The influence of ageing and culture conditions on limbal epithelial cells

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Abstract

Introduction. The use of limbal stem cells as a cellular therapy has expanded over the last decade. Currently, the success rate of limbal stem cell transplantations is around 76% with advanced donor age being a possible reason for this figure. Medium and oxygen concentration could also have a detrimental effect on the growth of these cells for transplantation. With age the limbus undergoes a topological change which could alter the stem cells surroundings. A mouse model of corneal ageing was also used to complement the human study and avoid culturing and tissue storage artefacts.

Aims. The main aim of this thesis was to assess the effect of age on corneal epithelial progenitor cells. This then led to the question; is the mouse cornea affected by age and can interventions mediate these outcomes? And finally do culture conditions affect the growth of corneal epithelial stem cell containing cultures, and how does this relate to changes to the niche with age?

Methods. Limbal derived progenitor cells were extracted from human corneosclearal disks donated for research. Cells were cultured either in LEM or DKSFM and either in hypoxia (3%) or normoxia (21%). Immunofluorescence, qPCR, TRAP assay, morphological and clonal analysis where used to assess progenitor composition. The mice used were strain B6.129S-Tert, tm1Yjc/J. Old/AL and DR mice were 22 months old, young were 3 months old. Rapamycin treated mice were 16 months old and were treated for four months, starting at 12 months of age. Dietary restriction was implemented for a period of 16 months from 6 months of age, additionally in the mouse eye a DNA damage marker was also assessed, both telomeric and non-telomericly.

Results and Conclusions. Age has a detrimental effect on the ability to culture a limbally derived clonal population. However age did not affect the levels of positive and negative gene expression markers or ΔNp63 protein level. Interestingly the time that corneal tissue is stored for did not affect the ability to isolate these progenitor cells. The growth of cells in hypoxia decreased negative marker *KRT3* and senescence marker *p21* regardless of culture medium.

In the mouse model, age had a detrimental effect on the cornea with decreased TAp63, increased ɣH2A.X and TAFs. DR however, tended to have a beneficial effect on the mouse cornea. Interestingly rapamycin seems to be detrimental to the mouse cornea, with similarities with the effects of age.
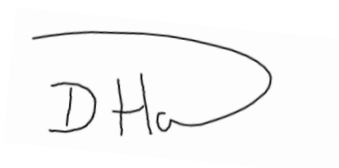
Dedication

For Eileen Thompson

1922-2012

Declaration

I, the undersigned, hereby declare that this thesis contains my own work and I have correctly acknowledged the work of collaborators. Any part of this work has not been submitted for fulfilment of a degree at this or any other University, and that all the sources I have used here have been indicated or acknowledged by means of references.

Dean Hallam

Acknowledgements

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List of Abbreviations

Abbreviation	Meaning
14-3-3	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
18s	18s ribosomal RNA
3T3	3-day transfer, inoculum 3×10^5
4E-BP1	4E-binding protein 1
ABBP1	Apobec-1 binding protein
ABCG2	ATP-binding cassette sub-family G member 2
ACTB	Actin β
ADP	Adenosine diphosphate
Akt	Protein Kinase B
AL	*ad libitum*
AMP	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of Variance
APES	Aminopropyltriethoxysilane
ARD1	Arrest-defective-1
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATP5B	ATP synthase subunit β
ATR	Ataxia telangiectasia and Rad3
BAX	Bcl-2-associated X protein
BMI1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenetic protein
BPE	Bovine pituitary extract
BRCA1-2	breast cancer 1-2, early onset
CBP	CREB-binding protein
CDC25B	M-phase inducer phosphatase 2
CDK	Cyclin dependent kinase
CESC	Corneal epithelial stem cell
cESC	*Callithrix jacchus* embryonic stem cells
CFE	Colony forming efficiency
CHAPS	3-[3-Cholamidopropyl dimethylammonio]-1-propanesulfonate
CREB1	cAMP response element-binding protein 1
C_t	Cycle threshold
Cx43	Connexin 43
DAPI	4',6-diamidino-2-phenylindole
DBS	Double strand break
DDR	DNA damage response
DEPTOR	DEP domain-containing mTOR-interacting protein

DIG-POD	Digoxigenin-peroxidase
DKSFM	Defined Keratinocyte serum free medium
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DPX	Distyrene, plasticizer and xylene
DR	Dietary restriction
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
eIF4-BP	Eukaryotic translation initiation factor 4E-binding protein 1
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular-signal-regulated kinase
ERRα	Estrogen-related receptor alpha
ESC	Embryonic stem cells
ETC	Electron transport chain
FANCD2	Fanconi anaemia group D2
FANCD2	Fanconi anemia group D2 protein
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridisation
FOV	Field of view
FOXO	Forkhead box
Gadd45α	Growth arrest and DNA-damage inducible gene 45 alpha
GAPDH	Glyceraldehyde-3-phopshate dehydrogenase
γH2A.X	Gamma H2A histone family, member X
GLUT1	Glucose transporter 1
GβL	G protein beta subunit-like
H2A.X	H2A histone family, member X
H3K9	Histone H3 lysine 9
HAM	Human amitotic membranes
HCL	Hydrochloric acid
Hes-1	Hairy and enhancer of split 1
Hey-1 & 2	Hairy/enhancer-of-split related with YRPW motif protein 1
HIF-1α	Hypoxia-inducible factor
HRE	Hypoxia response elements
HSC	Haematopoietic stem cell
Hsp90	Heat shock protein 90
HTCEpi	Human telomerase immortalised corneal epithelial cells
hVPS34	Human type III phosphatidylinositol 3-kinase
IAP	Inhibitor of apoptosis
IGF-1	Insulin-like growth factor 1
IHC	immunocytochemistry
IKK	IκB kinase
IKK	IκB kinase

JMJD3	Jumonji domain containing 3
K10	Keratin 10
K14	Keratin 14
K19	Keratin 19
Ki67	Kiel 67
KO	Tert$^{-/-}$
KRT12	Cytokeratin/Keratin 12
KRT3	Cytokeratin/Keratin 3
LEM	Limbal epithelial medium
LESC	Limbal epithelial stem cell
LSC	Limbal stem cell
LSCD	Limbal stem cell deficiency
LSCT	Limbal stem cell transplantation
LSD	Least significant difference
MDM2	Mouse double minute 2 homolog
MEK1	Dual specificity mitogen-activated protein kinase kinase 1
MK2	MAPK-activated protein kinase-2
MLST8	Target of rapamycin complex subunit LST8
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell
mSIN1	Mammalian stress-activated protein kinase interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non Homologous end joining
NO	Nitric Oxide
NRF1	Nuclear respiratory factor 1
OCR	Oxygen consumption rate
OCT	Optimum cutting temperature
ODDD	Oxygen dependent degradation domain
OXPHOS	Oxidative phosphorylation
P:O	Phosphate/oxygen ratio
p16	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor 1
p300	E1A binding protein p300
p38	Mitogen-activated protein kinase 38
p53	Tumour protein 53
p57	Cyclin-dependent kinase inhibitor 1C
PARN	Poly(A)-specific ribonuclease

PBG	Phosphate buffered saline, Foetal bovine serum, fish skin gelatin
PBS/DPBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PFA	Paraformaldehyde
PFKFB1-4	6-phospho-2-kinase/fructose 2, 6-biphosphatase
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide 3-kinase
Pins	Partner of Inscrutable
POLG	Polymerase gamma
POV	Palisades of Vogt
PRAS40	Proline-rich Akt substrate 40
PtdIns	Phosphatidylinositol
pVHL	von Hippel-Lindau ubiquitin E3 complex
qPCR	quantitate polymerase chain reaction
RAPTOR	Regulatory-associated protein of mTOR
Rb	Retinoblastoma protein
REDD1	Regulated in development and DNA damage responses 1
RELA	Transcription factor p65
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPL13A	60S ribosomal protein L13a
RT PCR	Reverse transcriptase polymerase chain reaction
S6K	Serine/threonine 6 kinase
SC1-3	Subculture 1-3
SDHA	Succinate dehydrogenase complex
SEM	Standard error of the mean
SHEM	Supplemental hormonal epithelial medium
SIPS	Stress induced premature senescence
SIRT	Sirtuin
SMAD	Mothers against decapentaplegic homolog 1
SOD2	Superoxide dismutase 2
SSB	Single strand break
STACs	Sirtuin activating compounds
TAC	Transit amplifying cells
TAF	Telomere associated foci
TDC	Terminally differentiated cell
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TFAM	Mitochondrial transcription factor A

TGFβ	Transforming growth factor beta
TMB	3,3',5,5' – tetramethylbenzidine
TP63	Tumour protein 63
TRAP	Telomerase repeat amplification protocol
TRF1-2	Telomeric repeat-binding factor 1-2
Tsc1	Tuberous sclerosis 1
Tsc2	Tuberous sclerosis 2
TWIST	Twist basic helix-loop-helix transcription factor 1
UV	Ultra violet
VEGF	Vascular endothelial growth factor
WRN	Werner syndrome ATP-dependent helicase
WT	Wild type
α-SMA	Alpha smooth muscle actin

Chapter 1 Introduction

In the UK 2 million people are living with some degree of sight impairment (RNIB, 2013). Of those 2,520 are related to an increase in corneal opacity, caused by chemical or thermal injury, Stevens Johnson syndrome (SJS), aniridia and ocular cicatricial pemphigoid (Shortt *et al.*, 2011). Loss of sight has a profound effect on an individual's mobility, independence and mental wellbeing. Corneal disease can often be painful and difficult to treat. Modern advances in cellular therapies are improving the prognosis of thousands of people, these therapies however require further refinement to achieve higher success rates. The current therapy success rate for one of the most severe corneal diseases, limbal stem cell deficiency, is around 76% (Baylis *et al.*, 2011). Little is known as to why some transplants fail. The effect of increased donor age and culture parameters are hypothesised to negativity impact upon the success rate of limbal stem cell transplantations.

