Characterisation of Limbal Stem Cells

A thesis submitted to Newcastle University
for the Degree of Doctor of Philosophy

by

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CONFIDENTIAL

Abstract

The cornea is the anterior most structure at the front of the eye and its most important function is to transmit light to the retina for a clear vision. The external surface of the cornea is composed of an epithelium which is continuous with the surrounding conjunctiva. The corneal limbus forms the narrow transition zone between the corneal and conjunctival epithelia and is believed to harbour the cornea stem cells. Limbal stem cell (LSC) deficiency (LSCD) is a painful and blinding condition of the eye. The recent technique of *ex vivo* expansion of healthy autologous limbal epithelia for transplantation is the mainstay of cellular therapy for this condition. Successful outcome of *ex vivo* expanded LSC transplantation is dependent on the quality of the transplanted tissues. The main aims of this thesis are; i) to characterise human LSC from both primary LEC (LEC) and from a human telomerase-immortalised corneal epithelial cell (HTCEC) line, ii) to enrich for LSC by utilising the side population (SP) discrimination assay. Further, adult stem cells are promising candidates for promoting donor-specific tolerance and adult stem cells have immunosuppressive mechanisms to protect them from immunological reactions which are damaging to the survival of the transplanted tissues. Therefore, another aim of the study was iii) to investigate the immunobiological aspects of LSC including HLA expression and cellular migration which can promote the success of cellular transplantation. LSC markers were characterised in both LEC and HTCEC. Using an optimised protocol, SP were identified in LEC and HTCEC. Isolated limbal mesenchymal stem cells (MSC) fulfil the minimum requirement of a human MSC. These limbal MSC (LMSC) exhibited plasticity, could maintain the expression of limbal markers and demonstrated viable growth on a biological substrate, qualities making them a suitable alternative to cultured limbal explants for clinical transplantation. SP cells in HTCEC and LMSC expressed known common limbal markers, stem cell antigens and the chemokine CXCR4. The presence of CXCR4 and CXCL12-mediated cellular migration were demonstrated in HTCEC. Further, HTCEC constitutively expressed HLA Class I antigens, while HLA Class II expressions were induced by Interferon-γ stimulation. In this study, HTCEC HLA-typing was presented and that HTCEC were in many ways comparable to LEC, therefore suitable as a LSC replacement or as a robust model for further understanding of LSC biology.

Acknowledgement

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Thank you from the bottom of my heart,
Bakiah Shaharuddin,
Newcastle Upon-Tyne,
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Table of Contents

List of Figures

List of Tables

Abbreviations

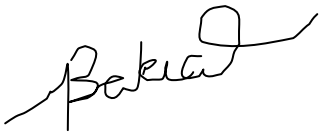
ABCG2	Adenosine tri-phosphate binding cassette G2
ABCB1	Adenosine tri-phosphate binding cassette B1
ABCC1	Adenosine tri-phosphate binding cassette C1
ACAID	anterior chamber induced immune deviation
AM	Amniotic membrane
APC	Antigen presenting cells
BRCP	Breast cancer resistance protein
BrdU	5-bromo-2'-deoxyuridine
CCL	C-C Ligand
CD	Cluster of differentiation
C/EBPδ	CCAAT/enhancer binding protein-delta
CF	Counting fingers
CFE	Colony forming efficiency
CK	Cytokeratin
CTL	Cytotoxic T cells
CTLA4Ig	Cytotoxic T lymphocyte antigen 4 immunoglobulin G
Cx	Connexin
CXCL	C-X-C Ligand
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DKSFM	Defined keratinocyte serum free medium
DMEM	Dulbecco's modification of Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide phosphate
DTH	Delayed type hypersensitivity
ECM	Extracellular matrix
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESC	Embryonic stem cell
FA	Formaldehyde

FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyonate
FSC	Forward scatter
FTC	Fumitremorgin C
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDNF	Glial derived neurotrophic factor
HESC	Human embryonic stem cell
HER-2	Human epidermal growth factor receptor (HER) factor-2
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HTA	Human Tissue Authority
HTCEC	Human telomerase-immortalised corneal epithelial cell
ICAM-1	Intercellular adhesion molecule-1
ICC	Immunocytochemistry
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL	Interleukin
IL-1Ra	IL-1 receptor antagonist
IPSC	Induced Pluripotent Stem Cells
KGF	Keratinocyte growth factor
LC	Langerhans cell
LEC	Limbal epithelial cell
LFA-1	Leucocyte function-associated antigen-1
LSP	Limbal SP
LSC	Limbal stem cell
LSCD	Limbal stem cell deficiency
MAPK	Mitogen-activated protein kinase
MIP-2	Macrophage inflammatory protein-2
mESC	Mouse embryonic stem cell
MHC	Major Histocompatibility Complex
MIP-2	Macrophage inflammatory protein-2
MSC	Mesenchymal stem cells
N	Number
NKT	Natural killer T cells
NSP	Non-side population

P	Probability
PBL	Peripheral blood leucocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1-B7-H1	Programmed death-1 Ligand-B7-H1 binding
PFA	Paraformaldehyde
PK	Penetrating keratoplasty
Rho-X	Rhodamine X
RNA	Ribonucleic acid
RT	Reverse transcription
SC	Stem cell
SP	Side population
SSC	Side scatter
T3	Tri-iodothyronine
TA	Transactivation
Ta	Annealing temperature
TAC	Transient amplifying cell
TBE	Tris/Borate/EDTA
TDC	Terminally differentiated cell
TGF-β1	Transforming growth factor β1
Th1	T helper type
Th2	T helper type 2
Tm	Melting temperature
TNF-α	Tumour necrosis factor-α
TSP-1	Thrombospondin-1
T reg	Regulatory T cells
VIP	Vasointestinal peptide
WHO	World Health Organisation

Declaration and Copyright Statement

Parts of the material offered have been presented at conferences or submitted and/or published as acknowledged or cited. Some contents of this thesis were taken from published materials where the writer was the first author. Material from the work of others has been acknowledged, and quotations and paraphrases suitably indicated.

Signature: 

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Publications, Abstracts and Awards

Publications*

"Limbal Side Population cells; a future treatment for limbal stem cell deficiency."
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"Immunological Properties of Ocular surface and Importance of Limbal Stem Cells for Transplantation". **Bakiah Shaharuddin**, Sajjad Ahmad, Annette Meeson, Simi Ali (2013). *Stem Cells and Translational Medicine*, 2:614-624.

"Optimisation of the Side population assay; for identification of limbal stem cells from both an immortalised corneal epithelial cell line and corneal limbal tissues."
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*Publications and abstracts are sourced from the research outlined in this thesis.

Chapter 1 LITERATURE REVIEW

1.1 Corneal Blindness

1.1.1 Definition, epidemiology and patho-physiology

According to the World Health Organisation (WHO) fact sheets on visual impairment and blindness, there are 285 million people who are visually impaired worldwide (WHO, 2014). Out of this, 39 million are blind, while 246 million have low vision. In the United Kingdom (UK), currently there are 2 million people living with sight loss. It was estimated that this will rise to 2.3 million by the year 2020 (Fight-for-Sight, 2014). There are 335,000 adults and 25,000 children in the UK who are registered as blind or partially blind according to "Fight for Sight" key facts (Figure 1.1).

Diseases affecting the cornea are the second most important causes of blindness after cataracts. The epidemiology of "corneal blindness" is complicated and covers a wide variety of patho-physiologies which mostly arise from infectious and inflammatory conditions and differ from one country to another. Corneal blindness carries a morbidity which affects quality of life and is often associated with an increased economic burden (Whitcher *et al.*, 2001).

1.1.2 Anatomy of the cornea and limbus

The cornea is the anterior most structure at the front of the eye (Figure 1.2). Its most important function is to transmit light onto the retina. Hence, the cornea has a smooth and transparent avascular surface. It also contributes as part of the physical, physiological and immunological defence mechanisms of the ocular surface. The cornea is tough with high tensile strength and flexibility to resist mechanical damage. It has inherent mechanisms to maintain the eye shape and eye pressure. The cornea has an overlying tear film which plays a major role in ocular surface immunology.

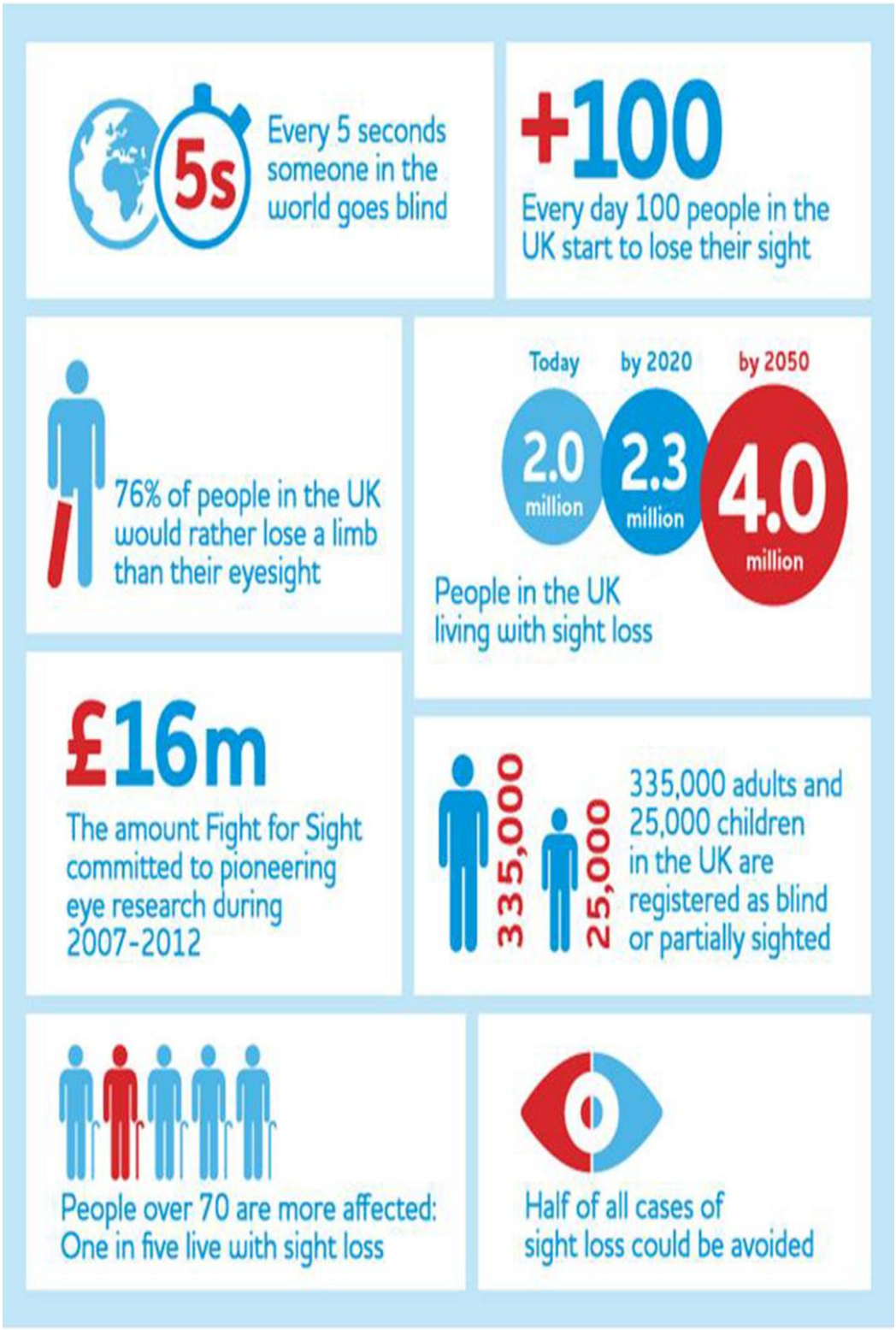


Figure 1.1 Key facts on sight loss and eye conditions. *[http://fightforsight.org.uk/sight-loss-facts]*

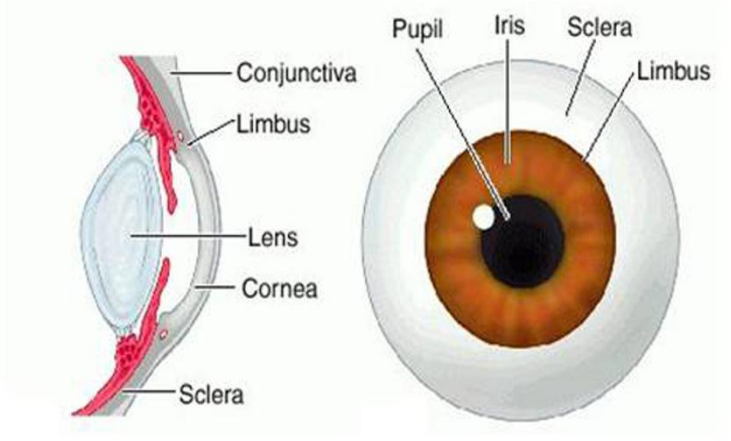


Figure 1.2 Anatomy of the eye and the limbus.

The cornea is a highly specialized structure with five distinct layers (Figure 1.3). The outermost layer of the cornea is the thin epithelial cell layer, which is composed of 5-6 layers of squamous, stratified, and non-keratinised cells (Sevel and Isaacs, 1988). The epithelium continues into the edge of the cornea towards the conjunctiva. The Bowman's layer is the basement membrane of the epithelium which is an acellular membrane, at the outer part of the corneal stroma.

The majority (80%) of corneal thickness is contributed by the stromal layer; the cornea enjoys beneficial physical properties which constantly keep it in a dehydrated state due to the organisation of tightly packed collagen lamellae and the presence of compact keratocyte networks within the stroma. The next layer is the thick Descemet's membrane, which is the basement membrane of the corneal endothelium - the most posterior part of the cornea. The endothelium faces the aqueous humour, and supplies the stroma with nutrients. It is equipped with physiological tight junctions with integral transport systems to maintain corneal functions. The corneal stroma continues up to the sclera and the endothelium is connected to the trabecular meshwork through a transition zone. The corneal limbus is a narrow strip of pigmented transitional zone between cornea and conjunctiva, located circumferentially peripheral to the cornea.

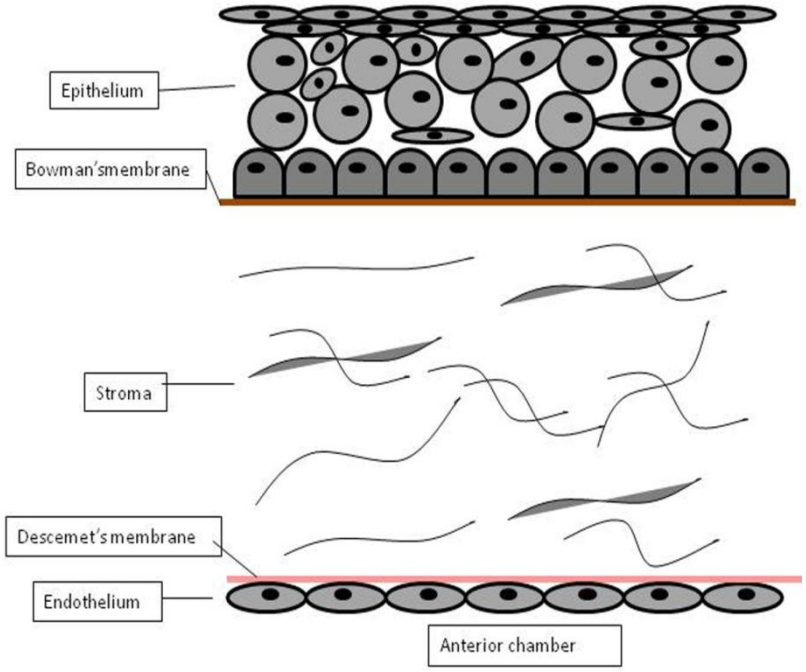


Figure 1.3 Diagrammatic representation of human cornea. The three main layers; epithelium, stroma and endothelium. The Bowman's membrane is an acellular layer which lies in the anterior stroma, just beneath the basement membrane of the epithelium. The Descemet's membrane is the basement membrane of the endothelia *(Shaharuddin et al., 2013b).*

1.1.3 Development of the human cornea

The fetal cornea can be identified at the 6th week of gestation; it is formed from the surface ectoderm overlying the lens (Figure 1.4). In the beginning, the cornea consists of two layers; the epithelium and endothelium with an acellular space in between, the primary stroma (Sevel and Isaacs, 1988). Studies in quails and chicks demonstrated a huge contribution of neural crest cells in periocular structures, refractive media, and the cornea (Creuzet *et al.*, 2005).

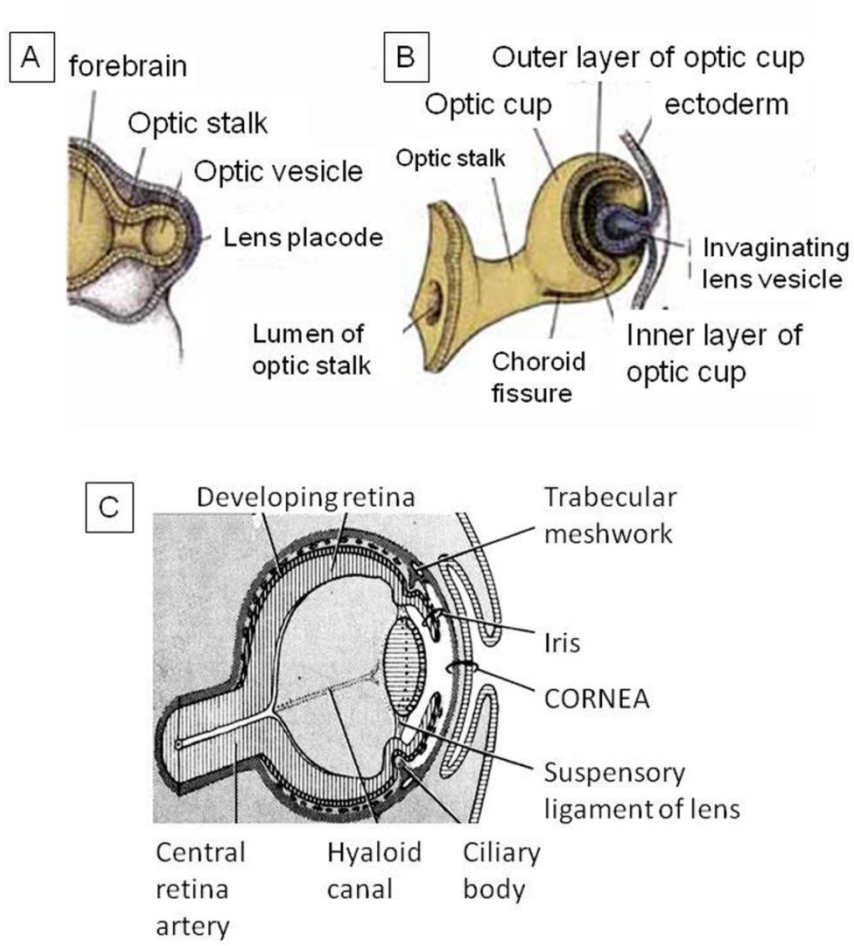


Figure 1.4 Fetal cornea development. (A) Forebrain and developing optic vesicles as seen in a 4-mm embryo. (B) Double-layered optic cup and invaginating lens vesicle as seen in a 7.5-mm embryo. The optic stalk connects the developing eye to the brain. (C) Ocular structures as seen in a 15-week fetus when all the layers of the eye are established. The hyaloid artery traverses the vitreous body from the optic disc to the posterior surface of the lens. *(Modified from Mann IC, The Development of the Human Eye. New York, Grune and Stratton, 1974)*

1.1.4 LSC niche

Adult stem cells are now believed to reside in most tissue populations for regenerative purposes and tissue repair. The defining characteristics of stem cells are self-renewal, which is the ability of the cells to replicate themselves and produce progenitor cells with high fidelity, and the capacity to differentiate into multiple different cell lineages. To ensure stem cells maintenance, stem cells are protected from hostile external factors in a specialized microenvironment i.e. stem cell niche. Within this protective niche, stem cells maintain cell-cell interactions with their neighbouring cells. They are able to secrete extracellular matrix and other factors that allow the resident stem cells to

maintain their stemness in an undifferentiated state. This potentially makes stem cells last as long as the life span of the organism.

There is mounting evidence that corneal LSC may reside in the basal layer of the limbus (Davanger and Evensen, 1971), (Schermer *et al.*, 1986), (Schlotzer-Schrehardt and Kruse, 2005),(Yeung *et al.*, 2008), (Sun *et al.*, 2010). When comparing the proliferative potential of limbal versus corneal cells, limbal cells showed greater growth rates than corneal cells when they were serially transplanted (Graziella Pellegrini *et al.*, 1999) (Wei *et al.*, 1993).

The limbus consists of multi-layers of cells, which are dispersed in the stroma, rich in blood vessels, contains melanocytes and have an abundant nerve supply (Figure 1.5). There are associated changes at the limbus; whereby the epithelium thickens from 5-6 layers at the corneal centre, to 10-12 layers in thickness. The Bowman's layer is also absent at this region, which results in the undulated corneal epithelium lying directly on the corneal stroma, in which the stromal collagen is also less organised and there is abundant fibroblast-like cells. These configurations are now referred as the "Palisades of Vogt" (Shanmuganathan *et al.*, 2007; Yeung *et al.*, 2008; Nubile *et al.*, 2012). The Descemet's membrane is also absent, and the endothelial cells appear bigger and flatter here.

There are others cells lying in close proximity to LSC, namely the transient amplifying cells (TAC), melanocytes and Langerhan's cell (LC) (Figure 1.5). It remains to be determined whether these cell types act as niche cells. It is believed that early TAC will differentiate into late TACs located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiated cells (TDC). Structurally, the basement membrane separates the epithelium from the underlying stroma. The limbal stroma contains mesenchymal cells, which may also serve as niche cells (Schlotzer-Schrehardt and Kruse, 2005).

Supporting evidence also come from the fact there is absence of the major corneal epithelial differentiation markers, CK3 and CK12 keratins in limbal basal cells. The exclusive location of slow-cycling and label retaining cells in the limbal basal layer was also observed (Cotsarelis *et al.*, 1989). The presence of 64K keratin in all layers of rabbit corneal epithelium but localized to the suprabasal area of the limbus showed that

the limbal epithelium is less differentiated (Schermer *et al.*, 1986). A seminal work by Pellegrini *et al*, also points to ocular surface epithelium consisting of holoclones with the properties of stem cells (Graziella Pellegrini *et al.*, 1999).

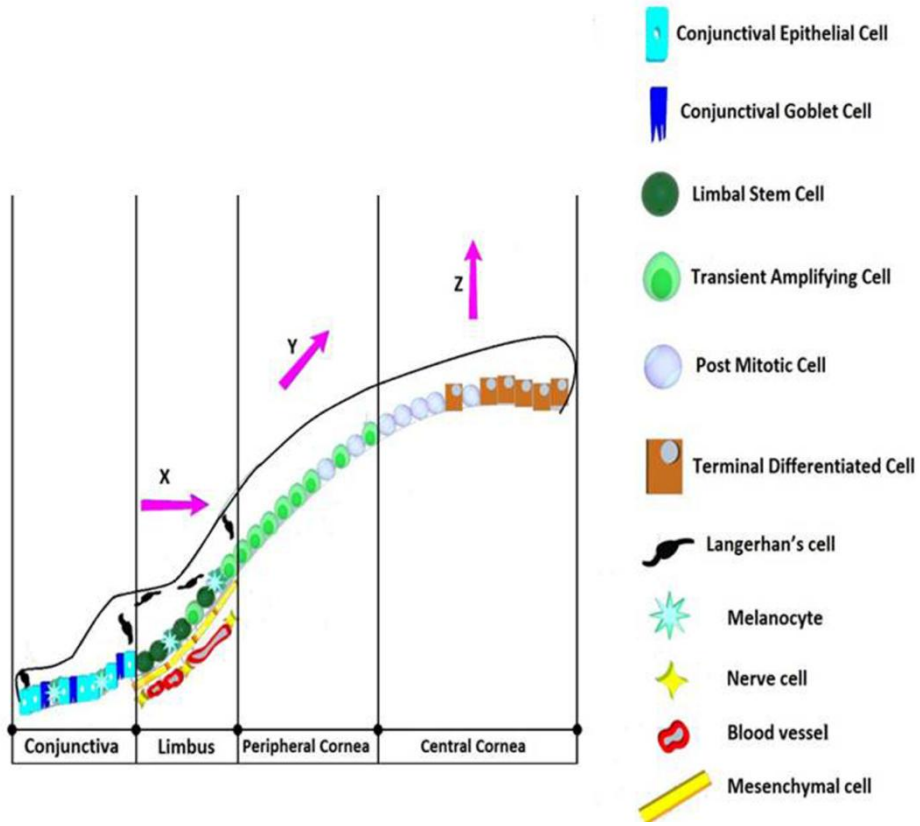


Figure 1.5 The corneal limbus and the LSC niche. (Shaharuddin *et al.*, 2013b).

The hypothesis that limbal stem cells are only confined to the limbus has been challenged by Majo *et al*, who suggested that the limbus is an equilibrium zone where the conjunctiva and corneal epithelia are confronted, similar to the tectonic plates (Majo *et al.*, 2008; Sun *et al.*, 2010). Factors like the dome-shaped of the cornea, lid blinking, ocular pressure and corneal stromal elasticity, hold the conjunctiva and corneal epithelia in two opposite directions, by way of confronting the limbus. In brief, disturbance to the equilibrium i.e limbal stem cell deficiency causes migration of limbal stem cells onto the cornea.

To support this, recent findings have demonstrated that self-renewal capacity of corneal epithelium is also shared by central corneal cells as a response to central corneal wounding (Ki-Sook Park *et al.*, 2006; Chang *et al.*, 2008). More interestingly, peripheral limbal and central corneal regenerative capacity remains the same with

limbal ablation for at least 24 hours after corneal wounding (Chang *et al.*, 2008). This clearly shows that oligopotent corneal stem cells are spread throughout corneal basal epithelium (Majo *et al.*, 2008) and not exclusive to the limbus. Despite this controversy, decades of scientific evidence and reports have localised the limbus as the location of corneal epithelial stem cells.

In response to corneal injury, limbal cells are induced to migrate and differentiate centripetally (Wei *et al.*, 1993). Removal of the limbus also diminishes the capacity of cornea to regenerate itself (Huang and Tseng, 1991). More recent advances using the lineage tracing technique in K14+ve Confetti mice supported the evidence that mouse limbus significantly contributed to self renewal and regeneration of the mouse cornea (Amitai-Lange *et al.*, 2015; Di Girolamo *et al.*, 2015). Limbal cells also responded rapidly to major wounding compared to the wound healing potential of the long-term corneal clones which mainly responded to minor injury (Amitai-Lange *et al.*, 2015).

It has been estimated that up to six limbal epithelial crypts may be present in the human cornea. The unique structure of the column and the undulating junctions between limbal epithelium and stroma also protect the cells from shear forces. These are papilla-like columns, richly vascularised and highly innervated. They extend radially into the conjunctiva or circumferentially at the periphery of the cornea (Dua *et al.*, 2005).

Melanin pigments which causes dark discolouration in these cells, offer a protection against possible insults of ultraviolet light and the generation of reactive oxygen species. The presence of limbal blood vessels close to the underlying stroma also provides nourishment to the cells. The limbal stroma is also highly innervated. These characteristics make the limbal region an ideal protective micro-environment and fulfil the requirements of a stem cell niche.

1.1.5 Molecular Characterisation of LSC

p63 is a homolog of transcription factors belonging to a family of tumour suppressor proteins which include p53 and p73 (Benard *et al.*, 2003). It is involved in Notch signalling and plays a role in the regulation of epithelial morphogenesis. All isoforms of the p63 protein consist of a DNA-binding domain and a carboxyl-terminal oligomerisation domain. There are 2 forms of p63; isoforms with an added amino-

terminal transactivation domain are termed TAp63 and those without are ΔNp63. In addition, there are three forms of the carboxyl-terminal oligomerisation domain – α, β, and γ. This results in a total of six p63 isoforms (Figure 1.6). p63 is expressed at the limbal basal epithelium, and co-expression of p63 with Connexin43 was evident in the central corneal epithelium (Du *et al.*, 2003). Although not specific to limbal cells, p63 may be a marker of undifferentiated precursor cells, either stem cells or TAC.

Mutations in the p63 gene have been associated with ectrodactyly, ectodermal dysplasia, and facial clefts syndrome (Celli *et al.*, 1999). The inheritance is by autosomal dominant and is characterised in part by the lack of normal stratified epithelium (Celli *et al.*, 1999; Mills *et al.*, 1999). p63 knockout mice show severe abnormalities of ectodermal differentiation (Mills *et al.*, 1999) where the skin in these mice is composed of a single layer, lacking any normal stratification.

Studies have localised p63, and in particular the ΔNp63α isoform, to the nuclei of progenitor cells in various epithelial structures (Yang *et al.*, 1998). Specifically in the case of corneal epithelial progenitors, immunohistochemistry and cell culture studies have localised this particular isoform of p63 to the LSC (G. Pellegrini *et al.*, 2001). There has however been growing evidence that ΔNp63α is also expressed by early TAC (Espana *et al.*, 2004; Harkin *et al.*, 2004a; Dua *et al.*, 2005; Anshu *et al.*, 2011), although these findings may be as a result of poor p63 isoform specificity of the antibody (Enzo Di Iorio *et al.*, 2005; Kawasaki *et al.*, 2005). In addition, Pellegrini and co-workers have recently shown using molecular biology techniques (rather than immunohistochemistry alone) that the ΔNp63α isoform is specific to LSC (Enzo Di Iorio *et al.*, 2005; E. Di Iorio *et al.*, 2006).

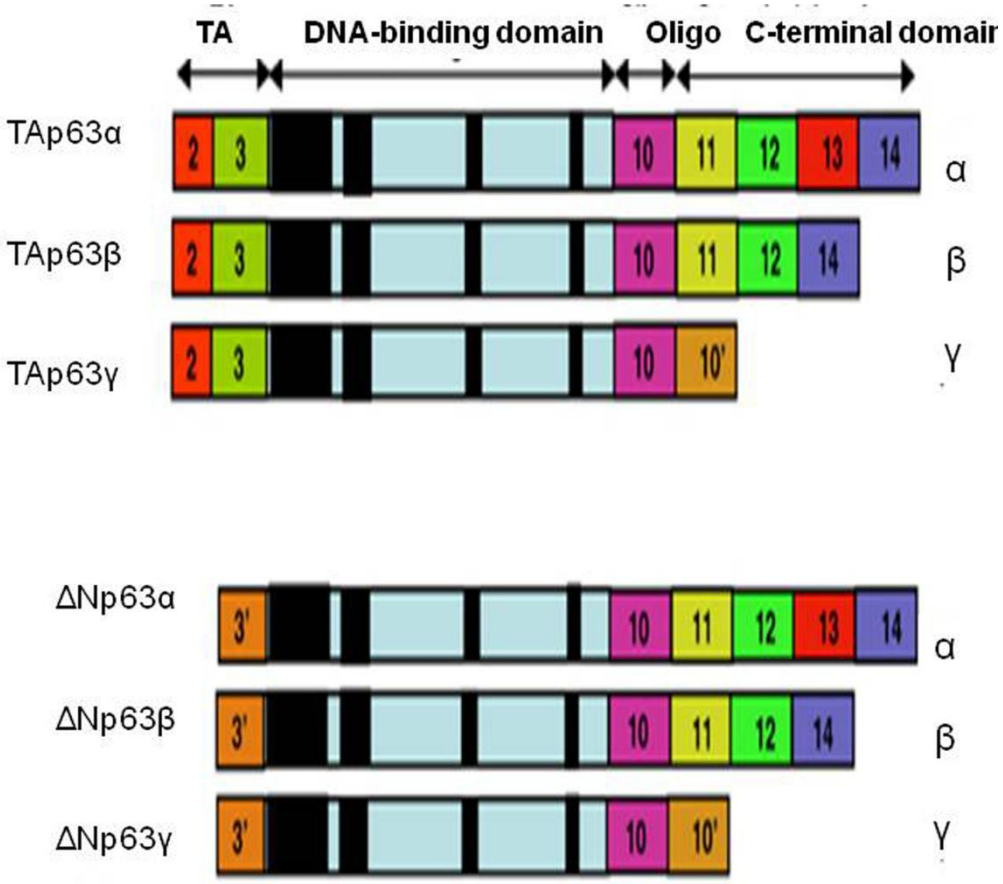


Figure 1.6 Six isoforms of the transcription factor p63. All isoforms consist of a DNA-binding domain. Some of these p63 isoforms have an added transactivation (TA) domain, the so-called TAp63 isoforms. Those without the TA domain are termed ΔNp63. All isoforms have an oligomerisation domain of which there are three different forms (α, β, and γ). These variations result in a total of six p63 isoforms – TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ. (Murray-Zmijewski *et al.*, 2006)

Integrins are integral cell membrane glycoproteins which aid cell-cell and cell-matrix interactions (Belkin and Stepp, 2000). Although previous studies have proposed α9β1 integrin as a putative marker for LSC (Stepp *et al.*, 1995), recent studies suggest that the α6β4 and α3β1 integrins are associated with BrdU label retaining basal cells of the limbal epithelium (Ahdeah Pajoohesh-Ganji *et al.*, 2006). This suggests α6β4 and α3β1 integrins as putative LSC markers, and the α9β1 integrin as a marker for early transient amplifying cells.

Adenosine tri-phosphate binding cassette G2 (ABCG2) is a cell surface transport protein, which is preferentially expressed in a variety of adult stem cells (Zhou *et al.*, 2001; Naylor *et al.*, 2004). This protein is a member of the White subfamily, and is alternatively referred to as a breast cancer resistance protein (BRCP). It functions as a

xenobiotic transporter which may play a major role in multi-drug resistance. It has recently been shown that ABCG2 expression can be localised to basal cells of the limbal epithelium and that approximately 3% of LEC express ABCG2 (Satoko Hori *et al.*, 2004; Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a).

The protein encoded by cytokeratin 3 (CK3) gene is a type II cytokeratin; consisting of basic or neutral proteins which are arranged in pairs of heterotypic keratin chains. It is co-expressed during differentiation of simple and stratified epithelial tissues. This type II cytokeratin is specifically expressed in the corneal epithelium with family member CK12. CK3 and 12 are intermediate filament, which together with microfilaments and microtubules, form the cytoskeleton of all vertebrate cells (Strelkov *et al.*, 2003). CK3 associates with CK12 to form the CK3/12 dimer. Using ICC, it has been shown that CK3/12 identifies the more differentiated cells in the corneal epithelium (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). This CK dimer is therefore absent in the LSC and early TACs of the limbal epithelium, and is therefore an important negative marker for LSC.

Connexin43 is an integral membrane protein of the connexin family, alpha-type (group II) subfamily. Functionally, Connexin43 is involved in gap junction formation, while the absence of functional gap junctions has been reported to be an important property possessed by stem cells in order to maintain a distinct intracellular environment (Matic *et al.*, 1997; Trosko *et al.*, 2000). It has been localized to the basal cells of the corneal epithelium (Matic *et al.*, 1997; Wolosin *et al.*, 2002), but shown to be absent from the basal cells of the limbal epithelium where both the LSC and the early TACs reside (Du *et al.*, 2003). It is therefore an important negative marker for LSC.

CCAAT/enhancer binding protein-delta (C/EBPδ) is an important transcriptional activator in the regulation of genes involved in cellular proliferation, differentiation, metabolism and inflammatory responses (Ramji and Foka, 2002). Recently, it has been shown that it plays an important role in the cell cycle and cell renewal of LSC. Co-expression of C/EBPδ, Bmi-1, and ΔNp63alpha was used to identify mitotically quiescent LSC (Barbaro *et al.*, 2007).

Bmi-1 (B lymphoma Mo-MLV insertion-region 1) is a component of a Polycomb group, a complex class required to maintain the transcriptional repressive state of many

genes, including Hox genes, throughout development and is believed to prevent unnecessary proliferation and differentiation of leukaemic stem cells (Lessard and Sauvageau, 2003) and thought to play a role in cell renewal (Molofsky *et al.*, 2003; In-kyung Park *et al.*, 2003).

Importin 13 is a recently investigated marker of LSC markers (Wang *et al.*, 2009). It is involved in the bidirectional transfer of substrates through the nuclear pore complexes between the nuclear and cytoplasmic compartments (Mingot *et al.*, 2001; Liang *et al.*, 2008; Bono *et al.*, 2010). Expression of Importin 13 in human limbal basal cells has been shown to be related to maintaining the properties of corneal progenitor cells.

Keratin 10 (K10) has been detected in the suprabasal limbal region in the late embryonic rat cornea at 20.5 days post conception and also in neonatal rats but was absent in adult rats (Waters *et al.*, 2009). K10 is hypothesized to be important in the development, but not the maintenance of the LSC niche. However, the role of K10 in the LSC niche remains unclear.

Identification and verification of LSC specific markers is still an ongoing challenging process. Isolation of LSC currently uses some non-specific markers. Often this would require a panel of positive and negative markers, a technique similarly observed in the haematopoietic and renal systems. Unfortunately, there are minimal cell surface markers that are associated with LSC. The use of specific cell surface markers would allow techniques such as fluorescence activated and magnetic cell sorting to be used for better isolation of LSC (Antonelli *et al.*, 2004). Enriched cell populations could then be further characterised by gene expression, immunofluorescence or protein expression studies.

1.1.6 Maintenance of corneal epithelium

Stem cells divide asymmetrically to maintain cellular homeostasis by the processes of self renewal and differentiation. In contrast to symmetric cell division, stem cells divide asymmetrically to produce two daughter cells with different cellular fates (Figure 1.7). One daughter cell has the same stem cell characteristics with the original stem cell including its proliferative capacity and maintenance of the dedifferentiation state, and the other daughter undergoes differentiation. The daughter stem cell's fate is normally to replenish the stem cell depot.

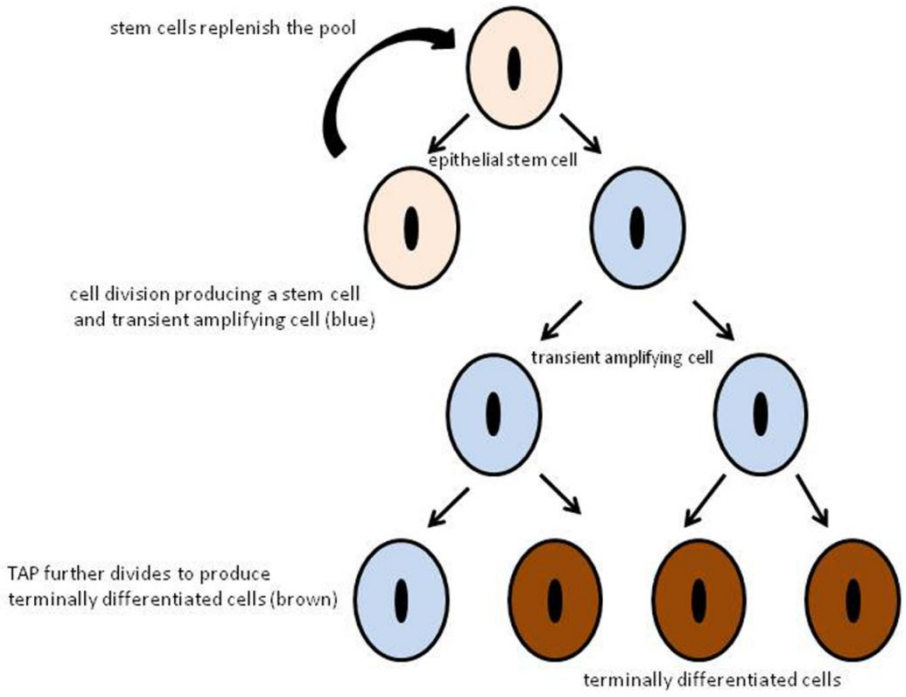


Figure 1.7 Asymmetric division of a corneal epithelial stem cell. The stem cell produced will replenish the pool, while the transient amplifying cell further divides and subsequently differentiate to terminally differentiated cells.

The XYZ hypothesis by (Thoft and Friend, 1983) has revolutionised the understanding on the LSC maintenance. It clarifies the corneal epithelial homeostasis in normal conditions and in response to injury, and aging. The hypothesis states that aged or damaged central corneal epithelial cells (through exfoliation, trauma or disease) are continuously replenished through centripetal migration of LSC.

The balance between cell proliferation and cell loss is directed by the equation $X + Y = Z$ where; X represents basal corneal epithelial cell movement towards the surface, Y represents movement of peripheral epithelial cells towards the central cornea and Z represents desquamation of the surface epithelium (Figure 1.8).

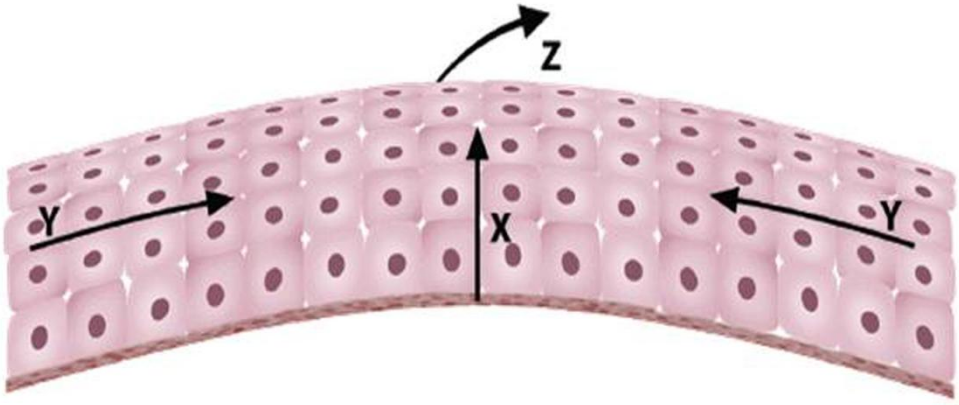


Figure 1.8 XYZ hypothesis on LSC maintenance explained by centripetal migration of LSC. The balance between cell proliferation and cell loss is directed by the equation X + Y = Z where; X represents basal corneal epithelial cell movement towards the surface, Y represents movement of peripheral epithelial cells towards the central cornea and Z represents desquamation of the surface epithelium (Fernández *et al.*, 2008).

1.1.7 Enrichment method for LSC – Side population assay

The side population (SP) discrimination assay is based on the differential potential of cells to efflux vital dyes; the most widely used of these is Hoechst 33342 dye. This efflux potential is due to expression of members of the ATP-binding cassette (ABC) family of transporter proteins expressed within the cell membrane (Zhou *et al.*, 2001).

When bone marrow cell populations were incubated with Hoechst dye and subjected to flow cytometry, active efflux of the dye caused a population of cells to appear as a segregated cohort known as SP. The SP phenotype of these cells was confirmed by the addition of a transporter inhibitor to cells from the same cell preparation that prevents dye efflux, FTC.

All ABC transporters contain two transmembrane-spanning domains (TMD) and ATP-binding domains (ABC). Typically, the transmembrane regions provide a translocation pathway for a specific substrate which allows transport of variety of substrates (Figure 1.9). The release of energy occurs intracytoplasmic which is a molecular compartment provided by the nucleotide binding domains. As the ABC cassettes bind and hydrolyze ATP, conformational changes occur and transmitted to the membrane-spanning domains, where they induce rearrangements that translocate the substrate from one side of the membrane to the other. The SP phenotype is lost upon inhibition of ABC transporter activity.

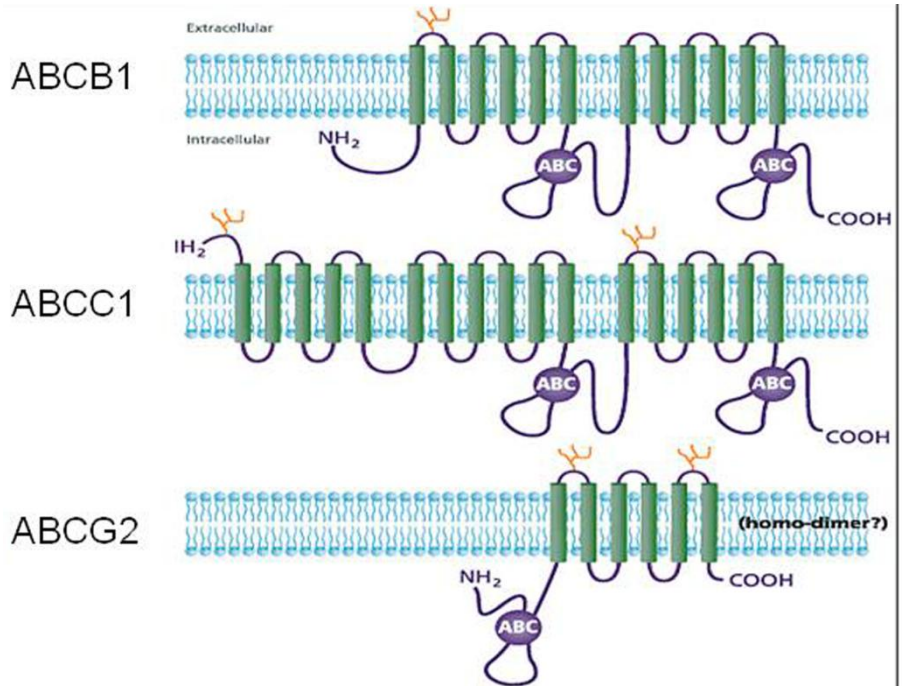


Figure 1.9 Membrane topology models for ABC transporters. The green bars represent predicted transmembrane helices, the purple circles represent the ABC domains, the gold trees are glycosylation sites at the extracellular surface. (Szakács *et al.*, 2008).

1.1.8 Alternative sources of stem cells at the limbus: MSC

The limbal epithelium also possesses stromal cells which are adherent to plastic, have fibroblastic morphology and are able to differentiate into multiple lineages suggestive of MSC (Branch *et al.*, 2012; Garfias *et al.*, 2012; Gui-Gang Li *et al.*, 2012). These cells can be isolated using protocols as described by previous investigators (Polisetty *et al.*, 2008). Human corneal mesenchymal stromal stem cells, unlike bone marrow-derived MSC, develop from neural crest cells (Hoar, 1982) and are believed to support the growth of limbal epithelial cells (Pinnamaneni and Funderburgh, 2012; Bray *et al.*, 2014).

1.1.9 Limbal stem cell deficiency

LSCD refers to a condition caused by abnormal maintenance of the LSC, as a result of a reduction in the frequency of LSC resulting in abnormal maintenance (Ahmad, 2012).

It can be broadly categorised into unilateral or bilateral involvement, acute or chronic conditions (Table 1.1). Among the causes are hereditary genetic disorders called aniridia, where there is developmental dysgenesis of the anterior chamber of the eye due to PAX6 gene mutation (He *et al.*, 2012; Smith *et al.*, 2012). Acquired causes of LSCD include chemical and thermal injury, inflammatory conditions i.e Steven-Johnson syndrome, ocular cicatricial phemphigoid and chronic limbitis. Cryotherapy to the limbus, radiation and topical instillation or subconjunctival injection of toxic drugs are some iatrogenic causes. Persistent traumatic friction to the limbus due to contact lens wear and chemical irritation due to contact lens solution can also contribute to this. Excessive surgery to the limbal region e.g. removal of pterygium is also a known cause for loss of LSC.

Table 1.1 Classification of causes of LSCD

PRIMARY (inherited)	SECONDARY (acquired)
Aniridia	**Injury**
Ectodermal dysplasias	Mechanical trauma
	Chemical or thermal burns
	Iatrogenic
	Extensive limbal surgery
	Cryotherapy
	Topical or subconjunctival application of cytotoxic agents
	Contact lens associated LSCD
	Inflammatory eye disease
	Stevens-Johnson syndrome
	Ocular cicatricial pemphigoid
	Chronic limbitis

Pathologically, the causes of LSCD may be the result of any one or a combination of these mechanisms; true loss of LSC, dysfunctional LSC, an alteration in the LSC niche micro-environment, or in the case of corneal inflammation or infection, a transient ‘distress’ or a disruption in the growth or proliferation of LSC.

Signs of LSCD include intense vascularisation, chronic inflammation, recurrent epithelial defects, and stromal scarring (Figure 1.10). Intense inflammation will inadvertently cause secondary problems like increased eye pressure, which can result in

the development of glaucoma and death of the optic nerve ganglion cells. Most patients will lose their vision due to the primary or secondary causes during the course of the disease. LSCD is usually clinically diagnosed although the use of confocal microscopy and impression cytology is substantially supported (Nubile *et al.*, 2012).

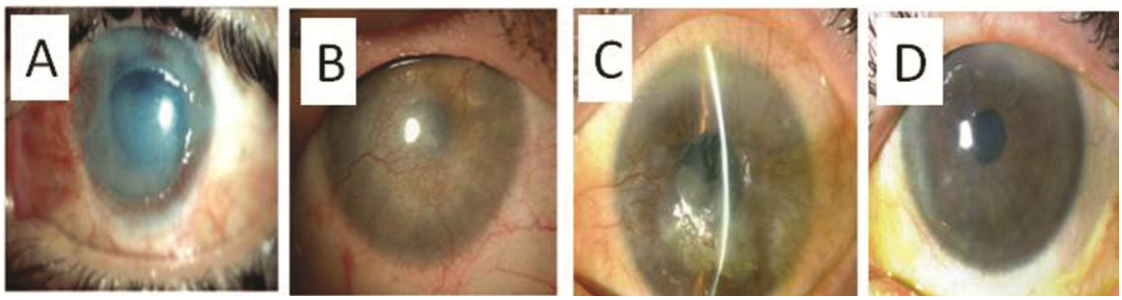


Figure 1.10 Clinical signs of LSCD. (A) Acute ocular changes showing limbal ischaemia, vascularisation, stromal opacity and epithelial erosion (B) and (C) chronic changes with conjunctivalisation of the cornea and corneal opacity. (D) Clearing of visual axis after treatment with cultivated corneal epithelium-AM scaffold. [Photos courtesy of (A) Sajjad Ahmad, (B) Scheffer Tseng *[Downloaded from http://www.revoptom.com/content/c/21674/ on 15 February 2013, (C) and (D) courtesy of Dr Tsutomo Inotomi]*

1.1.10 Diagnosis of LSCD

Patients with this condition present with redness in the eye, eye discomfort, excessive tearing, and reduced vision or blindness. Clinical signs of LSCD involve conjunctival epithelial outgrowth on the cornea or "conjunctivalisation" which gives rise to most of the symptoms. Other signs include recurrent epithelial erosion, corneal haziness or opacity and late fluorescence staining. Definitive diagnosis would require impression cytology to look for conjunctiva cells or goblet cells in the corneal surface. Immunofluorescence studies for CK3 and 19 would also support the diagnosis of LSCD.

1.1.11 Management of LSCD

Historically, LSCD was treated with whole cornea transplantation to replace the stem cell loss or dysfunction. However, whole cornea transplantation will only invoke failure in the absence of healthy limbal cells because of the absence of host stem cells and inadequate stem cells in the graft. Thus, re-epithelisation will only take place when

there are residual healthy limbal cells in the diseased eye, or a sufficient number of limbal cells are replaced.

Management of LSCD includes transplantation of healthy limbal tissue or cells to the damaged limbal areas. This is conventionally done by transplanting whole pieces of healthy limbal tissue (Kenyon and Tseng., 1989). However, this surgical method will usually involve a large area of graft taken from a donor site, thus rendering it susceptible to secondary LSCD.

In cases where cadaveric tissues or tissues taken from another healthy living donor are transplanted, measures to control graft allorejection have to be taken. Patients receiving allogeneic grafts have to be prescribed oral cyclosporine and prednisolone and put on topical steroids and antibiotic for extended period of time post-operatively. Limbal autografts also carry several adverse complications which are related to the grafts i.e. graft dislodging and misalignment. Inappropriate graft size leads to chronic ocular surface exposure, which potentially results in epithelial breakdown and conjunctivalisation of the cornea (Baradaran-Rafii *et al.*, 2012). The algorithm for the management for LSCD is described in Figure 1.11.

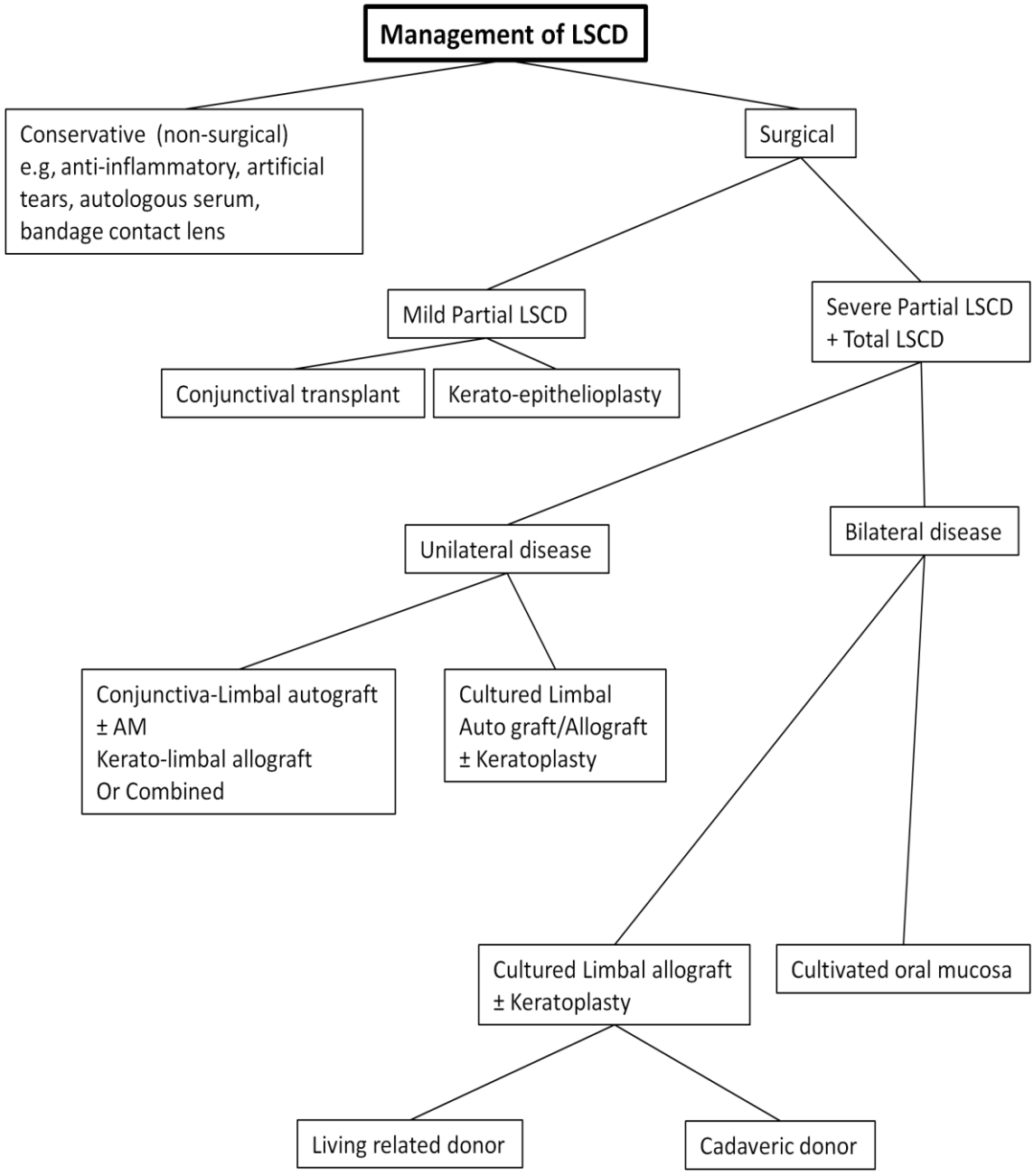


Figure 1.11 Management options of LSCD. This includes conservative (non-surgical) and surgical management. Surgical management strategies for mild partial LSCD and severe partial or total LSCD are highlighted.

1.1.12 New techniques in the treatment of LSCD (ex vivo expanded limbal epithelium)

A landmark report in 1997 by Pellegrini revealed a successful transplantation of *ex vivo* expanded limbal epithelium from a contralateral healthy limbal tissue grown on a fibrin carrier (Graziella Pellegrini *et al.*, 1997). The advantage of using autologous *ex vivo* expansion of limbal epithelium is the small sized-biopsy taken from the healthy eye

which will prevent secondary LSCD in the donor eye. The need for immune-suppression is also eliminated.

Since then, numerous reports have used the method of cultivating a corneal epithelial sheet on a substrate before transferring this tissue construct to the eye. In addition, there have also been reports on the use of cultivated oral mucosa epithelial transplantation for severe bilateral conditions (Nakamura *et al.*, 2004; Inatomi *et al.*, 2006). In the explant method, a small biopsy taken from the contralateral eye is placed on AM, which provides a surrogate environment for a stem cell niche.

1.1.13 Alternative sources for LSC transplantation

The lack of donor corneas in sufficient quantity and of sufficient quality to generate LEC for transplantation has motivated many clinicians and scientists to search for alternative sources of cells for cellular therapy. The option for replacement of adult limbal epithelial stem cells sourced from outside the cornea includes human embryonic stem cells (hESC) which can be directed to the corneal epithelial lineage (Kobayashi *et al.*, 2013).

