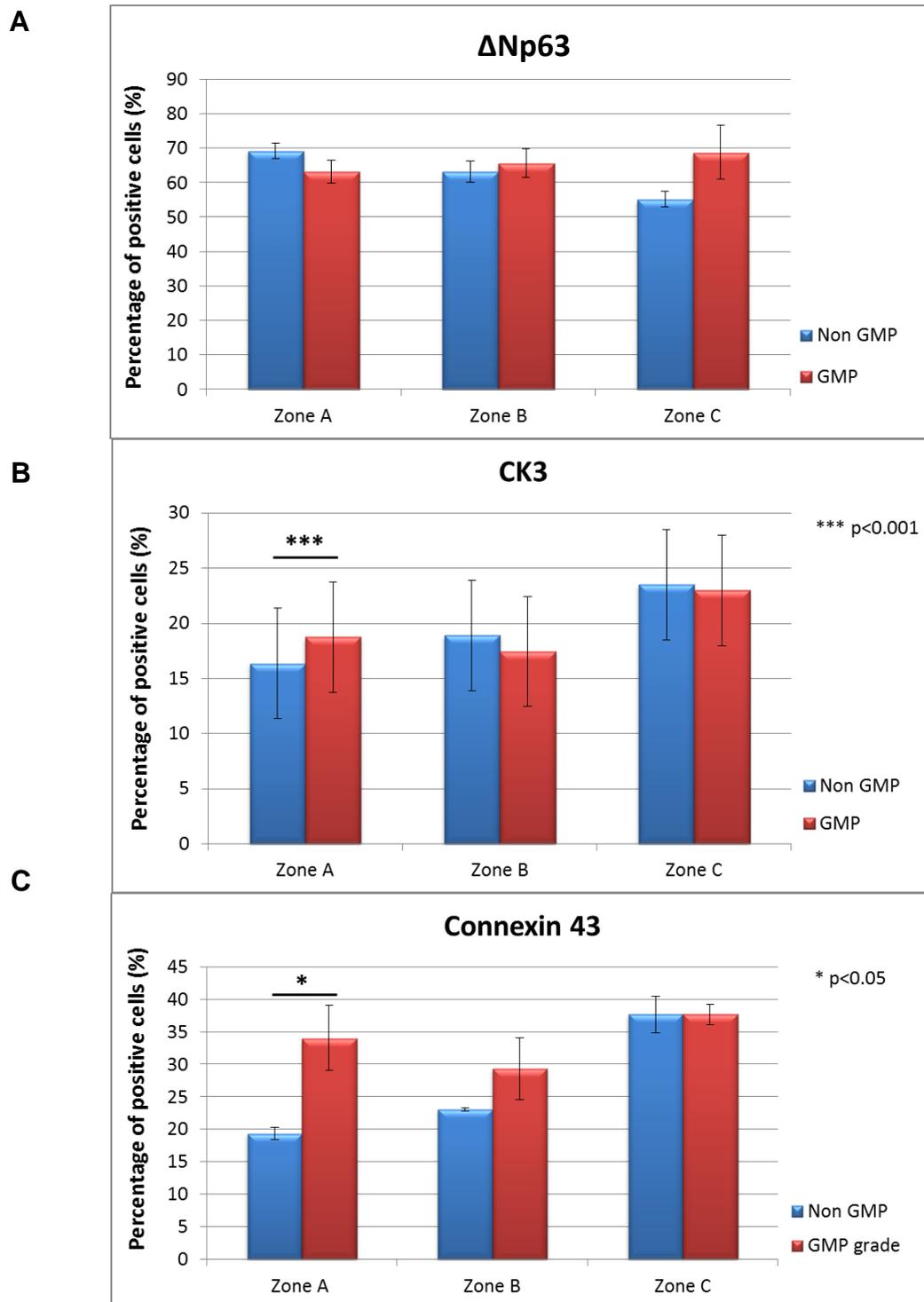
Figure 2.11 Expression of putative LSC and epithelial cell markers in non GMP and GMP grade media assessed by immunofluorescent microscopy. (A) Expression of ΔNp63 using the ΔNp63 antibody; (B) Expression of CK3; (C) Expression of Connexin 43. Data presented as mean ± SEM, * p < 0.05, *** p < 0.001.

2.3.6 Growth rate, CFE and expression of putative LSC and corneal epithelial cell markers in limbal explant epithelial cells expanded in different media

In order to assess the impacts of individual replacements of GMP grade ingredients, four additional complete media were prepared with replacement of just one ingredient in isolation (either Solu-Cortef®, Actrapid®, Liothyronine or Isoprenaline). Successful cultures for explant outgrowths and CFE assay were obtained in all different media formulations (**Figure 2.12**) (Yu *et al.*, 2016). Distribution of LSCs in non GMP medium followed a characteristic pattern, previously demonstrated by our group, with the highest percentage of progenitors in zone nearest to the explant (zone A) with successive decline in zones B and C which are situated further away from the explant, whilst distribution of progenitors in GMP grade medium did not follow this pattern (**Figure 2.13A**) (Yu *et al.*, 2016). Unusually, the percentage of progenitor cells in different zones of explants cultured in GMP grade media was the highest in zone C. Analysis of every substituted component in isolation (**Figure 2.13A-D**) showed that Liothyronine may be responsible for the altered distribution of progenitor cells in zones A to C as the similar "inverted" pattern with the highest percentages of progenitor cells in the zone C furthest from the explant was only seen in explant cultures with addition of Liothyronine in isolation (**Figure 2.13B**) (Yu *et al.*, 2016). Cultivation of explants in the presence of Liothyronine also led to different CFE per zone, where cells from zone C showed the highest CFE (**Figure 2.13A**) which is concordant with finding of the highest number of ΔNp63 positive cells in zone C using the same medium. Moreover, cells in zone A of the explants cultivated in Liothyronine medium showed higher expression of CK3 but not Connexin 43 compared to zones B and C (**Figure 2.13C-D**) (Yu *et al.*, 2016). Due to lack of HAM we were unable to repeat experiments with four additional media with replacement of only one ingredient in isolation (n=1) and no statistics has been done to further investigate effects of every single GMP ingredient in isolation.
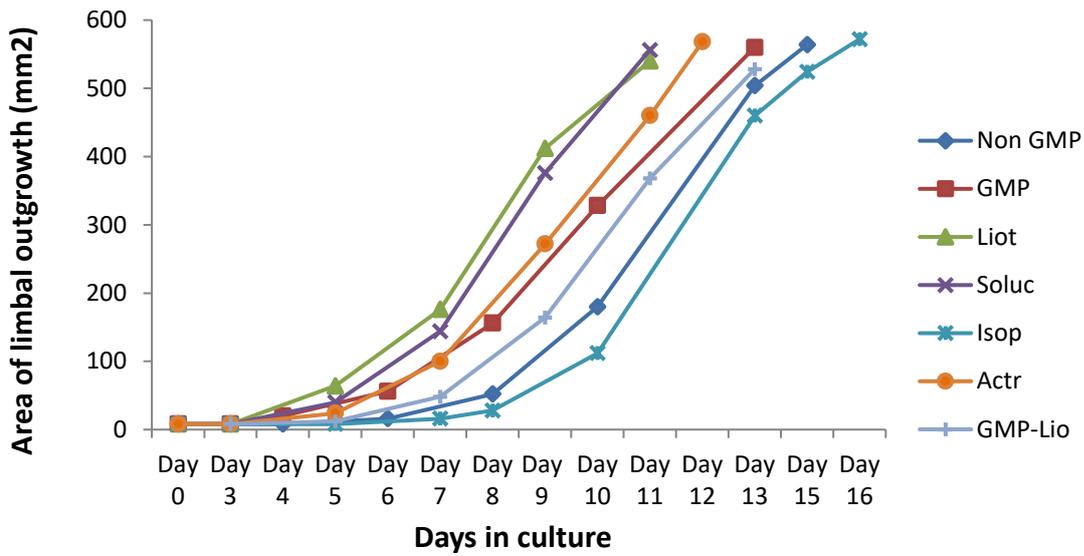
Figure 2.12 Schematic graph showing the area of explant outgrowth (mm^2) on different days of culture for the explants cultivated in non GMP grade medium, GMP grade medium, medium with replacement of Liothyronine in isolation, medium with replacement of Solu-Cortef® in isolation, medium with replacement of Isoprenaline in isolation and medium with replacement of Actrapid® in isolation (n=1).

CFE assay

ΔNp63

CK3

Connexin 43
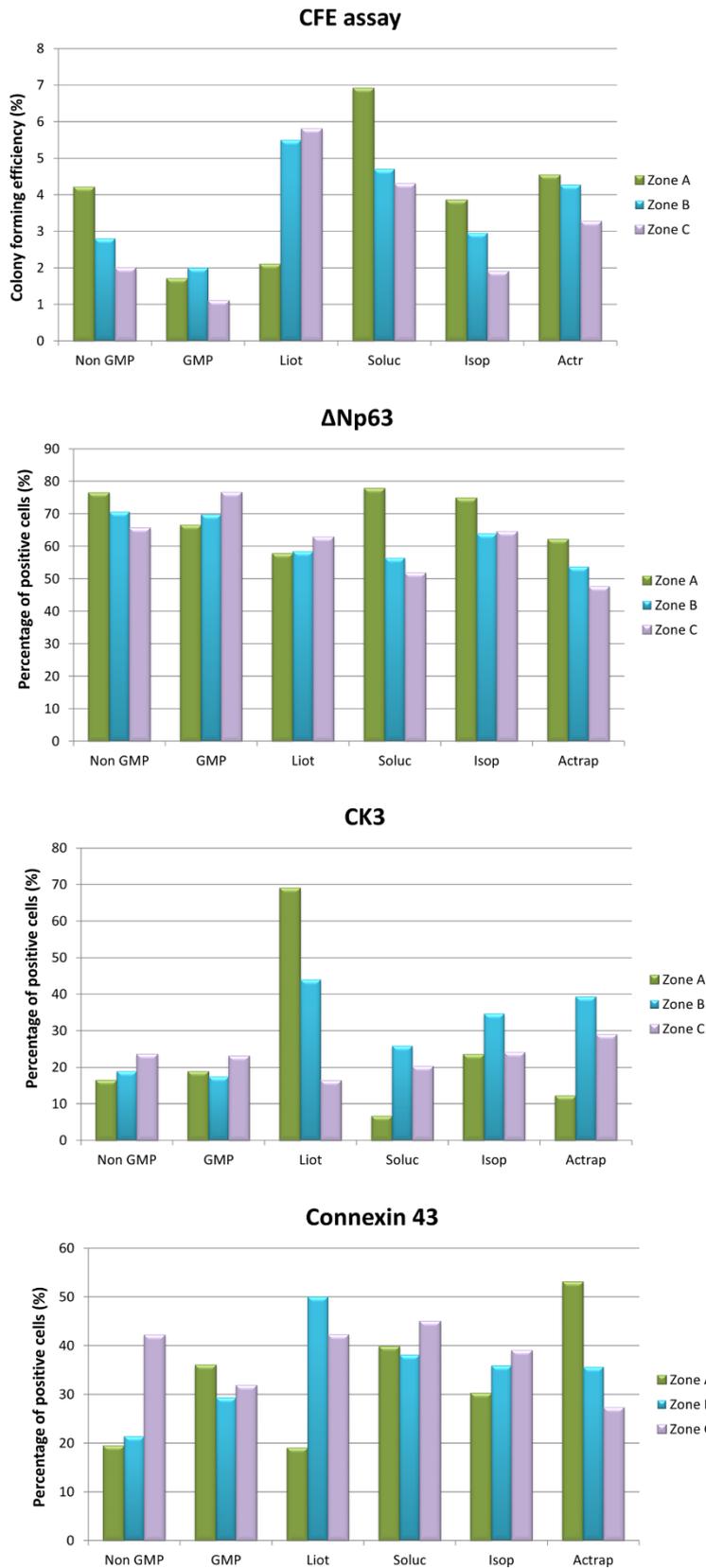
Figure 2.13 The colony forming efficiencies and expression of putative LSC and corneal epithelial cell markers of outgrowths cultivated in non GMP grade medium, GMP grade medium, medium with replacement of Liothyronine in isolation, medium with replacement of Solu-Cortef® in isolation, medium with replacement of Isoprenaline in isolation and medium with replacement of Actrapid® in isolation. (A) The colony forming efficiencies per zone of outgrowths

cultivated in different media; (B) Expression of ΔNp63 per zone of outgrowths cultivated in different media; (C) Expression of CK3 per zone of outgrowths cultivated in different media and (D) Expression of Connexin 43 per zone of outgrowths cultivated in different media (n=1).

2.4 Discussion

Limbal epithelial transplantation is a cell-based therapy with the goal to restore the limbal microenvironment enabling the cornea to regain a corneal epithelial phenotype. Since its introduction in 1997 by Pellegrini and coworkers the *ex vivo* expanded LSC transplantation has been successfully used to treat patients with LSCD. While it has numerous advantages, the *ex vivo* LSC culture protocol does introduce some risks related to the culture processing methods. This includes potential contamination with known and unknown infectious agents introduced by the use of human or animal tissue as well as the risk of immunogenic reactions. Moreover, GMP compliant cell production, careful and strict operative techniques and product traceability are further key elements that need to be considered when determining the safety of a stem cell and any other cell therapy.

Although many of the published cell culture protocols depend on the support of a 3T3 mouse cell feeder layer to nurture the graft there is still a risk of exposure to animal material during the culture period despite their inactivation. Therefore the use of 3T3 cells carries a risk in terms of microchimerism, graft rejection or infection with viral or prion agents (Schwab *et al.*, 2006; Lei *et al.*, 2007; Llames *et al.*, 2015). However, up to date such events have not been reported. Cells cultured under xeno-contaminated conditions can present a nonhuman sialic acid which has been reported as immunogenic to humans (Martin *et al.*, 2005; Heiskanen *et al.*, 2007). For all these reasons in our study we used HAM as a substrate for LSC cultivation, which acts as a surrogate environmental stem cell niche (Grueterich *et al.*, 2003a). HAM is the innermost layer of the placenta that has been extensively used in a treatment of ocular surface pathologies (Rahman *et al.*, 2009). The HAM exhibits several properties that make it suitable for use in tissue engineering (Niknejad *et al.*, 2008). The membrane has an immunomodulatory effect which explains why tissue rejection is not observed after its clinical use (Ueta *et al.*, 2002). Furthermore, cells in the epithelial layer of HAM have significant similarities to stem cells: expression of

pluripotent markers, ability to differentiate into all three germ layers and no need for feeder layer throughout their cultivation (Niknejad *et al.*, 2008). Its low immunogenicity together with anti-tumorigenic, anti-fibrotic, anti-inflammatory, anti-microbial, anti-scaring and useful mechanical properties make HAM suitable for use in tissue engineering (Niknejad *et al.*, 2008). Despite many advantages, its clinical use also carries theoretical risk of disease transmission therefore in Western countries strict legislation stipulates HIV, hepatitis B and C and HTLV tests on donor serum at the time of procuring the HAM (Rahman *et al.*, 2009).

Adenine, hydrocortisone, triiodothyronine and cholera toxin have been traditionally used in the skin and limbal epithelial explant cultures and shown to promote the growth and morphology of keratinocytes (Rheinwald and Green, 1975), the colony-forming ability of epithelial cells (Allen-Hoffmann and Rheinwald, 1984), proliferation of epithelial cells and the requirement for large amounts of fetal calf serum in the media (Allen-Hoffmann and Rheinwald, 1984) (Kolli *et al.*, 2010). Limbal epithelial explants could grow in media lacking any of these four components; however triiodothyronine stood out for its impacts on cell survival and adenine for increased explant surface area (Yu *et al.*, 2016). Our previous work has shown that self-renewal of LSCs, their migration and differentiation can be mimicked by our *ex vivo* culture system where the outgrowth zone nearer to explant contains the highest proportion of progenitor cells and as they move away from the explant they differentiate to acquire features typical of corneal epithelial cells (Kolli *et al.*, 2008). In accordance with this, removal of each of the four components reduced the self-renewal of LSCs in the outgrowth nearest to the explant (Yu *et al.*, 2016). Furthermore, the removal of adenine also led to increased percentage of differentiated cells in the zone closest to the explant as well as increased cell size showing that all four components are currently indispensable for the successful *ex vivo* expansion of limbal epithelial explants on HAM and for maintaining the highest number of proliferating LSCs. The balance between self-renewal and differentiation of LSCs is tightly regulated *in vivo* with LSCs moving centripetally towards the center of the cornea and differentiating to give rise to the corneal epithelial cells which are replaced approximately every 14 days (Thoft and Friend, 1983). At the same time, the limbal stem cell niche maintains the self-renewal of LSCs through uneven distribution of cell fate determinants across the corneal epithelium. ΔNp63a and C/EBPδ have been shown in asymmetric cell division and early cell fate decision of

human limbal stem cells (Mort *et al.*, 2012). Increasing evidence supports the theory that p63 promotes the maintenance of LSCs and also is a determinant of their proliferative potential. Pellegrini et al. has reported that the number of p63 bright cells is an important prospective measure of determining the clinical success of LSC transplantation (Pellegrini *et al.*, 2011). LSC culture system on HAM results in generation of a high percentage of p63 positive cells, namely: 78.1% in zone A, 67.8% in zone B and 49.4% in zone C; however the number of p63 positive cells significantly decreased to nearly half of that from standard medium when treated with media lacking any one of the four components tested in this study, thus suggesting that removal of each of these four components may pose a risk for clinical translations (Yu *et al.*, 2016).

Therapeutic use of LSCs must be performed in compliance with Good Manufacturing Practice as a quality assurance system to ensure highest quality and safety of cell product for transplantation in accordance with the European Union, Regulation (EC) No 1394/ 2007 of the European Parliament and of the Council, on Advanced Therapy Medicinal Products. As part of our study, we aimed at replacing all ingredients that were not produced according to GMP guidelines with GMP grade products. This is a rather important safety aspect as it has been shown that animal-derived components can cause severe immunologic reactions and potential transmission of microorganisms (Erickson *et al.*, 1991; Selvaggi *et al.*, 1997; Chachques *et al.*, 2004; Schwab *et al.*, 2006). We chose widely used clinical grade products Solu-Cortef®, Actrapid®, Liothyronine or Isoprenaline to replace non GMP grade ingredients. Isoprenaline, β-adrenergic agonist, is commonly used for the treatment of bradycardia and is known to increase intracellular calcium concentration of bovine corneal epithelial cells by inducing stimulation of cyclic AMP and thus it represents a safe alternative to cholera toxin for LSC cultivation (Reinach *et al.*, 1992; Akhtar and Choi, 1994). CT and L-isoproterenol are largely used to enhance cell proliferation (Ghoubay-Benallaoua *et al.*, 2012). It was particularly important to replace cholera toxin since this is obtained from bacteria cultured on bovine brain broth and fetal calf serum. While no data have been published for Solu-Cortef® and Liothyronine, Actrapid® and Isoprenaline were previously used for LSC cultivation with success (Ghoubay-Benallaoua *et al.*, 2012). These previous data indicated that isoprenaline-supplemented medium (2 mg/ml) is more efficient than cholera toxin for enhancing cell growth and decreasing cell size in two-week cultures (Ghoubay-Benallaoua *et*

al., 2012). In accordance with this, we observed a slightly faster growth rate of explants in the GMP culture media and a larger growth area at the end of culture period. Although CFE per zone did not differ significantly between the groups, the morphology of colonies was different. Cells cultivated in the GMP grade medium formed colonies with more differentiated appearance compared with cells cultivated in the non GMP grade medium. While the percentage of progenitor cells per zone did not differ between groups, distribution of progenitor cells per zone compared with explants cultivated in non GMP grade medium was unexpectedly altered. Furthermore, it seems that cultivation in GMP grade medium promoted differentiation of cells in the zone nearest to the explant. Analysis of every substituted component in isolation showed that Liothyronine may be responsible for the altered distribution of progenitor cells in zones A to C and for promoting the differentiation of progenitor cells in zone A. Notwithstanding these differences, LSCs expanded in LSC media with GMP grade reagents have not been tested *in vivo*. With this in mind, it would be useful to test their potential to engraft and reverse LSCD in animal model settings (for example rabbit) to investigate whether the molecular differences we have observed *in vitro* hold true *in vivo*.

2.5 Conclusion

Replacement of each of the research grade LSC media components with GMP grade reagents resulted in equal growth to non-GMP grade media; however an enhanced differentiation of progenitor cells in the outgrowth situated closest to the explant was observed, suggesting that additional combinations of GMP grade reagents need to be tested to achieve similar or better level of LSC maintenance in the same manner as the traditional LSC media. The research strategy of better refining the current stocks of materials and their possible replacement with GMP grade components provides a pathway that may be beneficial to other medicinal advanced cell therapy products currently being used in clinical trials.

Chapter 3. Transport

3.1 Introduction

Since 1997, when Pellegrini and colleagues first demonstrated transplantation of *in vitro* expanded limbal epithelium for treatment of LSCD from a small biopsy taken from the patient's contralateral healthy eye (Pellegrini *et al.*, 1997), transplantation of cultured limbal epithelial sheets has become the most successful alternative to corneal surface reconstruction in patients with unilateral LSCD (Burman and Sangwan, 2008). So far, more than 1000 transplantations have been performed worldwide (Utheim *et al.*, 2018a) with approximately 75% overall success rate (Utheim, 2013). In patients with LSCD, stem cell graft is required to replenish LSC reservoir, which is ultimately responsible for regeneration of the corneal epithelium. Current treatment options utilize limbal tissue biopsies that harbour LSCs as well as tissue culture expanded cells. The tissue is usually placed on a scaffold that supports the formation of so called "limbal epithelial cell sheets" which are then transferred and transplanted to diseased eye. A numerous biological and synthetic materials have been reported as carrier substrates for LSCs, some of which have been used in the clinic, including HAM, fibrin and silicone hydrogel contact lenses (Nguyen *et al.*, 2018).

Although a variety of expansion protocols for LSC culture showed good clinical outcomes, LSC-based therapy still faces challenges regarding tissue safety and sterility, tissue transportation, surgery logistics and availability of cultured tissue (Raeder *et al.*, 2007). As the European Union classified stem cell-based therapies as "medicinal products", their manipulation is strictly regulated and has to follow defined conditions of good manufacturing practice. The production of stem cell therapeutics is therefore limited exclusively to accredited production sites authorized by the national regulatory agencies (Di Iorio *et al.*, 2010; Massie *et al.*, 2014). The strict production requirements represent a barrier to a widespread use of stem cell-based therapies, despite the fact that demand is anticipated to increase due to successful clinical outcomes. For this reason, the need for safe, validated and reproducible techniques for *ex vivo* cultured tissue distribution and preservation are coming to the

forefront of research. Numerous new challenges for bio-banking industry such as retention of viability, sterility issues and good functionality of stem cells in the end product are still waiting to be fully scientifically addressed.

The cell-processing centre (CPC) is a clean room that serves as an essential area for aseptic culturing or processing of human cells for regenerative medicine (Oie *et al.*, 2014). Although many hospitals require tissue-engineered epithelial cell sheets for treatment, due to the high expense of a CPC, many hospitals need to share one CPC to standardize and spread regenerative therapy. Development of an efficient cell transportation technique is therefore vital for bridging many hospitals and CPCs (**Figure 3.1**).
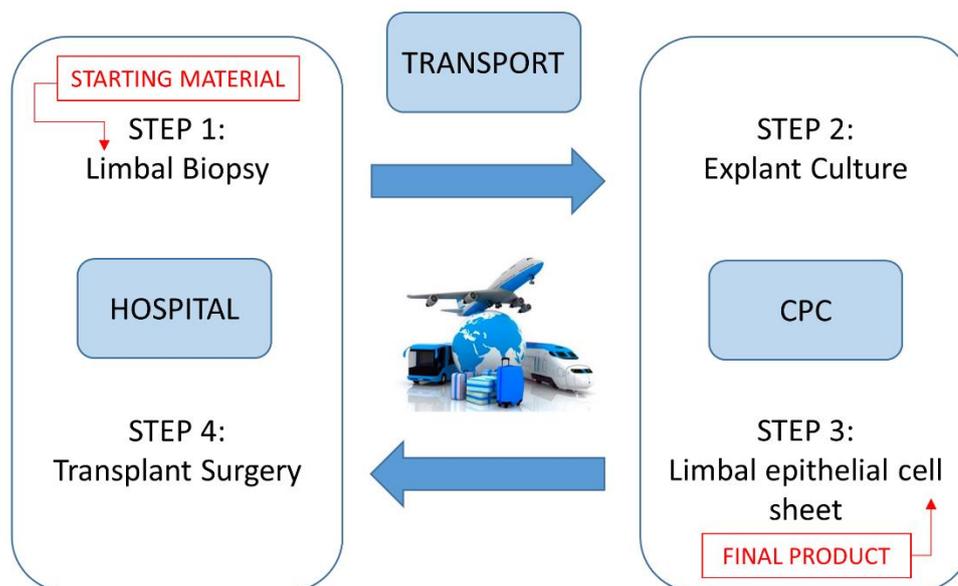
Figure 3.1 Simplified scheme of multi-centre study organisation. Limbal tissue biopsy and transplantation of cultured LECs on HAM are performed in a hospital, explant culture of limbal biopsies and preparation of limbal epithelial cell sheets in a CPC.

Connecting cell laboratories and eye banks is likely to increase the availability of regenerative medicine (Utheim, 2013). Growing interest in cultured limbal epithelial cells (LECs) is evident from the literature (Schwab *et al.*, 2000; Rama *et al.*, 2001; Kito *et al.*, 2005; Oh *et al.*, 2007; Raeder *et al.*, 2007; Utheim *et al.*, 2007; Yeh *et al.*,

2008; Utheim *et al.*, 2009; Raeder *et al.*, 2010; Rama *et al.*, 2010; Utheim *et al.*, 2015; Jackson *et al.*, 2016; Utheim *et al.*, 2018a). In a review on LSC therapy, Ahmad et al. suggested that the production of cultured limbal epithelial sheets for transplantation should be centralized for the following reasons: (a) strict regulatory demands make this kind of laboratories extremely costly and (b) requirement of expertise in culturing limbal tissue is of vital importance (Ahmad *et al.*, 2010). Due to the strict regulations of cell therapy, the number of small units producing tissue for clinical use will decrease (Daniels *et al.*, 2006b), therefore forcing centralization and forming of larger centres, which necessitate effective transportation strategies (Ahmad *et al.*, 2010).

Due to the limited insights into the LSC-based product's safety and stability after preservation, most of the clinical studies on LSC transplantation reported so far used non-preserved terminal products (fresh cultured LSC epithelial grafts on a carrier scaffold) applied directly to LSCD patients (Grueterich *et al.*, 2003a; Ti *et al.*, 2004). Without a reliable method of limbal epithelial sheets storage challenges regarding sterility, transportation, surgery logistic and availability of tissue cannot be overcome.

It is mandatory that the final products recovered from storage are physiologically and biochemically identical to its pre-preservation state, retaining high viability, good functionality and tissue morphology (Massie *et al.*, 2014). For example, retention of undifferentiated cell phenotype in cultured and stored cultured epidermal cell sheets is important for the treatment of patients with burns (De Luca *et al.*, 2006). Moreover, retention of a high percentage of progenitor cells within transplanted cultured limbal epithelial sheets in the treatment of LSCD results in a higher rate of clinical success (Rama *et al.*, 2010).

However, the current preservation methods still leave open fundamental questions regarding the optimal storage temperature and storage media formulations (short or long term) not only for single LSC suspensions but also for the final products (cultured epithelial sheets on different scaffolds). Thus, storage methods and preservation techniques for LSC delivery have recently been under investigation at an accelerated rate (Luznik *et al.*, 2016a).

To address these problems, Utheim *et al.* developed a method for short-term storage of cultured LSCs improving surgery logistics and enabling transportation and microbiological assessment using a closed container (Raeder *et al.*, 2007) whilst Oie

et al. designed a transport container for cell sheets with three basic functions: maintaining a constant interior temperature, air pressure and sterility (Oie *et al.*, 2014). Raeder *et al.* reported that organ culture storage of cultured human LECs in a closed container for 1 week at ambient temperature is superior to both organ culture storage at 31°C and Optisol-GS storage at 5°C (Raeder *et al.*, 2007). *Oie et al.* suggested that during 24 hour long transportation via an airplane, the temperature inside the container should be maintained above 32°C. They tested four kinds of transportation liquids (keratinocyte culture medium - KCM, KCM minus FBS and EGF, DMEM/F12 in 3:1 ration and HBSS) and concluded that HBSS was the best choice of transportation media (Oie *et al.*, 2014).

Appropriate tissue storage methods (both short and long term) would allow cultured tissue transportation from centralized laboratories to the operating theatre as well as between eye banks, offering the logistic flexibility in scheduling transplantation surgery (Raeder *et al.*, 2007). As cell cultures may fail at any time during cultivation, the planning of surgery becomes cumbersome without efficient storage technology (Utheim, 2013).