1.0   The eye, cornea and stem cells

1.0.1   Cornea, structure and function

The eye is a complex organ comprised of many cell types. Each region has a specific optical function to aid the transmission of light onto the retina. The cornea is an anatomical region at the front of the eye, at the anterior surface (figure 1.1). Being avascular in nature the cornea draws nutrients and oxygen from the tear film. The cornea is bordered by the conjunctiva, which functions to help lubricate the eye via mucus production and provide immune surveillance thanks to goblet cells within the epithelium.

The cornea is comprised of five layers: an epithelium, Bowman's layer, stroma, Descemet's membrane and the endothelium. The epithelium consist of around five layers, comprised of three cell types: a layer of columnar basal cells which are attached to the basement membrane, two to three layers of polyhedral wing cells and a squamous cell layer.
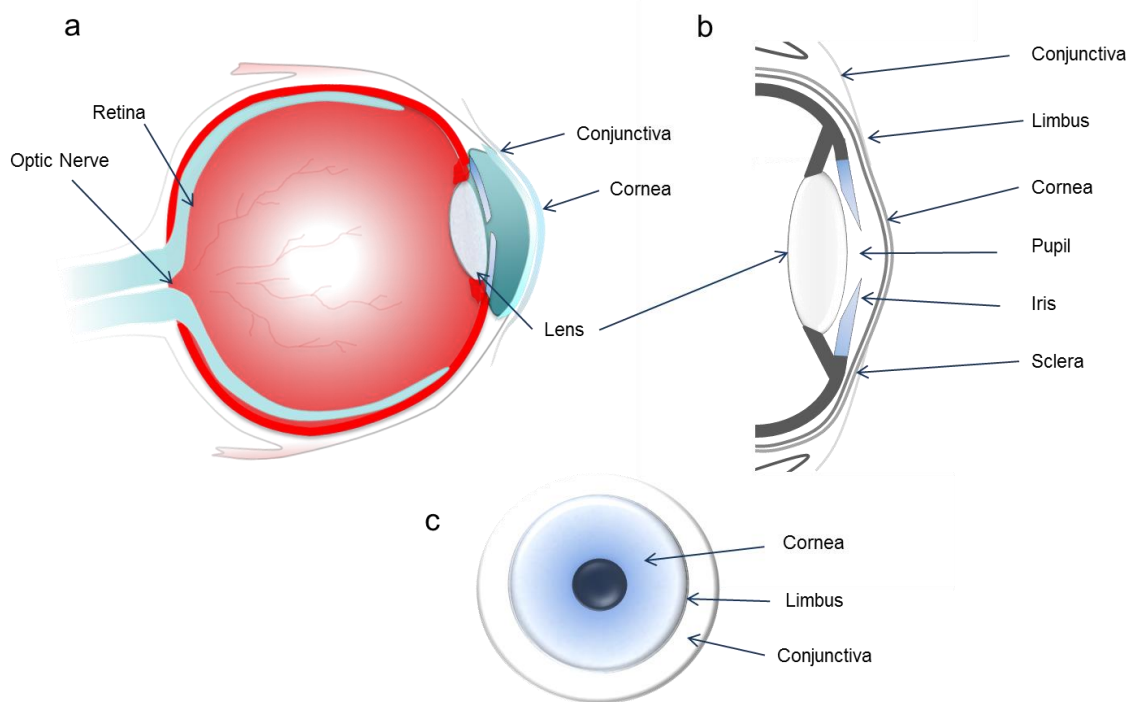
Figure 1.1 Diagrammatic representation of the eye
Panel a is a horizontal cross section through the eye. Light is refracted initially by the cornea (panel, b),
then by the lens focusing the photons on to the retina. Panel c depicts an anterior view of the eye.

The basal cell layer is a monolayer of mitotically active cells in the corneal epithelium
which provide the source of wing and squamous cells (figure 1.2 & 1.3). The wing cells
are around 30-45μm in size and have a high keratin content which aids in the
attachment of the cells to surrounding cells (Krachmer *et al.*, 2005). Close adhesion
prevents pathogens penetrating deeper into the tissue. It is presumed that wing cells are
mitotically inactive to prevent the propagation of DNA damage events accumulated
from ultra violet damage. UV irradiation causes the cross linking of pyrimidine bases.
The dimers that form inhibit DNA polymerase and result in misincorporation of
nucleotides, often initialling the DNA damage response (DDR) or even cell cycle arrest.
During normal tissue homeostasis the wing cells migrate towards the anterior surface
(Masters and Thaer, 1994). The surface of the epithelium is layered with flat scale like
cells called squamous cells. Squamous cells have cilia on their surface which may play a
role in acquiring nutrients from the tear film (Pfister, 1975). This arrangement classifies
the corneal epithelium as a stratified squamous epithelium.

Beneath the basement membrane is Bowman's layer, an acellular smooth,
collagen rich membrane which separates the epithelium from the stroma (Wilson and

Hong, 2000). The stroma makes up the largest proportion of the cornea, like Bowman's layer it is comprised of collagen fibres which are interwoven across the cornea (Coster, 2002). These filaments are produced by keratocytes which are distributed throughout the stroma (Bron, 2001). Descemet's membrane separates the stroma from the endothelium and is produced by endothelial cells. It is comprised of two main layers a posterior non-banded layer and an anterior banded layer, the main consistency of these layers is collagen IV and laminin (Kaufman *et al.*, 1998). The function of this layer is thought to be implicated in maintaining the curvature of the cornea (Pipe, 1984). The endothelium is a layer of mitochondria rich cells which separates the anterior chamber from the stroma (figure 1.3). The cell layers major function is to transport solutes to and from the cornea. The endothelium keeps the cornea in a dehydrated state which helps to increase the optical properties (Kaufman *et al.*, 1998).

The corneas' function, to focus light onto the retina, requires the tissue to be transparent in nature. This allows photons to pass through without being scattered, allowing for image formation. The corneal epithelium is one of the first barriers which photons encounter when entering the eye. It prevents microorganisms from penetrating the deeper corneal tissue and acts as a barrier to other tissues of the surrounding area. The conjunctival epithelium would otherwise repopulate the cornea reducing its opacity. The maintenance of the clarity of the corneal epithelium is crucial to its function. The corneal epithelium has an incredible regenerative capacity, which is thanks to a pool of stem cells within the tissue.
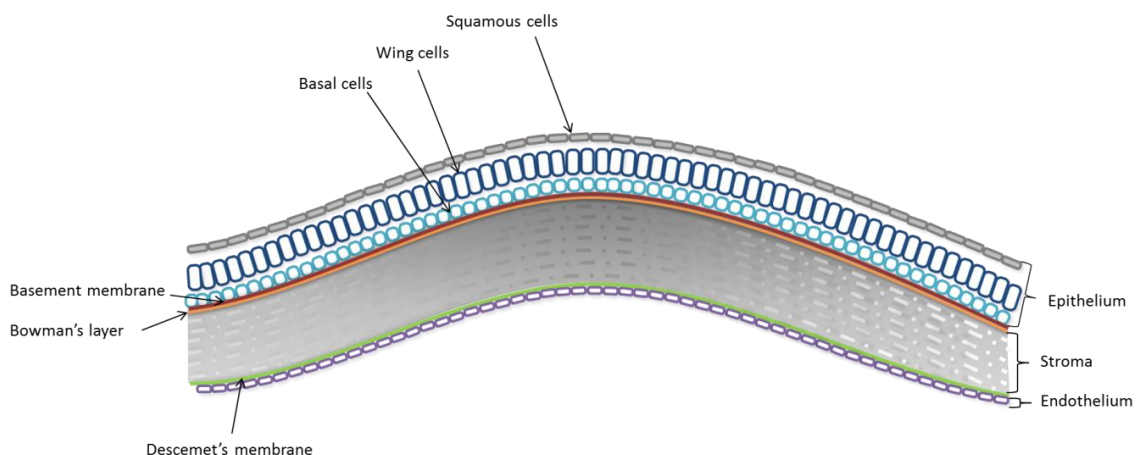
Figure 1.2 Schematic drawing of the human cornea
The squamous cell layer is depicted as a grey monolayer at the surface of the cornea, beneath this reside wing cells. Basal cells reside on the basement membrane The stoma, shown in grey is the thickest section of the cornea. The stoma is boarded by Bowman's layer and Descemet's membrane. Distally the endothelium, shown as purple, barriers the aqueous humour.
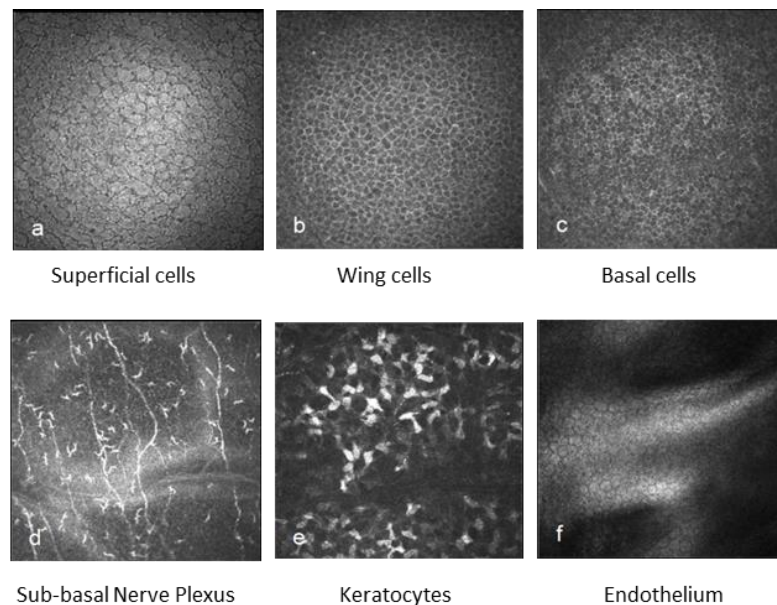
Figure 1.3 *In vivo* confocal microscope images of the cornea
a) Shows the large superficial cells on the corneal surface. B) Shows the wing cells beneath layer a-c) Depicts the basal cells d) Beneath the basal cells lie the nerve plexi. E) Keratocytes are present below the basal layer, these specialised fibroblasts are essential for the maintenance of the cornea. f) The final layer of the cornea is the endothelium. Not visible on these images is Bowman's layer, as it is completely transparent. Images taken by Dr Oliver Baylis using the In vivo confocal microscope at Newcastle Royal Victoria Infirmary.