The use of appropriate extra cellular matrix i.e. collagen IV, laminin or fibronectin in a differential protocol successfully directs hESC into corneal epithelia (Ahmad *et al.*, 2007). In a mouse derived ESC, the use of collagen IV as a culture substrate has resulted in corneal progenitors which expressed PAX6 and CK12 genes. PAX6 is important for ocular development while CK12 has been regarded as a specific marker of corneal epithelial differentiation. Indeed, transplantation of these corneal progenitors on denuded cornea produced epithelial surface re-epithelisation after 24 hours. However, restrictions surrounding ESC, namely ethical issues, technique of differentiation, accessibility and the costs, have limited the use of ESC for larger scale translational approach.

In the meantime, the advent of human induced pluripotent stem cells (iPSC) has partly resolved the ethical issues surrounding ESC. The use of transcription factors; Oct3/4, Sox2, c-Myc and Klf4 (Kazutoshi Takahashi and Yamanaka, 2006), on somatic cells can induce pluripotency in these cells, a process called “reprogramming”. Hayashi et al successfully induced cornea epithelial cells from human adult dermal fibroblast-derived iPSC and human adult corneal LEC-derived iPSC (Hayashi *et al.*, 2012) by using the

stromal cell-derived inducing activity method. In an animal model, mouse iPSC had been demonstrated to differentiate into corneal epithelial-like cells when co-cultured with a corneal stromal cells in the presence of additional factors such as β-FGF, EGF and NGF (Yu *et al.*, 2013).

Derivation of whole cornea from a parthenogenetically activated oocyst (hpSC) (Revazova *et al.*, 2007; Ostrowska *et al.*, 2011; Turovets *et al.*, 2011) is also an option. Parthenogenesis is asexual reproduction of eggs without contribution of the spermatozoa. hpSC lines have similar morphology to hESC, express pluripotent stem cell markers, have high alkaline phosphatase level and telomerase activity, and have the ability to differentiate *in vitro* and *in vivo* into derivatives of the three embryonic germ layers. Production of HLA-homozygous hpSC lines is also advantageous as being compatible with segments of human population, other than the 1
donor. This makes these cell lines a more favourable option as a transplantation therapy than ESC (Revazova *et al.*, 2007; Turovets *et al.*, 2011).

Likewise, transcriptionally induced pluripotent cells could also be a source of tumour formation (Hentze *et al.*, 2009; Cunningham *et al.*, 2012) and poses the problem of immunogenicity when used in transplantation (Boyd *et al.*, 2012). By re-programming stage-specific embryonic antigen-4 (SSEA4), SSEA4+ve limbal fibroblasts isolated from corneal stroma keratocytes and bone-marrow derived MSC could be differentiated into corneal epithelium (Katikireddy *et al.*, 2014). This was grown in under specific 3D culture system in embryonic stem cell media.

From the cord lining stem cells obtained from the waste birth products of fetal umbilical cord, investigators have isolated MSC and epithelial stem cells sourced from the external lining which comprises of umbilical cord AM. The bilayered lining of the membrane is advantageous for many epidermal and dermal keratinocytes derivatives. They also have potential to derive the epithelia for skin, liver and cornea(Matsuura *et al.*, 2014).

In the case of total and bilateral LSCD, cultivated oral mucosa epithelial transplantation on AM has also been clinically applied with promising results (Koizumi *et al.*, 2000; Nakamura *et al.*, 2004). This approach when reviewed for 15 treated eyes showed a success rate of 67% total re-epithelisation, without any major complications for a period

of at least 34 months. Similar method, but in the absence of 3T3 feeders and animal serum has also been trialled in two patients with successful regeneration of corneal epithelium (Kolli *et al.*, 2014).

1.1.14 Biological substrates for limbal stem cell transplantation

Historically, the strategy for LSC transplantation utilized fibrin sheet to treat LSCD (Graziella Pellegrini *et al.*, 1997). A mixture of fibrinogen and thrombin was placed on a plastic ring to allow a coagulation cascade. Primary limbal keratinocytes were grown on feeder layers on this fibrin sheet and the cell to matrix construct were then transplanted to patients' eyes (Rama *et al.*, 2001). A clinical trial involving larger number of patients showed a success rate of 76.6% up to 10 years (Rama *et al.*, 2010).

Amniotic membrane (AM) as part of the carrier system to transfer limbal epithelial sheets has been the substrate of choice to restore ocular surface disorders (Saw *et al.*, 2007). AM facilitates re-epithelisation and has been shown to have anti-inflammatory (Shimmura *et al.*, 2001), anti-angiogenic (Jae Chan Kim and Tseng, 1995; S. C. G. Tseng, 2001), and anti-scarring properties (Scheffer C. G. Tseng *et al.*, 1999).

To date, AM has been the most widely used substrate to deliver LSC to the ocular surface. Several modifications have been tried to provide different forms of AM to improve its quality as a carrier, including the use of denuded AM over an intact membrane (Koizumi *et al.*, 2000). However, the drawbacks are the difficulty to sustain the donor supply, ineffective and costly screening of the tissues, and clinical variations in the tissues which might affect the growth conditions. Hence, researchers have explored the potentials of other materials and used new strategies to develop tissue-engineered constructs to improve the outcome of limbal transplantation for ocular surface regeneration.

1.1.15 The outcome of ex vivo expanded limbal epithelial transplantation

Short term review of 28 clinical studies on cultivated corneal epithelial transplantation since 1997 to 2010 shows a success rate of 67% (Baylis *et al.*, 2011). This would probably be due to the majority of tissues used in these studies being autologous in nature (84%). Another long term study on the outcome of cultured limbal epithelial

transplantation using fibrin as a carrier gave 66% of full success, 19% for partial success and 15% of failure rate respectively (Graziella Pellegrini *et al.*, 2013).

In a review of clinical outcomes (Shortt *et al.*, 2007b), despite the heterogeneity of the type of grafts, the biological carrier to transplant LSC, culture methods, and the clinical cause of the disease, the overall outcome of 17 studies was 67% success rate.
It could not be emphasized enough that long term success of LSC transplantation depends on the quality of the grafts or the frequency of LSC on the grafts. This can be overcome by more effective identification or isolation of LSC, or the use of enrichment methods for LSC. It would also be expected that a major issue with allogeneic limbal tissue or cell transplants remains their immunogenicity.

1.1.16 Current prospective on tissue engineering for LSC transplantation

Tissue engineering was first introduced as an interdisciplinary approach to restore or enhance the biological functions of tissues and organs using substrates (Langer and Vacanti, 1993). The issue of cell sourcing was initially addressed by the use of adult stem cells or ESC. Although ESC have better differentiation and expansion potential than adult stem cells their use is hampered by ethical issues, regulatory problems and associated funding limitations. On the other hand, adult stem cells have limited differentiation capability. The advent of the Nobel Prize-winning development of human induced-pluripotent stem cells (IPSC) by Yamanaka group (Kazutoshi Takahashi and Yamanaka, 2006) seemed to offer a solution to the problems concerning the use of adult stem cells and ESC. However, the use of IPSC has been under scrutiny for possible genetic and epigenetic aberrations and tumorigenesis after transplantation.

The second major component in tissue engineering is the biomaterials used as the substrate or scaffolds for cell delivery. The initial focus was to replicate the physical and mechanical properties of the target tissues. Prospectively, more emphasis is being given to the integration of the substrates with the biological environment resembling closely the supportive extra cellular matrix (ECM).

The use of hydrogels which are biocompatible, inert and biodegradable is more attractive to replace or to complement AM. Hydrogels are more structurally uniformed and the physical and mechanical properties can be modified to suit cellular proliferation

(Wright *et al.*, 2013b). Hydrogels are made up of a 3D network of polymers and water, comprised of macro-molecules connected by electrostatic forces, hydrogen bond, or covalent links. As a scaffold, hydrogels can encapsulate cells and biomolecules as a cellular niche. Cross-linking of plastically compressed collagen hydrogels (Levis *et al.*, 2010; Mi *et al.*, 2011; Levis *et al.*, 2012) and alginate hydrogels are some of the examples useful for clinical epithelial transplantation (Wright *et al.*, 2013a; Wright *et al.*, 2013b; Wright *et al.*, 2014).

An artificial niche environment for LSC is an innovative mechanism to refine the LSCD treatment. Bioartificial alginate hydrogel systems provide a LSC microenvironment which allows slow release of therapeutic factors by the LSC to promote epithelisation and wound healing. In this model, growth factors such as epidermal growth factor, keratinocyte growth factor and hepatocyte growth factor migrate through the gel micro structure to facilitate the repair of the damaged corneal surface (Fritchley *et al.*, 2000).

Collagen forms the majority of constituents in the human cornea. Scaffolds of compressed collagen gel when compared to denuded AM have been demonstrated to be a better support for the growth of LEC (Groves and Jiang, 1995). This proved to be mechanically strong, thin and transparent. However, further investigations to study its immunogenicity based on its purity are needed. The use of recombinant collagen type I and IV has raised the issue of replacement of naturally occurring collagens, however this might come with increased costs (Feng *et al.*, 2014).

Other strategies like decellularisation of tissues and organs have emerged, where complete removal of cells and cellular materials is done to reduce immunogenicity (Lynch and Ahearne, 2013). This is performed by using chemical and enzymatic methods for decellularistion, followed by re-seeding with new cells, a process called "scaffold recellularisation". However, maintaining a close balance of preserving structural, mechanical and physiological properties of the scaffold and reducing cellular immunogenicity still remain the challenges in this area.

Fabricated nanofibers are a new substrate which is biocompatible with LEC and use of these with cells resulted in good cellular adhesion and cell proliferation (Sharma *et al.*, 2011). Fibrous nanoscaffolds from poly-ε-caprolactone (PCL) are highly porous, have

high surface: volume ratio and as a 3D biocompatible structure, this can mimic the physiological extra cellular matrix, used for synthetic ocular surface regeneration.

There is an increasing need to develop a synthetic, biocompatible and slowly biodegradable material which could be used as substitute for the AM. The use of a synthetic material would avoid the risk of infection. A biodegradable material has the advantage of variable degradation rate for the viable cells to be released at the site of injury. A contact lens which is resilient, biocompatible, and able to adapt to the surface of the eye is also an ideal carrier system for delivering of cultured corneal epithelium (Fiorica *et al.*, 2011). This is an interesting finding which needs further investigations to materialize as a cellular-based therapy.

A technique developed to escape from any use of carrier system is a new temperature-responsive polymer e.g. poly *N*-isopropylacrylamide (PIPAAm) as a cell sheet engineering system. This method allows transfer of cells and the extra cellular matrix to the ocular surface (Nagase *et al.*, 2009) at different temperature conditions in the absence of a scaffold (Kobayashi *et al.*, 2013). PIPAAm polymer and its co-polymer show a hydrophobic state at 37 °C and at this temperature cells would adhere and proliferate. At 32 °C and below, the cells are detached because of polymer hydration, which allows harvesting of the cells in a mono layer cell sheet while maintaining cell-to-cell and cell to-extra cellular matrix contact (Matsuura *et al.*, 2014).

Bioprinting is a new technology where scaffolding materials, living cells and extra cellular matrix can be delivered for regenerative purposes into a chosen material in an organized manner by a previously determined bio-printer (Pati *et al.*, 2014). As an example, human cardiomyocyte progenitor cells were successfully bio-printed on alginate scaffolds suitable as a cardiac construct (Gaetani *et al.*, 2012). This technique is still obscure in the field of LSC.

1.2 Immunological Aspects of Corneal Transplantation

1.2.1 Corneal transplantation

The cornea is the most transplanted organ in UK (with 3061 transplantations or 49.6 performed per million populations in 2009-2010 (Johnson, 2010)). This is the highest recorded rate of transplantation surpassing solid organ transplantation such as adult

kidney transplantation which was 892, pancreas – 200, cardiothoracic organ transplants - 272 and liver – 679 cases during the same period.

Corneal transplantation is the definitive therapy for most causes of corneal blindness. Importantly, transplantation is also performed to replace rejected or failed previous grafts in 20% of cases (K A Williams *et al.*, 2008). Approaches to corneal transplantation surgeries have evolved from whole cornea or partial thickness transplantation, to single layer keratoplasty, with very promising outcomes (Lee *et al.*, 2009; Borderie *et al.*, 2012).

However, corneal transplantation is contraindicated in the treatment of LSCD, which is an important cause of corneal blindness. Failure of grafts in this condition is due to loss of host stem cells and inadequate self renewing cells to replenish the surface of graft from the donor. In the case of LSCD, cellular therapy such as *ex vivo* limbal epithelial transplantation has shown impressive advances as future treatment in the past decade or so (Graziella Pellegrini *et al.*, 1997; S. C. G. Tseng, 2001; Shortt *et al.*, 2007b; Shortt *et al.*, 2008; Rama *et al.*, 2010).

The cornea is devoid of blood vessels and assumed to be protective of immune rejection of transplanted grafts, a condition termed "corneal immune privilege" (Azar, 2006). Its avascularity implies a lack of angiogenic factors or the possibility of it secreting anti-angiogenic factors. Vascularisation evokes an immune response and has implications for graft allorejection. In addition, absence of corneal lymphatics prevents the channelling of antigen presenting cells to the regional lymph nodes thus not allowing alloantigen-specific T cells to be activated. Activated T cells travel to the graft bed and initiate the crucial process of graft rejection. However, the relative ease of topical steroid application on the cornea and the immune tolerance in the anterior chamber also add to its relative success rate.

Despite being the most frequent organ transplanted, the immunology of corneal transplantation and rejection is not fully studied. For the most part, outcomes for whole cornea transplantation (penetrating keratoplasty) are dependent on the indications for surgery. The mechanism of rejection in cornea transplantation is a delayed type; T cell-mediated immune response which is accelerated by inflammatory process.

1.2.2 Mechanisms of immune privileges in the cornea

Cornea transplantation has been regarded differently from other organ transplants due to the immune privileged nature of the anterior chamber of the eye and the lack of direct allorecognition. Medawar revolutionised the concept of immune privilege in the cornea in 1940s (Medawar, 1948). He observed how skin grafts transplanted in the brain and the anterior chamber of the eyes could remain intact for extended periods of time in the absence of any vascularisation (Medawar, 1948). Medawar suggested that the immune reaction is mediated by blood plasma or by cells transported in it. During corneal transplantation, vascularisation caused the breakdown of the immunologically privileged status of the cornea, resulting in graft rejection. It was also suggested that the epithelium rather than the stroma played a role in the immune reaction (Billingham and Boswell, 1953).

The immune privilege of the cornea depends on multiple mechanisms to prevent immune destruction of grafts by allogeneic responses. At present, three major lines of thought prevail regarding the molecular mechanisms of immune privilege in the eye: (1) anatomical, cellular, and molecular barriers in the eye; (2) anterior chamber associated immune deviation (ACAID); and (3) immune suppressive microenvironment in the eye.

Firstly, there is a direct absence of anatomical lymphatic drainage and a physiological blood-ocular barrier in the cornea which defines the immunological tolerance. Without lymphatic drainage, the antigens are unable to leave the grafts to the draining lymph nodes. The blood-ocular barrier effectively presents a physiological block for the immune cells to enter the tissues. These factors collectively act as physical barriers to stimulate immune response to grafts.

ACAID was first described as an aberrant immune response of the eye (Kaplan and Streilein, 1977). This refers to the phenomenon where antigen-specific systemic immunological tolerance is induced to an antigen that has been introduced to the anterior chamber (Figure 1.12). In this situation, antibody responses are preserved while cellular responses such as delayed type hypersensitivity (DTH) and cytotoxic T cells (CTL) are suppressed. These include alloantigens involved in transplantation, soluble protein antigens, viral antigens, and tumour antigens (J. Y. Niederkorn and Mellon, 1996).

Vascularisation causes an allograft to be deemed high risk and robs its immune privilege status. Immunological rejections in high risk vascularised allografts occurs due to increased level of chemokines and inflammations at the site of the grafts, attracting allospesific activated T cells to the site (Y. Sano *et al.*, 1995; Azar, 2006; Amescua *et al.*, 2008). Chemokines are heparin-binding pro-inflammatory proteins that are responsible for directing leucocytes migration. Chemokines are categorised into CXC, CC, C and CX_3C based on the cysteine residue at the protein terminal. CXC chemokines i.e. CXCL1, play a major role in recruiting activated allospecific T cells, macrophages and other potent inflammatory mediators to the graft site, causing immunogenic rejection (Amescua *et al.*, 2008).

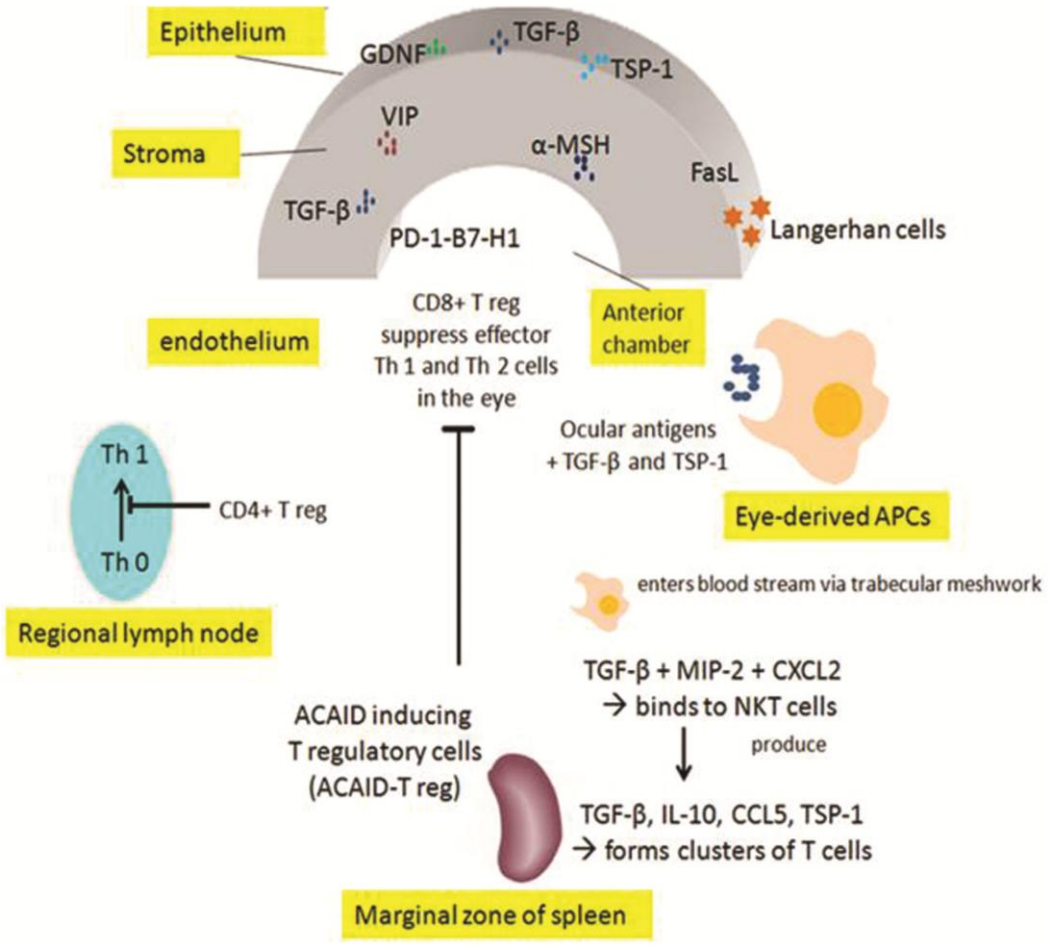


Figure 1.12 Components of ocular surface immunity. They include physiological barrier of the precorneal tear film, induction of ACAID and immunosuppressive environment in the eye. Immunomodulatory molecules in the cornea consist of neuropeptides, cytokines, growth factors and soluble immune peptides which have anti-inflammatory, anti-angiogenic, and pro-apoptotic activities that lead to death of allospecific T cells. *[Abbreviations: GDNF= glial derived neurotrophic factor, APC=antigen presenting cells, TGF-β=tumour growth factor-beta, VIP=vasointestinal peptide, TSP-1= thrombospondin-1, PD-1-B7-H1= programmed death-1 Ligand-B7-H1 binding, T reg= regulatory T cells, MIP-2= macrophage inflammatory protein-2, CXCL2= C-X-C*

ligand-2, NKT= natural killer T cells, IL-10=interleukin-10, CCL5=C-C ligand-5). (Shaharuddin et al., 2013b)]

The anterior chamber contains tumour growth factor (TGF)-β and thrombospondin-(TSP)-1, and antigen presenting cells which capture the antigens and secrete these into the blood stream through the trabecular meshwork and reach the marginal zone in the spleen. TGF-β stops proliferation of cells, induces differentiation, or promotes apoptosis while TSP-1 has an inhibitory effect on angiogenesis. Furthermore, there is production of TGF-β, macrophage inflammatory protein- (MIP) 2 and CXC-ligand 2 (CXCL2) in the spleen. These in turn attract and binds to NKT cells and stimulate the production of TGF-β, IL-10, TSP-1 and CC-ligand 5 (CCL5). In the spleen there is formation of clusters of T cells and subsequent induction of regulatory T cells, or ACAID-inducing-T regulatory cells (ACAID-T Reg). CD8+ T regulatory cells down regulate Th1 and Th2 effector functions in the eye, while CD4+ T regulatory cells inhibit differentiation of Th1 cells in the lymph node.

1.2.3 Rejection in corneal transplantation

Common indications for penetrating keratoplasty are keratoconus, corneal dystrophies, corneal opacities and post cataract surgeries. Comparisons on the outcome of corneal transplantation are difficult because of the non-uniformity of data on surgical indications, patient demography, type of grafts, survival analysis and range of follow-up. In general, uncomplicated low risk grafts give the highest survival rate with topical steroidal immune-suppression. Based on surgical indications, the Australian Corneal Graft Registry reported keratoconus has the highest 10-year survival rate (89%), compared to Fuch's corneal dystrophy (73%), non-herpetic corneal opacity (70%), herpetic corneal opacity (60%), pseudophakic and aphakic corneal oedema (40%), and regrafting (30%) (K A Williams *et al.*, 2008).

A breakdown of survival analysis shows the fate of allogeneic penetrating keratoplasty for correcting visual acuity was highest in the first year follow-up i.e. 87% and reduced to 52% after 10 years among Asians (Tan *et al.*, 2008). Generally, failed grafts are due to irreversible immunological rejection (34%), while corneal endothelial cell failure due to glaucoma (24%) and infection (14%) are common causes of failed grafts (K A Williams *et al.*, 2008). A similar trend is observed in a study of second grafts; in the

majority of cases immune rejection is the main cause of graft failure, although recurrence of disease and endothelial decompensation are also important causes (Kirkness *et al.*, 1990).

Clinically, other associated factors which are implicated include degree of pre-vascularisation of the recipient bed, size of donor corneal button, position of the donor corneal button in the recipient bed, preservation methods for the donor corneal button, choice of immunosuppression and immune status of the recipient.

Generally, the immune response to alloantigens is polarised in two directions; A) Activation of T helper type 1 (Th l) effector cells which results in production of interferon (IFN)-γ, introduction of CD4+ as a surface determinant and mediation of delayed immune response. B) Activation of the T helper type 2 (Th2) pathways, may produce cytokines which cross regulate Th1 factors and inhibit their mediator functions. IFN-γ is an important pro-inflammatory cytokine in the rejection of MHC-matched corneal allografts. It has been shown that manipulating the alloantigenic response from Th1 to Th2 pathways by blocking IFN-γ production in MHC-matched allografts significantly reduces graft rejection rate (Hargrave *et al.*, 2004).

The mechanism of rejection in cornea transplantation is a delayed type T cell- mediated immune response which is accelerated by inflammatory process. In a rat corneal transplantation, CD4+ cells had an important role in the mechanism of rejection, while the role of CD8+ T cells was not clearly shown. Interleukin (IL)-2, IL-4 and IFN-γ are regarded as important inflammatory markers for corneal rejection(Torres *et al.*, 1996).

This finding is in agreement with an experimental sheep corneal transplantation model, where acute rejection is associated with graft neovascularisation, infiltration of CD4+ and CD8+ T cells, and production of IFN-γ and IL-2 in the graft (Ka Williams *et al.*, 1999). But production of tumour necrosis factor (TNF)-α, IL-4 and IL-10 was not evident, which the investigators hypothesised might be due to rapid T cell death due to Fas-FasL interactions (Yamagami *et al.*, 1997). The expected result of this interaction would be induction of apoptosis in Fas bearing infiltrating leucocytes such as CD4+ T cells, CD8+ T cells, neutrophils, and macrophages (K A Williams *et al.*, 2005). The role

of TNF-α in initiating, maintaining and resolving inflammatory processes, has been observed to show dynamic changes in allograft transplantation (Chan *et al.*, 1999).

Corneal epithelium and stroma both peripherally and centrally, contain dendritic cells (DC) which undergo maturation by expressing MHC Class II and B7 co-stimulatory molecules during inflammation (Hamrah *et al.*, 2003). B7 co-stimulatory molecules provide potent stimulation for MHC/peptide-T cell receptor interaction to result in activation of T cells. B7 molecules are present as dimers on the surface of DC or other forms of APC.

A novel negative regulatory molecule has been described recently, which is a new member of the B7-CD28 super family, and it is referred to as programmed death-1 (PD-1) (Junko Hori *et al.*, 2006). B7-H1is a potential ligand for PD-1 (Freeman *et al.*, 2000). B7-H1 has been found in corneal epithelium and endothelium and is believed to have pro-apoptotic actions on T cells, thus prolonging the survival of corneal grafts (Junko Hori *et al.*, 2006).

1.2.4 Major Histocompatibility Complex (MHC) expression in cornea transplantation

The MHC gene complex present antigen to T cells and determines the compatibility of donors for organ transplants. The Human Leucocyte Antigen system (HLA) represents MHC which encodes for genes related to immune system and cell surface antigen presenting proteins. MHC class II antigen presentation pathways are expressed by professional antigen presenting cells (APC) e.g. dendritic cells, macrophages, B-cells, or epithelial LC.

A critical factor influencing corneal graft rejection is the presence and the high density of such dendritic cells in the graft (donor APCs). Avoiding placement of a graft near the limbal area where the APCs are abundant is a logical surgical strategy in this instance. An approach towards a donor specific-immnosuppression by regulatory T cells to achieve graft tolerance may be taken to eliminate the risk of a violent rejection when donor's and recipient dendritic cells coexist.

The most important antigen presenting cells in the cornea is bone marrow-derived MHC Class II+ LC which are responsible for the sensitisation of the host to alloantigens.

Under normal conditions LC reside in the limbal area but may migrate to the cornea in response to inflammatory conditions (Jerry Y Niederkorn, 1995). Activation of alloreactive T cells is dependent on the antigen presenting cells for the host to recognise foreign antigens on the grafts.

There is uneven distribution of LC which express HLA class II molecules in the cornea; this could possibly be the answer for prolonged survival of these grafts (Hamrah *et al.*, 2002). LC were originally thought to be exclusively abundant in the limbal and conjunctival region, but not at the central cornea (JY Niederkorn *et al.*, 1992; D. L. Williams, 2005). HLA class II expression in normal corneal stroma and endothelia is also lacking, and mostly confined to the limbal region of the epithelium (Foets *et al.*, 1991).

Expression of Class II is also absent without stimulation by inflammatory mediators (Nicholls *et al.*, 2006). However, more recent findings demonstrated that a heterogeneous population of immature and precursor dendritic cells also reside in the central corneal region (Hamrah *et al.*, 2002). These cells labelled as "MHC Class II-negative" are believed to be progenitor or immature LC. Although these immature cells are capable of antigen uptake and processing them, they are incapable to furnish naïve T cells with requisite B7 co-stimulatory molecules, thus unable to activate T cells (Thomson *et al.*, 1995). This mechanism of active suppression of LC maturation could be an important mechanism of corneal immune privilege.

Not surprisingly, there is low incidence of corneal graft rejection although HLA antigen matching of donor and recipient is not normally performed, especially in low risk grafts. In a study performed by The Collaborative Corneal Transplantation Studies Group in high risk corneal transplantation patients, HLA-A, B and HLA-DR matching did not reduce the rate of graft failure in this high risk group (1992). Additionally, a recent finding showed that although matching of HLA-A and -B did not significantly affect the rejection rate in low risk group, it certainly benefitted the high risk group. (Khaireddin *et al.*, 2003). HLA-DR matching was also demonstrated to significantly reduce allograft rejection by 40%, 3 years after surgery in the high risk group(Khaireddin *et al.*, 2003). The disadvantages of HLA matching include the costs and extended waiting time for suitable grafts. However, these need to be seriously weighed against the risk of failed

grafts and further therapeutic complications, regrafting and the overall impact on the pool of available donated corneas.

Despite encouraging results from MHC matching, grafts can still be rejected due to incompatibility in the minor MHC antigens. Th1 pathway and IFN-γ production was shown to be the mechanism involved in rejection of MHC-matched corneal allografts where minor MHC antigens are presented to the host (Hargrave *et al.*, 2004). Minor MHC antigens are naturally occurring polypeptides processed and presented by MHC molecules, shown to increase risk of grafts failure in grafts-versus-host disease. Minor MHC antigens induce activation of T cells and also play an important role in allograft rejection of solid organs following transplantations (Yoichiro Sano *et al.*, 2000; Kwun *et al.*, 2011; Pabón *et al.*, 2011). Minor H antigens are loaded to MHC molecules in corneal grafts allowing for indirect T cell allorecognition (Sonoda *et al.*, 1995). Alloreactive T cells recognise allogeneic peptides "indirectly" when they are introduced to self-MHC class I and II molecules. On the other hand, "direct" allorecognition occurs when T cells recognise donor alloantigens directly, irrespective of the peptides associated with the MHC molecules.

Experimental stimulation of human corneal epithelial cells with recombinant human IFN-γ demonstrated increased expression of MHC Class II antigens (Iwata *et al.*, 1992), (Iwata *et al.*, 1994) and significant ability to stimulate allogeneic lymphocytes (Iwata *et al.*, 1994). Another study revealed corneal epithelium and endothelium express MHC class II antigens when stimulated by IFN-γ, whereas it had no effect on the expression of MHC class I antigens (el-Asrar *et al.*, 1989).

1.2.4 Methods to prevent rejection

Strategies to reduce rejection rate must take into consideration immunosuppressive reagents and gene therapy. The use of anti-inflammatory mediators is an additional strategy to prolong survival of grafts. Soluble TNF-α receptor type I in a topical instillation form has shown favourable results in reducing murine allogeneic reactions (Qian *et al.*, 2000). TNF-α is a potent pro-inflammatory cytokine in alloimmune response. It is a macrophage derived cytokine responsible for expression of adhesion and co-stimulatory molecules, neutrophils activation, chemokine stimulation and inducing the nuclear factor-$_{K}$B signalling pathway.

More recently, administration of IL-1 receptor antagonist (IL-1Ra) was shown to be a promising as an effective modality for suppressing IL-1-mediated processes to counter rejection in corneal transplantation (Dana *et al.*, 1997; Stapleton *et al.*, 2008) to corticosteroid immune suppression in mice allogeneic grafts. IL-1 is involved in mediation of acute phase reactions, chemotaxis, stimulation of new vessels and most importantly, the migration and recruitment of LC. IL-1Ra is produced in abundance at the apical epithelial region in fresh cornea (Heur *et al.*, 2009). This potent inflammatory cytokine is derived from monocytes, macrophages and resident corneal cells and is expressed at high levels during corneal transplantation immune response. IL-1 receptor antagonist (IL-1Ra) has the effect of neutralising IL-1 by suppressing LC migration, thus prolonging survival of allografts. Interestingly, the success of experimental ex-vivo transduction of IL-1Ra in rat cornea will be a new landmark in corneal gene therapy to treat corneal inflammatory conditions and corneal transplantation (Yuan *et al.*, 2012).

NGF gene therapy in combination with a cytotoxic T lymphocyte antigen 4 immunoglobulin G (CTLA4Ig) has shown to prolong rodent corneal allografts survival by mediating its anti-inflammatory and anti-apoptotic pathways (Gong *et al.*, 2007). This is not surprising since NGF and its receptors are abundantly present in a densely innervated organ like the cornea and are responsible for the wound healing process (Kinoshita and Ueta, 2010).

In other pharmacological developments, suppression of inflammation by interleukin-10 (IL-10)(Boorstein *et al.*, 1994) and inhibition of intercellular adhesion molecule-1 or its ligand (Whitcup *et al.*, 1993) are prospective potential therapies.

1.3 Conclusion

A challenging aspect of LSC is the absence of specific markers to uniquely identify these stem cells and to distinguish them from other adult stem cells, or LSC derived from TAC. This necessitates the characterization process involving several modalities i.e. RT-PCR, immunochemistry or cell cycling methods. A consistent isolation method for LSC should also be instigated, to include protocols to enrich for LSC such as functional phenotypic assays like the SP assay.

Ex vivo expanded limbal epithelial transplantation has proven to be able to reconstruct the ocular surface in LSCD eyes. However, this method needs to be further refined to include protocols to assess tissue viability, the quality of tissue constructs and safety assessment. In order for LSC to be successfully translated into favourable clinical outcomes, a good manufacturing practice (GMP)-compliant protocol would be a positive step towards achieving high quality tissues to fulfil the requirements for clinical transplantation(DiIorio *et al.*, 2010).

As the most transplanted organ, the importance of understanding corneal immunology is of considerable interest. A major issue with whole cornea transplantation, allogeneic limbal tissues or *ex vivo* LSC transplants remains their immunogenicity. Questions arise whether the ocular immune system is similar in all regions of the eye, and does the immune privilege status of the anterior chamber apply to other ocular tissues? It would be interesting to explore immunogenicity of grafts placed on the cornea in an ocular reconstruction surgery where no breach of the anterior chamber happens at all.

Further investigation on the immunological factors in the cornea that allow tolerogenic potential of the organ to respond to ocular antigens is necessary to exploit corneal immune privilege to its full advantage. Identification of inflammatory molecules and apoptotic markers, their signalling pathways, and role of HLA matching, are some of the strategies to promote the survival of whole corneal and LSC transplantation. Furthermore, differential expressions of HLA antigens during inflammation and rejection process may elucidate the role of antibodies alone, or in combination with other soluble factors in donor antigen-specific immune suppression. This approach of immune suppression by regulatory T cells may potentially achieve graft tolerance to eliminate the risk of a violent rejection.

1.4 Objectives

This project will aim to address a deficit in our knowledge of LSC biology, namely the characterization of LEC from both primary LEC and a human telomerase-immortalized corneal epithelial cell (HTCEC) line, limbal SP cells (as an enriched adult stem cell population) and studying the biological and immunological properties of corneal epithelial cells with implications on LSC transplantation. A novel therapeutic approach

to culture an alternative stem cell population sourced from the corneal limbus on cryopreserved AM, thawed from frozen will be tested as a preferred biodegradable scaffold.

1.4.1 Experimental Approaches and Techniques

Aim 1

To achieve these objectives, specific aims will involve these techniques;

1) Isolation of human LEC from primary donor corneas.
2) Characterisation methods for both human LEC and HTCEC.
3) Optimisation of LSP protocol for both human LEC and HTCEC.
4) Characterisation of human limbal SP (LSP) cells.

Aim 2

To study the biological and immunological properties of corneal epithelial cells with implications on LSC transplantation, these techniques will be employed;

1) Examine the expression of HLA class I & II molecules in corneal epithelial cells.
2) Determine the HLA expression under stimulation of pro-inflammatory cytokines.
3) Study cellular migration in corneal epithelial cells.

Aim 3: From the results defined in Aim 1, research grade cryopreserved AM obtained from NHS Blood and Transplant will be used as a substrate to promote cellular proliferation and differentiation of LMSC. .

Chapter 2 GENERAL MATERIAL AND METHODS

2.1 Ethics and Research Approval

This project was conducted according to the ethical principles of the Declaration of Helsinki for human experiments. Project and ethical approvals received for this project were as listed:

2.2 Project Approval

This project had received project and ethical approval from the Ethics Committee, Faculty of Medical School, Newcastle University on 18 January 2013 (No. 00617/2012) and amended on 24 April 2013 (N0. 00617_1/2013) (Appendix 1).

The corneal tissues were harvested by the The Eye Bank and stored in Optisol media contaning dextran. All requests for human cells, tissues and organs from the bank were peer-reviewed for merit and procurement feasibility and in order to apply for tissues we provided proof that we had ethical approval from Newcastle University for this research. We did not require any identifiers to the donors, other than age, gender, time of death to preservation in the media mentioned above and death to delivery interval. Therefore there was no issue with storage of personal data.

Data generated from our study were kept on computers housed within Newcastle University. Results of our studies were available to other researchers and the public via publication in peer reviewed journals, the university website and by presentation at conferences.

As a general rule, we received donor corneas which had satisfied the General Organ Donor Criteria, with the exception of age and blood type. All tissues had undergone screening for infective diseases e.g. HIV, Hepatitis B or C, syphilis, cytomegalovirus and Epstein Barr Virus. In addition, the donors did not have known corneal trauma or diseases or intra-ocular eye surgery. Additional exclusion criteria were as at the discretion of the Eye Banks. Alternatively, donor cornea which was not suitable for transplantation was assigned for research and training.

2.3 Human Tissue Authority License

For use of human tissues in research, the Institute of Genetic Medicine has been approved for handling, storage and research on human disorders and functions in the body (Appendix 2).

2.4 Material Transfer Agreement (MTA)

The approval for use of human corneascleral rings was obtained through a material transfer agreement with the Central Manchester and Manchester Children's University Hospitals (NHS Trust) (Appendix 3). Donor corneas were either unused whole cornea from clinical transplantation, or whole cornea/corneoscleral rims suitable for research and training.

2.5 Ethics/Project approval

The ethical approval for the use of human AM was obtained through Newcastle Uteroplacental Bank Ethics Committee, application reference number 13.04 (Appendix 4).

2.6 Ethics/Project approval

Human cryopreserved AM were supplied by the National Health Service (NHS) Blood and Tissue bank, Liverpool, United Kingdom. Donors had undergone medical and social history interviews and tissue screening for infective diseases as mentioned above.

2.7 General Laboratory Practice

All experiments were performed according to the Control of Substances Hazardous to Health (COSHH and BIOCOSHH) regulations. All procedures were in compliance with Newcastle University current safety policies. Tissue culture was carried out in compliance with regulations for containment of Class II pathogens.

2.8 Tissue Culture

2.8.1 3T3 Fibroblast culture

Immortalised Swiss mouse embryonic fibroblasts from the J2 strain (a gift from Dr. Fiona Watt originally from Howard Green's Laboratory, Harvard) were thawed from frozen. The resulting cell suspension was centrifuged with 3T3 medium (Table 2.1) at 1,000 revolutions per minute (rpm) for 3 minutes at room temperature.

2.8.2 3T3 Fibroblast Culture medium

3T3 fibroblasts were routinely grown in T25cm^2 flasks [Iwaki/VWR] at 37°C, 5% CO_2 in a humidified incubator until 80-100% confluency determined microscopically (Figure 2.1). Medium was removed and cells washed with Dulbecco's Phosphate Buffer Saline (DPBS) [Sigma]. 3ml 0.05% trypsin-EDTA was added into the flask and incubated for 2 minutes at 37°C. Flask was examined for cell detachment and subsequently agitated. Cell suspension was then removed and transferred to a 15ml centrifuge tube and centrifuged at 1,000 rpm for 3 minutes. The supernatant was discarded and the cell pellet re-suspended in 5ml 3T3 medium and subcultured into a new T75cm^2 flask in a total medium volume of 10ml. Medium was changed every other day.

Table 2.1 Composition of the medium for 3T3 fibroblast culture

Reagent	Composition	Supplier
High glucose DMEM (4,500mg/l)	89%	Gibco
FCS	10%	Gibco
Penicillin/Streptomycin	1%	Gibco

2.8.3 Cryopreservation of 3T3 fibroblast

Cells were frozen using Recovery Cell Culture Freezing Medium [Gibco], which contains DMSO 10%, high glucose DMEM, and fetal bovine serum. The medium was first removed from flask and washed with DPBS. 5ml of 0.05% trypsin-EDTA was added to the flask and incubated for 3 minutes at 37°C. Cells were agitated collected in a 15ml centrifuge tube which was then centrifuged at 1,000 rpm for 3 minutes. The supernatant was removed and 1ml of freezing medium was added to cell pellet containing about 1 x 10^6 cells and transferred to a cryogenic vial and put in a cell freezing container [Nalgene], which contains 250ml of 100% isopropyl alcohol [VWR BD Prolab]. This provides -1°C/minute cooling rate required for successful cell cryopreservation and recovery, and then frozen at -80°C.

2.8.4 Re-culturing of cryopreserved 3T3 fibroblast

Frozen cell suspensions were rapidly thawed and cytotoxic cryopreservants were inactivated by washing in 3ml of 3T3 media in a 15ml conical centrifuge tube at 1,000

rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended with 1 ml of 3T3 media and transferred to a T25cm^2 or 75cm^2 flask containing 10ml 3T3 medium. The media was changed on the next day and every other day following that.

2.8.5 Inactivation of mouse 3T3 fibroblast feeder layer with mitomycin C (MMC)

When cells reached 80% confluence, MMC [Sigma] was added at the concentration of 10µg/ml of the volume of medium. The tissue culture flask was then incubated for 2 hours at 37°C. After which, 5ml of 0.05% trypsin-EDTA was added and incubated at 37°C for 3 minutes. The detached cells were harvested, into 3T3 media and centrifuged at 1,000 rpm for 3 minutes at 4°C and the cell pellet was then resuspended in 2ml 3T3 medium and counted. The cells were plated at 2.4 x 10^4/cm^2 in a 6-well plate. The cells are amitotic but still living and can be used as a feeder layer.

2.8.6 Inactivation of 3T3 fibroblast feeder layer with X-irradiation

Alternatively, confluent 3T3 were exposed to 60 Gy units at 0.01 Gy units per second [Astrophysics Research Corporation X-Ray Inspection System]. The medium was removed and briefly washed with DPBS, and replaced with 10ml 3T3 medium. The cells were then detached from the flaks using 5ml 0.05% trypsin-EDTA and centrifuged and the cell pellet then re-suspended in 3T3 media and plated at a density of 2.4 x 10^4/cm^2 in a 6-well plate.

2.8.7 Cell counting

Cells were counted prior to experiments/cryopreservation using a haemacytometer [Scientific Laboratory Supplies]. 10µl of cell suspension was pipetted onto the haemacytometer and allowed to diffuse under the cover slip and examined under a light microscope. Cells in the 25 squares of the grid were counted and the total multiplied by 1 x 10^4 to obtain the number of cells/ml. To ascertain cell viability, 20µl sample of the cell suspension to be counted was placed in a solution containing 50µl 0.4% trypan blue solution [Sigma] and 30µl DPBS [Gibco] and incubated for 10 minutes at room temperature. This resulting cell suspension was counted as above. Blue cells were non-viable cells and excluded from the count. The final count was multiplied by a factor of 5 to allow for the dilution of volume of cells to volume of trypan blue and PBS (1:5).

2.8.8 Plating of 3T3 fibroblast as a feeder layer

After performing a count of the viable cells, 24,000 cells/cm^2 in 3T3 medium were added to the 9.6cm^2 tissue culture wells [VWR], containing inactivated 3T3 fibroblasts as feeder cells (refer section 2.8.2 and 2.8.5 on culture and preparation of 3T3 feeder layer).

2.9 Primary LEC Culture

2.9.1 Isolation of LEC

LEC culture was conducted according to (Graziella Pellegrini *et al.*, 1999) – Cadaveric limbal tissues were obtained in agreement with the UK Eye Bank. All tissue culture work was routinely carried out in a containment level II microbiological safety cabinet. Limbal tissues were sourced from discarded corneo-scleral rings as left over from penetrating keratoplasty surgeries, or harvested as research grade whole corneas (Figure 2.2).

The tissue was cut into 4 quadrants and cut further into 1 mm^2 pieces. These limbal pieces were incubated with 0.05% trypsin-EDTA [Gibco] for 20 minutes at 37°C, 5% CO_2 in a humidified tissue culture incubator with gentle agitation of the suspension at the 10 minute point of a 20 minute cycle. The resulting cell suspension was removed from the limbal pieces, and 1ml limbal epithelial medium (Table 2.2) was added to this suspension. The cell suspension was then centrifuged for 3 minutes at 1,000 rpm at 4°C and the supernatant discarded. The remaining cell pellet was re-suspended in limbal epithelial medium (LEM). This process of trypsinisation of the limbal pieces and centrifugation of the resulting cell suspension was repeated for a further three times using the same limbal tissue. Finally, the resulting limbal cell suspensions were pooled together and centrifuged.

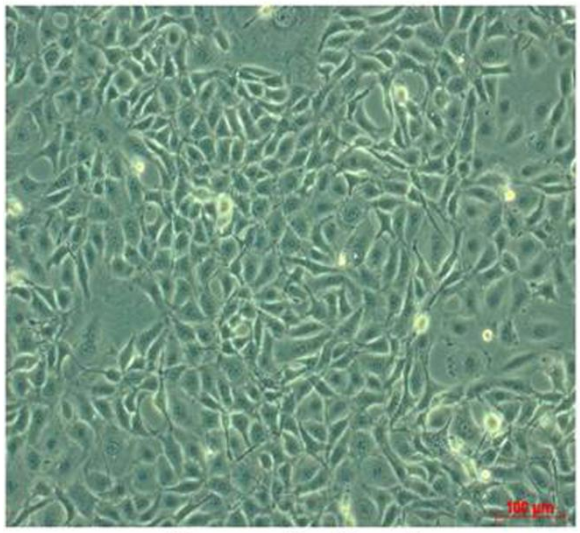


Figure 2.1 Confluent 3T3 fibroblast cultures.

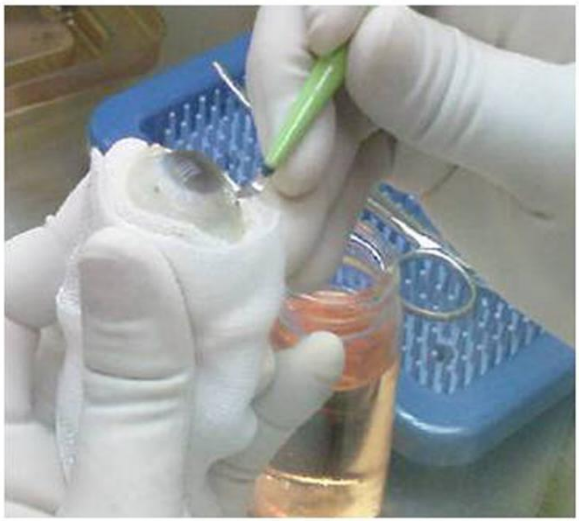


Figure 2.2 Harvesting a cornea from an enucleated cadaveric eye.

2.9.2 LEC medium

The composition of reagents and additives for LEC media is listed in Table 2.2.

Table 2.2 Composition of limbal epithelial medium (LEM).

Reagent	Quantity	Supplier
DMEM (Low Glucose 1g/L)	375 ml (75%)	Invitrogen
Ham's F12 Medium	125 ml (25%)	Invitrogen
Foetal calf serum	10%	Invitrogen
Penicillin/Streptomycin	1%	Invitrogen
Hydrocortisone	0.4µg/ml	Sigma
Insulin	5µg/ml	Sigma
Triiodothyronine	1.4ng/ml	Sigma
Adenine	24µg/ml	Sigma
Cholera Toxin	8.4ng/ml	Sigma
Epidermal Growth Factor	10ng/ml	Sigma

2.9.3 Plating and subculturing of LEC

The cells in the limbal media suspension were counted for viable cells. 30,000 viable LEC in epithelial medium were added to the 9.6cm² tissue culture wells [VWR], containing inactivated 3T3 fibroblasts as feeder cells.

Sub-culturing was done according to (Rheinwald and Green, 1975a) - To expand the number of LEC, the primary cultures were sub-cultured prior to confluence of the colonies (Figure 2.3). The medium was removed from the culture well and the culture was briefly irrigated with PBS. The culture was incubated with 0.05% trypsin-EDTA solution for 5 minutes in a tissue culture incubator. Epithelial medium was then added to the resulting cultured LEC suspension. The suspension was centrifuged for 3 minutes at 1,000 rpm at 4°C and the supernatant was removed. The resulting cell pellet was re-suspended in epithelial medium. After performing a count of viable cells, the cells were plated in a 9.6cm² well containing 3T3 feeder cells. The sub- cultures were then treated in the same way as primary cultures above (refer section 2.8.4).

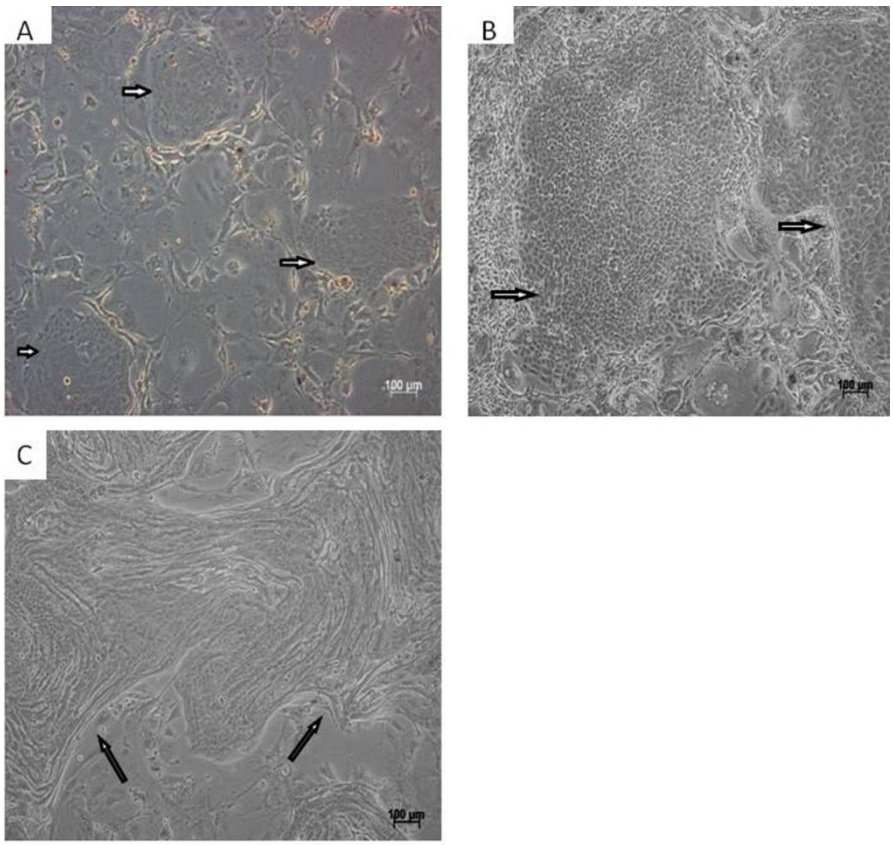


Figure 2.3 LEC grown on mitotically inactivated 3T3 fibroblast in different stages of cultures. (A) Formation of holoclones on Day 3 (white arrows) (B) enlarging colonies on Day 7 (C) spindling of cells at a differentiated stage on day 12 (black arrows).

2.9.4 Culture of LEC on coating matrix using serum-free medium

Subcultures of LEC were plated in tissue culture well 9.6cm^2 or 25cm^2 flask without feeder-cells and under serum-free culture conditions (Figure 2.4A, 4B, 4C). Wells and flasks were Coated using Coating Matrix Kit [Gibco] which contains 0.5ml sterile recombinant human Type-1 collagen as a Coating Matrix (Item # 50-9700) and 50ml Dilution Medium (Item # 50-9701) were used. Dilution Medium was added to each flask (5ml per each 75cm^2 flask, or 1.7ml per each T25cm^2 flask or 0.65ml for a 9.6cm^2 well). Coating Matrix was directly added to the Dilution Medium in each flask (50 µl per each T75cm^2 flask, or 17µl per each T25cm^2 flask or 6.5µl per each 9.6cm^2 well). To ensure uniform distribution of the coating matrix over the surface of the flask, the flasks were rocked back and forth gently. The flasks were capped and incubated for 30 minutes at room temperature. Excess Coating Matrix/Dilution Medium was removed from each flask. The flasks could be used immediately, or stored at 2° to 8° C for short periods of time.

Prior to plating of LEC the flasks and wells were briefly washed with PBS and cells plated in defined keratinocyte serum free medium (DKSFM) [Gibco]. This is a medium optimised for expansion of keratinocytes without supplementation of bovine pituitary extract or the use of feeder layers. Growth promoting agents were added to enhance consistent product performance. Plating of cells was performed as described above.

2.10 Human telomerase-immortalised cornea epithelial cell line (HTCEC)

We received a frozen vial of HTCEC from Professor Winston Kao's laboratory in University of Cincinnati, USA. The cell line was originally derived by Professor James Jester (University of California, Irvine, USA). We successfully expanded HTCEC and maintained this cell line in feeder- and serum-free conditions (Figure 2.4D).

Frozen suspensions of HTCEC were rapidly thawed and mixed and washed with DKSFM [Gibco] for 3 minutes at 1,000 rpm at 4°C. The supernatant was discarded and the resulting pellet was re-suspended in DKSFM into a T25 cm^2 tissue culture flask [Scientific Laboratory Supplies] and maintained in a tissue culture incubator at 37ºC, 5% CO_2. The medium was changed on the next day and then every three days.

2.10.1 Maintenance of human telomerase-immortalised cornea epithelial cell line

For the purpose of maintaining HTCEC, the medium was removed from subconfluent cell cultures and the cells washed with DPBS. The cells were detached from the flask with a tissue dissociation buffer [Gibco] (the buffer is an enzyme-free aqueous formulations of salts, chelating agents, and cell-conditioning agents in either Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution which is suitable for gentle dissociation of cells

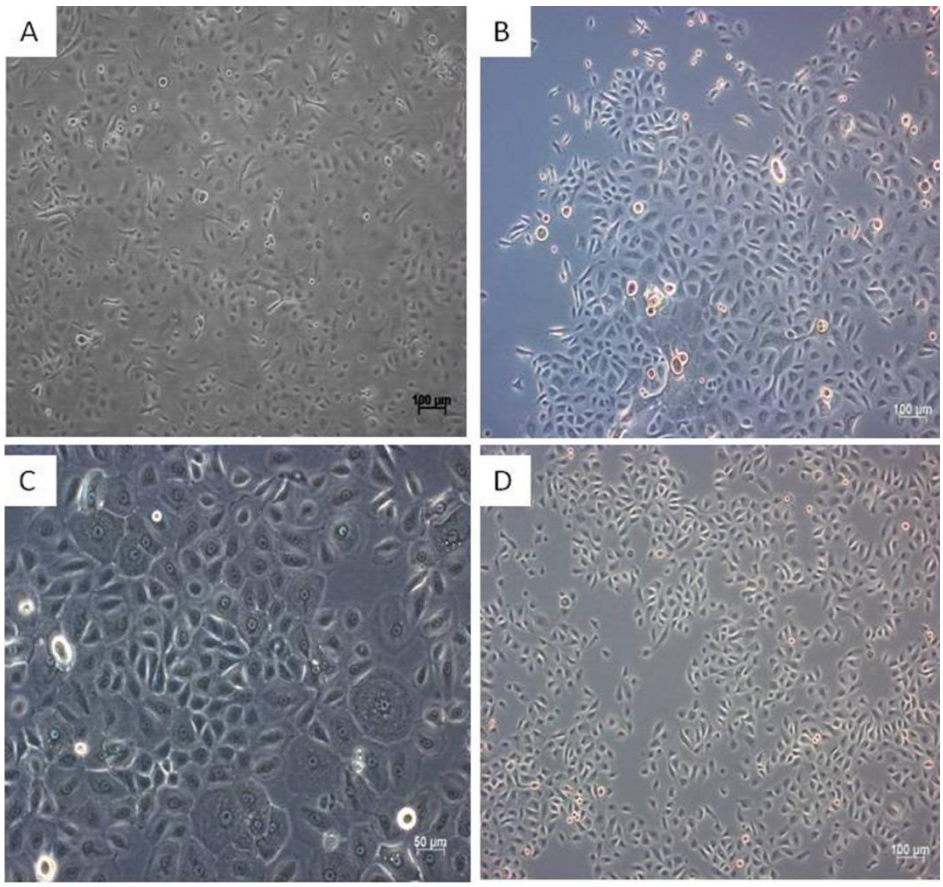


Figure 2.4 Human primary limbal cultures (Passage 2) grown on a collagen II coating matrix at different stages. (A) Day 2 (B) Day 8 (C) Day 15 (D) HTCEC in serum-free and feeder free conditions on Day 3.

from support substrates and each other, or in this case, firmly adherent cells) for 20 minutes at 37ºC and then incubated with 0.05% trypsin-0.53 mM ethylene diaminetetraacetic acid (EDTA) solution [Gibco] for 3 minutes in a tissue culture incubator also at 37ºC. The trypsin was then inactivated by the addition of DMEM containing 10% fetal bovine serum. The resulting cell suspension was centrifuged for 3 minutes at 1,000 rpm. The supernatant discarded and the cell pellet re-suspended in DKSFM. The cell suspension was then split into two T25 cm² flasks or being put into one T75 cm² tissue culture flask [Scientific Laboratory Supplies] containing DKSFM. The HTCEC cultures were maintained in a tissue culture incubator and the medium was changed every three day. The HTCEC was repeatedly sub-cultured for a maximum of 20 passages.