Importantly, tissue storage would increase the time for quality control of LEC cultures (microbiological testing, stem cell identification etc.) (Jackson *et al.*, 2014). Daniels and colleagues proposed that control measures should be employed, whenever possible, to determine the quality of the final cell product (Daniels *et al.*, 2006b). Microbial analyses of LECs cultures are mandatory part of the quality control measures conducted before these cells are applied to treat patients with LSDC. Bacterial infection of LEC cultures may be avoided by using a hermetically sealed container for storage prior to clinical use (Utheim *et al.*, 2009). Long term corneal epithelium renewal after *ex vivo* cultured limbal epithelial transplantation depends mainly on the sufficient number of LSCs transferred in the cultured epithelial sheets (Rama *et al.*, 2001). To date, transcriptional factor ΔNp63α is the commonly used putative marker for determination of percentage of stem cells in limbal epithelial cultures, as a positive clinical correlation was already reported between ΔNp63α expression and clinical success rate (Pellegrini *et al.*, 1999a; Di Iorio *et al.*, 2010). Pellegrini et al. suggested that stringent quality criteria should be adopted for LEC cultures to ensure that they contain a sufficient number of stem cells vital for long term epithelial survival (Pellegrini *et al.,* 2011). Quality control should include rigorous clonal analysis or the evaluation of cell doublings generated during serial cultivation

of LECs (Pellegrini *et al.*, 2011). Another important measure for ensuring quality of cultivated cells is regulating levels of contaminating fibroblasts in LEC cultures. Di Iori and colleagues evaluated the percentage of contamination of murine fibroblasts in cultured LECs and estimated that >5% contamination was unsuitable for clinical use (Di Iorio *et al.*, 2010). Proulx *et al.* emphasized the importance of viability assessment of LECs prior to clinical use (Di Iorio *et al.*, 2010) while other authors highlighted the need for standardization and validation of LEC cultures for clinical use (Higa and Shimazaki, 2008; Hayashi *et al.*, 2010).

Additionally to short-term preservation, efficient long-term cryopreservation of surplus cultured tissue could enable consecutive surgeries in case of unsuccessful primary treatment (Mohamed-Noriega *et al.*, 2011).

For all these reasons accredited centres for LSC culture are challenged to further develop efficient, standardized and validated transport and preservation methods.

3.1.1 Current preservation strategies in different stages of the production process of bio-engineered limbal epithelial sheets

Current preservation methods fall into two main categories: preservation of LSCs before *in vitro* limbal tissue generation (e.g. preservation of donor corneas, cryopreservation of suspended amplified LSCs after several passages) or preservation of limbal bio-engineered sheets on different scaffolds.

3.1.1.1 Preservation of donor corneas and single LSCs

Most of the tissue used for penetrating keratoplasty is issued through eye banks that store the corneo-scleral button either in organ culture at 31-37ºC introduced in 1976 or in hypothermic storage at 2-6ºC introduced in 1974 (Pels *et al.*, 2008).

The two preservation techniques differ in technical aspects, tissue evaluation methods, storage time and microbial safety. Hypothermic storage is a simple technique that allow storage for up to 7-10 days whilst organ culture is a relatively complicated technique but allow storage for longer period of time, up to 4 weeks.

Both preservation techniques seem to result in similar graft survival. Organ culture, although it originates from United States (Doughman *et al.*, 1976; Doughman, 1980), is now widely applied only in Western Europe but not commonly used elsewhere (Pels *et al.*, 2008).

A longer storage time permits a greater flexibility in the use of donor tissue and prevents wastage. Currently, only cryopreservation methods offer truly long-term storage of living cells and tissues (Woods *et al.*, 2004; Armitage, 2009). Long-term preservation is especially important for preventing the loss of outdated material (Corydon *et al.*, 2009), particularly in countries where shortage of donor corneas exists. However, bio-banking of donor corneas by cryopreservation is technically challenging and still in experimental phase. It's been shown that only corneas obtained immediately after death from young donors were suitable for cryopreservation (Corydon *et al.*, 2009) as freezing led to damage of the corneal endothelial cells (Armitage, 2009). On the other hand, cryopreserved whole donor corneas could be potential source of LSCs (Mohamed-Noriega *et al.*, 2011). Bratanov and colleagues successfully cultured limbal explants from cryopreserved corneas (Bratanov *et al.*, 2009). Albeit the limbal tissue was subjected to long lasting storage in liquid nitrogen, it enabled proliferation of epithelial cells with phenotypically identified LSCs (p63 positive cells) present in culture. However, no further functional test for LSCs identification was performed and a different growing pattern of expanded cells from the cryopreserved limbal explants was reported, most likely due to destabilization of the extracellular matrix during freezing-thawing procedures (Bratanov *et al.*, 2009).

Another possible strategy of long-term LSCs preservation is cryopreservation after *in vitro* expansion. Several studies reported cryopreservation of the suspension LSC culture (Corradini *et al.*, 2012; Pellegrini *et al.*, 2014). Schrader and colleagues reported that conjunctival epithelial cells could be efficiently cryopreserved with successful maintenance of progenitor cell-like characteristics and function *in vitro* over several culture passages (Schrader *et al.*, 2009). Importantly, cryopreservation of suspended LSCs after *in vitro* expansion showed a non-immunogenic nature of defrosted limbal cells (Vasania *et al.*, 2011). After thawing, human LSC cultures retained the expression of LSC markers but no HLA-DR gene expression was observed (Vasania *et al.*, 2011). Moreover, cultured LSCs were also unable to stimulate allogenic T cell proliferation *in vitro*, even in the presence of pro-

inflammatory cytokines (Vasania *et al.*, 2011). Thus, cryopreserved LSC cultures could express negative immunoregulatory molecules, which may be critical for their survival in an allogenic environment and would enable better allograft survival (Vasania *et al.*, 2011).

However, the exact cryopreservation details are often not explicitly reported in clinical case publications and further evaluation of the impact of freezing/thawing procedures is often missing (Luznik *et al.*, 2016a). Therefore, further experimental studies are needed to elucidate this important issue.

So far, only one experimental study reported successful reconstruction of corneal epithelium using cultured epithelial grafts generated from long-term cryopreserved LSCs in a goat model of LSCD (Mi *et al.*, 2008). However, it is yet not proven how stable these limbal epithelial sheets will be over time.

3.1.1.2 Preservation of bio-engineered limbal epithelial sheets on different scaffolds

Optimal physiological approach to preserve cultured limbal epithelial sheets at the genomic, proteomic, structural and functional levels is still under active investigation. Before widespread clinical use there are several issues to be solved regarding the optimal preservation temperature and storage media composition.

Recent studies were focusing on the optimal temperature for efficient preservation of limbal epithelial cell sheets (Jackson *et al.*, 2014; Eidet *et al.*, 2015; Jackson *et al.*, 2016; Jackson *et al.*, 2017).

Utheim and colleagues first reported a method for short-term storage of cultured corneal sheets (Raeder *et al.*, 2007; Utheim *et al.*, 2007). They showed successful maintenance of the original multi-layered structure and undifferentiated phenotype after 1 week in organ culture at 23ºC of limbal epithelial sheets obtained from explant culture on intact HAM (Raeder *et al.*, 2007; Utheim *et al.*, 2007). Raeder et al. showed morphological corneal epithelial changes such as epithelial detaching and

intracellular vacuoles after 1 week of organ culture preservation of cultured corneal epithelium at 31ºC. On the other side, hypothermic storage of corneas at 5ºC in Optisol-GS also showed separation of cells below the superficial epithelial layer and pronounced intracellular oedema (Raeder *et al.*, 2007). Taken together, their data indicated that storage of cultured limbal epithelial cells on HAM at ambient temperature is superior to organ culture storage at 31ºC and Optisol-GS storage at 5ºC, with minimal apoptosis after storage (Raeder *et al.*, 2007; Utheim *et al.*, 2007). Later studies from the same research group showed successful storage of cultured human conjunctival epithelial sheets on HAM for at least 4 days under serum free conditions (Eidet *et al.*, 2012).

For long-term preservation of the end cell product, as previously mentioned, the only effective method is cryopreservation. However, cryopreservation of adherent fully stratified ocular epithelium has been shown more challenging compared to cryopreservation of suspended cells. Up to date, there is no consensus regarding the functional and morphological outcomes after cryopreservation of cultured limbal epithelial sheets based on previous *in vitro* studies (Luznik *et al.*, 2016a).

Kito *et al.* reported that rabbit limbal epithelial cells stored at a lower storage temperature (-196ºC) showed an improved survival compared with samples stored at a higher temperature (-80ºC) (Kito *et al.*, 2005). Although the structural integrity of the cultured limbal epithelial sheets was destroyed in all tested cryopreservation protocols, cell viability was reported to be up to 70% and the remained cells were able to regenerate a new cell sheet (Kito *et al.*, 2005). Yeh and colleagues reported successful 8 weeks long cryopreservation of limbal explant cultures expanded on HAM (Yeh *et al.*, 2008). Using their method, cell viability was reported to be around 50% and good growth ability of remained cells was proven (Yeh *et al.*, 2008). Oh and colleagues cryopreserved human limbal and rabbit conjunctival cultured epithelial sheets for 1 week at -196ºC (Oh *et al.*, 2007).

3.1.2 Aims of the study

As strict regulations for cell therapy promote centralization of culture units (Daniels *et al.*, 2006b), the treatment remains limited to a few centres of expertise (Utheim *et al.*, 2015). For this reason, definition of reliable and practical transportation strategies is vitally important. This study aims to optimize a transport conditions of the starting material (limbal biopsies - LBs) as well as the conditions of transport and storage of the final cell product (cultured LECs on HAM - limbal epithelial sheets), which have not been precisely define yet, for their widespread distribution and use in regenerative medicine.

To access the best transport conditions for the starting material, short (30 minutes) and prolonged (24 hours) transport at both room temperature (RT) and 4°C will be compared, whilst the best transport and storage conditions of the final cell product will be accessed by comparison of 24h transport in culture media and prolonged 4 days and 7 days transport in serum-free storage media. The best transport conditions will be defined by comparison of explants growth rates, morphology, phenotype, and viability of cultured human LECs (**Figure 3.2**).
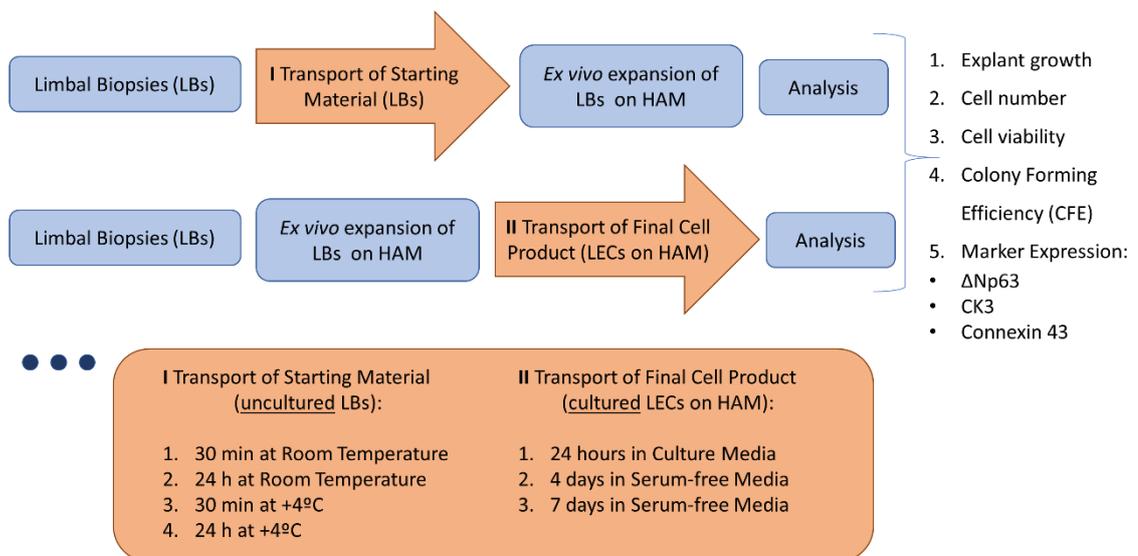
Figure 3.2 Experimental design.

3.2 Material and Methods

3.2.1 Human donor tissue

Cadaveric adult human limbal tissue was obtained from the corneo-scleral rings remaining (2 males and 1 female, average age 62, SEM 3.84, range from 55 - 68 years), after removal of the central cornea for transplantation supplied by the NHS Blood and Transplant (NHSBT) Cornea Transplantation Service eye banks based in Manchester and Bristol, UK. Human amniotic membranes were provided as individual units of 3 x 3 cm^2 mounted on nitrocellulose paper by NHSBT, Tissue Services. Human tissue was handled according to the tenets of the Declaration of Helsinki and informed consent was obtained for research use of all human tissue from the next of kin of all deceased donors, with approval from the local Research Ethics Committee. The study was approved by the NRES Committee North East - Newcastle & North Tyneside 1 (REC number: 11/NE/0236, protocol number 5466) on the 29th October 2013.

3.2.2 Transport of the starting material: limbal biopsies' transport simulation

Having in mind that actual LBs represent a very precious tissue taken only in very special cases (such as a need for limbal epithelial cell transplantation) we used 4 mm^2 segments of cadaveric corneo-scleral tissue as their alternative (tissue from three different donors, n=3). Segments of cadaveric corneo-scleral tissue were prepared as previously described in Chapter 2. Four different transport conditions were compared: the first segment of each corneo-scleral ring was kept in a traditional LSC culture medium filled tube for 30 minutes at RT, the second segment of each ring for 30 minutes at 4ºC, third segment for 24 hours at RT and finally fourth segment of each ring for 24 hours at 4ºC. After transport simulation all the segments were cultivated on HAMs as described in Chapter 2 (using "LSC explant culture") until they've reached 90% confluence. The epithelial outgrowths were then divided into three equal zones depending on proximity to the explant (A, B and C zone) as previously described and used for further analyses.

3.2.3 Transport of the final cell product: limbal epithelial sheets' transport simulation

Following LSC explant culture four different dishes containing a cultured limbal epithelial cell sheet at 90% confluence from each of three donors were randomly selected for transport and storage simulation (three LSC explant cultures from each donor) or used as a non-stored control (one LSC explant culture from each donor). All experimental groups contained identically cultivated cell sheets at the start of storage. Control cultured limbal epithelial sheets from each of three donors were processed immediately after reaching 90% confluence while the rest of cultured limbal epithelial sheets were stored in dishes sealed by parafilm for 24 hours, 4 days and 7 days at 23ºC.

Cultured LECs on HAM were shaken at 200 orbital rotations per minute (rpm) on Titramax 100 (Heidolph Instruments, Germany), multi-purpose mixer shaker, for 2 hours before they were stored for 24 hours, 4 and 7 days in order to expose the tissue to transport simulation.

Limbal epithelial sheets were stored in either traditional LSC culture medium (for the simulation of short 24 hour transport) or in serum-free storage medium (for long term 4 and 7 days transport simulation).

3.2.4 Tissue analysis after transport simulations

All other techniques including monitoring of explant outgrowth, cell counting and viability, CFE and immunofluorescent staining of putative stem cell marker ΔNp63 and markers of corneal differentiation CK3 and Connexin 43 are described in details in Chapter 2.

3.2.5 Statistical analysis

Data are presented as mean ± SEM. All experiments were performed in biological replicates of three or more. To access the difference between the groups, one-way

analysis of variance (ANOVA) were performed. A significance level of p < 0.05 was used throughout the study. GraphPadPrism 7.0 (San Diego, CA, https://www.graphpad.com/scien-tific-software/prism/) was used to perform all statistical analyses.

3.3 Results

3.3.1 Limbal explant growth rate, cell number, viability and CFE after LBs transport simulation

Successful explant cultures were obtained after all four LBs transport condition simulations (**Figure 3.3**). A trend of earlier onset of cell growth, faster growth throughout the culture and earlier confluence reach were observed in RT groups compared to 4ºC groups in all three donors. No difference in growth rates between short 30 min and prolonged 24h transport was present at RT. On the other hand, there was a visible difference in growth rates between short and prolonged LBs transport at 4ºC across all three donors, with 24h transport at 4ºC showing the latest onset of growth and the slowest growth throughout the culture (**Figure 3.3**).
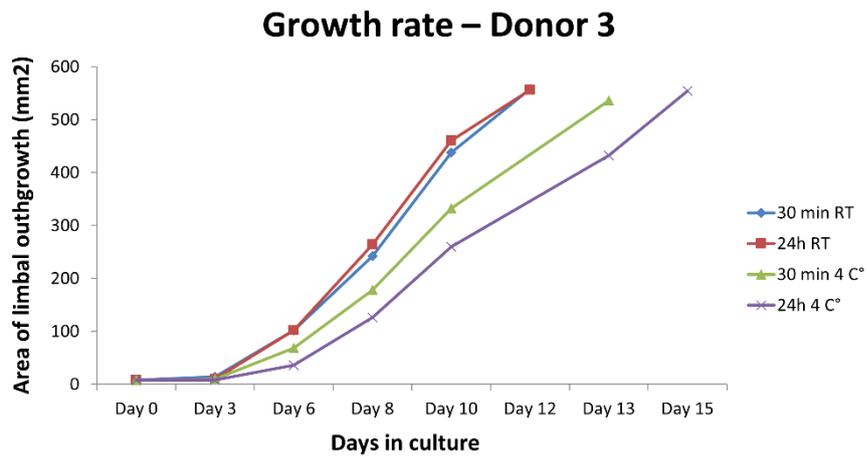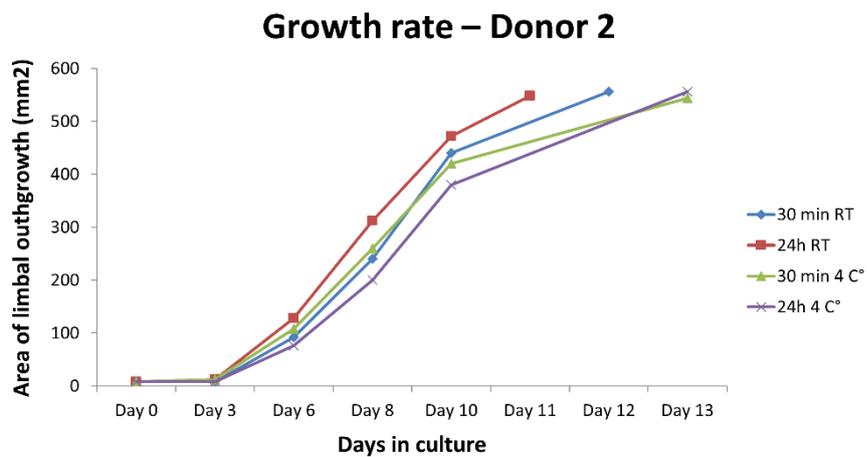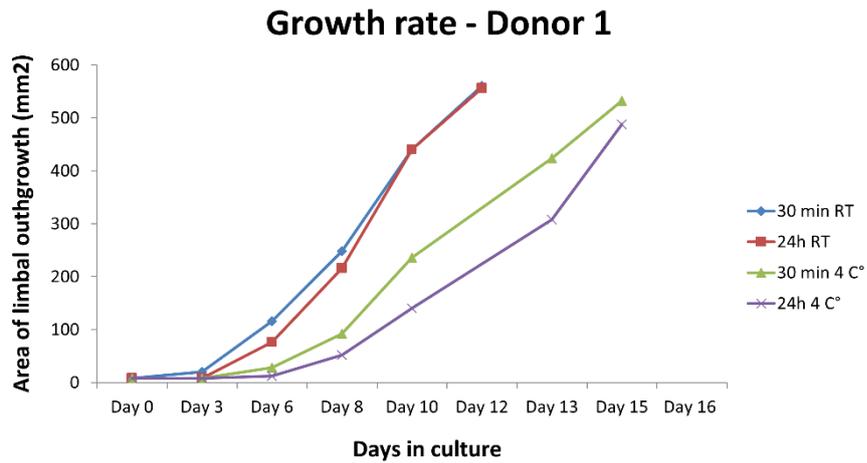
Figure 3.3 Limbal epithelial cell growth across different LBs transport condition groups. Schematic graphs are showing the area of explant outgrowth (mm^2) on different days of culture; (n=3).
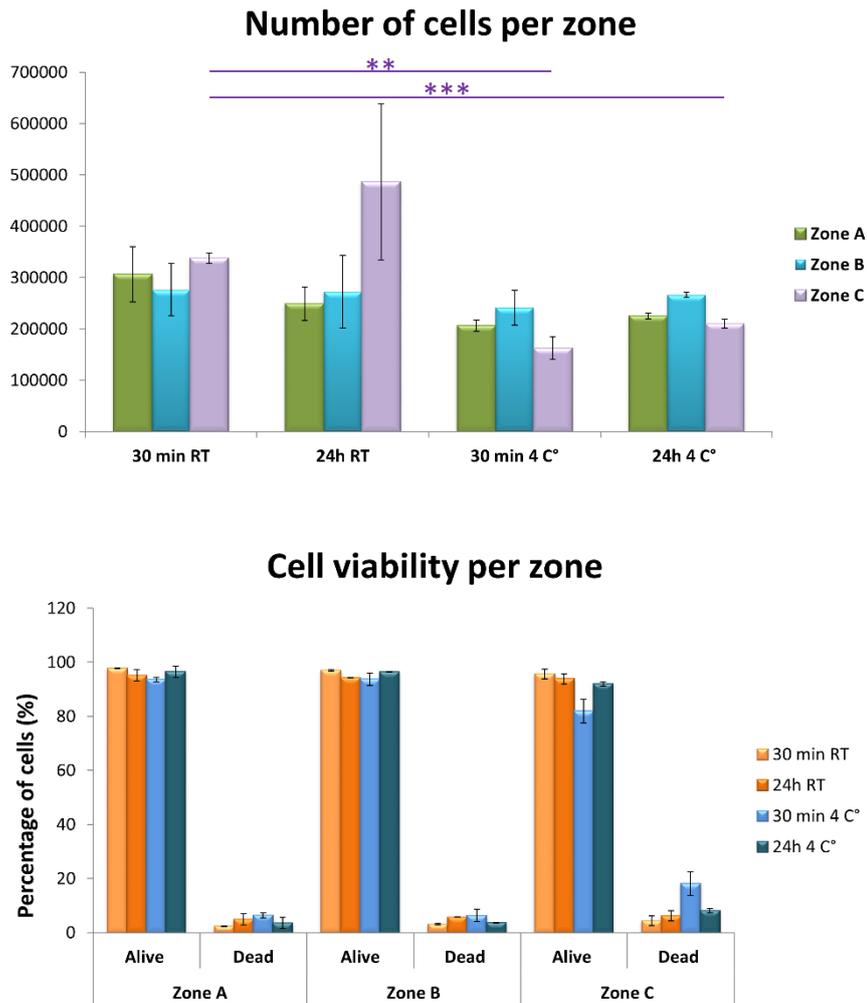
Figure 3.4 Number of cells and cell viability per zone of limbal outgrowths after limbal biopsies transport simulation. Data are presented as mean ± SEM, ** $p < 0.01$, *** $p < 0.001$ (n=3).

In terms of cell number per zone, no significant differences were found with the exception of zone C which contained higher number of cells in RT groups compared to 4°C groups (**Figure 3.4**). This difference was significant between 30min RT and both 30min 4°C and 24h 4°C groups ($p<0.01$ and $p<0.001$ respectively). A similar trend was observed in 24h RT compared to both 4°C transport groups but without any significance found ($p>0.05$).

Cell viability was well preserved and there were no significant differences between the groups in any of the zones (**Figure 3.4**).

CFE was significantly higher in zone B and zone C of limbal outgrowths grown from LBs transported over 30min at RT compared to LBs transported over 24h at 4°C (p<0.05). An overall trend of higher CFEs in RT compared to 4°C groups was present. No differences were found between short and prolonged transport between either RT or 4°C groups (**Figure 3.5**).

CFE Zone A

CFE Zone B

CFE Zone C
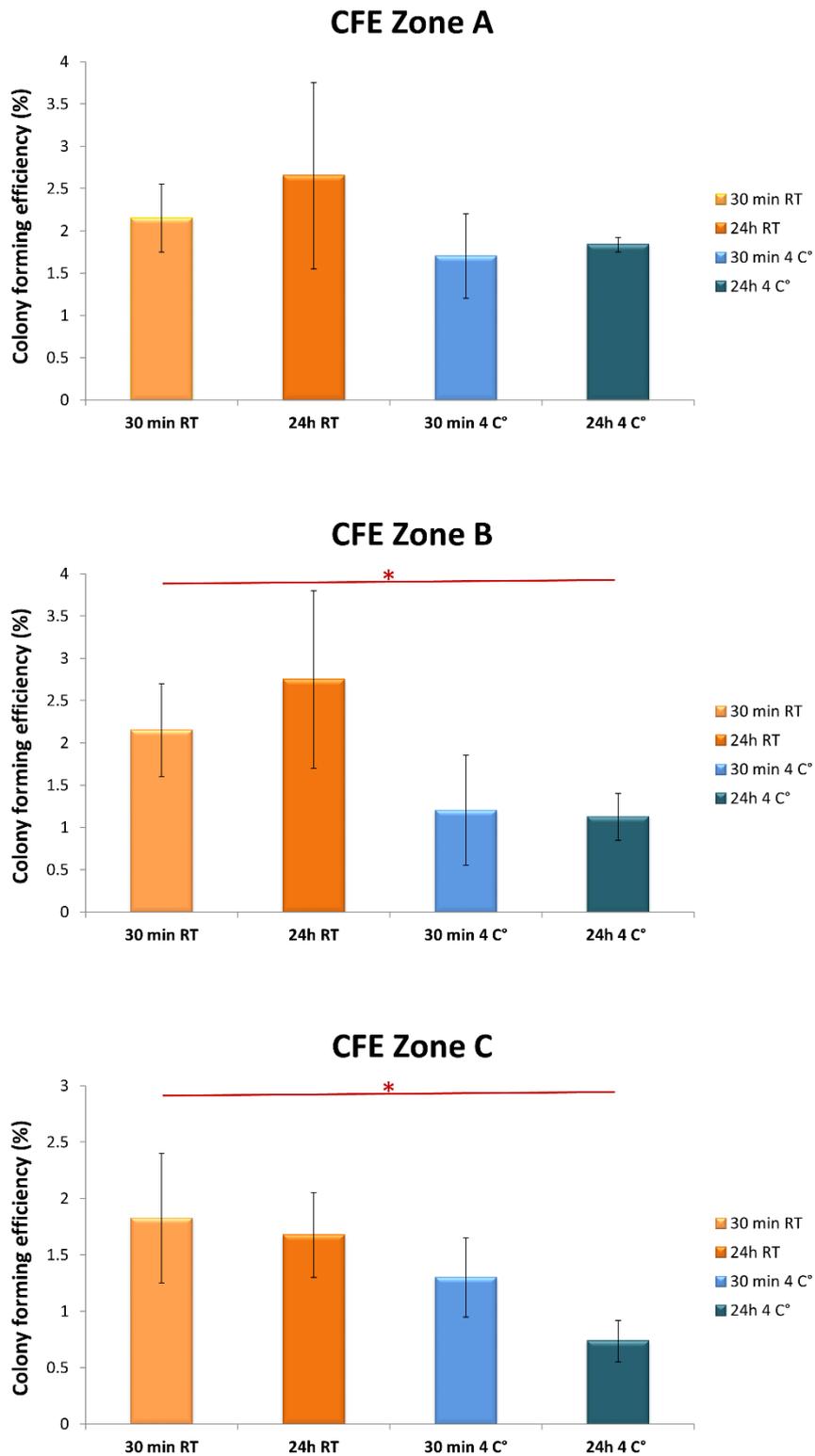
Figure 3.5 Colony forming efficiencies per zone of limbal outgrowths after limbal biopsies transport simulation. Data are presented as mean ± SEM, * $p < 0.05$ (n=3).

3.3.2 Limbal epithelial cell marker expression after LBs transport simulation

Expression of putative limbal stem cell marker ΔNp63 in zone A of explant outgrowths is significantly higher in both 30min and 24h RT groups compared to 24h 4°C group ($p < 0.01$ and $p < 0.05$ respectively). 24h RT group show also significantly higher expression of ΔNp63 in zone A compared to 24h 4°C transport group (**Figure 3.6**).

In terms of ΔNp63 expression in zones C and B, similar pattern of differences between the groups was observed. Prolonged 24h transport on 4°C showed significantly lower expression of ΔNp63 compared to 30min RT, 24h RT and 30 min 4°C groups ($p < 0.05$ in all three cases in zone B; $p < 0.001$; $p < 0.001$ and $p < 0.05$ in zone C respectively) (**Figure 3.6**).
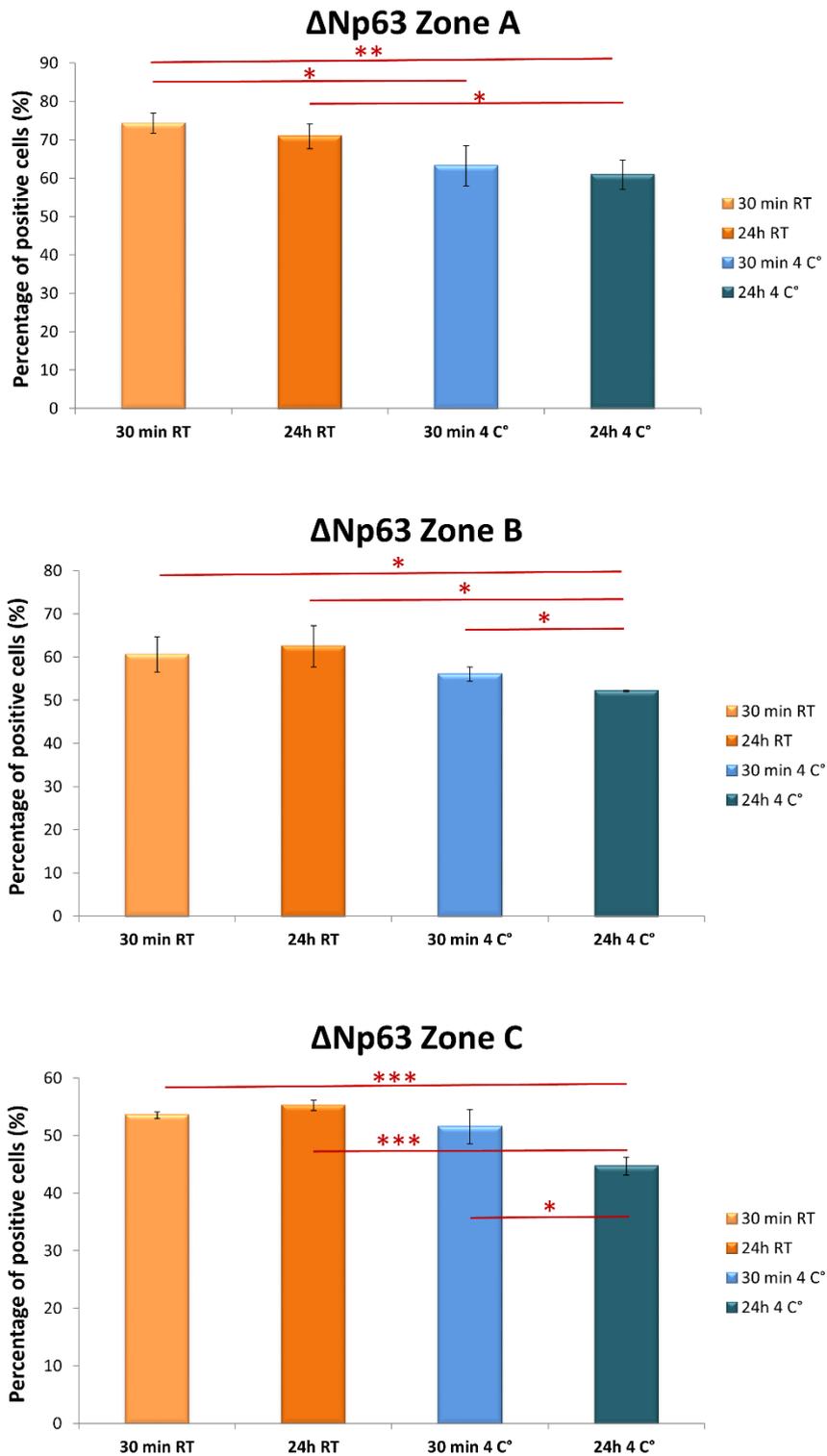
Figure 3.6 Expression of putative limbal stem cell marker ΔNp63 per zone of limbal outgrowths after limbal biopsies transport simulation. Data are presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n=3).