1.0.1 Stem cells

Stem cells were first defined in 1987 as cells which are capable of self renewal, producing exact replicas but also a large number of daughter cells which are able to differentiate into multiple cell types(Potten and Loeffler, 1987).
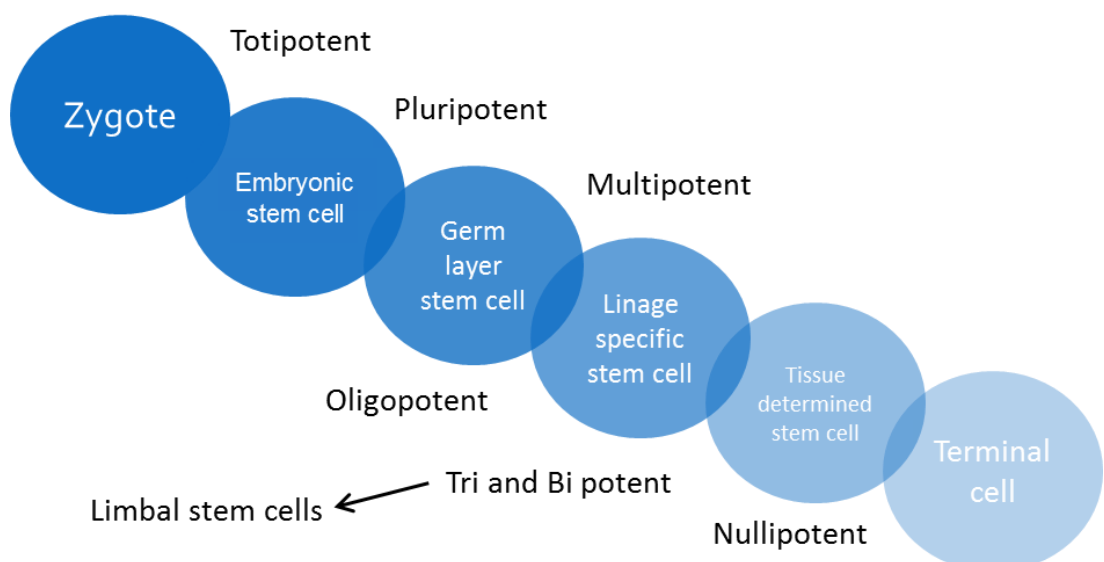
Figure 1.4 Diagram showing the potency of stem cells
Adapted from (Sell *et al.*, 2004) Trophoblast stem cells have the highest level of potency (not shown), giving rise to both embryonic and placental linages. Embryonic stem cells give rise to the three germ layers, ectoderm, mesoderm and endoderm. Less potent stem cells within these layers then specialise to maintain specific tissues and organs.

Stem cells can be classified in a hierarchical manner (figure 1.4), firstly, totipotent stem cells give rise to both placental and embryonic lineages. Pluripotent stem cells give rise to only embryonic lineages, which later differentiate into stem cells and give rise to the three germ layers, the mesoderm, endoderm and ectoderm. Within these three germ layers multipotent cells give rise to different stem cells of differing organ origins. Haematopoietic stem cells are an example of multipotent stem cells, giving rise to lymphoid and myeloid progenitors (Ugarte and Forsberg, 2013). Another example of multipotent stem cells are mesenchymal stem cells which can differentiate into osteoblasts, chondrocytes and adipose (Uccelli *et al.*, 2008). Oligopotent stem cells only give rise to the cells of one tissue type, be that through the multiple stem cell types. Bi and tri potent cells can produce two-three different cell types within a tissue. Limbal stem cells generate exclusively epithelial cells, which produce basal, wing and squamous cells, as shown by serial transplantations of the mouse cornea (Majo *et al.*, 2008). These epithelial cells produced by stem cells where the focus of this thesis. The final classification is nullipotent; it refers to the non-stem cells which make up the majority of an organisms cell populations. These classifications have been challenged recently, with some evidence that lineage specific stem cells can actually give rise to other tissues (Mezey, 2011) with examples of bone marrow derived stem cells able to transdifferentiate into other germ layer derived cells such as the epithelial cells of the intestinal tract. Traditionally though, adult stem cells (or somatic stem cells), such as stem cells of the corneal epithelium, are present throughout the life of an organism and are responsible for the continuous maintenance of specific tissues and organs (Tarnok *et al.*, 2010). Fibroblasts originating from the stromal limbus, however have been shown to have the potential to transdifferentiate, transforming from a mesoderm linage into ectoderm, eventually differentiating into corneal epithelial cells (Hashmani *et al.*, 2013). This has implications for the specificity of limbal progenitor cell extractions. The methods by which limbal fibroblasts are transformed however, requires the use of specific embryonic stem cell medium (Katikireddy *et al.*, 2014). The presence of these cells is significant as it is thought that they aid in the modelling of the limbal niche. This is postulated from their role in assisting other stem cells, such as mesenchymal stem cells (MSCs) whereby these cells aid the proliferation and differentiation of hematopoietic stem cells (HSCs) (Lu *et al.*, 2006).

1.0.2 Corneal epithelial stem cells

Corneal epithelial stem cells are often referred to as limbal stem cells (LSC), a name which originates from the anatomical region were these cells are thought to reside. Limbus, in medical terminology simply refers to a border between two parts. In the case of the anterior eye, the limbus is the region where the cornea and conjunctiva meet. Although not conclusively identified, there have been several key studies which link limbal stem cells to this region. Cultured cells from the epithelial surfaces of the eye show that cells derived from this region have the highest proliferative capacity (Ebato *et al.*, 1988). Removal of the limbal region following wounding of the central cornea also leads to a lack of corneal re-epithelisation (Kruse *et al.*, 1990; Chen and Tseng, 1991; Huang and Tseng, 1991). One of the most compelling studies in mice, used radio-labelled thymidine to show that cells in the limbus were slow cycling, however upon wounding began to cycle rapidly (Cotsarelis *et al.*, 1989).