2.10.2 Cryopreservation and reculturing of HTCEC

For the purposes of maintaining stocks, 1 x 10^6 cells was suspended in 1ml of Recovery Cell Culture Freezing Medium [Gibco], the suspension was then transferred to a cryogenic vial and put in a cell freezing container [Nalgene], which contains 250ml of

100% isopropyl alcohol [VWR BD Prolabo]. This provides -1°C/minute cooling rate required for successful cell cryopreservation and recovery, and then frozen at -80 °C or in liquid nitrogen.

The frozen cell suspension was rapidly warmed and washed in DKSFM and centrifuged at 1,000 rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended in 1ml of DKSFM and transferred to a $25cm^2$ or $75cm^2$ flask containg a further 10ml DKSFM. The media was changed on the next day and then every three days.

2.11 Mycoplasma detection and treatment

Mycoplasma are small and simple prokaryotes which depend on their host for nutrients due to their limited biosynthesis. As they compete with the cells for the nutrients in the culture media, this will affect cellular growth and proliferation and cause changes in the gene expression. Cells were regularly tested for mycoplasma contamination every six months. Mycoplasma detection was carried out using the MycoAlert™ assay [Lonza] according to the manufacturer's protocol. In principle, this assay is a selective biochemical test that exploits the activity of certain mycoplasma enzymes which upon lysis, reacts with the MycoAlert™ substrate and catalyses the conversion of ADP to ATP. The level of ATP before and after the addition of the MycoAlert™ substrate indicates the presence or absence of mycoplasma and can be measured using a luminometer 'Luminoskan' [Thermo Scientific]. The bioluminescent reaction involved is as follows:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{LIGHT}$$

Briefly, after dissociation, cells were centrifuged and 100µl of cleared fresh supernatant was suspended with 100µl reconstituted MycoAlert™ reagent in assay buffer, and left for 5 minutes in a luminometer cuvette. Luminescence was then measured using a Luminometer TD-20/20 [Turner Biosystems, Inc.USA] and labelled as Reading A. Following that, 100µl MycoAlert™substrate was added to the sample and left for 10 minutes, after which the second luminescence reading was labelled as Reading B. The ratio of Reading B/Reading A is used for the interpretation of mycoplasma contamination. The ratio of <0.9 is negative for mycoplasma, 0.9-1.2 is borderline, and

> 1.2 signifies mycoplasma contamination. Cells represented with borderline results were quarantined and retested within the next 24 hours. Cells with mycoplasma infection were discarded and fresh stocks obtained.

2.12 Other Cell Lines

Other cell lines were grown in appropriate culture conditions:

2.12.1 *Breast cancer cell MCF-7*

This is a human breast adenocarcinoma cell line. MCF-7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman. The cell line retains several characteristics of mammary epithelium. It is an oestrogen receptor (ER) positive progesterone receptor positive cell line **(Yamaguchi *et al.*, 2005)**.

MCF-7 cells were grown in complete Dulbecco's Modified Eagle's medium (cDMEM) that contains 10% fetal bovine serum (v/v) and 1% Penicillin/Streptomycin under standard TC conditions, while still subconfluent the medium was removed and the adherent cells washed briefly with with PBS, incubated with 0.05% trypsin-0.53 mM ethylene diaminetetraacetic acid (EDTA) solution [Gibco] for 5 minutes in a tissue culture incubator at 37ºC with 5% CO_2. The trypsin was then inactivated by the addition of DMEM containing 10% fetal bovine serum. The resulting cell suspension was centrifuged for 3 minutes at 1,000 rpm. The supernatant was removed and the resulting pellet was resuspended in cDMEM for subculturing. For cryopreservation, 1.0×10^6 cells were cryopreserved as described previously (refer section 2.8.3).

2.12.2 Breast cancer cell MDA-MB-231

This cell line was obtained from pleural effusion of a 51-year old patient at M.D. Anderson Cancer Centre, Texas, USA in 1973. It appears spindle-shaped and has epithelial morphology, with an invasive phenotype. These cells are oestrogen and progesterone receptor negative. The cells were propagated in DMEM. Subculturing, cryopreservation and revival of cells follows similar steps as described previously (refer section 2.8.4).

2.12.3 Ovarian cancer cell HeyA8MDR

These malignant ovarian epithelial cancer cells are counterpart to HeyA8. It is taxane-resistant and was recently demonstrated as secreting endoglin, an angiogenic marker

which is associated with chemo-resistance (Ziebarth *et al.*, 2013). The cells were propagated in RPMI 1640 Complete Culture medium [Sigma Aldrich] with NaHCO3 and without L-glutamine. Subculturing, cryopreservation and revival of cells follow similar steps as described above in section 2.8.4.

2.12.4 Human Epstein-Barr Virus (EBV)-negative cell line (Ramos)

These cells were derived from a Caucasian Burkitt's lymphoma patient. It does not possess the Epstein Barr Virus (EBV) genome, however these cells may be infected *in vitro* with a prototype strains of EBV (Trivedi *et al.*, 2001). These are non-adherent cells and propagated in RPMI 1640 Complete Culture medium [Sigma Aldrich] with NaHCO3 and without L-glutamine.

2.13 Primary cells – Other

2.13.1 Human peripheral blood leukocytes (PBL)

Peripheral blood leukocytes were used as negative control gating in flow cytometry for optimisation of antibodies. PBL isolation was conducted together with Dr Gendie Lash (Institute of Cellular Medicine, Newcastle University). Briefly, venous blood was acquired from the antecubital vein and collected in a heparinised universal tube. 10ml PBS was added to 10ml of blood. In a new universal tube, 5ml lympholyte-H [CedarLane Laboratories] was added. Following this, diluted blood was carefully layered on top of the lymphoprep. The tube was then centrifuged at 800G for 20 minutes with no brake (set at 0). This separated the blood into layers, the serum was gently removed. The leukocytes were then harvested into a fresh universal tube and washed with PBS 25ml, followed by centrifuged at 400G for 10 minutes, with the brake set at 3., this step was repeated a second time and the final cell pellet was resuspended in 5ml of media ready for use.

2.13.2 Human bone marrow (BM)-derived MSC

BM-derived MSC [courtesy of Dr Rachel Oldershaw] was used as positive control for immunofluorescence. The cells were maintained in MSC-enriched media and incubated in hypoxic condition with 5% oxygen, 5% CO_2 incubator at 37°C, prior to use.

2.14 Flow cytometry

2.14.1 Definitions and principles

Flow cytometry is a technological process that involves light scattering, excitation and emission of fluorochromes to provide data of cells or particles. Samples were hydro-dynamically focused in a sheath of PBS before intercepting a light source (usually a laser) (Figure 2.5).

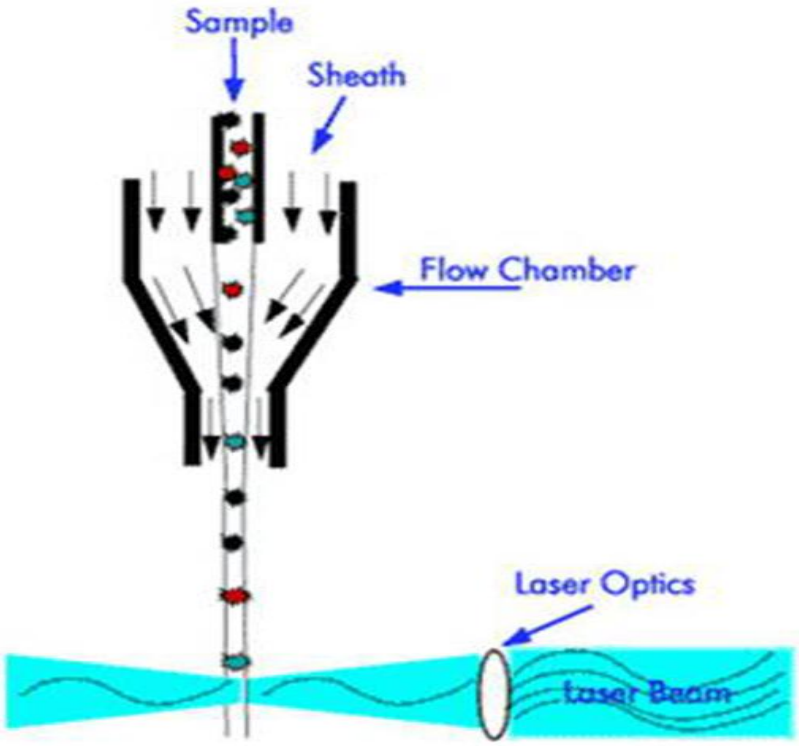


Figure 2.5 Principles of flow cytometry-1. The sample is injected into the sheath and hydro-dynamically focused at the centre of the stream. When it intercepts a laser source, they scatter light and the fluorochrome is excited to a higher energy state. When the molecule goes to a relaxed state, it will release a photon of light that has unique specific spectral properties to the fluorochrome *(From Current Protocols In Cytometry, Unit 1.2, p 1.2.2).*

When a cell intercepts a laser source, light is scattered and the fluorochrome is excited to a higher energy state. When the molecule goes to a relaxed state, it will release a photon of light that has unique specific spectral properties to the fluorochrome (Figure 2.6).

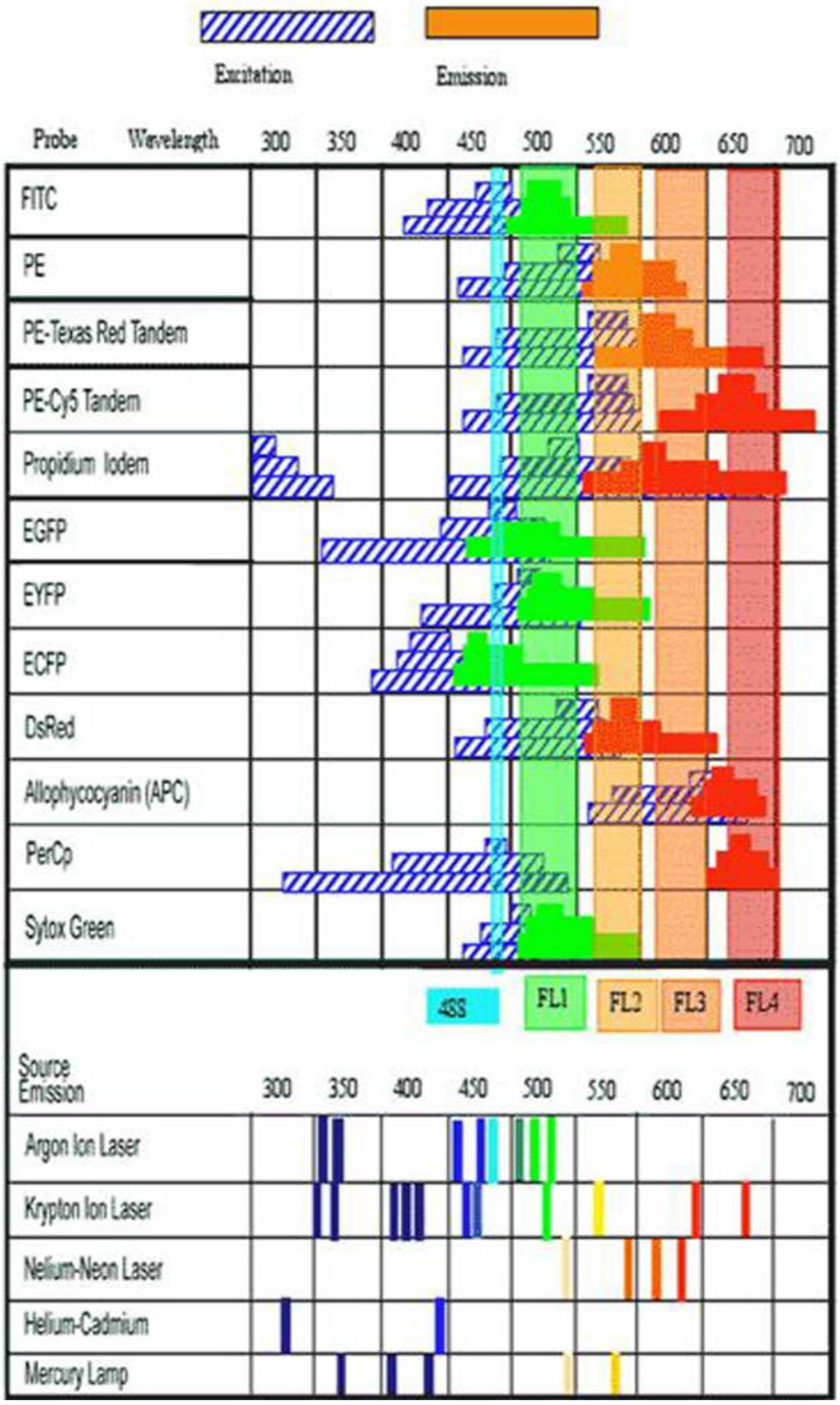


Figure 2.6 Fluorescence spectra of commonly used fluorochromes. The bottom part of the table summarizes the emission wavelengths of various light sources used in flow cytometry. The 488nm line of the argon ion laser is extended over the spectra. *(From Practical Flow Cytometry, Third Edition, Howard M. Shapiro. P. 245)*

Several dichroic mirrors and filters separate the scattered and emitted light into different wavelengths which divide the light to a series of photomultiplier tubes (PMT) (Figure 2.7). PMT processed the potential difference by a series of linear and log amplifiers

which give signal pulses proportional to the number of photons reaching the photodetector.

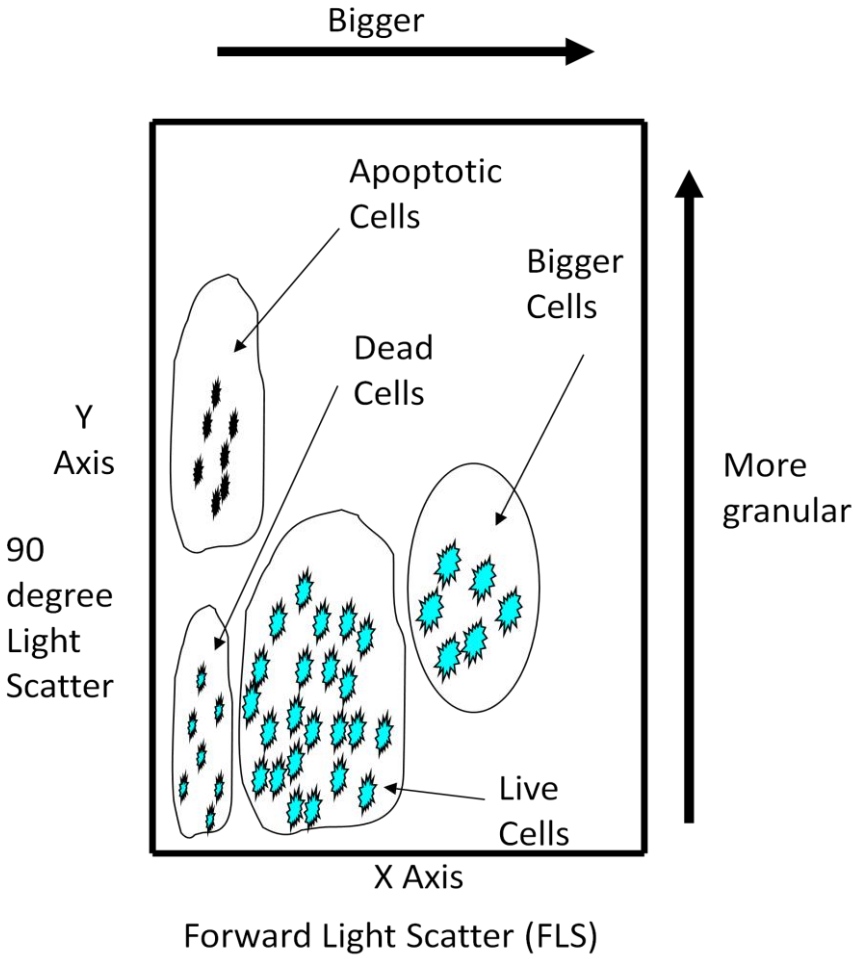


Figure 2.7 Light Scattering, 2-parameter histogram. Logarithmic amplification is used to measure fluorescence in cells. Different signals or pulses are amplified to allow for events to be plotted on histograms.

Logarithmic amplification is most often used to measure fluorescence in cells. After the different signals or pulses are amplified they are processed by an Analog to Digital Converter (ADC) which in turn allows for events to be plotted on a graphical scale e.g. One Parameter or Two parameter Histograms (Figure 2.8).

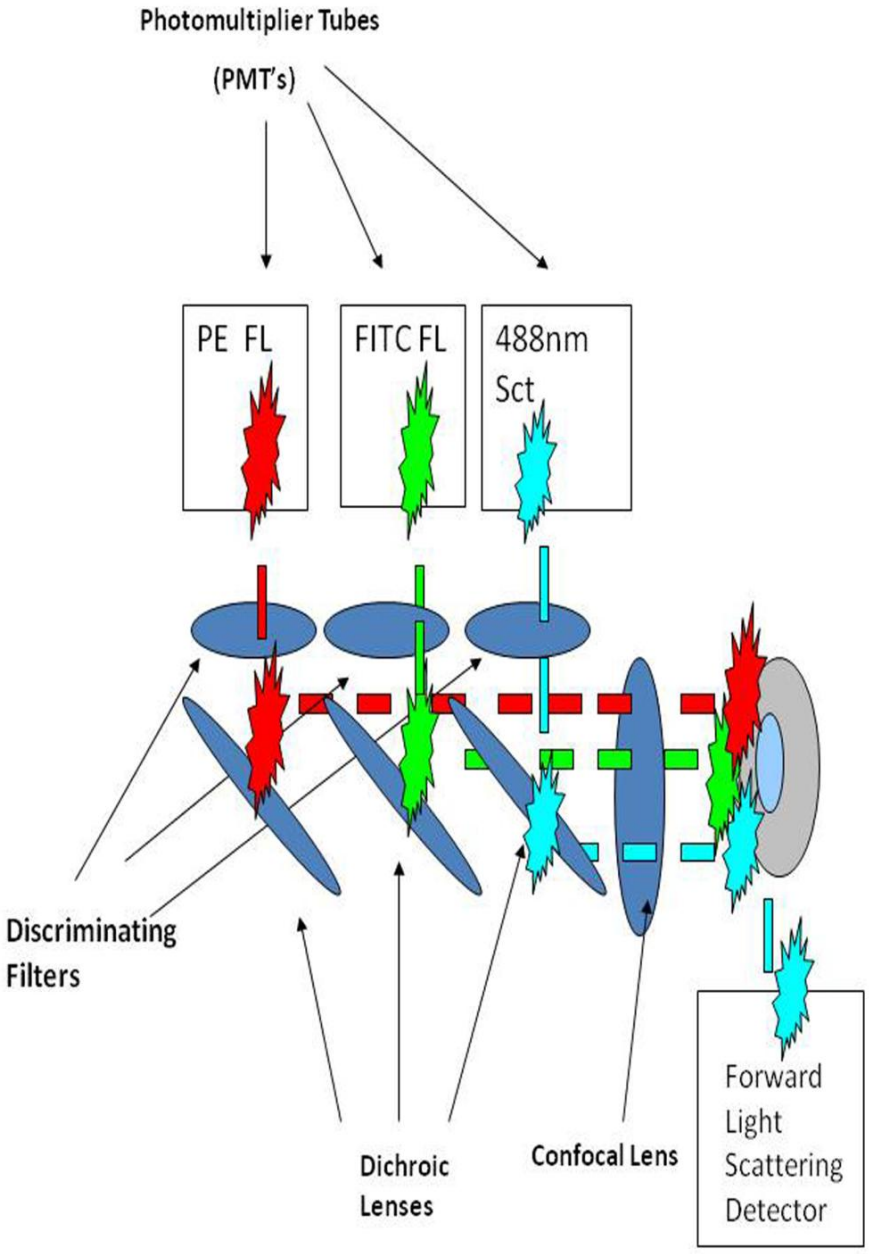


Figure 2.8 Principles of flow cytometry-2. Several dichroic mirrors and filters separate the scattered and emitted light into different wavelengths which divide the light to a series of photomultiplier tubes (PMT). PMT processed the potential difference by a series of linear and log amplifiers which give signal pulses proportional to the number of photons reaching the photodetector.

This information can be used to sort subpopulations of cells. Data generated from flow cytometry are available in a multiparameter acquisition display software programmes. Flow cytometry analysis data of a single suspension yields multi parameter data which correspond to Forward Light Scatter and 90° Light Scatter. This information allows characterisation of subpopulations and this separation process is called "sorting".

2.14.2 Forward scatter and side scatter

Forward scatter or low angle scatter is detected in the plane of incident light. The amplitude of the signal often corresponds to cell size or cell diameter of cells with the same refractive index.

While SSC or the 90˚ light scatter or "right angle scatter" refers to the internal granularity or complexity of a cell. Light tends to scatter at larger angles to the incident beam by cells that are more dense or granular.

2.14.3 Principles of cell sorting

A cell sorter can rapidly separate the cells in a suspension on the basis of size and the properties of the fluorochromes. A cell sorter like FACS DiVa [BD] uses the droplet method, where a cell suspension containing cells which are labelled with a fluorescent dye is hydronamically focused in the cell sheath to allow single passage of droplets. This stream emerges from a nozzle vibrating at some 40,000 cycles per second which breaks the stream into 40,000 discrete droplets each second. A laser beam is directed at the stream just before it breaks up into droplets. As each labelled cell passes through the beam, its resulting fluorescence is detected by a photocell which is electrically charged.

If the signals from the two detectors meet either of the criteria set for fluorescence and size, an electrical charge (+ve or -ve) is given to the stream. The droplets retain this charge as they pass between a pair of charged metal plates. Positively-charged drops are attracted to the negatively-charged plate and vice versa. Uncharged droplets (those that contain no cell or a cell that fails to meet the desired criteria of fluorescence and size) pass straight into a third container and are later discarded (Figure 2.9).

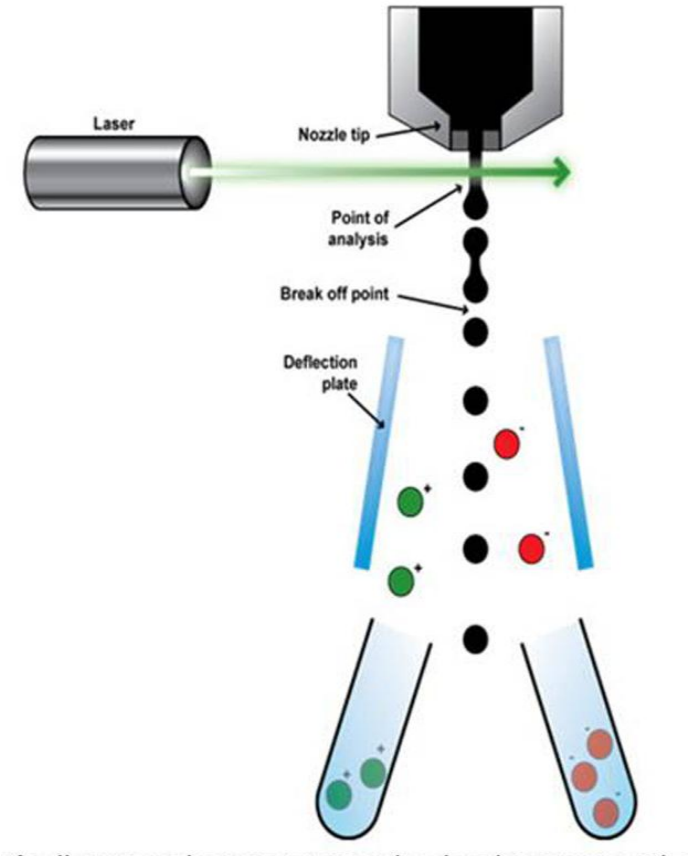


Figure 2.9 Principles of cell sorting. The suspension in the sheath intercepts a laser beam, becoming droplets which then pass an electrical field which causes the droplets to retain positive or negative charges. Positively-charged drops are attracted to the negatively-charged plate and vice versa, the cells are "sorted" according to the desired criteria of fluorescence and size.

2.15 Immunocytochemistry (ICC)

2.15.1 Titration of antibodies

For antibody optimisation, primary antibodies were diluted in 0.5% Normal goat serum (NGS) [Sigma]/PBS to 1:50, 1:100, 1:300, and 1:500 dilutions. For transcription factor p63 and ABCB5 antibodies, optimum antibody concentration was determined by performing ICC on HTCEC. The optimum expression for p63 was at 1:50 dilution. For ATP-binding casette (ABC)-G2 transporter antibody, optimised expression was obtained using a rabbit polyclonal anti-human ABCG2 antibody at 1:100 dilutions [Novus Biologicals]. In the case of stem cell markers NANOG, Oct 3/4 and Sox2, antibody concentrations were optimised using embryonic stem cell (ESC) courtesy of

Dr Rachel Oldershaw (Institute of Genetic Medicine). The optimum expression for these primary antibodies was observed at 1:100 dilutions.

2.15.2 Staining for markers

40,000 cells were seeded on an 8-chamber slide [BD Falcon] in appropriate tissue culture medium and allowed to proliferate until 80% confluence in 5% CO_2 incubator at 37°C. Upon reaching confluence, culture medium was removed and cells washed with PBS three times. Cells were then fixed with cold methanol [BDH Laboratory Supplies] at-20°C for 10 minutes. Slides were then washed with PBS and then permeabilised for 10 minutes in 0.3% (v/v) Triton X-100 [Fisher Scientific] diluted in PBS, and then washed again with PBS. Blocking of non-specific binding sites in 5% normal goat serum and PBS was performed for 30 minutes in a humid chamber in the dark. Cells were incubated with primary antibodies in 0.05% FBS/PBS overnight at 4°C. Cells were then washed with PBS 3 x 5 minutes. Cells were incubated with secondary antibody for 30 minutes in a humid chamber in the dark. Following that, slides were washed with PBS three times/5 minutes and the chamber was removed from the slide.

The slide was then mounted in vectashield anti-fading medium [Vector Laboratories] and slides were covered with a cover slip. Slides were stored at 4°C in the dark or taken immediately for viewing under a fluorescence microscope. For longer term, storage slides were kept at -20°C. Negative controls were cells stained without primary antibody, but with secondary antibody. A list of primary and secondary antibodies is presented in Table 2.3.

Table 2.3 Primary and secondary antibodies used for ICC.

Antibody	Cellular localisation of antigen	Optimal Dilutions	Supplier
PRIMARY			
Mouse monoclonal anti-human p63 (clone 4A4)	Nuclear/ cytoplasmic	1:50	Santa Cruz Biotechnology
Mouse monoclonal anti-human ABCB5 (clone 5H3C6)	Transmembrane	1:100	Abcam
Mouse monoclonal anti-human Oct3/4	Intracellular	1:100	BD Biosciences
Mouse monoclonal anti-human NANOG	Intracellular	1:100	Abcam
Rabbit polyclonal anti-human ABCG2 (clone 5D3)	Transmembrane	1:100	Novus Biologicals
Rabbit polyclonal anti-human Sox2	Nuclear	1:100	Abcam
SECONDARY			
FITC-conjugated secondary goat anti-mouse immunoglobulins	Intracellular	1:25	Jackson Immunology Research Laboratories
FITC-conjugated secondary goat anti-rabbit immunoglobulins	Intracellular	1:25	Jackson Immunology Research Laborotories
Rhodamine-conjugated secondary goat anti-rabbit immunoglobulins	Intracellular	1:25	Jackson Immunology Research Laborotories

2.16 RNA isolation

RNA isolation was performed using RNEasy Plus Micro Kit [Qiagen] as per manufacturer's instructions on 500,000 – 1,000,000 cells. Briefly, 350 µl Buffer RLT Plus was added to the cells. The lysate was then homogenized by centrifuge at 11,000 rpm for 30 seconds. The lysate was transferred to gDNA Eliminator spin column in a 2 ml collection tube. This will remove the genomic DNA. It was then centrifuged for 30

seconds at 11,000 rpm. The spin column was discarded and total RNA was collected in the 2 ml collection tube.

350µl 70% ethanol was added and mixed by pipetting. It was then transferred to an RNEasy MinElute spin column in a 2ml collection tube, and centrifuge at 11,000 rpm for 15 seconds. The flow through was discarded. 700µl Buffer RW1 was added to the spin column, centrifuged for 15 seconds at 11,000 rpm and the follow through discarded. 500µl Buffer RPE was then added to the spin column, and centrifuged at 11,000 rpm for 15 seconds. The flow through was discarded. 500µl 80% ethanol was added to the spin column, and centrifuged at 11,000 rpm for 2 minutes. The flow through was then discarded.

In a new 2ml collection tube, then cap of the spin column was opened, and centrifuge at 11,000 rpm for 5 minutes to dry the spin column membrane. The collection tube was then discarded. The spin column was then placed in a 1.5ml collection tube. 14µl RNase-free water was added directly to the centre of the spin column membrane, and centrifuged for 1 minute at full speed (13,000 rpm) to elute the RNA.

2.16.1 RNA quantification

RNA was quantified using Nanodrop Spectrophotometer 2000 [Thermo Scientific]. Initially the spectrophotometer was calibrated with 1µl blank solution and followed by the RNA sample. A260/280 is the absorbance ratio for nucleotides:protein, and used to determine the purity of RNA and DNA. A value of ~1.8 is acceptable for DNA purity while a slightly higher level ~2.0 is acceptable for RNA purity. A260/230 which is the absorbance ratio for nucleotide:chemical contamination i.e phenols, alcohol, was also documented.

2.16.2 RNA integrity

As a measure of RNA integrity, RNA were electrophoresed on 1% agarose gel [NBS Biologicals] at 90V for 45 minutes in tris-borate-EDTA (TBE) buffer [Sigma]. Agarose gel electrophoresis is a technique used to separate DNA or RNA fragments by size. The sugar-phosphate backbone results in the nucleic acids being negatively charged. When a current is applied, the RNA or DNA migrates towards the positive electrode and the rate of migration is dependent on the nucleic acids size, the smaller fragments will migrate

faster than the larger fragments. The RNA sample was examined for 28S and 18S bands.

To make 50 ml 1% agarose gel, 50 ml 1X Tris/Borate/EDTA buffer was added to 0.5g agarose to dissolve it by heating. The solution was allowed to cool down prior to addition of 2µl of 0.5µg/ml ethidium bromide [Bioline]. Ethidium Bromide is a fluorescent compound which intercalates into DNA and fluoresces when a UV light is exposed. The gel was poured into a cast and allowed to solidify for approximately 30 minutes. The gel was then placed in an electrophoresis tank and covered with 1X TBE buffer.

5µl hyperladder IV [Bioline] was loaded to determine the size of products for each sample. 0.5µg – 1.0µg of samples were added to 2µl of 5X Blue DNA loading buffer [Bioline] and the mix was made up with RNAse-free water to 20µl final volume. A loading dye allows the progress of DNA to be tracked during electrophoresis and the presence of glycerol in this solution ensures the DNA remains at the bottom of the well prior to electrophoresis. The gel was then visualized under a UV gel documentation system [UVP Ltd, Cambridge, UK].

The ratio of 28S to 18S is approximately 2.1. This 2:1 ratio is a good indication that the RNA is completely intact. Partially degraded RNA will have a smeared appearance, will lack the sharp RNA bands, or will not exhibit the 2:1 ratio of high quality RNA (Figure 2.10).

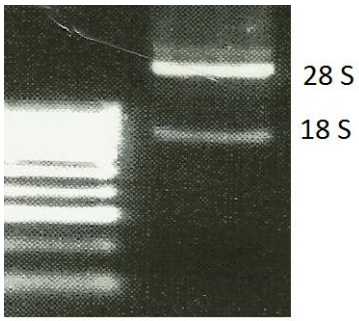


Figure 2.10 Gel electrophoresis of an RNA sample from LEC to examine RNA integrity. *[Lane 1 DNA hyperladder, Lane 2 template RNA].*

2.17 Reverse transcription

cDNA was synthesised using a cDNA Synthesis Kit [Bioline]. The following was prepared on ice:

(1 µg) RNA	n µl
Oligo $(dT)_{18}$	1 µl
10 mM dNTP	1 µl
DEPC-treated water	Up to 10 µl

The samples were incubated at 65°C for 10 minutes. Then the samples were cooled on ice for 2 minutes. After which, the following reaction mix were prepared:

5x RT Buffer	4 µl
RNase inhibitor	1 µl
Reverse Transcriptase (200u/ µl)	0.25 µl
DEPC-treated water	Up to 10 µl

10µl of the above reaction mix was added to the tube containing the primed RNA. The samples were incubated at 42°C for 50 minutes. The reaction was terminated by incubating at 70°C for 15 minutes and then chilled on ice.

2.18 Semi-quantitative Polymerase chain reaction (sq-PCR)

2.18.1 Primer design

Gene sequences were obtained from National Centre for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool (BLAST®) databases. Invitrogen OligoPerfect™, a designer application tool was used to obtain the forward and reverse primers. Sequences were designed from two different exon sequences in order to avoid genomic contaminants being identified. Designed primers were generally 17-25 nucleotides in length within the 100-300bp product size, containing 40-60% genomic content. The melting temperature (Tm) was determined for both the forward and reverse primers using the formula:

$$Tm = 4(G+C) + 2(A+T).$$

The initial annealing temperature (Ta) used for each PCR reaction was generally 5°C below the lowest Tm of the primer pair (forward and reverse primers). However, for each primer pair the Ta was optimised by performing serial PCR at temperatures varying around the initial Ta.

2.18.2 Semi quantitative-PCR (sq-PCR)

Sq-PCR was performed using a PCR master mix [Promega]. This master mix is a ready-to-use solution containing *Taq* DNA polymerase (50 units/ml) in a pH 8.5 reaction buffer, dNTPs, and 3mM $MgCl_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates. A 25µl final solution containing the following was prepared in a PCR tube [Scientific Laboratory Supplies] as listed in Table 2.4.

Table 2.4 Components of an sq-PCR reaction

Component	Volume (µl)
PCR Master Mix, 2X	12.5
Forward primer, 10µM	0.5
Reverse primer, 10µM	0.5
cDNA	2.5
Nuclease-Free Water to	25

Denaturation was set at 94°C for 30 seconds and elongation took place at 72°C for 1 minute at primer pair specific annealing temperature, and maintained at 4ºC. The final PCR product mixture was either stored at +4ºC or electrophoresed on an agarose gel.

For each primer pair the annealing temperatures were optimised by performing serial PCR at gradient temperatures from 51°C to 60.0°C using G-storm thermocycler for 30, 35 or 40 cycles. For the purpose of primer annealing temperature and cycles optimisation, template cDNAs were derived from different sources e.g. breast cancer cell line (MCF-7), ovarian cancer cell line (HayA8 MDR) and LEC where appropriate. Optimum amplification conditions for oligonucleotide primers are provided in separate relevant chapters.

2.18.3 Gel Extraction for DNA sequencing

Gene sequencing was performed to validate detection of target genes. PCR was performed and products were electrophoresed and the gel extracted for DNA elution. Briefly, 50µl PCR reactions were prepared containing 25µl Master Mix, 1.0µl forward

and reverse primers, 5µl cDNA template and 18 µl nuclease-free water. PCR was performed at this volume for 35 cycles with annealing temperature of 55.0°C.

Subsequently, 1% agarose gel containing 5µl of Gel Green Nucleic Acid stain [Biotium] was prepared in 1x TBE buffer. 2µl of Gel Green loading buffer [Biotium] was mixed with PCR product then loaded on the gel and electrophoresed at 85V for 45 minutes. 5µl of DNA Hyperladder IV [Promega] was also electrophoresed. Gel was viewed on a blue illuminator [Claire Chemical Research] and bands removed for gel extraction.

DNA was eluted using QIAquick Gel Extraction Kit [Qiagen] according to the manufacturer's protocol. In brief, DNA fragments were excised from the agarose gel and weighed. 3 volume of buffer GQ was added to 1 volume of gel, and incubated at 50°C for 10 minutes. To ensure complete solubilisation, samples were vortexed every 2-3 minutes. The reactions were checked for colour changes, to ensure an appropriate pH of ≤7.5 (yellow colour) was maintained. 1 gel volume of isopropanol was mixed to the reactions.

For DNA binding, the reactions were placed in a spin column in a 2ml collection tube, and centrifuged at 13,000 rpm for 1 minute. Flow through was discarded and spin column placed back in the collection tube. 0.5ml Buffer GQ was added the reaction centrifuged again. To wash, 0.75ml buffer PE was added and centrifuged again for an additional 1 minute. Then the spin column was placed in a clean 1.5ml centrifuge tube. To elute DNA, 50µl buffer EB was added to the centre of column membrane for 1 minute and centrifuge at 13,000 rpm for 1 minute. Eluted DNA was sent for sequencing to Source BioScience.

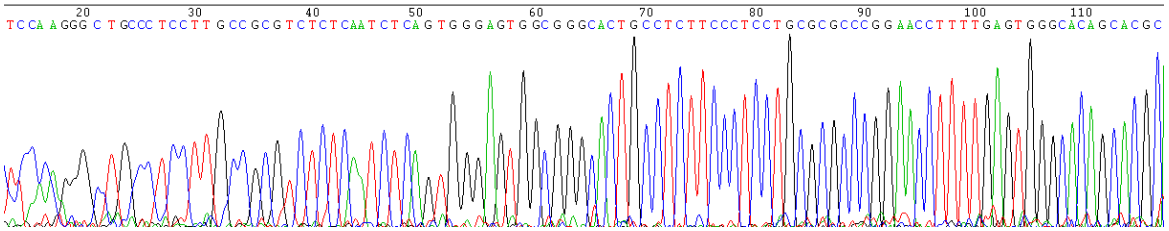
2.18.4 Validation of target gene primer(s) by gene sequencing

Chromatograph of the gene sequences for both forward and reverse primers were examined for evenly-spaced peaks, minimal noise, signal intensity (>100) and mis-spaced nucleotides which are characteristics of unacceptable "noisy" chromatogram.

Gene chromatographs:

a. ABCG2_Forward
   Signal: G:124 Gene sequences:

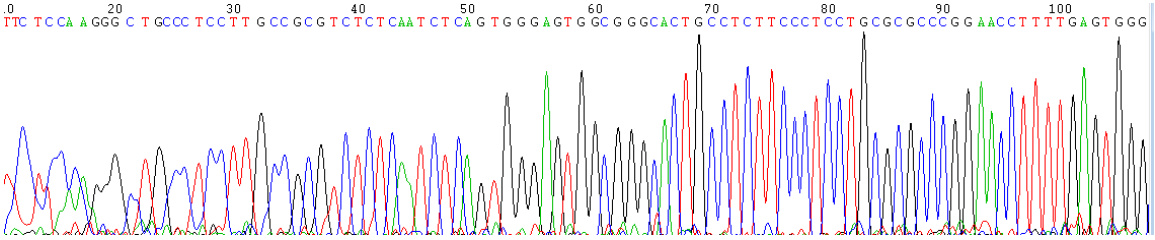
GGGCTTTGGCCGAAGAAGGGAGAGGCAGTGCCCGCCACTCCCACTGAGATTGAG
AGACGCGGCAAGGAGGCAGCCTGTGG
AGGAACTGGGTAGGATTTAGGAACGCACCGTGCACATGCTA



b. ABCG2_Reverse

Signal: G:143 Gene sequences:

ATAATAGGTTTCTCCAAGGGCTGCCCTCCTTGCCGCGTCTCTCAATCTCAGTGG
GAGTGGCGGGCACTGCCTCTTCCCTC
CTGCGCGCCCGGAACCTTTTGAGTGGGCACAGCACGC



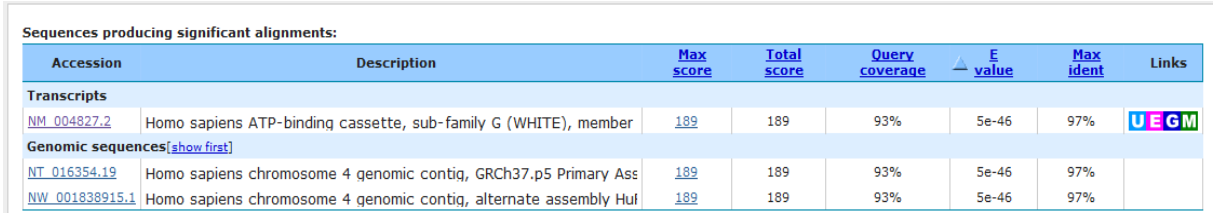
Gene sequences obtained from the above was entered into the National Centre for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool (BLAST®) databases and the results showed the sequences were described for ABCG2 gene as follow:

i. Sequences producing significant alignment with ABCG2 forward primer (121 bases) shows 94% coverage for ABCG2 gene transcript no. NM_004827.2

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
Transcripts							
NM_004827.2	Homo sapiens ATP-binding cassette, sub-family G (WHITE), member	180	180	94%	4e-43	96%	U E G M
Genomic sequences [show first]							
NT_016354.19	Homo sapiens chromosome 4 genomic contig, GRCh37.p5 Primary Ass	180	180	94%	4e-43	96%	
NW_001838915.1	Homo sapiens chromosome 4 genomic contig, alternate assembly Hul	180	180	94%	4e-43	96%	

ii. Sequences producing significant alignment with ABCG2 reverse primer (117 bases) shows 93% coverage for ABCG2 gene transcript no. NM_004827



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
Transcripts							
NM_004827.2	Homo sapiens ATP-binding cassette, sub-family G (WHITE), member	189	189	93%	5e-46	97%	UEGM
Genomic sequences [show first]							
NT_016354.19	Homo sapiens chromosome 4 genomic contig, GRCh37.p5 Primary Ass	189	189	93%	5e-46	97%	
NW_001838915.1	Homo sapiens chromosome 4 genomic contig, alternate assembly Hul	189	189	93%	5e-46	97%	

Other target genes that were validated by sequencing are listed in Table 2.5:

Table 2.5 List of other primers validated by gene sequencing.

Genes	Primers
Cytokeratin 3 (CK3)	Forward: GGATGTGGACAGTGCCTATATG Reverse: AGATAGCTCAGCGTCGTAGAG
C/EBPD	Forward: ACTCAGCAACGACCCATACC Reverse: CGCTCCTATGTCCCAAGAAA
Connexin 43 (Cx43)	Forward: ATGAGCAGTCTGCCTTTCGT Reverse: TCTGCTTCAAGTGCATGTCC
Bmi-1	Forward: CTGGAGAAGGAATGGTCCAC Reverse: GCCTTGTCACTCCCAGAGTC

2.18.5 Gel electrophoresis

PCR products were electrophoresed on 2% agarose gel. To prepare 50ml 2% (w/v) agarose gel, 50ml 1X Tris/Borate/EDTA buffer was added to 1.0g agarose and dissolved by heating. The solution was allowed to cool down prior to addition of 5µl of Gel Green [Bioline] following the steps as previously described (refer section 2.16.2).

2.19 Quantitative Real-timer PCR (QRT-PCR)

2.19.1 Primer design

Primer probes were designed and commercially purchased from Life Technologies. The probes were customised using the Taqman® Gene Expression Assays. Probes would be exon-spanning in order to avoid genomic contaminants, and selected for best coverage.

2.19.2 Primer validation

Table 2.6 Efficiency values for validation of Taqman primers.

Assay ID	Gene symbol	Efficiency (%)
Hs02758991_g1	GAPDH	88.0
Hs01053790_m1	ABCG2	60.0
Hs00184500_m1	ABCB1	74.1

Efficiencies for qRT-PCR primer probes as listed in Table 2.6 were validated using cDNA template from MCF-7 cell line diluted in 1:10 fold dilutions in triplicates. Average of cycle threshold (CT) values was plotted against $\log_{10}$ [concentration of cDNA]. Linear regression analysis was performed with 95% confidence interval for the slope of the validation curve, where y is expressed as $mx + c$, m is the value for the slope and c was the value where it intercepts at the y axis. Slopes between -3.1 and -3.6, giving reaction efficiencies between 90 and 110% are typically acceptable. The value for the slope was then entered into the efficiency formula;

$$E= -1+10^{(-1/slope)}.$$

Efficiency graphs and values are presented in Figure 2.11.

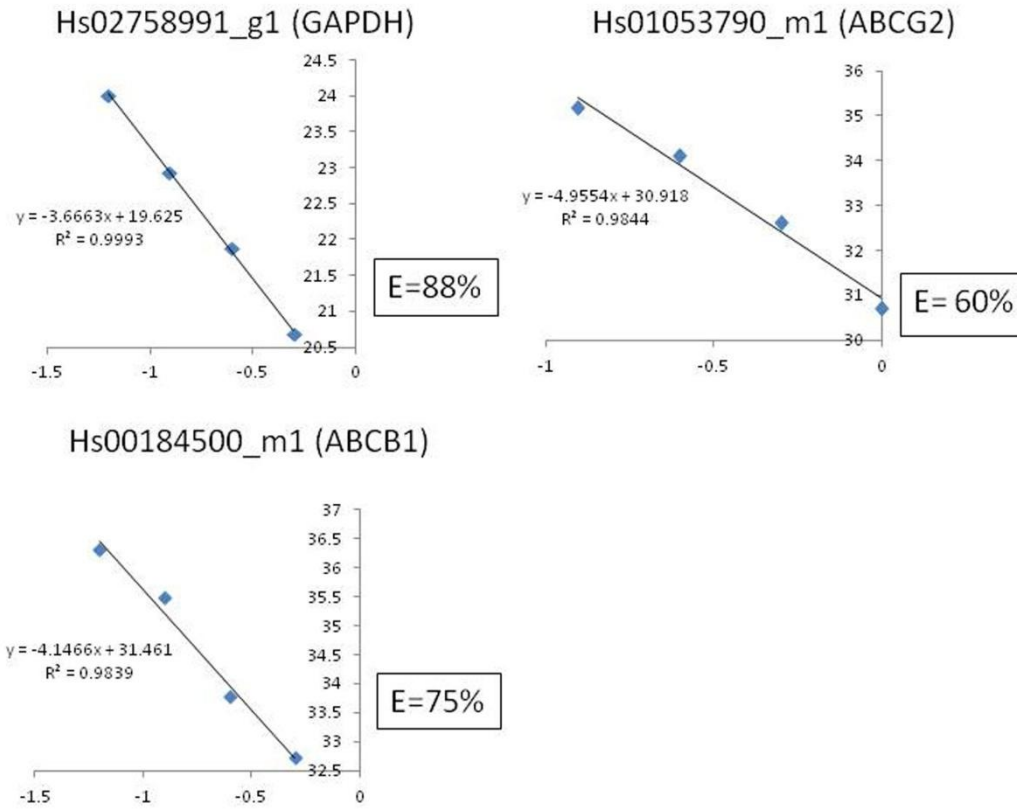


Figure 2.11 Efficiency values (E) for validation of Taqman probes. Linear regression analysis gave a standard curve $y=mx + c$. The slope of a standard curve (m) indicates PCR efficiency. Y axis represents CT value, X axis represents log [10] cDNA concentration.

2.19.3 Quantitative Real-time polymerase chain reaction (qRT-PCR) and analysis

For qRT-PCR reactions, each sample was prepared in triplicates. Each reaction contains 2µl cDNA, 1µl Taqman Gene Expression Assay [Applied Biosystems], 10µl Brilliant II QPCR master mix with High ROX [Agilent] and DNase-free water [Promega/Sigma] to make up 20µl of reaction mix. No template reaction (NTR) for negative control for each gene expression was also included. All reactions were prepared in nuclease-free 0.2 ml microcentrifuge tubes. Tubes were capped and inverted several times to mix the reactions. Samples were then loaded onto a 96-well plate [Applied Biosystem], and sealed with a plastic cover and centrifuged briefly. The plate was then placed in a Light Cycler [Applied Biosystems]. qRT-PCR was performed at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute, with a single data

acquisition step for comparative cycle threshold (ΔΔCT). Values for CT, ΔCT, ΔΔCT and relative expression to genes/fold change were calculated and plotted on a graph.

2.20 Imaging methods and quantification software

2.20.1 Phase contrast inverted microscopy

For the purpose of examining cells in cultures, plates, and flasks, we used an Axiovert 200M microscope [Zeiss] which allows the performance of transmitted light brightfield, phase contrast and epifluorescence technique. Images were then processed using the AxioVision40 version 4.8.2.0 software [Zeiss]. We also used Nikon Digital Sight–DSFi1 camera and Nikon NIS-Elements D software [Nikon Metrology] for brightfield, phase contrast, and differential interference contrast (DIC).

2.20.2 Fluorescence microscopy

Fluorescence microscopy was undertaken using an Axiovert 200M [Zeiss] for cells in wells and by Axioplan F [Zeiss] for cytological slides. Images were then processed using the AxioVision40 version 4.8.2.0 software [Zeiss].

2.21 Statistical Analysis

Descriptive statistics were used to handle data according to the location, dispersion or shape. Measurement of location was described as means, median, or mode. Dispersion of data where described in range, variance or standard deviations (SD). Standard error (SE) of means was measured to show the precision of the sample means. The choice of the most appropriate statistical methods often depends on the shape of distribution. A normal distribution assumes a "bell-shaped" symmetrical data, which is often used in many statistical tests.

Quantitative data which were normally distributed were analysed using parametric statistical tests including independent t-test and one way ANOVA.
For 2 groups of independent data, independent t-test was used to compare the means between the groups. For 3 or more groups of univariate data with single factor, one-way analysis of variance (ANOVA) was used to obtain p values. Results with p values of less than 5% ($p < 0.05$) were considered statistically significant.

For hypothetical testing, the null hypotheses (H_0) are tested when the difference in means equals to zero (no effect). The alternative hypotheses (H_1) is defined and holds when the null hypotheses is rejected at a significance level of less than 5% ($p < 0.05$). Data which are not normally distributed were analysed using non-parametric tests which make no assumption about the probability distribution. These tests include: sign test,Wilcoxon signed-ranks test, Mann-Whitney U test or Kruskal-Wallis test. Sign test and Wilcoxon signed-ranks test were used for measurement of mean in one sample. Mann-Whitney is the equivalent of independent t-test, where means are measured in 2 samples. Kruskal-Wallis is equivalent to one-way ANOVA and used when there were three or more groups of variables. Results with p values of less than 0.05 ($p < 0.05$) were considered statistically significant.

Chapter 3 **CHARACTERISATION OF CORNEAL LIMBAL STEM CELLS AND HUMAN LIMBAL MESENCHYMAL STEM CELLS**

3.1 Introduction

Most LSC characterisation techniques have involved *in vitro* animal studies and/or the use of histological specimens (Zhuo Chen *et al.*, 2004; Hyun-Seung Kim *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005). A limited knowledge of LSC identification has been produced using *in vivo* techniques such as live imaging (Shortt *et al.*, 2007a) or observation of label retaining cells (Cotsarelis *et al.*, 1989), which have been critical in studying LSC biology.

In the last twenty years, a lot of studies have focused on trying to identify molecular markers for LSC, and the transient amplifying cells of the limbus. However, there are species variations in what marker expression clarify and variations in the techniques used for these analysis. To this date, there are still no specific markers for LSC and the molecular characteristics of LSC remain poorly understood.

To summarise findings to date, basal cells of limbal epithelium strongly express cytokeratin 19, vimentin, α-enolase, p63, β1 integrin, ABCG2, TGF-β and EGF-R (Zhuo Chen *et al.*, 2004; Hyun-Seung Kim *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005). The putative polycomb proto-oncogene, Bmi-1 (Lessard and Sauvageau, 2003; Molofsky *et al.*, 2003) and C/EBPδ (Barbaro *et al.*, 2007) have also been suggested to be potential LSC markers. On the other hand, cytokeratin 3 and 12, gap junction protein Connexin 43 and involucrin (Zhuo Chen *et al.*, 2004; Hyun-Seung Kim *et al.*, 2004; Schlotzer-Schrehardt and Kruse, 2005) have been put forward as negative markers.

The location of the LSC has been identified under the surface of Palisades of Vogt, now termed as the “limbal epithelial crypts”. In addition, limbal epithelium also possess stromal cells which are adherent to plastic, have fibroblastic morphology and are able to differentiate into multiple lineages, characteristics of a human MSC (MSC) (Branch *et al.*, 2012; Garfias *et al.*, 2012; Gui-Gang Li *et al.*, 2012). These MSC like cells can be isolated using protocols as described previously (Polisetty *et al.*, 2008).

As described in Chapter 1, there are three major layers of the cornea which are derived from different germ layers. The epithelial layer of the cornea develops from ectoderm, while the stroma and endothelium are mesenchymal in origin. Corneal stroma makes up 90% of corneal volume, is populated by mesenchymal keratocytes which are quiescent after birth, but undergo differentiation in response to wound, trauma, inflammation or infection (Darby *et al.*, 1990). This suggests plasticity of the cells to adapt to its environment.

The corneal limbus has been reported to possess stromal cells which have fibroblastic morphology, are adherent to plastic and able to differentiate into multiple lineages, suggestive of a MSC population (Branch *et al.*, 2012; Garfias *et al.*, 2012; Gui-Gang Li *et al.*, 2012). Human corneal mesenchymal stromal stem cells, unlike bone marrow-derived MSC, develop from neural crest cells (Hoar, 1982) and are believed to support the growth of limbal epithelial cells (Pinnamaneni and Funderburgh, 2012; Bray *et al.*, 2014).

MSC are non-haemopoietic stem cells with regenerative ability and can differentiate into cell lineages of mesenchymal origin i.e. adipocytes, chondrocytes and osteocytes. Due to variable defining characteristics for MSC, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has defined several minimal requirements (Dominici *et al.*, 2006): 1) MSC must be plastic adherent, 2) they must express CD105, CD73 and CD90, but lack of expression to CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR surface molecules. The third minimal criteria 3) they have to satisfy the multi potentiality to differentiate into adipocytes, osteoblast and chondroblast *in vitro*.

Paracrine secretions of many bioactive molecules i.e. cytokines, trophic and growth factors, by human MSC have been well documented making it an important player in regenerative medicine and transplantation (Langer and Vacanti, 1993; Miguel *et al.*, 2012; Levis *et al.*, 2013; Graziella Pellegrini *et al.*, 2013).

Bone-marrow-derived MSC (BM-MSC) have shown excellent anti-inflammatory and wound healing properties when introduced to corneal injury models in animal studies (Ma *et al.*, 2006; Augello *et al.*, 2007; Omoto *et al.*, 2009; Jiang *et al.*, 2010; Hsu *et al.*,

2012; Jia *et al.*, 2012). In support of that, BM-MSC has been investigated in the repair of corneal damage (Ma *et al.*, 2006), and the proposed mechanism is suppression of new vessel formation and inflammation post-transplantation.

In this study we look at HTCEC line as a model for primary limbal cultures, by looking at molecular markers, stem cell properties, immune-biological response and functional assays i.e. side population protocols and migration analysis. The use of primary limbal cultures in many optimisation assays is limited due to low availability, especially in countries with limited access to donor corneas. There is also the issue of donor-to-donor variations which may interfere with optimisation steps or screening protocols.

HTCEC exhibit genetic stability, as evident by normal cell cycle kinetics in the presence of high telomerase activity. It could maintain the capacity to differentiate into normal human corneal epithelium *in vivo* (Robertson *et al.*, 2011). Cell immortalisation is done to extend cells' finite life span by eliminating a critical cell cycle point or suppress cellular tumorigenesis by incorporating an oncogene (Robertson *et al.*, 2008). Telomerase is a specialized cellular reverse transcriptase that can compensate for the erosion of telomeres by synthesizing new telomeric DNA. Infection with human telomerase reverse transcriptase (hTERT) leads to the activation of telomerase, preventing telomere erosion and subsequent telomere-dependent senescence (Morales *et al.*, 2003). Side by side comparison of HTCEC with normal primary corneal epithelium validated this cell line as a viable model for the study of ΔNp63 isoforms (Robertson *et al.*, 2008).

This study aims to improve the current methods of *ex vivo* expanded corneal epithelial transplantation by investigating alternative stem cells sourced from the limbal region. LMSC were characterised by immunophenotyping, histology and mRNA expression of selected MSC markers for lineage differentiation. LMSC were grown on AM; a biological substrate used in limbal transplants which was thawed from frozen.

3.2 Specific Aims

1. To isolate LEC andLMSC from whole corneas or corneo-scleral rings by serial trypsinisation method.
2. To establish LEC cultures from cell suspension by co-culture method with inactivated mouse 3T3 fibroblasts.

3. To establish LMSC cultures from cell suspension by growing in MSC-growth promotion media (MGPM) in low oxygen (5%).
4. To establish HTCEC cultures in serum-free and feeder-free conditions.
5. To characterise LEC and HTCEC by immunohisto/cytochemistry and mRNA expression.
6. To screen for stem cell antigens expression in LEC and HTCEC cultures by immunohisto/cytochemistry and mRNA expression.
7. To characterise LMSC using LSC markers ABCG2, ABCB5 and p63.
8. To perform immunophenotyping in LMSC and characterise LMSC by tri-lineage differentiation by histological analysis and mRNA expression.
9. To study growth of LMSC plated on cryo-preserved AM and characterise LMSC using limbal markers and stem cell antigen expression.