Differentiation marker CK3 differs significantly between the groups across all the zones (**Figure 3.7**), while there were no significant differences or trends observed in expression of the other corneal differentiation marker, Connexin 43 (**Figure 3.8**).

In zone A, CK3 expression was the lowest in 30min RT group. 24h RT, 30min 4°C and 24h 4°C group all showed higher CK3 expression compared to 30min RT ($p < 0.05$; $p < 0.05$ and $p < 0.01$ respectively). Moreover, both 30 min and 24h 4°C groups also showed higher expression of CK3 in zone A from 24h RT transport group (**Figure 3.7**). Similarly, in zone B and zone C, 4°C transport led to higher CK3 expression in comparison with RT conditions. In zone B, 30min 4°C transport showed higher expression of CK3 than 30min and 24h RT group ($p < 0.001$) and the difference between 24h 4°C and both RT groups was even more significant ($p < 0.0001$). Finally in zone C, CK3 expression was significantly higher in 30min 4°C and 24h 4°C compared with 30min RT ($p < 0.0001$ and $p < 0.05$ respectively) (**Figure 3.7**).
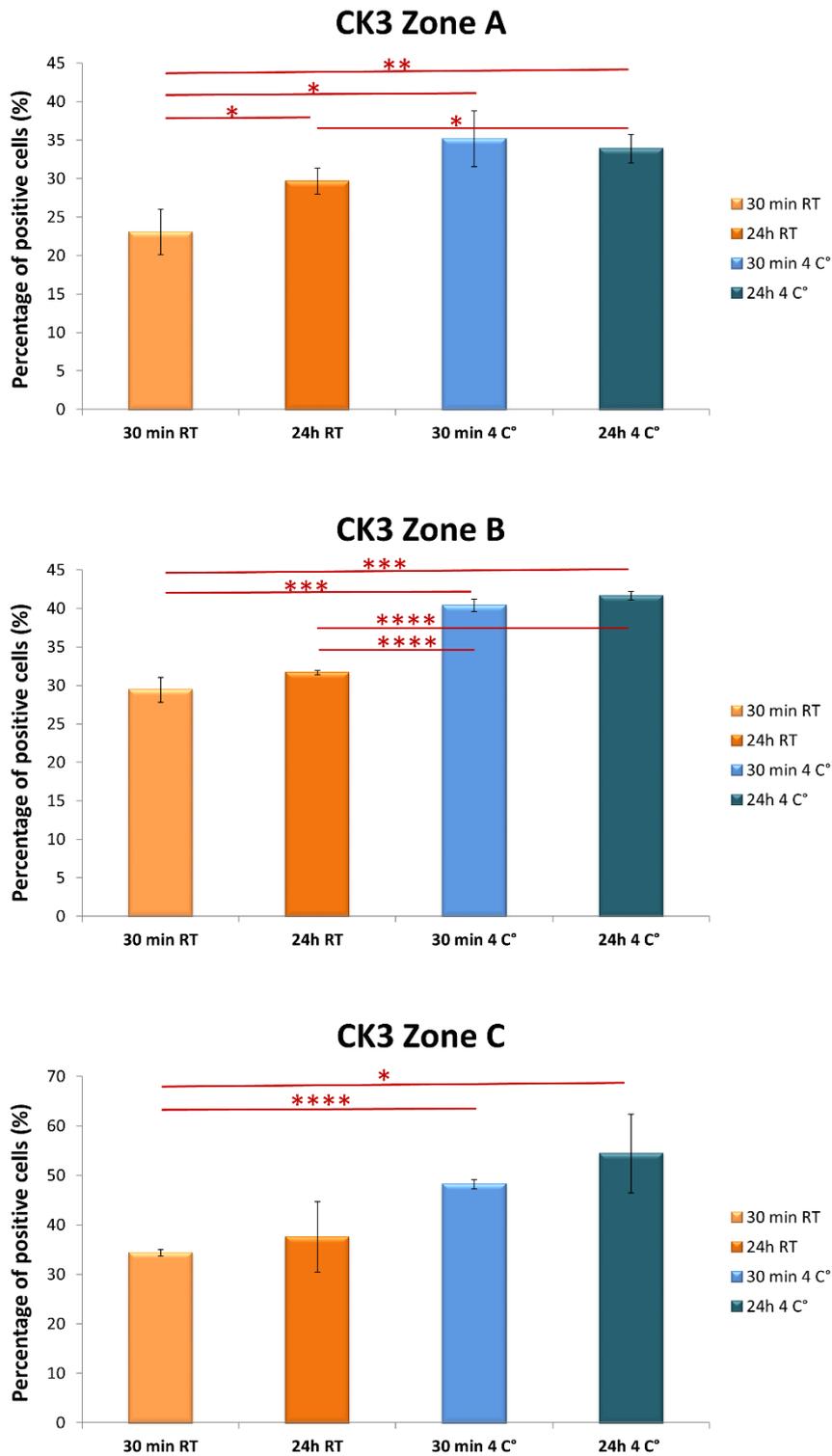
Figure 3.7 Expression of corneal differentiation marker Cytokeratin 3 per zone of limbal outgrowths after limbal biopsies transport simulation. Data are presented as mean ± SEM, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 (n=3).
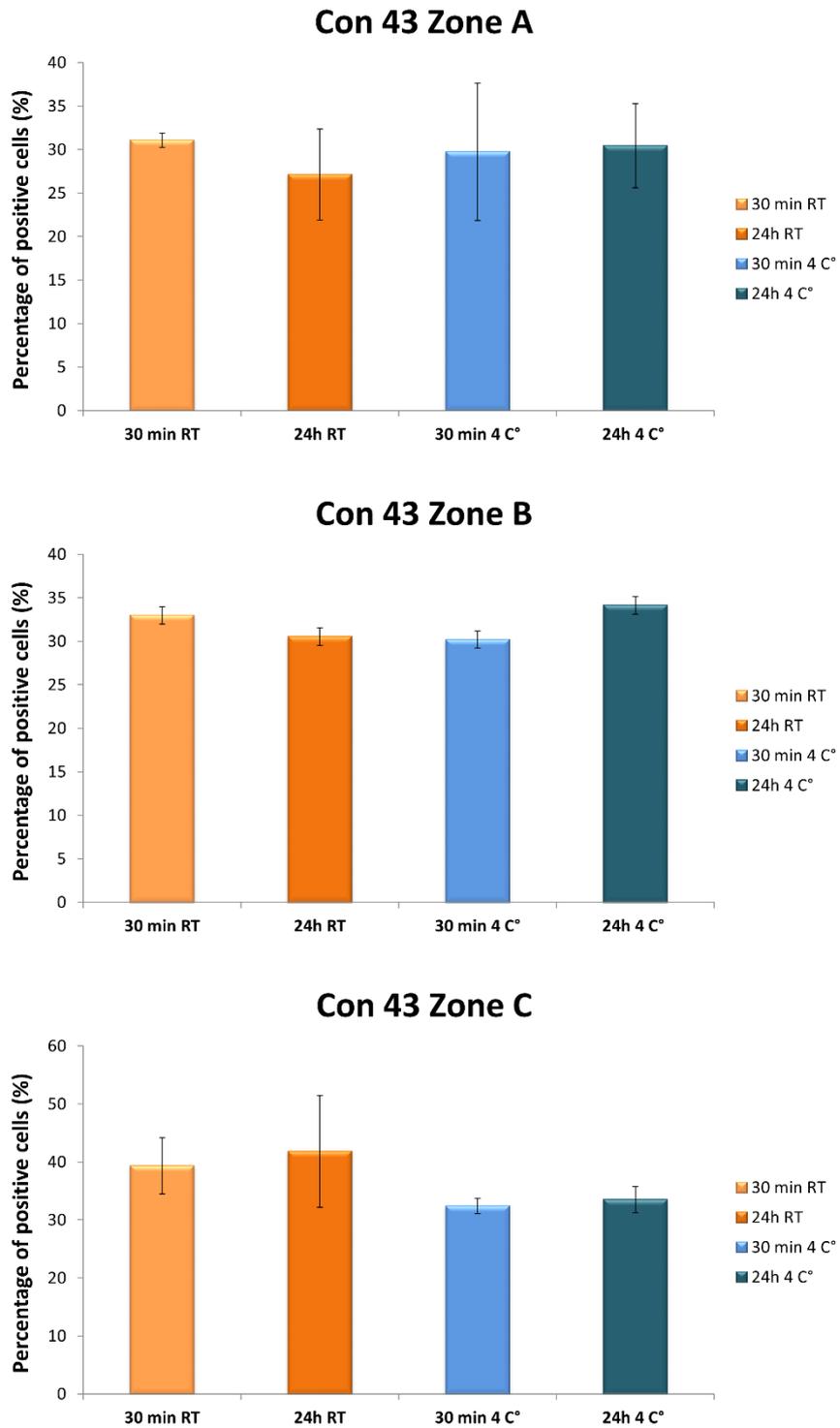
Figure 3.8 Expression of corneal differentiation marker Connexin 43 per zone of limbal outgrowths after limbal biopsies transport simulation. Data are presented as mean ± SEM (n=3).

3.3.3 Limbal explant growth rate, cell number, viability and colony forming efficiency after final product transport simulation

After successful explant cultures were obtained from all limbal biopsies (**Figure 3.9**), expanded limbal epithelial cells on HAM were used for final product transport simulation. All visible differences in growth rate were non-significant, most likely depending on the number of stem cells in the particular piece of cadaveric limbal tissue used for the respective explant culture.

Growth rate - Donor 1
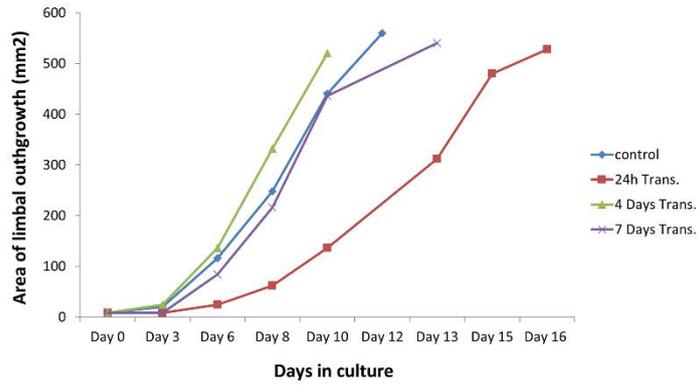
Growth rate - Donor 2
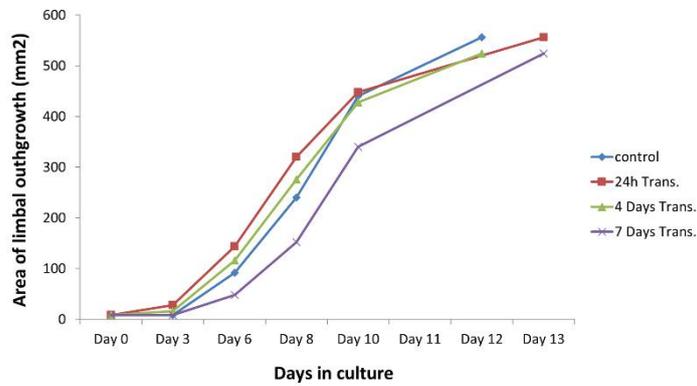
Growth rate - Donor 3
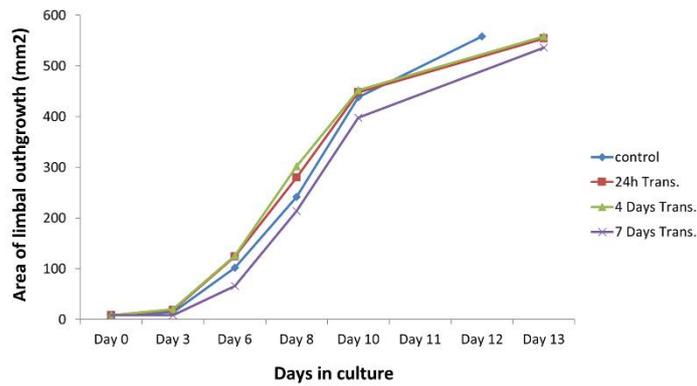
Figure 3.9 Limbal epithelial cell growth across different final product transport condition groups. Schematic graphs are showing the area of explant outgrowth (mm^2) on different days of culture; (n=3).

With regard of number of cells per zone, control had significantly higher number cells in zone A and zone C compared to 4 days transport group (p<0.05 and p<0.001 respectively) and in zone B and zone C compared to 7 days transport group (p<0.05 and p<0.0001 respectively). There was no significant difference between cell numbers between control and 24h transport group in any of the zones. 4 days compared to 7 days transport group showed higher number of cells in zone B (p<0.01) (**Figure 3.10**).

Cell viability was significantly lower in 4 and 7 days transport groups compared to control across all the zones (p<0.05) whilst viability in 24h transport group was affected only in zone A compared to control (p<0.05). However, viability in zone A of 24h transport group was significantly lower than in 7 days transport group (p<0.05) (**Figure 3.10**).

Number of cells per zone

Cell viability per zone
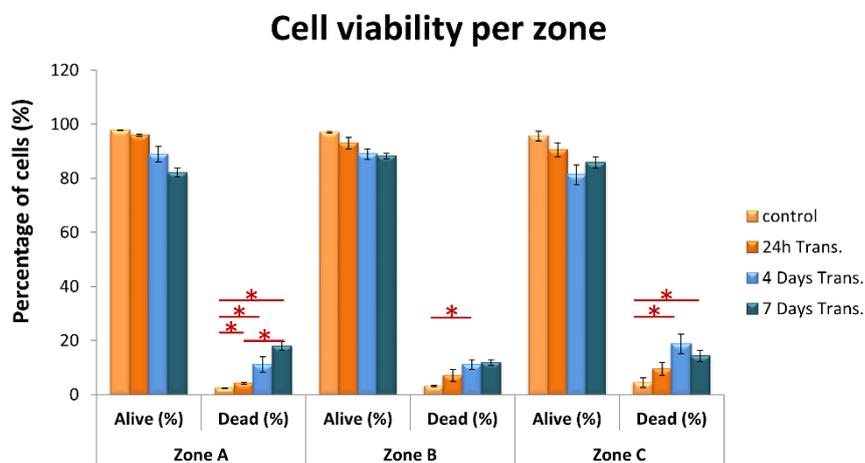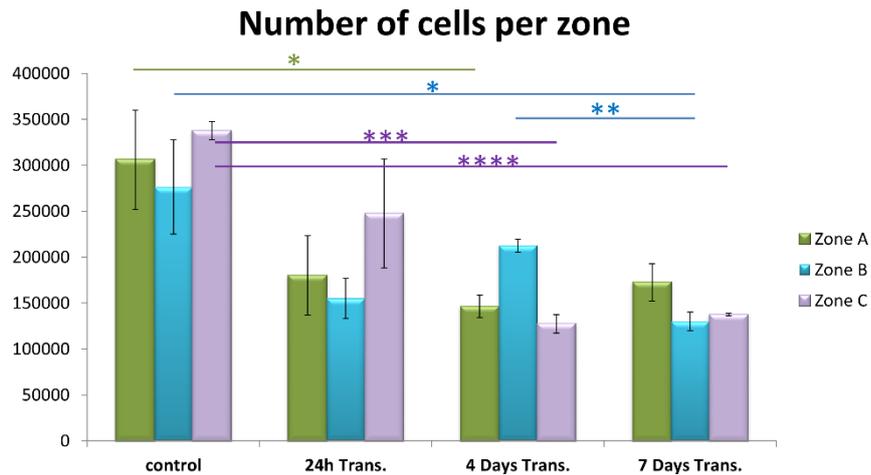
Figure 3.10 Number of cells and cell viability per zone of limbal outgrowths after final product transport simulation. Data are presented as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (n=3).

Interestingly, CFE was higher in 4 and 7 days transport groups in all the zones compared to control and 24h transport, with the highest CFE showed after 7 days of transport simulation. The difference was significant between 7 days and 24h transport in all three zones ($p<0.05$) and 4 days and 24h transport in zones A and B ($p<0.05$). 24h transport group had the lowest CFE, significantly lower not only from 4 and 7 days transport groups but also lower than control especially in zone B where the difference is significant ($p<0.05$) (**Figure 3.11**).
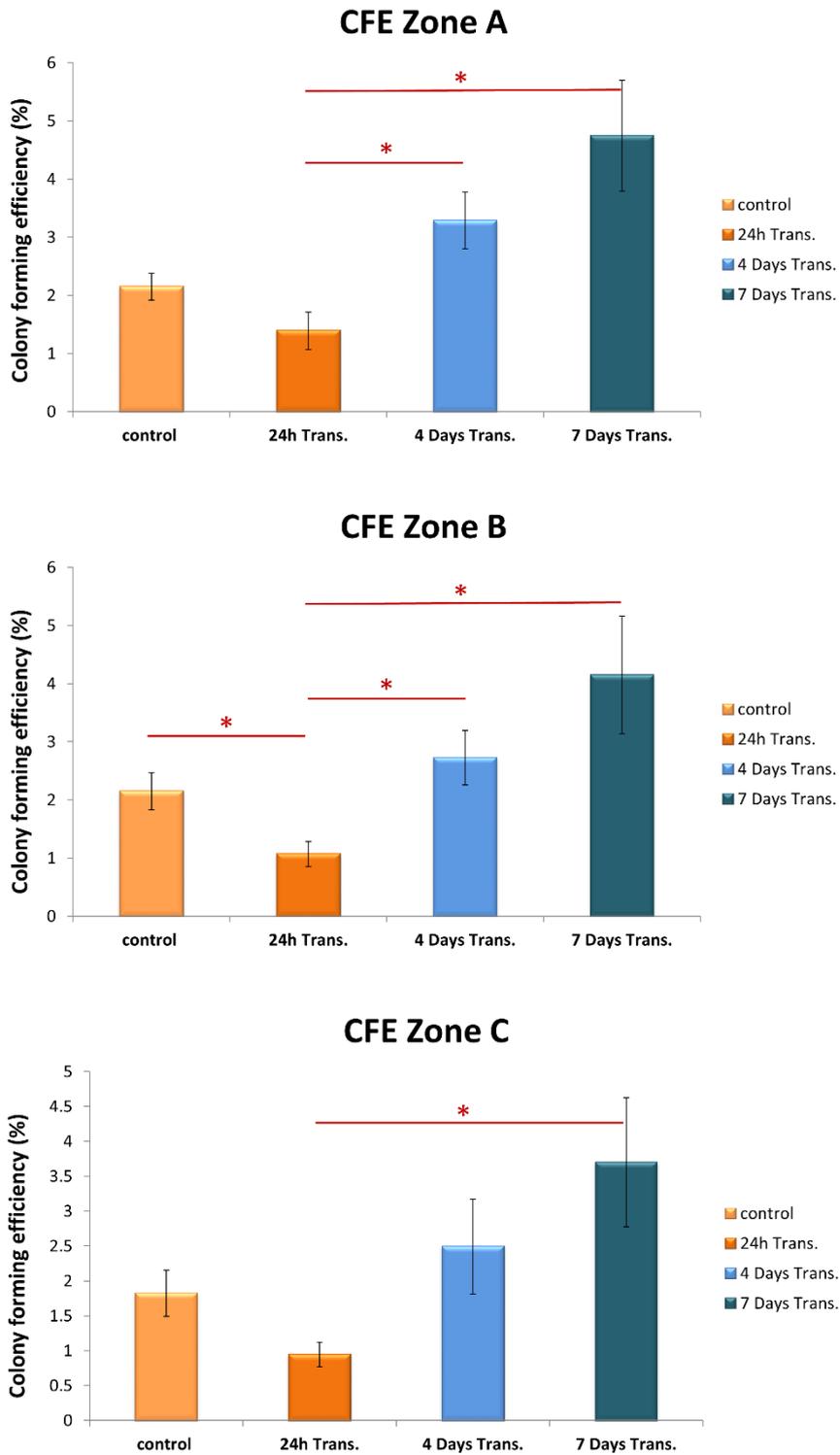
Figure 3.11 Colony forming efficiencies per zone of limbal outgrowths after final product transport simulation. Data are presented as mean ± SEM, * $p < 0.05$ (n=3).

3.3.4 Limbal epithelial cell marker expression after final product transport simulation

Several significant differences were found between the groups related to marker expression but scattered throughout zones and not logically correlated. For example significantly higher expression of limbal stem cell marker ΔNp63 was found in control compared to 24h transport group ($p<0.05$) but on the same time expression of Connexin 43 which is a marker of corneal differentiation was also significantly higher in control compared to 24h transport group ($p<0.01$) (**Figure 3.12 and 3.13**). Other differences found for the expression of putative stem cell marker ΔNp63 (**Figure 3.12**) and corneal differentiation markers CK3 (**Figure 3.13**) and Connexin 43 (**Figure 3.14**) were also inconsistent and for that reason they will not be commented in details.
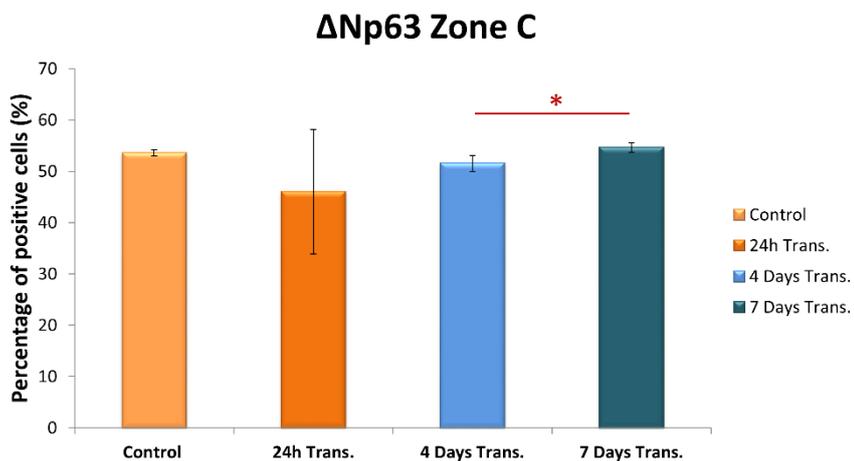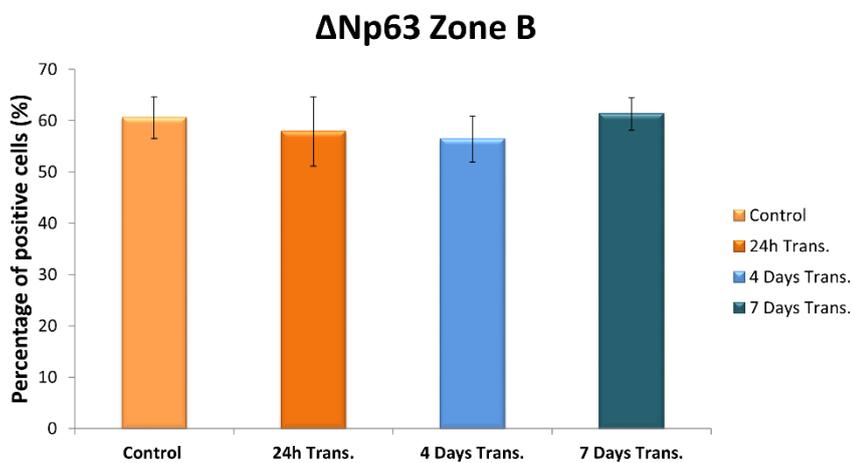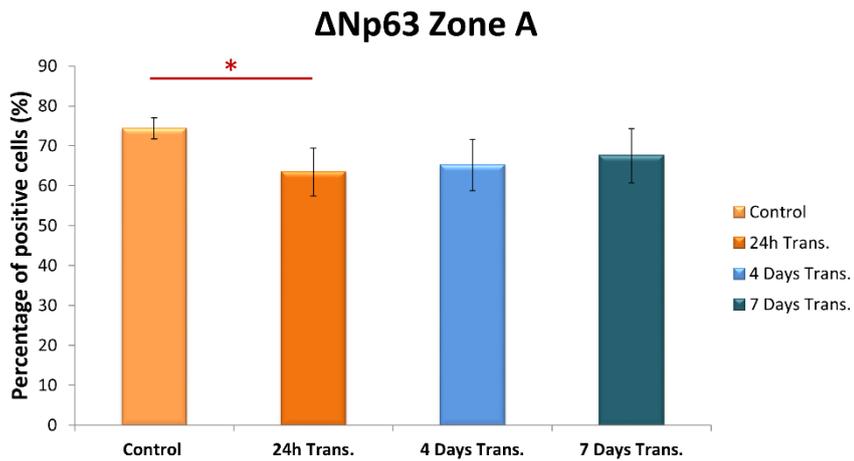
Figure 3.12 Expression of putative limbal stem cell marker ΔNp63 per zone of limbal outgrowths after final product transport simulation. Data are presented as mean ± SEM, * p < 0.05 (n=3).

CK3 Zone A

Percentage of positive cells (%)

45
40
35
30
25
20
15
10
5
0

Control   24h Trans.   4 Days Trans.   7 Days Trans.

Control
24h Trans.
4 Days Trans.
7 Days Trans.

CK3 Zone B

Percentage of positive cells (%)

45
40
35
30
25
20
15
10
5
0

*

Control   24h Trans.   4 Days Trans.   7 Days Trans.

Control
24h Trans.
4 Days Trans.
7 Days Trans.

CK3 Zone C

Percentage of positive cells (%)

60
50
40
30
20
10
0

*

Control   24h Trans.   4 Days Trans.   7 Days Trans.

Control
24h Trans.
4 Days Trans.
7 Days Trans.
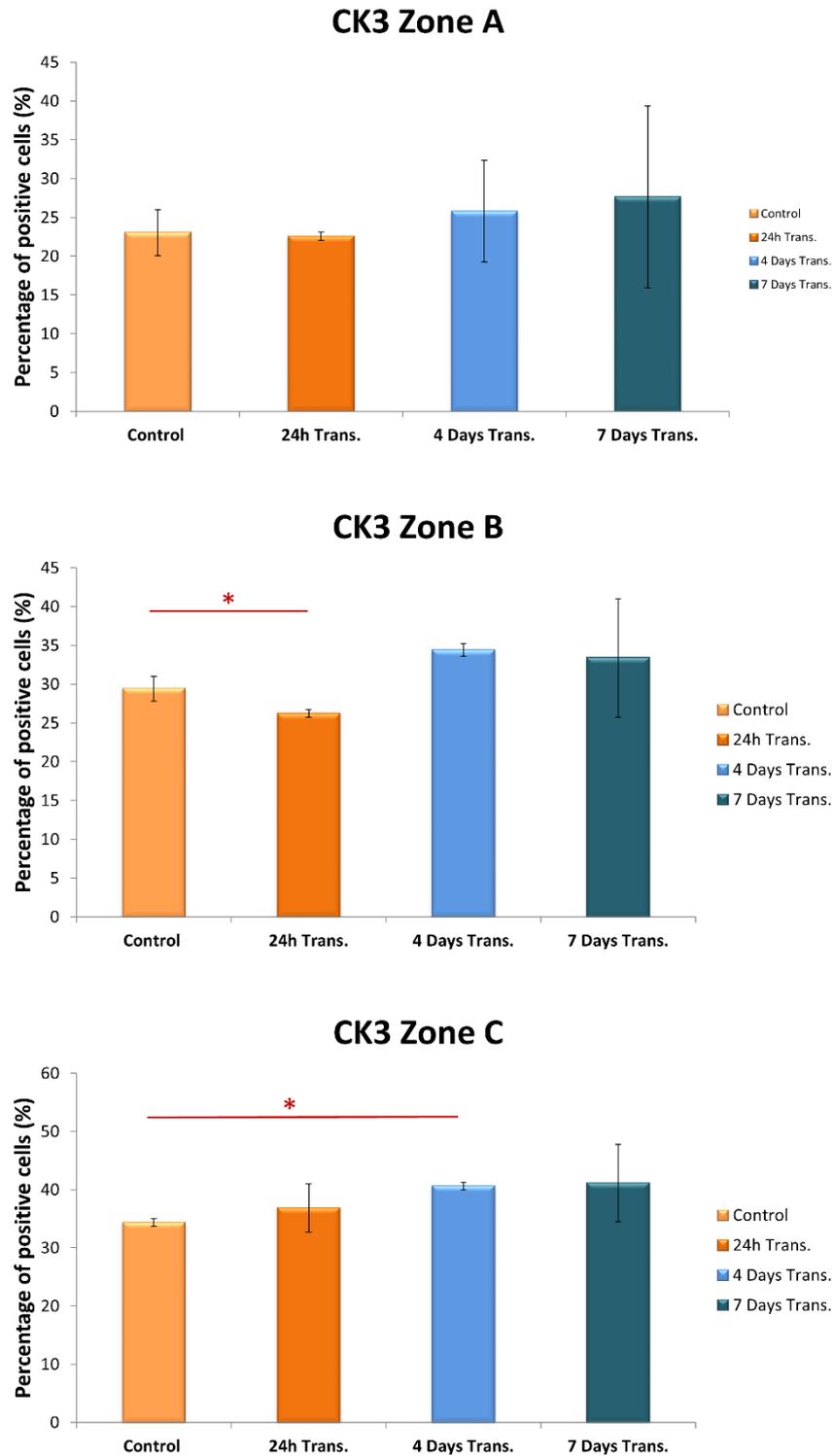
Figure 3.13 Expression of corneal differentiation marker Cytokeratin 3 per zone of limbal outgrowths after final product transport simulation. Data are presented as mean ± SEM, * $p < 0.05$ (n=3).