1.0.2.1 Limbal stem cell deficiency

Limbal stem cell deficiency (LSCD) is caused by a loss of LSCs, often the result of chemical or thermal injuries, directly destroying the stem cells or their niche. In some cases LSCD can be the result of congenital factors such as aniridia (absence of the iris), SJS or ectodermal dysplasia. Without these progenitor cells the cornea cannot be replenished. This in turn leads to cells of conjunctival origin migrating over the cornea and colonising. LSCD is characterised by a clouding and conjunctivalisation of the cornea accompanied by vascularisation (Puangsricharern and Tseng, 1995) (figure 1.5). Historically, LSCD was treated by cornea transplantation, this provided improved sight for a limited amount of time, however conjunctival ingrowths persisted, as the deficiency of limbal stem cell is not corrected (Dua *et al.*, 2010). Today patients can be treated with autolimbal transplantations, either autografts (from fellow eye) or allografts (from relative or cadaver). The process involves removing a small 5mm x 2mm section of the conjunctiva/corneal limbus and expanding the cells from the tissue piece in culture (Pellegrini *et al.*, 1997). These sections are called explants and are placed on human amitotic membranes (HAM), initially a monolayer of cells grow outward from the tissue section (Grueterich *et al.*, 2003). Amniotic membranes are comprised of a simple epithelial cell layer which is anchored to a basement membrane, separating it from the stromal tissue. HAM is used to provide a base for cells to expand into, extracellular

complexes and cytokines (Koizumi *et al.*, 2000). Once the explant has expanded to around 4cm^2 it is transplanted along with the HAM which then acts as a biological dressing.
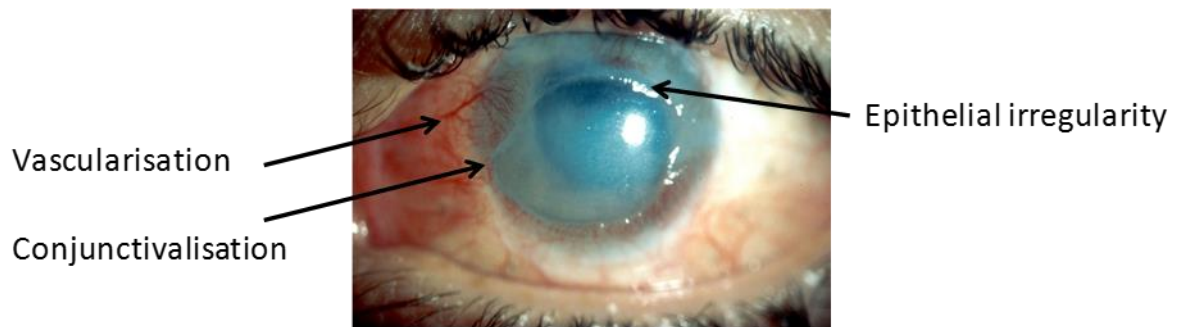
Figure 1.5 An eye afflicted with LSCD
Picture adapted from Ahmad *et al.,* 2006. Conjunctival tissue migrates to cover the cornea reducing the opacity. The ingrowth of conjunctival cells brings vascularisation.

1.0.2.2   The corneal epithelial stem cell hypothesis

The corneal epithelial stem cell (CESC) hypothesis suggests that stem cells are distributed over the entire corneal epithelial basal layer and that stem cells in the limbus are only activated during injury. This hypothesis was suggested following experiments on transgenic β-gal-*ROSA26* mice whereby tissue was excised from both the limbus and central corneal regions and transplanted into other mice. The mouse corneas were then wounded and the donor cells tracked after sacrifice using β-gal staining. Those donor cells from the limbal region only migrated during wound healing, in unwounded mice however there was little migration of the cells from the limbal area. Suggesting that maintenance of the corneal epithelium is dictated by progenitors throughout the entire cornea (Majo *et al.*, 2008) (figure 1.6, b). No experimental evidence however was presented which stated that these cells could be isolated and expanded in culture. In humans evidence exists which shows that ex-vivo wound healing is independent of the limbus and occurred when the limbus was destroyed (Chang *et al.*, 2008). The time period in which this was performed however was rather short (12 hours). Any limbal involvement could have taken longer than the test period. The findings Cheng and colleagues observed may have been mediated by transit amplifying cells (TAC), present in the peripheral cornea (Lavker *et al.*, 1991). TACs expand rapidly a finite number of times producing large numbers of cells before differentiating (Tseng, 1989).

The ability to establish successful cultures from the cornea is essential for cellular

therapeutic reasons. The central cornea was shown to possess clonogenic cells which could be cultured suggesting that this region possesses oligopotency (Chang *et al.*, 2011). In clinical practice, patients with total LSCD still have selected regions of corneal epithelium which persists after the limbus has been destroyed (Dua *et al.*, 2009). Currently the CESC hypothesis is gaining some ground, however it remains to be seen if true progenitors exist within the central cornea or if transit amplifying cells (TAC's) have a greater potency than previously accepted.

1.0.2.3　The limbal epithelial stem cell hypothesis.

The limbal epithelial stem cell hypothesis (LESC) suggests that corneal epithelial stem cells are located in the limbus (as stated previously). Subsequent progeny migrate centripetally replenishing the cornea. This hypothesis is also referred to as the XYZ hypothesis (Thoft and Friend, 1983) (figure 1.6, a), whereby X+Y=Z, X represents the proliferation of basal cells and subsequent movement towards the tear film. Y is the migration of cells from the peripheral cornea (limbus) inwards towards the central region. Z is the loss of squamous cells from the surface. The movement of cells up through the strata has been documented a number of times (Hanna and O'Brien, 1960; Hanna *et al.*, 1961; Schermer *et al.*, 1986; Lavker *et al.*, 1991). In rabbits the replacement of a host corneal epithelium after donor grafts occurs from the limbal zone (Kinoshita *et al.*, 1981).

Using a thymidine label in mice and wounding the central cornea indicated a pool of label retaining cells within the limbus (Cotsarelis *et al.*, 1989) which was later confirmed using a non-wounding model (Lehrer *et al.*, 1998). In *KRT5$^{LacZ/-1}$* mice, β-galactosidase positive cells have been shown to migrate in stripes originating from the limbus moving centripetally.

In humans Pellegrini and colleagues proved that holoclones, colonies which contain the highest proportion of progenitor cells in epithelia, could only be isolated from the limbus (Pellegrini *et al.*, 1999). In treatment of LCSD, a replacement of the central cornea does not recover the limbus, however small pieces of the limbus can eventually regenerate other limbi and the central cornea (Kenyon and Tseng, 1989; Frucht-Pery *et al.*, 1998).
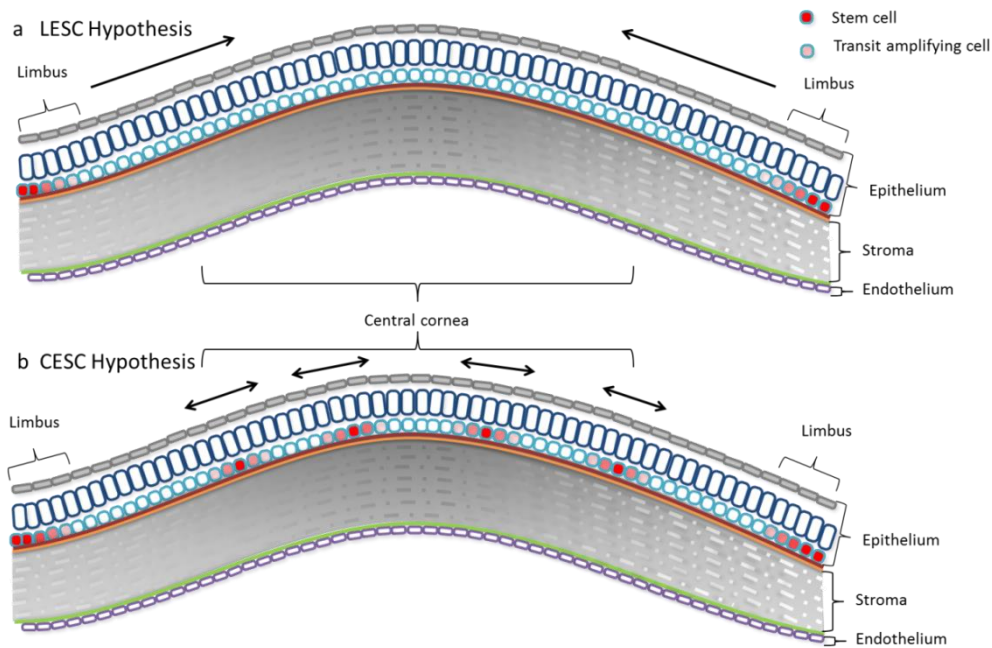
Figure 1.6 Corneal epithelial stem cell hypotheses
Cross sectional view of the cornea. The LESC hypothesis, a, implicates the movement of TAC cells centripetally, shown as the reduction red colouration of the basal cells. The CESC hypothesis on the other hand suggests that progenitor cells are located in the central regions as well as peripherally (b).

The LESC hypothesis is currently the most widely accepted theory due to the wealth of publications in favour of this paradigm.