3.3 Materials and Methods

3.3.1 Primary LE) and HTCEC Cultures

Isolation of LEC and HTCEC were conducted as previously described (Shaharuddin *et al.*, 2013c) and detailed in Chapter 2. LEC were isolated and the pooled cell suspensions were centrifuged and the resulting cell pellet containing a mixed heterogeneous cell population was then resuspended in a selective MSC growth promotion media (MGPM) which was made up of; alpha-modified Eagle's medium (α-MEM) [Lonza], L-glutamine [Sigma],10% (vol/vol) FBS [Lonza], 5ng/ml human recombinant fibroblast growth factor-2 (FGF2) [Pepro Tech] and seeded at a cell density of 1×10^6 cells in a T75cm^2 flask, incubated at 37°C, 5% CO_2, and 5% oxygen tension. Non-adherent cells were removed the next day and media was changed every 3 days. Established cultures of LMSCs were passaged at 90 % confluence by enzymatic digestion using TrypLE™ Express [Gibco].

3.3.2 LMSC tri-lineage differentiation

Osteogenic differentiation of LMSC

LMSC were seeded in MGPM into 12-well cell culture at a cell density of 2.5×10^4 cells/well until confluence. Media was then removed and replaced with osteogenic differentiation medium (α-MEM, 10% (vol/vol) FBS, 10nM dexamethasone, 10mM β-glycerophosphate and 100μM L-ascorbic acid-2-phosphate; all supplementary reagents purchased from [Sigma Aldrich], UK. Cultures were incubated at 37°C, 5% CO_2, under low oxygen tension (5%) O_2 for 4 weeks. Media was changed every 3 days.

Chondrogenic differentiation of LMSC

LMSC were seeded into three 15 ml centrifuge tubes at a cell density of 500,000 cells/tube in chondrogenic differentiation medium (α-MEM, 100μM L-ascorbic acid-2-phosphate [Sigma], 10nM dexamethasone [Sigma], 400μl ITS^{+1} (10 μg/ml insulin, 5.5μg/ml transferrin, 5ng/ml selenium, 0.5mg/ml bovine serum albumin, 4.7μg/ml linoleic acid) [Sigma] and 10ng/ml transforming growth factor (TGF)-β1 [R&D Systems]. Cell suspensions were centrifuged at 1,000 rpm for 3 minutes to form spherical pellets and left incubated at 37°C, 5% CO_2, under low oxygen tension (5%) O_2 for 2 weeks. Media was changed every 3 days.

Adipogenic differentiation of LMSC

LMSC were seeded in MGPM into a 12-well cell culture plate at a cell density of 5×10^4 cells/well until confluence. MGPM was then removed and replaced with osteogenic differentiation medium, STEMPRO® adipogenesis differentiation kit [Gibco]. From this point onwards, cultures were incubated in atmospheric oxygen (22%), 5% CO_2 at 37°C. Cells were cultured in this condition for 30 days with media change every 3 days.

3.3.3 Immunocytochemistry

This was carried in HTCEC, LEC and LMSC to characterize for limbal and stem cell markers as detailed in Chapter 2.

3.3.4 Semi-quantitative Polymerase Chain Reaction (sq-PCR)

sq-PCR reactions were assembled with 12.5 μl PCR master mix [Promega, UK], 0.5 μl forward primer, 0.5 μl reverse primers and 2 – 2.5 μl cDNA template and DEPC-treated water to a final volume of 25 μl reactions. PCR reactions were amplified for each gene and the conditions are summarised in Table 3.1.

PCR was performed on this final reaction mixture for 30-35 cycles. Denaturation was set at 94°C for 30 minutes and elongation took place at 72°C for 1 minute at primer pair specific annealing temperature, and maintained at 4ºC. The final PCR product mixture was either stored at +4ºC or electrophoresed on 2% agarose gel, at 90V for 60 minutes.

Table 3.1 List of oligonucleotides primers and amplification conditions.

Gene	Primers	Sequences	Product Length	Annealing temperatu	Amplification

			(bp)	re (°C)	Cycles
CHONDROGENIC					
Collagen X			730	55	35
	Forward	CAA GGC ACC ATC TCC AGG AA			
	Reverse	AAA GGG TAT TTG TGG CAG CAT ATT			
Aggrecan			129	55	35
	Forward	TGA GTC CTC AAG CCT CCT GT			
	Reverse	TGG TCT GCA GCA GTT GAT TC			
Sox9			150	55	35
	Forward	GACTTCCGCGACGTGGAC			
	Reverse	GTTGGGCGGCAGGTACTG			
OSTEOGENIC					
Bone sialoprotein-1			161	55	35
	Forward	AAA GTG AGA ACG GGG AAC CT			
	Reverse	GAT GCA AAG CCA GAA TGG AT			
Alkaline phosphatase			187	55	40
	Forward	GGT GAA CCG CAA CTG GTA CT			
	Reverse	CCC ACC TTG GCT GTA GTC AT			
ADIPOGENIC					
SREBP-1			230	55	35
	Forward	GGA GCC ATG GAT TGC ACT TTC			
	Reverse	ATC TTC AAT GGA GTG GGT GCA G			
Adipocyte binding protein-2 (aP2)			130	55	35
	Forward	ATGGGATGGAAAATCAACCA			
	Reverse	GTGGAAGTGACGCCTTTCAT			
Adipophilin			200	55	35
	Forward	CGCTGTCACTGGGGCAAAAGA			
	Reverse	ATCCGACTCCCCAAGACTGTGTTA			
LIMBAL STEM CELL MARKERS					
ΔNp63α			143	55	35
	Forward	GTGATGATGGTTCACGTTGG			
	Reverse	ACATGACGTCGGGTGTTTTT			
CK3			145	53	35
	Forward	GGATGTGGACAGTGCCTATATG			
	Reverse	AGATAGCTCAGCGTCGTAGAG			
ABCG2			143	55	35
	Forward	GCGTGCTGTGCCCACTCAAA			
	Reverse	AGCATGTGCACGGTGCGTTC			
ABCB5* Hs 02889060_m1		N/A	98	55	35
GAPDH			100	55	35
	Forward	ATG GGG AAG GTG AAG GTC G			
	Reverse	TAA AAG CAG CCC TGG TGA CC			

**Taqman probe [Applied Biosystems].*

3.3.5 Immunophenotyping

LMSC from passage 1 were characterised for CD19, CD44, CD45, CD90, CD105, CD146, CD166, MHC Class I and MHC Class II which were FITC-conjugated [R&D System]. Cells were detached from tissue culture flasks with 3ml trypLE express and resuspended in flow cytometry buffer solution (PBS/0.1% bovine serum albumin). For direct immunofluorescence, 200,000 cells were added into each tube and stained with 5 µl primary antibodies in 100µl cell suspension. After incubation for one hour in 4^0C, cells were washed with buffer solution and resuspended in 200µl and ran through BD FACSCanto™ II [BD Biosciences].

For indirect immunofluorescence, 200,000 cells were added into each tube and stained with 5µl primary antibodies in100µl cell suspension. After incubation for one hour in 4^0C, cells were washed. Following that, goat anti-mouse IgG FITC-conjugated secondary antibody was added at 1:25 dilution in 100µl cell suspension; this was incubated again for 30 minutes at 4^0C. At the end of incubation, cells were washed and then resuspended in 200 µl of flow cytometry buffer solution before running through FACS Canto II and analysed with FACS Diva software {BD Biosciences].

Flow cytometry was performed using BD FACSCanto™ II and analysed using BD FACSDiva™ software [BD, Oxford,UK]. Antibodies used for flow cytometry analysis were purchased from R&D Systems. Cell populations were labeled with antibodies specific for cell surface antigens expressed by human MSC (CD44, CD90, CD105, CD106, CD146 and CD166), haematopoietic cell surface antigens (CD19 and CD45), MHC Class I and Class II, and murine Ig G as negative control.

3.3.6 Histological analyses

LMSC from passages 2-3 were used from three biological replicates for tri-lineage differentiation:

Chondrogenic differentiation

Cell pellets were removed from chondrogenic differentiation media after two weeks, and covered with an embedding media to ensure optimal cutting temperature (OCT). Blocks were wrapped in aluminium foil and stored at -80°C prior to sectioning which was done using a cryostat [Leica CM3050]. 5µm sections were placed on histology glass slides for staining with Safranin O to determine the presence of collagen

deposition. Briefly, 0.1% (vol/vol) Safranin O was prepared with 0.1% acetic acid; 0.010g Safranin O powder [Sigma] was added to 1ml 1% acetic acid in 10ml water, and filtered through a 0.45µm filter [Sartorius]. At room temperature, OCT was dissolved by rinsing the sections with 70% ethanol followed by PBS washes. Slides were flooded with Safranin O for 5 minutes and then rinsed with deionised water.

Osteogenic differentiation

Cell layers cultured in osteogenic differentiation media was stained with Alizarin Red to determine areas of mineralisation. Briefly, Alizarin Red stain was prepared by dissolving 2g of Alizarin Red in deionised water over night. To adjust the pH of the solution between 4.2 and 4.4, 10% ammonium hydroxide in deionised water or HCl were used. The required amount of Alizarin Red was filtered through a 0.45µm pore size syringe filter [Sartorius] prior to use. The culture media were removed and the wells were covered with Alizarin Red and left for 2 hours at room temperature and then rinsed with PBS ready for viewing.

Adipogenic differentiation

Media was removed from the cultures and cells were rinsed with PBS for staining with Oil Red O. A stock solution of Oil Red O was prepared by dissolving 150mg Oil Red O powder with 50ml 99% Isopropanol. To prepare a working solution of the stain, 6 ml of the stock solution was diluted with 4 ml deionised water and pre-filtered using a 0.45µm filter and left at room temperature for 10 minutes before use. Subsequently, the cell layers were covered with 2ml of Oil Red O working solution and left for 5 minutes at room temperature. The working solution was then removed and rinsed with tap water until the water rinses were clear. 2ml Haematoxylin was then added to cover the monolayer for 1 minute and rinsed off with tap water before viewing.

3.3.7 Growing LMSC on AM

LMSC from passages 4-5 were cultured in MGPM in a T75cm^2 tissue culture flask in low oxygen (5%) until 80% confluence. Research grade cryopreserved AM obtained from NHS Blood and Transplant was used as a substrate for growth of LMSC. The AM was stretched under tension with two 22mm x 22mm cover slips placed in a 6-well plate (Figure 3.1). 24,000 cells/cm^2 were plated directly onto the membrane, initially suspended in MGPM. On the following day the media was partially substituted 50:50

with DKSFM and on the third and subsequent days, with DKSFM only. 100µl DKSFM was supplemented daily directly onto the AM. Cultures were placed in 5% CO_2 incubator with normal oxygen (20%) at 37°C for 5 days. At the end of 5 days, the media was drained from the well and the AM was rinsed with PBS and embedded in OCT for cryostat sectioning.

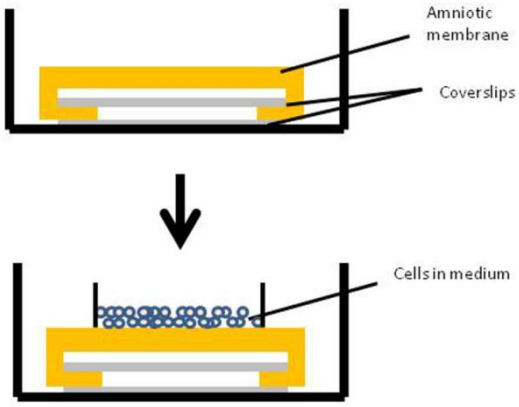


Figure 3.1 Plating of LMSC on cryopreserved AM. The membrane was kept stretched and taut using two 22mm x 22mm cover slips. The cells were plated into an area confined to an area within a make shift plastic ring, suspended in culture media.

3.3.8 Haematoxylin and eosin (H&E) staining

Cryostat sections on silane-adhesion pre-coated slides [CellPath] were placed in 95% ethanol for 2 minutes to dissolve OCT then fixed with cold methanol for 30 minutes. Slides were then placed in 70 % ethanol for 2 minutes and placed in running tap water for 5 minutes. Subsequently, the slides were flooded with haematoxylin for 4 minutes and washed in running tap water for 5 minutes. Slides were then placed in Schott's water for 2 minutes and washed in running tap water for 5 minutes. Next, the slides were flooded with eosin for 2 minutes and washed in running tap water for 5 minutes. Following this, slides were briefly placed in 70% ethanol, 95% ethanol and 100% ethanol for 30 seconds each. Finally the slides were mounted in vectashield and coversliped ready for viewing using brightfield microscopy.

3.3.8 Harvesting cells for RNA extraction

Cells grown on AM were detached from the membrane using 0.05% trypsin-EDTA for 3 minutes, deactivated with 10% fetal bovine serum/PBS and centrifuged to get a cell pellet **(**labeled **L-AM).** LMSC were detached from T75cm^2 flask using TrypLE express

and centrifuged to get a pellet and subsequent RNA extraction **(**labeled **LMSC).** An area devoid of any LMSC growth (based on microscopy) but only the epithelia of AM **(**labeled **AM)** was cut separately. The cells were detached using 0.05% trypsin-EDTA for 3 minutes, deactivated with 10% fetal bovine serum/PBS and centrifuged to get a cell pellet. Subsequent steps for RNA extraction followed the manufacturer's instructions using RNEasy Plus Micro Kit [Qiagen]. PCR was performed as described in Chapter 2.

3.3.9 Immunohistological analysis of LMSC on AM

The LMSC-AM construct was covered with an embedding media (OCT) and then frozen in a -80°C freezer before taking to the cryostat. 5 μm sections were placed on silane-adhesion pre-coated slides for optimal histology staining [CellPath]. Slides were air-dried overnight and stored at -20°C. Before staining, slides were flooded with PBS for two minutes to wash away the OCT. Subsequent steps followed the ICC protocol (as described earlier. Due to the strong background fluorescence from the tissue, Sudan Black [Fisher Scientific] was used to reduce autofluorescence, this was done by incubating the sections in 0.1% (w/v) Sudan Black for 10 minutes. The slides were then washed with PBS and cover slipped for imaging.

3.3.10 Microscopy and Imaging

All images were taken using a Nikon Digital Sight–DSFi1 camera and Nikon NIS-Elements D software [Nikon Metrology U.K. Ltd., Derby, UK]. Images were assembled using Adobe Photoshop® CS3 [Adobe Systems]. Fluorescence microscopy was undertaken using Axioplan F [Zeiss]. Images were then processed using the AxioVision40 version 4.8.2.0 software [Zeiss].

3.3.11 Statistical analysis

Descriptive statistics were used to describe means, median, or mode. Quantitative data which were normally distributed were analysed for comparison between 2 groups using independent t-test. Results with *P* values of less than 5% ($\boldsymbol{P} < 0.05$) were considered statistically significant.

3.4 Results

3.4.1 HTCEC as a viable model for LEC

LEC have been successfully carried out using cell suspension by the trypsinisation method as described previously (Shaharuddin *et al.*, 2014). It is well established that epithelial cells can be grown successfully on a "feeder" layer of 3T3 fibroblasts. Feeder layers are prepared from inactivated 3T3 fibroblast. Inactivation was carried out by the using mitomycin C. Mitotic inactivation of the 3T3 fibroblasts is essential to prevent them from overgrowing the epithelial cells in culture.

Cultured human LEC expressed ABCG2, Sox2 and p63 (Figure 3.2). Comparably, some HTCEC also expressed ABCG2 and p63, in addition to expressing ABCB5 (Figure 3.3). Screening for the stem cell antigens NANOG and Oct3/4 antibodies in HTCEC also showed some cells expressed these markers (Figure 3.4).

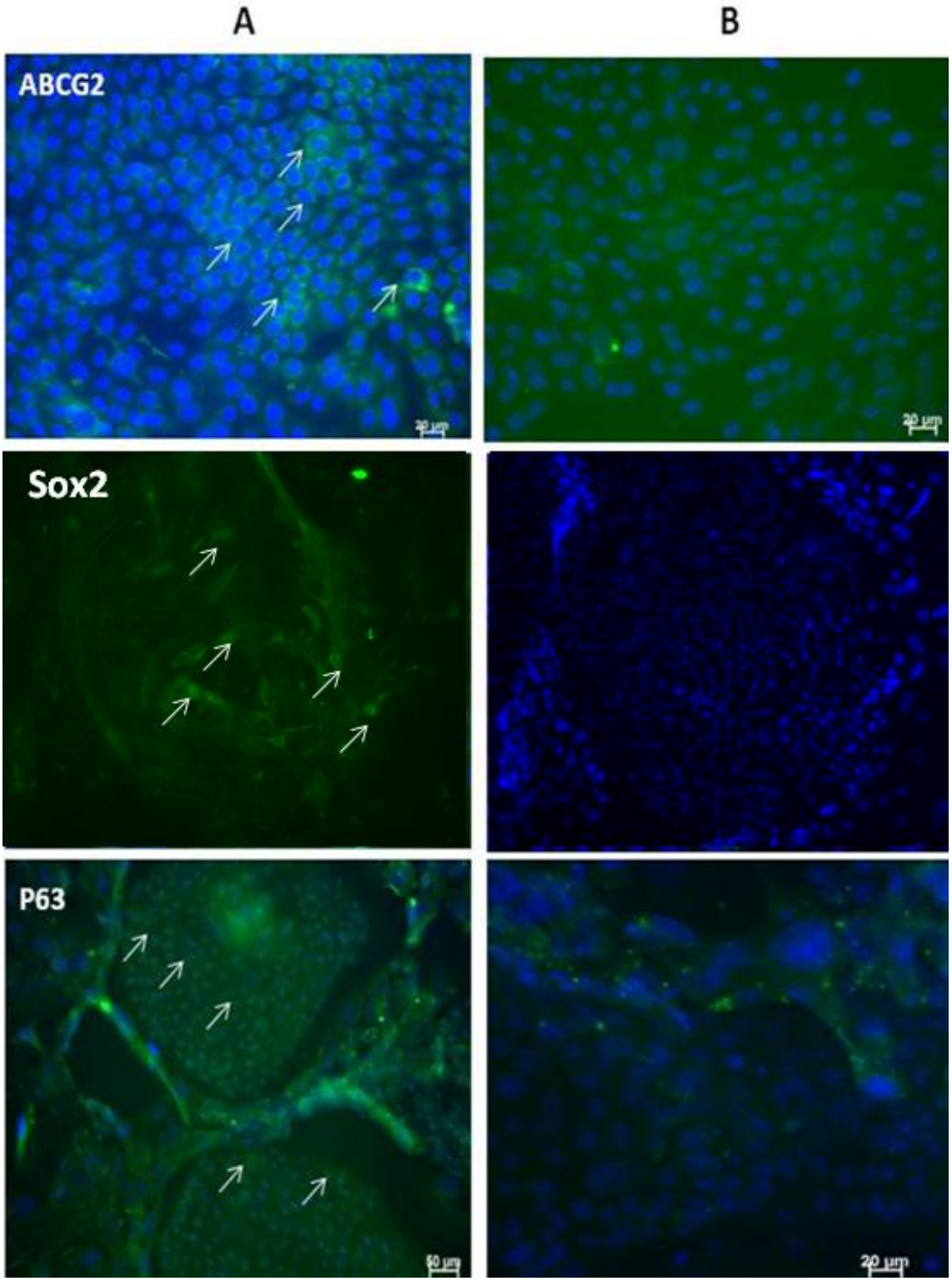


Figure 3.2 Results of immunocytochemical analysis of limbal markers in LEC. Note the presence of cells expressing of anti-human ABCG2, Sox2 and p63 antibodies (arrows) in panel A, negative control (secondary IgG) on panel B. *(DAPI=blue (nuclei), FITC-conjugated secondary antibody= green) N=3.*

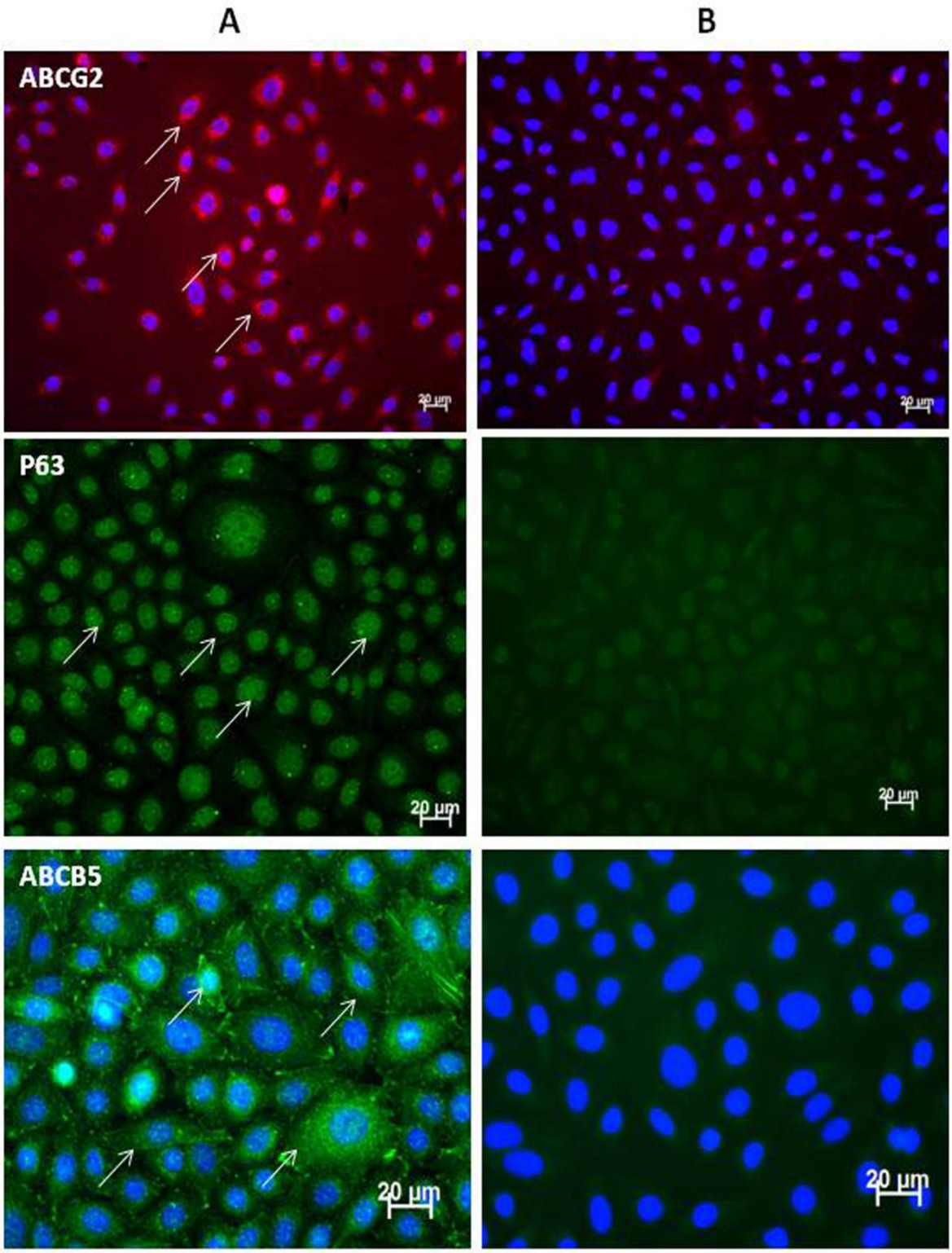


Figure 3.3 Results of immunocytochemical analysis of limbal markers in HTCEC. Note the presence of cells expressing of anti-human ABCG2, p63 and ABCB5 antibodies (arrows) in panel A, negative control (secondary IgG) on panel B. *(DAPI=blue (nuclei), FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody= red),, N=3.*

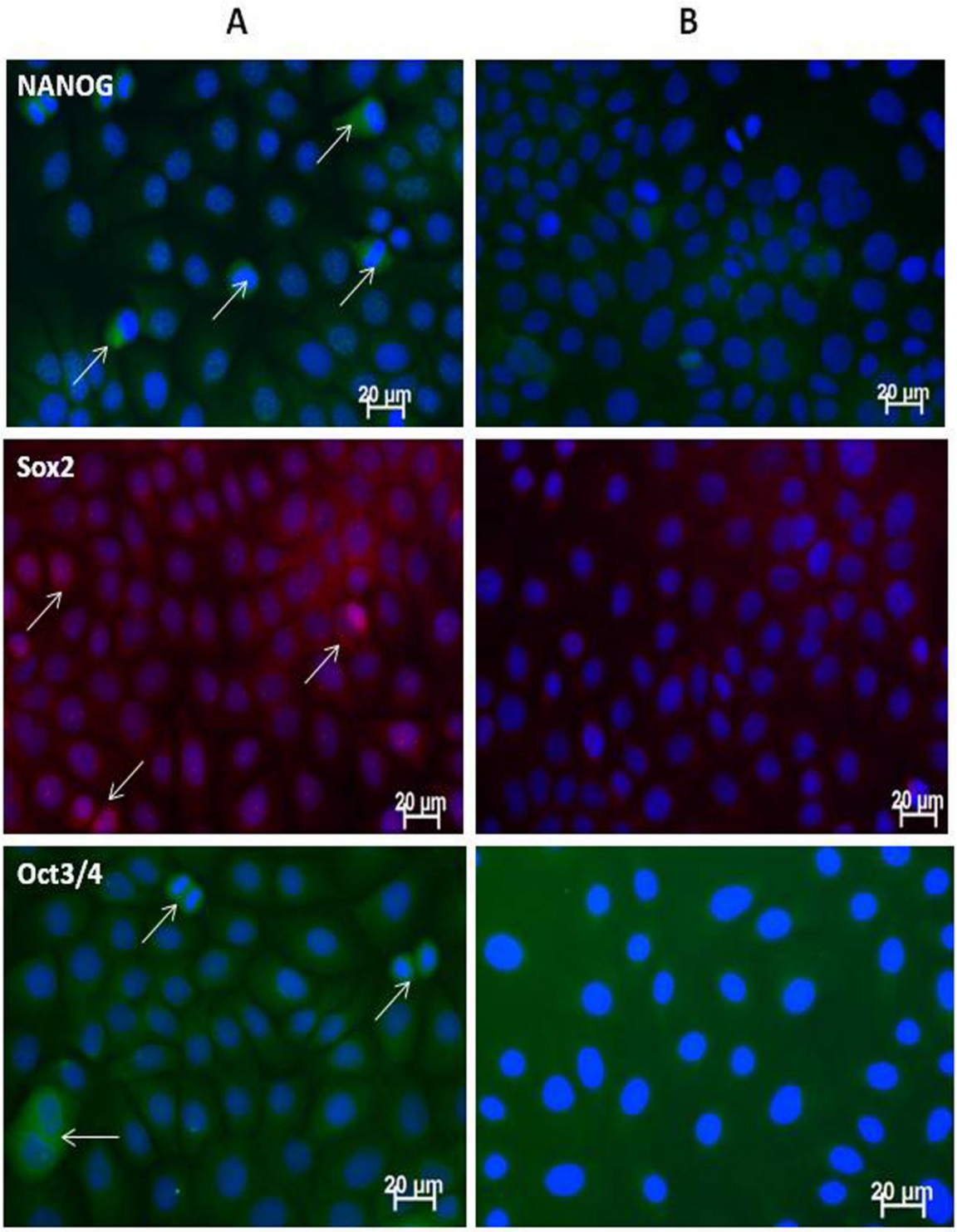


Figure 3.3 Results of immunocytochemical analysis of stem cell markers in HTCEC. Note the presence of cells expressing of anti-human NANOG, Sox2, and Oct3/4 antibodies(arrows) in panel A, negative control (secondary IgG) on panel B. *(DAPI=blue(nuclei), FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody= red), N=3.*

Results of PCR analysis showed mRNA expression of the stem cell genes NANOG, Sox2 and Oct 4, both in HTCEC and LEC (Figure 3.5A). ABCB5 was positively expressed in HTCEC and in four human LEC samples (L1-L4) as presented in Figure 3.5B.

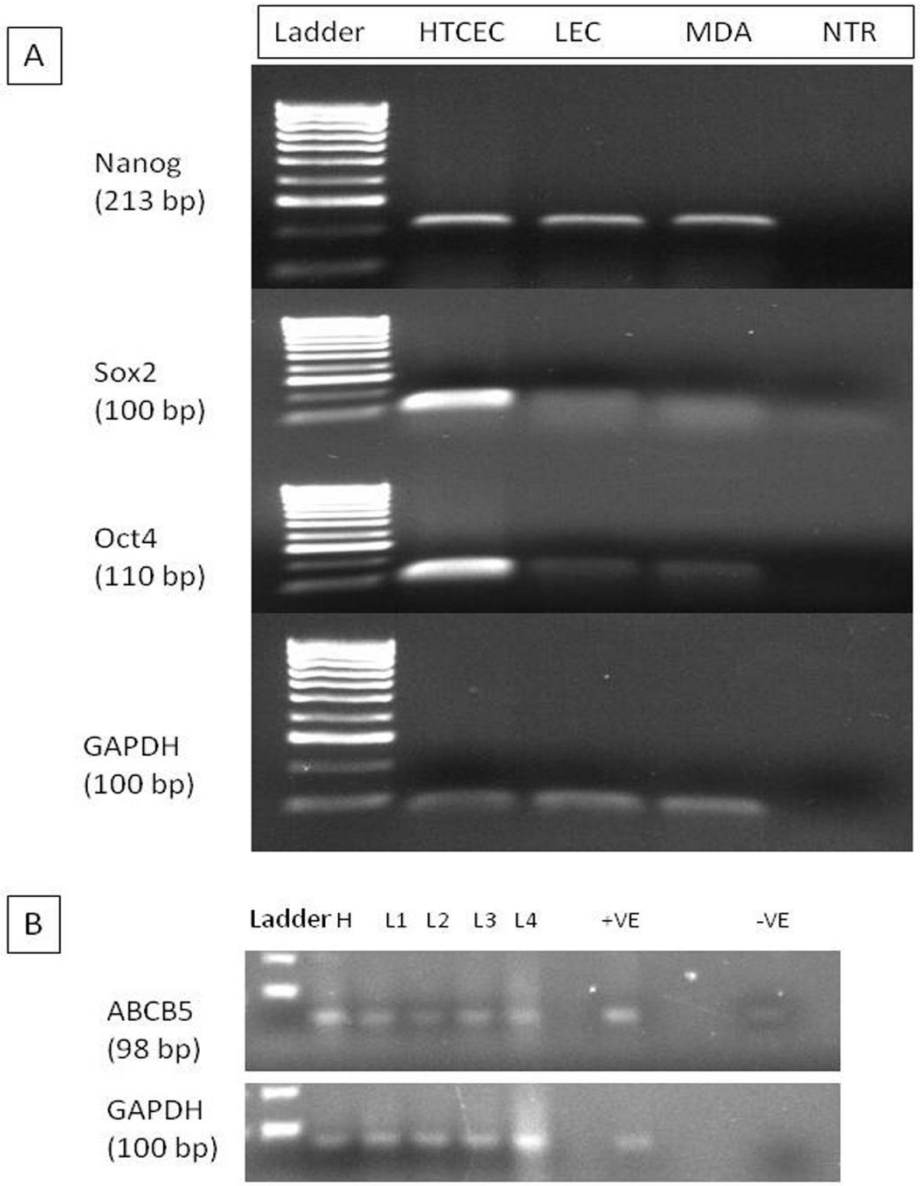


Figure 3.4 mRNA expression of stem cell markers and ABCB5. (A) Expression in HTCEC and LEC. (B) ABCB5 expression in HTCEC and LEC, N=3. [*Abbreviations: MDA – MDAMB-231 breast cancer cell line as positive control, H – HTCEC, L – LEC , L1-L4 – four LEC samples, NTR – no template reaction].*

Subsequently, we showed comparable expressions at mRNA level of common limbal markers in both LEC and HTCEC, which were ABCG2, ABCB1, p63, C/EBPδ, putative oncogene BMi-1 and CK3 (Figure 3.6). CK3 expression in LEC is a marker

for corneal epithelial differentiation, which is poorly expressed in HTCEC in our culture conditions.

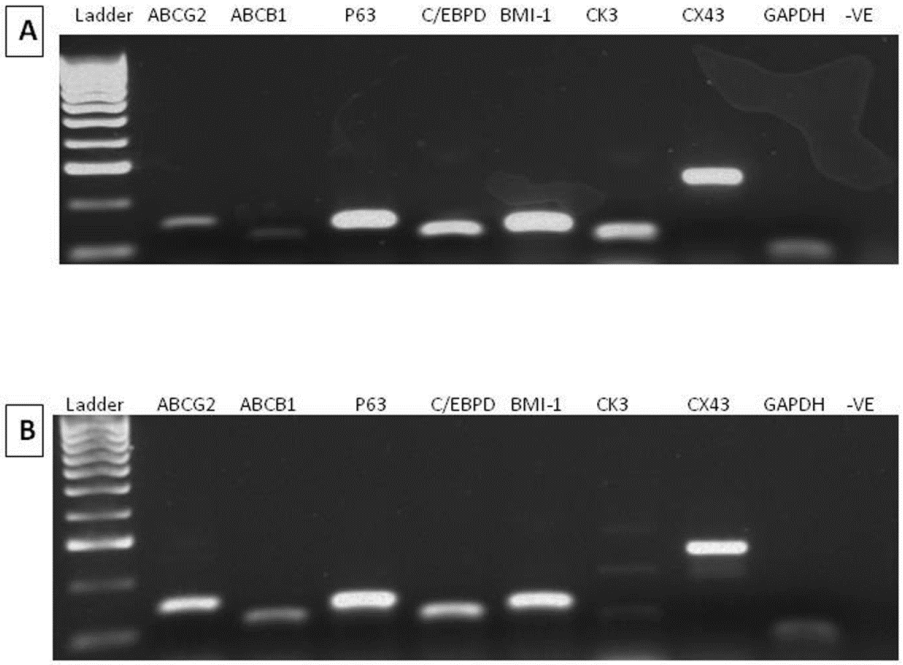


Figure 3.5 mRNA expression of common LSC markers and markers for differentiated cells CK3 and Connexin43 (A) Cultured LEC (LEC) and (B) HTCEC. Lane 1 DNA ladder, lane 2 ABCG2 (143bp), lane 3 ABCB1 (116 bp), lane 4 ΔNp63 (143 bp), lane 5 C/EBPδ (111 bp), lane 6 bmi-1 (132 bp) lane 7 cytokeratin3 (125 bp), lane 8 connexin43 (249 bp), lane 9 GAPDH (100 bp), lane 10 –ve control, N=3.

3.4.2 Characterisation of LMSC

LMSC cultures were expanded from plastic adherent cell populations in MGPM for 5-7 days or until 90% confluent and subsequently passaged. ICC analysis for common limbal markers revealed expression of ABCG2, ABCB1 and p63 in some cells in standard LMSC cultures (Figure 3.7A). Molecular analysis revealed that LMSC also expressed ABCB5 as did LEC and HTCEC. MDA-MB-231 cells were used as a positive control (Figure 3.7B).

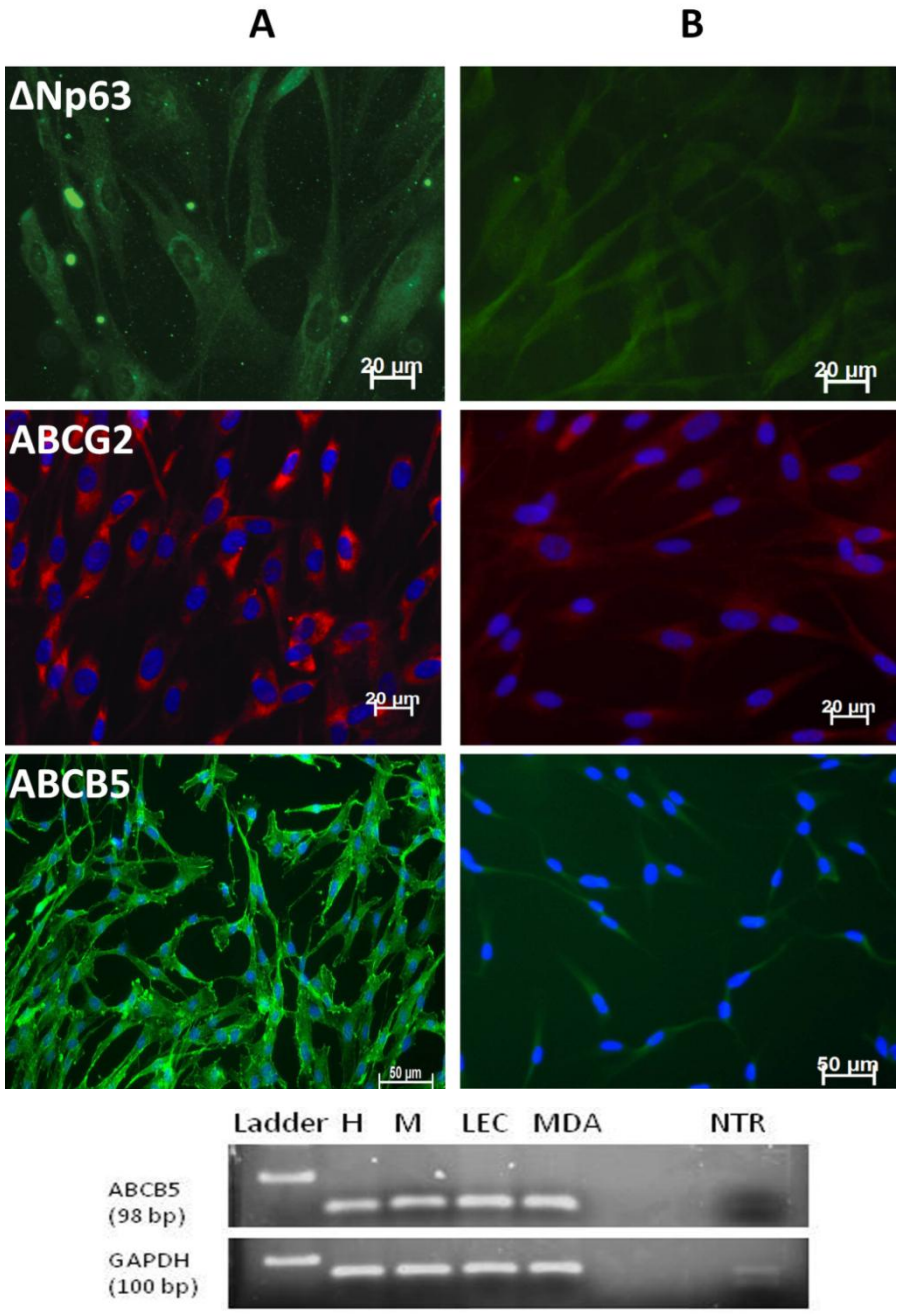


Figure 3.6 Characterisation of common limbal stem markers and ABCB5 in LMSC. (Top) Results of immunocytochemical analysis of stem cell markers in HTCEC. Note the presence of cells expressing of anti-human ABCB5, ABCG2 and p63 antibodies in LMSC antibodies in panel A, negative control (secondary IgG) on panel B. *[DAPI=blue(nuclei), FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody=red], N=3.* (Bottom) mRNA expression of ABCB5 in HTCEC, LMSC and LEC. *[Abbreviations: H – HTCEC, M – LMSC, LEC - primary limbal cultures, MDA – MDA-MB-231 as +VE control and -VE (no template reactions), N=3.*

Using FACS analysis in combination with fluorophore labelled antibodies, we observed that LMSC expressed the human MSC surface antigens CD44, CD90, CD105, CD146 and CD166), but were negative for expression of the haematopoietic cell surface

antigens CD19 and CD45. LMSC also expressed MHC Class I and had no expression of MHC Class II (Figure 3.8A). Graphical representation of percentage of stained-positive cells, from three biological replicates (N=3) showing more than 50% of cell population stained positively for CD44, CD90, CD105, CD166 and CD146 (Figure 3.8B).

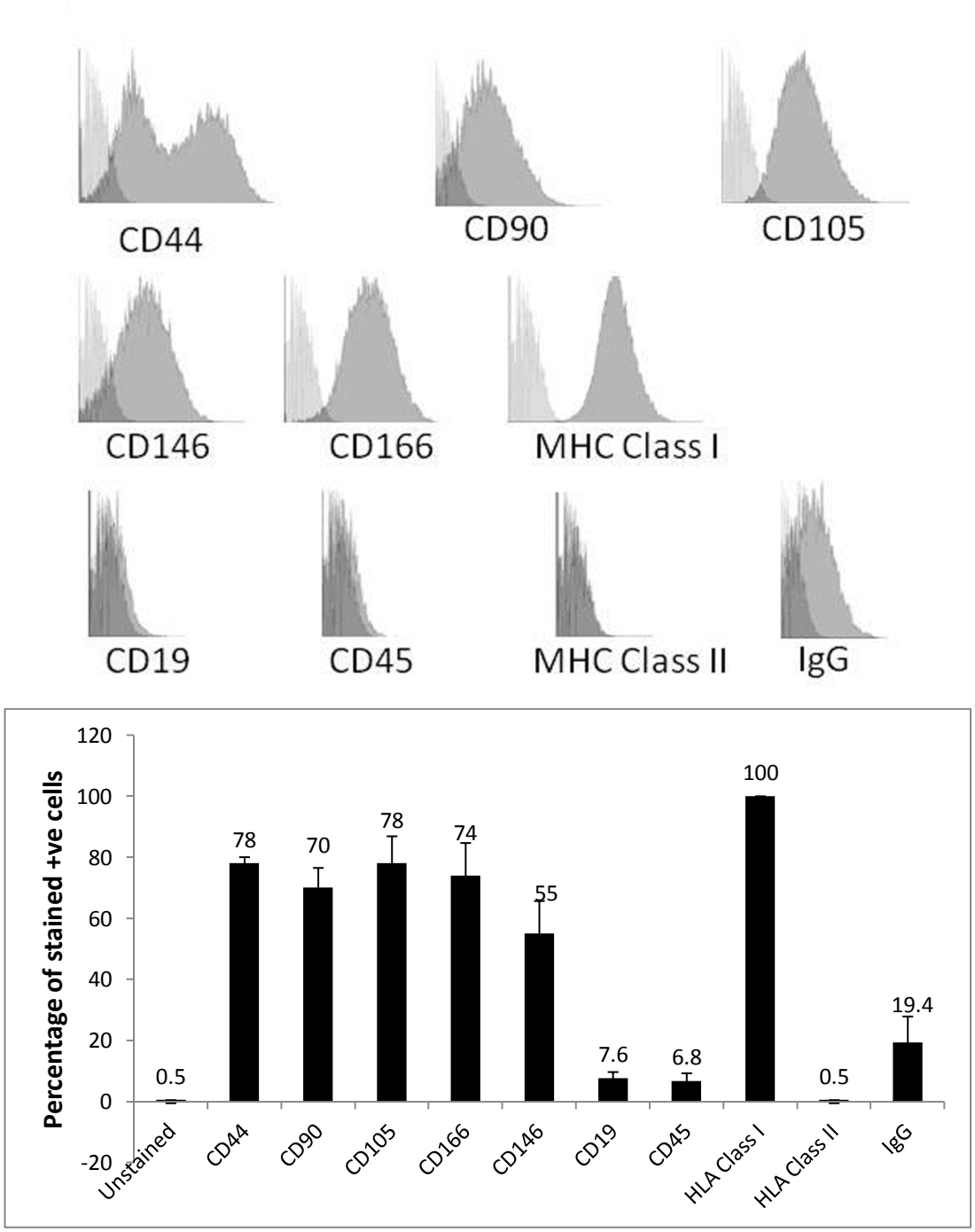


Figure 3.7 Results of LMSC immunophenotyping (Top) Cell populations labelled for surface antigens expressed in human MSC; CD44, CD90, CD105, CD146, CD166. Negative for haematopoietic stem cells; CD19, CD45, CD106. They are also HLAClass I-positive and HLAClass II-negative cells. Murine IgG was used as control. Antibody profiles (dark grey) were over layered onto the unlabelled population (light grey). (Bottom) Percentage of stained-positive cells, results are from three biological replicates (N=3).

LMSC were capable of tri-lineage differentiation as assessed by staining for adiopogenic differentiation using Oil Red O to detect lipid vesicles (Figure 3.9A), chondrogenic sections with Safranin O for cartilaginous deposits (Figure 3.9C) and osteogenic differentiation was indicated by Alizarin Red staining for matrix mineralisation (Figure 3.9 E). These were compared to cultures with respective stains in un-differentiated cells as negative control.

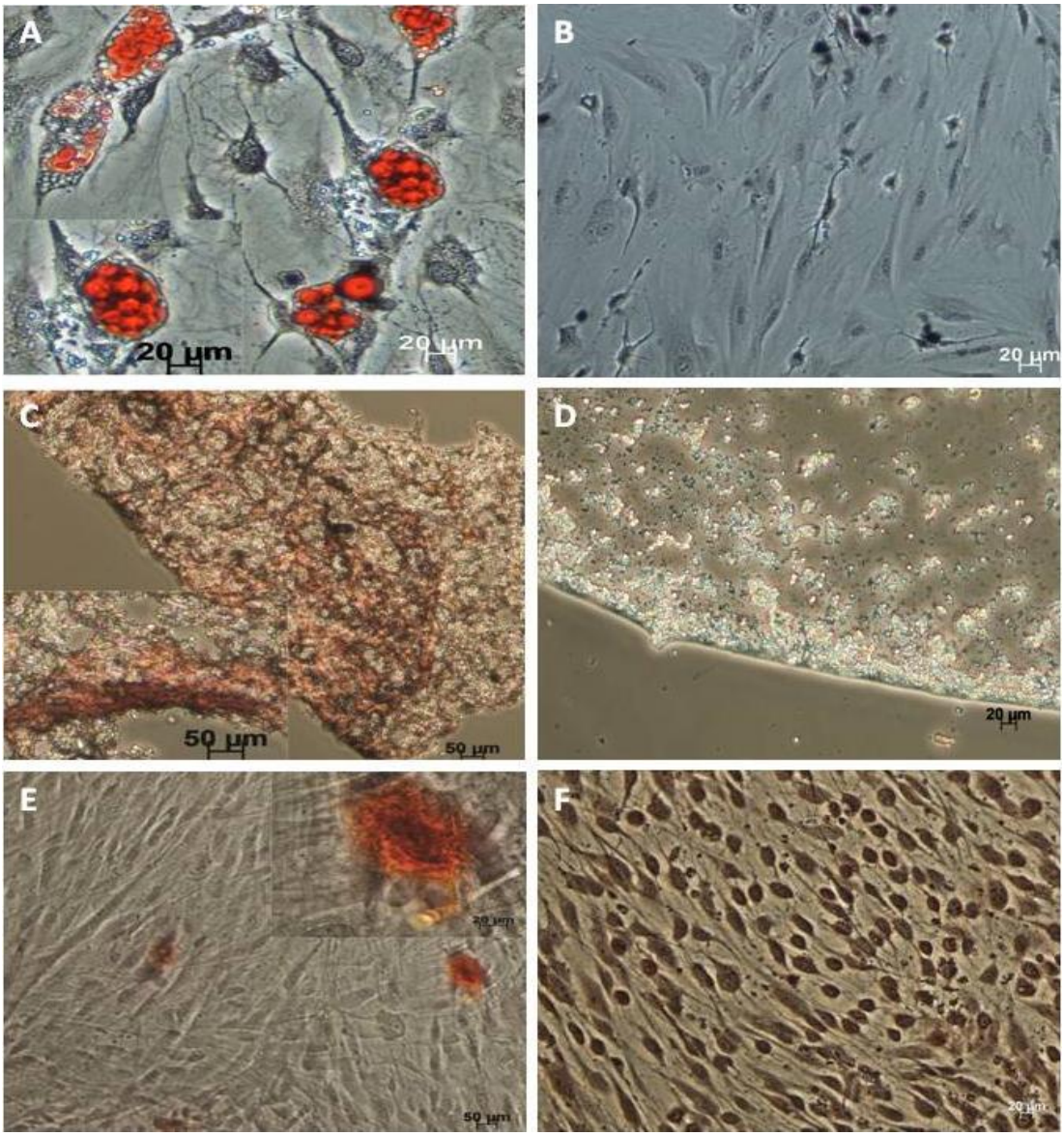


Figure 3.8 Histological analysis for LMSC differentiated cells. (A) Oil Red O staining of adipogenic differentiated cells showing lipid vesicles stained bright red, nuclei blue, after 30 days in culture, photo inset is a magnification of the same image (C) Safranin O staining of cartilaginous deposition (orange) on a representative section of a pellet generated after 2 weeks culture in chondrogenic differentiation media, photo inset is a magnification of the same image. (E) Alizarin Red staining of mineral deposition in osteogenic differentiated cells after 3 weeks in culture, photo inset is a magnification of the same image. (B), (D) and (F) are cultures with respective stains in un-differentiated cells as negative control. (N=3).

We demonstrate further here adipogenic differentiation cultures showing vacuolation after 7 days, which started to enlarge and developed lipid vesicles from the second week onwards (Figure 3.10).

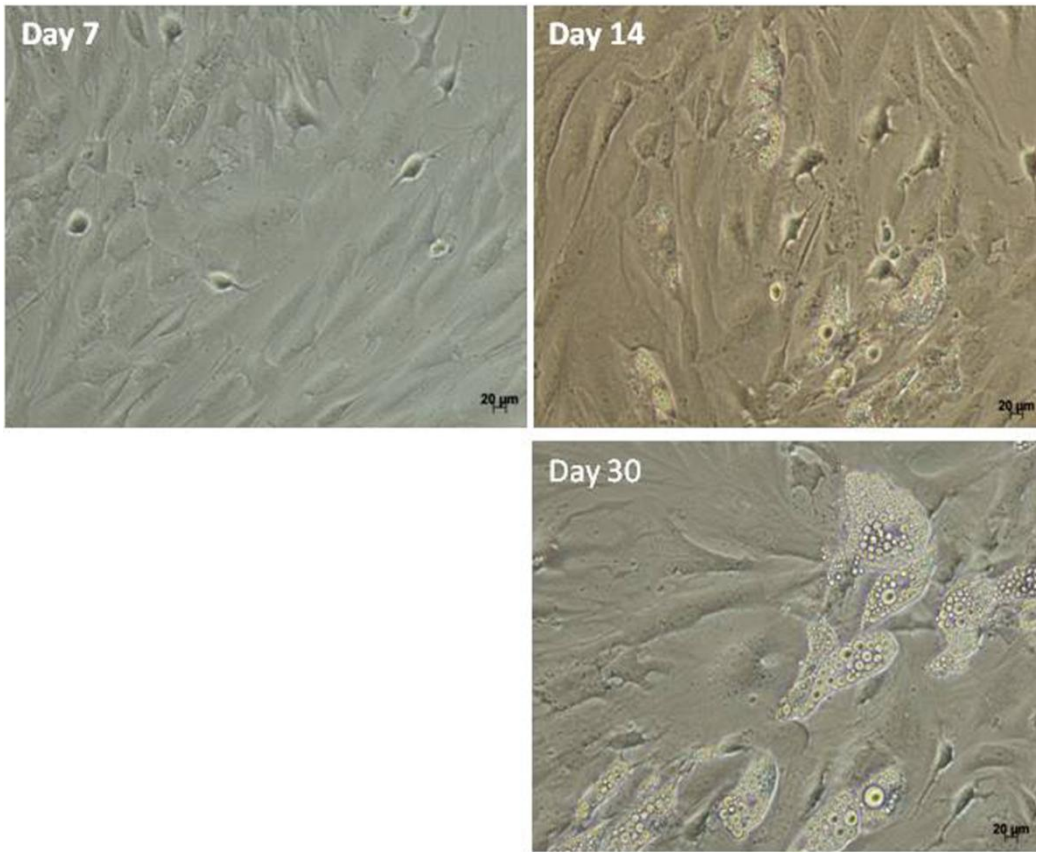


Figure 3.9 Adipogenic differentiation of LMSC. Cultures were grown in adipogenic differentiation media over 30 days. Enlarging size of vesicles containing lipid droplets evident from day 7 onwards, N=3.

Standard PCR was performed to study mRNA expression of tri-lineage commitment of differentiated LMSC (Figure 3.11). Adipogenic differentiated cells from LMSC showed expression of adipophylin, adipocyte binding protein-2 and sterol regulatory-element-binding protein-1. While chondrogenic differentiation was marked by expression of aggrecan and Sox9 but cells failed to express collagen X. Osteogenic differentiation was marked by expression of alkaline phosphatase and bone sialoprotein-1.

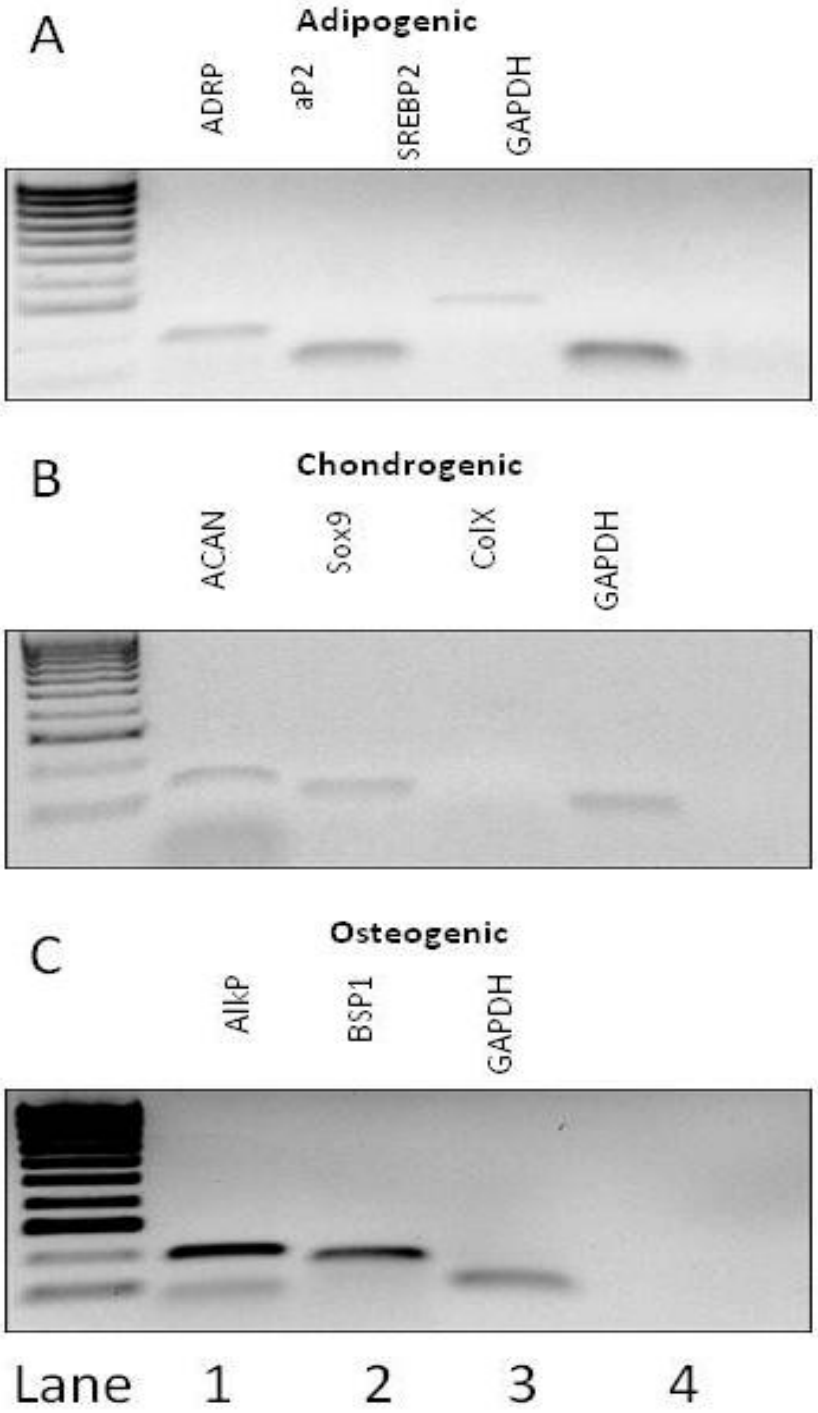


Figure 3.10 mRNA expression of tri-lineage committed cells. (A) Adipogenic: Lane 1 Adipophylin (150 bp), lane 2 Adipocyte binding protein-2 (130 bp), lane 3 Sterol regulatory element-binding protein-1 (230 bp) (B) Chondrogenic: Lane 1 Aggrecan (170 bp), lane 2 Sox9 (150 bp), lane 3 – no expression for Collagen X (730 bp) (C) Osteogenic: Lane 1 Alkaline phosphatase (187 bp), lane 2 Bone sialoprotein-1 (161 bp). GAPDH was used as housekeeping gene. (N=3).

3.4.3 Growth of LMSC on AM

LMSC were plated onto AM thawed from frozen and grown in MGPM. The media was then changed to serum-free keratinocyte medium once the cells had adhered to the surface of the membrane on the next day. The growth of LMSC were recorded daily or every other day (Figure 3.12). LMSC were adherent on the membrane and observed to proliferate and achieved confluence.