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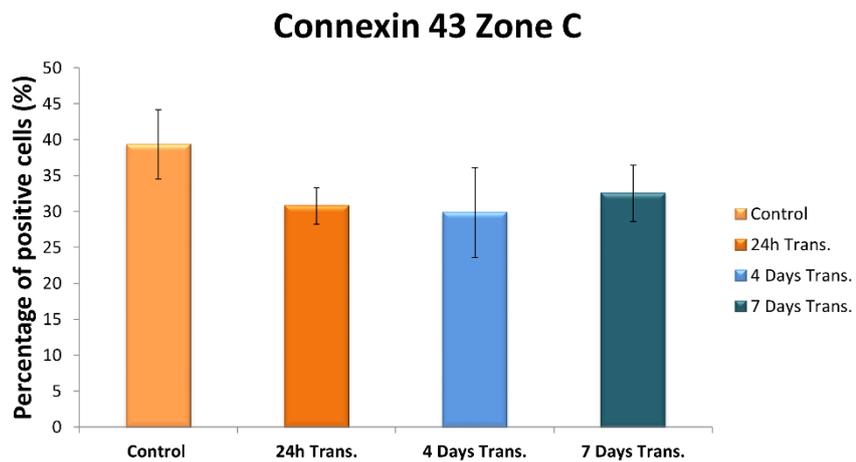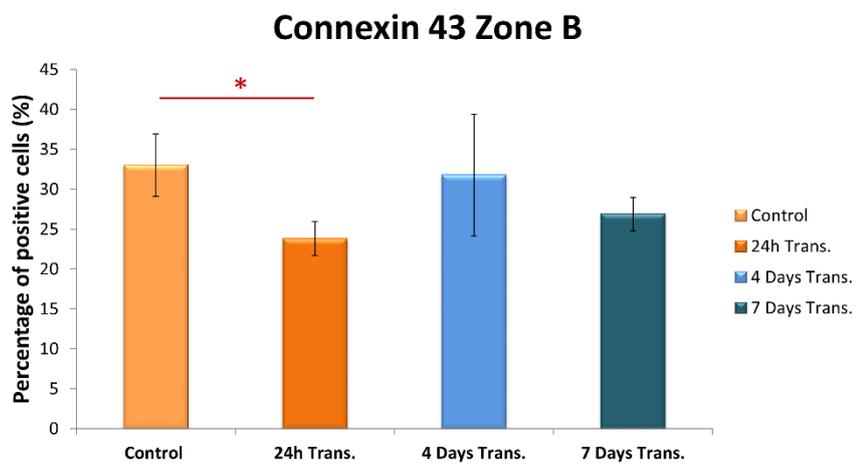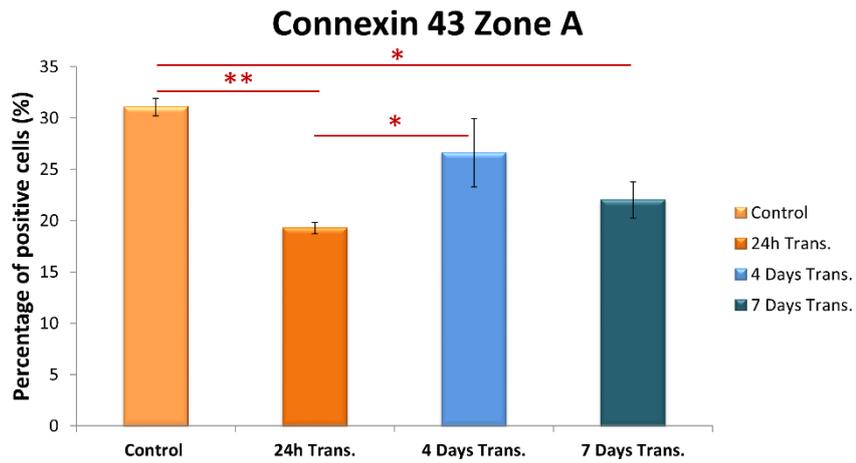
Figure 3.14 Expression of corneal differentiation marker Connexin 43 per zone of limbal outgrowths after final product transport simulation. Data are presented as mean ± SEM, * p < 0.05, ** p < 0.01 (n=3).

3.4 Discussion

LSC transplantation has been shown to reverse the signs of deficiency of these cells by restoring a corneal epithelial phenotype. Unilateral LSCD can be treated with autologous LSC transplantation, but in case of bilateral LSCD, only allogeneic transplantation is possible and the donor limbal tissue must be taken from a living relative or a cadaver (Tan *et al.*, 1996; Djalilian *et al.*, 2005; Fernandez-Buenaga *et al.*, 2018). In our study we used human cadaveric limbal tissue as an alternative to real LBs obtained from living donors, which are taken only in strictly indicated occasions.

The two basic approaches to store whole corneas for grafting are hypothermia and organ culture. Procedural simplicity and the immediate availability of tissue for transplantation make hypothermia the most widely used method (Pels *et al.*, 2008). The recommended maximum storage time under hypothermic conditions (2–6°C) is up to 14 days. Organ culture storage is used in about 65% of European eye banks because of the possibility of performing a detailed assessment of the corneal endothelium and extending storage time up to 4–5 weeks (Pels *et al.*, 2008).

The effect of different storage temperatures on the maintenance of LEC phenotype and function was studied by Raeder *et al.* The research group evaluated effects of 1 week storage of human limbal epithelial cultures in conventional organ culture (at 31°C or 23°C) and hypothermic storage (at 5°C) in Optisol-GS (Raeder *et al.*, 2007). They reported that although cultured human LECs remained undifferentiated in all storage conditions, the ultrastructure was better preserved at 23°C, while storage at 31°C and 5°C was associated with enlarged intercellular spaces, separation of desmosomes, and detachment of epithelial cells (Raeder *et al.*, 2007). After they identified the temperature of 23ºC as an optimal for LECs storage they evaluated viability, morphology and phenotype of cultured human LECs on HAM following 2 and 3 weeks of organ culture storage (Utheim *et al.*, 2009). Although a less differentiated phenotype was maintained, the multi-layered structure was lost after 3 weeks whilst preserved in around 70% of cultures following 3 week storage along (Utheim *et al.*, 2009). They concluded that even with a slight decrease in viability the human LEC sheets remain acceptable, which was not the case with 3 week storage which results was unsatisfactory (Utheim *et al.*, 2009). Furthermore, they reported that the storage conditions with 23ºC temperature in either serum-free medium

(Quantum 286) or xenobiotic-free medium (Minimal Essential Medium-MEM) protected against cell death, loss of ultrastructure and differentiation of cells for 4-7 days period (Utheim et al., 2015).

The same group also studied effect of different storage temperatures on cultured human conjunctival (Eidet et al., 2012; Eidet et al., 2015) and epidermal cell sheets (Jackson et al., 2014; Jackson et al., 2016; Jackson et al., 2017). They reported that human conjunctival epithelial cells may be stored for at least 4 days in serum free conditions at 23ºC while maintaining the cell phenotype and viability (Eidet et al., 2012). Furthermore, they later evaluated effect of nine different storage temperatures between 4ºC and 37ºC for 4 days and 7 days storage of cultured human conjunctival epithelium (Eidet et al., 2015) and cultured epidermal cell sheets (Jackson et al., 2014). Storage at 12ºC appeared to be optimal for preserving the morphology, viability and total cell number of cultured human conjunctival epithelium. These superior cell preservation on 12ºC may be related to temperature associated effects on cell metabolism (Eidet et al., 2015). In terms of preservation of cultured epidermal cell sheets their initial results indicated that 12ºC and 24ºC storage temperature represent the prospective optimum temperature for short term storage of these cell sheets (Jackson et al., 2014). 24ºC temperature showed the clear advantage on cell viability whilst 12ºC were particularly favourable in terms of maintenance of proliferative capacity and morphology of cultured cells after storage (Jackson et al., 2014). In their next study, they further reported that lower storage temperatures, in particular 12ºC, showed to be superior in terms the maintenance of the original phenotype of cultured epidermal cells (Jackson et al., 2014). Their third study of different storage temperatures from 2017 reported retention of undifferentiated cell phenotype on 12ºC, transition to differentiation at 16ºC and increased differentiation at 24ºC and confirmed that 12ºC temperature may be ideal for storage of cultivated epidermal cell sheets (Jackson et al., 2017).

These studies clearly show that the choice of storage temperature has an important role in preservation of epithelial morphology on cultivated epithelial cells. In our study, we were interested in effect of the choice of different temperatures even before LEC cultivation, during transportation of LBs. As most eye banks in the world use either hypothermic storage (2-6ºC) or organ culture storage (31-37ºC) we decided to compare two different temperatures for LBs transportation, 4ºC and room temperature, similarly to storage temperatures in the two main approaches of the

corneal storage. The reason behind choosing RT instead of 31ºC was the fact that in 2007 two studies reported that organ culture storage of cultured LECs on HAM at ambient temperature is superior to organ culture storage at 31ºC with minimal apoptosis after storage (Raeder *et al.*, 2007; Utheim *et al.*, 2007). We compared effects of these temperatures during short (30 minutes) transport and prolonged (24 hours) transport of LBs.

We demonstrated that cultivation of LBs transported at either RT or 4ºC will results in their successful growth on HAM after both short and long term transportation with no difference in regard of cell viability of the outgrowths. However, RT transport proved to be superior compared to 4ºC transport in terms of growth rate and preservation of undifferentiated cell phenotype. Besides better CFEs, LBs transported at RT showed presence of higher percentage of putative stem cells (higher expression of ΔNp63) and lower percentage of differentiated cells (lower CK3 expression) in comparison with LBs transported at 4ºC. Short and long transportation at RT proved to be equally efficient, whilst between short and long transportation at 4ºC there was a significant difference in expression of ΔNp63 suggesting that long 4ºC transportation is the least convenient option for preservation of LSCs within LBs.

Our results are in accordance with a study that showed retention of proliferative function at 4ºC but also showed low cell viability, as well as cell-cell contact disruption and cell shrinkage, indicative to apoptosis (Jackson *et al.*, 2014). Thus, possible explanation for slower growth in 4ºC transport groups, especially in 24 hours transportation group, may be the lower number of LSCs in LBs transported at low temperature due to increased apoptosis. We could further hypothesize that increased differentiation also may be the result of lower number of LSC in LBs after transportation but this needs to be further investigated.

Further study from the same research group investigated the molecular mechanisms underlying activation of cell death pathways using genome-wide transcriptional analysis in human LECs following 2, 4 and 7 days storage at 4ºC (Utheim *et al.*, 2016). The most upregulated genes after 4 and 7 days hypothermic storage were the histone coding genes HIST1H3A and HIST4H4, involved in a functional network highly associated with cell death, necrosis, and transcription of RNA. The most down regulated gene, HDAC1, encoding histone deacetylase 1, is involved in a regulating

network associated with cellular function and maintenance, differentiation of cells and DNA repair (Utheim *et al.*, 2016).

Additionally, together with the choice of storage temperature, the choice of storage media also plays a role in the preservation of epithelia. For example, Utheim at al. showed that storage in MEM, simple storage medium preserve immature phenotype better than more complex Quantum 286. The possible reason might be the fact that simple media reduces metabolism and therefore potentially decreases differentiation during storage to a higher extent than media with added growth factors, such as Quantum 286 (Utheim *et al.*, 2015). Their findings are in accordance with the other study demonstrating stimulated differentiation of cultured human LECs over time using a complex culture medium (Ghoubay-Benallaoua *et al.*, 2013). Similarly, Oie *et al.* used Hanks' Balanced Salt Solutions (HBSS) as the transportation liquid for human and rabbit cell epithelial sheets air transportation using their special transportation container (Oie *et al.*, 2014). HBSS is a relatively simple buffered solution that does not contain any growth factors but provides cells with water and certain bulk inorganic ions as well as the carbohydrates and glucose essential for cell metabolism.

Development of efficient short-term storage can expand the utility of cultured limbal epithelial sheets by providing an extended window for quality control and sterility testing in centralized culture facilities, wider distribution, flexibility in timing of transplantation surgeries and back-up sheets for repeated operations.

In our study we compared short one day (24 hours) storage in traditional LSC culture medium, with FBS and growth factors added, with prolonged 4 and 7 days storage in simple MEM medium without serum and growth factors.

Cell number and viability of LECs in our study did not differ significantly between 24 hours and 4 and 7 days storage, but the trend of increased cell death with longer storage was observed. Previously it has been shown that both apoptosis and necrosis occur in cells during corneal storage, with apoptosis appearing to predominate (Komuro *et al.*, 1999). The viability of LECs stored for 4 and 7 days was significantly lower in comparison with non-stored controls in all the zones, but still around 80%. Similarly to our findings, Utheim and al. reported that viability of cells after 4 days serum free storage was 97% (Utheim *et al.*, 2015).

Beside the maintenance of cell viability as the first general priority in storage of cells, maintenance of stemness is equally important during cultivated limbal epithelium storage and transportation.

Highly proliferative cycling epidermal progenitor cells are the first to contribute to regeneration following transplantation, while quiescent stem cells provide long-term renewal (Schluter *et al.*, 2011). As the same principle applies to the limbal epithelium, our objective was to maintain an undifferentiated cell phenotype and proliferative capacity within limbal epithelial cell sheets during storage.

In terms of expression of putative stem cell markers and markers of corneal differentiation we didn't find any consistent difference between three different storage groups and control. It seems that all storage conditions preserved stemness in a similar manner.

In terms of colony forming ability, another important aspect of LSCs present in limbal epithelial sheets, the trend of lower CFE was obvious in cells stored for 24 hours in culture media compared to non-stored control cells, with significant difference in zone B. Surprisingly both 4 days and 7 days storage groups showed significantly higher CFE than 24 hours storage group in all three outgrowth zones. These phenomenon may be explained by serum starvation of stored LECs on HAM in serum free media for 4 and 7 days and their consequential arrest in the G1 phase of the cell cycle which was reversed upon returning into FBS supplemented culture media. Currently, this is hypothesis that needs to be verified experimentally.

Multiple studies have been published on the various aspects of storage of cultured LECs (Kito *et al.*, 2005; Oh *et al.*, 2007; Raeder *et al.*, 2007; Utheim *et al.*, 2007; Yeh *et al.*, 2008; Utheim *et al.*, 2009; Wright *et al.*, 2012; Utheim *et al.*, 2015) while transportation of epithelial sheets for ocular surface reconstruction has been studies to a limited extent. Transport is different from storage in the sense that the tissue is exposed to movement that unlike other environmental factors cannot be eliminated by a sealed transport container (Utheim *et al.*, 2018b). Vasania *et al.* tested in-house designed transportation container for cultured conjunctival epithelial cell sheets on HAM (Vasania *et al.*, 2014). They reported presence of viable, intact epithelial sheets upon arrival and good post-operative outcome for pterygium surgery (Vasania *et al.*, 2014). Oie *et al.* developed a transportation container with three basic functions: the maintenance of interior temperature, air pressure and sterility (Oie *et al.*, 2014).

Cultured human oral mucosa and rabbit LECs were successfully transported in the container for 5 hours in the airplane. After the transport, lower expression of zonula occludens-1 (ZO-1) was observed, suggesting that transportation may cause a reduction in intercellular adherence and barrier function (Oie *et al.*, 2014). Utheim *et al.* simulated extreme transport conditions followed by a storage period and found that transport simulation of up to 36 hours appeared not to be critical to the viability, ultrastructure and phenotype of human LECs with a completely filled transportation container (Utheim *et al.*, 2018b).

3.5 Conclusion

The choice of transportation temperature has an important role in preservation of LECs proliferation and phenotype. Here we demonstrated that LBs, as a starting material for limbal epithelial sheets production, may be efficiently transported within 24 hours at room temperature from hospital where the biopsy was taken to CPC facility for further cultivation.

Additionally, we showed that the final product, limbal epithelial sheets, may be stored up to 7 days in simple serum free medium without significant decrease in cell viability and proliferative capacity and without any negative effect on their undifferentiated phenotype.

Chapter 4. Towards identification of novel limbal epithelial stem cell surface markers

4.1 Introduction

4.1.1 Challenges in LSC therapy

As discussed in previous chapters, transplantation of autologous *ex vivo* expanded LSCs is an established and European Medicines Agency authorised treatment for patients with total/severe unilateral LSCD due to ocular surface burns. Currently however, *ex vivo* expansion strategies of limbal and other autologous epithelial stem cell are labour intensive and often lack standardization, largely because it is currently impossible to prospectively isolate pure populations of these cells for research or clinical use. Until this occurs, different centres will likely use specific techniques for isolation and *ex vivo* culture of LSCs in their respective institutions that have been developed and investigated in their individual basic laboratories, rendering it impossible to compare clinical success rates between clinical trials performed in different centres around the world.

4.1.1.1 Identification of LSCs

A major challenge in corneal stem cell biology is the ability to identify stem cells *in vitro* and *in situ* and one of the main controversies in the field relates to the issue of reliable stem cell markers. The ideal LSC marker should not only be able to pinpoint the location of LSC within the epithelium but should also allow isolation, enrichment and molecular characterisation of viable LSC.

LSCs play the vital role in the regeneration of damaged corneal tissue, as discussed in details in Chapter 1. Due to the limited options available to treat corneal scars,

their isolation and identification received much attention as they have potential for autologous, cell-based approaches for treatment of damaged cornea. In the last decade LSCs transplantation has been successfully applied in the clinic. The LSC frequency significantly influences the outcome of corneal transplantation (Rama *et al.*, 2010). The study by Rama *et al.* showed that more than 3% of p63 positive cells out of the total number of transplanted cells led to a successful rate of 78%. Less than 3% of p63 positive cells, on the other hand, led to a much lower successful rate of only 11% (Rama *et al.*, 2010). The accuracy of LSC identification is therefore urgent for successful corneal transplantation. Up to date many putative LSC markers have been reported in the literature (page 33, **Table 1.1**), including cytoskeletal proteins, cell adhesion molecules, cell cycle regulators, enzymes, growth factors and their receptors, ATP-binding cassette transporters and differentiation associated markers (Chen *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005). However, there are still no LSC reliable specific biomarkers reported for accurate identification yet.

LSC identification is usually based on the following methods:

➢ Co-expression of putative positive LSC markers (such as p63, ABCG2, ABCB5, integrin α9, vimentin etc.) and negative LSC markers (Connexin 43, CK3, CK12, involucrin etc.);

➢ Morphologic criteria (cell size and nucleocytoplasmic ratio (N/C) ≥ 0.7): As shown by flow cytometry and confocal microscopy, the smallest cells are located in the limbal basal epithelium compared to the basal corneal epithelium (10.1±0.8 vs. 17.1±0.8 µm) (Romano *et al.*, 2003). Similarly, it has been shown that N/C ratio of LSCs lying on the basal membrane was higher than N/C ratio of TACs and corneal epithelial cells (Schlotzer-Schrehardt and Kruse, 2005). Moreover, LSCs were successfully identified and quantified based on ABCG2 expression and N/C ≥ 0.7 (Priya *et al.*, 2013). Additionally using the same principle, Kasinathan *et al.* established a two-step protocol for LSC enrichment by combining basal cell isolation and laser capture microdissection of small cells with N/C ≥ 0.7. Using the protocol they achieved 76-78% enrichment of LSCs from 2% LSCs in total limbal epithelial cells (Kasinathan *et al.*, 2016);

➢ Label retaining property: The BrdU-based "pulse-chase" experiment has been widely used for stem cell identification. Using the fact that BrdU can be incorporated into newly synthetized DNA molecule instead of thymidine, after

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a period of BrdU pulse all cells with different degree of differentiation can be labelled by BrdU. As a result of slow cell cycling of stem cells, fewer divisions of labelled LSCs compared to differentiated cells will happen in the same time. Hence, after a period of BrdU chase, the BrdU retaining cells can be considered as LSCs (Arpitha *et al.*, 2008). From the percentage of radiolabelled thymidine retaining cells present in the limbal zone, it has been concluded that stem cells may represent less than 10% of the total limbal basal cell population (Lavker *et al.*, 1991). In 1998 Lehrer *et al.* showed that the limbal basal epithelium contains both slow-cycling stem cells and early TACs;

➢ Side population (SP) phenotype: Most cells accumulate fluorescent dyes such as Hoechst 33342, but a subset of "dull cells" is often found and termed the "side population". The SP cells are identified according to their ability to efflux the Hoechst dye at a higher pace than the remaining cells termed the main population (non-SP). The Hoechst 33342 efflux activity results from the specific stem cell protein expression, such as ATP binding cassette transporters expression (Goodell, 2005). The SP assay has emerged as a promising method for identifying stem cell and progenitor populations in different tissues, particularly in the absence of specific cell-surface markers (Golebiewska *et al.*, 2011). Goodell *et al.* successfully isolated mice bone marrow stem cells using FACS based on Hoechst-SP method (Goodell, 2005) while Shaharuddin *et al.* successfully isolated LSCs (Shaharuddin *et al.*, 2014).

4.1.1.2 Difficulties in the discovery of specific LSC biomarkers

4.1.1.2.1 Particularity of limbal structure and phenotype

Limbus represents the narrow band of tissue with unclear boundaries with to cornea and conjunctiva. Although there are some histological differences between the cornea and limbus, such as the presence of palisades of Vogt in limbal epithelium and more layers of epithelial cells compared to cornea (Mariappan *et al.*, 2010), it is

still difficult to distinguish corneal epithelium from limbal epithelium. Moreover, limbal epithelial cells share some molecular markers with both corneal and conjunctival epithelial cells that together with many controversial reports in the literature make their identification a very difficult task. For example, as reported by different authors CK19 and CK13 can serve as markers of conjunctival epithelial cells (Donisi *et al.*, 2003; Poli *et al.*, 2015), but other author showed that CK19 expression was also detected both in limbal and corneal epithelial cells (Chen *et al.*, 2004). Similarly, although Ramirez-Miranda *et al.* showed that CK13 was more specific than CK19 in terms of identification of conjunctival epithelial cells (Ramirez-Miranda *et al.*, 2011), Poli *et al.* showed that CK13 was expressed in both the conjunctival epithelium and the suprabasal and superficial layers of the limbal epithelium (Poli *et al.*, 2015). Although limbus could be distinguished from conjunctiva based on the presence of goblet cells, which are used to diagnose LSCD (Puangsricharern and Tseng, 1995), some conditions such as Stevens-Johnsons syndrome and long-term administration of glaucoma drugs could lead to LSCD with no goblet cell invasion (Poli *et al.*, 2015).

While CK12 is expressed in corneal but not in conjunctival epithelium (Chen *et al.*, 1994) and may be used to distinguish them, it is not clear if its expression is limited to central corneal epithelium or presents also in the limbal epithelium. Namely, Latta *et al.* showed that CK12 was specifically expressed in corneal epithelial cells but not in limbal epithelial cells (Latta *et al.*, 2018) but, on the other hand, Ramirez-Miranda *et al.* reported that CK12 was not only expressed in corneal epithelial cells but also in limbal epithelial cells except in the basal layer (Ramirez-Miranda *et al.*, 2011).

4.1.1.2.2 Enzyme digestion impact

The limbal epithelial suspension used for the isolation of LSCs is usually prepared from whole mass limbal tissue by enzymatic digestion using either dispase II, collagenase A or trypsin (Espana *et al.*, 2003; Chen *et al.*, 2011; Tovell *et al.*, 2015; Shirzadeh and Heidari Keshel, 2018). Dispase II separates the limbal epithelium from the stroma by destruction of the BM, collagenase A disrupts the cellular connection in a limbal niche by digestion of extracellular matrix while trypsin separates a cells mass into a single cell suspension. Enzyme treatment may significantly influences gene and protein expression profile of LSC. Optimization of digest conditions including

digestion time and concentration of enzymes to avoid enzyme-induced destruction of the LSC integrity has been reported in many studies (Stasi *et al.*, 2014; Lopez-Paniagua *et al.*, 2016).

4.1.1.2.3 Stem cell heterogeneity

Heterogeneity has been reported in different type of stem cells, such as embryonic stem cells (Luo *et al.*, 2018), mesenchymal stem cells (Du *et al.*, 2016) but also LSCs (Hayashida *et al.*, 2010). An obvious problem with current LSC clinical treatments is that transplanted cells are a heterogeneous cell population containing many cell types in addition to LSCs (ranging from epithelial, stromal stem and progenitor cells, conjunctival and corneal epithelial cells and blood or vascular cells) that significantly affect safety and efficiency of treatment. This was best highlighted by a landmark study published by Rama *et al.* who showed that successful corneal regeneration was strongly correlated with the presence of more than 3% holoclone-forming (ΔNp63α-bright) cells in *ex vivo* expanded cultures used for grafting of patients with LSCD (Rama *et al.*, 2010). Various studies have described morphological characteristics of LSCs (i.e. small cell size, pigmentation and high nuclear to cytoplasmic ratio) (Chen *et al.*, 2004), their slow cycling nature and location within clusters at palisades of Vogt (Dua *et al.*, 2005); however these factors have not been linked with LSC function and outcome of transplantation; hence harvesting a specific and purified sub-population of these cells remains a major challenge. Several key putative markers have already been identified including ΔNp63α, ABCG2, ABCB5, C/EBPδ, Bmi1 and Notch-1 among others (Pellegrini *et al.*, 2001; Schlotzer-Schrehardt and Kruse, 2005; Joe and Yeung, 2014; Ksander *et al.*, 2014); however it is unclear whether these proteins are expressed by different LSC sub-populations or different LSC subsets within each population marked by a single putative marker.

Stem cell heterogeneity has been well described in various stem cell compartments including blood, skin and intestinal epithelium pointing to the concomitant existence of multiple types of stem cells with distinct everyday roles (Goodell *et al.*, 2015). From these studies it has also emerged that these different stem cell types are more adaptable than previously thought, in that they have a 'default' role under normal conditions, however following perturbation, such as stimulation by injury, they can

fulfil distinct functions when required (Goodell *et al.*, 2015). Some tissues may contain rapidly-cycling, committed progenitors which are responsible for the majority of tissue maintenance, as well as a population of slow-cycling stem cells which maintain a higher degree of stemness and can act as alternative source of stem cells in response to injury and stress (Li and Clevers, 2010). To date, it is not yet known whether corneal epithelium is also maintained by a combination of such quiescent and cycling progenitors, however it is interesting to note that in the human cornea two different sub-populations have been identified: (1) Bmi1$^+$, C/EBPδ$^+$ and ΔNp63α$^+$ mitotically quiescent LSCs which generate holoclones in culture and (2) Bmi1$^-$, C/EBPδ$^-$ and ΔNp63α$^+$ population which respond to injury (Barbaro *et al.*, 2007). It is not known whether LSC heterogeneity extends beyond the presence of these two LSC sub-populations and whether cell surface markers to distinguish between these two subpopulations can be identified.

4.1.1.2.4 Lack of robust LSC isolation technology

Among the total number of limbal epithelial cells there is a small amount of LSCs present, accounting only 0.5 to 10% of cells according to Latta *et al.* (Latta *et al.*, 2018). Existence of such a complex limbal epithelial suspension makes isolation of LSCs in low frequency very complicated task. Some traditional methods of LSCs isolation were previously published, such as FACS (Shaharuddin *et al.*, 2017), magnetic-activated cell sorting (Polisetti *et al.*, 2016) and gradient centrifugation (Krulova *et al.*, 2008). However, all of these methods include enzyme digestion which may influence changes in gene expression patterns and protein profile of LSCs and increase the risk of suspension contamination due to complicated process involved (Emmert-Buck *et al.*, 1996; Espina *et al.*, 2006; Espina *et al.*, 2007).