1.0.2.4 Corneal epithelial stem cell culture

The culture of LSCs is performed using adherent cell culture or organotypic systems. Cells are normally isolated from a heterogeneous population of cells. Early attempts to cultivate skin and corneal epithelium failed because of the heterogeneity, cultures would become contaminated with fibroblasts from other areas of the tissue (Flaxman *et al.*, 1967; Fusenig and Worst, 1974; Newsome *et al.*, 1974). Fibroblasts have a higher growth rate compared to epithelial cells and overwhelmed the cultures. The major advance in epithelial cell culture arose when mouse fibroblasts (3T3) were co-cultured with epithelial cells (Sun and Green, 1977). These 3T3 cells were generated from randomly bred Swiss mouse embryos (Todaro and Green, 1963). Unlike native fibroblasts in the culture the mouse fibroblasts are mitotically inactivated with mitomycin C or irradiation (Rheinwald, 1980; Freshney *et al.*, 2002). Mitomycin creates DNA crosslinks targeting guanine residues, this leads to interference at CpG islands which normally sit upstream of promoter regions. Such major genetic disruption activates DNA damage proteins initiating cell cycle arrest, preventing any further proliferation. The 3T3 "feeder" layer is thought to perform a number of functions: firstly,

it inhibits the growth of native fibroblasts from the primary isolate. Fibroblasts are sensitive to contact inhibition, whilst epithelial cells are not (Abercrombie, 1970). This creates a selection pressure favouring epithelial cells. Secondly, it is thought that the extracellular matrices which are produced by the mouse fibroblasts allow for easy anchorage to the culture surface, aiding in initial cellular growth (Alitalo *et al.*, 1982). Finally, while the fibroblasts are mitotically inactive they are still metabolically active, secreting growth factors which could be beneficial to epithelial cell growth. In recent years the move away from animal/human co-cultures and the use of animal derivatives has driven the culture of epithelial cells into xenobiotic free conditions (Sangwan *et al.*, 2011). These conditions allow direct comparison with clinical cell culture practices, and promote the standardisation of medium. In particular the removal of serum, a cocktail of undefined substances, from the medium is a big step for standardisation.

1.0.2.5   Methods for determining culture quality

The isolation and subsequent culture of single epithelial cells can yield three different types of colony. The study of these clonally derived colonies is called clonal analysis (Barrandon and Green, 1985; Barrandon and Green, 1987). The three clone types are holoclones, meroclones and paraclones. Holoclones produce the largest colonies, which have smooth defined edges. Holoclones are thought to contain the highest proportion of progenitors, as subcultures of these clones produce the least number of terminal colonies. Meroclones give rise to more irregular colonies which are smaller than holoclone colonies. When cultured, they produce more terminal colonies than holoclones. Meroclones are hypothesised to consist of mainly transit amplifying cells. Finally, paraclones produce small, highly irregular colonies, which have a low ability to be subcultured. These colonies are thought to contain mainly terminally differentiated cells. It has previously been shown that holoclones are limited to the limbus and have high expression of the marker gene $\Delta Np63\alpha$ (Pellegrini *et al.*, 2001; Di Iorio *et al.*, 2005). Clonal analysis can be a useful tool for determining the proportion of progenitor cells within a culture.

Other methods which could be used are side population analysis to determine the number of cells which have an increased ability to efflux exogenous dyes, an upregulated property of stem cells which helps to exclude potentially damaging extracellular molecules. Another method of determining if a culture contains stem cells

is to exploit their normal slow cell cycling rate by adding bromodeoxyuridine (BrdU) or tritiated thymidine by incorporating into DNA replacing thymidine nucleobases. The greater number of replications the more diluted these nucleobases become in the genome replaced with endogenous thymine, as shown recently by Ksander and colleagues in limbal progenitor cells (Ksander *et al.*, 2014). Due to the low cycling of stem cells, these cells are often described as "label retaining cells". Another observation can be made referring to the morphology of stem cells, by measuring the nucleus to cytoplasm ratio. Stem cells and cancer cells have a large nucleus to cytoplasm ratio, often distinqishing them from other cells. Arpitha and colleagues used this technique to try to characterise limbal stem cells, however limbal progentors are indistinquishable from the transit amplifying cell population (Arpitha *et al.*, 2005).

1.1 The stem cell niche and putative limbal epithelial stem cell markers.

1.1.1 Corneal stem cell niche

Regions in which stem cells reside are called niches. A stem cell niche is a specialised microenvironment containing extracellular matrix components which can influence stem cell function. Niches are normally a combination of anatomical architecture and biochemically distinct regions. The limbal niche is not a clear defined area, unlike the hair follicle bulge and intestinal crypts. Suggestions have been made that limbal epithelial crypts and focal stromal projections have niche like qualities (Dua *et al.*, 2005; Shortt *et al.*, 2007) (figure 1.6). The projections bring the potential stem cells closer to stromal vasculature (Gipson, 1989). Another area considered as potentially harbouring limbal stem cells are the palisades of Vogt. The palisades of Vogt are fibrovascular ridges at the corneoscleral limbus (Goldberg and Bron, 1982; Townsend, 1991). Their structure resembles other niches morphologically and contains a high proportion of melanocytes suggesting a possible protective role against UV in the form of melanin (figure 1.7). However there is no conclusive evidence which puts limbal stem cells within these structures (Li *et al.*, 2007).
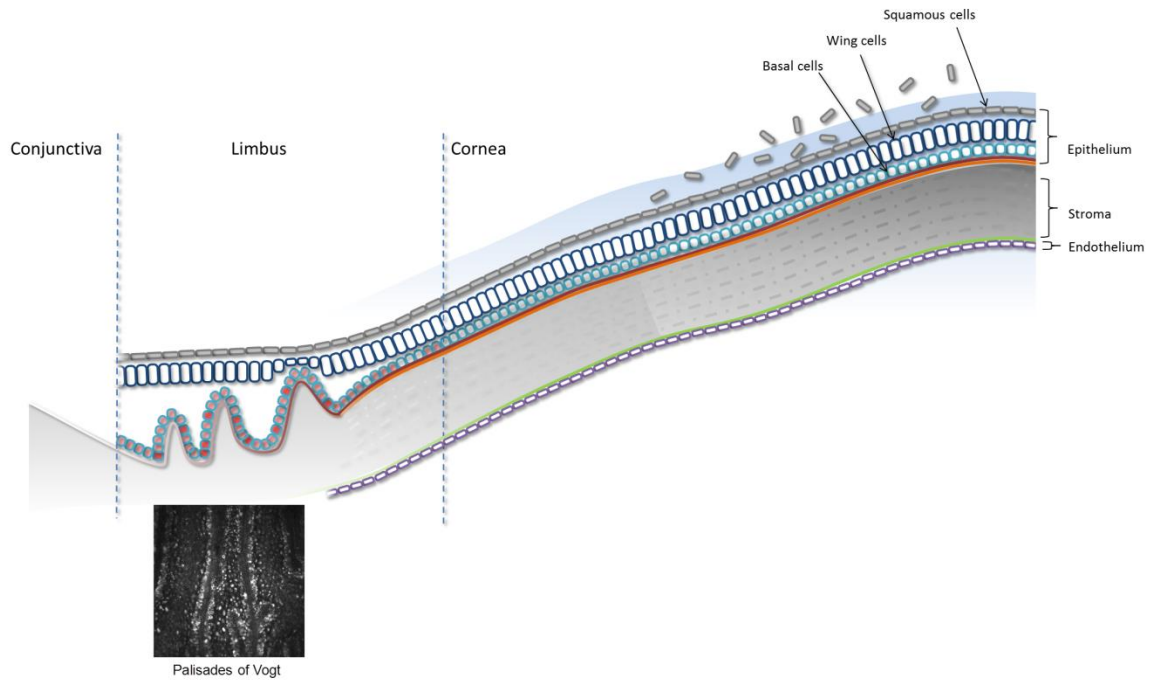
Figure 1.7 The hypothetical limbal stem cell niche
It is hypothesised that limbal stem cells reside within limbal crypts or the palisades of Vogt (inlayed image). *In vivo* confocal microscope image taken by Dr Oliver Baylis.

1.1.2 Corneal marker genes.

One of the main reasons for the lack of clarity on niche position is down to the lack of corneal stem cell specific markers. In this study the following genes were chosen: *TP63 (ΔN- α isoform), ABCG2, BMI1, TERT, KRT3, KRT12* and *p21 (CDKN1A)*.