There was a change in cellular morphology from the slender shaped, fibroblastic morphology of LMSC (day 3) to rounded cell bodies (day 5). Cryostat sections of LMSC on AM at day 5 were stained with haematoxylin and eosin (Figure 3.13), note the presence of LMSC on the AM (asterisks) compared with AM alone.

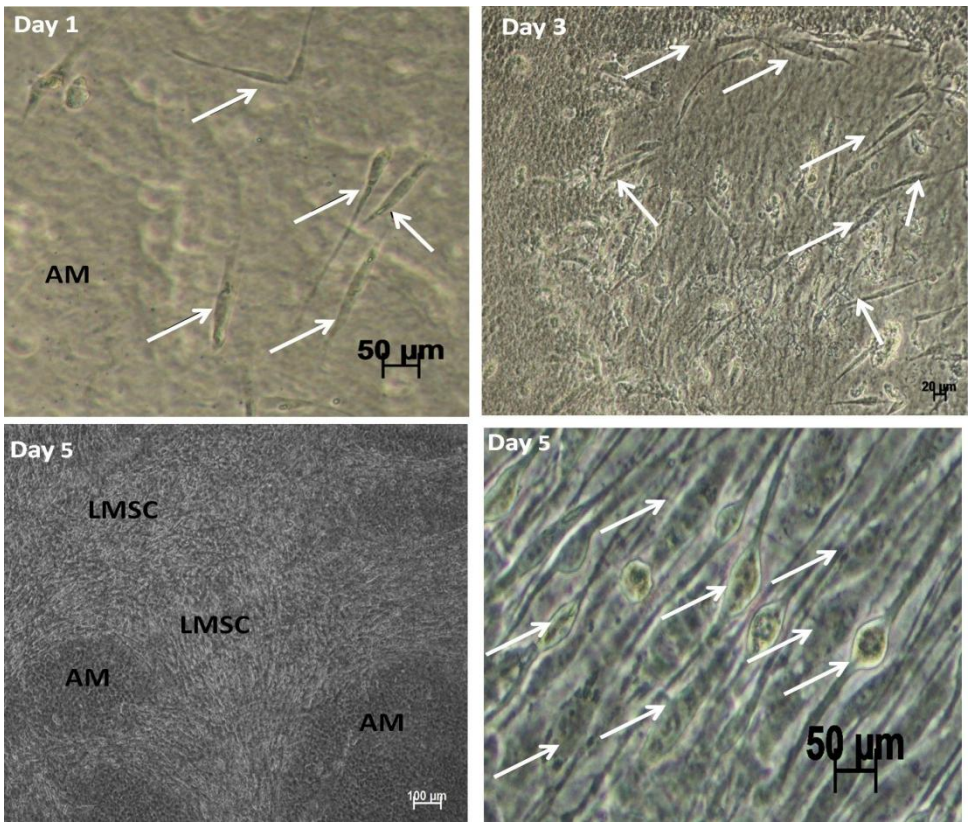


Figure 3.11 Growth of LMSC (white arrows) on AM. Representative culture images from Day 1 until Day 5. On Day 5, there was distinct LMSC colonies growing on AM, a magnified image shows changes in cellular morphology (white arrows) from spindle-shaped cells to rounded bodies with bipolar projections, N=3.

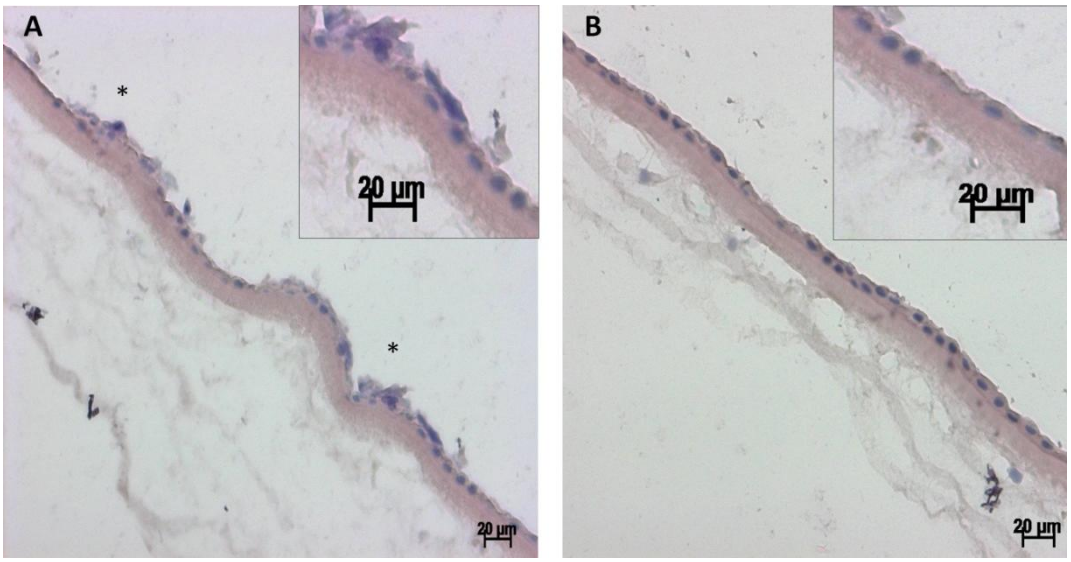


Figure 3.12 Haematoxylin and eosin staining of a cross section of AM. (A) with areas of LMSC growth (asterisks) (B) a cross section of AM without LMSC for comparison, photo insets show a magnified view. *[Blue –cell nuclei, scale bar: 20 µm]*.

Molecular analyses for common limbal markers were investigated in cells grown on AM (L-AM), LMSC and epithelial cells of the AM (Figure 3.14). L-AM and LMSC showed high expression of the transcription factor p63 similar to AM, but low/no expression of cytokeratin3 (CK3). CK3 was detected in some LMSC culture, but absent in other biological replicates (Figure 3.15). Another corneal differentiation marker CK12 was also not expressed. GAPDH was used as a loading control. ICC analysis of the LMSC grown on AM construct showed some cells expressed ABCG2 (6A), p63 (6C) and ABCB5 (6C), compared to the sections devoid of MSC (Figure 3.16).

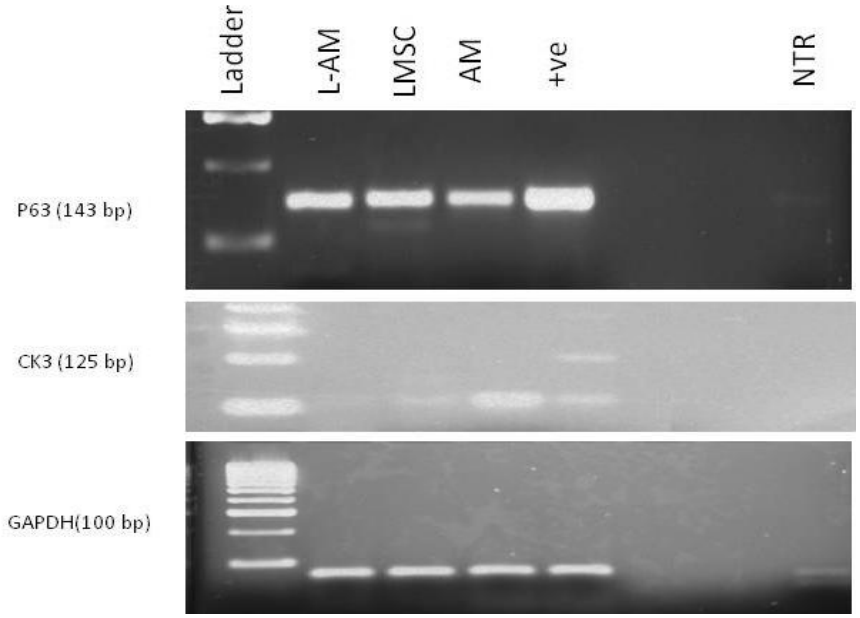


Figure 3.13 mRNA expression of CK3 and p63 in LMSC on AM, GAPDH as the house keeping gene. *[Abbreviations: L-AM: LMSC on AM, LMSC LMSC alone, AM alone, +ve control and NTRm-no template reactions]*.

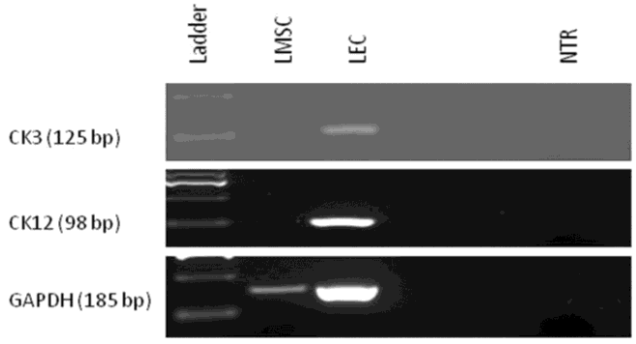


Figure 3.14 mRNA expression of CK3 and CK12 in LMSC and LEC, GAPDH was used as the house keeping gene. *[Abbreviations: LMSC – LMSC, LEC - Limbal epithelial cells, NTR - no template reaction]*.

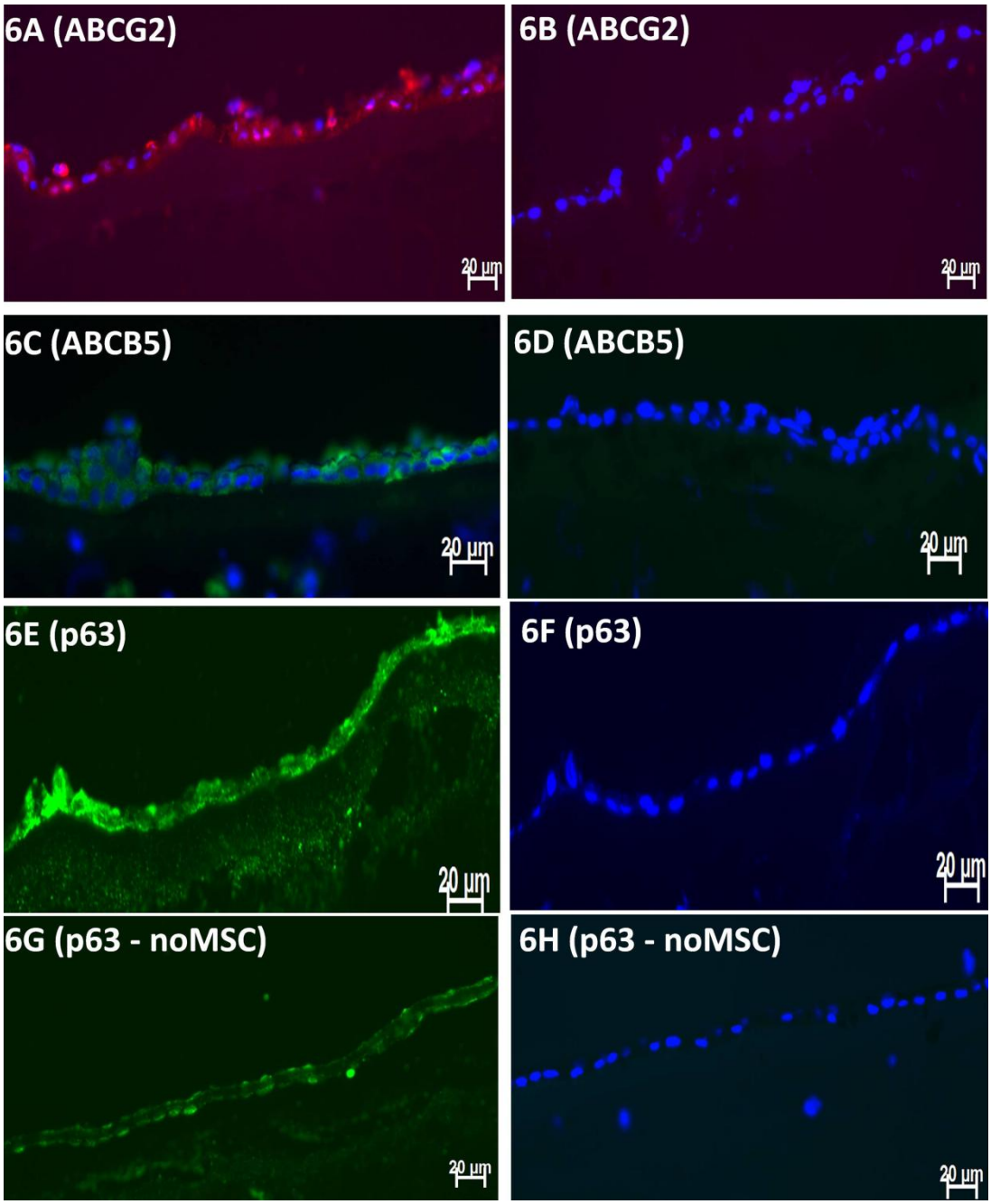


Figure 3.15 Results of immunohistochemistry of AM sections with LMSC growth. (6A) LMSC expressing ABCG2, (6C) ABCB5 and (6E) p63 (6B) and (6D) are negative control sections stained with secondary IgG only (6F) the same cross section used for p63 staining with DAPI only (6G) and (6H) are cross sections of AM without LMSC growth; (6G) p63 staining (6H) negative control with secondary IgG only. *[DAPI=blue, FITC-conjugated secondary antibody= green, Rhodamine-conjugated secondary antibody=red], N=3.*

3.5 Discussion

There are still no specific markers for LSC and the molecular characteristics of LSC remainpoorly understood. There are molecular markers for cells of the basal layer of the limbus and the clusters of cells within this anatomical region that potentially characterised the LSC and the limbal progenitor cells, these are categorised into: (1) nuclear proteins – transcription factor i.e ΔNp63 (2) drug resistance transporters i.e ABCG (3) cytoplasmic proteins – cytokeratins i.e CK3, 12, 19. (4) cell membrane or transmembrane proteins – integrin β1, β4, α6 receptors i.e epidermal growth factor receptor, and (5) Other genes include C/EBPδ and putative oncogenes that modulate stem cell pluripotency, BMI-1.

A new marker, ABCB5 which was recently reported to be an important molecular limbal marker for corneal development was also looked at. ABCB5 was first identified in skin progenitor cells (Frank *et al.*, 2003) and malignant melanoma cells (Frank *et al.*, 2005). ABCB5 is an ATP binding cassette membrane transporter P-glycoprotein, that plays a significant role in cell fusion. Cell fusion is a molecular mechanism implicated in cellular differentiation (Frank *et al.*, 2003). ABCB5 has been associated with tumour progression and resistance to cancer treatment in melanoma, colorectal and liver carcinomas (Frank *et al.*, 2005; Wilson *et al.*, 2011; Linley *et al.*, 2012; Lin *et al.*, 2013; Wilson *et al.*, 2014).

In the human cornea, ABCB5 has been identified as an important marker for LSC (Ksander *et al.*, 2014). It has been reported that ABCB5 has an important role in LSC maintenance and corneal wound healing. In this study we showed mRNA expression of ABCB5 in HTCEC and four sample donors of LEC, in addition our ICC analysis demonstrated expression of ABCB5 in some cells of the HTCE cell line.

In addition, ICC analysis of HTCEC showed some cells expressed of ABCG2, ΔNp63 and the stem cell marker Sox2. This is comparable with ABCG2, p63 and Sox2 expressions in primary LEC. In addition, HTCEC also expressed the stem cell markers NANOG and Oct3/4. Analysis at the mRNA level of both HTCEC and |LEC revealed both cell populations expressed stem cell markers NANOG, Sox2 and Oct4.

We compared mRNA expressions of common limbal markers ABCG2, p63, C/EBPδ, BMI-1, and cytokeratins 3 (CK3) between HTCEC and LEC. CK3 is a marker for

corneal epithelial differentiation was robustly expressed in the LEC cultures, while its expression was low in HTCEC; this may be evidence of that HTCEC cells are poorly differentiated in the cell culture conditions employed in this study.

Previous studies have demonstrated the isolation of LMSC from corneal tissue although protocols varied among the investigators. Polisetty et al described harvesting spindle-shaped outgrowths from primary limbal cultures after growing them on AM (Polisetty *et al.,* 2008). What where termed "limbal niche cells" by Tseng group, where cells isolated from corneo-scleral rings using collagenase, or with dispase and grown on coated matrigels (Gui-Gang Li *et al.*, 2012). The Funderburgh group used dispase and collagenase digestion on minced pieces of stromal tissues and successfully grew these cells in a selective media containing 2% FBS; incubated in humidified atmosphere, at 5% CO_2 (Du *et al.*, 2005; Branch *et al.*, 2012). These cells have all been shown to express MSC markers (Du *et al.*, 2005; Polisetty *et al.*, 2008; Branch *et al.*, 2012; Gui-Gang Li *et al.*, 2012) and have been described as having angiogenic potential (Gui-Gang Li *et al.*, 2012).

Our group used serial trypsinisation which we believe effectively cleaved the epithelial layers from the stroma as shown by haematoxylin and eosin staining on cross sections of limbal tissues taken from each cycle (unpublished data). Further use of trypsin allowed separation of the epithelial layer into single cells. In this study LMSC were cultivated using an MSC-growth promotion media (MGPM) containing α-MEM, FBS and FGF_2, according to previous literature (Barbero *et al.*, 2003; Knuth *et al.*, 2013). Addition of growth factor(s) in a basic media was found to enhance multi potentiality of MSC (Barbero *et al.*, 2003; Du *et al.*, 2005).

LMSC cultures were propagated in a hypoxic condition, with the exception of those being analysed for adipogenic differentiation which requires normal oxygen levels. Hypoxia or low oxygen tension has been shown to be a critical regulator of stem cell biology though the activation of hypoxia inducible factor pathways and the maintenance of cellular health (Mohyeldin *et al.*, 2010). Hypoxia or low oxygen tension is more physiologically relevant to maintenance of stem cell function and differentiation (Bath, 2013) and in addition has been shown to be physiologically effective for greater cell vitality, enhance tri-lineage differentiation and modulates the paracrine activity of MSC (Das *et al.*, 2009).

Immunophenotyping was used to demonstrate that the cells isolated in this study had the characteristics of human MSC; these cells were positive for expressions of cell surface antigens CD44, CD90, CD105, CD146 and CD166. They also had low/no expression for the haematopoietic commitment markers CD19 and CD45. This is comparable to findings of LMSC immunophenotyping reported in previous studies (Polisetty *et al.*, 2008; Polisetti *et al.*, 2010; Wilson *et al.*, 2011; Branch *et al.*, 2012; Garfias *et al.*, 2012). It has been noted that there were variations of cell surface antigen presentation in LMSC in different culture media or with addition of growth factors (Du *et al.*, 2005). For example, CD146+ve was expressed in cultures with serum supplement, but no expression in commercially available serum-free media (Bray *et al.*, 2014). A CD34+/CD105+ve LMSC population has also been shown to be stem-like and have tri-lineage differentiation capabilities (Wilson *et al.*, 2011).

Human MSC showed high expression of MHC Class I, but low/no expression of Class II, similar to previous findings (Garfias *et al.*, 2012; Bray *et al.*, 2014). Therefore, human MSC should be recognised by alloreactive T cells, however transplantation of mismatched MSC in non-human primates did not show proliferation of allogeneic lymphocytes (Bartholomew *et al.*, 2002). Further, undifferentiated and lineage-differentiated bone marrow-MSC suppressed proliferation of reactive allogeneic lymphocytes (Le Blanc *et al.*, 2003) making these cells attractive for HLA-mismatched clinical cellular therapy (Ringden *et al.*, 2007).

Both in rabbit and human studies, LMSC have also been reported to immunosuppress T cell proliferation in two-way mixed donor leukocyte assays (Bray *et al.*, 2014). This suppression could be mediated by soluble factor like TGF-β, that are secreted by LMSC (Garfias *et al.*, 2012). This support human MSC has a highly suitable cell population for cellular therapy and transplantation.

The current study showed that LMSC fulfils the criteria for tri-lineage differentiation. LMSC were committed to be osteogenic, adipogenic and chondrogenic by histology. Oil Red O staining shows lipid laden cells undergoing adipogenesis, while calcium deposition was indicated by Alizarin Red staining, similar to previous findings in LMSC (Polisetty *et al.*, 2008; Wilson *et al.*, 2011). We also showed sections from a cell pellet grown in chondrogenic media to have cartilage deposition which was indicated by

Safranin O staining. Alternatively, characterisation of chondrogenesis has also been performed by using Alcian Blue as reported in other studies (Wilson *et al.*, 2011; Gui-Gang Li *et al.*, 2012).

Further, LMSC showed mRNA expressions to genes known to be expressed by these cell types, except for lacking expression of collagen X. Collagen X is normally expressed by hypertrophic chondrocytes during endochondral ossification (Gaetani *et al.*, 2012), and the expression might need cellular maturity not observed in our cell population. Tri-lineage commitment in LMSC shows a similar multipotentiality possessed by human BM-MSC (Polisetty *et al.*, 2008; Wilson *et al.*, 2011; Branch *et al.*, 2012).

AM has been widely used for ocular surface diseases in the UK since 1998, the outcome of AM transplantation has given varying results under different conditions (Saw *et al.*, 2007). The beneficial effects of AM include the anti-angiogenic, anti-inflammatory and wound healing properties, making it the most common biological substrate for tissue constructs. The long term storage of AM in cryopreserved media also did not alter the sterility, histology or biological properties (Thomasen *et al.*, 2011). In the case of bilateral LSCD, cultivated oral mucosa epithelial transplantation on AM has also been clinically applied with promising results (Koizumi *et al.*, 2000; Nakamura *et al.*, 2004).

LMSC grown on AM rapidly became a confluent cell layer and in addition, underwent morphological changes from a fibroblast-like morphology (Barbero *et al.*, 2003; Gui-Gang Li *et al.*, 2012) to cells with a more rounded cell body with bipolar protrusions. A series of morphological changes have previously been observed from outgrowths of limbal epithelial cells grown on intact versus denuded AM by Tseng group (Wei Li *et al.*, 2006) which might be pertinent to the changes observed in our cultures. In their publication, they described the morphological changes of small, cuboidal cells (intact AM) versus large, spindle-shaped cells (denuded AM), stratification (intact AM) versus two-layer cells (denuded AM), etc. These changes were attributed to a process of cellular maturation and differentiation by limbal epithelial cells as they dissolved and then reassembled AM basement membrane as they grew on the AM.

In this study we show that LMSC share some expression of limbal markers that have been widely used to characterise LSC at both the level of gene expression and protein

expression. p63 and ABCG2 have been identified as the two most common limbal markers (Zhuo Chen *et al.*, 2004; Enzo Di Iorio *et al.*, 2005; Kawasaki *et al.*, 2005; Fight-for-Sight, 2014). p63 is responsible for limbal epithelial proliferation (Hsueh *et al.*, 2012) and the isomer ΔNp63α of the transcription factor plays a significant role in corneal regeneration and wound healing (Enzo Di Iorio *et al.*, 2005). While expression of p63 is generally considered to be nuclear we observed that LMSC grown in culture and on AM exhibited both punctuate nuclear staining and cytoplasmic staining, this may be indicative of cellular differentiation. Galli et al (Galli *et al.*, 2010) reported that certain genes like MDM2 can mediate translocation of ΔNp63 from the cell nucleus to the cytoplasm under appropriate stimuli that can include cellular differentiation.

CK3 distinguishes undifferentiated LMSC from epithelial cells, and is a good marker for corneal epithelium and also as a negative marker for limbal stem cells (Harkin *et al.*, 2004b). We found mRNA expression of CK3 was not consistent in all our samples which may suggests variability between donors. Variability in patient-derived MSC has been reported in other MSC derived from other tissues (D'Ippolito *et al.*, 1999).

3.6 Conclusion

HTCEC is comparable to primary LEC in the expressions of important molecular markers for limbal epithelial stem cells, corneal progenitor cells and stem cell antigens. Due to existing problems of primary tissues, donor availability and donor-to-donor-variability, HTCEC is attractive to provide consistent and reproducible results. This study demonstrates a simpler way of isolating LMSC from primary corneal tissues by serial trypsinisation alone. The cells grew in MSC-growth promotion media in hypoxic condition. The advantages of these conditions include shorter duration of reaching cell confluence due to the rapid growth of LMSC (5-7 days) compared to 10-14 days for proliferation of outgrowths from limbal explants (Polisetty *et al.*, 2008) or using cell suspension from primary tissues (Wilson *et al.*, 2011; Branch *et al.*, 2012). LMSC is a vital component of the LSC niche. The LMSC-AM tissue engineered construct has great potential for ocular surface regeneration and more importantly in the cellular treatment of LSCD.

Chapter 4 CHARACTERISATION OF LIMBAL SIDE POPULATION CELLS

4.1 Introduction

Characterisation of LSC remains challenging in part due to the absence of specific LSC markers. Successful outcome of *ex vivo* expanded LSC transplantation is dependent on the frequency of stem cells or limbal progenitors in the transplanted tissues (Rama *et al.*, 2010). One of the ways to isolate an enriched population of putative stem cells is by utilising ATP- binding cassette (ABC)-transporter mediated efflux of a DNA-binding dye, such as the most commonly used Hoechst 33342. A seminal work by (Goodell *et al.*, 1996), demonstrated a population of stem cell enriched cells from within the haematopoetic cells of the mouse, these were isolated using high-speed dual wavelength flow cytometry analysis combine with vital dye efflux (Figure 4.1).

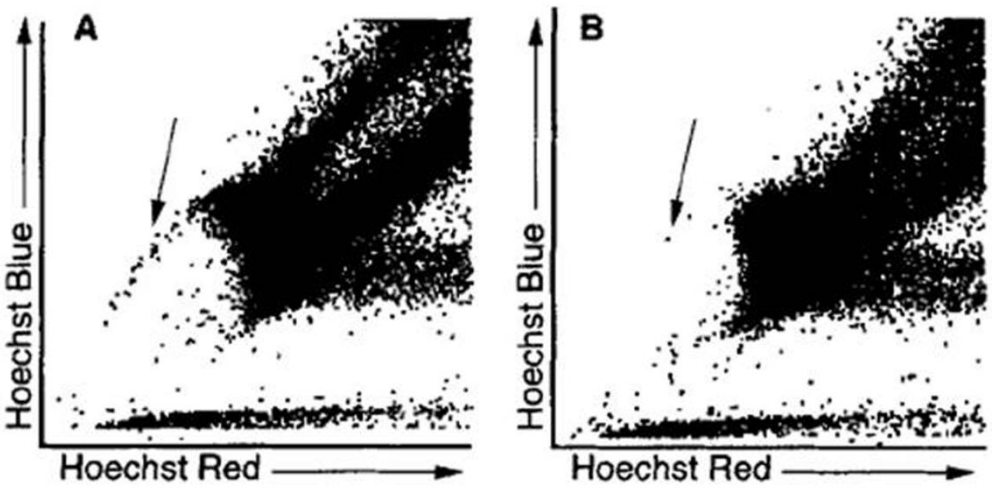


Figure 4.1 Flow cytometry profiles of murine bone marrow side population. (A) SP cells are indicated with arrows. (B) Loss of SP cells on addition of verapamil (Goodell *et al.*, 1996).

Currently, SP assays have been used to identify stem cell and progenitor populations in various tissues i.e. umbilical cord blood, skeletal muscle (Annette P. Meeson *et al.*, 2004), kidney, liver, pancreas (Lechner *et al.*, 2002), cardiomyocytes (Cindy M. Martin *et al.*, 2004), mammary glands, lung, brain and cancer cells (Kelly M. Britton *et al.*, 2011). In most cases it has been shown that the ABC transporter, ABCG2 is over-expressed in LSP cells, and is the cause of the SP phenotype (Zhou *et al.*, 2001; Scharenberg *et al.*, 2002; Savary *et al.*, 2007). ABCG2 has been implicated as the determinant for SP phenotypes in other systems i.e. musculo skeletal (Annette P.

Meeson *et al.*, 2004; Doyle *et al.*, 2011), cardiac cells (Annette Meeson *et al.*, 2013), hepatocytes (Shimano *et al.*, 2003), and the airway epithelia (Hackett *et al.*, 2008). Another ABC transporter, ABCB1 has been identified as determining the SP phenotype in ovarian cancer cell lines and is responsible in part for resistance to chemotherapy (Wright *et al.*, 2013b).

Hoechst 33342 is a fluorescent dye which binds to DNA in live cells. When excited by ultra violet light, Hoechst 33342 has an emission peak at 450nm which can be detected in what is commonly referred to as the Hoechst Blue channel , in this case using a 450/50 band pass filter. Hoechst dye also shows a secondary emission defined as Hoechst Red detected in this case by a 675nm long pass filter. This appears to be partially dependent on the DNA conformation; as the intensity of the dye signal in the blue channel is proportional to the DNA content of the cell.

The SP is detected as a discrete "tail" attached to the G0/G1 subpopulation in a bivariate plot of these two signals. In order to determine that this "tail" of cells are indeed SP cells confirmation of the SP phenotype is necessary. This can be determined using an ABC-transporter inhibitor which needs to be added to the cells (from the same cell preparation) to prevent dye efflux. Some of the ABC-transporter inhibitors responsible for preventing vital dye efflux are verapamil which blocks ABCB1 efflux activity and partially blocks ABCG2 efflux, cyclosporine A which inhibits ABCC1 and FTC that is a specific inhibitor for ABCG2 (Dean, 2002).

ABC transporters are proteins which are embedded in lipid membranes and they facilitate the import of nutrients into cells or the release of toxic products into the surrounding medium. The largest and most important family of membrane transport proteins is the ABC transporters. ABC transporters belong to the super family of membrane pumps that catalyse ATP-dependent transport of various endogenous compounds and xenobiotics out of the cells. This often occurs against a concentration gradient, requiring hydrolysis of ATP.

ABC transporters contain a number of membrane-spanning domains that include a translocation pathway for a specific substrate. As the ABC cassettes bind and hydrolyze ATP, conformational changes occur and are transmitted to the membrane-spanning domains, where they induce rearrangements that translocate the substrate from one side

of the membrane to the other. The SP phenotype is lost upon inhibition of ABC transporter activity.

It should however be noted that ABCG2 expression alone may not be solely responsible for conferring on stem cells the ability to efflux vital dyes. Phylogenetic analysis places 48 known human ABC transporters into seven superfamilies of proteins (Dean, 2002), several of which have been shown to confer on cells the ability to efflux various unrelated compounds. The ABC genes represent the largest trans-membrane proteins and these include the ATP-binding cassette sub-family B member 1 (ABCB1), ABCG2 and ABCC1 genes.

ABCG2, ABCB1 and ABCC genes are the most commonly reported genes that confer multi drug resistance phenotypes to cancer stem cells. ABCG2 overexpression has been associated with development of many solid tumours; such as laryngeal squamous cell carcinoma (Diestra *et al.*, 2003), bladder carcinoma (Sauerbrey *et al.*, 2002), lung cancer and leukaemia (Brangi *et al.*, 1999). ABCG2 overexpression is known to cause resistance to some currently in use chemotherapeutics which include mitoxantrone and camptothecin analogues (Partanen *et al.*, 2012). In laryngeal squamous cell carcinoma, down regulation of ABCG2 through the MAPK pathway reduced cellular proliferation, and led to cancer cell apoptosis (Diestra *et al.*, 2003). Further, amplification and overexpression of ABCC3 gene has been implicated in human epidermal growth factor receptor (HER)-2 positive breast cancer cell lines (Jin Xie *et al.*, 2014), as well as conferring resistance to the chemotherapeutic drug "paclitaxel" in ovarian cancer (Eyre *et al.*, 2014).

However, not all cancer SP cells show characteristics of cancer stem cells (CSC), these are termed "CSC-like" cells which do not show stem cell activities. SP cells from glioblastoma multiforme did not enrich for neurospheres, were not able to self renew and had lower tumorigenic potential than the NSP cells (O'Brien *et al.*, 2008). Furthermore, SP cells from adrenocortical cancer cell lines did not show a superior cellular proliferation, self renewal capacity or chemoresistance than the NSP cells (Broadley *et al.*, 2011).

4.1.1 Limbal side population assay

Studies of ABCG2 expression at mRNA and protein levels have provided convincing evidence that ABCG2 is the main transporter implicated in determining the LSP phenotype (Zhuo Chen *et al.*, 2004; Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Umemoto *et al.*, 2005).

Comparable to other adult tissues, corneal limbal and conjunctival epithelial cells derived cells contain SP fraction which is sensitive to the ABCG2-inhibitor FTC (Budak *et al.*, 2005). A number of studies have now reported the presence of LSP in several species, although the concentration of Hoechst dye used and incubation time of cells in the presence of dye are variable between studies, which may reflect on the varied percentage of SP populations reported, as summarised in Table 4.1.

Table 4.1 Summary of publications on LSP.

Type of tissues	Inhibitors	Hoechst incubation	% SP cell yield	References
Human -Limbal epithelium -Corneal epithelium	(R)+-Verapamil Tryprostatin A (30min 37°C)	Concentration: 3 µg/ml),Incubation: 90 min at 37 °C.	Limbal: 0.20% gated cells. Corneal :0.02% gated cells).	(Watanabe *et al.*, 2004)*
New Zealand White rabbits -Limbal epithelium -Corneal epithelium	R(+)-verapamil (Sigma) or tryprostatin-A (Alexis Biochemicals, Carlsbad, CA)	Concentration: 3 µg/ml),Incubation: 90 min at 37 °C.	Limbal :0.40% gated cells Corneal:0.01% gated cells	(Umemoto *et al.*, 2005)
Wistar rats and New Zealand White rabbits -Limbal epithelium -Corneal epithelium	R(+)-verapamil (Sigma) or tryprostatin-A (Alexis Biochemicals, Carlsbad, CA)	Concentration: 3 µg/ml), Incubation: 90 min at 37 °C.	Limbal: Rabbit: 0.56% Rat: 0.40% Corneal: Rabbit: barely Rat: 4.6% (contaminant)	(Umemoto *et al.*, 2006)
Rabbit - limbal epithelial cell	Tryprostatin-A (Alexis Biochemicals, Carlsbad, CA)	Concentration: 5 µg/ml),Incubation: 90 min at 37 °C	0.40% gated cells	(Kusanagi *et al.*, 2009)
Human -corneoscleral tissue *(primary cultured cells)*	50 µM of verapamil	Concentration: 5 µg/ml), Incubation: 37°C for 120 minutes	2.1 % gated cells	(Cintia S. de Paiva *et al.*, 2005a)*
Rabbit and Human – cornea and Conjunctiva	10 µM reserpine (Sigma), 10-100 µM verapamil (Sigma) or 10 µM Fumitremorgin C	Conjunctival epithelium: 3.0-3.5 µM for 90 minutes Limbal epithelium: 2.0-2.5 µM for 60 minutes at 37°	SP (% of total) Human limbus: 0.49% Human conjunctiva: 0.58% Rabbit limbus: 0.88% Rabbit conjunctiva: 0.96%	(Budak *et al.*, 2005)*
Pig limbal epithelium *(overnight culture)*	Fumitremorgin C	Concentration: 5 µg/ml 1.5 hours	Limbal: 0.89 %	(Akinci *et al.*, 2009)
HTCEC Human primary limbal epithelial cultures	Verapamil (20µM) 30 minutes	3 µg/ml 45 minutes	0.2% 0.8%	(Shaharuddin *et al.*, 2013c)*

[studies involving human tissues. Part of this table was taken from (Shaharuddin et al., 2013a)*]

4.1.2 Are LSP cells stem cells?

Limbal side population assays have been performed using both animal and human cornea tissues. It has been reported that SP cells are present in the conjunctival and limbal epithelia of both rabbits and humans but absent from the corneal epithelium (Watanabe *et al.*, 2004; Umemoto *et al.*, 2005). A number of studies of LSP cells have also determined that the ABC transporter ABCG2 is the main transporter responsible for conferring on SP cells their ability to efflux Hoechst 33342 dye (Watanabe *et al.*, 2004; Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Ki-Sook Park *et al.*, 2006; Umemoto *et al.*, 2006; Akinci *et al.*, 2009).

Previous investigations of both human and animals showed LSP cells had features consistent with stem cells. In a study of human LSP cells, it was observed that the SP cell fraction expressed higher levels of ABCG2 than the NSP using real time PCR and immunohistochemical analysis on limbal and corneal tissue sections. ABCG2 was expressed in the LEC but not in the corneal epithelial cells (Watanabe *et al.*, 2004). Other reported properties of LSP cells include the slow cycling nature, higher *in vitro* proliferation and p63 expression (Wolosin *et al.*, 2000; Cintia S. de Paiva *et al.*, 2005a) and data from flow cytometry showed that LSP cells have features which are consistent with stem cells; they showed low forward scatter (FSC) and extremely low side scatter (SSC) (Budak *et al.*, 2005).

Murine keratinocyte stem cells have also been reported to be slow cycling (Cotsarelis *et al.*, 1990; Tani *et al.*, 2000) as have rat conjunctival stem cells (Wensheng Chen *et al.*, 2003) and LEC (Cotsarelis *et al.*, 1989). These reports support small cell size and slow cycling as characteristics of stem cells.

In addition, LSP cells were shown to express elevated levels of ABCG2, Bmi-1 and nestin but lacked expression of CK3 and 12 compared with NSP cells (Umemoto *et al.*, 2006) (77). CK3 and CK12 are markers of differentiated corneal epithelial cells (Chaloin-Dufau *et al.*, 1990; Budak *et al.*, 2005), whereas Bmi-1 has been reported to be a marker of a number of stem cell populations (Lessard and Sauvageau, 2003; In-kyung Park *et al.*, 2003) including but not exclusive to: neural crest stem cells (Molofsky *et al.*, 2003), intestinal stem cells (Sangiorgi and Capecchi, 2008.), hematopoietic stem cells and neuronal stem cells (Molofsky *et al.*, 2003; In-kyung Park *et al.*, 2003), where it is thought to be an important regulator of stem cell renewal. While nestin has been

reported to be important for self renewal of neural stem cells (Donghyun Park *et al.*, 2010).

LSP cells have also been reported to have a higher colony forming efficiency than NSP cells (Cintia S. de Paiva *et al.*, 2005a). Colony forming efficiency is also considered a stem cell characteristic and has been demonstrated in numerous studies of many different stem cell populations including but not limited to retinal stem cells (Tropepe *et al.*, 2000), bone marrow stem cells (Friedenstein *et al.*, 1987), and dental pulp stem cells (Gronthos *et al.*, 2000).

As LSP cells are small in size, slow cycling, express stem cells markers and are capable of colony formation, this would suggest that they are indeed stem cells. However, to be useful in the clinical setting LSP cells need to be expanded *in vitro* while maintaining their stem cell characteristics. A relatively low percentage of SP cells within a large NSP cell population pose a challenge in determining the best culture system for cell expansion. In a study by Park et al, 2006, post sorted-SP cells were able to show stratification and proliferative capacity in a modified 3-D raft culture (Ki-Sook Park *et al.*, 2006). Swiss 3T3 fibroblasts were plated on a type I collagen gel matrix within a 12-mm culture insert in supplemented Eagle's medium to mimic an *in vitro* stromal equivalent. Immunohistochemistry performed on the sorted-SP cells successfully demonstrated a high proportion of cells expressing p63, which is a marker for cell proliferation.

From wound healing models of the cornea, *in vivo* ability of LSP cells to increase in number in response to injury was observed (Ki-Sook Park *et al.*, 2006). It is also possible to expand SP cells *ex vivo* while maintaining their stem cell characteristics as demonstrated in articular synovial tissues (Teramura *et al.*, 2008). Proliferative capacity of cochlear SP cells were also studied in adherent versus non-adherent conditions, in which they favour the former (Chao *et al.*, 2013). Based on these published reports, expansion of LSP in culture is something that is probable, once the optimal culture conditions have been determined.

SP cells have demonstrated regenerative properties in several studies. Jackson et al, 2001 transplanted highly enriched haematopoietic SP cells into the ischaemic heart in lethally irradiated mice and showed these SP cells were capable of differentiating into

cardiomyocytes and endothelial cells (Jackson *et al.*, 2001). This was later emphasized in another study where cardiac SP cells were able to differentiate into multiple lineages including cardiomyocytes, fibroblasts, endothelial cells and smooth muscle cells (Oyama *et al.*, 2007). The role of SP cells in repair and regeneration of skeletal muscle has also been widely investigated, these cells proliferate in response to injury and some participate in regeneration while others are thought to replenish the SP cell population (Annette P. Meeson *et al.*, 2004; Tanaka *et al.*, 2009; Doyle *et al.*, 2011). Similarly, in response to an alkali burn central cornea wound, the SP cell population showed a transient increase in cell numbers after 24-hours of injury (Ki-Sook Park *et al.*, 2006). Further investigations into the role of SP cells in wound healing are warranted.

4.2 Specific Aims

In this chapter we aim to;

1. To describe the methodology for the identification and isolation of corneal epithelial SP in both primary LEC and HTCEC.
2. To describe the optimisation of a protocol for SP isolation from both LEC and HTCEC.
3. To investigate expressions of ABC-transporters in LEC.
4. To investigate the association between cell confluence and SP percentage
5. To characterise LSP cells using LSC markers
6. To study the association of cell diameter and colony formation and LSP.

4.3 Materials and methods

4.3.1 LEC and HTCEC

Isolation and cultivation techniques of limbal epithelial sheets followed the protocol described in Chapter 2 and published earlier (Shaharuddin *et al.*, 2013c). It was based on the keratinocyte culture system by Rheinwald and Green (Rheinwald and Green, 1975a; Rheinwald and Green, 1975b; Green *et al.*, 1977) and described in many reports (Notara *et al.*, 2007; Shortt *et al.*, 2007b; Osei-Bempong *et al.*, 2009; Meyer-Blazejewska *et al.*, 2010). The cells were propagated in a co-culture system with murine 3T3-J2 fibroblast feeder layer (a kind gift from Professor Fiona Watt, King's College, London, United Kingdom).

Our stock of HTCEC line (Robertson *et al.*, 2005; Robertson *et al.*, 2008; Robertson *et al.*, 2011) was generously contributed by Dr James Jester and Winston Kao, of the University of Cincinnati, Cincinnati, Ohio, USA. We successfully expanded HTCEC and maintained this cell line in feeder- and serum-free conditions using defined keratinocyte serum free medium (DKSFM) [Gibco].

4.3.2 Reagents

Hanks balanced salt solution (HBSS) medium

This was prepared as follows; 500ml HBSS [Sigma] containing 2% fetal bovine serum (FBS) [Lonza], and 5ml of 5,000 units of penicillin/streptomycin [Gibco].

Verapamil [Sigma Aldrich]

Verapamil is a general calcium channel inhibitor, which inhibits efflux of drugs at the ATP-dependent pumps e.g. Hoechst 33342 dye in SP discrimination assay. Verapamil is made into a 5mM stock solution in H_2O and stored frozen at -20^{0}C, once thawed it can be stored at 4^{0}C for 1 month and is used at 20 – 40μM concentration.

Fumitremorgin-C [Axxora]

FTC is a specific inhibitor of ABCG2. FTC is diluted in DMSO to make a 10mM stock and stored at -20^{0}C and is used at a final concentration of 5μM.

Hoechst 33342 [Sigma]

25mg Hoechst 33342 in powder form was dissolved in 25ml H_2O to a final concentration of 1mg/ml, filter sterilized and aliquots frozen at-20°C. Once thawed Hoechst was kept at 4°C for 1-2 months.

Propidium iodide (PI)

25mg PI powder was dissolved in 2.5ml H_2O to a final concentration of 10mg/ml, filter sterilised and aliquots were stored at -20°C, once thawed vials were kept at 4°C.

4.3.3 LEC and HTCEC sample preparation

On Day 10, cells from LEC were detached from tissue culture wells by first adding 1ml of Ethylenediaminetetraacetic acid (EDTA) for 3 minutes to remove any residual feeder

cells, and subsequently adding 1 ml of 0.05% trypsin-EDTA and placed in a CO_2 incubator at 37°C for 3 minutes. To inactivate trypsin, 1ml of medium containing 10% Fetal Bovine Serum (FBS) [Lonza] was added to the well subsequently. The cell suspension was then centrifuged and the cell pellet resuspended in Hank's medium.

For HTCEC sample preparation, DKSFM was first removed and cultures irrigated with Dulbecco's Phosphate Buffer Saline (DPBS) [Sigma]. Cells in a $75cm^2$ tissue culture flask were then treated with a cell dissociation buffer – enzyme free, Hank's based [Gibco] in a CO_2 incubator at 37°C for 20 minutes and then rinsed with PBS. 5ml 0.05% Trypsin-EDTA was then added and incubated for another 3 minutes. The steps as described above were repeated.

Cells were counted manually using an improved Neubauer haemocytometer [Reichert], 1.0×10^6 cells/ml were resuspended in Hank's media and placed into micro centrifuge tubes [Fisher] to be stained with either Hoechst 33342 dye alone or with Hoechst dye in the presence of ABC-transporter inhibitors. As a control to the confirm the SP phenotype, cells from the same cell preparation as those being analysed (prior to addition of Hoechst)were incubated in the presence of inhibitor for 30 minutes in a 5% CO_2 incubator at 37°C in a MACsMix rotator [Miltenyi Biotec]. After this, Hoechst dye was then added to all tubes as described above.

All reactions were terminated by placing the contents of the tubes in a 15ml centrifuge tube [Fisher] containing 10ml ice cold Hank's/FBS medium. The tubes were then centrifuged at 2,500 rpm for 5 minutes and cells resuspended with 500µl Hank's medium and passed through a 70µm pore size cell strainer [BD Falcon] and stored on ice prior to FACS analysis.

4.3.4 Flow Cytometry Analysis and Fluorescence Activated Cell Sorting

FACSanalysis and cell sorting was done using either an LSR2 SORP analyser or a FACSAria IIu sorter [Becton Dickinson]. Both machines used identical filter sets and laser configurations. Propidium iodide (PI) was added immediately prior to analysis at a final concentration of 2µg/ml. Initial gating was designed to exclude non viable cells, these were defined as PI positive and were detected using 488nm excitation and a 610/20 emission filter (data not shown). SP cells were identified on a bivariate plot

showing Hoechst Blue (355nm excitation, 450/50 emission) versus Hoechst Red (355nm Excitation, 675LP emission). For analysis, at least 50,000 events were collected. For sorting SP and NSP, cells were collected into either culture media for molecular characterisation and cell culture or directly onto microscope slides for immunofluorescence studies.

4.3.5 ICC of LSP and NSP cells

100 HTCEC SP and NSP cells were sorted directly onto glass slides. Slides were fixed with cold methanol [BDH Laboratory Supplies] at -20 °C for 20 minutes and then washed with PBS. Cells were permeabilised in 0.3% (v/v) Triton X-100 [Fisher Scientific] in PBS for 10 minutes and then washed again with PBS. Non-specific binding sites were blocked with 5% normal goat serum [Invitrogen] in PBS for 30 minutes in a humid chamber in the dark. Cells were then incubated with primary goat anti-mouse monoclonal antibody to ABCB5 (1:100 dilution) [Abcam], CXCR4 (1:100 dilution) [R&D Systems], p63 (1:50 dilution) [Santa Cruz biotechnology] and goat anti-rabbit polyclonal antibodies to ABCG2 (1:100 dilution) [Novus Biologicals] in 0.05% FBS/PBS overnight at 4°C. Cells were then washed 3x5 minutes PBS and incubated with appropriate secondary antibody either goat anti-mouse FITC or anti-rabbit Rhodamine [Jackson Immunology Research Laboratory] 1:25 dilution for 30 minutes in a humid chamber in the dark, then washed with PBSX3 5 minutes and mounted in vectashield anti-fading media containing DAPI [Vector Laboratories], cover slipped and examined using an Axioplan F microscope [Carl Zeiss]. In the case of ΔNp63 slides, vectashield without DAPI was used. Negative controls were cells incubated without addition of primary antibody, but with secondary antibody.

4.3.6 Image quantification

Under 20X magnification, the collection site for cells on the slides were arbitrarily divided into 5 quadrants (Figure 4.2). For each quadrant, all cells were selected for image quantification using image quantification software [Image J version 1.44]. The means (± standard deviation) of fluorescence signal intensities were averaged and analysed to compare the means between SP and NSP cell fractions, using independent t-test [IBM SPSS Statistics Version 19]. *P* value of < 0.05 was taken as a significant difference in means between two groups.

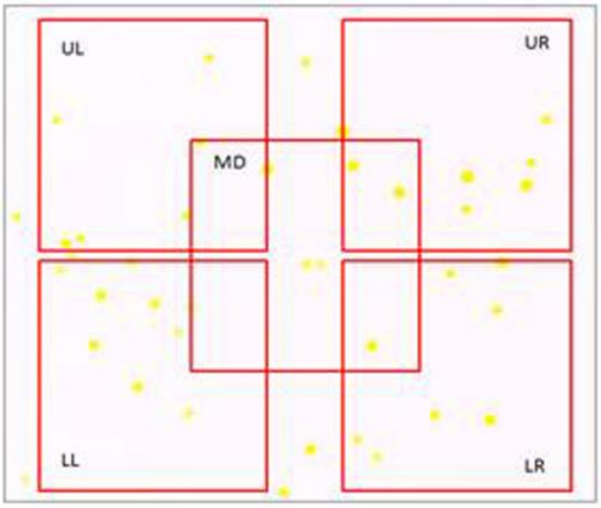


Figure 4.2 Schematic diagram for arbitrary division of areas for microscopic quantification of markers-positive cells in NSP and SP cells.
[Abbreviations: UL – Upper left quadrant, UR – Upper right quadrant, MD – Middle quadrant, LL – Lower left quadrant, LR – Lower right quadrant].

4.3.7 Measurement of cell diameter

Cell diameter was measured using the Axiovision software version 4.8.2.0 [Zeiss], by drawing a straight line at the longest diameter across a cell. The fluorescent filter was switched off during this measurement. The mean values for SP and NSP cells were then entered into IBM SPSS statistical software Version 19 and analysed with independent t-test for difference in means between two groups. *P* value of < 0.05 was taken as significant.

4.3.8 Semi quantitative-Polymerase chain reaction (sq-PCR)

sq-PCR reactions were assembled with 12.5µl PCR master mix [Promega, UK], 0.5µl forward primer, 0.5µl reverse primers and 2 – 2.5µl cDNA template and DEPC-treated water to a final volume of 25µl reactions. Denaturation was set at 94°C for 30 minutes and elongation took place at 72°C for 1 minute at primer pair specific annealing temperature, and maintained at 4ºC or electrophoresed on a 2% agarose gel [NBS Biologicals]. PCR reactions were amplified for each gene and conditions are summarised in Table 4.2.

Table 4.2 Oligonucleotide sequences and amplification conditions.

Gene	Primers	Oligonucleotide Sequences	Product Length (bp)	Cycles	Annealing temperature °C
ABCG2			143	35	55.5
	Forward	GCG TGC TGT GCC CAC TCA AA			
	Reverse	AGC ATG TGC ACG GTG CGT TC			
ABCB1			116	35	55
	Forward	GAC GTC ATC GCT GGT TTC GAT			
	Reverse	TCA TTT CCT GCT GTC TGC ATT GT			
GAPDH			150	30	55
	Forward	GCA CCG TCA AGG CTG AGA AC			
	Reverse	GCC TTC TCC ATG GTG GTG AA			

4.4 Results

4.4.1 Optimisation of LSP protocol

Due to the limitations associated with primary limbal tissues and its culture conditions which involve co-culture expansion with animal-sourced feeders or AM, and often limited cell numbers, optimisation for LSP was performed using the HTCEC line based on bone marrow SP as established by Goodell (Goodell *et al.*, 1996). The steps involved optimising for Hoechst dye concentration and duration of incubation, and verapamil concentration. FTC was used at a concentration of 5µM based on pre-optimisation by other investigators (Rabindran *et al.*, 2000) (Figure 4.3). We achieved a final LSP protocol using Hoechst dye concentration 3.0µg/ml for 1.0 x 10^6 cells/ml, 45 minutes incubation at 37^{O}C, using Verapamil 20µM as an SP-inhibitor for 30 minutes.

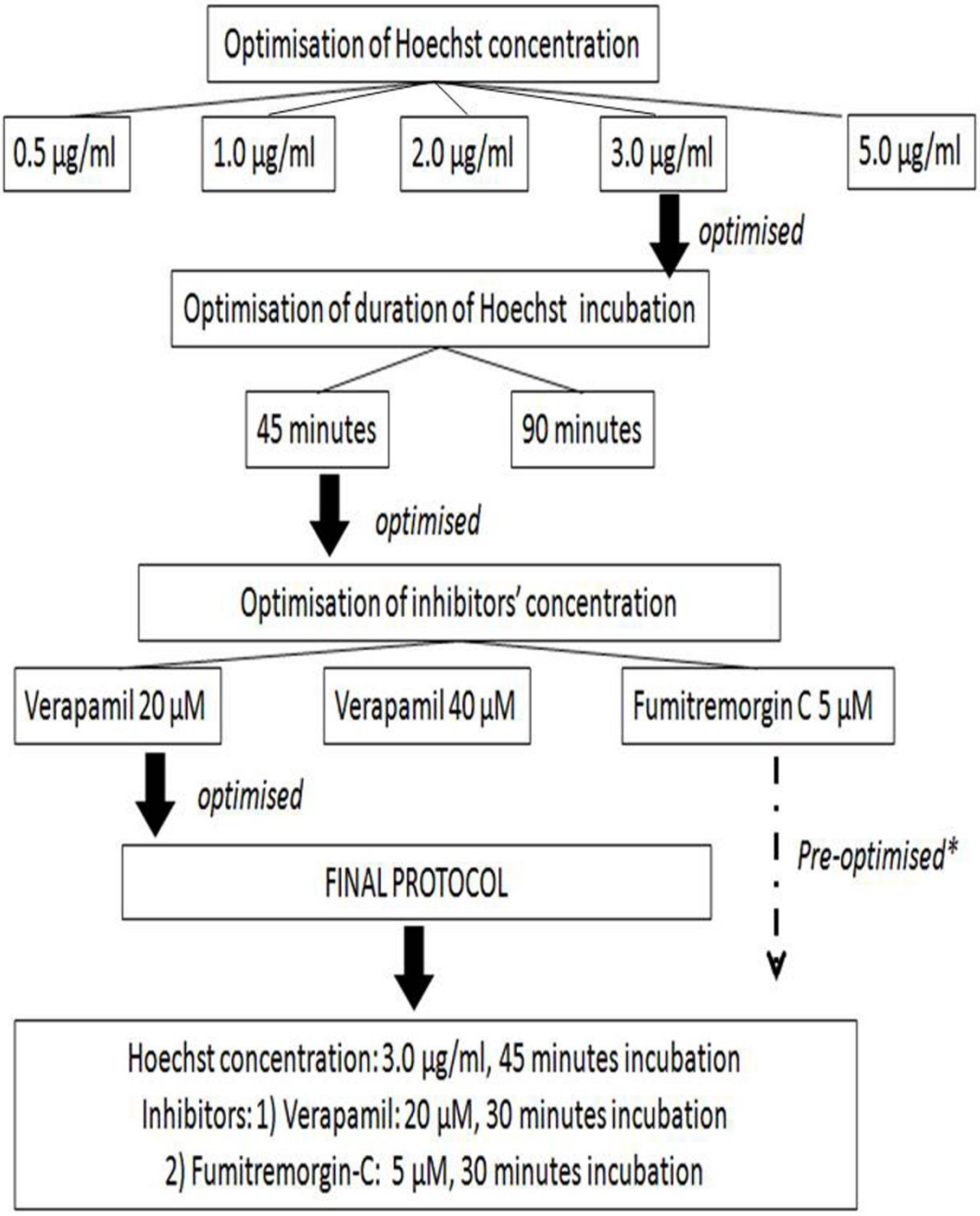


Figure 4.3 Optimisation steps to limbal side population protocol.
*[*pre-optimised in previous literature (Rabindran et al, 2000). This figure was previously published (Shaharuddin et al., 2013c)]*

To determine the optimal Hoechst concentration, a Hoechst concentration curve was first determined. Cells were incubated in the presence of a range of concentrations from 0.5µg/ml to 5.0µg/ml. In the presence of 3µg/ml of Hoechst dye, an SP population of 0.2% could be detected with cell death of 37% (Figure 4.4A), furthermore the efflux ability of these cells could be blocked in the presence of verapamil (Figure 4.4B). A higher concentration e.g. 5µg/ml of Hoechst dye led to reduced SP resolution or loss of

the SP phenotype. Cell viability was severely reduced at 47% (Figure 4.4C), and in the presence of verapamil further cell death occurred - 54% (Figure 4.4D). Using a lower concentration of Hoechst than the optimum concentration, will lead to under saturated SP profile where NSP cells will be introduced into the SP gate giving a high, but unlikely SP phenotype (Golebiewska *et al.*, 2011).