Isolation of target cells from complex and heterogeneous tissues became possible with a discovery of the laser capture microdissection (LCM) technique by Emmert-Buck *et al.* (Emmert-Buck *et al.*, 1996). Furthermore, a combination of LCM with next generation sequencing (NGS) has become a hot topic of heterogeneity and specific biomarker research (Guo and Zhang, 2018). Bath *et al.* collected cells for NGS from

four different human limbal compartments using LCM providing plentiful information for the study of LSC markers (Bath *et al.*, 2013). Using the same technique Polisetti *et al.* proved the connection between LSC niche cells and cell adhesion molecules (Polisetti *et al.,* 2016). However, LCM poses also some limitations. As thin sections (5-15 μm) cut the cell into several parts resulting in incomplete RNA information that cannot represent a full cell, large number of sections is needed to obtain enough RNA for future studies (Fend *et al.*, 1999; Datta *et al.*, 2015). Moreover, pre-treatment of sections including fixation, dehydration and staining can significantly influence the quality of RNA (Fend *et al.*, 1999) reducing efficiency for subsequent experiments such as NGS or microarray analysis (Gautam *et al.*, 2016).

4.1.1.3 Prospective in LSC biomarker identification

Recently, many reports have demonstrated new biomarkers at the RNA level. Techniques such as NGS opened possibility to obtain mRNA and microRNA information enabling development of mRNA or micro RNA based biomarkers (Guo and Zhang, 2018), providing a new direction for LSC specific biomarkers study.

Previously, the in situ hybridization has been used to localize LSCs (Chen *et al.*, 2004). In 2013 Ke *et al.* developed a new technique for RNA analysis known as *in situ* sequencing (Ke *et al.*, 2013) which enables gaining of target RNA sequence with the information of original morphology and location of its hosting cells. Therefore this technology may be used to solve the LSC location and heterogeneity issues.

Process of LSC isolation may destroy LSC niche that plays the vital role in maintaining LSC stemness. Study of LSCs in the transgenic animal system represents the excellent method to avoid LSC niche and specific microenvironment destruction. For example, Sartaj *et al.* developed a tetracycline-inducible (tet-off) double transgenic "pulse-chase" mouse system that accurately identified LSCs, and then used isolated LSCs in combination with NGS technology to analyse the expression profile of candidate LSC biomarkers (Sartaj *et al.*, 2017). Similar transgenic mouse system has also been reported to demonstrate the mechanism of

LSC-promoted wound healing (Kasetti *et al.*, 2016) (Nasser *et al.*, 2018) and the homeostasis of corneal epithelium (Richardson *et al.*, 2017).

Combination of traditional biotechnologies with the virtual simulation technology represents also an useful tool for the study of LSCs. Molvaer *et al.* simulated the 3D computer model of the human corneo-limbal region by 3D visualization software based on series hematoxylin and eosin (HE) stained paraffin sections (Molvaer *et al.*, 2013). Using the technique, they successfully identified three niche types of LSCs (limbal epithelial crypts, limbal crypts and focal stromal projections), and their distribution in superior, inferior, nasal, and temporal regions. Furthermore, Lobo *et al.* successfully demonstrated the mechanism of self-organization centripetal migration of LSCs to repair the wounded corneal epithelium without external cues by using the virtual digital model (Lobo *et al.*, 2016).

Importantly, virtual simulation technology may lead to reduction of the amount of animals used in experiments. Moreover, the computer systems provide advantages to execute multiple complex bio-hypotheses at the same time (Guo and Zhang, 2018).

4.2 Material and methods

4.2.1 Corneal tissue

Cadaveric adult human limbal tissue was obtained from the corneo-scleral rings remaining (9 females, 15 males, average age 69.42 years, SEM 2.99, range 28 - 83 years) after removal of the central cornea for transplantation supplied by the NHS Blood and Transplant (NHSBT) Cornea Transplantation Service eye bank in Manchester and Bristol, UK. Average time from death to retrieval of corneo-scleral tissue was 16.1 ± 1.99 hours (mean±SEM). Average time tissue spent in organ culture was 36.55 ± 7.8 days (mean±SEM). Human tissue was handled according to the tenets of the Declaration of Helsinki and informed consent was obtained for research use of all human tissue from the next of kin of all deceased donors. The

study was approved by the NRES Committee North East - Newcastle & North Tyneside 1 (REC number: 11/NE/0236, protocol number 5466) on the 29th October 2013.

Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

4.2.2 Single cell culture of human limbal epithelium on 3T3-J2 feeder layers

Twenty four hours before limbal epithelial cell isolation from corneo-scleral tissue, mitotically inactivated J2–3T3 mouse fibroblasts were suspended in high-glucose DMEM supplemented with bovine calf serum (10%) (Hyclone, USA) and penicillin/streptomycin (1%) (Thermo Fisher Scientific, USA) and plated in a 9.6 cm^2 tissue culture well at the final density of 2.4×10^4 cells per cm^2 as previously described (Yu *et al.*, 2016). The use of bovine calf serum instead of fetal calf serum was recommended by the manufacturer of the 3T3-J2 cell line (Karafast, USA). The 3T3 cell suspension was placed in a tissue culture incubator at 37°C overnight to allow the establishment of a 3T3 feeder layer. On the following day, limbal stem cells were harvested from cadaveric corneo-scleral rims as previously described (Ahmad *et al.*, 2007) (**Figure 4.1**). The deeper layers of the corneo-scleral rings were dissected away together with excess sclera leaving a ring containing approximately 2 mm of peripheral cornea and 2 mm of adjacent sclera. The remaining tissue containing limbal epithelium was then cut into smaller 1 mm^2 pieces. The limbal epithelial cells were isolated from these pieces using serial trypsinization with 0.05% trypsin-EDTA solution (Thermo Fisher Scientific, USA). After 20 minutes incubation in a tissue culture incubator, the resulting cell suspension was removed from the limbal pieces and epithelial medium was added to this suspension. After the cell suspension was centrifuged for 3 minutes at 1000 rpm in Heraeus Megafuge 16R Centrifuge (Thermo Fisher Scientific, USA), the supernatant was removed and the remaining cell pellet was re-suspended in epithelial medium containing 3:1 mixture of low-glucose DMEM:F12 supplemented with fetal calf serum 10%, penicillin/streptomycin 1% (all Thermo Fisher Scientific, USA), hydrocortisone 0.4 µg/ml, insulin 5µg/ml, triiodothyronine 1.4 ng/ml, adenine 24 µg/ml, cholera toxin 8.4 ng/ml and EGF 10

ng/ml (all Sigma-Aldrich, UK). The trypsinization and centrifugation process was repeated a further three times using the same limbal tissue and the same centrifuge and settings. The resulting cell suspensions were pooled together. After counting, 30,000 of viable limbal epithelial cells (trypan blue exclusion test) in epithelial medium were added to one 9.6 cm^2 tissue culture well containing the growth arrested 3T3 fibroblast and placed in a tissue culture incubator at 37°C with a humidified atmosphere containing 5% CO_2. The medium was exchanged on the third culture day and every other day thereafter. Several days after, LSC colonies with typical morphology started to appear (**Figure 4.2**) and were cultured until became sub-confluent. After 3T3 feeder cells were detached and removed using 0.02% EDTA (Lonza, Switzerland), sub-confluent primary cultures were dissociated with 0.5% trypsin-EDTA (Santa Cruz, USA) to single cell suspension and passaged at a density of 6 × 10^3 cells/cm^2. For serial propagation, cells were passaged and cultured as above, always at the stage of sub-confluence, until they reached passage 3.
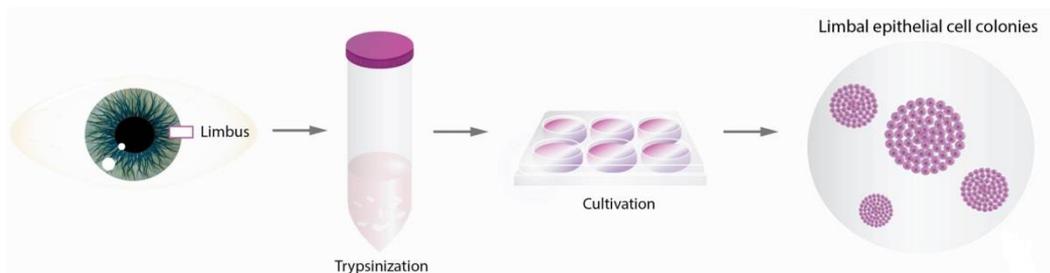
Figure 4.1 Schematic representation of LSC isolation process. The corneo-scleral rings were cut into 1 mm^2 pieces, trypsinized, centrifuged and obtained cells seeded onto mouse 3T3 feeder cells. Several days later LSC formed colonies with characteristic morphology.
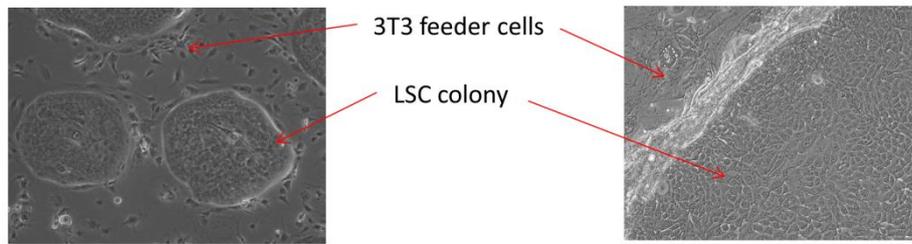
Figure 4.2 Microphotograph of human LSC colonies surrounded by 3T3 mouse feeder cells. Characteristic appearance of LSC colonies with well-defined edges and cobblestone-like epithelium.

4.2.3 Limbal epithelial cell surface marker screening

Limbal epithelial cell cultures (passage 1) were dissociated as described above to a single cell suspension. Limbal epithelial cells were stained with 361 different phycoerythrin (PE) labelled antibodies and 10 immunoglobulin (Ig) isotype controls using the LEGEND Screen™ Lyophilized Antibody Panel Human Cell Screening (PE) Kit (700007, BioLegend, USA) (**Figure 4.3**). After the staining, cells were washed and analysed by LSR Fortessa (BD, USA) flow cytometer. Data were analysed with FCS Express 6 Flow Cytometry Software (De Novo Software, USA). The screening was repeated three times, for each experiment corneo-scleral rings from seven donors were pooled (21 donors in total; 8 females, 13 males, average age 70.50 years, SEM 2.06, range 55 - 83 years).
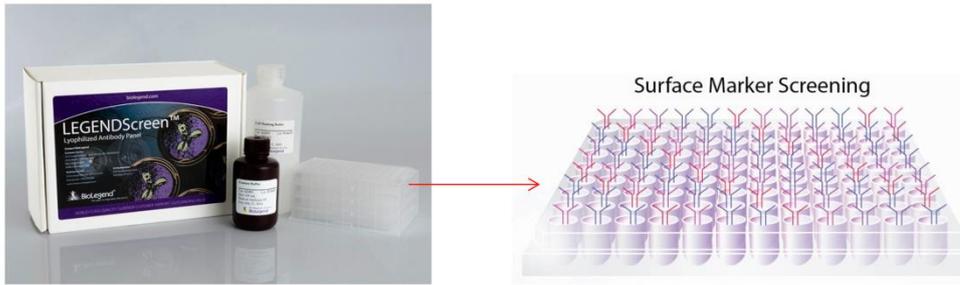
Figure 4.3 LEGEND Screen™ Lyophilized Antibody Panel Human Cell Screening Kit and schematic representation of a 96 well plate with different surface antibody in each well.

After we identified candidate markers using LEGEND Screen™ Lyophilized Antibody Panel Human Cell Screening Kit we further investigated these markers by flow cytometry (after passage induced and calcium induced differentiation) and immunohistochemistry in order to perform further selection. Only the final two candidate markers were sorted using fluorescence-activated cell sorting (FACS) and positive and negative populations were investigated by qRT-PCR, clonal assay, proliferation and CFE assay (**Figure 4.4**).

1. Monitoring through passage and calcium induced differentiation by flow cytometry

2. IHH staining of corneal sections

3. Cell sorting:

Sorted (+) and (-) cell populations

❖ Clonal assay
❖ Colony forming efficiency assay
❖ Proliferation assay
❖ qRT-PCR
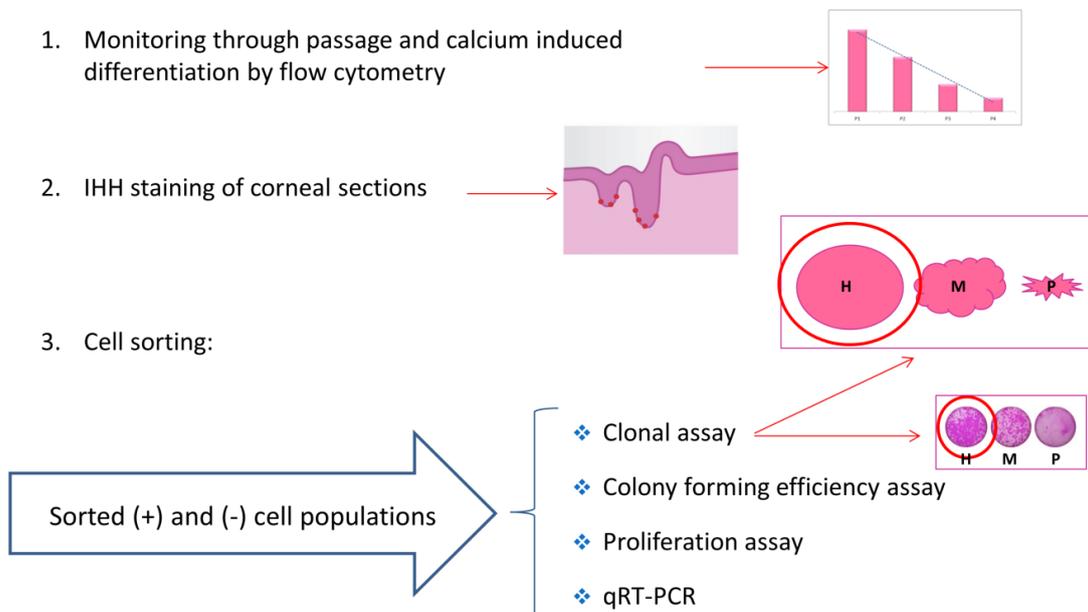
Figure 4.4 Schematic representation of the study research design. The aim of the study was to discover the best candidate marker through multi-step examination. Firstly, potential candidate's expression should decrease through differentiation process, secondly it should be expressed exclusively by epithelial cells in the basal layer of the limbus and thirdly sorted positive cell population for the given marker should be able to form holoclones. If all the conditions were fulfilled further analysis such as qRT-PCR and proliferation assay were performed. Abbreviations: H-holoclones, M-meroclones, P-paraclones.

4.2.4 Calcium induced differentiation

Limbal epithelial cells from three different donors (n=3, passage 1) were plated at a density of 200,000 cells per well in a 6 well plate and cultured in EpiGRO™ Human Ocular Epithelia Complete Media Kit (SCMC001, Merck Millipore, USA) without 3T3-J2 feeders or any plate coating. The medium contained basal medium, supplements mix (L-Glutamine 6 mM, Epinephrine 1.0 μM, Insulin 5 μg/mL, Apo-Transferrin 5 μg/mL, Hydrocortisone 100 ng/mL, EpiFactor O proprietary and EpiFactor P 0.4%), 150μM calcium and 1% penicillin/streptomycin. When the cells reached 80% confluence, calcium was added to a final concentration of 1.2mM, for the induction of

differentiation. Cells were differentiated for up to 1 week and collected for flow cytometry analysis.

4.2.5 Flow cytometry analysis and FACS

The expression of selected markers in limbal epithelial cell cultures was monitored through subsequent passages, from passage one to passage four, and during calcium induced differentiation using cells from three different donors (n=3) to provide biological triplicates. After trypsin dissociation, limbal epithelial cells re-suspended in flow buffer (1% Bovine Serum Albumin in PBS) were stained for 20 minutes with different selected antibodies on ice and analysed by flow cytometry (FACSCanto II, BD, USA). A minimum of 10,000 events were recorded for each sample. Antibodies used for FACS were PE-conjugated anti-human CD200 (329205, BioLegend, USA, dilution factor 1:100), PE-conjugated anti-human CD109 (323305, BioLegend, USA, 1:100) and APC-conjugated anti-human p63 delta (NBP2-33090, Novus Biologicals, USA, 1:100).

FACS was carried out using a FACSAria II sorter (BD, USA) (**Figure 4.5**). Limbal epithelial cells used for the cell sorting experiments were passage 1. The limbal epithelial cell staining was performed as above using FACS buffer (1% FBS in PBS) under aseptic conditions for both final candidate markers, CD200 and CD109. The stained cell suspension was then filtered through a 40 µm nylon filter to remove any cell clumps. 10% DAPI stain was added to a final cell suspension to eliminate dead cells. Side scatter and forward scatter profiles were used to eliminate cell doublets. Positive and negative sorted cells were used for CFE, clonal assay, and qRT-PCR.
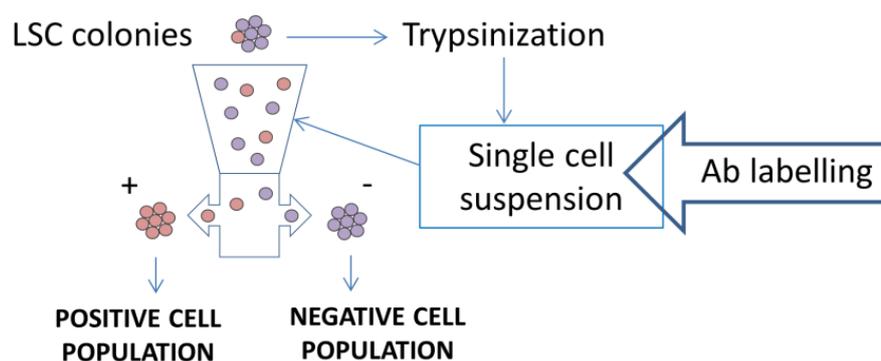
Figure 4.5 Schematic representation of a cell sorting process. Firstly, sub-confluent LSC colonies were enzymatically digested to single cell suspension by trypsin. The cell suspension obtained was then labelled by fluorescent antibody of interest and used for cell sorting.

4.2.6 Colony-forming efficiency and clonal assay

Colony-forming efficiency assay (CFE) was performed as previously described by Yu *et al.,* 2016 (Yu *et al.*, 2016). Following staining with 1% Rhodamine B, colonies were counted under dissecting microscope (SMZ645, Nikon, Japan). The CFE was calculated as number of colonies formed/number of cells plated×100 for both positive and negative cell populations for three different donors (n=3). Each donor served as a biological replicate. Sorted limbal epithelial cells from three different donors were also plated for clonal assay (n=3) performed as described by Dziasko *et al.*, 2014 (Dziasko *et al.*, 2014). Limbal epithelial cells used for the cell sorting experiments were passage 1. The sorted populations were re-plated for CFE and clonal assay. The clonal type was determined by (1) the morphology of colonies and (2) the percentage of aborted colonies as follows: when <5% of the total colonies were terminally differentiated, the clone was scored as a holoclone; when more than 95% of colonies were terminally differentiated, the clone was scored as a paraclone and finally, when >5% but <95% of colonies were terminally differentiated, the clone was classified as a meroclone (Barrandon *et al.*, 1989; Pellegrini *et al.*, 1999a).

4.2.7 Fluorescence Immunohistochemistry and Microscopy

Cultured limbal epithelial cells, human frozen corneal sections and paraffin sections of mouse and human cornea were fixed for 15 minutes either in 4% paraformaldehyde (CD200 staining) or in ice-cold methanol (CD109 staining). For human sections blocking step was performed by incubation in antibody diluent containing 1% bovine serum albumin (Sigma-Aldrich, UK) with 5% normal goat serum (Thermo Fisher Scientific, USA) for 30 minutes prior to staining. Permeabilization with 0.2% Triton X-100 in PBS was performed prior to staining with antibodies for internal cell markers. Cells were incubated with primary antibodies at 4°C overnight and further incubated with secondary antibodies for 1 hour. The following primary antibodies were used at the indicated dilutions: anti CD109 (sc-271085, Santa Cruz, USA, 1:200), anti-human CD200 (329201, BioLegend, USA, 1:200), anti-mouse CD200 (AF3355, Novus Biologicals, USA, 1:100), anti p63 delta (NBP2-29467, Novus Biologicals, USA, 1:200), anti-cytokeratin 15 (ab52816, Abcam, UK, 1:100)and anti Ki67 antibody (ab15580, Abcam, UK, 1:100). Sections were mounted in Vecta shield (Vector Labs, USA) with Hoechst 33342 (1:1000, Thermo Fisher Scientific, USA). Images were obtained using Axio Imager microscope with ApoTome accessory equipment and AxioVision software (Zeiss, Germany).

Immunostaining of mouse sections was performed on paraffin sections (5-7µm) of C57BL/6 mouse tissues, as described previously (Nasser *et al.*, 2018) by our collaborators from Department of Genetics and Developmental Biology, The Ruth and Bruce Rappaport Faculty of Medicine, Technion- Israel Institute of Technology, Haifa, Israel (Prof. Ruby Shalom-Feuerstein and Dr. Aya Amitai-Lange).

4.2.8 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

As in previous experiments, passage one of cultured limbal epithelial cells obtained from three different donors were used for the cell sorting (n=3). The sorted cell populations were then subjected to qPCR analysis. cDNA was synthesised using the

Cells-to-cDNA™ II kit (AM1723, Ambion, Thermo Fisher Scientific, USA) directly from cell lysates as per the manufacturer's protocol. Each reaction was set up using Go-Taq® qPCR Master Mix (Promega, USA) and was composed of 5 µl 2X Master Mix buffer, 0.4 µl forward primer, 0.4 µl reverse primer, 0.8 µl template cDNA, 3.7 µl RNAse-free water and 0.1 µl COX. All reactions were analysed on a QuantStudio™ 7 Flex Real Time PCR System (Thermo Fisher Scientific, USA) according to the manufacturer's instructions using SYBR® Green as the detection dye, and ROX™ channel to detect COX as the reference dye. A standard, 40-cycle qPCR was performed for each sample. The primer sequences used for qRT-PCR are listed in **Table 4.1**. The data was analysed using the $2^{-\Delta\Delta Ct}$ calculation method.

Table 4.1 qRT-PCR Primers.

Gene	Direction	Primer Sequence
GAPDH	Forward	GTCAGTGGTGGACCTGACCT
	Reverse	CACCACCCTGTTGCTGTAGC
CD109	Forward	GCCCGGAGGAAATGTGACTA
	Reverse	TTGGGGTCTGATGGAAGAGTA
CD200	Forward	TGGGGACTGTGACCGACTTT
	Reverse	TATTTAGGGCTCTCGGTCCTGA
ΔNp63	Forward	CTGGAAAACAATGCCCAGAC
	Reverse	GGGTGATGGAGAGAGAGCAT
ABCB5	Forward	TACTCTTCCCACTGCCATTG
	Reverse	CAATTATCCATCAAGACCATCTATCAAG
C/EBPδ	Forward	GGACATAGGAGCGCAAAGA
	Reverse	GCTTCTCTCGCAGTTTAGT
BMI1	Forward	CTGGTTGCCCATTGACAGCG
	Reverse	AAATCCCGGAAAGAGCAGCC
AXIN2	Forward	AGCCAAAGCGATCTACAAAGG
	Reverse	GGTAGGCATTTTCCTCCATCAC
FZD7	Forward	GCCGCTTCTACCACAGACT
	Reverse	TTCATACCGCAGTCTCCCC
CDH3	Forward	GGCGCTGGGGAAAGTATTCA
	Reverse	GGAGCAACCACCCAATCTCT
PAX6	Forward	TCTTTGCTTGGGAAATCCG
	Reverse	CTGCCCGTTCAACATCCTTA
WNT7A	Forward	TGCCCGGACTCTCATGAAC
	Reverse	GTGTGGTCCAGCACGTCTTG
Ki67	Forward	CGTCCCAGTGGAAGAGTTGT
	Reverse	CGACCCCGCTCCTTTTGATA
CK3	Forward	CGTACAGCTGCTGAGAATGA
	Reverse	CTGAGCGATATCCTCATACT
CK14	Forward	TTCTGAACGAGATGCGTGAC
	Reverse	GCAGCTCAATCTCCAGGTTC
CK15	Forward	ACCACCACATTTCTGCAAACT
	Reverse	AGCTGAGATACTTCGGCTTCC

4.2.9 Cell proliferation assay

Passage one of limbal epithelial cells from three different donors (n=3) at 60-70% confluence were exposed to BrdU at a final concentration of 10 µM in cell culture medium and incubated for one, four and eight hours. Control cells were cultured without BrdU. After incubation, cells were stained with PE conjugated anti-CD200 antibody (329205, BioLegend, USA) for 20 minutes on ice, then were washed, fixed

and permeabilized before DNAse treatment. Following BrdU epitope exposure cells were stained with PerCP-Cy™5.5 conjugated anti-BrdU antibody (560809, BD, USA, 5 µl per test) and DAPI stain for cell cycle analysis and analysed by LSR Fortessa (BD, USA) cell analyser.

4.2.10 Hoechst 33342 and Pyronin Y Staining for G0/G1 Separation

Quiescent cells, which are arrested in G0 phase, have lower level of RNA compared to active cells (G1 phase). Hoechst is an exclusive DNA dye while Pyronin Y reacts with both DNA and RNA. However, in the presence of Hoechst, Pyronin Y reaction with DNA is blocked, and Pyronin Y stains RNA only. When cells are stained first with Hoechst 33342 and then with Pyronin Y it is possible to distinguish DNA from RNA. Limbal epithelial cells from three different donors (n=3) were stained with APC conjugated anti-CD200 antibody for 20 minutes (329207, BioLegend, USA). For the separation of G0 and G1 cell cycle phases, limbal epithelial cells were stained with Hoechst 33342 (Sigma-Aldrich, UK) in a final concentration 10µg/ml and incubated at 37°C for 45 minutes. After 45 minutes, 5µl of 100µg/ml Pyronin Y (Sigma-Aldrich, UK) was added directly to the cells and incubated at 37°C for a further 15 minutes. Single colour controls and negative control were also prepared. LSR Fortessa (BD, USA) flow cytometer was used to analyse cells.

4.2.11 siRNA Transfection

To investigate the impact of *CD200* downregulation on the clonal ability of limbal epithelial cells, RNA interference (RNAi) was performed using small interfering RNA (siRNA). Passage one human limbal epithelial cells from 3 different donors were grown on 3T3 feeder layer in complete epithelial medium supplemented with EGF, adenine, cholera toxin, hydrocortisone, insulin and triiodothyronine. A day before transfection, limbal epithelial cells (150×10^3) were re-seeded in 12-well plate without feeders in order to increase transfection efficiency. The day after re-seeding cells were transfected with CD200 Human Stealth siRNAs (set of 3: HSS106678,

HSS106679, HSS181160; 1299003, Thermo Fisher) and Stealth RNAi™ siRNA Negative Control Lo GC (12935200, Thermo Fisher) using Lipofectamine™ RNAiMAX Transfection Reagent (13778030, Thermo Fisher) according to the manufacturer's protocol. The transfected cells were incubated for 48 hours for CFE and clonal assay.

After 48h incubation with CD200 siRNA and control siRNA, cells we re-seeded back to 6 well plates in different densities (500 and 1000 cells/well) and cultured on 3T3 feeders for next 14 days. The rest of the cells were used for RNA extraction and qRT-PCR to confirm *CD200* downregulation.

4.2.12 Statistical Analysis

GraphPadPrism 7.0 (San Diego, CA, https://www.graphpad.com/scien-tific-software/prism/) was used to perform all statistical analyses. The data showed normal distribution therefore Student's t-test was used to analyse differences between groups and $p \leq .05$ was considered statistically significant. All experiments were performed in biological replicates of three or more, and data are presented as mean ± SEM.

4.3 Results

4.3.1 Flow cytometric based cell surface screening of limbal epithelial cell cultures

After removing 3T3 feeder cells with EDTA, passage one sub-confluent limbal epithelial cells were lifted from the tissue culture plates using Trypsin-EDTA and stained with 361 human surface proteins and analysed by flow cytometry. The cell surface marker screening was performed three times and in each case, limbal epithelial cells from seven different donors were pooled to obtain sufficient cell number for this type of analysis. A summary of these results is shown in **Appendix 1**. LEGEND Screen™ analysis confirmed high expression of the commonly cited limbal epithelial cell markers: EGFR (88.81±6.02) (Zieske and Wasson, 1993; Chen *et al.*, 2004; Kim *et al.*, 2004), SSEA-4 (54.02±5.93%) (Truong *et al.*, 2011), CD71 (88.76±5.92) (Chen *et al.*, 2004; Hayashi *et al.*, 2008), integrin β5 (91.45±1.24) (Stepp *et al.*, 1993), integrin α6 (92.54±6.41) (Hayashi *et al.*, 2008), E-cadherin (88.48±6.06) (Schlotzer-Schrehardt and Kruse, 2005) as well as many other general markers of corneal epithelium. The presence of other markers previously related to limbal epithelial cells was also confirmed: CD40 (26.00±6.94) (Iwata *et al.*, 2002), CD117 (c-kit) (8.22±2.56) (Albert *et al.*, 2012; Luznik *et al.*, 2016b), CD146 (67.04±2.87) and CD166 (95.08±0.97) (Albert *et al.*, 2012) as well as the presence of putative LSC marker integrin α9/β1 (4.85±1.98) (Jones and Watt, 1993; Chen *et al.*, 2004; Kim *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005; Albert *et al.*, 2012). The expression of the autophagy marker LAMP1 (84.82±11.89) was also high, corroborating with previously reported data on limbal epithelial cultures (Dhamodaran *et al.*, 2015).