1.1.2.1 TP63

p63 or TP63 is a tumour suppressor protein related to the transcription factor *p53* in its structure and function. The p63 protein was first identified as an important factor in epidermal development by Yang and Mills independently in the late 1990's in mouse models (Yang *et al.*, 1998; Mills *et al.*, 1999). The knockout mice contained terminally differentiated suprabasal keratinocytes but no stem cells in the basal layers. At the molecular biological level p63 has two promoters, encoding for two unique groups of proteins (Levrero *et al.*, 1999).
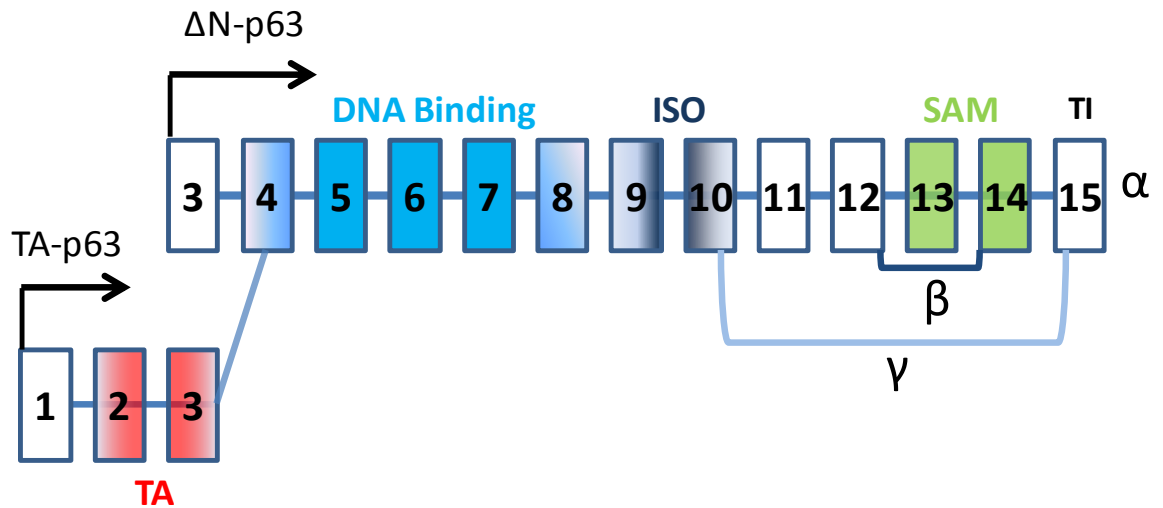
Figure 1.8 The alternative promoters and splicing of p63 adapted from Levrero *et al* (1999)
Exons are numbered 1 to 15, with different splice variants connected by alternating connecting lines. TA-p63α is composed of all 15 exons, TA-p63β contains all exons excluding 13, TA-p63γ is the shortest TA-p63 isoform missing exons 11 to 14. TA-p63 and ΔNp63 proteins contain the DNA binding domain and tetramerization domain (ISO). The DNA binding domain interacts with promoter sequences driving the expression of other genes. Tetramerization domains help with the formation of the proteins tertiary structure. Variation arises in the presence of a sterile alpha-motif (SAM), these domains are protein interaction modules which can homo or hetero oligomerise (Peterson *et al.*, 1997). SAM domains have also been shown to interact with RNA and other molecules. In addition to the afore mentioned domains the ΔNp63α protein also contains a transactivation-inhibitory domain, this domain can bind to the transactivation domain rendering the TA-p63 protein non functional (Koster *et al.*, 2004). The TA-p63α also contains this domain and can directly inhibit its own activity.

The two distinct forms of *p63* are *TAp63* and *ΔNp63*, distinguished by their alternative N-terminus arrangements (figure 1.8). Each of these two proteins has three splice variants α, β and γ which alter the C-terminus. Alpha isoforms include a transactivation-inhibitory domain which can inhibit TAp63 proteins. In humans inhibition of TAp63 promotes the maturation and subsequent differentiation of epidermal cells (Mills *et al.*, 1999).

Up-regulation of *p63* does not necessarily lead to enhanced transcriptional activation of other genes. Cleavage of α subunits from both proteins must take place for any wide scale transcriptional shift. During apoptosis caspases (3,6,7 and 8) can cleave the α subunit allowing *p63* to activate genes such as *BAX*, *MDM2* and *p21* (Yang *et al.*, 1998), leading to cell cycle arrest at G1 and G2 checkpoints by activating *Cdk4,2* and *6*. Interestingly, all *ΔNp63* isoforms can inhibit *p53* which is upstream of *p21* in most damage induction pathways (Serber *et al.*, 2002). However it has also been suggested that an isoform of *p63* interacts with p57^{Kip2} which allows cell cycle progression at this point (Su and Flores, 2009).

P63 is debated as a true stem cell marker with a number of papers questioning its specificity. Di Iorio and colleagues stated that only *ΔNp63α* is present in limbal epithelial cells and only when stimulated by wounding *in vivo* (Di Iorio *et al.*, 2005). It is thought that as cells migrate from the limbal region, their gene expression changes to allow for a higher proliferative rate (figure 1.9).
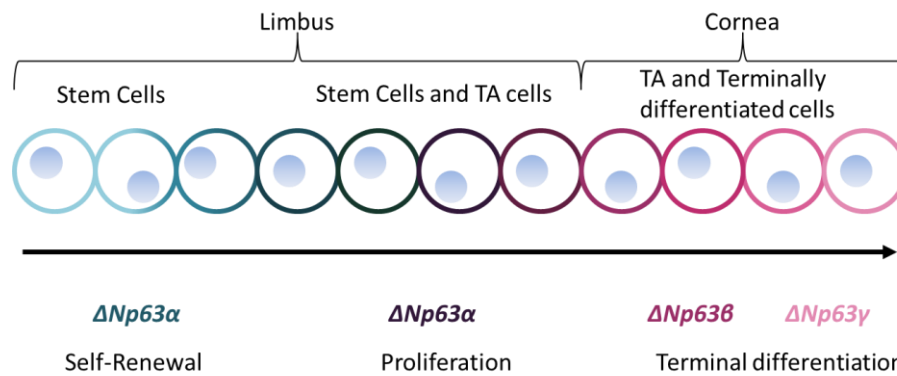
Figure 1.9 Diagram showing the differential expression of *p63* and its isoforms throughout the limbus and cornea adapted from Pellegrini *et al* (2009)
ΔNp63α is expressed in resting stem cells and in early transiently amplifying cells (TA). *ΔNp63β* and *ΔNp63γ* on the other hand, are expressed in the terminally differentiated cells of the cornea.

Senoo *et al* (2007) found that 94% of rat thymic epithelial cells, cultured in a similar medium composition, e.g. insulin, EGF, 3,3-5-triiodo-L-thyronine, hydrocortisone, adenine, cholera toxin in DMEM/Ham's F12, 10% fetal bovine serum and on 3T3's, similar to limbal epithelial stem cell culture, expressed *p63*. The authors suggested that the conditions in which epithelial cells are grown have a major impact on the gene expression, however the specific *p63* isoform was not stated (Senoo *et al.*, 2007).

1.1.2.2 ABCG2

ABCG2 is a cell membrane protein found on most cells however it is upregulated in adult stem cells, and as such a common marker. *ABCG2* belongs to a large group of ABC transporters which are highly conserved within the eukaryote kingdom. The transporters use the hydrolysis of adenosine triphosphate as an energy source to translocate large molecules such as lipids and mRNA molecules. *ABCG2* codes for protein ATP binding cassettes which homodimerise (Krishnamurthy and Schuetz, 2006) to form pumps which move various molecules out of the cell. This ability to pump exogenous xenotoxins and endogenous compounds out of the cell leads to the classification of ABCG2 as drug resistance protein. One such role is in the transport of porphyrins such as haem which are endogenous compounds. Haem is pumped out of the cell in hypoxic conditions,

without oxygen very little haem is produced and intermediary compounds build up, such as protoporphyrin, leading to toxicity. The up-regulation of *ABCG2* in stem cells is thought to serve in the protection of the genome from mutagenic factors, such as free unpaired cations, in these slow cycling cells. *ABCG2* was demonstrated as being expressed in basal limbal cells *in vivo* and at high levels in primary limbal epithelial cultures (de Paiva *et al.*, 2005). ABCG2 can be used to identify a sub-population of cells via fluorescence activated cell sorting, this population is called a side population as it is distinguished via a differential staining or lack of it, relative to the main population of cells. Recently, it has been shown that limbal epithelial cell side populations positive for ABCG2 decrease with age, suggesting that this gene is a good marker of stem and TAC in the context of ageing (Chang *et al.*, 2011). It is important to consider however, that all cells have ABCG2 on their surface and can efflux staining dyes, so identification of cells via ABCG2 requires additional confirmatory markers.

1.1.2.3   BMI1

BMI1 is a polycomb ring finger oncogene. Polycomb proteins are transcriptional repressors able to silence epigenetic signals which help to determine cell identity (Sparmann and van Lohuizen, 2006). *BMI1* can also be used to distinguish senescence from quiescence, as *BMI1* is down regulated in senescence but not in quiescence (Park *et al.*, 2004). *BMI1* regulates the expression of $p16^{INK4A}$ and $p19^{Arf}$ (Lessard and Sauvageau, 2003) which are important cell cycle checkpoint proteins. Inactivation of *p19* has the downstream effect of down regulating *p53*, preventing cell cycle arrest, through the *p21* mediated pathway. Inactivation of $p16^{INK4A}$ affects the G_0 to G_1 cell cycle checkpoints. Without $p16^{INK4A}$ cyclin D can accumulate and complex with CDK4/6 inhibiting its kinase ability, pRb can then become hyperphosphorylated and is unable to bind to the E2F transcription factor resulting in cell cycle progression. So far BMI1 has been shown to be involved in the self-renewal of intestinal, haematopoietic (HSC) and neural stem cells (Molofsky *et al.*, 2003; Iwama *et al.*, 2004; Sangiorgi and Capecchi, 2008). Importantly, *BMI1* was found to identify potential limbal stem cells in a quiescent state (Barbaro *et al.*, 2007).

In addition to its cell cycle roles *BMI1* may also regulate telomerase reverse transcriptase (*TERT*) expression (see 1.1.2..4). In human mammary epithelial cells lines over expression of *BMI1* leads to extended replicative lifespan in culture. It is likely that

BMI1 acts by down regulating a mediator of telomerase (Pietersen *et al.*, 2008). Interestingly the effect seems to be cell type specific with over expression of *BMI1* not inducing *TERT* in human fibroblasts (Dimri *et al.*, 2002).