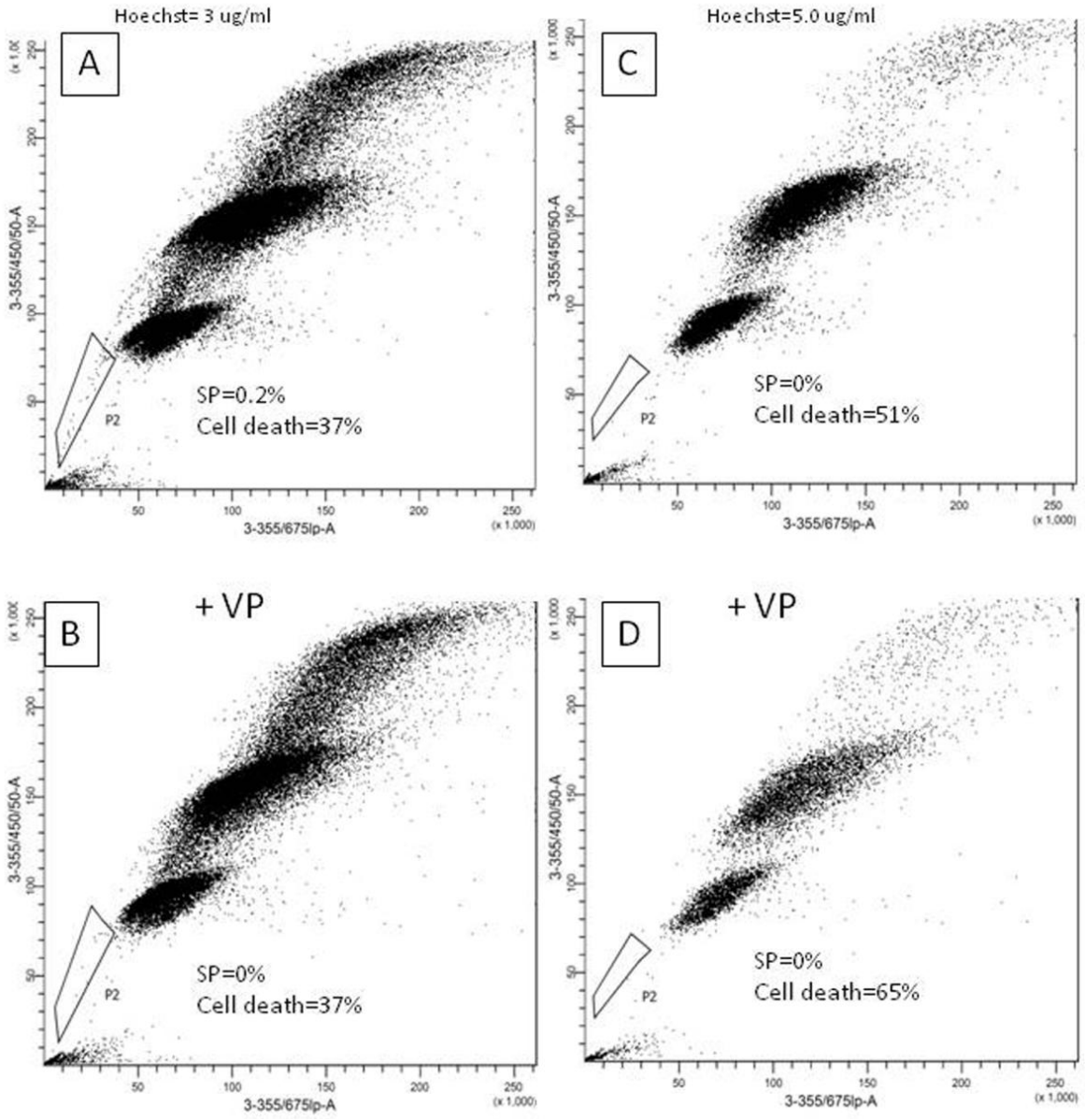
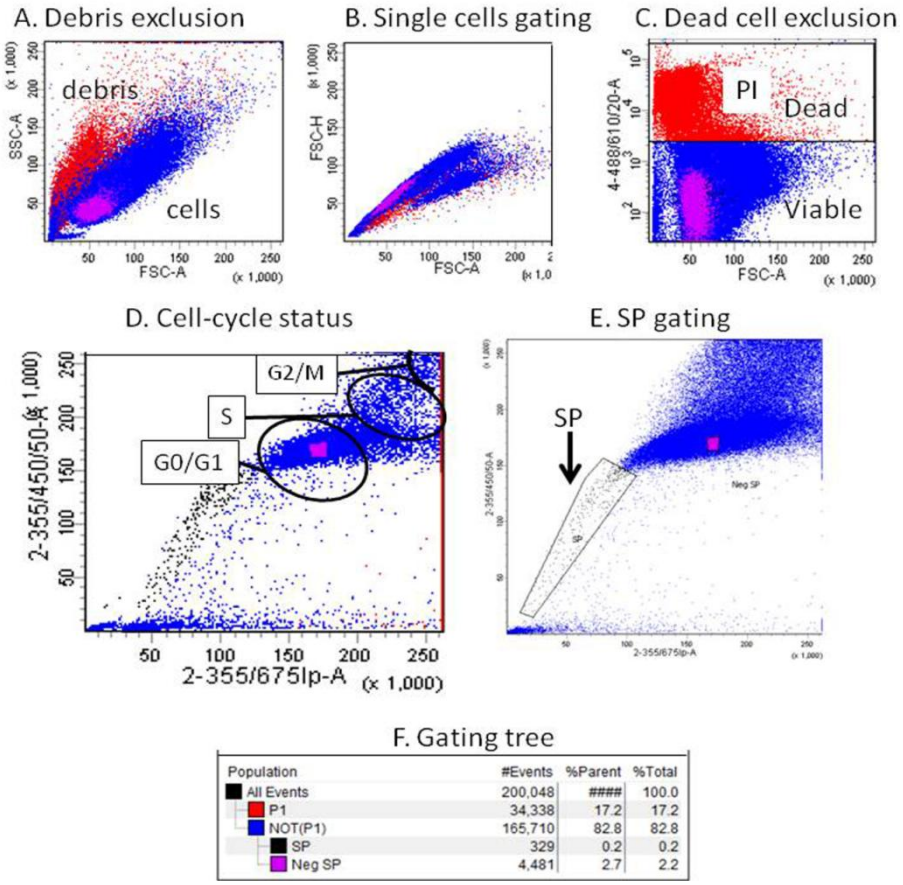


Figure 4.4 Representative FACS data plots showing SP protocol optimisation. Gating indicates SP cells. (A) Cells stained using Hoechst concentration of 3µg/ml, note the presence of SP cells in the gated region (B) Absence of cells in the SP gate in the presence of VP. (C) Cells incubated with 5µg/ml of Hoechst and (D) Cells from the same preparation but with the addition of VP. In both C and D no SP phenotype was observed. *[Abbreviations: SP – side population, VP – Verapamil. Part of this figure was previously published (Shaharuddin et al., 2013c)]*

The gating strategy for isolating a final population of cells by SP discrimination is presented in Figure 4.5. The steps involved initial debris exclusion, followed by a strategy to gate out cell doublets and cellular aggregates using the properties displayed by FSC and SSC. Next, dead cells were distinguished by its affinity to a cell death discrimination marker e.g. propiodium iodide (PI). Due to the function as a DNA-binding dye, Hoechst dye fluorescent signals give indication for DNA content in the cells, thus specific cellular phase i.e G0/G1, S and G2/M can be identified. SP cells appear as the "tail" at the left hand side of the G0/G1 cell population. A gating tree gives information to the final SP population as a percentage to the parent gated events or as a percentage of the total recorded events.



Population	#Events	%Parent	%Total
All Events	200,048	####	100.0
P1	34,338	17.2	17.2
NOT(P1)	165,710	82.8	82.8
SP	329	0.2	0.2
Neg SP	4,481	2.7	2.2

Figure 4.5 Step by step gating strategy for SP. (A) Cells are distinguished from debris by FSC (based on cell size) and SSC (based on granularity) (B) To ensure signals are directed from single cells, cell doublets and aggregates are singled out based on FSC-Area (FSC-A) or SSC-A against FSC-Height (FSC-H) or SSC-H.(C) Dead cells are gated out by its positivity to a dead cell discrimination marker e.g. Propiodium iodide (PI) (D) DNA-binding property of Hoechst gives off fluorescent signal which gives information on the specific cell cycle indicated by the DNA content of the cells (E) SP cells are recognised as a dim "tail" which appears first at the left hand side of the G0/G1 population (F) The gating tree is a sequential events leading to isolation of an SP population. %Parent refers to percentage of gated events relative to the preceding gate. %Total refers to percentage of gated events relative to total events recorded.

4.4.2 LSP in primary LEC cultures

Using our optimized SP protocol, harvested limbal colonies from co-cultures with 3T3 feeder layer were analysed for the presence of SP. SP cells were present as shown in Figure 4.6A. Cells from the same culture but incubated in the presence of verapamil were used to confirm the SP cell phenotype (Figure 4.6B). Samples from 11 donor corneas were processed and the epithelia cultured and prepared for SP assays with the final SP phenotype being confirmed by addition of either verapamil or FTC. SP yields from these are presented in Figure 4.7. Range of SP yields was between 0.1 to 0.8% with a median value of 0.2 (SE 0.07).

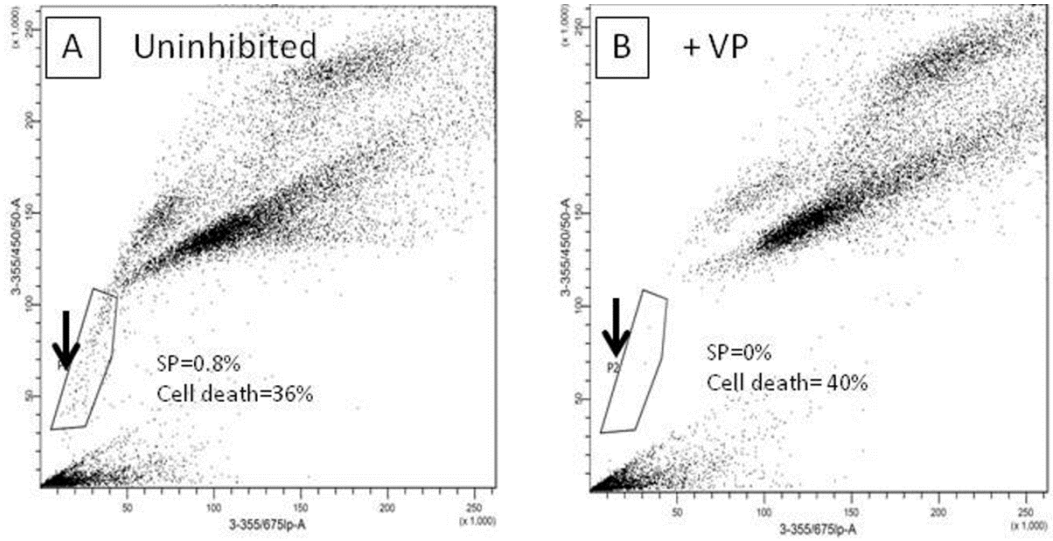


Figure 4.6. Representative FACS dot plots showing SP cells in LEC. (A) SP in primary LEC (SP gate indicated by arrows). (B) The same preparation but with the addition of Verapamil . Note the absence of SP cells in the gated region on addition of verapamil. (Shaharuddin *et al.*, 2013a; Shaharuddin *et al.*, 2013c).

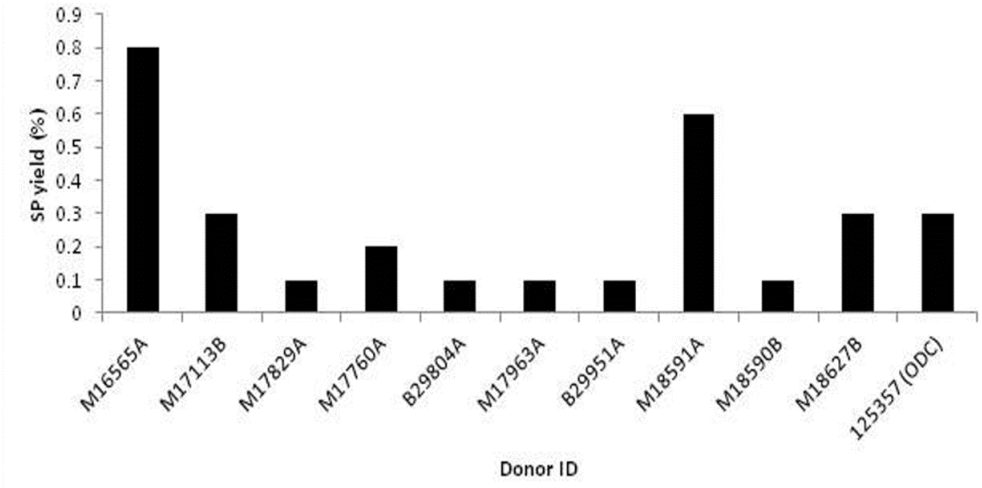


Figure 4.7 SP yields in primary LEC, N=11. SP yield ranges from 0.1 – 0.8%

4.4.3 ABC-transporters in LSP cells

The ability of both primary limbal epithelial SP and HTCEC to efflux Hoechst dye could be inhibited by the addition of verapamil or FTC alone. This inhibition was either complete or partial, which was the same for both cell line and primary epithelial cultures, but partial inhibition is a phenomenon more frequently associated with primary epithelial cultures, an example is given in Figure 4.8A, 4.8B and 4.8C. In the presence of 3 μg/ml of Hoechst dye, an SP population of 0.4% could be detected (4.8A), which was partially inhibited by verapamil, note the reduced number of SP cells in the SP gate (4.8B), with the addition of FTC note no SP cells can now be seen in the SP gate (4.8C).

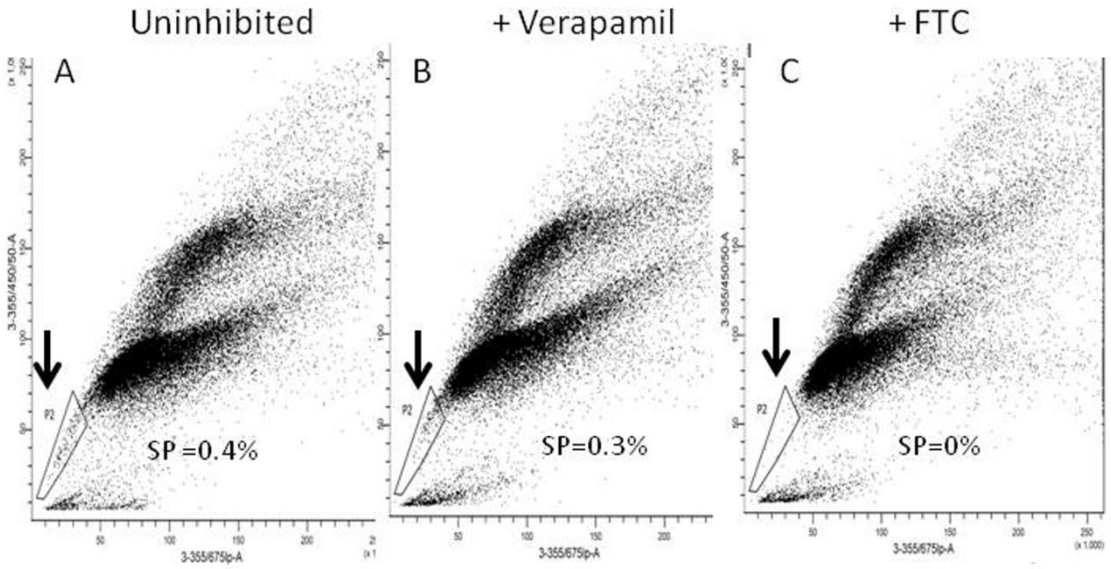


Figure 4.8. Representative FACS dot plots showing SP cells in HTCEC (A) SP gates are indicated with arrows (SP=0.4%). (B) Note reduction of SP on addition of Verapamil 0.3%), and (C) Loss of SP cells on addition of FTC (Shaharuddin *et al.*, 2013c).

When a partial inhibition was observed we hypothesised this was due to there being more than one ABC transporter being responsible for the SP phenotype. Both ABCG2 and ABCB1 transporter genes were expressed by both primary LEC and HTCEC, as evident by sq-PCR results (Figure 4.9A and 4.9B). Results from immunocytochemistry showed ABCG2 was expressed in HTCEC and LEC (Figure 4.9C and 4.9D). The co-culture system for culture of primary epithelial cells called for further investigations on the possible role of 3T3 fibroblast to contribute to the SP cell populations. However sq-PCR and ICC results suggest this is not the case as no gene (ABCG2 and ABCB1) or protein expression (ABCG2 ICC only) could be detected (Figure 4.9E and 4.9F).

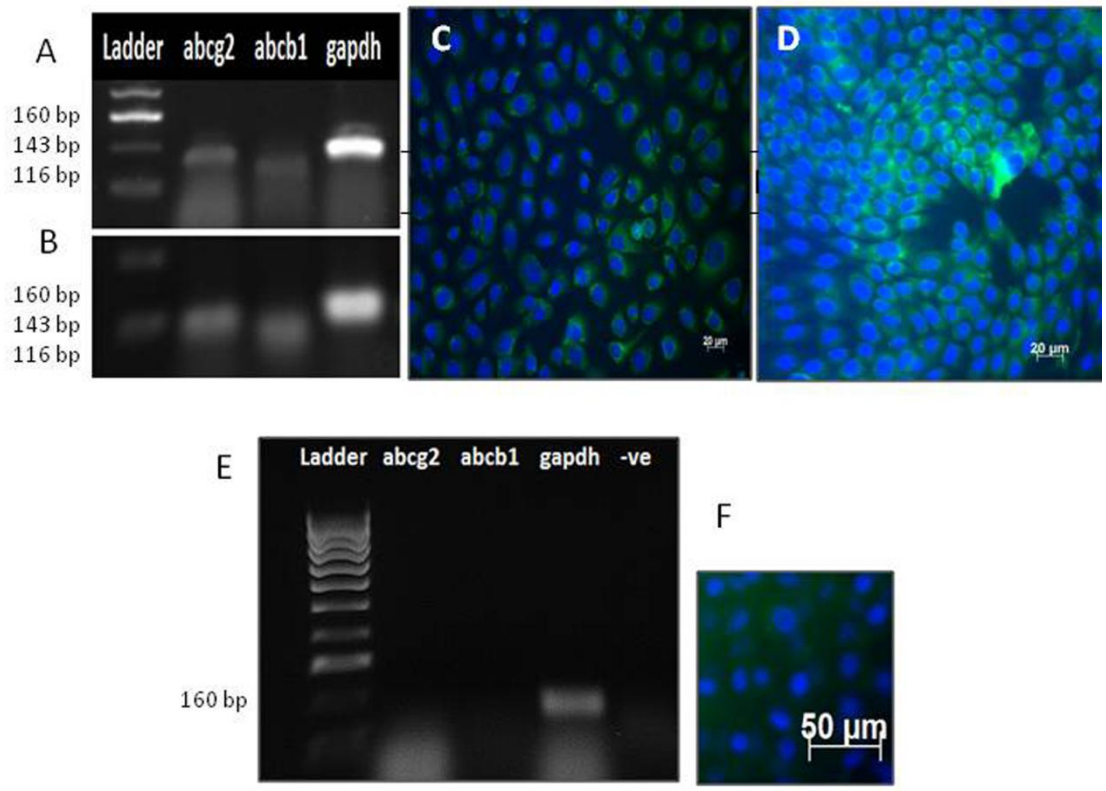


Figure 4.9. Representative image of gene expression of ABCG2 and ABCB1 and protein for ABCG2 in unsorted HTCEC and LEC (A) sq-PCR results for mRNA expression in HTCEC and (B) primary LEC, (N=3). Note both expressed ABC G2 and ABCB1 (C) Results of immunocytochemical analysis showing representative images of ABCG2 expression in HTCEC (N=3), and (D) primary LEC (N=2). (E) sq-PCR results for mRNA expression showed no expression of ABCG2 and ABCB1 in 3T3 murine fibroblast (feeder layer), (N=2) (F) No ABCG2 expression could be detected in 3T3 fibroblast using ICC (N=2). *[DAPI=blue, ABCG2 green. Part of this figure was previously published]..*

Quantitative RT-PCR was performed using primary LEC (N=5) to study the expression of ABCG2 and ABCB1.A differential expression of ABC transporter genes in the LEC samples was observed (Figure 4.10). Results were normalised to GAPDH and HTCEC was used as a reference sample. Statistics for relative expression, standard error and P values for up- and down-regulated genes are presented in Table 4.3.

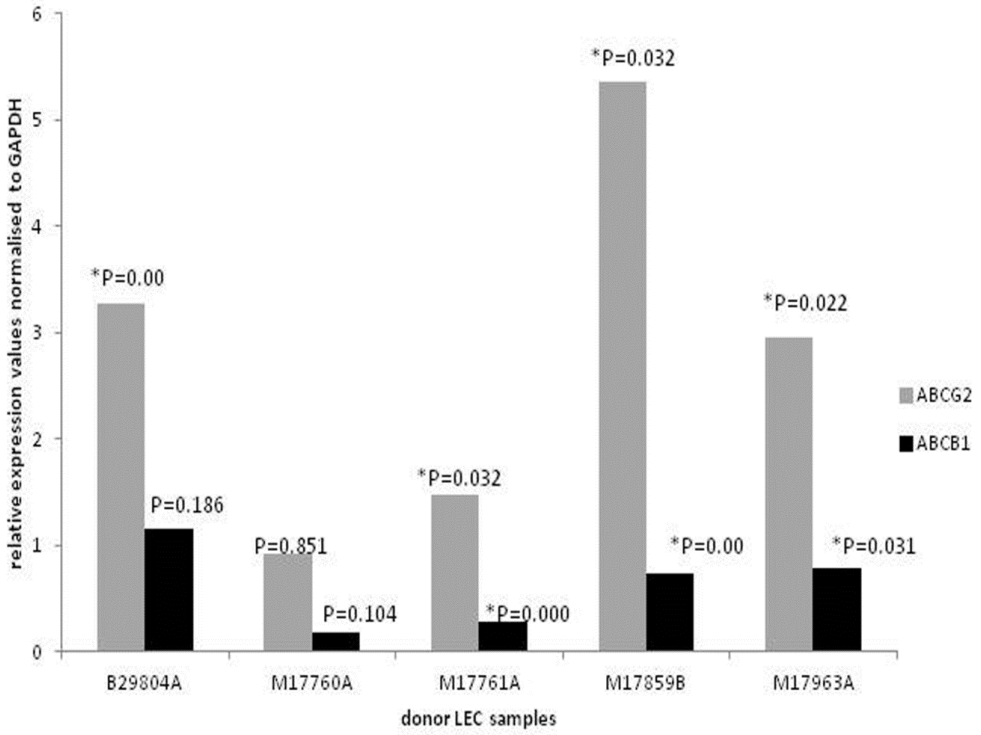


Figure 4.10. RT-QPCR showed variability in relative expression of ABCG2 and ABCB1 in 5 samples of LEC, data normalised to GAPDH, using HTCEC as reference (control).

Table 4.3 Statistics for ABCG2 and ABCB1 expression in LEC by RT-PCR.

Donor ID	Genes	Relative expression	SE	P value	Results
B29804A	ABCG2	3.268	3.0-3.6	*0.000	UP
	ABCB1	1.147	1.0-1.2	0.186	
M17760A	ABCG2	0.910	0.6-1.2	0.851	
	ABCB1	0.181	0.1-0.2	0.104	
M17761A	ABCG2	1.48	1.2-1.6	*0.032	UP
	ABCB1	0.27	0.1-0.2	*0.000	DOWN
M17859B	ABCG2	5.356	4.5-6.1	*0.032	UP
	ABCB1	0.733	0.6-0.7	*0.000	DOWN
M17963A	ABCG2	2.952	2.5-3.4	*0.022	UP
	ABCB1	0.785	07-0.8	*0.031	DOWN

*Significant difference in relative gene expression compared to control.

In four samples of LEC (B29804A, M17761A, M17859B and M17963A) significant upregulation of ABCG2 gene was demonstrated compared to control group. In three LEC samples (M17761A , M17859B and M17963A) there was significant downregulation of ABCB1 gene compared to control group.

4.4.4 Effects of cell confluence on SP yields

Analysis of the impact of low cell confluence on SP cell yields has been reported to increase SP yields in cancer cell cultures (Tavaluc *et al.*, 2007). We further investigated this in LSP. HTCEC were plated at different cell densities; 250,000, 500,000, and 1,000,000 cells in T75cm^2 flasks and incubated in 5% CO_2, 37°C.

After four days, different cell confluence was observed using brightfield microscopy (Figure 4.11). The cells were then harvested and the SP assay performed. Cell density was counted as total cell count divided by 75cm^2 tissue culture flask area to obtain the number of cells/cm^2.

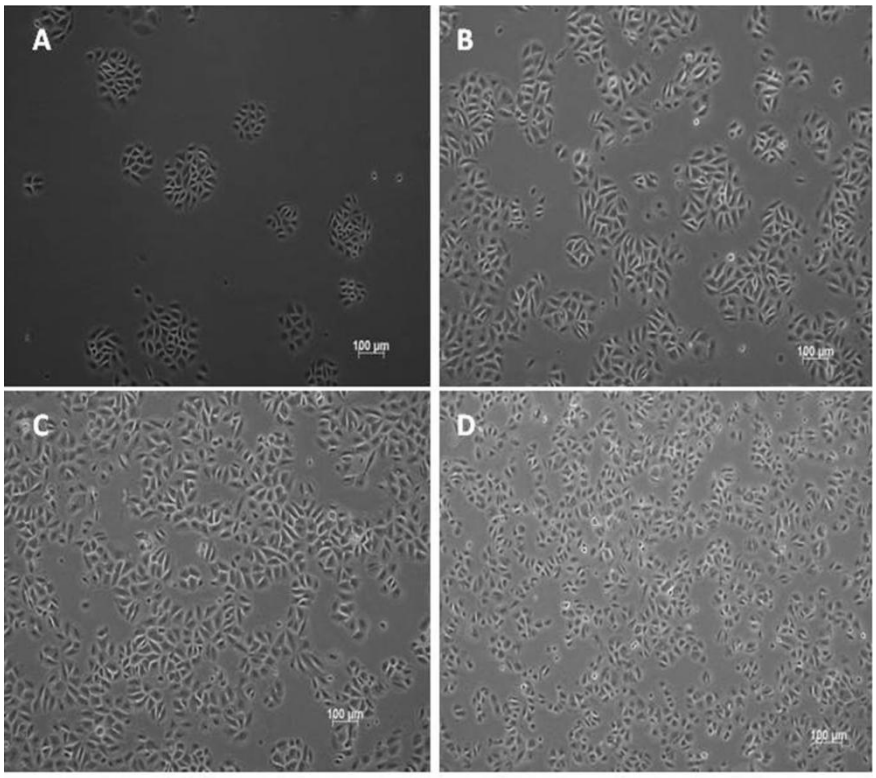


Figure 4.11 SP and cell confluence. Representative photo micrographs of cultures at day 4. Each figure represents different cell densities plating (A) 250,000 cells, (B) 500,000 cells, (C) 1,000,000 and (D) 2,000,000 cells.

Experiments were performed twice (Figures 4.12. and 4.13). Harvesting densities (number of cells/cm^2) were calculated and plotted against SP yields. We found the cell populations with lower cell confluence gave higher SP percentages (Figure 4.14).

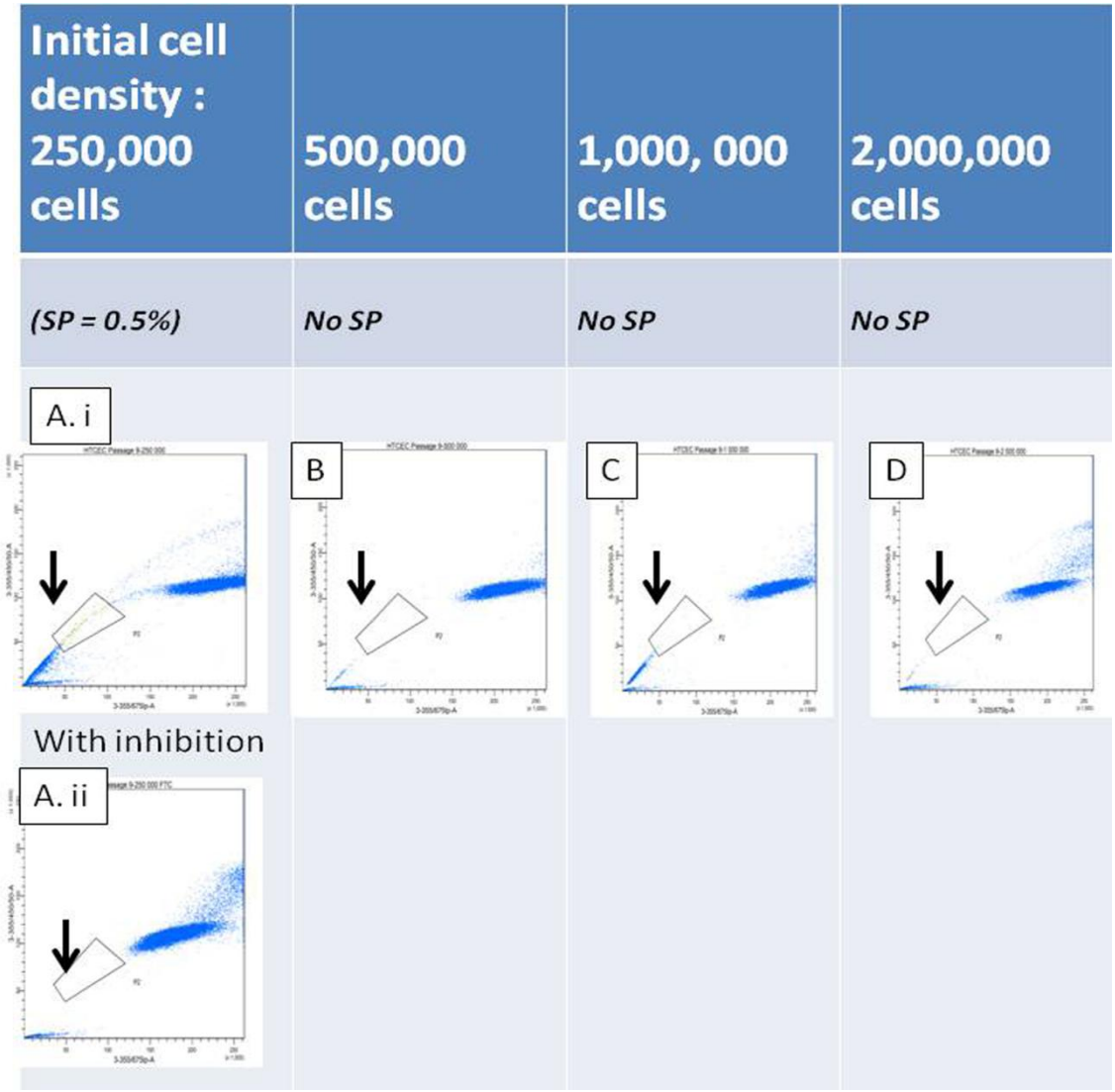


Figure 4.12. Representative FACS dot plots showing cell confluence and SP yield on day 4 of culture. SP gates are indicated with arrows. [A(i)] initial plating density of 250,000 cells/cm2: note SP 0.5% was detected, [A(ii)] Cells from the same preparation lost the SP phenotype on addition of verapamil (B) Cultures with initial cell density of 500,000 cells (C) 1,000, 000 cells/cm2 and (D) 2,000,000 cells; note no SP cells are detected in B, C and D.

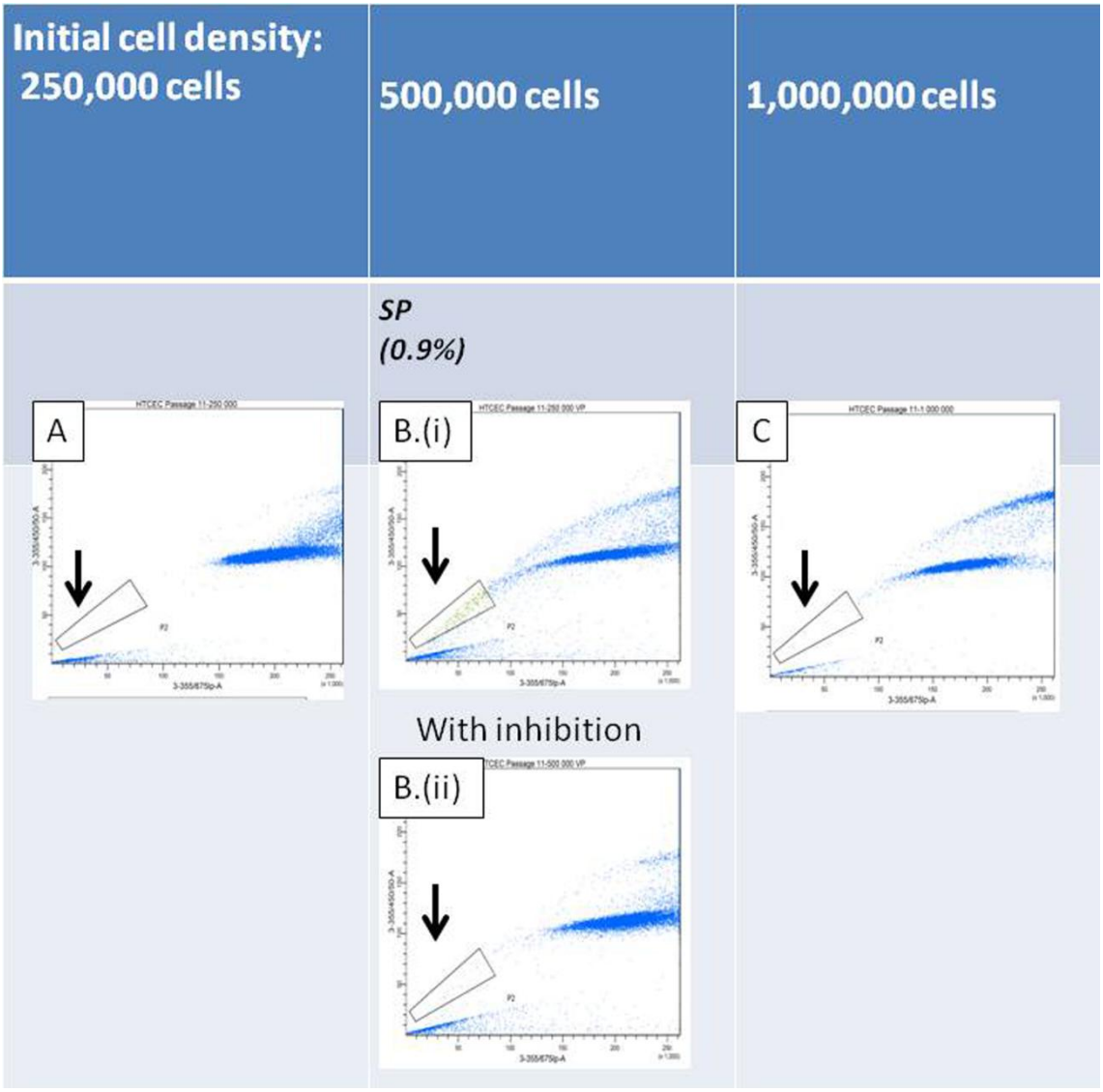


Figure 4.13 Representative FACS dot plots showing cell confluence and SP yield on day 4 of culture. SP gates are indicated with arrows. (A) Initial plating density of 250,000 cells/cm^2 [B(i)] Cultures with initial cell density of 500,000 cells: note SP 0.9% was detected, [B(ii)] Cells from the same preparation lost the SP phenotype on addition of verapamil (C) Cultures with initial cell density of 1,000, 000 cells/cm2. Note no SP cells are detected in A and C.

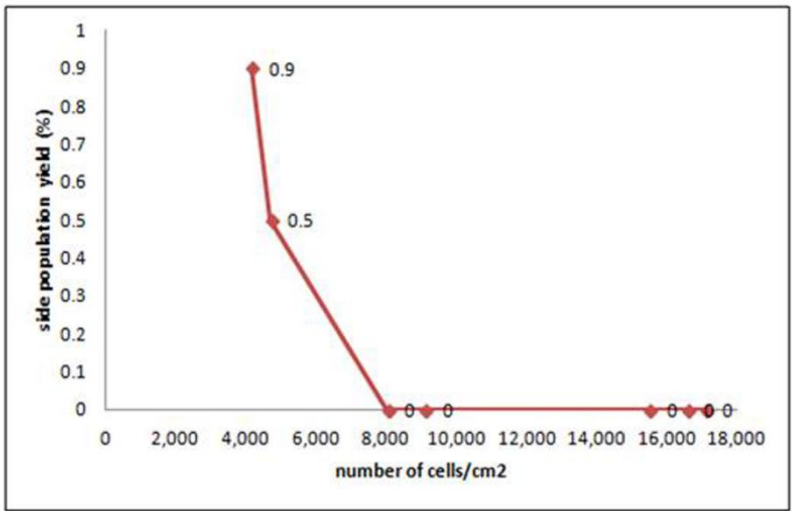


Figure 4.14. Cell confluence and SP in HTCEC. SP percentage (Y axis) plotted against harvesting densities (X axis).

4.4.5 Side population and hypoxia

We prepared HTCEC from cultures growing under normal atmospheric oxygen level (20%), 5% oxygen and 1% oxygen for side population assays (N=2). There was no significant difference between SP in the two oxygen levels (Table 4.4).

Table 4.4 SP yields in cultures grown at 5% and 1% oxygen levels.

Oxygen (%)	SP (%)	Cell death (%)
5	0.4	15.3
	0.3	6.3
1	0.4	9.9
	0.4	10.8

4.4.6 Characterisation of SP cells from HTCEC

Using our optimised LSP protocol, we sorted SP and NSP cells of the HTCEC directly onto slides and stained them with antibodies to p63, Sox2, ABCG2 and ABCB5. Under 20x magnifications, areas were arbitrarily divided into five fields for image quantification.

Using Image J software, the fluorescence intensity for each marker in SP and NSP fractions were quantified, values were analysed using independent t-test for parametric data by IBM SPSS software.

For ABCG2, ABCB5 and Sox2, the expression of all three markers was significantly higher in the SP versus NSP cells, while NSP signal intensities for p63 were significantly higher than in SP fraction. Statistics for signal intensity (ICC) quantification for SP and NSP cells with limbal markers are tabulated in Table 4.5.

Table 4.5 Statistics for fluorescence intensities (arbitrary units) for positively stained cells with LSC markers.

Markers	N	Means	(±) SD	*P* value*
ABCG2				
-SP	131	18.43	7.40	0.012
-NSP	131	16.33	5.91	
Sox2				
-SP	33	29.17	4.19	0.000
-NSP	34	18.36	7.16	
ABCB5				
-SP	160	26.35	8.70	0.020
-NSP	160	24.17	8.07	
p63				
-SP	170	40.17	7.10	0.000
-NSP	165	44.25	7.31	

[Abbreviations: ABCG2= ATP-binding cassette sub-family G member 2, ABCB1= ATP-binding cassette sub-family B member 1, ABCB5 = ATP-binding cassette sub-family B member 5, SP= side population, NSP= non side population].
**Independent t-test.*

Representative ICC images for SP and NSP cells stained positive for limbal markers ABCG2, ABCB5, p63 and stem cell marker Sox2 are shown in Figure 4.15.

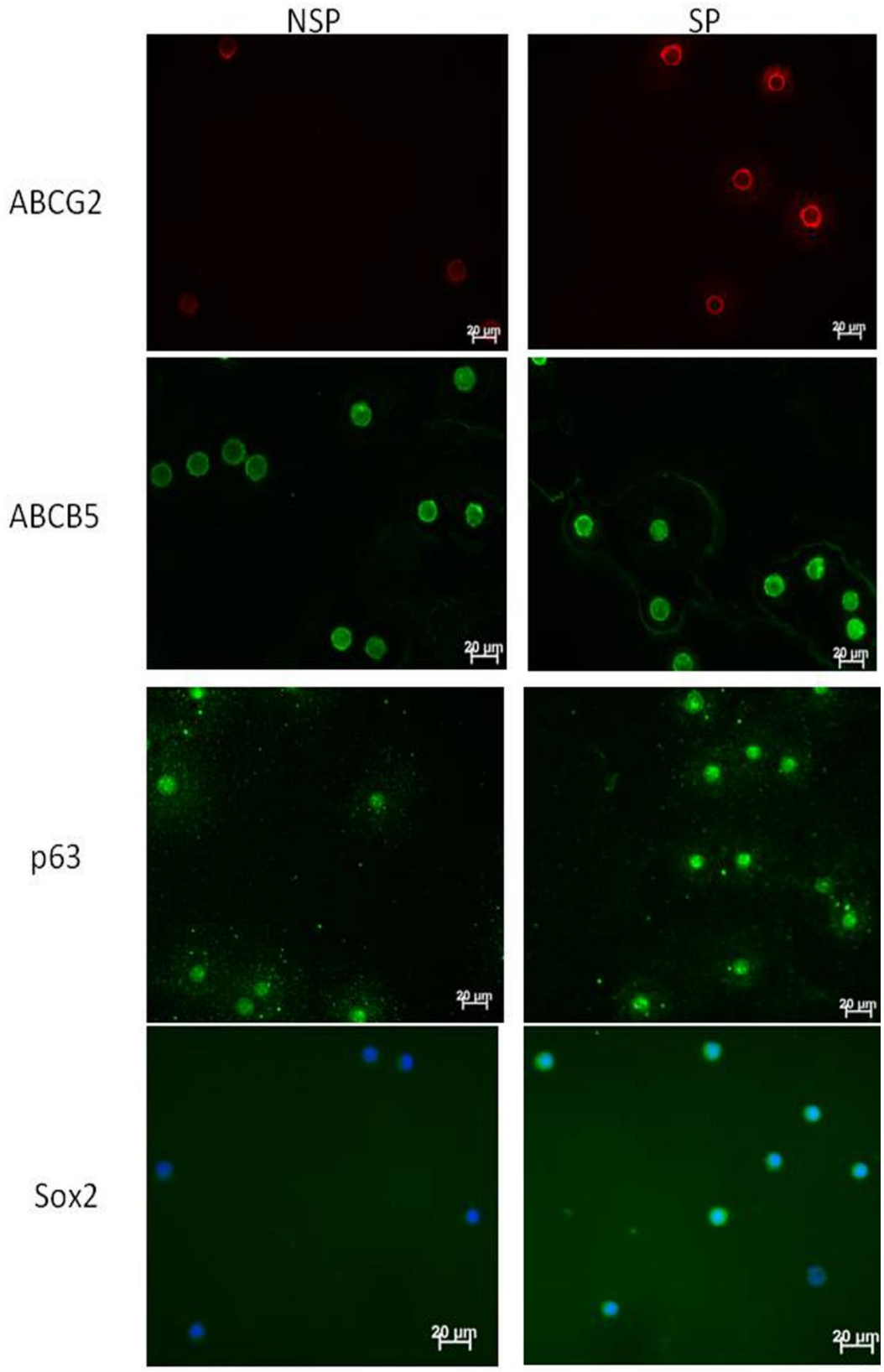


Figure 4.15. Representative images of ICC analysis of SP and NSP cells. Note Expression of ABCG2, ABCB5, p63 and Sox2 antibodies in both SP and NSP cel populations, N=3. *[FITC-conjugated secondary anti-mouse antibody= green, Rhodamine-conjugated anti-rabbit secondary antibody = red. DAPI = blue. Part of this figure was previously published (Shaharuddin et al., 2013c)].*

FACS-sorted LSP cells were stained using ICC to examine the expression of ABC transporters ABCG2 and ABCB5 in HTCEC. In addition to ABCG2 expression, SP-sorted HTCEC also expressed ABCB5 (Figure 4.16A). An overlay of these images showed a number of cells had dual expression of these transporters in the SP fraction (Figure 4.16B).

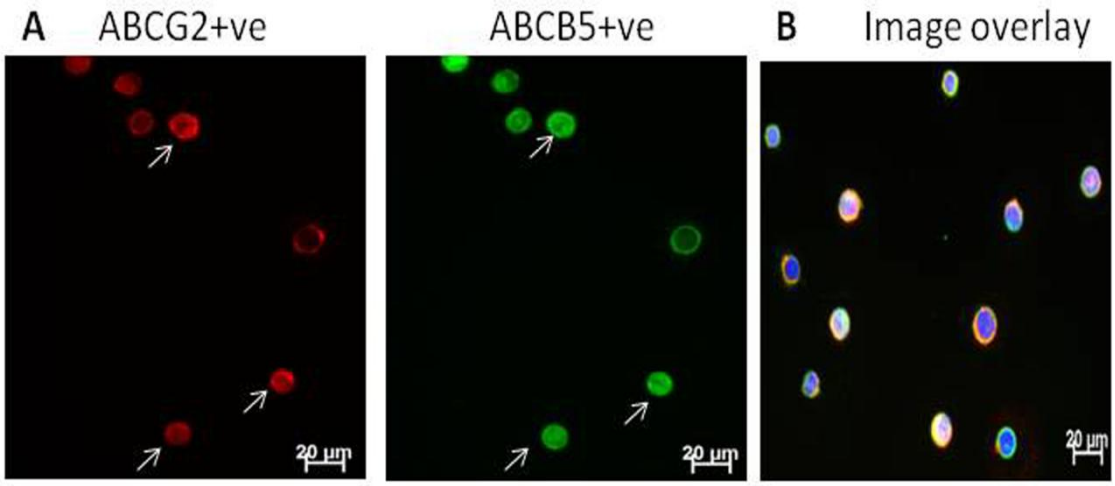


Figure 4.16 Representative images of dual labelling ICC analysis for ABCB5 and ABCG2 expression in SP-sorted HTCEC, N=3. (A) Expression of ABCG2 and ABCB5 in SP cells. Arrow heads indicate cells that express both. (B) Image overlay showing co-expression by some cells in the SP fraction of both transporters. (*[FITC-conjugated secondary anti-mouse antibody= green, Rhodamine-conjugated anti-rabbit secondary antibody = red, DAPI –nuclear, stain blue].*

4.4.7 Cell diameter and SP

Analysis of ABCB2 stained HTCEC derived SP and NSP showed SP cells were smaller in size compared to the NSP cells (Figure 4.17) Image J was the used to to measure diameter of stained cells in both population fractions and values were analysed using independent t-test for parametric data by IBM SPSS software. The cell diameter was significantly smaller for SP fraction 11.49 (±1.59) µm, than the NSP fraction 11.94 (±1.91) µm, as presented in Table 4.6.

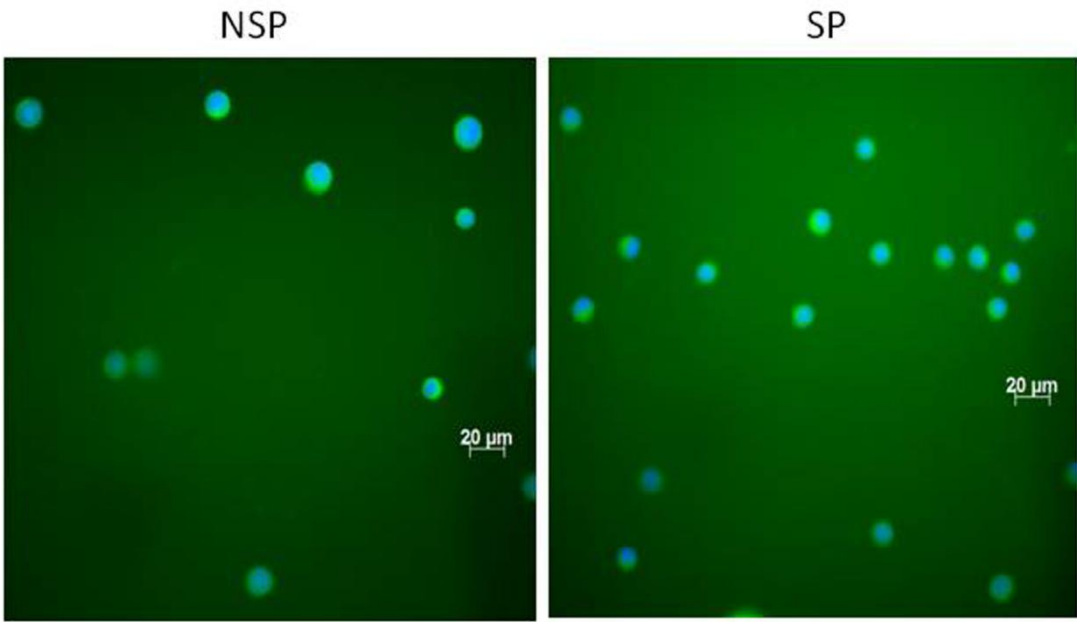


Figure 4.17 Representative images of HTCEC stained with ABCG2 antibody demonstrating SP cells to have smaller cell diameter compared to NSP cells., (N=3). *[ABCG2 FITC = green, DAPI = blue (cell nuclei)]*

Table 4.6 Cell diameter from SP- and NSP-sorted cells.

	N	Means (µm)	(±) SD	*P* value
SP	179	11.49	1.59	0.019*
NSP	163	11.94	1.91	

[Abbreviations: SP = side population, NSP = Non-side population.This table was previously published (Shaharuddin et al., 2013c)]
**Independent t-test*

4.4.8 Colony formation of SP cells

Next we examined the colony forming potential of SP and NSP cells of the HTCEC line in a plastic adherent mono layer culture conditions which demonstrated SP-sorted HTCEC formed colonies in tissue culture wells (Figure 4.18A). The NSP cells also had colony formation (Figure 4.18B) but the colonies were smaller and contained fewer cells/colony than the SP counterpart. The difference in the cell number per colony between the SP and NSP cell fractions was calculated by using the cell counter in Image J and statistically analysed by IBM SPSS. There was a significantly higher number of cells/colony in SP than NSP, *P*=0.01 (Mann-Whitney) (Figure 4.18C).

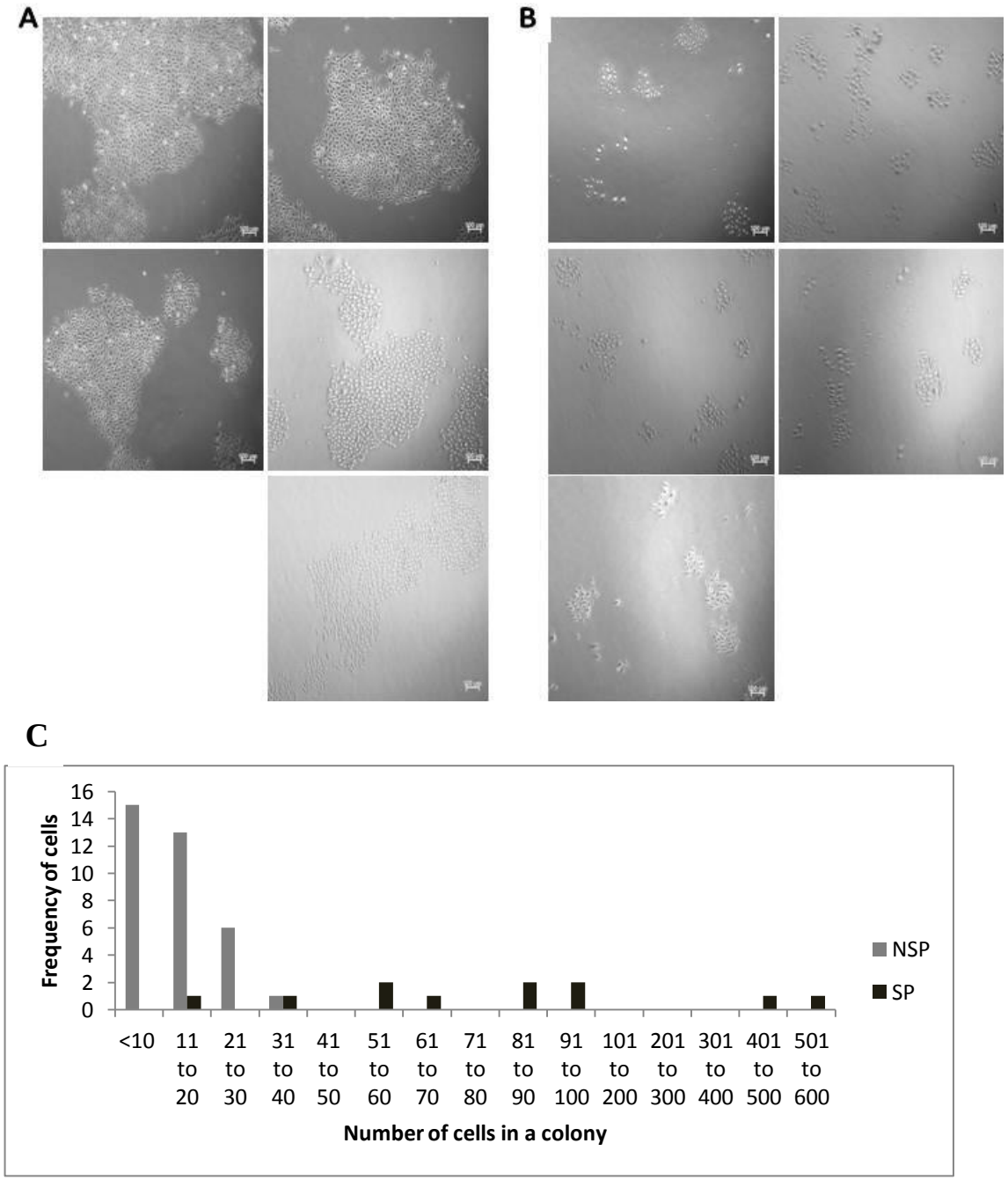


Figure 4.18 Colony formation of SP and NSP-sorted cells on Day 5 of culture. (A) SP fraction (B) NSP fraction (C) Number of cells per colony plotted against cell count in SP and NSP cells in HTCEC.

4.5 Discussion

One of the advantages of SP assay is the feasibility for further analysis on the viable cells such as *in vitro* and *in vivo* functional characterisation, some of the examples are molecular characterisation using stem cell markers (Pfister *et al.*, 2010). After an initial wash out with PBS, the two cell fractions may further be characterised by PCR (Watanabe *et al.*, 2004; Cintia S. de Paiva *et al.*, 2005a), immunophenotyping by FACS (Pfister *et al.*, 2010), propagated for proliferative studies such as colony forming efficiency (Cintia S. de Paiva *et al.*, 2005a) or spheroid formation (Ostrowska *et al.*, 2011).

Optimisation of the SP protocol

There are critical parameters that can challenge the design of experiments and interpretation of the results which necessitates the process of optimising an SP protocol. When an established or stable cell line is used, factors that needed to be looked into are the cell density, nutrient used, serum or oxygen levels (Tavaluc *et al.*, 2007), therefore it is advisable to keep to the same culture conditions. It is also important to maintain similar cell concentration/number of cells to gather the equilibration of the dye with the intracellular components.

It is crucial to obtain a single cell suspension with limited cellular damage because the assay is based on active physiological process which depends on viability and protein integrity at the cellular membrane. This poses a challenge when processing tissues using enzymatic digestion which is potentially damaging to the cells.

In a critical appraisal for SP methods, Golebiewska et al listed a number of parameters that need to be optimised to ensure reproducibility and standardisation of SP data (Golebiewska *et al.*, 2011). The optimisation included several steps such as appropriate dye concentration, stable incubation conditions such as incubation time, temperature, darkness and inhibition controls all essential to obtain a high resolution SP FACS plot and to allow for discrimination between the SP and NSP fraction.

Cumulative data from previous SP protocols showed differences exist between various tissues of the same species, or the same tissues from different species. Furthermore, human tissue sourced SP cells often required different Hoechst concentration and contact times from those of their animal counterparts. For example, in murine bone

marrow and skeletal muscle assay, 5 μg/ml Hoechst dye was used, while in liver, spleen and kidney, 10 μg/ml of dye was used (Asakura and Rudnicki, 2002). The SP assays for murine cardiac progenitor cells, has been reported to use between 1 – 5 μg/ml of Hoechst dye and a 90 minute incubation period (Pfister *et al.*, 2010; Annette Meeson *et al.*, 2013). However, a study in a human developing heart used 1.25 μg/ml of Hoechst dye and a 60 minutes incubation time (Alfakir *et al.*, 2012).

Although the cell count of 1.0×10^6 cells/ ml is standard for SP cell analysis, it is particularly important especially when doing a profile on primary tissues/cultures to include only viable cells or nucleated cells. Therefore, adequate gating during analysis, paying attention to single, viable and nucleated cells is crucial when determining an SP phenotype. Hoechst dye stains all DNA regardless if cells are alive or dead, therefore dead cell discrimination is an important step in FACS analysis. Appropriate gating for cell debris and dead cells should be integrated during analysis.

For a consistent temperature control, Hoechst incubation should be carried out under stable temperature control. We advise the use of a 37°C incubator using a gentle shaker or rotator. It is also important to note that to maintain Hoechst within cells after staining is complete, all activities post staining which include washing, centrifugation and data analysis should be performed cold (4°C).

LSP in LEC and HTCEC

In the rat, SP cells have been shown to be present in both limbal and central cornea (Umemoto *et al.*, 2005) suggesting species variations as this contradicts findings from rabbit and human LSP studies. A number of studies have demonstrated the presence of LSP cells in both animal and human limbus. They have provided evidence that these SP cells have some stem cell characteristics; however few have looked at optimisation of the SP assay.