Marker selection for further investigation was based on three criteria: (1) presence in a small subpopulation of cells (up to 10%) in accordance with label retaining cells in the limbal zone making up less than 10% of the total population (assessed on the basis of the percentage of radiolabelled thymidine retaining cells present in the limbal zone (Cotsarelis *et al.*, 1989) and the studies of Umemoto and co-workers showing that approximately 10% of total limbal epithelial cells expressed the putative LSC marker ABCG2 (Umemoto *et al.*, 2005); (2) passage or calcium differentiation

induced reduction in expression frequency in limbal epithelial cells and (3) presence in other epithelial stem or progenitor cells. CD200 (2.25±0.69%, n=3) was one of the few markers that fulfilled all these three criteria (Rosenblum *et al.*, 2004; Ohyama *et al.*, 2006; Ohyama, 2007; Gerhards *et al.*, 2016) and was selected for further characterisation. In addition to LSC markers, we also selected putative transient amplifying cell surface markers based on similar expression to ΔNp63 (assessed by our group to be expressed in 45-60% of *ex vivo* expanded limbal epithelial cells (Yu *et al.*, 2016) as well as passage and differentiation induced reduction in expression frequency in limbal epithelial cells. CD109 (56.29±13.96%, n=3) was amongst the cell surface marker that fulfilled these criteria and was selected for further characterisation (Bojic *et al.*, 2018).

4.3.2 The expression of CD109 in human limbal epithelial cell cultures, human and murine corneas

The LEGEND Screen™ results were confirmed by flow cytometric analysis which showed CD109 to be expressed in a relatively high percentage of limbal epithelial cultures in p1 (47.51±9.35%, n=5) (**Figure 4.6A**) (Bojic *et al.*, 2018). The expression of CD109 did not vary significantly through the first four passages (*p>0.05*) (**Figure 4.6A**) (Bojic *et al.*, 2018). Nonetheless, the expression of CD109 decreased significantly (*p<0.05*) after 5 days of calcium-induced differentiation under feeder-free conditions similarly to ΔNp63 expression (*p<0.05*) (**Figure 4.6B**) (Bojic *et al.*, 2018).
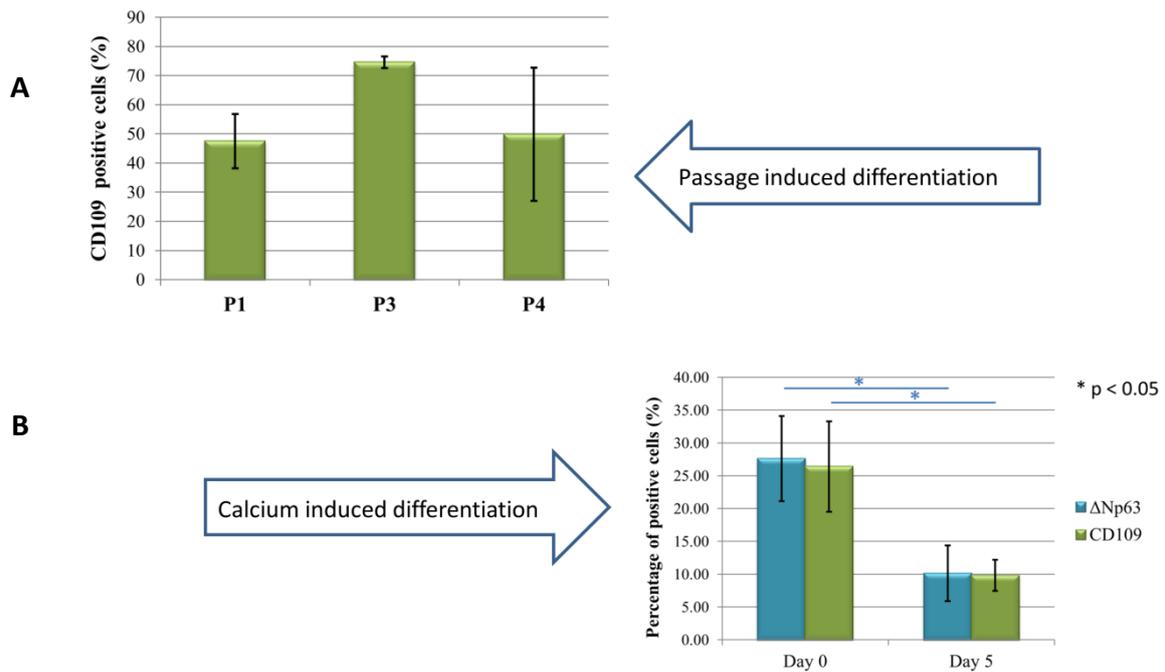
Figure 4.6 CD109 expression during *ex vivo* differentiation of human limbal epithelial cells. (A) Quantification of CD109 expression through different passages of limbal epithelial cells by flow cytometry. Values represent mean ± SEM, n=3-5. (B) Quantification of ΔNp63 and CD109 expression during calcium induced differentiation of limbal epithelial cells by flow cytometry. Values represent mean ± SEM, n=3, *p<0.05*.

Using immunostaining, we determined the localisation of CD109$^+$ cells in human ocular surface epithelial tissues (**Figure 4.7A**) (Bojic *et al.*, 2018). CD109$^+$ cells were exclusively located at the limbus and co-localised with ΔNp63 (**Figure 4.7A**), whilst undetectable in the suprabasal and superficial layers of limbal epithelium as well as in the all layers of central corneal epithelium (**Figure 4.7A**) (Bojic *et al.*, 2018). *In vitro*, CD109$^+$cells were present predominantly on the outer border of colonies (**Figure 4.7B**) (Bojic *et al.*, 2018).

A

B
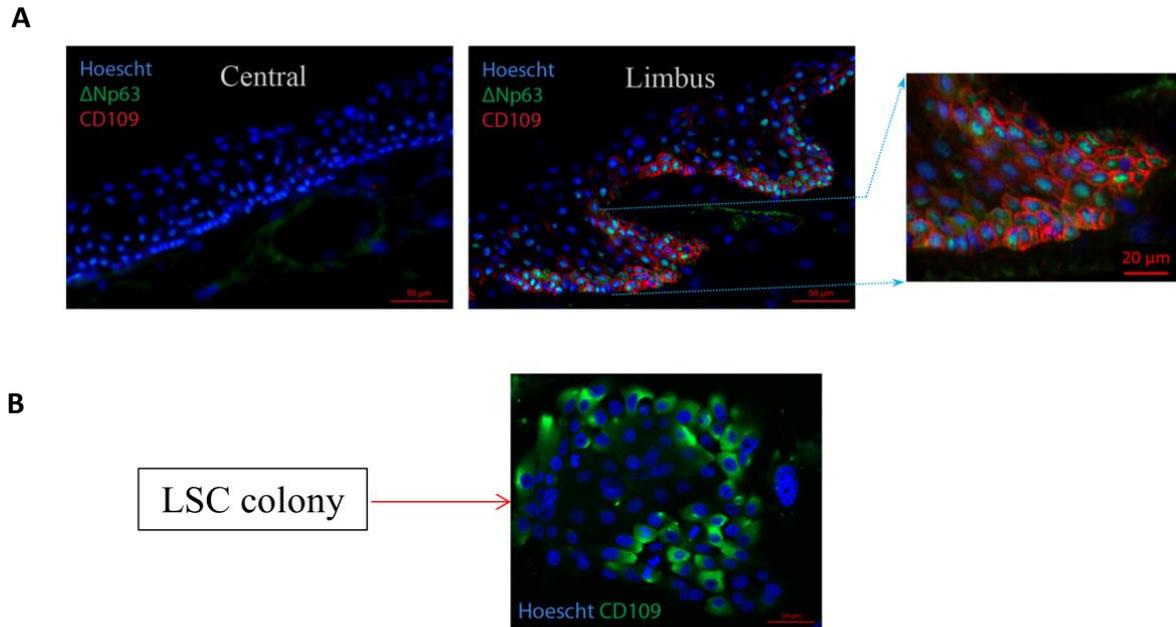
Figure 4.7 CD109 expression in human cornea *in vivo* and during *ex vivo* expansion of human limbal epithelial cells. (A) Immunohistochemical staining of human corneal tissue cryosections for ΔNp63 and CD109 within the central cornea and limbus. Nuclei are shown by Hoechst counter staining. Scale bars 50 μm with exception of additional inset with higher magnification with scale bar of 20 μm. (B) CD109 immunohistochemical staining of human limbal epithelial colony *in vitro.* Nuclei are shown by Hoechst counter staining. Scale bar 50 μm.

In murine corneal tissue, CD109 (**Figure 4.8**) was also exclusively located at the limbus and co-localised with ΔNp63 and CK15 while absent in central corneal epithelium (Bojic *et al.*, 2018).
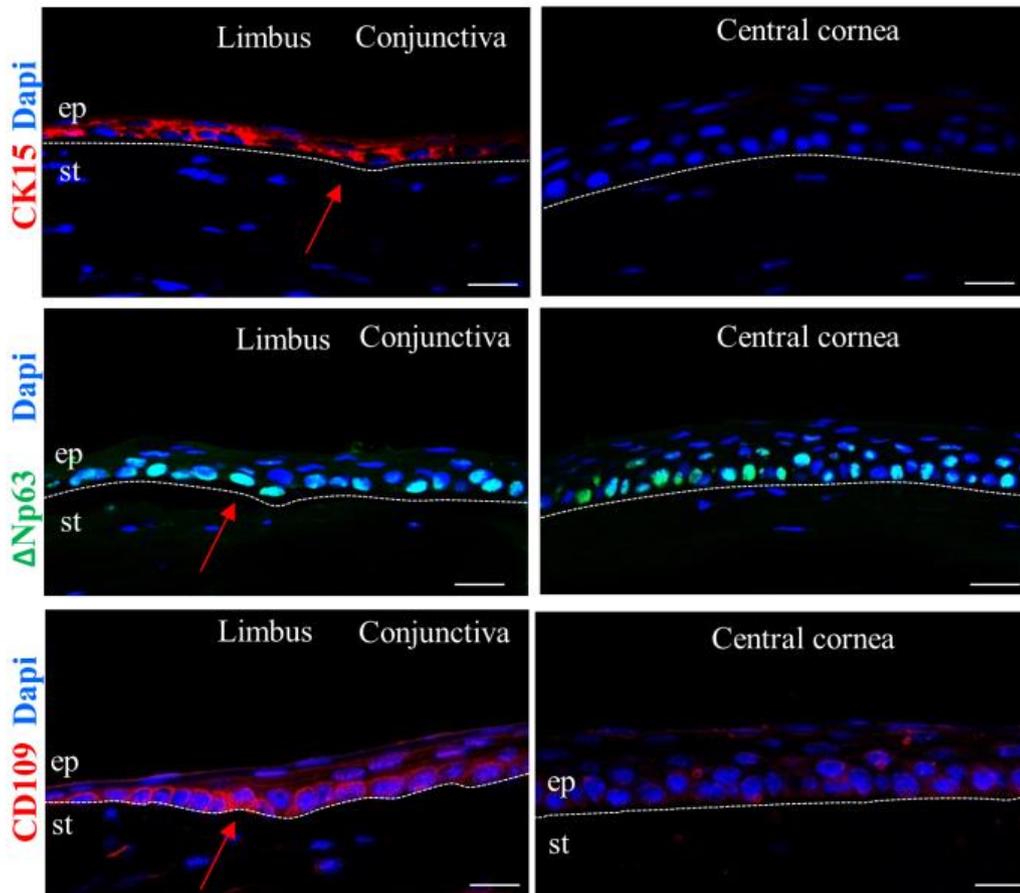
Figure 4.8 CD109 expression in mouse cornea *in vivo*. Immunohistochemical staining of murine corneal tissue cryosections for CK15, ΔNp63 and CD109 within the central cornea and limbus. Nuclei are shown by DAPI counter staining. The dashed line indicates the stromal-epithelial junction. Red arrows point at the limbal region. Scale bars 20 µm. Abbreviations: st – stroma, ep – epithelium.

4.3.3 Colony forming efficiency and proliferative ability of CD109⁺ cells

To identify actively replicating cells, and thereby assess cellular proliferation, BrdU was applied to cells in culture and the number of cells in the S phase was monitored after 1 hour, 4 hours and 8 hours incubation with BrdU by flow cytometry. No statistically significant differences were found in the percentage of cells in the S phase of the cell cycle for CD109⁺ or CD109⁻ population after incubation with BrdU for 1 hour and 4 hours. However, after 8 hours incubation with BrdU, there was a

significantly higher number of CD109$^+$ cells in the S phase compared do CD109$^-$ cells (**Figure 4.9A-B**) (Bojic *et al.*, 2018).
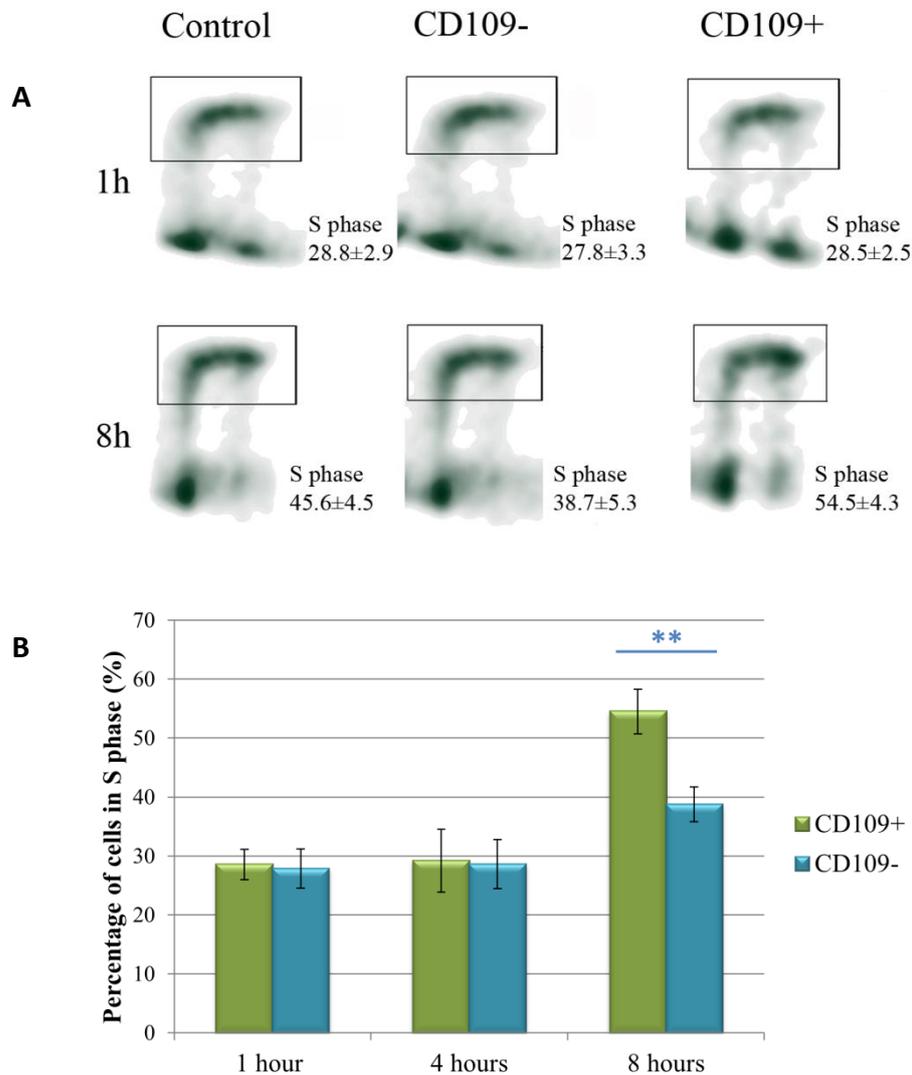
Figure 4.9 Proliferative potential of sorted CD200 positive and negative population. (A) BrdU cell proliferation assay of CD109 negative and positive limbal epithelial cell population after 1-hour and 8-hours incubation with BrdU. Values represent mean ± SEM, n=3. (B) Quantification of cells in the S phase of the cell cycle in CD109+ and CD109- population after 1-hour, 4-hours and 8-hours incubation with BrdU. Values represent mean ± SEM, n=3, **p<0.001.

Sorted positive and negative cells for both markers were tested for their colony forming efficiency and clonal potency (n=3). There were no significant differences between the positive and negative cells in colony forming efficiency (CFE) (**Figure 4.10C**); however the relative colony-covered-area size was significantly greater in CD109$^+$cells ($p<0.01$), meaning they formed larger colonies (**Figure 4.10D** and **E**) when compared to CD109$^-$ cells. Despite the fact that both CD109$^+$ and CD109$^-$ cells formed colonies classified as meroclones, number of aborted colonies was significantly higher ($p<0.01$) in CD109$^-$ population (**Figure 4.10A** and **B**) (Bojic *et al.*, 2018).
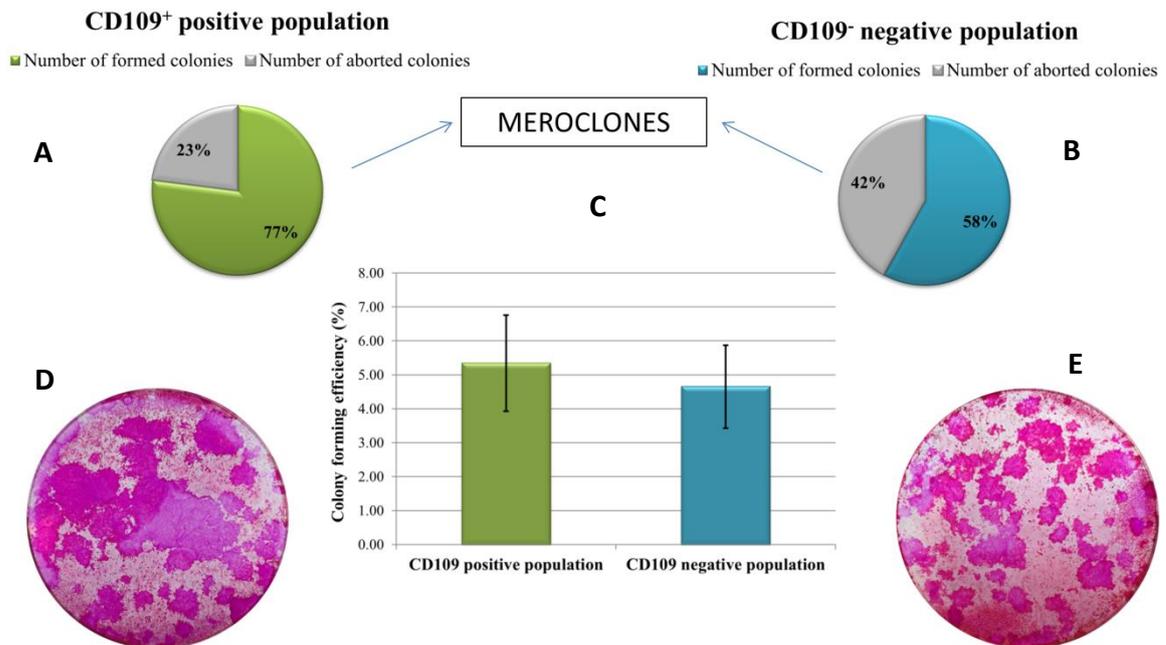
Figure 4.10 Colony forming efficiency and clonal potential of sorted CD109 positive and negative population. (A) Pie chart showing the distribution of formed and aborted colonies in CD109$^+$ population. (B) Pie chart showing the distribution of formed and aborted colonies in CD109$^-$ population. (C) Comparison of colony forming efficiency between CD109$^+$ and CD109$^-$ cell populations. (D) Colonies of CD109$^+$ limbal epithelial cells stained with 2% rhodamine. (E) Colonies of CD109$^-$ limbal epithelial cells stained with 2% rhodamine. Values represent mean ± SEM, n=3-5.

4.3.4 The expression of CD200 in human limbal epithelial cell cultures, human and murine corneas

CD200 was expressed in a small percentage of limbal epithelial cultures in p1 (4.13±1.10%, n=10) (**Figure 4.11A**). Furthermore, the expression of CD200 decreased significantly ($p<0.05$) and rapidly through subsequent passages (**Figure 4.11A**). During calcium induced differentiation, the expression of CD200 disappeared from the culture after 5 days ($p<0.05$) (**Figure 4.11B**). For all the markers, including CD109, CD200 and ΔNp63, a lower expression was observed under feeder-free culture conditions used for the calcium-induced differentiation assays, which may suggest that the feeder-free culture is less conducive to LSC maintenance (Bojic *et al.*, 2018).
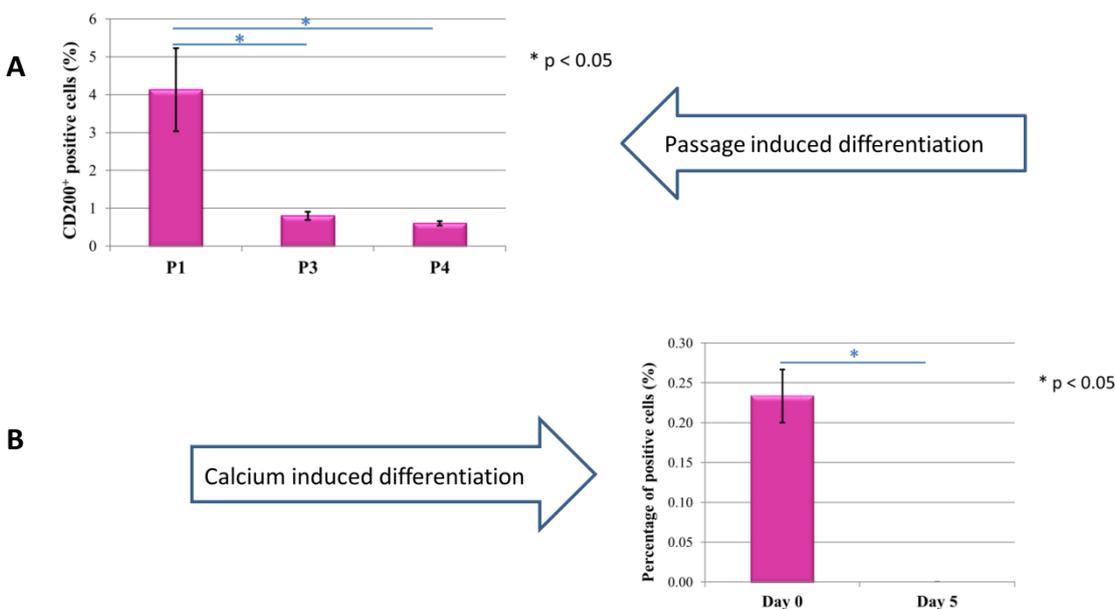
Figure 4.11 CD200 expression during *ex vivo* differentiation of human limbal epithelial cells. (A) Quantification of CD200 expression through different passages of limbal epithelial cells by flow cytometry. Values represent mean ± SEM, n=3-10, *$p<0.05$. (B) Quantification of CD200 expression during calcium induced differentiation of limbal epithelial cells by flow cytometry. Values represent mean ± SEM, n=3, *$p<0.05$.

The presence of CD200^{+} cells was confirmed in the basal layer of the limbal epithelium, whilst its expression was absent in all the other layers of limbal and corneal epithelium (**Figure 4.12**). In murine corneal tissue, CD200 (**Figure 4.13**) was

exclusively located at the limbus and co-localised with ΔNp63 and CK15 while absent in other parts of corneal epithelium (Bojic *et al.*, 2018).
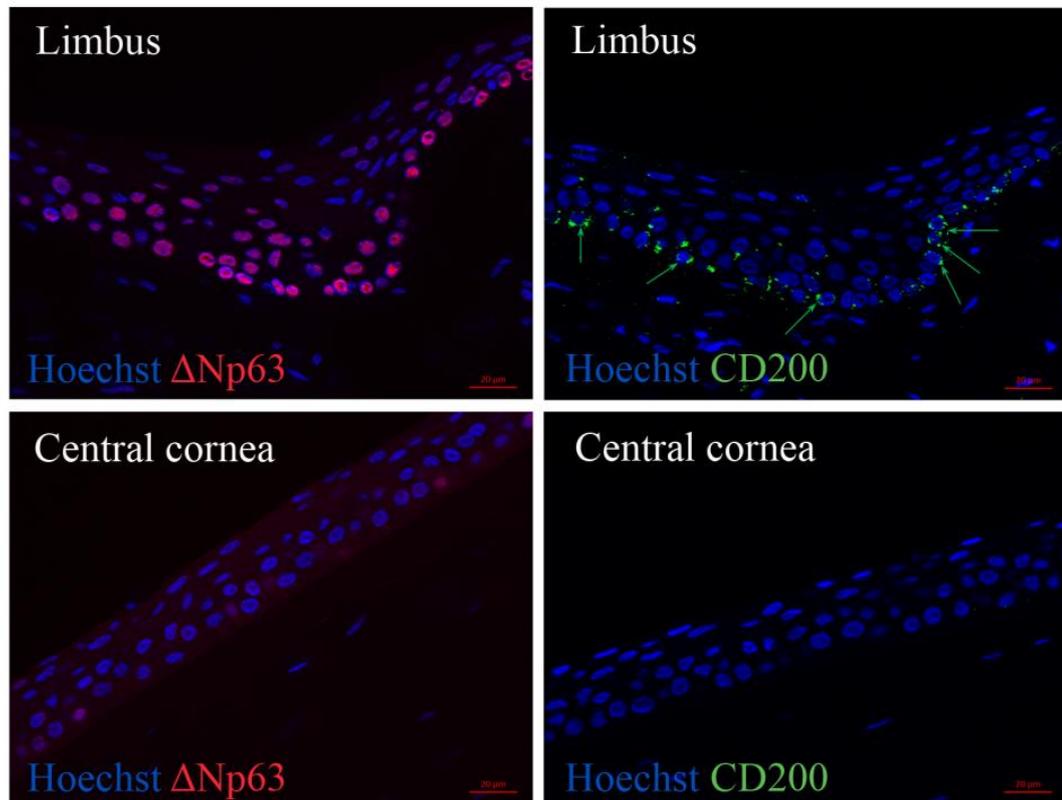
Figure 4.12 CD200 expression in human cornea *in vivo*. Immunohistochemical staining of human corneal tissue paraffin sections for ΔNp63 and CD200 within the central cornea and limbus. Nuclei are shown by Hoechst counter staining. Scale bars 20 μm.
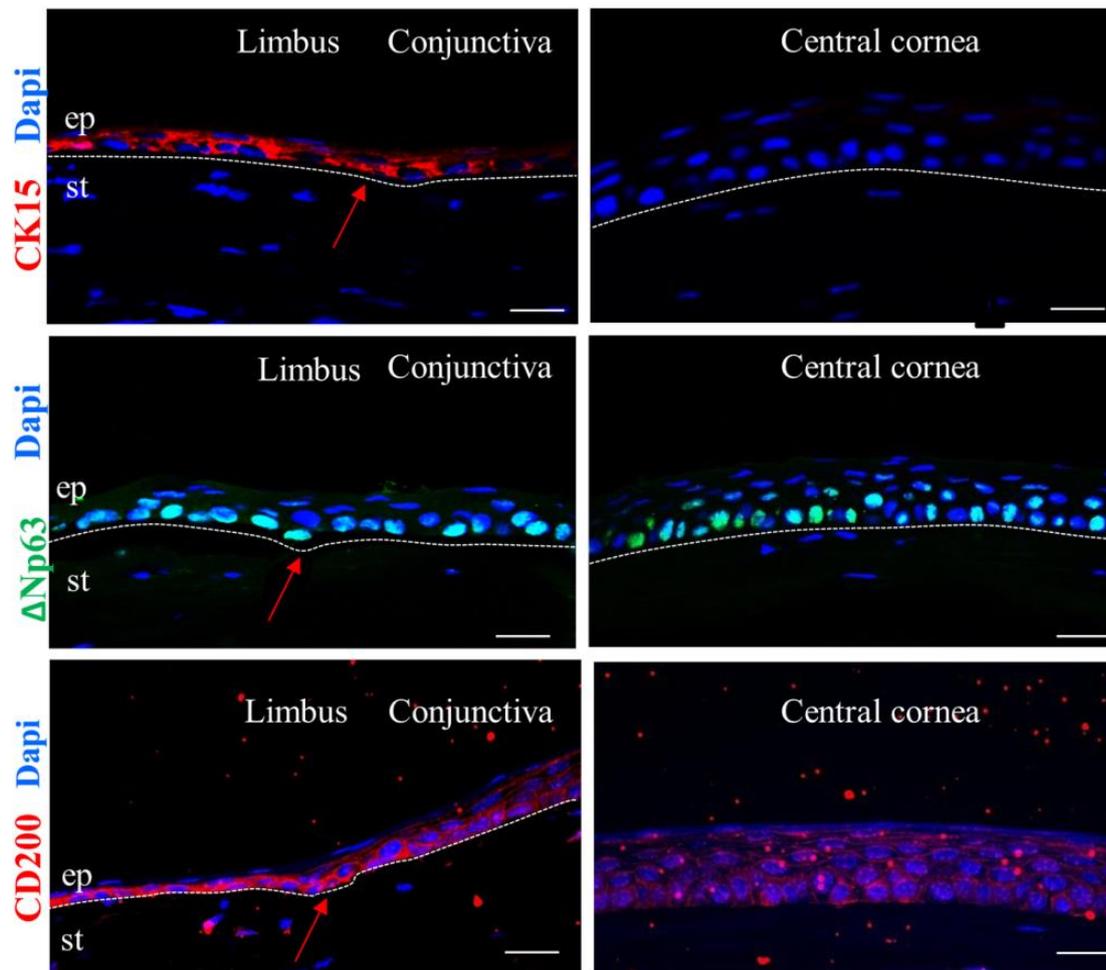
Figure 4.13 CD200 expression in mouse cornea *in vivo*. Immunohistochemical staining of murine corneal tissue cryosections for CK15, ΔNp63 and CD200 within the central cornea and limbus. Nuclei are shown by DAPI counter staining. The dashed line indicates the stromal-epithelial junction. Red arrows point at limbal region. Scale bars 20 µm. Abbreviations: st – stroma, ep – epithelium.