1.1.2.4 TERT

Telomerase consists of a catalytic subunit TERT and an RNA component TERC, the enzyme uses the RNA template to reverse transcribe terminal regions of chromosomes. In somatic cells, the telomeres are eroded due to the "end replication problem" as well as oxidative stress (von Zglinicki, 2000). The end replication problem is a phenomenon whereby terminal telomeric sequences are omitted from synthesis due to the binding of an RNA primer, during DNA replication of the lagging strand. DNA polymerase works exclusively in a 5'-3' direction, to replicate the lagging strand. RNA primers are needed to allow the polymerase to synthesise small sequences called Okazaki fragments (Okazaki and Okazaki, 1969). These fragments are then ligated together by DNA ligase. However during this process the terminal binding of the RNA primer prevents addition of nucleotides to the section, resulting in exposed overhangs and the loss of the section during consecutive replication events.

In vivo, adult stem cells are slow dividing cells, however often long lived. Telomerase is thus required to maintain the telomeres and plays a major role in the self-renewal properties of stem cells (Hiyama and Hiyama, 2007). The importance of telomerase in stem cells is highlighted by knockdown of the RNA component (TERC) in embryonic stem cells, which leads to a loss of their immortality (Niida *et al.*, 1998). There is substantial evidence of non-telomere related functions of *TERT*, so called non-canonical functions. These functions include the activation of Wnt, Myc signalling (Park *et al.*, 2009) and improved DNA damage repair kinetics (Sharma *et al.*, 2003). TERT has also been shown to reduce ROS production and increase the mitochondria membrane potential (Ahmed *et al.*, 2008; Singhapol *et al.*, 2013). All of these factors help to maintain the integrity of the genome in stem cells.

In the corneal epithelium, *TERT* is thought to be expressed in limbal epithelial cells and immediate TAC, similar to what is observed in the haematopoietic system (Chiu *et al.*, 1996; Umemoto *et al.*, 2006). Specifically in cultured limbal epithelial cells telomerase activity was not affected by advanced age (Notara *et al.*, 2013), however assessing *TERT* at the mRNA level was neglected.

1.1.2.5   KRT3 and 12

Keratin (K) 3 and K12 are intermediate filament proteins which form heterodimers and polymerise to the intermediate filaments (Moll *et al.*, 1982). K3 is a basic type intermediate filament protein, while K12 is acidic, both K3 and K12 correlate with more differentiated cells of the corneal epithelium (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). *In vivo*, KRT3 is expressed throughout the corneal epithelium, but is absent in the basal layer of the limbus. During the process of airlifting, differentiation is induced, encouraging stratification of a culture. This is done by reducing the medium level, exposing cells to the overall result of which leads to increased expression of *KRT12* and *3* (Osei-Bempong *et al.*, 2009). In this study, expression of *KRT12* and *KRT3* were used as negative markers of limbal progenitor cells.

1.1.2.6   p21

Cyclin dependent kinase inhibitor 1 (*CDKN1A)* or p21 is a key cell cycle progression protein. G_1 is the cell cycle phase in which cyclin D/CDK4 and E/CDK2 promote further proliferation. The protein p21 regulates cell cycle progression at the G_1-S phase by inhibiting the kinase component and preventing subsequent activation of cyclins (Gartel and Radhakrishnan, 2005). CDK4 phosphorylates the retinoblastoma gene product (Rb). The phosphorylation of Rb causes the release of the normally tightly bound E2F. E2F is a major transcription factor which triggers downstream initiation of DNA synthesis proteins and other cell cycle related cyclins.

One of the classical methods by which p21 is activated is in response to DNA damage, mediated through p53 (Eldeiry *et al.*, 1993; Dulic *et al.*, 1994). This response protects the genome from propagating potentially deleterious mutations. It is at this point which p21 can initiate apoptosis or senescence, thus increases in p21 expression are indicative of increased DNA damage and subsequent senescence induction.

1.1.3   Oxygen tension, the culture of limbal epithelial cells and the niche

Human LSCs are conventionally grown in atmospheric oxygen conditions (21%). In recent years the importance of oxygen in the regulation of cultured cells has been recognised (Csete, 2005; Saretzki, 2011). Physiologically the concentration of oxygen is significantly different from atmospheric conditions. It has been suggested that the oxygen tension for the culture of stem cells should mimic those with which these progenitor cells would normally encounter *in vivo*. Internally normal physiological oxygen concentrations vary

dependent on the tissue, in bone barrow for example 0% can be found, whereas (Tondevold *et al.*, 1979) 14% is common in the lungs (Saltzman *et al.*, 2003). These tensions are both much different from atmospheric conditions.

A number of stem cells are already cultured in hypoxic conditions, those include embryonic, neural, haematopoietic and mesenchymal (Danet *et al.*, 2003; Ezashi *et al.*, 2005; Prasad *et al.*, 2009; Estrada *et al.*, 2012). Culturing LSCs in hypoxia is still highly controversial. Currently there are only five papers which have studied the effects. The papers cover a diverse number of organisms and medium types, with studies in mice, rabbits and humans in defined keratinocyte serum free medium (DKSFM), limbal epithelial medium (LEM) and Epilife (See 2.2.1 and appendix D). In addition to this each study varied in the oxygen tensions used for hypoxic cultivation of LSCs (2-15%). The earliest paper which studied the effect of hypoxia on human cells in DKSFM, initially indicated that hypoxia is beneficial for the growth of these cells (Miyashita *et al.*, 2007). Miyashita and colleagues showed that the number of colonies formed in hypoxia was greater than those in normoxia. In addition, the expression of the negative marker gene *KRT3* was also shown to decrease in hypoxia compared to normoxia using RT-PCR. Immunofluorescence of the positive marker gene *K15* however showed no change between oxygen tensions (Miyashita *et al.*, 2007). Four years later, three papers where published, one of which contradicted the findings of Miyashita and colleagues. Colony forming assays on rabbit corneal epithelial cells showed a decreased in hypoxia. RT-PCR however was unable to show any significance between the conditions with both ABCG2 and *ΔNp63α*. These results were however generated using corneal epithelial cells from rabbit cultured in LEM (O'Callaghan *et al.*, 2011). By contrast, in mice, colony forming ability was greater in hypoxia, with DKSFM as the medium used in this paper (Ma and Liu, 2011). The group also noted that cells cultured in hypoxia reached confluence quicker than those in normoxia. Immunofluorescence of ΔNp63 and K19 were used as positive markers, however it was not commented upon if there was a statistical difference between the conditions (Ma and Liu, 2011). Cells isolated from mice, however tend to grow better in hypoxic conditions, namely due to their reduced tolerance to oxidative stress (Parrinello *et al.*, 2003). To add to the plethora of experimental conditions in which hypoxia has been tested, Li and colleagues studied the effects of different oxygen tensions on limbal explants from both rabbit and human (Li *et al.*,

2011). The proliferation marker Ki67 was more prominent in hypoxic cultured airlifted explants compared to those in normoxia. The positive stem cell markers p63 and K14 on the other hand increased in normoxia. K14 is a type I intermediate filament protein which is associated with progenitor cells (Zhao *et al.*, 2008). The authors noted that in hypoxia the localisation of p63 became more nuclear. Greater accumulation of p63 in the nucleus suggests that the protein is having a more prominent effect on the expression of other genes in hypoxia. Confusingly, the paper then goes on to state that Western blotting confirmed the up-regulation of Ki67, p63 and K14 in hypoxia contradicting there previous data on p63 and K14. Overall, the paper states that human explants undergoing airlifting have enhanced proliferation compared to normoxic counterparts (Li *et al.*, 2011). This paper also studied epithelial sheets derived from rabbit corneas, these were also airlifted to induce stratification (Kinoshita *et al.*, 2004). Immunofluorescence was used to study the proteins K10 and K12, which are both makers of epithelial differentiation. K10 was expressed in epithelial sheets cultured under normoxia, however it was not present in hypoxia. K12 on the other hand, was present in both conditions. These results were confirmed using RT-PCR, indicating that hypoxia has a clear effect on the expression of certain keratins in the cornea.

The most recent study by Bath and colleagues was the most comprehensive, with experiments in both a serum free condition (Epilife) and using the traditional LEM. In LEM they noted that colony forming ability tended to increase in hypoxia. This agrees with both Miyashita and Ma, who however, grew their cells in DKSFM. Colony forming efficiencies were not performed under serum free conditions by Bath and colleagues boding to an objection to the ability to distinguish individual colonies (Bath *et al.*, 2013). Immunofluorescence for the positive markers ΔNp63, ABCG2 and the negative marker K3 was used in their study. Generally, in LEM no detectable difference was observed in the positive markers, except at 15% oxygen which decreased both ΔNp63 and ABCG2 at the protein level. Interestingly K3 was increased at this oxygen tension, while further reductions in oxygen decreased the amount of K3. Serum free conditions differed from those observed in LEM. The positive marker ΔNp63 increased in hypoxia, ABCG2 on the other hand had a more complicated expression pattern, initially decreasing with reducing oxygen, however increasing at 2% oxygen. The converse was true for K3 which increased initially, decreasing at 5% and 2%. It is clear from these results that more

research is required to determine if hypoxia is a beneficial condition in which to culture limbal epithelial cells.