In the field of limbal corneal SP, a noticeable variation among species was also observed. As examples, in the pig – Hoechst concentration of 5 μg/ml for 90 minutes was used (Akinci *et al.*, 2009) and rabbit –3 μg/ml for 90 minutes (Umemoto *et al.*, 2006). Budak and colleagues (Budak *et al.*, 2005) commented that optimisation of their protocol for LSP cells (rabbit and human) was initially done on rabbit cells and from the results of these they concluded what the optimal Hoechst dye concentration and

incubation time would be for the human cells. Whereas de Paiva and colleagues (Cintia S. de Paiva *et al.*, 2005a) used both freshly isolated primary human cell cultures as a source of LSP cells but did not optimise the SP cell assay, instead they based their SP protocol on a method previously used to isolate SP cells from hematopoietic stem cells. Watanabe and colleagues (Watanabe *et al.*, 2004) also reported on the identification of human LSP cells from human limbus but again did not report on optimisation of the SP assay used. However they did use a Hoechst concentration of 3µg/ml and an incubation time of 90 minutes.

In this study we optimised our SP protocol using HTCEC and achieved an optimal Hoechst concentration of 3µg/ml which gave a consistent and reproducible SP percentage of 0.2%. The optimal incubation time for Hoechst dye was 45 minutes, as more HTCEC death occurred at a longer incubation times (90 minutes) (data not shown). The shorter exposure of cultures to Hoechst dye was preferred to prevent cellular toxicity as Hoechst 33342 has proven to cause cytotoxicity by causing single and double strand breaks in DNA and markedly increase sensitivity of DNA and cells to ultra violet damage (R. F. Martin and Denison, 1992; Singh *et al.*, 2004).

A number of studies have determined that the ABC transporter ABCG2 is the main transporter responsible for conferring on SP cells their ability to efflux Hoechst 33342 dye (Watanabe *et al.*, 2004; Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Ki-Sook Park *et al.*, 2006; Umemoto *et al.*, 2006; Akinci *et al.*, 2009). Confirmation that the cells identified are SP cells is important due to issues that can be caused by under or over saturation therefore the inhibitor and its concentration need to be validated. We opted for the use of verapamil, a calcium channel which blocks both ABCG2 and ABCB1 instead of FTC which is a specific inhibitor for ABCG2.

In our laboratory, a median value of 0.2% of SP yield was obtained from 11 primary LEC samples (range: 0.1 – 0.8%), which is comparable to previous investigations on human LSP (summarised in Table 4.1). Data in previous literatures showed a broad range of SP yields in human LSP from 0.2% to 2.1%. Differential expression of ABCG2 and ABCB1 has also been demonstrated in the SP cells derived from fine needle aspiration biopsy of the breast (K. M. Britton *et al.*, 2012).

We demonstrated here SP yields in HTCEC were consistent at the value of 0.2% for cells cultured under normal atmospheric oxygen levels. The use of established cell lines as sources of SP cells have been reported previously (Hirschmann-Jax *et al.*, 2005; Yamazaki *et al.*, 2008; Kelly M. Britton *et al.*, 2011; Rizzo *et al.*, 2011).

While the percentage of SP cells is relatively small in the HTCEC, stem cells are in many systems rare cells, moreover it was also demonstrated that as few as 500 rabbit LSP cells were able to regenerate cornea like epithelium which was p63+ve in a 3-D culture system (Ki-Sook Park *et al.*, 2006). The convenient use of an established cell line surpasses the difficulty in the isolation and identification of LSC from primary cultures and enables studies to be performed when valuable primary tissue is unavailable.

ABC-transporters and inhibition of SP phenotype

We observed that both LEC and HTCEC could be inhibited by verapamil and FTC, and that both the ABCG2 and ABCB1 transporters were expressed in primary LEC and HTCEC by ICC and PCR. Data on ABCG2 and ABCB1 expression in primary cultures of LEC showed co-expressions of both genes at variable levels of expressions. However, the ABCG2 gene was more upregulated and ABCB1 gene more down regulated in LEC compared to HTCEC. This explains the partial inhibition of SP profile by verapamil which blocks ABCB1 but partially blocks ABCG2 efflux activity. This effect was more evident in LEC compared to HTCEC line. Incomplete inhibition by verapamil is not exclusive to LSP cells, it has also been observed in the SP assays of murine skin and muscle (Montanaro *et al.*, 2004).

Cell confluence and hypoxia in SP

We also show that cell confluence affects SP yields in our results, which is an important parameter when a cell line is being used in SP protocols. Lower cell confluence has shown a higher SP yields in colonic and breast cancer cell lines (Tavaluc *et al.*, 2007). Cell confluence was demonstrated to be more important than the duration of cultures for cellular differentiation (Tavaluc *et al.*, 2007). Reduced cell density would be perceived as a stressor which stimulate proliferative and renewal capacity of stem cells which directly results in increased expression of ABC transporters and Hoechst dye efflux. In the current study we demonstrated harvesting densities between 4,000 to 6,000 cells/cm^2 gave increased SP percentages above that of higher densities.

Hypoxia was also a factor that gave an increased SP yield in malignant colonic and breast cancer stem cells (Tavaluc *et al.*, 2007). Hypoxia is a significant factor towards stabilisation of the hypoxia inducible factors (HIF). HIF targets the ABC transporter genes, among others; which leads to more efflux of Hoechst dye and increased SP percentages. In a malignant condition, this confers the cancer SP cells to be more malignant.

We studied HTCEC cultures incubated at O_2 level of 1% and 5%, as this closely mimics the physiological relevant state of cells, as demonstrated by (Bath, 2013), in a series of oxygen levels from 2% to 20%, they found cultures grown at 2% oxygen had higher proliferative capacity, higher expression of ABCG2 and p63, higher colony formation potential and lower number of cells in the S/G2 cell cycle.

In our project, at the two oxygen levels tested, we did not find any significant difference in SP percentage. However, there was an increased of SP to 0.4% compared to 0.2% at normal atmospheric oxygen of 21%, although this result was inconclusive due to limited number of biological repeats. Further investigations on different hypoxia levels i.e. 0.2%, 0.5%, 1%, 2% and/or 5% needs to be conducted to see the true effects of hypoxia. Due to logistic and cost limitations, this has not been performed in the current study.

LSP characterisation

Studies of ABCG2 expression at mRNA and protein levels have provided convincing evidence that ABCG2 is the main transporter implicated in determining the LSP phenotype (Zhuo Chen *et al.*, 2004; Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Umemoto *et al.*, 2005). However, in previous studies of human LSP cells, while ABCG2 expression has been reported to be upregulated in SP cells versus NSP cells, this has been focused on gene expression (Watanabe *et al.*, 2004; Cintia S. de Paiva *et al.*, 2005a).

Several studies have used immunohistochemical staining to examine tissue sections to show the location of ABCG2 expressing cells in the human eye and have reported that ABCG2 positive cells can be found in the limbal epithelial basal layer (Zhuo Chen *et al.*, 2004; Watanabe *et al.*, 2004; Cintia S. de Paiva *et al.*, 2005a), however this does not prove that they are stem cells.

de Paiva and colleagues also selected out ABCG2-positive and ABCG2-negative cells using an anti-ABCG2 antibody combined with flow cytometry from their primary limbal cell cultures (Cintia S. de Paiva *et al.*, 2005a). They showed that these cells expressed ABCG2 and ΔNp63 mRNA whereas the ABCG2-negative cells did not, moreover the positive expressing cells had a great colony forming efficiency than the negative cells, suggesting that within the ABCG2-positive population there were stem cells. Moreover they also selected SP and NSP cells from the primary human limbal cell cultures and showed that these SP cells showed upregulated expression of ABCG2 and ΔNp63 mRNA and that these SP cells had a greater colony forming efficiency than NSP; however they did not look at protein expression for either of these markers.

In our study we not only looked at protein expression for ABCG2, ABCB5, ΔNp63 and Sox2 in SP cells and NSP cells from the HTCEC, we also quantitated the level of expression, this is important as NSP cells can also express some level of all these markers.

We showed that SP cell fraction when stained with ABCG2 antibody also presented with significantly higher expression compared to NSP , supporting previous results by mRNA expression in both human and animals (Watanabe *et al.*, 2004). The functional role of ABCG2 as a putative marker for limbal progenitor cells has been explored in a study of oxidative stress in ABCG2-knock out mice and immortalized corneal epithelial cells (Kubota *et al.*, 2010). Although ABCG2-knock out mice had morphologically normal corneal epithelium, the cornea cells were susceptible to oxidative stress and prone to cellular damage induced by mitoxantrone in a dose and time dependent manner. Further, we also provide evidence of expression of mRNA for another ABC transporter ABCB5, in HTCEC which co-localised with ABCG2. The expression of ABCB5 in SP fraction was also significantly higher than in NSP. This is the first data which relates co-localisation of ABCG2 and ABCB5 in LSP phenotype, which supports further the evidence of LSP as an enrich population of LSC.

However, LSP in the HTCEC line showed that the mean fluorescent intensity in the SP cell fraction was significantly lower than NSP for the transcription factor p63. p63 is a common molecular marker present at the nucleus of basal cells in stratified epithelial cells in the skin, oral mucosa, cervix, vaginal epithelium, urothelium, prostate and

others (Di Como *et al.*, 2002). p63 marks for cellular proliferation and its expression is lost with reduced DNA-content (cell cycle arrest) and decreased cellular proliferation. It is also downregulated during terminal differentiation (Westfall *et al.*, 2003). A study in the rat LSP showed that the percentage of p63-+ve expression was low at the start of cultures in a day-to-day analysis of LSP cells (Epstein *et al.*, 2005). We conclude that LSP cells from HTCEC are more quiescent, slow cycling in nature and lack cellular proliferation, which are defining characteristics of stem cells.

Cell diameter

We observed the SP cell fraction of HTCEC contained cells with a smaller cell diameter compared to NSP cells. This supports previous finding in rabbit LSP which contained a high proportion of cells with a smaller diameter then the NSP cells (Umemoto *et al.*, 2006). The same findings were also observed in skeletal muscle SP, where the SP cells had a relative small diameter and a high nuclear to cytoplasmic ratio than NSP (Annette P. Meeson *et al.*, 2004). Cell size was a parameter used to describe stem cell populations, including epidermal stem cells (Juxue Li *et al.*, 2008) and epithelial stem cells (Tani *et al.*, 2000).

Colony formation

LSP cells have also been shown to be enriched for stem cell activities such as increased colony-forming and high proliferative capacity (Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Akinci *et al.*, 2009) than NSP cells. Colony forming efficiency is considered a stem cell characteristic in other adult stem cells such as retinal stem cells (Tropepe *et al.*, 2000), bone marrow stem cells (Friedenstein *et al.*, 1987), and dental pulp stem cells (Gronthos *et al.*, 2000). Our SP cells had a higher colony forming potential that the NSP cells.

4.6 Conclusion

The sensitivity of SP assay to different species and cell types makes standardisation of SP protocols mandatory to ensure reliability and reproducibility of SP data. This will ultimately result in better interpretation of SP results and enable more accurate comparison between data generated in multiple studies. In the past 17 years since Goodel, *et al* demonstrated SP in murine haematopoietic stem cells, accumulating evidence has shown this assay to be useful for functional characterization of putative stem cells and cancer stem cells, especially in the absence of specific molecular

markers. Characterisation of LSP using ABCG2, ABCB5, p63 and stem cell antigen Sox2 in HTCEC show that SP cells mark for LSC and HTCEC is a useful and robust model for LSC. An enrichment method by SP has a lot of potential for increasing the quality of LSC in a cellular-based therapy to treat LSCD or to be used in ocular surface regenerative surgery.

Chapter 5 IMMUNOBIOLOGICAL CHARACTERISATION OF CORNEAL LIMBAL EPITHELIAL CELLS; IMPLICATIONS ON LIMBAL STEM CELL TRANSPLANTATION

5.1 Introduction

The cornea has a potentially proliferative and self-renewal capacity due to the presence of corneal stem cells which are believed to be located at the limbal region (reviews (Daniels *et al.*, 2001; Limb and Daniels, 2008)). Replacement of donor cells by the host-derived cells is a feature of tissue transplantations, which takes place in the cornea 1 – 2 years after surgery (Wollensak and Green, 1999). In most cases, all types of donor cells; epithelial, stromal keratocytes and endothelial layers, were observed to be taken over by the host cells (Hatanaka *et al.*, 2013) .

However, corneal transplantation is contraindicated in the treatment of LSCD an important cause of corneal blindness. Failure of grafts in this condition is due to loss of host stem cells and inadequate self renewing cells to replenish the surface of the graft from the donor. In the case of LSCD, cellular therapy such as *ex vivo* limbal epithelial transplantation has shown impressive advances as a treatment in the past decade or so (Graziella Pellegrini *et al.*, 1997; S. C. G. Tseng, 2001; Shortt *et al.*, 2007b; Shortt *et al.*, 2008; Rama *et al.*, 2010). In addition, endothelial cell transplantations have also shown promising results in animal studies (Okumura *et al.*, 2009; Okumura *et al.*, 2011; Koizumi *et al.*, 2012; Okumura *et al.*, 2012).

Tolerance towards allogeneic organ grafts represents one of the major aims of transplantation medicine. Stem cells are promising candidates for promoting donor-specific tolerance (Imberti *et al.*, 2011). Adult stem cells have immune-suppressive mechanisms to protect them from immunological reactions and apoptosis which are damaging to the survival of the transplanted tissues (review (Baraniak and McDevitt, 2009).

5.1.1 MHC Gene Complex

The human MHC is located on chromosome 6. MHC genes control several antigens most of which influence allograft rejection. These genes or antigens are divided into

three classes; Class I, II and III. Class I and II are expressed on cells and tissues while Class III are associated with serum proteins and body fluids.

The Class I gene complex has three significant major loci antigens; A, B and C, and some other less significant undefined loci. Each loci codes for antigenic polypeptide α chains which have many alleles (polymorphic) and each are associated with β chains e.g β-2-microglobulins. A non-functional α-β-chain causes non-expression of Class I antigens on the cell surface and deficiency in cytotoxic CD8 T cells.

The MHC Class II gene complex also contains three major loci; DR, DP and DQ. Each of these loci is associated with one α and variable β polypeptide chains, making up the Class II antigens. MHC Class II genes feature polymorphism. HLADRB has more than 700 alleles at population level, HLADRA has three variants. Both chains of HLADP and HLADQ are also polymorphic. For HLADP, a few alleles are present in the heterodimer DPA1/DPB1.

MHC Class II molecules play an important role in the immune system. They are essential in the immune system for defence against infection and are the main player in transplantation immunology. MHC Class II present antigens from extracellular sources to CD4+ T cells and also mediate thymic selection of helper T cells.

The Human Leucocyte Antigen system (HLA) represents MHC which encodes for genes related to immune system and cell surface antigen presenting proteins. MHC class II antigen presentation pathways are expressed by professional antigen presenting cells (APC) e.g. dendritic cells, macrophages, B-cells, or epithelial LC.

5.1.2 Stem cells as a potential therapeutic strategy to overcome rejection

Evidence for immunological privilege of corneal stromal stem cells was supported by the absence of T cell mediated immune rejection, when these stem cells were injected in mouse corneal stroma (Du *et al.*, 2009). Proliferative capacity of corneal stroma stem cells and its immune privilege clearly play an important role in corneal stromal tissue engineering (Alaminos *et al.*, 2006; Jian Wu *et al.*, 2012)' (Zajicova *et al.*, 2010).

In a study conducted in adult mice, a small population of LSC effectively inhibited lymphocyte proliferation and modulated cytokine production. LSC were also noted to display an enhanced expression of genes for the anti-apoptotic proteins like induced myeloid leukaemia cell differentiation protein (Mcl-1), B cell lymphoma-2 (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP), and survivin. The LSC were more resistant to CTL-mediated or apoptotic cell death compared to other limbal cells which are necessary for tissue survival (Holan *et al.*, 2010). These regulatory properties are important to safeguard LSC from the damaging effects of inflammatory processes and cell toxicity in the anterior chamber of the eye.

5.1.3 Pro-inflammatory cytokines

Interferon-gamma (IFN-γ)

IFN-γ is a Type II interferon secreted by thymus-derived T cells. It is secreted under certain activated conditions, by CD4+ T helper cells (Th1 lymphocytes), CD8+ cytotoxic lymphocytes, natural killer (NK) cells, B cells, NKT cells and professional APCs.

IFN-γ potentially upregulates antigen presentation through Class I and II MHC pathways. IFN-γ may induce the pro-apoptotic effect of TNF-α by increasing surface expression of TNF-α receptors in human endometrial stromal cells (Fluhr *et al.*, 2007) and human foetal astrocytes (Wosik *et al.*, 2001).

Tumour necrosis factor-alpha (TNF-α)

TNF-α is a cytokine involved in general inflammation and induces acute phase reactions. It is chiefly produced by activated macrophages, although other cells like CD4+ lymphocytes, endothelial cells, mast cells, fibroblast and NK cells also produce it. The main function of TNF-α is to regulate the immune response. It also produces a synergistic anti-proliferative effect when combined with IFN-γ on the growth of human salivary gland (HSG) cell line (AJ Wu *et al.*, 1994) due to apoptotic cell death (Kamachi *et al.*, 2002) . This synergistic effect was also observed in the IFN-γ mediated epithelial dysfunction in the intestinal epithelial mucosa of patients with inflammatory bowel disease (Fish *et al.*, 1999).

5.1.4 Chemotaxis

Chemokines are important molecules for leukocyte migration and immune cell trafficking in pathological and physiological conditions. Chemokines mediate their effect through G-protein coupled 7-transmembrane chemokine receptors (Trosko *et al.*, 2000) on the cell surface of specific and well-defined leucocyte subsets (Groves and Jiang, 1995). However, some chemokine receptors are found on non-haematopietic cells, including neuron and microglia (Cheung *et al.*, 2011), epithelial (Lin *et al.*, 2013) and endothelial cells (Linley *et al.*, 2012).

Chemokines are especially important in endothelial cell functions for proliferation, migration and differentiation and wound healing process, whereby CXCR4 has been shown to play an important role in vascularisation during angiogenesis (Linley *et al.*, 2012), migration of neural crest cells to the dorsal root ganglia (A. Pajoohesh-Ganji *et al.*, 2005), homing mechanism of renal transplanted cells (C. S. de Paiva *et al.*, 2005b) and adhesion of MSC to target sites (Budak *et al.*, 2005). CXCR4 signalling was also an important indication of growth and metastases of germ cell tumours (Gilbert 2009). In the cornea, CXCR4 proved to be important in maintaining the physical contact between corneal stem cells with the subjacent stromal mesenchymal cells or the "niche cells" for the maintenance of LSC (Hua-Tao Xie *et al.*, 2011).

5.2 Specific Aims

The aims in this chapter are:

1. To present data on HLA typing in HTCEC.
2. To optimize HLA antibodies concentration by flow cytometry.
3. To optimize concentration and duration of pro-inflammatory cytokines treatment in HTCEC for HLA expression.
4. To study HLA expression in unstimulated and stimulated HTCEC with pro-inflammatory cytokines.
5. To examine CXCR4 expression in HTCEC and LMSC
6. To examine cellular migration in HTCEC and LMSC in CXCL12-mediated migration.

5.3 Materials and methods

5.3.1 Human Leucocyte Antigen (HLA) typing for HTCEC

The HTCEC line was HLA typed, the procedure was outsourced to NHS Blood and Tissue Bank (Newcastle), courtesy of Dr Vaughan Carter.

5.3.2 Optimisation of HLA antibodies and treatment with pro-inflammatory cytokines

Anti-human HLA antibodies to Class I (A,B,C) and Class II HLA-DR were optimized for concentrations using HTCEC stimulated with IFN-γ. Anti-human HLA-DP and HLA-DQ antibodies were optimized using peripheral blood leukocytes (PBL) because of very low expression of these antibodies in HTCEC at initial optimisation methods. The concentrations used for the antibodies were 0.05, 0.25, 0.5 and 1.0μg/ml.

Briefly, 100,000 HTCEC were plated in T25 cm^2 tissue culture flasks and incubated in 5% CO_2. On day 2 of culture, at approximately 20-30% confluence, the cells were treated with either interferon-γ (IFN-γ) or tumour necrosis factor-α (TNF-α) or with a combination of both. For optimization of dose and time of stimulation with the cytokines, each treatment lasted for 1 day, 3 days or 5 days. The concentrations used were 0.25ng/ml, 0.5ng/ml and 10ng/ml for IFN-γ, and 5ng/ml, 10ng/ml and 20ng/ml for TNF-α. At cut-off points, cells were detached from tissue culture flasks and resuspended in flow cytometry buffer solution (0.1% (w/v)/FBS/PBS, ready to run through flow cytometry (Figure 5.1).

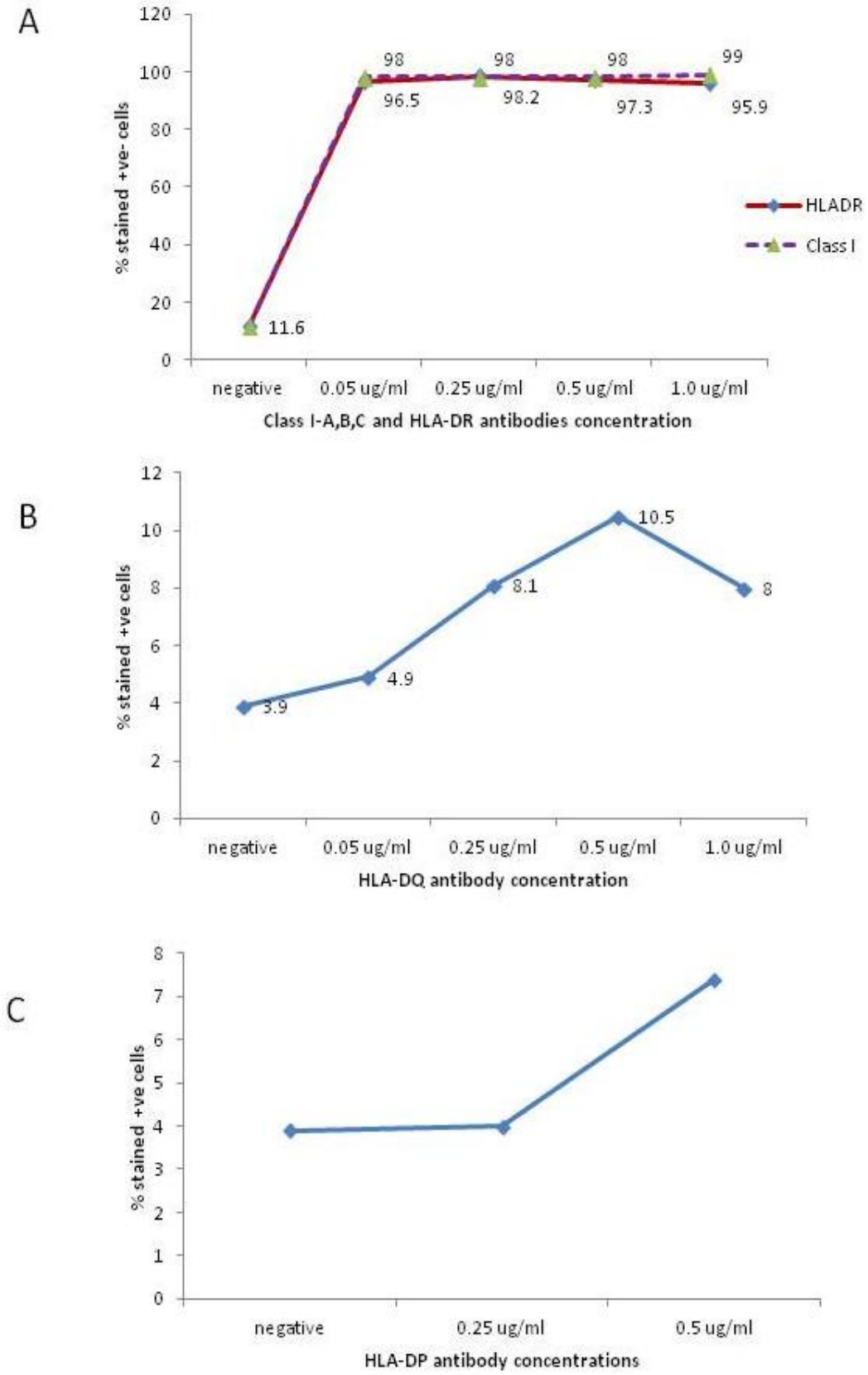


Figure 5.1 Results of HLA antibody titrations. (A) Percentage of stained positive-cells for Class I (HLA-A,B,C) and Class II HLA-DR antibodies using IFN-γ stimulated HTCEC cells. (B) Anti-HLA-DQ and (C) HLA-DQ antibody titration using unstimulated peripheral blood leucocytes.

In the case of peripheral blood leukocytes, titrations for anti- HLA-DQ antibodies were performed at 0.05, 0.25, 0.5 and 1.0µg/ml and for HLA-DP, the concentrations used were 0.25 and 0.5µg/ml. CD45+ve and secondary IgG only populations were used as negative controls to isolate only the signals from HLA-DP antibody (Figure 5.2).

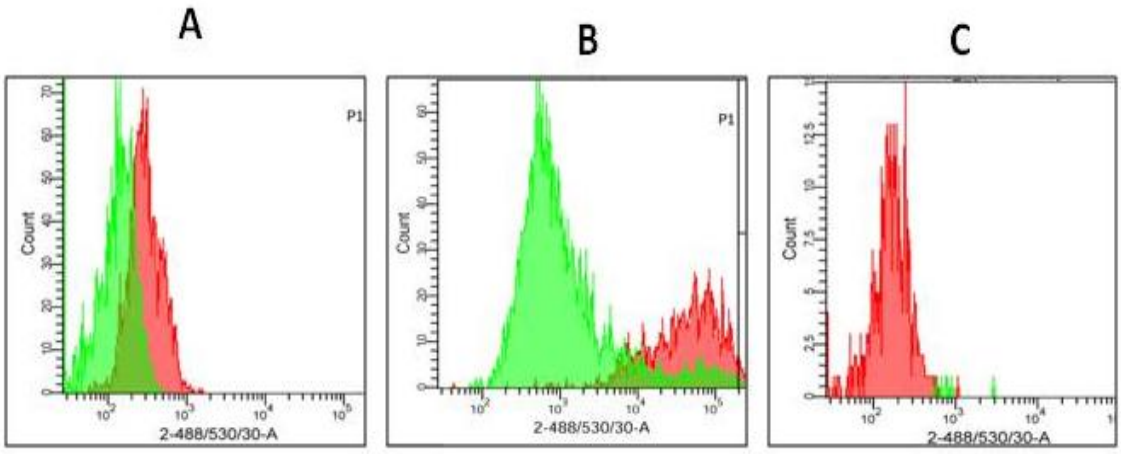


Figure 5.2 Histograms showing detection of anti human HLA-DP antibody in (unstimulated) peripheral blood leucocytes. (A) CD45+ve antibody expression (red) is distinguished from unstained (control) population (green) (B) Anti-HLA-DP antibody expression (red) as a distinct population from CD45+ve cells (green) (C) CD-45+ve population only.

Finally, the individual concentration of IFN-γ and TNF-α which gave the highest expression of HLA antibodies, was used in the combined stimulation experiment and subsequent repeats. This concentration was 10ng/ml, for the duration of 3 days. Direct and indirect immunofluorescence was performed using FACS Canto [BD Sciences] and analysed with FACS Diva software [BD Sciences] to obtain median fluorescence index (MFI). Experiments were performed using two biological replicates to get average MFI values.

5.3.3 Flow cytometry

For screening of purified antibody on HTCEC, cells were detached using cell dissociation buffer and trypsin/EDTA, centrifuged and resuspended in 100µl 2%FBS/PBS. 200,000 cells were added into each tube and stained with primary monoclonal antibody conjugated to Allophycocyanin [R&D system] at 10µl, 20µl and 30µl for antibody optimisation.

For HLA expression, HTCEC were incubated with anti-HLA Class I-A,B,C [BioLegend], HLADR [BioLegend] or HLADQ [Biolegend] monoclonal antibodies in 100 µl 2%FBS/PBS. After 1 hour incubation at 4ºC, cells were washed twice with 2% FBS/PBS and resuspended into 200µl of FBS/PBS before running through FACS Canto II [BD Biosciences] and analysed using a FACS Diva software [BD Biosciences].

For indirect immunofluorescence, cells were stained with primary antibody and incubated for 30 minutes at 4°C. After incubation, cells were washed twice with 2% FBS/PBS and stained with secondary antibody for 20 minutes at 4^0C. After incubation, cells were washed twice with 2% FBS/PBS and resuspended into 200µl of FBS/PBS before run through FACS Canto II [BD Biosciences] and analysed using FACS Diva software [BD Biosciences]. A list of antibodies used in flow cytometry is listed in Table 5.1.

Table 5.1 HLA antibodies used in flow cytometry.

Antibodies	Optimum dilution	Manufacturer
PRIMARY		
Mouse monoclonal FITC anti-human HLA-DR (Clone L243)	1:20	Biolegend
Mouse monoclonal FITC anti-human HLA-A,B,C (Clone W 6/32)	1:20	Biolegend
Mouse FITC anti-human HLA-DQ (Clone HLADQ1)	1:20	Biolegend
Mouse monoclonal Anti-human HLA-DP (Clone HI43)	1:20	Abd Serotec
Mouse monoclonal Allophycocyanin anti-Human CXCR4- (Clone 12G5)	1:20	R & D Systems
SECONDARY		
Goat anti-mouse FITC-conjugated IgG	1:25	Jackson Immuno Research Lab

5.3.4 Chemotaxis assay: Transwell Migration Analysis

For the purpose of optimization for CXCL12 concentration and duration of treatment, 100,000 cells were suspended in 300µl of media and added to the upper chamber of a 5 um pore diameter 24-well plate transwell filter. The lower chamber was filled with 200 µl media without cells but supplemented with CXCL12 at 0ng/ml (internal control), 200ng/ml and 300ng/ml. Treatment was repeated for 3, 5 and 7 hours in 5% CO_2 at 37°C (Figure 5.3).

At the end of incubation, the lower chamber was removed. The inner part of the insert was swabbed with a cotton bud to remove non-migrant cells. Following that, the filters were washed in tap water and stained in haematoxylin for 1 minute, then washed by submerging in Scott's water to help develop the colour. The migrant cells were captured in 5 randomly selected fields under 20X magnification and cells were counted using a light microscope [Nikon Digital Sight–DSFi1]. The optimum concentration of CXCL12 used was 300ng/ ml for 5 hours incubation.

5.3.5 Side Population assay

Following our optimised side population protocol, cells were counted manually using an improved Neubauer haemocytometer [Reichert], 1.0 x 10^6 cells/ ml were resuspended in Hank's media and placed into micro centrifuge tubes [Fisher] to be stained with either Hoechst 33342 dye alone or with Hoechst dye in the presence of ABC-transporter inhibitors. Prior to addition of Hoechst the cells were incubated in the presence of inhibitor for 30 minutes in a 5% CO_2 incubator at 37°C in a MACs Mix rotator [Miltenyi Biotec]. After this, Hoechst dye was then added to both tubes. All reactions were terminated by placing the contents of the tubes in a 15 ml centrifuge tube [Fisher] containing 10 ml ice cold Hank's medium/2% FBS. The centrifuge tubes were then centrifuged at 2,500 rpm for 5 minutes and the cells resuspended with 500µl Hank's medium and passed through a 70µm pore size cell strainer [BD Falcon] and stored on ice prior to FACS analysis.

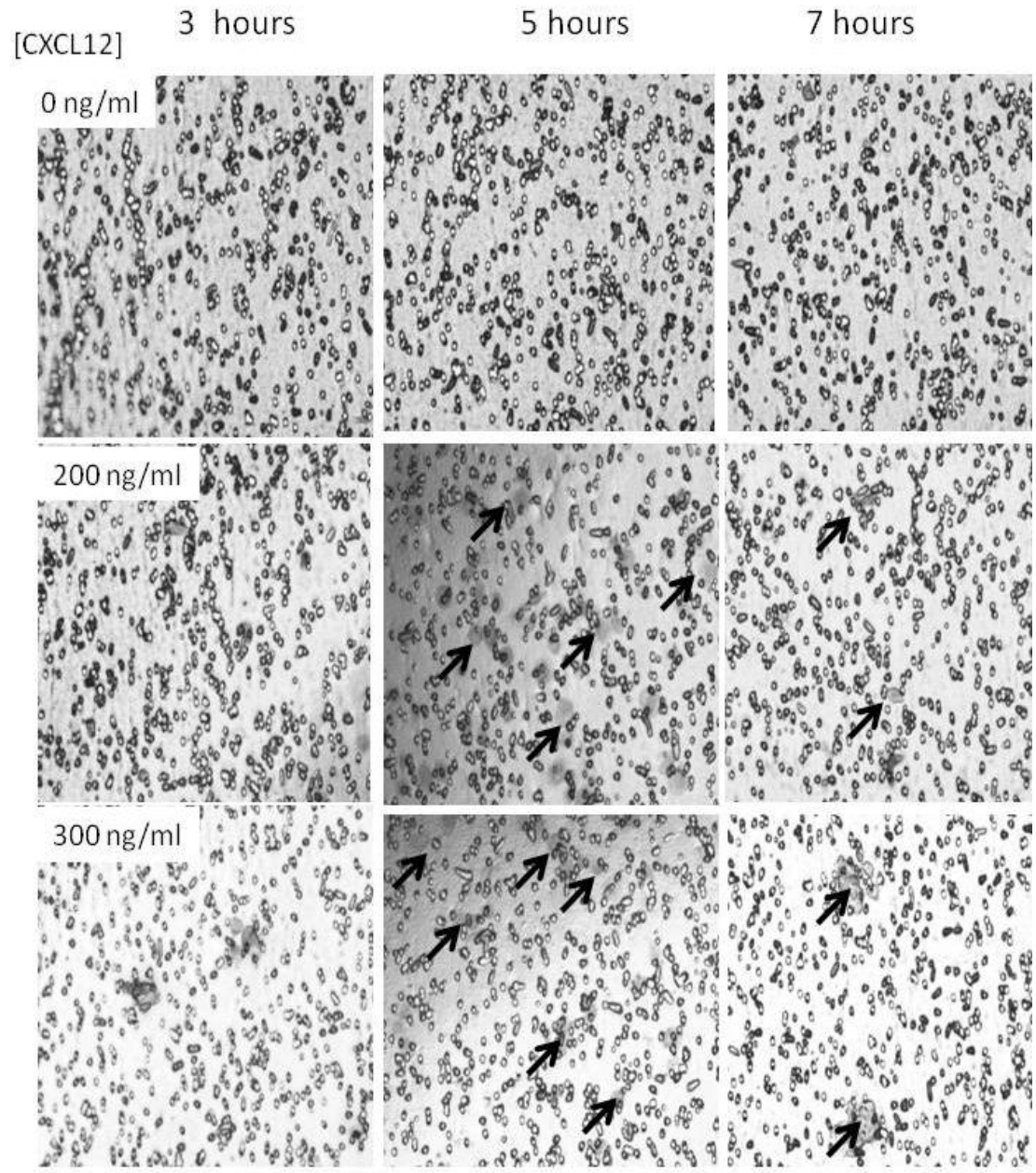


Figure 5.3 Representative images of transwell migration test for chemotaxis assay. Cells were treated with 200 and 300ng/ml for 3, 5 and 7 hours or in DKSFM as negative control (0ng/ml CXCL12). Migrant cells (arrows) were examined under at least 5 high power fields, photographed and analysed.

5.3.6 ICC of LSP and NSP cells

Post sorting, 100 HTCEC from each SP and NSP fractions were directly concentrated onto glass slides. Slides were fixed with cold methanol [BDH Laboratory Supplies] by incubation at -20°C for 20 minutes and then washed with PBS. Cells were permeabilised in 0.3% (v/v) Triton X-100 [Fisher Scientific] in PBS for 10 minutes and then washed again with PBS. Non-specific binding sites were blocked with 5% normal goat serum in PBS for 30 minutes in a humid chamber in the dark. Cells were then

incubated with primary goat anti-mouse monoclonal antibody to ABCB5 (1:100 dilution) [Abcam], CXCR4 (1:100 dilution) [R&D Systems], p63 (1:50 dilution) [Santa Cruz biotechnology] and goat anti-rabbit polyclonal antibodies to ABCG2 (1:100 dilution) [Novus Biologicals] in 0.05% FBS/PBS overnight at 4°C. Cells were then washed 3x5 minutes PBS and incubated with appropriate secondary antibody, either goat anti-mouse antibodies conjugated with FITC or anti-rabbit secondary antibodies conjugated to rhodamine [Jackson Immunology Research Laboratory] at a dilution of 1:25 for 30 minutes in a humid chamber in the dark, then washed with PBS three times, 5 minutes and mounted in vectashield anti-fading media containing DAPI [Vector Laboratories], cover slipped and examined using an Axioplan F microscope [Carl Zeiss]. Negative controls were cells incubated without addition of primary antibody, but with secondary antibody only. Antibodies used in ICC are listed in Table 5.2.

Table 5.2 List of antibodies used in immunocytochemistry.

Antibody	Cellular localisation of antigen	Dilution	Manufacturer
PRIMARY			
Mouse monoclonal anti-human ABCB5 (clone 5H3C6)	Transmembrane	1:100	Abcam
Mouse monoclonal anti-human CXCR4 (clone 44716.111)	Cell surface	1:100	R & D Systems
SECONDARY			
FITC-conjugated secondary goat anti-mouse immunoglobulins	Intracellular	1:25	Jackson Immunology Research Laboratories

5.3.7 Imaging methods and quantification software

For the purpose of examining cells in cultures, plates, and flasks, Axiovert 200M [Zeiss] microscope which allows the performance of transmitted light bright field, phase contrast and epi-fluorescence technique were utilised. Images were then processed using the AxioVision40 version 4.8.2.0 software [Zeiss]. Nikon Digital Sight–DSFi1 camera

and Nikon NIS-Elements D software [Nikon Metrology] for bright field and phase contrast were also used. Fluorescence microscopy was undertaken using Axiovert 200M [Zeiss] for cells in wells and by Axioplan F [Zeiss] for immunocytological slides.

5.3.8 Image quantification

All images were taken using a Nikon Digital Sight–DSFi1 camera and processed with Nikon NIS-Elements D software [Nikon Metrology U.K. Ltd., Derby, UK]. Images were assembled using Adobe Photoshop® CS3 [Adobe Systems. Cell count or cell signal intensities were performed using Image J [Image J version 1.44].

5.3.9 Statistical Analysis

IBM SPSS statistics processor (Version 19) was used for descriptive and statistical analysis. A normal distribution assumes a "bell-shaped" symmetrical data. Quantitative data which were normally distributed were analysed using parametric statistical tests and the equivalent non-parametric tests were used for data which were skewed or outside the normality curve. For 2 groups of independent data, independent t-test or Mann-Whitney U test was used to compare the means between the groups. Results with p values of less than 5% ($P < 0.05$) were considered statistically significant. Data requiring comparison between more than two groups were analysed by one way ANOVA.

5.4 Results

5.4.1 Human Leucocyte Antigen (HLA)-typing and HLA expression in HTCEC

HLA-typing for HTCEC demonstrated expression of both Class I-A,B,C and Class II (HLA-DR and HLA-DQ) antigens:

MHC Class I: **A*02, A*26, B*38, B*56, BC*01, C*12.**

MHC Class II: **DR B1*01, DR B1*13, DQ B1*05, DQ B*06**

We further examined HLA expressions in HTCEC when stimulated with IFN-γ and TNF-α using flow cytometry. HTCEC expressed HLA Class I and II and are upregulated, especially in response to IFN-γ stimulation. We examined HTCEC for HLA expression in unstimulated and stimulated by interferon (IFN)-γ, tumour necrosis factor (TNF)-α and combination of both at 10 ng/ml for 3 days (pre-optimised).

In unstimulated condition, there was constitutive expression of HLA Class I-A,B,C but very low/no expression of Class II antigens compared to unstained (control) population, N=3 (Figure 5.4). Statistics for Median Fluorescence Index (MFI) for expression of HLA in unstimulated HTCEC is presented in Table 5.3.

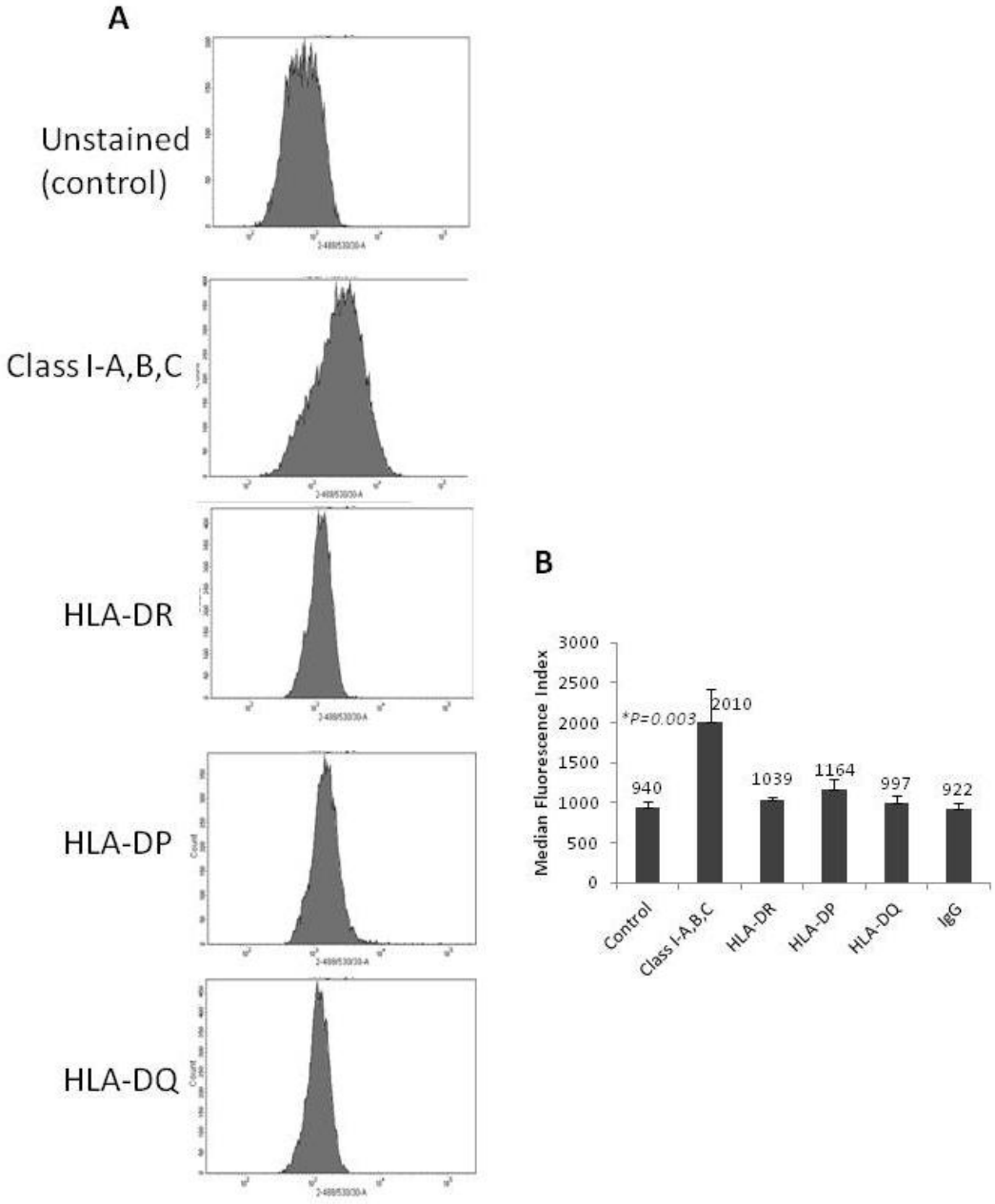


Figure 5.4 FACS analysis of HLA expression in HTCEC of unstimulated cell populations (A) Histograms showing unstained population (control) and cell populations stained with HLA Class I-A,B,C and Class II antibodies. (B) Median Fluorescence Index of HLA expression for control and stained populations without cytokines stimulation (N=3). Mean MFI for Class I was significantly different to control but not for other Class II molecules (O*ne-way ANOVA- Fisher's LSD test).*

Table 5.3 Statistics for HLA expressions in unstimulated HTCEC.

	N	Mean MFI	SD	*P value
Control	3	890.67	211.86	
Class I-A,B,C	3	2171.33	938.68	0.003
HLA-DR	3	1039.0	57.19	0.668
HLA-DP	3	1164.67	238.70	0.433
HLA-DQ	3	997.33	145.55	0.758
IgG	3	922.0	138.17	0.928

*One way ANOVA *(Fisher's Least Significant Difference test)*

HLA expression in HTCEC after cytokine stimulation is presented in Figure 5.5 and Figure 5.6. After stimulation with IFN-γ 10 ng/ml for 3 days, HTCEC expressed high levels of Class I and Class II antibodies compared to unstimulated control samples. The highest expression was observed for HLA-Class I and HLA-DR, followed by lower expression of HLA-DP and very low expression of HLA-DQ. MFI values were 34,560 (Class I-A,B,C), 162,000 (HLA-DR), 46,187 (HLA-DP), 17,250 (HLA-DQ) and 1,135 (Ig G – internal control).

The treatment with TNF-α at the same concentration and duration showed low expressions for all HLA antibodies, except for a slight rise in Class I expression compared to control, followed by HLA-DR. MFI values were 5,906 (Class I-A,B,C), 1,129 (HLA-DR), 1,292 (HLA-DP), 1,217 (HLA-DQ) and 1,175 (Ig G). When treated with combination of IFN-γ and TNF-α at the same concentration and duration, HLA expression for all antibodies were higher than that observed when treated with TNF-α alone, especially in HLA-DR (MFI 9,329) although this did not surpass the levels observed in INF-γ treatment (Figure 5.5). MFI values for HLA Class I (1,450), HLA-DR (9,329), HLA-DP (3,464), HLA-DQ (3,043) and IgG (1,378).

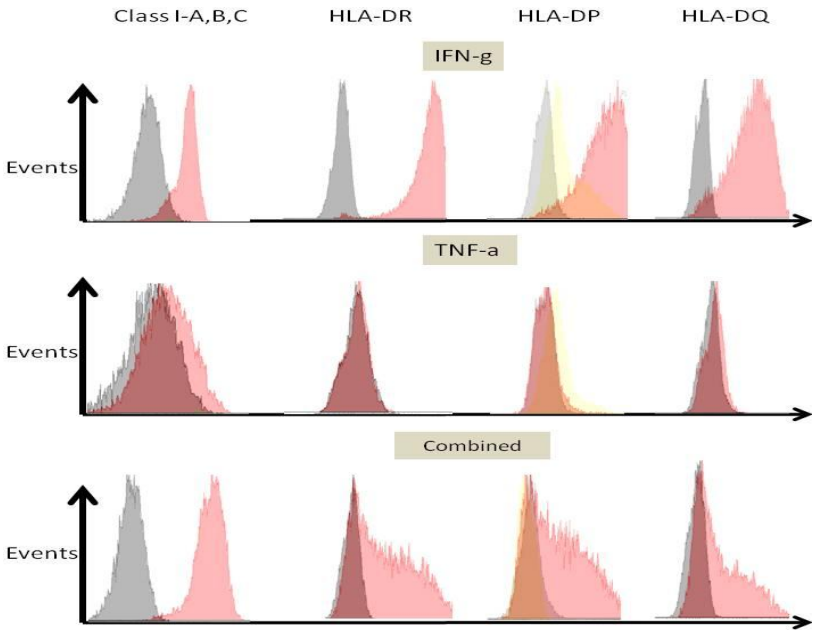


Figure 5.5 Representative FACS histograms out of 3 replicates showing HLA expression of Class I and Class II antigens in HTCEC under stimulation of Interferon-γ, tumour necrosis-α and combined stimulation of both. Cells were stained with FITC-conjugated Class I, HLA-DR, HLA-DQ antibodies and HLA-DP. Negative control for HLA-DP was secondary IgG only *[Light grey – unstimulated cells, red – stimulated cells, yellow – IgG only stimulated cells]*.

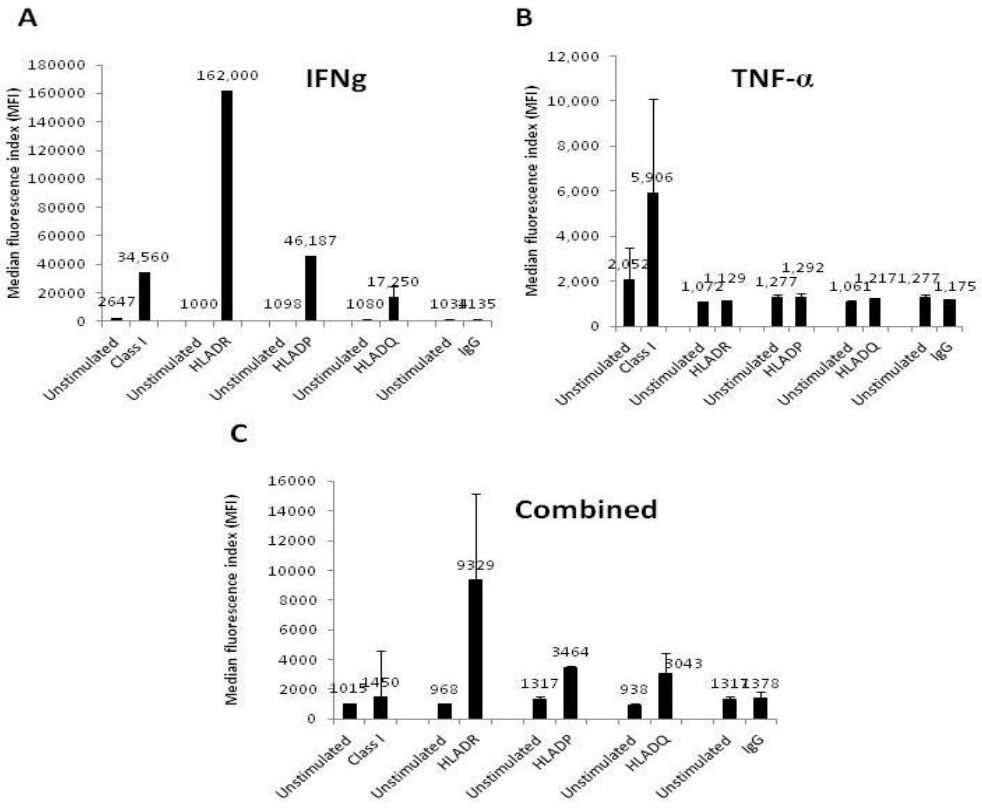


Figure 5.6 Median Fluorescence Index of HLA Class I-A,B,C and Class II expression in HTCEC for control (unstimulated) and stimulated populations in response to cytokines treatment at 10ng/ml for 3 days (N=3). Treatment by (A) Interferon-γ alone (B) Tumour necrosis-α alone (C) Combined treatment. Results are from two biological replicates.

5.4.2 Expression of CXCR4 in HTCEC and LMSC

CXCR4 expression in HTCEC and LMSC using ICC were examined. HTCEC and LMSC expressed anti human CXCR-4 antibody which was localised at the cell surface (Figure 5.7). Bone-marrow derived-MSC and breast cancer cell line MDA-MB-231 were used as positive controls. Staining with secondary IgG only was used as negative control and did not show any staining.

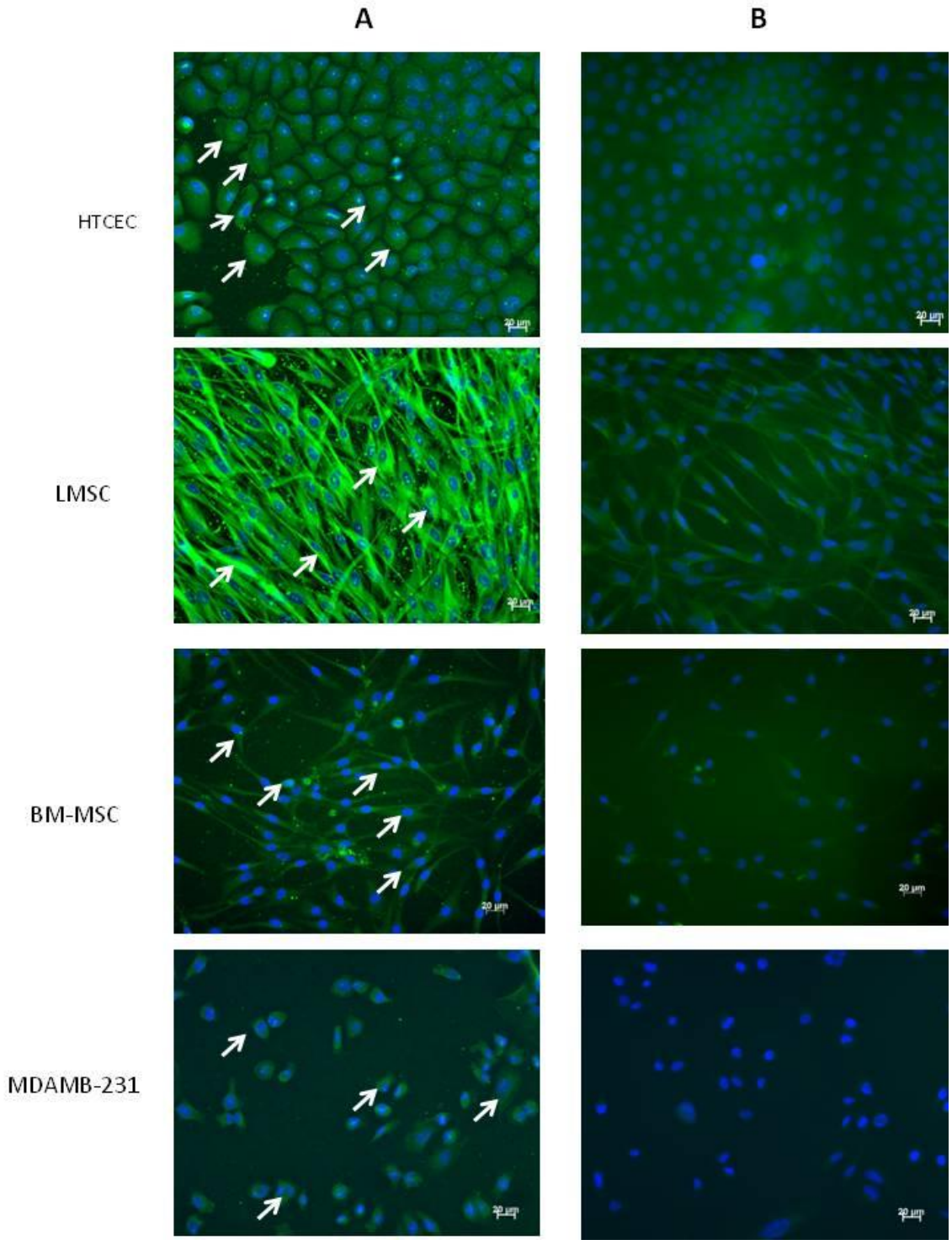


Figure 5.7 Results of immunocytochemical analysis for expression of CXCR4 in unsorted HTCEC and LMSC. Bone marrow-MSC and MDAMB 231 were used as positive control, N=3. Note presence of cells expressing CXCR4 (arrows) in panel A, negative control (secondary IgG) on panel B. IgG-only negative control shows no staining *(DAPI=blue, CXCR4 FITC-conjugated anti-mouse secondary antibody= green).*

Flow cytometry to look for CXCR4 expression in HTCEC and LMSC using unstained cells as negative controls were performed. FACS histogram showed right shift from the unstained (control) population (Figure 5.8). Median fluorescence index (MFI) which is the median or the mid-point of the generalised population of events; for anti-human CXCR4 antibody titration in HTCEC was also performed for objective quantification and showed increased MFI from 258 (no antibody) to 651, for three CXCR4 antibody concentrations used for HTCEC staining LMSC gave higher expression with MFI values increased from 600 (no antibody) to 1652 (Figure 5.9).

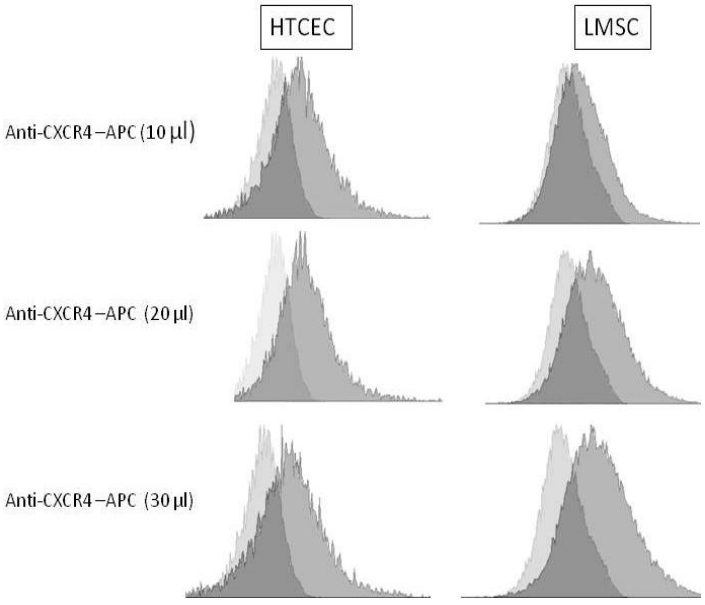


Figure 5.8 FACS histograms for anti-human CXCR4 antibody expression at 10, 20 and 30µl of antibody in unsorted HTCEC and LMSC. *[Light grey -unstained cells (negative control), dark grey – stained cells].*

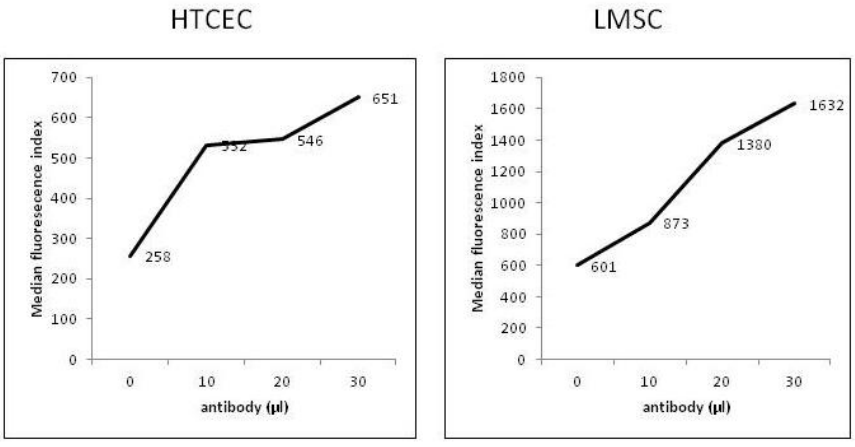


Figure 5.9 Median fluorescence index for expression of anti-hCXCR4 at three antibody concentrations in HTCEC and LMSC.