CD200$^+$ cells were also present in *ex vivo* expanded limbal epithelial cell cultures, but in lower number compared to CD109$^+$ cells and moreover were found scattered throughout the colonies (**Figure 4.14A**). CD200$^+$ cells (3.66±0.25%) were much less abundant than ΔNp63$^+$ cells (47.65±3.01%) (**Figure 4.14A** and **B**). All CD200$^+$ cells were also ΔNp63$^+$; however ΔNp63$^+$ CD200$^+$ cells represented only 6.23±0.97% of all ΔNp63$^+$ cells (**Figure 4.14C**) (Bojic *et al.*, 2018).
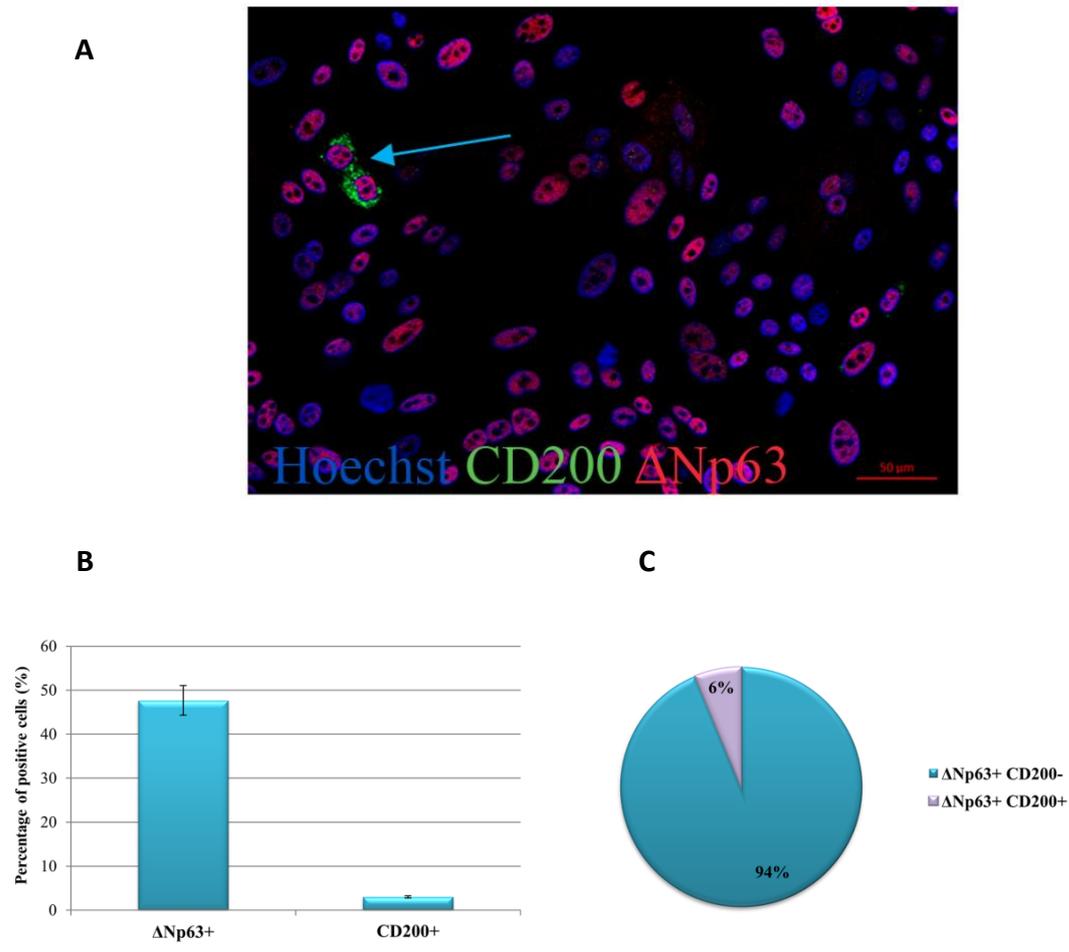
Figure 4.14 CD200 and ΔNp63 expression during *ex vivo* expansion of human limbal epithelial cells. (A) Immunohistochemical staining of limbal epithelial cell colonies *in vitro* for CD200 and ΔNp63. Blue arrow points CD200$^+$ cells. Nuclei are shown by Hoechst counter staining. Scale bar 50 μm. (B) Quantification of ΔNp63$^+$ and CD200$^+$ cells in limbal epithelial cell culture by immunohistochemistry. Values represent mean ± SEM, n=3. (C) Pie chart showing the distribution of CD200$^+$ and CD200$^-$ cells within ΔNp63$^+$ cells.

4.3.5 Colony forming efficiency and proliferative ability of CD200⁺ cells

The proliferative potential of CD200$^+$ cells was examined by Ki67 immunofluorescent staining of limbal epithelial cells cultured *in vitro* (**Figure 4.15A**). Interestingly, while some CD200$^+$cells were Ki67$^+$ (41.67±0.22%), there were more Ki67$^-$ cells in the CD200$^+$ population (58.33±0.22%) (**Figure 4.15B**) (Bojic *et al.*, 2018).
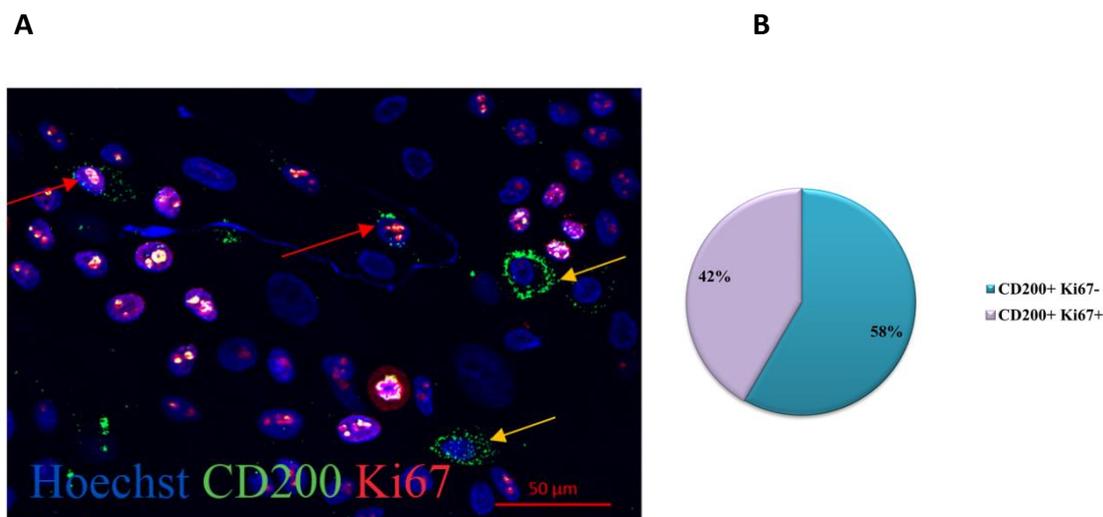
Figure 4.15 CD200 and Ki67 expression during *ex vivo* expansion of human limbal epithelial cells. (A) Immunohistochemical staining of limbal epithelial cell colonies *in vitro* for CD200 and Ki67. Red arrows point to CD200$^+$ Ki67$^+$ cells; orange arrows point to CD200$^+$Ki67$^-$ cells. Nuclei are shown by Hoechst counter staining. Scale bar 50 µm. (B) Pie chart showing the distribution of Ki67$^+$ and Ki67$^-$ cells in CD200$^+$ cell population.

There were no statistically significant differences in CFE between CD200$^+$ and CD200$^-$ groups (**Figure 4.16B**), however CD200$^+$ cells were exclusively able to form holoclones - large colonies with smooth, thick borders (**Figure 4.16D**), while CD200$^-$ cells formed meroclones which were characterised by irregular borders (**Figure 4.16E**). The number of aborted colonies was significantly higher in CD200$^-$ population (*p<0.01*). Using the percentage of terminal colonies described in the methods, CD200$^+$ colonies were scored as holoclones (**Figure 4.16A**) while CD200$^-$ colonies were scored as meroclones (**Figure 4.16C**) (Bojic *et al.*, 2018).
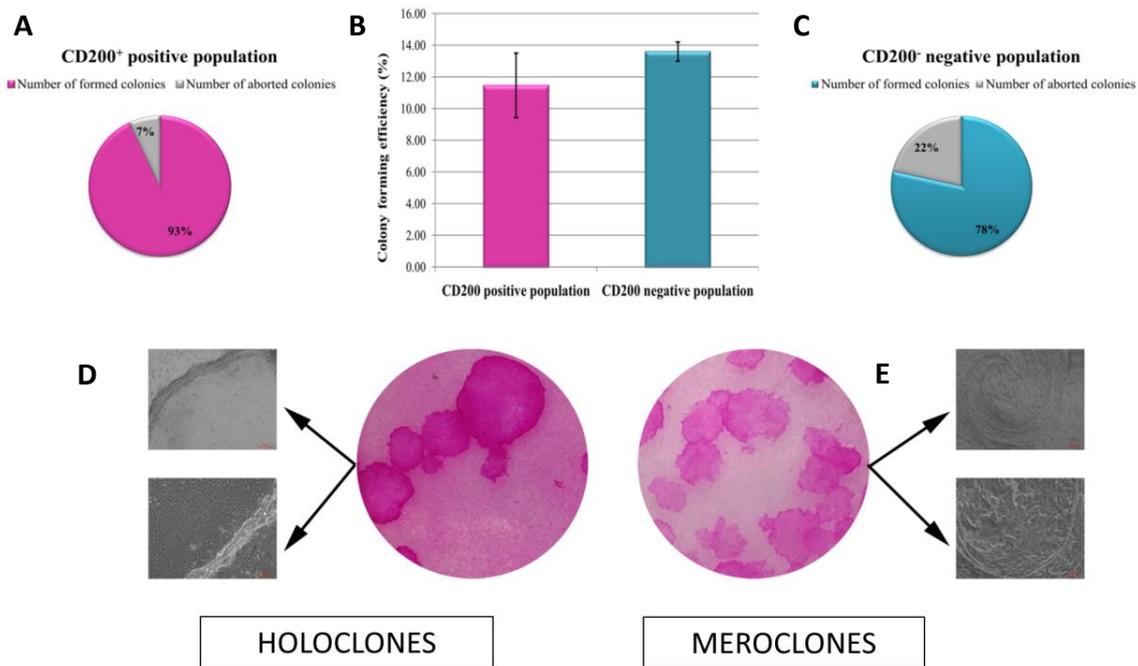
Figure 4.16 Colony forming efficiency and clonal potential of sorted CD200 positive and negative population. (A) Pie chart showing the distribution of formed and aborted colonies in CD200$^+$ population. (B) Comparison of colony forming efficiencies of CD200$^+$ and CD200$^-$ cells. Values represent mean ± SEM, n=3. (C) Pie chart showing the distribution of formed and aborted colonies in CD200$^-$ population. Values represent mean ± SEM, n=3. (D) Microscopic and macroscopic appearances of colonies formed by CD200$^+$ cells. Scale bars 100 μm. (E) Microscopic and macroscopic appearances of colonies formed by CD200$^-$ cells. Scale bars 100 μm.

CD200$^+$ cells were slow to enter S phase: at 1 and 4 hours there were significantly less CD200$^+$ in S phase when compared to CD200$^-$; these differences became non-significant at 8 hours (**Figure 4.17 A** and **B**). For that reason we used Hoechst 33342 and Pyronin Y staining for G0/G1 separation. A larger part of G0 subpopulation was made up by CD200$^+$ cells (59.30±3.12%) than CD200$^-$ cells (40.70±2.11%) (**Figure 4.17C**). Interestingly, we also found that majority (78.66±3.20%) of side population (SP) cells were in the CD200$^+$ population (**Figure 4.18**) whilst the CD200$^-$ population contained less SP cells (21.34±3.20%) (**Figure 4.17D**) (Bojic *et al.*, 2018).
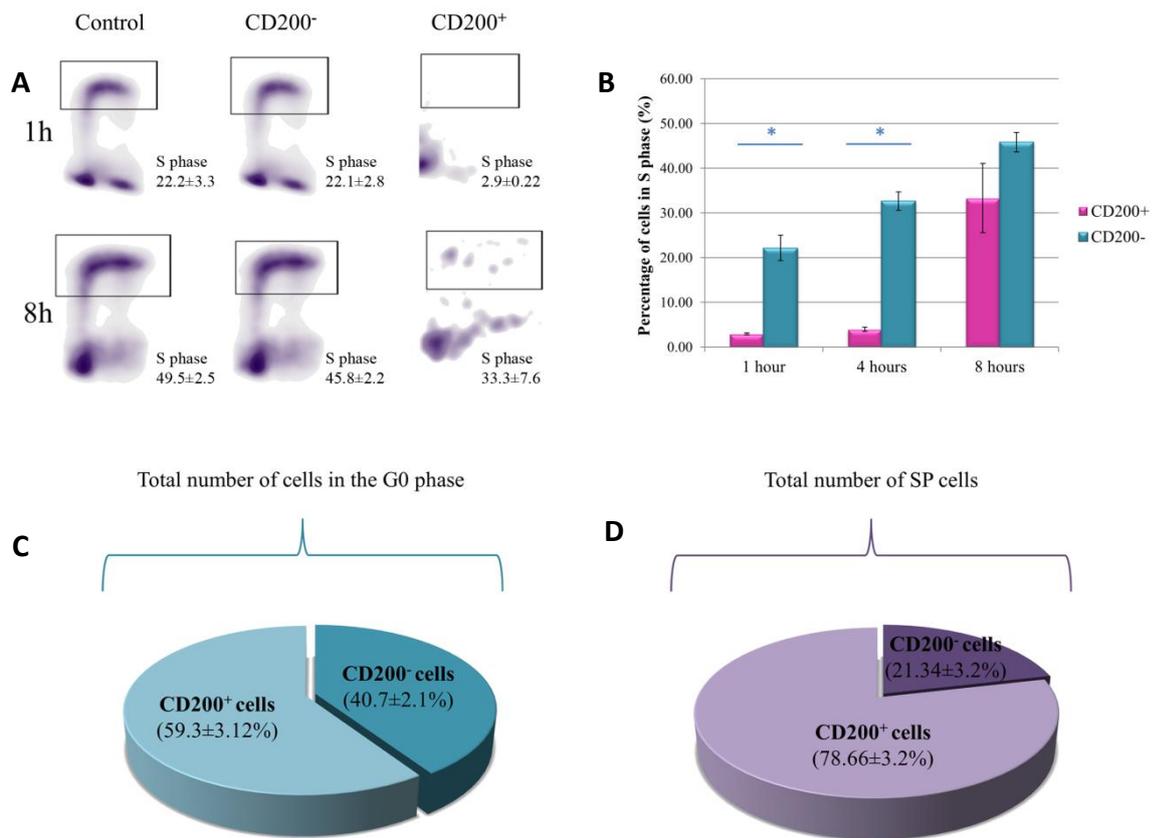
Figure 4.17 Proliferative potential of sorted CD200 positive and negative population. (A) BrdU cell proliferation assay of CD200 negative and positive limbal epithelial cell population after 1-hour and 8-hours incubation with BrdU. Values represent mean ± SEM, n=3. (B) Quantification of cells in the S phase of the cell cycle in CD200$^+$ and CD200$^-$ population after 1-hour, 4-hours and 8-hours incubation with BrdU. Values represent mean ± SEM, n=3, *$p<0.05$. (C) The contribution of CD200$^+$ and CD200$^-$ cell population to the total number of cells in the G0 phase of the cell cycle. Values represent mean ± SEM, n=3. (D) The contribution of CD200$^+$ and CD200$^-$ cell population to the total number of SP cells. Values represent mean ± SEM, n=3.
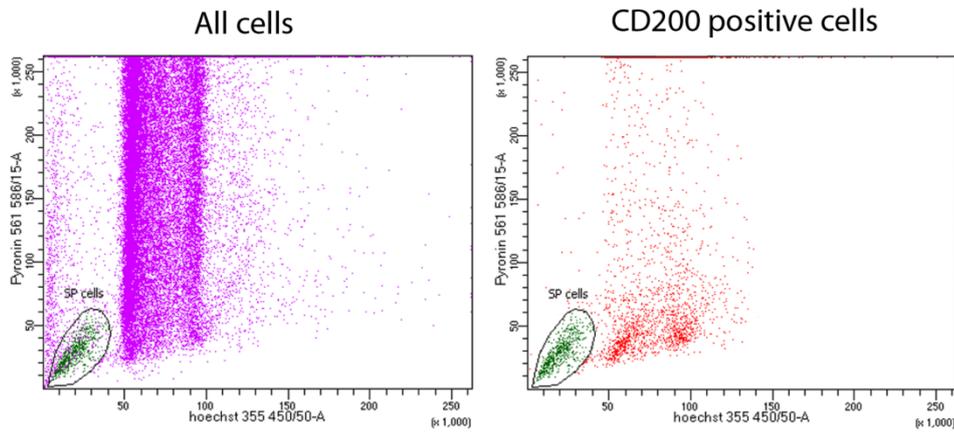
Figure 4.18 Side population cells. Representative dot plot showing gated SP cells and cells in the G0 phase of the cell cycle in whole population and CD200 positive population of cells.

4.3.6 The expression of LSC markers in the CD109 and CD200 positive and negative populations

To investigate the transcriptional profile of CD109$^+$ and CD200$^+$ cells, expression of putative LSC markers *ΔNp63*, *ABCB5*, *C/EBPδ*, *BMI1*, *AXIN2*, *FZD7*, *CHD3*, *WNT7A*, *CK14*, and *CK15* (Yoshida *et al.*, 2006; Barbaro *et al.*, 2007; Figueira *et al.*, 2007; Ksander *et al.*, 2014; Mei *et al.*, 2014; Sartaj *et al.*, 2017; Kalha *et al.*, 2018), corneal epithelial differentiation marker *CK3 (Merjava et al., 2011)* and marker of proliferative cells *Ki67 (Sun et al., 2015)* was assessed by qRT-PCR.

The expression of *CD109,* was significantly higher ($p<0.01$) in CD109$^+$ group compared to CD109$^-$ group, thus validating the flow activated cell sorting strategy. In addition, the expression of LSC markers *PAX6* ($p<0.05$) and C*K14* ($p<0.01$) and proliferative marker *Ki67* ($p<0.001$) was also higher in the CD109$^+$ group when compared to the CD109$^-$ (**Figure 4.19**). No statistically significant differences were found in the expression of other LSC markers *ΔNp63*, *ABCB5*, *C/EBPδ*, *BMI1*, *AXIN2, FZD7, CHD3, WNT7A,* and C*K15* and corneal differentiation marker *CK3* between the CD109$^+$ and CD109$^-$ group (**Figure 4.19**) (Bojic *et al.*, 2018).

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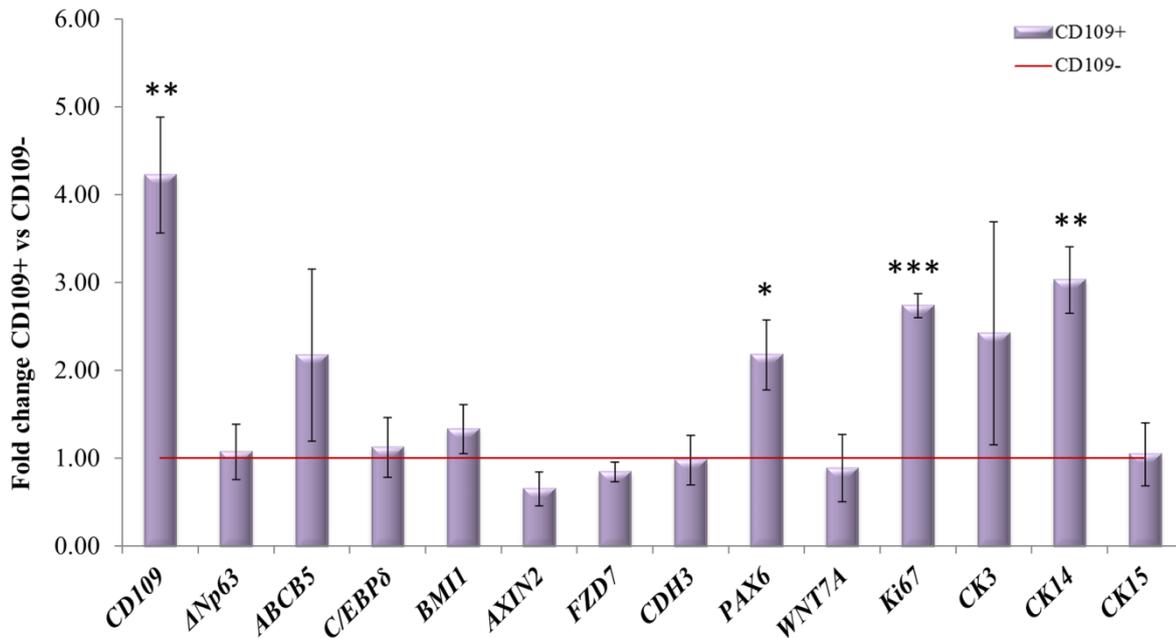
Figure 4.19 Expression of putative LSC and corneal epithelial cell markers in the sorted CD109 positive and negative cell populations. Quantitative reverse transcriptase polymerase chain reaction expression data for CD109$^+$ limbal epithelial cell population versus CD109$^-$ limbal epithelial cell population represented by the red line (value 1). Values represent mean ± SEM, n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CD200 was significantly upregulated in CD200$^+$ cell population ($p<0.001$) along with the putative LSC markers *ABCB5* ($p<0.001$), *CDH3* ($p<0.001$), *PAX6* ($p<0.01$), *WNT7A* ($p<0.01$), *CK14* ($p<0.01$), and C*K15* ($p<0.001$). On the other hand, *ΔNp63* and *Ki67* ($p<0.05$) were significantly downregulated in CD200$^+$ cell population compared to the CD200$^-$ cell population. There were no significant differences in the expressions of *C/EBPδ, BMI1, AXIN2, FZD7* and *CK3* between the CD200$^+$ and CD200$^-$ groups (**Figure 4.20**) (Bojic *et al.*, 2018).
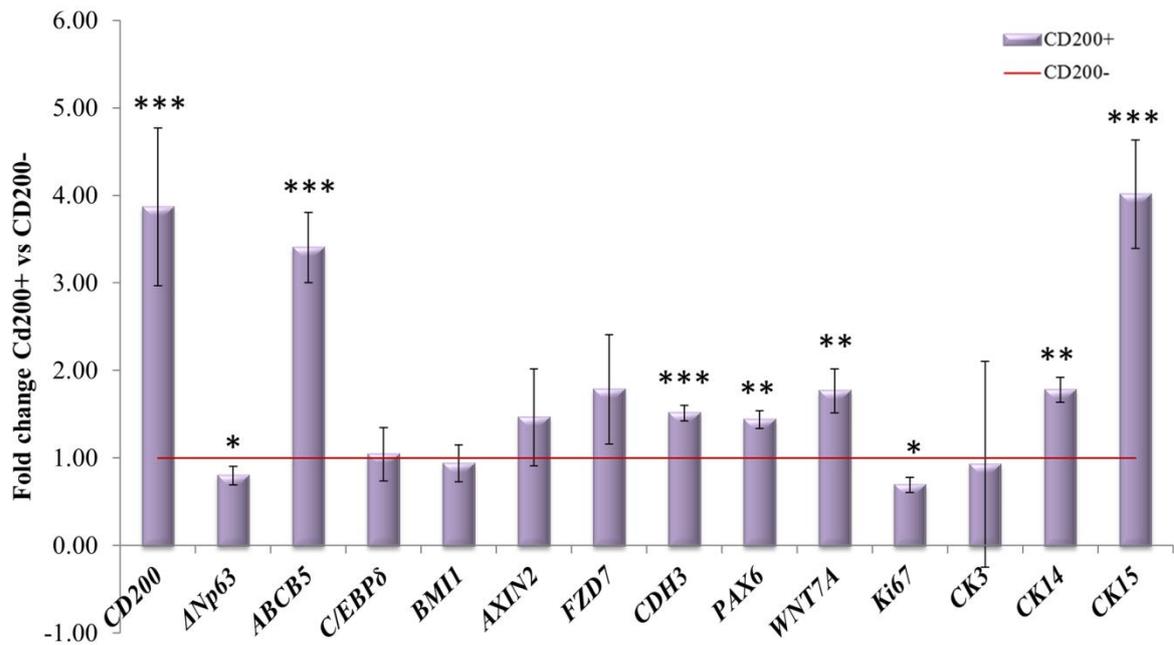
Figure 4.20 Expression of putative LSC and corneal epithelial cell markers in the sorted CD200 positive and negative cell populations. Quantitative reverse transcriptase polymerase chain reaction expression data for CD200$^+$ limbal epithelial cell population versus CD200$^-$ limbal epithelial cell population represented by the red line (value 1). Values represent mean ± SEM, n=3, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.3.7 CD200 siRNA transfection

To investigate the impacts of *CD200* downregulation on limbal epithelial cell cultures, RNAi was carried out using a pool of three different siRNAs as detailed in the materials and methods section. Quantitative RT-PCR analysis confirmed downregulation of *CD200* in the group treated with *CD200* siRNA compared to control group (*$p<0.05$*) (**Figure 4.21**) (Bojic *et al.*, 2018).
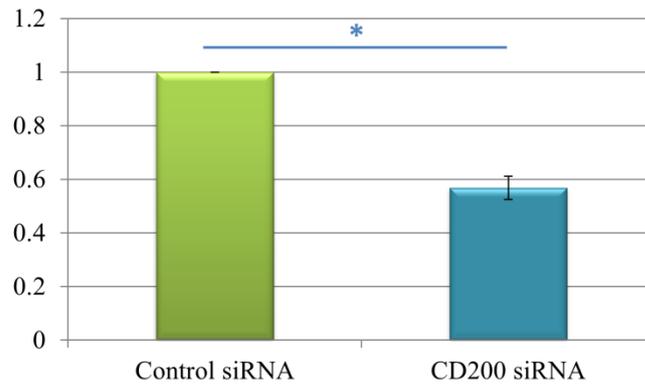
Figure 4.21 CD200 knockdown. Quantitative reverse transcriptase polymerase chain reaction expression data for control siRNA versus *CD200* siRNA treated limbal epithelial cells. Values represent mean ± SEM, n=3, *p<0.05*.

Interestingly, the colony forming efficiency assay showed no significant difference in the percentage of formed paraclones or meroclones between the two groups (**Figure 4.22A** and **B**), but holoclones completely disappeared from the siRNA transfected group (**Figure 4.22B** and **C**), leading to a significant difference of the percentage of holoclones formed between the groups (*p<0.05*) (Bojic *et al.*, 2018).

A **Control siRNA**

■ Paraclones ■ Meroclones ■ Holoclones

11%
13%
77%

B **CD200 siRNA**

■ Paraclones ■ Meroclones ■ Holoclones

11%
89%

C

	Control siRNA	CD200 siRNA
500 cells per well		
1000 cells per well		
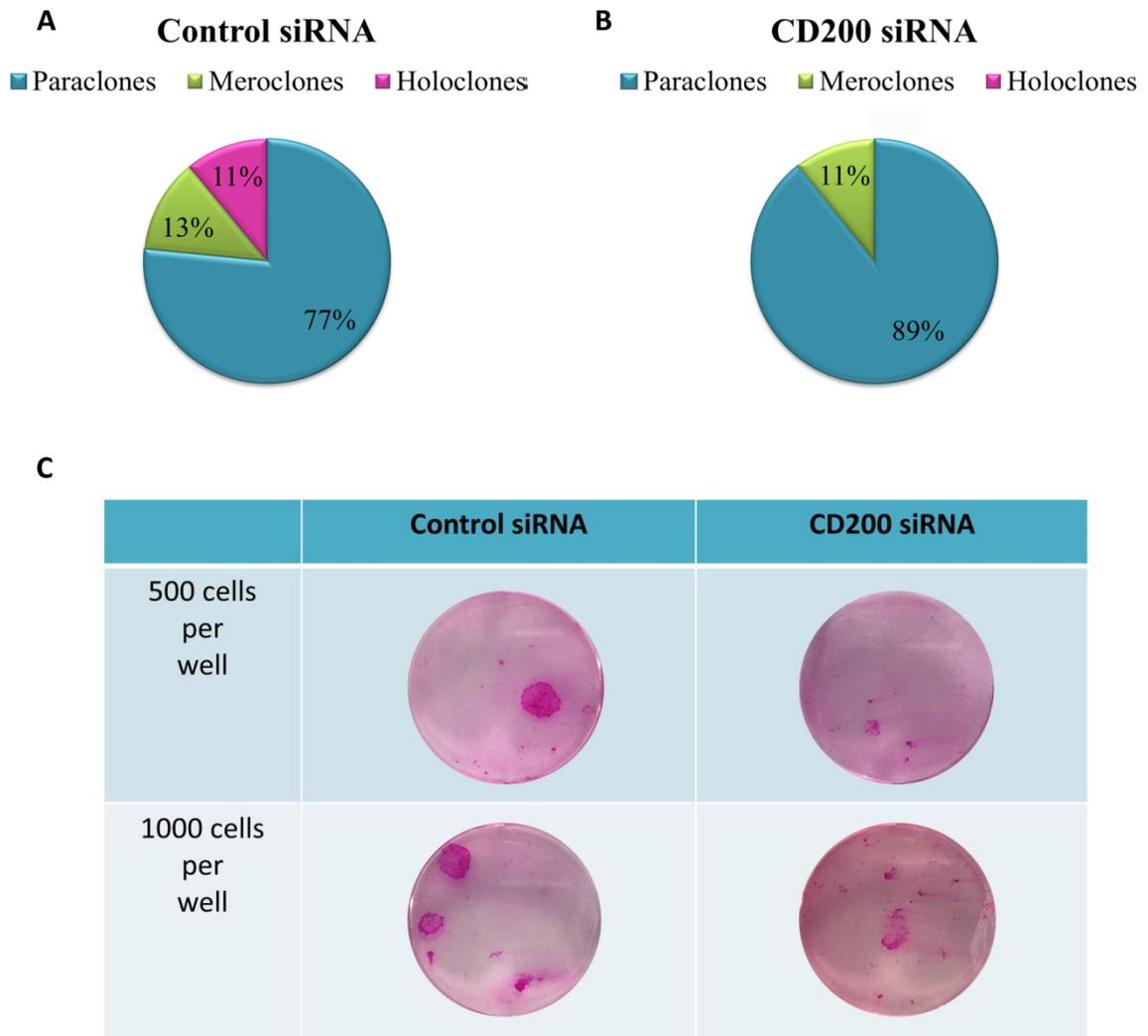
Figure 4.22 Effect of *CD200* knockdown on clonal ability of limbal epithelial cells. (A) Pie chart showing distribution of paraclones, meroclones and holoclones formed by control siRNA treated cells and (B) *CD200* siRNA treated cells. (C) Representative images of colonies formed in control and *CD200* siRNA group, with 500 or 1000 cells seeded per well.

4.4 Discussion

To date, a few putative LSC markers (e.g. *ΔNp63, ABCG2, C/EBPδ, BMI1, PAX6, WNT7A, ABCB5*) have been associated with LSCs, however, amongst these, only ABCB5 represents a cell surface marker that enables enrichment of viable LSCs. Commonly used putative LSC marker, ΔNp63α is shown to be expressed not only in LSCs but also in early TACs. Moreover, as an internal marker it doesn't allow isolation of viable LSCs. ABCB5, on the other hand, as a cell surface marker, appeared to be more promising in terms of LSC isolation and purification but recent study by Liu *et al.* showed that p63 is superior to ABCB5 as a marker for stemness, while ABCB5, either alone or in co-expression patterns with p63 and CK3, identifies more committed progenitor cells (Liu *et al.*, 2018). Moreover, ABCB5 marks around 25% of limbal epithelial cells, which is much more than LSC compartment hence we tried to find a new marker specific to LSCs. Having in mind that viable LSCs could be isolated only using a cell surface marker we focused on LEC surface marker screening in the process of identification of potential candidate markers. In this study we used the LEGEND Screen™ Lyophilized Antibody Panel to assess the expression of 361 cell surface markers in *ex vivo* expanded limbal epithelial stem cells and selected CD200 and CD109 as cell surface markers of interest for further investigation.

Up to date, there are no reports of either CD109 or CD200 expression or functional significance in the corneal epithelium. CD109 is a glycosylphosphatidylinositol (GPI)–anchored glycoprotein whose expression is upregulated in several types of human cancers, particularly squamous cell carcinomas, whilst in normal human tissues CD109 expression is limited to certain cell types including myoepithelial cells of mammary, lacrimal, salivary, and bronchial glands, basal cells of the prostate and bronchial epithelium (Mii *et al.*, 2012), human hepatic progenitor cells (Li *et al.*, 2014a), endothelial cells and a subpopulation of bone marrow CD34^{+} cells enriched in hematopoietic stem and progenitor cells (Murray *et al.*, 1999). CD109 has been shown to enhance EGF-signalling in the SK-MG-1 glioblastoma cell line through the interaction of membrane anchored N-terminal CD109 fragment with EGFR (Zhang *et al.*, 2015), and to negatively regulate TGF-β1 signalling in keratinocytes by either directly modulating receptor activity or by binding of soluble CD109 to type I TGF-β

receptor (Finnson *et al.*, 2006; Hagiwara *et al.*, 2010). TGF-β is an important cytokine that negatively regulates proliferation of different cell types including primary cultured human limbal epithelial cells (Chen *et al.*, 2006). Mii *et al.* reported that CD109-deficient mice exhibit epidermal hyperplasia and chronic skin inflammation, and CD109 regulates differentiation of keratinocytes *in vivo* (Mii *et al.*, 2012). Taken together these data show that the CD109 molecule plays an important role in epithelial cell proliferation through the positive regulation of EGF and negative regulation of TGF-β signalling as well as being involved in epithelial cell differentiation.

Our results showed that CD109 is expressed in both human and mouse corneal epithelium and is co-localised with ΔNp63 in the basal layer of the limbal epithelium whilst is absent in the other layers of the limbal epithelium and all layers of the central corneal epithelium. *In vitro*, CD109$^+$ cells were located at the edge of growing colonies, similar to ΔNp63 expression in proliferating cells at the periphery of holoclones as previously reported (Meyer-Blazejewska *et al.*, 2010). Moreover, CD109 expression decreased during calcium-induced differentiation in a similar manner to ΔNp63 expression. There were more CD109$^+$ cells in S phase of the cells cycle after 8 hours incubation with BrdU. This observation together with the higher Ki67 expression and larger colony area formed by the CD109$^+$ cells suggest that CD109 represents a cell surface marker for proliferating corneal epithelial progenitor cells.

CD200 (also known as OX-2) is a 45 kDa transmembrane immune-regulatory protein that belongs to the immunoglobulin superfamily (Barclay *et al.*, 2002; Gorczynski, 2005). The human CD200 cDNA encodes a 278 amino acid (aa) precursor that includes a 30 aa signal sequence, a 202 aa extracellular domain (ECD), a 27 aa transmembrane segment, and a 19 aa cytoplasmic domain. The ECD is composed of one Ig-like V-type domain and one Ig-like C2-type domain (McCaughan *et al.*, 1987a; McCaughan *et al.*, 1987b). A splice variant of CD200 has been described and has a truncated cytoplasmic tail. Within the ECD, human CD200 shares 76% amino acid sequence identity with mouse and rat CD200.

CD200 is widely distributed across tissues, including lymphocytes, endothelial and neuronal cells but not ubiquitously expressed (Wright *et al.*, 2001). Its cognate receptor (CD200R) is also an immunoglobulin transmembrane glycoprotein restricted

primarily to mast cells, basophils, macrophages, and dendritic cells, which suggests myeloid cell regulation as the major function of CD200 (Fallarino *et al.*, 2004; Cherwinski *et al.*, 2005; Shiratori *et al.*, 2005). CD200 transmits an immuno-regulatory signal through its receptor (CD200R) to attenuate inflammatory reactions and promote immune tolerance (Rosenblum *et al.*, 2004). CD200/CD200R mediated intracellular communication among different epidermal cell sub-populations may have an important role in preventing undesired immune responses in the skin (Matsue, 2005).

Previous studies have suggested the presence of a stem cell niche at the bulge region of the hair follicle, which contains CD200^{+} cells (Rosenblum *et al.*, 2004; Rosenblum *et al.*, 2005; Ohyama *et al.*, 2006; Kloepper *et al.*, 2008; Gerhards *et al.*, 2016) and have shown enrichment of human bulge stem cells by positive selection using CD200 as a cell surface marker (Ohyama and Kobayashi, 2012). Hair follicles represent one of the few sites of "immune privilege" (Meyer *et al.*, 2008), possibly with the aim of preserving keratinocyte stem cells (Paus *et al.*, 2003). The CD200 molecule therefore may play a vital role in this "protection" since CD200/CD200R interaction attenuates perifollicular inflammation and prevents hair follicle specific autoimmunity, thereby protecting the epidermal stem cell reservoir from autoimmune destruction (Rosenblum *et al.*, 2006). Additionally, CD200 has a clinical importance in allo- and xenotransplantation (Gorczynski *et al.*, 1999). CD200 overexpression in transgenic mice increases skin, cardiac and renal allograft survival (Gorczynski *et al.*, 2013) by suppression of inflammation and acquired immunity. Apart from normal tissues, high CD200 expression was found in colon cancer, myeloma, breast and brain cancer, melanoma and normal mesenchymal stem cells (Zhang *et al.*, 2016). It is closely related to tumour immunosuppression and has been proposed as a cancer stem cell marker in colon cancer (Zhang *et al.*, 2016). CD200 has also been proposed as a putative marker of corneal endothelial cells that enables their differentiation from stromal keratocytes and corneal stromal fibroblasts (Cheong *et al.*, 2013). We also observed CD200^{+} corneal endothelial cells in human corneal sections corroborating data published by Cheong *et al.* (Cheong *et al.*, 2013) (data not shown). Recently Rauner *et al.* showed that expression of CD200 and its receptor CD200R1 marks distinct mammary repopulating units subpopulation with stem and progenitor characteristics (Rauner *et al.*, 2018).

Taking into consideration this published literature and the low frequency of CD200$^+$ in our limbal epithelial cultures (< 5%), we hypothesised that CD200 may represent a potential cell surface marker of LSCs. Using immunostaining in human and mouse corneal tissue we showed that CD200 is exclusively located at the base of the limbal epithelium. In addition, its expression is significantly and rapidly decreased upon subsequent passaging and calcium induced differentiation of limbal epithelial cells in keeping with a stem/transient amplifying cell phenotype. CD200$^+$ cells obtained from hair follicle have been shown to possess a high CFE potential (Ohyama *et al.*, 2006); however our findings do not support these results. We found no significant difference between the CFE of CD200 positive and negative populations. However, we showed that only CD200$^+$ cells were able to form holoclones which are derived from LSCs, whilst CD200$^-$ cells produced meroclones which are known to descend from transient amplifying cells. Moreover, we showed that CD200$^+$ cells are slow cycling and only start to enter the S phase of the cells cycle after eight hours long incubation with BrdU, whereas CD200$^-$ cells enter the S phase one hour after incubation with BrdU. Importantly, downregulation of *CD200* by RNAi led to complete loss of holoclones, thus indicating an important role for CD200 in the maintenance and /or self-renewal of LSCs from which the holoclones are derived.

Both quiescent and active stem cell subpopulations coexist in several tissues, in separate yet adjoining locations (Li and Clevers, 2010). Moreover, mammalian adult stem cells are predominantly detected in quiescent state (Cotsarelis *et al.*, 1990; Potten *et al.*, 1997; Arai *et al.*, 2004). We observed a higher number of Ki67$^-$ cells and lower expression of *Ki67* within the CD200$^+$ population when compared to CD200$^-$ cells, suggesting that CD200$^+$ may represent the quiescent LSCs. Indeed, a larger part of cells in G0 phase was made up with CD200$^+$ cell population which in itself contained 79% of the side population cells, corroborating previously published findings by Umemoto *et al.* that limbal epithelial side population are quiescent and do not demonstrate proliferative capabilities in *ex vivo* culture conditions (Umemoto *et al.*, 2006). Several studies showed that only 0.3-0.5% of cells in the limbal epithelium exhibit the SP phenotype (Watanabe *et al.*, 2004; Budak *et al.*, 2005; Umemoto *et al.*, 2006). However, as with cell surface markers, possession of an SP phenotype is not a universal property of stem cells, in some tissues the SP fraction may not contain the stem cells. Combining SP determination with cell-surface marker phenotyping

has led to efficient and reliable characterization of one of the most pure and potent adult stem cell populations, the HSC subset (Shaharuddin *et al.*, 2014).

We also observed a consistently higher expression of putative LSC markers including *WNT7A, PAX6, ABCB5, CDH3, CK14* and *CK15* (Yoshida *et al.*, 2006; Chen *et al.*, 2010; Meyer-Blazejewska *et al.*, 2010; Ksander *et al.*, 2014; Eghtedari *et al.*, 2016; Lopez-Paniagua *et al.*, 2016; Richardson *et al.*, 2017; Sartaj *et al.*, 2017) in the CD200$^+$ subpopulation.

4.5 Conclusion

In summary, we report herein the identification of a new cell surface marker for LSCs (CD200) as well as a cell surface marker for proliferating progenitor cells (CD109). We believe that the identification of these two new cell surface markers will significantly aid live enrichment of these two cell types and their biological and clinical applications with potential benefits for patients suffering with limbal stem cell deficiency.

Apendix 1

The expression of various surface markers on limbal epithelial cells.

	Average (%)	SD	SEM
CCR10	9.88	6.08	3.51
CD278 (ICOS)	0.54	0.44	0.25
IFN-γRβ chain	4.09	0.71	0.41
CD46	89.41	10.00	5.77
CD70	75.17	6.31	4.46
CD1a	2.55	0.68	0.39
CD2	0.52	0.31	0.18
β2- microglobulin	96.39	6.21	3.58
B7-H4	1.61	0.57	0.40
Cadherin 11	9.52	5.56	3.94
CD10	4.75	2.27	1.31
CD100	15.67	4.35	2.51
CD103	0.40	0.05	0.04
CD105	25.92	2.58	1.83
CD106	8.33	11.19	7.92
CD107a (LAMP-1)	84.82	16.81	11.89
CD107b (LAMP-2)	52.27	20.80	14.71
CD109	56.29	24.19	13.96
CD111 (Nectin 1)	89.02	9.24	5.33
CD112 (Nectin-2)	94.99	1.55	1.10
CD114	2.96	0.60	0.35
CD116	5.45	5.12	2.96
CD117 (c-kit)	8.22	3.61	2.56
CD119 (IFN-γR α chain)	82.47	3.18	2.25
CD11a	1.07	0.18	0.10
CD11b	2.11	0.48	0.28
CD122	1.03	0.58	0.33
CD123	4.88	5.78	4.09
CD126 (IL-6Rα)	3.83	2.03	1.17
CD127 (IL-7Rα)	4.21	2.52	1.45
CD13	8.40	4.42	2.55
CD131	1.95	1.04	0.60
CD134	2.27	0.32	0.18
CD135	3.98	2.40	1.38
CD137 (4-1BB)	4.66	4.81	2.78
CD137L (4-1BB Ligand)	45.49	3.09	2.19
CD138	40.68	0.81	0.57
CD14	5.91	4.27	3.02
CD140a	3.97	1.74	1.00

	Average (%)	SD	SEM
CD140b	16.94	4.62	3.27
CD141	91.27	4.61	3.26
FCD142	93.07	1.10	0.78
CD143	5.26	2.77	1.60
CD146	67.04	4.05	2.87
CD148	87.18	7.33	5.18
CD15	1.82	0.19	0.14
CD150 (SLAM)	4.43	5.70	3.29
CD151	34.92	26.45	18.71
CD154	2.28	0.18	0.13
CD156c (ADAM10)	95.26	1.20	0.84
CD158e1 (KIR3DL1, NKB1)	3.50	0.57	0.40
CD16	1.09	0.20	0.12
CD161	1.44	0.57	0.33
CD162	1.90	0.62	0.36
CD163	4.45	3.64	2.58
CD164	82.83	20.96	12.10
CD165	86.01	8.51	4.91
CD166	95.08	1.37	0.97
CD169	0.46	0.65	0.46
CD170 (Siglec-5)	86.58	2.69	1.90
CD172a/b	77.01	19.31	13.66
CD172g	7.25	4.35	2.51
CD178 (Fas-L)	2.49	0.90	0.52
CD179a	33.90	20.41	14.43
CD179b	7.81	9.28	5.36
CD18	2.68	1.36	0.79
CD180 (RP105)	2.70	3.14	1.81
CD182 (CXCR2)	9.52	9.80	5.66
CD183	10.44	12.99	7.50
CD185	1.67	0.33	0.23
CD19	3.53	4.02	2.32
CD191	1.47	0.68	0.48
CD194	1.56	0.78	0.55
CD1b	9.76	9.52	5.50
CD1c	5.92	8.43	4.86
CD200	2.25	0.97	0.69
CD200 R	3.66	1.77	1.26
CD202b (Tie2/Tek)	3.22	1.18	0.68
CD203c (E-NPP3)	1.36	0.49	0.35
CD205	5.17	3.68	2.13
CD206 (MMR)	1.34	0.52	0.30
CD207 (Langerin)	1.14	0.52	0.30
CD21	0.78	0.25	0.14
CD213α1	3.54	0.80	0.57

	Average (%)	SD	SEM
CD213α2	17.22	18.25	10.53
CD218a (IL-18Rα)	7.87	8.92	5.15
CD221 (IGF-1R)	84.03	18.08	10.44
CD223	2.53	0.65	0.46
CD226 (DNAM-1)	9.08	5.90	3.41
CD227	85.43	13.71	9.69
CD229 (Ly-9)	1.11	0.30	0.17
CD23	2.61	3.49	2.01
CD231 (TALLA)	15.77	12.52	7.23
CD244 (2B4)	1.21	0.45	0.26
CD245 (p220/240)	14.58	5.04	2.91
CD25	1.64	0.69	0.40
CD252 (OX40L)	80.15	4.98	2.88
CD261 (DR4,TRAIL-R1)	36.67	12.04	8.51
CD262 (DR5,TRAIL-R2)	88.98	9.99	5.77
CD263 (DcR1,TRAIL-R3)	10.23	14.34	8.28
CD266 (Fn14,TWEAK Receptor)	82.43	21.74	12.55
CD268 (BAFF-R, BAFFR)	1.24	0.56	0.32
CD27	1.58	0.81	0.47
CD271	78.24	8.38	4.84
CD275 (B7-H2, B7-RP1,ICOSL)	12.99	14.29	8.25
CD276	90.00	8.92	5.15
CD277	15.60	3.07	1.77
CD279 (PD-1)	1.06	0.53	0.31
CD28	66.33	6.73	4.76
CD29	89.56	9.18	5.30
CD290	3.21	1.55	1.10
CD298	89.72	9.47	5.47
CD3	1.79	0.97	0.56
CD30	2.38	0.76	0.44
CD300c	3.04	1.98	1.40
CD309/VEGFR2	2.16	1.04	0.74
CD31	1.44	0.51	0.29
CD314 (NKG2D)	2.36	0.83	0.48
CD317	3.16	0.56	0.32
CD324 (E-Cadherin)	88.48	10.49	6.06
CD325	6.64	5.85	3.38
CD328 (Siglec-7)	1.37	0.61	0.43
CD33	3.33	1.37	0.79
CD334 (FGFR4)	1.09	0.57	0.33
CD335 (NKp46)	0.77	0.57	0.33
CD336 (NKp44)	1.42	0.88	0.51
CD337 (NKp30)	1.03	0.49	0.28
CD34	2.25	1.64	0.95
CD340 (erbB2/HER-2)	82.58	20.37	11.76

	Average (%)	SD	SEM
CD344 (Frizzled-4)	8.77	3.50	2.02
CD35	1.30	0.55	0.32
CD354 (TREM-1)	1.97	0.97	0.56
CD360	4.36	1.92	1.11
CD365 (Tim-1)	5.05	1.20	0.69
CD366 (Tim-3)	1.61	0.64	0.37
CD367 (CLEC4A)	2.22	0.16	0.12
CD36L1	92.76	1.87	1.33
CD38	1.76	1.11	0.79
CD39	0.72	0.23	0.16
CD4	1.12	0.68	0.39
CD40	26.00	12.02	6.94
CD41	0.50	0.33	0.19
CD42b	1.35	0.28	0.20
CD43	21.87	1.41	0.99
CD44	87.57	11.29	6.52
CD45	0.48	0.20	0.11
CD47	88.18	11.48	6.63
CD48	2.86	1.32	0.76
CD49a	45.98	34.13	19.70
CD49b	94.98	1.20	0.84
CD49c	84.20	14.17	8.18
CD49d	19.80	20.60	11.90
CD5	1.41	0.66	0.38
CD50 (ICAM-3)	1.44	1.44	1.02
CD54	37.33	14.57	8.41
CD55	84.41	15.51	8.95
CD56	4.83	2.49	1.44
CD58	90.10	10.03	5.79
CD6	1.04	0.82	0.47
CD61	2.84	2.97	1.71
CD62E	1.59	0.99	0.57
CD62L	2.39	2.86	1.65
CD62P (P-Selectin)	3.47	3.67	2.12
CD63	88.99	9.01	5.20
CD64	2.97	1.48	0.85
CD69	1.44	1.34	0.77
CD73	90.19	10.44	6.03
CD74	11.49	4.81	2.78
CD79b	3.83	3.05	1.76
CD8	24.09	33.55	23.72
CD80	1.84	0.03	0.02
CD81	87.97	9.49	5.48
CD82	90.20	9.23	5.33
CD83	9.20	7.52	4.34

	Average (%)	SD	SEM
CD85g (ILT7)	1.24	0.86	0.50
CD85k (ILT3)	1.27	0.32	0.18
CD87	39.07	10.93	7.73
CD89	9.21	11.53	6.66
CD8a	1.47	0.99	0.57
CD9	89.54	8.93	5.16
CD90 (Thy1)	23.76	3.16	1.82
CD93	5.50	6.77	3.91
CD94	1.45	0.47	0.27
CD95	88.73	10.10	5.83
CD96	7.16	5.82	3.36
CD97	42.88	1.67	1.18
CD99	72.68	24.04	13.88
CXCL16	1.36	0.54	0.39
DLL1	0.56	0.38	0.22
DLL4	0.55	0.31	0.18
DR3 (TRAMP)	2.61	1.63	0.94
EGFR	88.81	10.42	6.02
GITR	1.29	0.78	0.55
GPR19	2.59	1.25	0.89
GPR56	6.31	0.79	0.56
HLA-E	22.87	3.11	2.20
HVEM (TR2)	6.89	4.22	2.99
Ig light chain k	0.34	0.36	0.21
IgM	0.43	0.23	0.13
IL-21R	1.87	1.17	0.83
Integrin α9β1	4.85	3.43	1.98
Jagged 2	9.83	7.29	4.21
Ksp37	0.91	0.43	0.31
LAP	0.90	0.16	0.12
LY6G6D	1.52	0.71	0.50
MERTK	7.20	0.76	0.54
MSC (W7C6)	61.89	11.78	8.33
MSC and NPC (W4A5)	7.67	7.78	4.49
MSCA-1 (MSC, W8B2)	1.11	0.30	0.17
MUC-13	1.63	0.57	0.41
NKp80	3.06	3.03	1.75
Notch 1	34.03	16.13	9.31
Notch 3	4.74	2.25	1.30
Notch 4	44.84	16.48	9.52
NPC (57D2)	11.47	5.49	3.17
NTB-A (NTBA)	1.48	0.83	0.59
PSMA	0.75	0.83	0.48
ROR1	30.05	12.17	8.61
Siglec-10	2.95	1.95	1.13

	Average (%)	SD	SEM
Siglec-7	2.03	1.64	1.16
Siglec-8	3.13	2.66	1.53
Siglec-9	1.28	0.51	0.29
SSEA-5	14.48	4.35	2.51
SUSD2	20.11	8.42	5.95
TCR a/ß	4.49	5.01	2.89
TCR g/d	10.80	12.05	8.52
Tim-4	3.21	2.67	1.54
TLT-2	1.79	0.80	0.46
TM4SF20	0.75	0.25	0.18
TRA-2-49	2.65	1.60	1.13
TRA-2-54	1.42	0.29	0.21
TSLPR (TSLP-R)	1.20	0.56	0.32
VEGFR3	2.51	2.19	1.55
APCDD1	1.43	0.92	0.65
BTLA	6.33	7.47	4.31
CCR8	12.04	12.49	8.83
CCRL2	5.84	6.02	4.26
CD102	1.82	1.26	0.73
CD104	94.75	0.04	0.03
CD124	3.19	2.18	1.26
CD130	1.05	0.18	0.13
CD144	3.12	2.61	1.85
CD152 (CTLA-4)	2.85	2.75	1.59
CD155 (PVR)	88.77	10.78	6.22
CD158b (KIR2DL2/L3,NKAT2)	4.70	4.34	2.51
CD184 (CXCR4)	2.65	1.59	0.92
CD186	2.01	2.05	1.45
CD192	1.75	1.36	0.97
CD197 (CCR7)	3.53	3.30	1.90
CD199	7.59	7.81	5.52
CD209 (DC-SIGN)	4.38	3.84	2.72
CD217	11.89	4.31	3.05
CD230 (Prion)	94.36	1.41	1.00
CD24	86.76	10.67	6.16
CD243	3.11	2.09	1.20
CD26	1.67	0.37	0.21
CD269	2.52	1.90	1.34
CD282 (TLR2)	2.91	2.39	1.38
CD284 (TLR4)	9.84	9.13	6.46
CD301	18.78	17.88	10.32
CD303	1.12	0.48	0.28
CD304	4.29	0.15	0.11
CD307	1.70	1.48	0.86
CD323	85.30	2.31	1.63

	Average (%)	SD	SEM
CD357 (GITR)	3.10	3.23	1.86
CD36	1.16	0.84	0.49
CD369 (Dectin-1/CLEC7A)	3.06	2.31	1.64
CD370 (CLEC9A/DNGR1)	1.42	0.63	0.36
CD371 (CLEC12A)	0.76	0.44	0.25
CD45RO	1.26	0.54	0.31
CD51	88.38	10.66	6.16
CD59	92.05	10.32	5.96
CD7	1.97	0.63	0.36
CD71	88.76	10.26	5.92
CD84	6.99	5.93	3.42
CD88	2.78	1.76	1.01
CRTAM	3.78	3.24	1.87
erbB3/HER-3	7.62	2.22	1.28
FPR3	10.45	12.25	8.67
Ganglioside GD2	1.72	0.96	0.68
GPR83	9.98	8.37	5.92
HLA-A,B,C	89.33	9.33	5.39
HLA-DR	0.71	0.20	0.12
Ig light chain λ	0.52	0.08	0.05
IgD	1.66	0.28	0.20
IL-28RA	1.25	1.12	0.79
integrin b5	91.45	1.75	1.24
KLRG1	3.86	1.98	1.40
LOX-1	3.25	1.68	1.19
MICA/MICB	33.12	7.48	4.32
MSC (W3D5)	18.53	8.58	4.95
Notch 2	3.02	1.32	0.76
TACSTD2	94.24	0.71	0.50
TIGIT	0.82	0.66	0.47
C3AR	2.70	1.62	1.15
CCX-CKR	2.56	0.62	0.44
CD11c	2.30	0.91	0.53
CD129 (IL-9 R)	6.29	4.82	2.78
CD158	2.34	0.38	0.27
CD181 (CXCR1)	17.50	2.16	1.53
CD193 (CCR3)	3.64	1.04	0.60
CD196	13.70	4.70	3.32
CD1d	7.54	6.44	3.72
CD20	0.71	0.51	0.30
CD22	1.42	0.22	0.13
CD220	5.23	1.50	1.06
CD235ab	0.52	0.51	0.30
CD258 (LIGHT)	12.51	12.37	8.75
CD274 (B7-H1, PD-L1)	89.02	10.52	6.07

	Average (%)	SD	SEM
CD319 (CRACC)	9.52	4.58	2.64
CD32	1.85	1.25	0.72
CD326 (Ep-CAM)	89.35	8.23	4.75
CD338 (ABCG2)	23.13	17.96	10.37
CD368 (CLEC4D)	4.83	2.45	1.74
CD45RA	0.73	0.10	0.06
CD45RB	0.45	0.23	0.13
CD49e	91.04	12.99	7.50
CD52	4.31	2.13	1.23
CD66a/c/e	34.26	10.27	5.93
CD85h (ILT1)	4.29	2.04	1.18
CD85j (ILT2)	4.30	0.27	0.15
CD86	5.00	5.55	3.21
CD92	93.29	2.72	1.93
CXCR7	35.08	8.00	5.66
Delta Opioid Receptor	3.32	0.67	0.39
Dopamine Receptor D1 (DRD1)	1.74	0.49	0.35
EphA2	95.24	1.53	1.08
FceRIa	0.49	0.43	0.25
GARP (LRRC32)	1.29	0.54	0.31
IL-15Rα	1.84	0.68	0.48
Lymphotoxin β Receptor (LT-βR)	85.41	15.89	9.17
MRGX2	2.74	0.24	0.17
TMEM8A	14.46	14.29	10.11
CD254	5.06	1.75	1.01
CD318	93.86	0.77	0.55
CD255 (TWEAK)	8.21	7.64	5.40
SSEA-4	54.02	10.27	5.93
Sialyl Lewis X	81.43	13.63	9.64
TRA-1-81	2.14	1.12	0.65
CD160	0.77	0.22	0.16
CD57	5.94	7.94	4.59
CD66b	1.50	1.07	0.62
TRA-1-60-R	0.74	0.27	0.16
CD115	14.67	16.34	9.44
CD201 (EPCR)	88.53	1.36	0.96
CD120b	3.02	0.33	0.23
CD210 (IL-10 R)	13.15	13.81	9.77
CD267 (TACI)	4.04	2.58	1.49
CD294	25.34	11.99	8.48
CD49f	92.54	11.10	6.41
CD85a (ILT5)	1.55	1.02	0.72
CD85d (ILT4)	5.78	1.79	1.03
integrin b7	1.06	0.22	0.13
XCR1	1.95	0.57	0.41

	Average (%)	SD	SEM
Podoplanin	85.82	9.68	5.59
CD132	4.05	1.71	1.21
CD195 (CCR5)	2.57	0.72	0.41
CX3CR1	3.24	1.11	0.64
SSEA-3	0.96	0.25	0.18

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Publication and Abstracts

Published Papers

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* These papers are directly related to the research outlined in this thesis and can be found attached at the end of the thesis.

Abstracts at International Meetings:

Bojic S, Figueiredo F, Lako M. Optimisation of Transportation for Limbal Biopsies and Cultured Limbal Epithelial Cells for Worldwide Treatment of Limbal Stem Cell Deficiency. Presented as a poster at the Association for Research in Vision and Ophthalmology 2019

Bojic S, Hallam D, Sagoo P, Alcada N, Buck H, Figueiredo G, Amitai Lange A, Ljujic B, Shortt A, Figueiredo F, Lako M. Towards identification of novel limbal stem cell surface markers. Presented as a poster at the Association for Research in Vision and Ophthalmology 2018

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