5.4.3 mRNA expression of CXCL12 in HTCEC and LEC

Real time quantitative PCR was performed to examine CXCL12 expression in HTCEC and LEC. Breast cancer cell lines MDAMB-231 and MCF-7 were used as positive controls. Delta CT values were calculated normalised to GAPDH. Delta Delta CT values were calculated using MCF-7 as a calibrator (reference) sample (Table 5.4).

Table 5.4 mRNA expression of CXCL12 in HTCEC and LEC

	Mean Δ CT	Δ CT SE	ΔΔ CT	Fold change
HTCEC	Undetermined	Undetermined		
LEC	16.5	0.27	11.9	0.00026
MDAMB-231	18.7	0.11	14.1	0.000056
MCF-7	4.6	0.07		

mRNA expression of CXCL12 was not detected in the triplicate samples of HTCEC. A low level of CXCL12 RNA expression was detected in LEC and MDAMB-231 compared to MCF-7.

5.4.4 Chemotaxis in HTCEC and LMSC

In order to examine CXCL12-mediated cellular migration in HTCEC and LMSC, chemotaxis experiments were performed. As a control (background migration) the equivalent number of cells as used for the experimental samples but without addition of CXCL12 in the media was used. There was a minimal/negligible number of migrant cells in the control samples. Following stimulation with 300ng/ml CXCL12 for 5 hours (pre-optimised), HTCEC and LMSC were found to be migratory in response to CXCL12 (Figure 5.10).

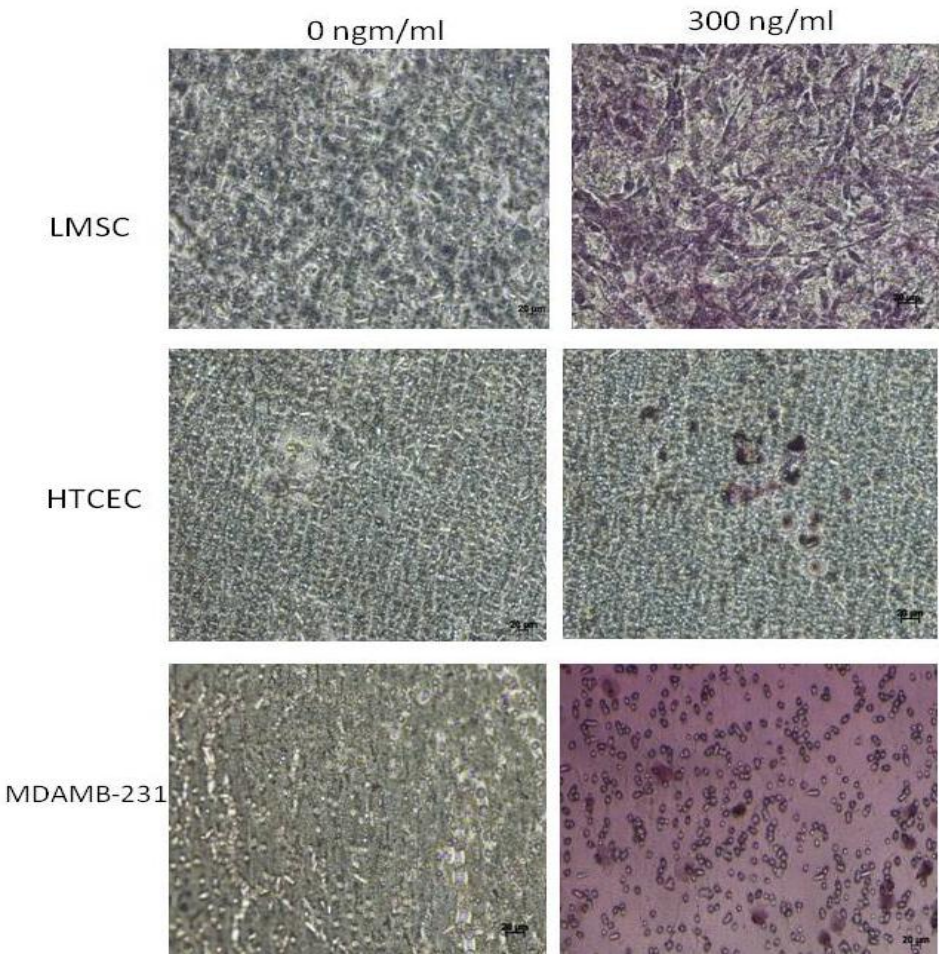


Figure 5.10 Transwell chemotaxis assay. Brightfield images showing cells incubated for 5 hours with 0 ng/ml (negative control or background migration) and presence of migrant cells in transwells with 300 ng/ml CXCL12, 5 hours stimulation in LMSC, HTCEC and breast cancer cell line MDA-MB-231, N=3.

The difference in the means of migrant cells/high power fields in comparison to background migration (negative control) were statistically significant for HTCEC (P=0.009), LMSC (P=0.01) and MDAMB231 (P=0.01) (Figure 5.11).

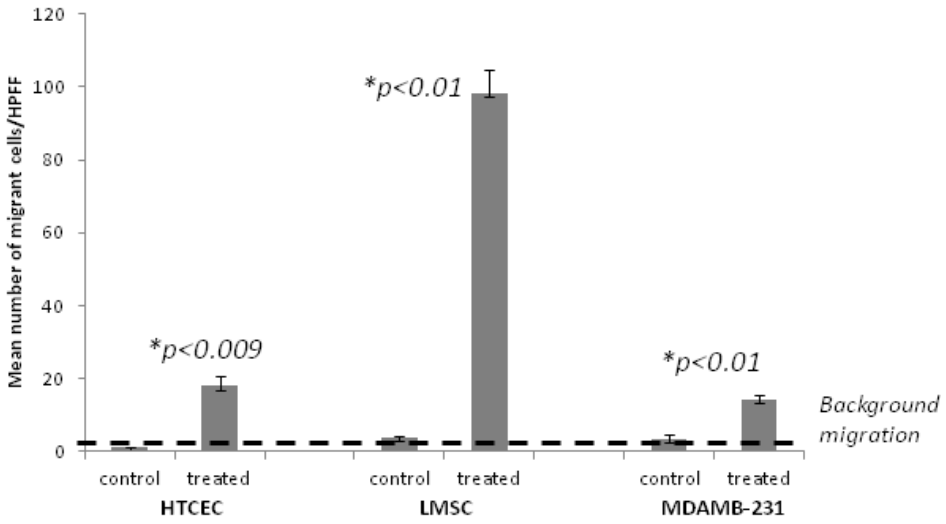


Figure 5.11 Mean number of migrant cells/high power fields from three biological replicates for CXCL12-mediated migration of LMSC, HTCEC and MDAMB231 in comparison to background migration (control). Treatment with CXCL12 ligand was at 300ng/ml for 5 hours. *[* Difference in mean values between control and CXCL12 treated groups (Mann Whitney test)]*

5.4.5 Expression of CXCR4 in SP-sorted HTCEC

To examine the expression of CXCR4 in SP and NSP, HTCEC was sorted into SP and NSP fractions and stained with anti- human CXCR4 antibody (Figure 5.12).

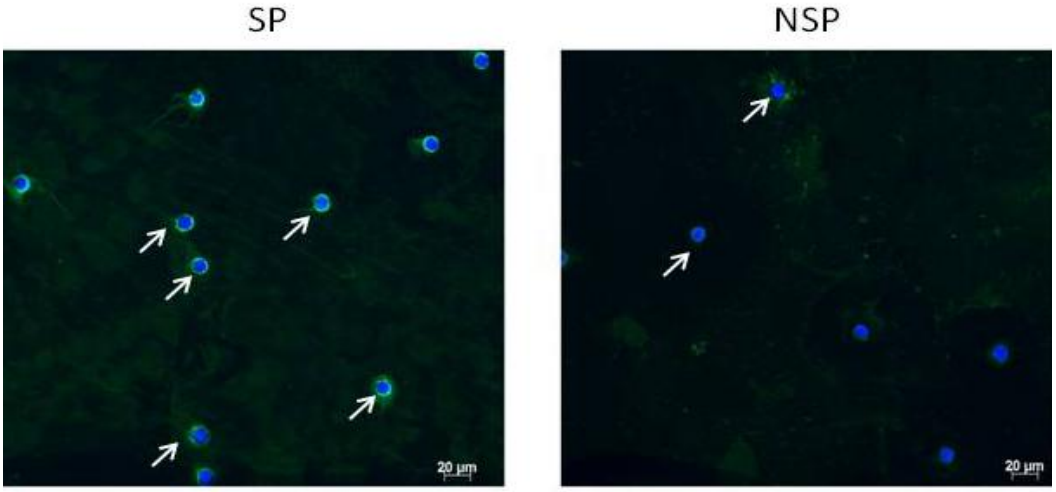


Figure 5.12 Results for CXCR4 expression (arrow heads) in SP cells and NSP of HTCEC [*DAPI=blue, CXCR4 FITC-= green]*.

Following that, fluorescence signal intensities (arbitrary units) were measured in both populations using Image J software. There was a statistically significant difference in means for fluorescence signal intensity in SP= 18.0, NSP=9.0, *P*=0.001 (Table 5.5).

Table 5.5 Statistics for CXCR4 expression in SP and NSP-sorted cells

Group	N	Mean	Std. Deviation	*P* values
SP	46	18.1650	7.21965	0.001*
NSP	39	8.9036	3.48029	

**Independent t-test*

5.5 Discussion

Firstly, HLA typing of HTCEC was performed to study the potential of HTCEC line for LSC transplantation and to investigate further the immunological response in HTCEC. As detailed in the Results section, this cell line expressed HLA class I – A,B,C and HLA Class II molecules –DR and DQ. HLA-DP was not expressed in this cell line.

HLA Class I-A,B,C could be detected in the unstimulated HTCEC population, however, Class II antigens HLA-DR, HLA-DP and HLA-DQ expression was low/minimal compared to control. This finding was similar to previous reports describing Class I expression in unstimulated human corneal epithelial cultures where only Class I antigens were detected (Whitsett and Stulting, 1984; el-Asrar *et al.*, 1989; Iwata *et al.*, 1994).

HLA Class II expression in HTCEC was inducible using pro-inflammatory cytokines stimulation in a dose and time-dependent manner. Interferon (IFN)-γ in particular, upregulated HLA Class I-A,B,C and Class II-HLA-DR, HLA-DP and HLA-DQ expression with the highest expression being HLA-DR and HLA-Class I, followed by HLA-DP and a lesser expression of HLA-DQ.

In human cornea, HLA Class I antigens were readily detected in the epithelium and keratocytes but not HLA Class II antigens. On the other hand, HLA-DR antigen has been detected in dendritic cells at the limbus (Whitsett and Stulting, 1984). Induction of HLA-DR expression by IFN-γ stimulation in human corneal epithelial and endothelial cultures has been demonstrated previously (el-Asrar *et al.*, 1989) (Iwata *et al.*, 1992)'(Iwata *et al.*, 1994). Induction of HLA-DR antigen expression by IFN-γ stimulation is not exclusive to the cornea and has been observed in other unstimulated cell populations such as thyroid epithelium and melanoma cells (Tsujisaki *et al.*, 1987; Yang *et al.*, 1999; Fernández *et al.*, 2008; Yu *et al.*, 2013), liver cells, endothelial cells and human fibroblasts (Maurer *et al.*, 1987; Espana *et al.*, 2004; Harkin *et al.*, 2004a).

Importantly, IFN-γ also induced HLA-DP antibody expression in this HLA-DP-negative cell line. There is limited literature on induction of HLA-DP in non-marrow derived cells or HLA-DP-negative population. However, experimental induction of Class II antigens by IFN-γ stimulation in human endothelial cells and dermal fibroblasts showed comparable expression of HLA-DR in all endothelial cells and dermal fibroblasts followed by a smaller increase in HLA-DP and a negligible level of HLA-DQ expression (Maurer *et al.*, 1987; Espana *et al.*, 2004). In contrast, our study showed HTCEC expressed HLA-DQ after IFN-γ stimulation.

When both IFN-γ and TNF-α were combined, expression of all HLA were very much lower than of IFN-γ alone. The inhibitory effect on IFN-γ induced uregulation of HLA-DR expression has been demonstrated in cultured human gingival fibroblasts (Keiso Takahashi *et al.*, 1994). Here the investigators had proposed the endogenous secretion of prostaglandin E_2 to have immunosuppressive effect on HLA-DR expression. Alternatively, the suppressive effect when both cytokines were combined together has previously been attributed to the pro-apoptotic effect of TNF-α through the Fas signalling pathway in human endometrial stromal cells (Fluhr *et al.*, 2007), human salivary gland cell line (AJ Wu *et al.*, 1994) and human foetal astrocytes (Wosik *et al.*, 2001).

Our results show that HTCEC closely mimics the immunogenicity of human corneal epithelium as demonstrated by Iwata et al, 1992 where HLA Class II (-DR and –DP) expression was inducible by IFN-γ treatment, and a very low but concomitant HLA-DQ expression was related to cellular differentiation. HLA-DP antigen is not naturally

occurring in this cell line but was shown to be inducible under IFN-γ stimulation. IFN-γ stimulated HTCEC expressed HLA-DP and HLA-DQ in monolayer cell cultures making it a unique and excellent model for inflammatory eye conditions, auto-immune diseases or transplantation.

IFN-γ induces expression of HLA class II antigens through the activation of Janus kinase (JAK)/signal transducers and activator of transcription (STAT) of the JAK/STAT pathway (Watling *et al.*, 2008). Janus kinase is a non-receptor intracellular tyrosine kinase that transduces the cytokine-mediated pathway (Figure 5.13). After the cytokine binds to the receptor, JAK phosphorylates and activates downstream proteins involved in the signal transduction pathway.

The JAK autophosphorylation induces an internal conformational change which attracts the phosphorylated STAT proteins to bind to each other forming dimer pairs. The dimer moves into the nucleus, binds to the DNA, and causes transcription of genes. Kamper et al 2012 have also shown involvement of B-associated transcript-3 (BAT3) in the expression of HLA Class II whereby they observed strongly elevated levels of BAT3 transcription in various tumour cell lines and macrophages following IFN-γ treatment (Kämper *et al.*, 2012). Expression of Class II antigens was also linked with the expression of a Class II transactivator detected within hours of IFN-γ treatment of endothelial cells (Fritchley *et al.*, 2000).

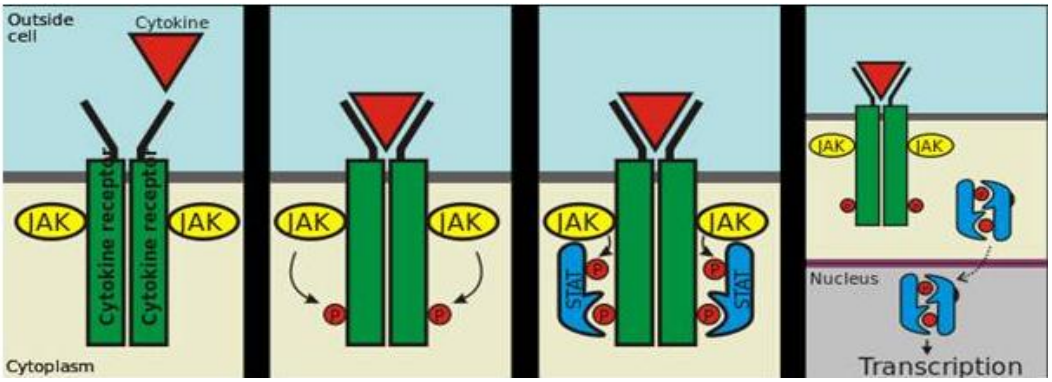


Figure 5.13 The key elements in the JAK/STAT pathway. The JAK-STAT system consists of three main components: (1) a receptor (green), which penetrates the cell membrane (2) Janus kinase (JAK) (yellow), which is bound to the receptor and (3) Signal Transducer and Activator of Transcription (STAT) (blue), which carries the signal into the nucleus and DNA. Red dots -phosphates. *(Image from Peter Znamenkiy - All right released, donated to public domain. Downloaded on 08.01.2015).*

In the cornea, LC, which are professional antigen presenting cells (APC) reside in the limbal region, but migrate to the cornea in inflamed conditions (Jerry Y Niederkorn, 1995). LC trigger HLA class II antigen presentation pathways by sensitising the host's alloreactive T cells to recognise foreign antigens on the grafts. Indeed this is an important mechanism to explain activation of T lymphocytes in inflammatory eye disease such as herpetic keratitis (Yang *et al.*, 1998) and corneal allograft rejection (Khaireddin *et al.*, 2003; Joseph *et al.*, 2004).

However, there are evidences that LC are also present in the central cornea (Hamrah *et al.*, 2002; Hamrah *et al.*, 2003) and these dendritic cells are capable to express Class II antigens after surgery and drain into the regional lymph nodes of the allografted hosts.

The role of corneal epithelial cells to function as APC and produce T-cell response needs further investigation. It would also be useful to study whether Class II-bearing corneal epithelial cells associate with other signalling factors like intercellular adhesion molecules (ICAM) to interact with APC. The role of ICAM-1 and its ligand leukocyte function –associated antigen-1 (LFA-1) has been implicated in inflammation, immunologic response and wound repair. For example, upregulation of ICAM in activated cornea fibroblasts caused adhesion of eosinophils and subsequent fibroblast damage in severe allergic corneal ulcers (Okada *et al.*, 2005).

Chemokine CXCL12 ligand secretion in LMSC has been published previously (Polisetti *et al.*, 2010) and in corneal fibroblast (Wilson *et al.*, 2014). However, our results showed that mRNA expression of CXCL12 in the HTCEC cell line was not detected by q-RT-PCR, and the fold change expression in LEC was low compared to the breast cancer cell line MCF-7. CXCL12-mediated cellular migration in HTCEC could probably not be directed due to the unsupported expression of CXCL12 in this cell line, as opposed to LMSC. This finding alludes to the role of other potential factors being in play to direct cellular migration for corneal repair.

MSC are non-haemopoietic stem cells which have regenerative ability and can differentiate into cell lineages of mesenchymal origin i.e. adipocytes, chondrocytes and osteocytes. In our project we have successfully isolated and expanded LMSC which fulfil the minimum criteria defining them as human MSC. Bone-marrow-derived MSC has shown excellent anti-inflammatory and wound healing properties when introduced

to corneal injury models in animal studies (Ma *et al.*, 2006; Augello *et al.*, 2007; Omoto *et al.*, 2009; Jiang *et al.*, 2010; Hsu *et al.*, 2012; Jia *et al.*, 2012). Bone-marrow-derived MSC has been investigated in the repair of corneal damage (Ma *et al.*, 2006), and the proposed mechanism is suppression of new vessel formation and inflammation post-transplantation. The role of human LMSC for transplantation and ocular regeneration is an avenue to be further explored.

Previously we described HTCEC cell line contains side population (SP) cells which proved to be enriched for expression of LSC markers ABCG2, p63 and the stem cell antigen Sox2 using an optimised human LSP protocol (Shaharuddin *et al.*, 2013c). SP phenotyping can lead to further functional characterization of putative stem cells as demonstrated in both humans and animals. Studies have shown that LSP cells had features consistent with stem cells (Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Umemoto *et al.*, 2005; Umemoto *et al.*, 2006; Selver *et al.*, 2011). In addition, here we demonstrated evidence that SP-sorted HTCEC showed higher expression of CXCR4 compared to the NSP fraction.

Corneal wound healing can be alluded to involve new vascular formation and cellular migration. This is possibly regulated by pro-inflammatory cytokines via the CXCR4 - CXCL12 axis; similar to that observed to occur in bone marrow-MSC migration for bone regeneration (Feng *et al.*, 2014) and MSC migration to sites of injury in cord blood (Deshpande *et al.*, 2013). Additionally, Polisetti et al has demonstrated vascular endothelial growth factor (VEGF) was also found in abundance in LMSC (Polisetti *et al.*, 2010).

5.6 Conclusion

To our knowledge, this is the first study to provide data on HTCEC HLA-typing which is an important step to study immunogenicity in this cell line. HTCEC expressed HLA expression when stimulated with pro-inflammatory cytokines which suggests that HTCEC is a suitable candidate to be an *in vitro* and *in vivo* model to study corneal inflammation, auto immune diseases and allograft corneal transplantation. Both HTCEC and the limbal sourced-LMSC exhibited cellular migration in response to CXCL12 ligand stimulation which is an important property for angiogenesis and cellular regeneration. SP-sorted HTCEC also contained a significant number of CXCR4-positive cells which may also be useful for studying stem cell migration.

Chapter 6 GENERAL DISCUSSION

6.1 Summary of aims

At the beginning of this study, these specific aims were outlined;

- To characterise LEC and HTCEC by LSC markers and stem cell antigens
- To isolate human LMSC
- To study growth and proliferation of LMSC on AM and characterise LMSC using limbal markers and stem cell antigens.
- To isolate and identify LSP phenotypes in both primary LEC and HTCEC
- To characterise LSP cells using LSC markers and stem cell antigens
- To study the constitutive HLA expression in HTCEC both unstimulated and stimulated by pro-inflammatory cytokines
- To examine CXCR4 expression in primary HTCEC and LMSC in both SP and NSP cells of HTCEC
- To study chemotactic potential of HTCEC and LMSC in CXCL12-mediated cellular migration

6.2 Summary of outcomes

1). HTCEC was comparable to primary LEC in the expressions of common molecular markers for LSC and stem cell antigens.

The challenges in LSC biology and its applications revolve around the identification, isolation and expansion of the stem cell phenotype. Characterisation of LSC is made more difficult with absence of specific LSC markers.

2). MSC isolated from the limbus "LMSC" fulfils the minimum requirement defining them as human MSC, and they characterised for LSC markers.

This study demonstrates a simple way of isolating LMSC from primary corneal tissues by serial trypsinisation alone. The cells grew in MSC-growth promotion media in 5% O_2, 5% CO_2 incubator at 37°C. LMSC have the characteristics of human MSC being plastic adherent and were positive for expressions of cell surface antigens CD44, CD90, CD105, CD146 and CD166. They also had low/no expression for the haematopoetic

commitment markers CD19 and CD45. LMSC have the tri-lineage commitment to be osteogenic, adipogenic and chondrogenic.

3). LMSC on AM tissue construct showed expression of important limbal markers, in addition to rapid growth without the use of feeder layers.

The advantages of these culture conditions include shorter duration of cell cultures (5-7 days) compared to 10-14 days for using outgrowths of limbal explants or using cell suspension from primary tissues. LMSC characterised for p63, ABCG2 and ABCB5.

4). HTCEC has shown a side population which proved to be enriched for LSC.

The fact that ABC transporters are located transmembranously allows a prospective strategy for LSC enrichment using SP protocols. The sensitivity of SP assay to different species and cell types makes standardisation of SP protocols mandatory to ensure reliability and reproducibility of SP data. This will ultimately result in better interpretation of SP results and enable more accurate comparison between data generated in multiple studies.

5). LSP cells were characterised by common limbal markers, stem cell antigen Sox2 and also ABCB5.

Here we provide the first data on ABCB5 characterisation in LSP cells which support the importance of this marker as an LSC marker.

6). HTCEC was HLA-typed, and they constitutively expressed HLA Class I antigens.

7). HLA Class II expressions were inducible and upregulated in HTCEC after stimulation with pro-inflammatory cytokines.

Expression of HLA Class II molecules in HTCEC had similar trends as observed previously in primary human corneal cultures.

8). HLA-DP expression was inducible and upregulated in HTCEC

From the results of HLA typing, it was noted that this cell line does not possess allelles for HLA-DP, but the expression was inducible after stimulation with IFN-γ. Due to the limited published data on induced HLA-DP expression, this provides new insight to Class II antigen-specific immunological response in the cornea and also other systems.

9). CXCR4 was expressed in HTCEC and LMSC and both these cell types were migratory in response to CXCL12 ligand stimulation.

10). The high expression of CXCR4 in SP cells is useful to study CXCL12-mediated cellular migration of LSC for re-epithelisation during cornea injury.
As an example, CXCR4 and its specific ligand CXCL12 has been implicated in the "homing mechanism" to sites of injury in the renal system. This is an important mechanism for LSC to migrate to the site of corneal injury for repair and reconstruction.

The main outcomes of this thesis are graphically represented in Figure 6.1.

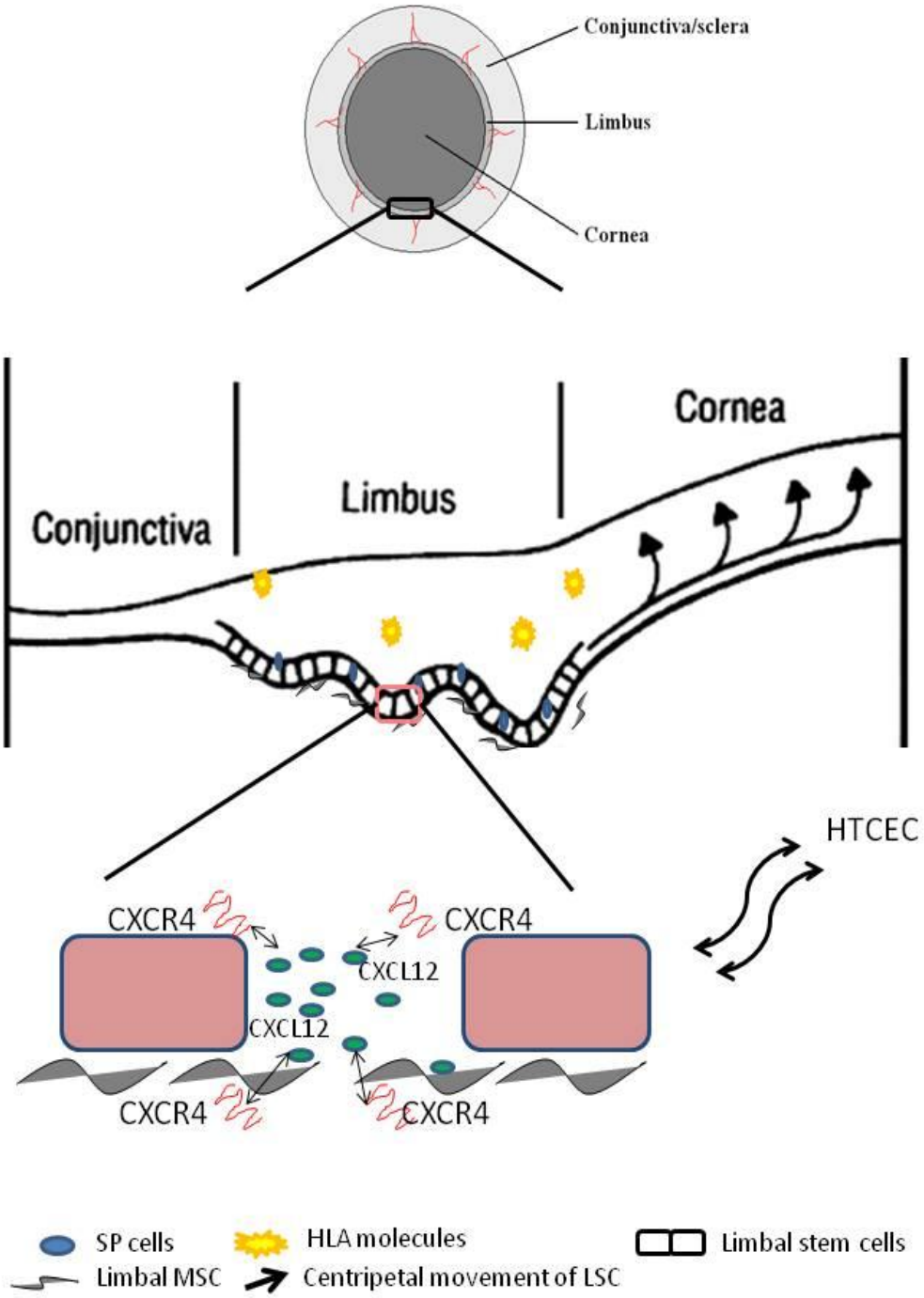


Figure 6.1 Graphical presentation of important outcomes. Limbus is hypothesised to be the location of LSC (i) LEC and HTCEC contain SP cells that characterised for LSC markers and stem cell antigens (ii) LMSC are multipotent stem cells sourced at the corneal limbus which characterised for LSC markers(iii) HLA molecules are also abundant at the limbus and the Class II expression is inducible following IFN-γ stimulation (iv) LMSC express LSC markers (v) LMSC and HTCEC express CXCR4 and migrate in response to CXCL12.

6.3 Implications of the project and recommendations for future work

A major requirement for successful LSC transplantation is viable isolation of these cells by identification of its molecular markers. It has been shown that there are no specific markers which mark LSC. Thus a panel of positive and negative molecular markers would be necessary for isolation of LSC.

An alternative approach to the reliance on absent/presence of surface markers would be the utilization of a stem cell enrichment strategy for LSC such as SP discrimination assay. This would also allow for the isolation of more stem cells for transplantation. The use of SP assay with an immortalized corneal cell line that contains LSC would be useful in addressing questions about LSC biology.

The LSP discrimination assay could be used as an enrichment or purification method to further characterise and/or enrich for LSC. Studies have shown that LSP cells were enriched for stem cell activity had an increased colony forming and proliferative capacity (Budak *et al.*, 2005; Cintia S. de Paiva *et al.*, 2005a; Akinci *et al.*, 2009). In addition, SP cells represent a source of stem cells that does not rely for identification on the use of a panel of defined stem cell markers and lineage negative markers and can be sourced from the host if healthy corneal tissue remains.

The LMSC-AM tissue engineered construct has great potential for ocular surface regeneration. Currently there are no clinical trials using LMSC for LSC transplantation despite many clinical trials using MSC in other organs such as in the lungs, intestine and liver. LMSC also have immunomodulatory effects e.g. trophic and growth factors, and these cells can be expanded readily in culture, stored frozen and brought back up in culture and are therefore a potential cellular tool.

The challenge would be to achieve an effective SP cells expansion system. The problems exist in the low yield of fresh SP-sorted cells, and absence of molecular LSC markers. SP cells need to be grown successfully to be transferred on an appropriate scaffold system for clinical use. It is reasonable to believe that a stem cell-enriched population of cells might proliferate rapidly in appropriate and supportive culture conditions. More evidence to address the variations in percentage of SP populations across mammalian species needs to be collected, which might be directly or indirectly related to SP protocols. However, culture confluence can be a tool for isolation of higher numbers of SP. SP cells could readily expand in numbers and colony size

compared to NSP. Further, reduced cellular confluence and hypoxic culture condition were two factors which might be strategies for SP cellular expansion.

Roles of ABCB1, ABCG2 and ABCB5 transporters with LSP and possible mechanisms of signalling factors have been investigated in other systems and shown to confer the SP phenotypes. The importance of ABC transporters responsible for the SP phenotype in LSC has been previously investigated. Here we have shown the inhibition of efflux activity of SP cells by verapamil (which mainly blocks ABCB1 and ABCG2). We have also demonstrated mRNA and protein expression of ABCG2, ABCB1 (mRNA) and also ABCB5 expression by PCR and ICC in HTCEC and LMSC. Further investigations with knock out animal models would reaffirm the role of these genes in LSP phenotypes, and their links to LSC maintenance and regeneration.

To our knowledge, this is the first study to provide data on HTCEC HLA-typing which is an important step to study immunogenicity in this cell line. HTCEC also expressed similar trend of inducible HLA expression as demonstrated in previous human corneal cultures when stimulated with inflammatory cytokines, especially by IFN-γ. On top of that, there is a limited knowledge in previous literature on inducible HLA-DP expression, such as provided in our results. This alludes to the suitability of HTCEC for LEC replacement and as a robust model for corneal inflammations, auto immune diseases and allograft corneal transplantation.

Both HTCEC and the limbal sourced-LMSC exhibited cellular migration in response to CXCL12 ligand stimulation in the presence of CXCR4 receptors. However, it was demonstrated that LMSC was more responsive than HTCEC, which could attribute to the presence of CXCR4 and secretion of CXCL12 in LMSC as previously reported. Conversely, although CXCR4 was expressed by HTCEC, our results showed this cell line did not exhibit CXCL12 gene expression. This finding suggests the role of other potential factors such as inflammatory mediators being in play to direct cellular migration for corneal repair.

The recruitment of adult stem cells to the site of injury for cellular repair, loosely termed as the “homing” mechanism has been observed in the haematopoietic (Deshpande *et al.*, 2013) and renal systems and is regarded as essential for tissue engraftment (C. S. de Paiva *et al.*, 2005b). Using knock out gene models or neutralizing

antibodies to CXCR4, or CXCR7 receptor, a novel endothelial receptor for ligand CXCL12, we could further explore into their role in corneal epithelial regeneration in response to injury as demonstrated in the renal system (C. S. de Paiva *et al.*, 2005b). The role played by adhesion molecules in this signalling mechanism is also an attractive area to investigate.

Due to existing problems in donor availability and donor-to-donor-variability, HTCEC is an attractive consistent and a reproducible source of corneal stem cells and can be used for refinement of methods or optimisation protocols and useful for experiments requiring large number of cells. Although cell lines could not be more superior to donor samples, the use of cell lines have proven to be economical in term of reducing animal experiments and ultimately the results could be translated for therapeutic potentials. The outcomes of this project have shown HTCEC has remarkable biological properties as a robust model for further understanding of LSC biology.

Future studies will focus on the proliferation and maturation of HTCEC and the limbal derived-MSC on biological and non-biological substrates for ocular surface reconstruction such as *in vivo* transplantation for LSCD. Alternatively, to study the usefulness of these cells to address a basic problem such as cell signalling and the factors involved in LSC biology. Bone-marrow-derived MSC, these cells have shown excellent anti-inflammatory and wound healing properties when introduced to corneal injury models in animal studies (Ma *et al.*, 2006; Augello *et al.*, 2007; Omoto *et al.*, 2009; Jiang *et al.*, 2010; Hsu *et al.*, 2012; Jia *et al.*, 2012) and in the repair of corneal damage (Ma *et al.*, 2006). The proposed mechanism is suppression of new vessel formation and inflammation post-transplantation.

Further investigation into the immunological factors in the cornea that allow tolerogenic potential of the organ to respond to ocular antigens is necessary to exploit corneal immune privilege to its full advantage. Identification of inflammatory molecules and apoptotic markers, their signalling pathways, and role of HLA matching, are some of the strategies to promote the survival of whole corneal and LSC transplantation. Tolerance towards allogeneic organ grafts represents one of the major aims of transplantation medicine.

Ex vivo expanded LSC transplantation has been proven to be able to reconstruct the ocular surface in LSCD eyes using a biological scaffold system which provides transfer of proliferative cells to the target site. Alternately, producing a scaffold which incorporates stem cell-enriched population from a corneal cell line could be the answer to ensure uniformity in the quality of the grafts. However, these methods need to be further refined to include protocols to assess tissue viability, the quality of tissue constructs and safety assessment. Needless to say, the quality of cells in the tissue construct is vital to ensure permanent tissue repair and successful engraftment. In order for LSC to be successfully translated into favourable clinical outcomes, a good manufacturing practice (GMP)-compliant protocol would be a positive step towards achieving high quality tissues to fulfil the requirements for clinical transplantation.

Currently, it is still unclear how restoration of the damaged ocular surface takes place after LSC transplantation. It is very unlikely that it is due to replacement of stem cell numbers alone. Studying the LSC fate in different aetiologies of stem cell deficiency and the types of tissue transplantation would be a future direction to explain the process of cellular restoration. At present, there is no consensus on LSC fate in different types of transplantation (Shimazaki *et al.*, 1999; Daya *et al.*, 2005; Djalilian *et al.*, 2005) such as in penetrating keratoplasty, alone or in combination with limbal allograft transplantation, or in the case of *ex vivo* LSC transplantation. The problem for case to case comparison exists in multiple diagnosis of LSCD, tissue transplantation types, methods for cell tracking to determine the cellular fate, and non-standard cut off points for cellular analysis to take place.

Molecular mechanisms regulating the immune plasticity of corneal stem cells could be relevant in clinical setting and may explain the differences in the outcomes between murine and human systems. Thus understanding these mechanisms will contribute to the development of better therapeutic approaches for transplantation of not only the cornea, but other organs as well.

The role of CXCR4/CXCR7 and their unique ligand CXCL12 in the endothelial cells has been previously investigated (So and Epstein, 2004). It would be interesting to study the roles of these receptors to address the process of angiogenesis and corneal vascularisation which occur in LSCD. Corneal conjunctivalisation is due to the encroachment of vascularised tissue into the cornea which leads to blindness and

ocular pain. The role of vascular endothelial growth factor receptors and its inhibitors to prevent vascularisation is also an interesting area to investigate in the quest for improved outcomes of cellular therapy for LSCD.

During the course of this project, the field of LSC has dramatically advanced with a lot of research on basic and fundamental sciences and clinical application. LSC has acquired a niche in cornea biology and drawn tremendous interest from research scientists. Publications in this field have soared remarkably which draws the necessity for more translational approach to address the gap in the management of LSCD and ocular surface regeneration. This project offers strategies to improve the outcome of LSC transplantation by offering a cellular selection strategy, alternative cellular tools and also a robust cellular model for further understanding of LSC biology.

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APPENDIX

Appendix 1

Newcastle University

24 April 2013

Dr Annette Meeson
Institute of Genetic Medicine
International Centre for Life

Faculty of Medical Sciences

Newcastle University
The Medical School
Framlington Place
Newcastle upon Tyne
NE2 4HH United Kingdom

Professor Michael Whitaker
FIBiol FMed Sci
Dean of Research & Innovation

FACULTY OF MEDICAL SCIENCES: ETHICS COMMITTEE

Dear Annette

Title: Characterisation of limbal corneal stem cells
Application No: 00617_1/2013 (Amendment)
Start date to end date: 01 December 2012 – 26 September 2015

On behalf of the Faculty of Medical Sciences Ethics Committee, I am writing to confirm that the ethical aspects of your proposal have been considered and your study has been given ethical approval.

The approval is limited to this project: **00617_1/2013**. If you wish for a further approval to extend this project, please submit a re-application to the FMS Ethics Committee and this will be considered.

During the course of your research project you may find it necessary to revise your protocol. Substantial changes in methodology, or changes that impact on the interface between the researcher and the participants must be considered by the FMS Ethics Committee, prior to implementation.*

At the close of your research project, please report any adverse events that have occurred and the actions that were taken to the FMS Ethics Committee.*

Best wishes,

Yours sincerely

M. Holbrough

Marjorie Holbrough
On behalf of Faculty Ethics Committee

cc.
Professor Michael Whitaker, Dean of Research & Innovation
Ms Lois Neal, Assistant Registrar (Research Strategy)

*Please refer to the latest guidance available on the internal Newcastle Biomedicine web-site.

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Appendix 2

HTA
Human Tissue Authority

Licensing Number	**12534**
Licensed Premises	**Institute of Genetic Medicine** **International Centre for Life** **Newcastle University** **Newcastle upon Tyne** **NE1 3BZ**
Licence Holder	**Newcastle University**
Designated Individual	**Professor Andrew G Hall**

This licence is granted under Section 16 (2) (e) (ii) of the Human Tissue Act 2004.

This licence authorises the storage of relevant material which has come from a human body for use for the following scheduled purposes:

- Determining the cause of death
- Establishing after a person's death the efficacy of any drug or other treatment administered to him
- Obtaining scientific or medical information about a living or deceased person which may be relevant to any other person (including a future person)
- Public display
- Research in connection with disorders, or the functioning, of the human body
- Clinical audit
- Education or training relating to human health
- Performance assessment
- Public health monitoring
- Quality assurance

The licensed activity should be carried on only at the licensed premises specified above, and under the supervision of the Designated Individual.

This licence is subject to the conditions set out in the Annexes accompanying this licence as may be subsequently varied pursuant to an application under paragraph 8 of Schedule 3 to the Human Tissue Act 2004.

This licence is valid from the date specified below and will remain in force until revoked.

.....
Baroness Diana Warwick
Chair

.....
Craig Muir
Chief Executive

Dated **22 June 2011**

HUMAN TISSUE AUTHORITY HTA

An independent statutory regulator sponsored by DH Department of Health

Appendix 3

Central Manchester and Manchester Children's University Hospitals NHS Trust

Material Transfer Agreement, governing supply of biological Relevant Material by Corneal Transplant Service Manchester Eye Bank (" CTS-MEB ") for Research Purposes.

Requestor's Name (block letters please): ANNETTE MEESON

Address: University of Newcastle Upon Tyne, Kings Gate Newcastle Upon Tyne NE1 7RU **Tel: 0191 241 8856** **E-mail: Annette.meeson@ncl.ac.uk**	**CTS-MEB contact:** Dr. Isaac Zambrano. Designated Individual Corneal Transplant Service Manchester Eye Bank Manchester Royal Eye Hospital M13 9WH **Tel:** 0161 2765623 **FAX:** 01612765610 **Email:** isaac.zambrano@cmft.nhs.uk

BACKGROUND:-

(A) The CTS-Manchester Eye Bank, through the Designated Individual ("DI") for Human Tissue Authority ("HTA") Licence Number 11056, is the custodian of certain Relevant Material as defined in the Human Tissue Act 2004 ("HT Act").

(B) The Recipient, named in the Appendix, wishes to use Relevant Material to conduct research in the Recipient's laboratories.

(C) The DI is willing to supply the Recipient with the required Relevant Material for an agreed period to conduct the Research under the terms and conditions of this Agreement.

MATERIAL:- Corneal Tissue.

Terms and conditions:

1. In this Agreement reference to (a) "Relevant Material" means such tissues or other materials agreed to be supplied by CTS-MEB to the Recipient when available in writing (or any part) and shall include any and all derivatives of the same; (b) "Purpose" means such proposed research use(s) for the applicable relevant material as may be agreed by CTS-MEB and the Recipient when agreed in writing; and (c) "Completion" means the anticipated date for completion of the applicable Purpose in relation to the applicable Relevant Material as may be agreed by CTS-MEB and the Recipient in writing. The DI will supply the relevant Material on a non-exclusive basis after this agreement is entered into. The quantity and frequency of delivery of Relevant Material specified by the Recipient are for guidance only, and will not be binding on the CTS-MEB DI or the Central Manchester Foundation Trust (CMFT). All future correspondence pertaining to the Relevant Material

and the Research should be addressed to the DI. If the DI changes, the CMFT undertakes to inform the Recipient.

The Relevant Material and any related information ("Confidential Information") disclosed by CTS-MEB will remain the property of CTS-MEB . The Recipient will act as the custodian for the Relevant Material and, where necessary, will provide evidence of:

(a) Ethics Approval Committee favourable opinion for the Research; and/or

(b) an HTA licence;

to confirm that the storage and use of the Relevant Material is lawful. This Agreement shall not be construed to grant any right or licence in or to any intellectual property which is the property of CTS-MEB . Upon Completion, the Recipient shall (at the request of CTS-MEB and in accordance with all appropriate guidelines and legislation) dispose of the Relevant Material and keep records which will be made available at the request of the CTS-MEB.

3. The Recipient will use the Relevant Material for the Purpose and for no other purpose. The legal responsibility for storage and use falls on the Recipient rather than CMFT. The Recipient agrees that it shall not transfer (directly or indirectly) the Relevant Material, nor disclose the Confidential Information to any party other than those of its employees who have a need to use the Relevant Material or know of the Confidential Information in the carrying out of the Purpose. The Recipient will ensure that each of its employees who need to use the Relevant Material and know of any Confidential Information is advised of and accepts the terms of this Agreement. No modification of the Relevant Material or use other than that expressly specified in this Agreement will be permitted without the prior written consent of CTS-MEB .

4. The Relevant Material and the accompanying Information is supplied in confidence. On CTS-MEB's request, Recipient shall delete any Confidential Information that unintentionally be received with the tissue. The confidentiality obligations under this Agreement shall continue in force notwithstanding the completion of the Purpose.

5. Notwithstanding the provisions of clause 4, the Recipient undertakes to comply with the Data Protection Act 1998 (as amended) where applicable to the transfer of the Relevant Material and (where relevant) to observe the recommendations of "The Caldicott Committee : Report on the Review of Patient-identifiable Information" and such other relevant reports as CTS-MEB may reasonably specify.

6. The Recipient may disclose any confidential Information if required by a government body or court order provided that it gives a written notice to CTS-MEB of such request or order at least thirty (30) days prior to the date it is required to disclose.

7. The Relevant Material is provided "as is" and CTS-MEB expressly disclaims all warranties, express or implied, including without limitation warranties of satisfactory quality, fitness for a particular purpose and non-infringement with regard to the Relevant Material. The Recipient shall use the Relevant Material in compliance with all laws and governmental guidelines applicable to the Relevant Material and will comply with all relevant statutory or other regulatory guidelines and such other guidelines as may be issued from time to time by CTS-MEB. In particular, the Recipient agrees to effect robust mechanisms for traceability of Relevant Materials and to enable access by

representatives of CTS-MEB to all documentation (or supply copies of all such documentation) relevant to such mechanisms on reasonable request.

8. The Recipient shall be solely responsible for any claims or liabilities which may arise in relation to, the use, handling, storage and disposal of the Relevant Material by the Recipient or any third party to whom the Recipient transfers the Relevant Material.

9. The Recipient covenants and undertakes that the Relevant Material will not be administered to humans and in so far as it is administered to animals, no animal to which the Relevant Material is administered, or animal product derived there from, will be used for food, therapeutic or diagnostic purposes, or kept for any other purpose different for that specified in this agreement.

10. Where the Relevant Material consists of or incorporates "relevant material" as defined by the Human Tissue Act 2004 ("the Act"):

10.1 the Recipient warrants and undertakes that (where applicable):

10.1.1 it is and will remain, properly licenced under the provisions of the Act;

10.1.2 The Relevant Materials in the possession, or under the control of the Recipient shall be immediately returned or lawfully disposed of

(i) on the request of the DI or the University; or

(ii) on termination of this Agreement; or

(iii) on completion of the Research; or

(iv) in the event that either the Investigator or the Recipient being in breach of any of the conditions of this Agreement.

10.1.3 it will at all times comply with the requirements of the Act relating to the taking, storage and disposal and use of relevant material as defined in the Act and, in particular, will comply with all Codes of Practice issued by the Human Tissue Authority, insofar as they relate to the Relevant Material

10.1.4 it has appointed a Designated Individual who will supervise the use, storage and disposal of the Relevant Material and will ensure compliance by the Institution with the requirements of the Act so far as it applies to the Relevant Material; the Recipient will store, use or return or dispose of the Relevant Material in accordance with good laboratory practice good clinical laboratory practice and with the highest standards of skill and care and shall ensure compliance with all applicable laws, regulations and research governance pertaining to the Research, including but not limited to the HT Act, the Human Tissue Authority Codes of Practice, and all ethical guidelines relating to the use, storage, transportation and disposal of the Relevant Material. The Recipient must therefore have in place and abide by a policy for the disposal of Relevant Material that complies with the HT Act and the HTA Codes of Practice

10.1.5 it will ensure that any third party to whom the Relevant Material is transferred with the consent of CTS-MEB enters into obligations in identical terms as set out in this clause 10. The Recipient agrees to provide indemnity to the CTS-MEB against any claims or liabilities which CTS-MEB suffers or incurs arising from any breach by the third party of its obligations under the Act; and

11. This Agreement shall be govern by and construe in accordance with English Law. Each Party hereby submits to the exclusive jurisdiction of the English Courts.

SIGNED
For and on behalf of
CTS- Manchester Eye Bank

.....
Duly Authorised Signatory

NAME: Dr. Isaac Zambrano

POSITION: Designated Individual
DATE: 17th July 2013

SIGNED
Recipient of Tissue

.....
Duly Authorised Signatory

NAME: Dr Annette Meeson

POSITION: Recipient of tissue
DATE:14th May 2013

Responsibilities of Designated Individual

- To ensure that lawful consent has been obtained for the Relevant Material
- To check that all relevant information has been obtained including NRES favourable opinion and/or HTA licence
- To prepare the Relevant Material for transport to the Recipient
- To answer specific technical questions about storage and disposal of the Relevant Material
- If the DI changes, the new DI undertakes to inform the Recipient

Responsibilities of Recipient

- To provide the DI with accurate and relevant information including evidence of NRES favourable opinion and/or HTA licence
- To use the Relevant Material for the specified purpose only
- To act as custodian for the Relevant Material in accordance with the HTA Act 2004
- To work within the framework of the HTA Codes of Practice
- To dispose of the Relevant Material, where necessary, in a lawful manner that complies with the HT Act and keep appropriate records.

Appendix 4

Newcastle
Uteroplacental Tissue Bank

Professor John M Davison
Chair of Uteroplacental Tissue Bank Committee
Institute of Cellular Medicine
Reproductive and Vascular Biology Group
3rd Floor, William Leech Building
Medical School
Framlington Place
Newcastle University
Newcastle upon Tyne
NE2 4HH

email: j.m.davison@ncl.ac.uk

5 August 2013

Dr Gendie Lash
Institute of Cellular Medicine
Newcastle University

Dear Gendie

Title of the Research Project: **Cultivated limbal stem cells on amniotic membrane for ocular surface reconstruction**

Application reference: **13.04**

Thank you for your recent application to access material from the Uteroplacental Tissue Bank for the above project. The Committee considered your application on 26 July 2013. On behalf of the Committee, I am pleased to confirm that your project has been approved.

I enclose a copy of the Uteroplacental Tissue Bank Ethical Approval for your records. Details of your project will be included in the forthcoming Annual Report which will be sent to the Newcastle and North Tyneside 1 Ethics Research Committee and the R&D Department at Newcastle Hospitals NHS Foundation Trust.

You must inform the Committee of any amendments made to the protocol of your project. Please note that approval can only be considered for amendments which remain within the conditions outlined in the Uteroplacental Tissue Bank Protocol. As a user of the Bank you are required to cooperate with any sample auditing requests. At the end of your project, 26 September 2015, you are required to submit a completion report which will be included in the Annual Report. You are encouraged to acknowledge the Tissue Bank in any presentations or publications arising from your project.

Please do not hesitate to contact the Bank Manager (Professor SC Robson s.c.robson@ncl.ac.uk) if you have any further queries relating to further use of the Bank.

Yours sincerely

John

Professor John M Davison
Chair - Uteroplacental Tissue Bank Committee

Tel: +44 (0) 191 282 4132
Fax: +44 (0) 191 222 5066
http://www.ncl.ac.uk/icm/research/areas/reproductive

Appendix 5

	Tissue ID	Tissue bank No	Age of donor	Date of use	Date of disposal	Use
1	M16295A	92831	71	30/11/2011	30/11/2012	RNA
2	M16246A	92651	88	25/10/2011	14/11/2011	culture
3	B28555A	92762	85	09/11/2011	16/11/2011	culture
4	B28588B	92905	70	09/11/2011	20/11/2011	culture
5	B28555B	92762	85	18/11/2011	18/11/2011	RNA (Trizol)
6	M16295B	92831	71	04/01/2012	07/01/2012	culture
7	M16102A	92049	85	04/01/2012	07/01/2012	culture
8	B28605A	NA	NA	15/01/2012	18/01/2012	culture
9	M16589B	NA	NA	15/01/2012	18/01/2012	culture
10	M16409B	93215	NA	30/01/2011	31/01/2011	culture
11	M16628A	93925	NA	31/01/2012	06/02/2012	culture
12	M16577B	93715	NA	31/01/2012	09/02/2012	culture
13	M16590B	93752	71	07/12/2011	07/12/2011	RNA
14	M16565A	93694	NA	07/12/2011	07/12/2011	RNA
15	M16568A	76893	NA	06/03/2012	04/04/2012	culture
16	B28594A	NA	NA	02/12/2011	02/01/1900	culture
18	M16133A	NA	NA	23/01/2012	23/01/2012	RNA
19	M16565A	NA	NA	07/02/2012	21/02/2012	SP
20	M16590B	NA	NA	07/02/2012	07/02/2012	RNA
21	M16579A	NA	NA	23/02/2012	24/02/2012	culture
22	M16750B	NA	NA	24/02/2012	25/02/2012	culture
23	M16718B	NA	NA	29/02/2012	19/03/2012	culture
24	M16568A	NA	NA	06/03/2012	20/04/2012	culture
25	M16843B	NA	NA	28/03/2012	03/04/2012	SP
26	M16920B	NA	NA	24/03/2012	29/03/2012	SP
27	M16942A	NA	NA	03/04/2012	22/04/2012	ICC , RNA
28	M16947B	95054	71	19/04/2012	05/05/2012	RNA
30	B29097A	95594	88	30/05/2012	30/05/2012	RNA (trizol)
31	B29139A	NA	NA	01/05/2012	11/05/2012	RNA
32	M17113B	95591	70	31/05/2012	11/06/2012	SP
33	M17259B	96058B	NA	17/07/2012	24/07/2012	culture
34	M17640A	97270A	NA	28/08/2012	17/09/2012	culture
35	B29464A	NA	NA	18/09/2012	18/09/2012	RNA (trizol)
36	M17829A	98000	NA	25/09/2012	10/10/2012	SP
36	M17760A	97685	50	02/10/2012	15/10/2012	SP, RNA
37	B29804A	98379	NA	23/10/2012	31/10/2012	SP, RNA
38	M17963A	98494	NA	06/11/2012	20/11/2012	SP
39	M17859B	NA	NA	05/11/2012	05/11/2012	RNA
40	M17761A	NA	NA	05/11/2012	05/11/2012	RNA
41	B29951A	99072	NA	05/12/2012	18/12/2012	SP
42	M18307B	99565	80	14/01/2013	28/01/2013	RNA
43	M18158A	99092	86	14/01/2013	28/01/2013	culture
44	M18534B	82748	NA	08/02/2013	23/02/2013	RNA
45	M18488A	100228	90	06/03/2013	22/03/2013	culture
46	M18452A	100135	41	06/03/2013	27/03/2013	ICC
47	M18574B	82857	NA	14/03/2013	14/03/2013	ICC
48	M18591A	100630	NA	12/03/2013	22/03/2013	SP

49	M18590B	NA	78	02/03/2013	25/03/2013	SP
50	M18627B	NA	NA	02/03/2013	25/03/2013	SP
51	M18751B	101163	77	12/04/2013	23/04/2013	culture
52	M18747B	101171	75	12/04/2013	22/04/2013	culture
53	125357 (O	NA	73	19/04/2013	01/05/2013	SP
54	125357 (OI	NA	73	23/04/2013	03/05/2013	SP
55	M19028B	101976	80	04/06/2013	14/06/2013	SP
56	M19447A	103414	83	08/09/2013	10/09/2013	Culture
57	M19447B	103414	83	08/09/2013	10/09/2013	Culture
58	M19425A	103326	79	31/08/2013	11/09/2013	Culture - AM
59	M19425B	103326	79	31/08/2013	11/09/2013	Culture - AM
60	M19430A	103344	59	04/09/2013	16/09/2013	Culture
61	M19430B	103344	59	04/09/2013	16/09/2013	Culture
62	M19450A	103454	80	04/09/2013	18/09/2013	Culture - LMSC
63	M19450B	103454	80	04/09/2013	18/09/2013	Culture - LMSC
64	M19580A	103996	70	03/10/2013	14/10/2013	Culture
65	M19580B	103996	70	03/10/2013	14/10/2013	Culture
66	M19935A	105321	46	11/12/2013	16/12/2013	Culture
67	M19935B	105321	46	11/12/2013	16/12/2013	Culture
68	M19918A	105224	79	14/12/2013	16/12/2013	Culture
69	M19914B	105197	78	14/12/2013	16/12/2013	Culture
70	M20087B	NA	69	22/01/2014	04/02/2014	Culture - LMSC - AM
71	M20028B	NA	69	22/01/2014	04/02/2014	Culture - LMSC - AM
72	M20008B	NA	NA	29/01/2014	02/02/2014	Culture/ICC
73	M20098A	NA	75	29/01/2014	02/02/2014	Culture/ICC

Abbreviations;

LMSC	Limbal mesenchymal stem cells
AM	amniotic membrane
SP	side population
NA	data not available