The limbal niche, as mentioned previously, cannot be firmly identified due to a lack of true stem cell marker genes. However, if one assumes that the stem cells originate from the limbus, it is possible to extrapolate the hypothetical oxygen concentration to which these cells may be exposed to *in vivo* (figure 1.10).
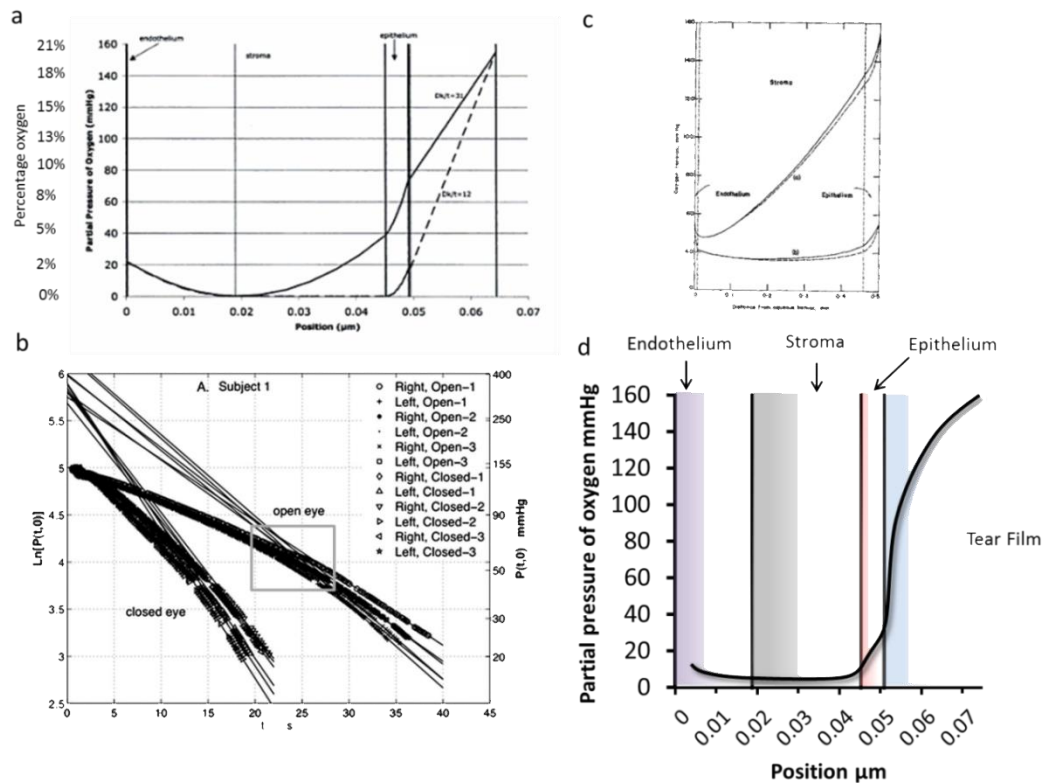
Figure 1.10 The *in vivo* concentration of oxygen in the cornea
Panel a and c desribe existing theories of oxygen permeability (Lin, 1976; Brennan, 2005). Panel b, shows the surface oxygen concentration, the partial pressure of oxygen highlighted in the grey box is around 60mmHg (7.8%) (Takatori *et al.*, 2012). Panel d, extrapolates the effect the new values discovered in panel b,.Towards the basal layer the mmHg of oxygen could reach as low as 10-15, resulting in a concentration of around 1.3-2%.

The oxygen tension of the cornea is difficult to measure, as it fluctuates with the opening and closing of the lid. Traditionally mathematical equations predicted the partial pressure of oxygen (Lin, 1976) (figure 1.10, b) or crude invasive measures were used (Kwan *et al.*, 1972). One of the major draw backs of Kwan's method was that breaching the tissue allowed oxygen to contaminate the true *in vivo* measurements. In 2002 Stucker and colleagues used a fluxoptode to measure the oxygen supply to the dermis and epidermis. Their results suggested that the epidermis received around

140mmHg (18%) while the dermis is around 50mm Hg (6%) (Stucker *et al.*, 2002). Recent studies used advanced microelectrodes (Clark) to measure corneal surface oxygen in a less invasive manner (Takatori *et al.*, 2012). Using this method the authors found the surface of the corneal epithelium had an oxygen level of around 60mm Hg (7.8%). These values are representative of central corneal region while, the limbus is juxtaposed to the conjunctiva, thus it is closer to vasculature of this region (figure 1.11).
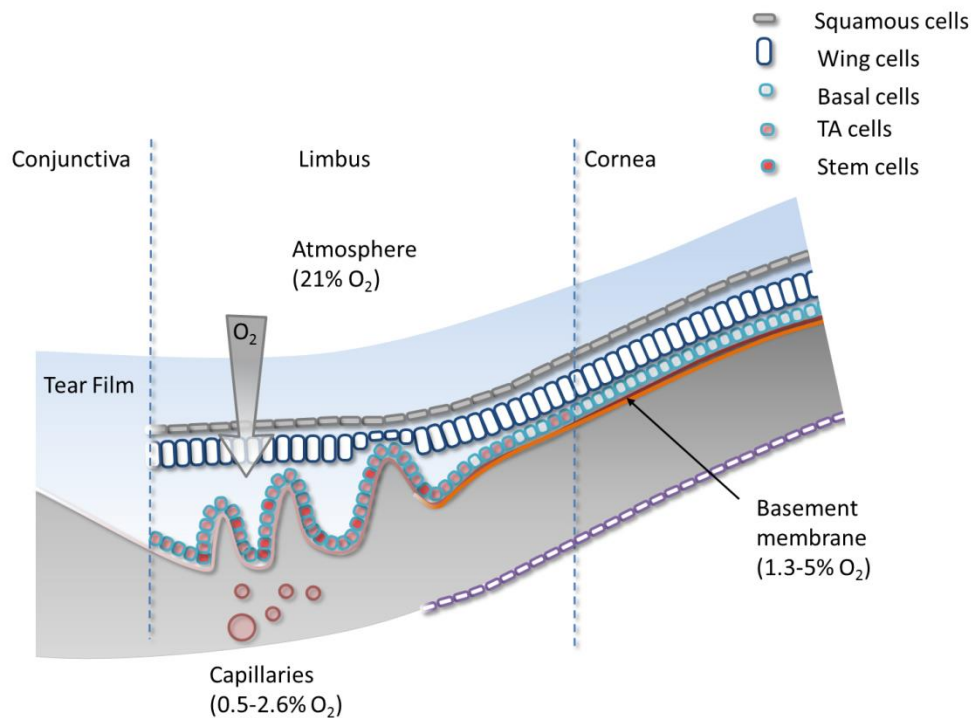
Figure 1.11 Hypothetical oxygen tension in the limbal niche
The basement membrane is hypothesised as being at around 1.3-5% oxygen (Takatori *et al.*, 2012), whilst peripheral capillaries contain about 0.5-2.6% oxygen (Brahimi-Horn and Pouyssegur, 2007).

Hypoxia triggers the formation of a heterodimer consisting of the constitutively active aryl hydrocarbon receptor nuclear translocator (ARNT) (Kallio *et al.*, 1997) and hypoxia inducible factor HIF-1α (Huang *et al.*, 1996). HIF-1α is the limiting protein in HIF-1 formation. In hypoxia, HIF-1α can accumulate because of the reduction in hydroxylation at specific proline residues and acetylation of lysine amino acids. These modifications occur in the oxygen dependent degradation domain (ODDD) of HIF-1 α by 2-oxoglutarate (2-OG)-dependent dioxygenase via hydroxylation (Srinivas *et al.*, 1999) and arrest-defective-1 (ARD1) via acetylation (Jeong *et al.*, 2002). In normoxia these residues would elicit the von Hippel-Lindau ubiquitin E3 complex (pVHL) causing ubiquitination and subsequent proteasomal degradation (Kamura *et al.*, 2000).

HIF1 induces an array of promoters to up regulate or down regulate important pathways. One of the genes which are transactivated by HIF-1α is telomerase reverse transcriptase (TERT), in placental JAR, JEG-3 cells (Nishi *et al.*, 2004) and cervical cancer cells (Yatabe *et al.*, 2004). Knockdown of HIF-1α reduces the expression level of *TERT* and causes a subsequent reduction in telomere length (Coussens *et al.*, 2010). HIF-1α often interacts with the promoter regions of genes to directly regulate them, the sequences which are recognised are called hypoxia response elements (HRE). *TERT* has two of these regions located within the promoter region (Nishi *et al.*, 2004; Yatabe *et al.*, 2004). The further roles of *TERT* in stem cells were discussed in section 1.1.1. It is debated as to the importance of HIF-1α in stem cells. Over expression of HIF-1α can lead to differentiation of embryonic stem cells (Jeong *et al.*, 2007). HSCs however have been shown to exist in an hypoxic environment (Kubota *et al.*, 2008). Additionally in mesenchymal stem cells, hypoxia was shown to maintain the stem cell properties and inhibit senescence through the down regulation of p21 by the HIF-TWIST complex (Tsai *et al.*, 2011). Mesenchymal and HSC are not the only adult stem cells to benefit from hypoxic culture, neuronal stem cells are also grown in this condition, and has been shown to promote self-renewal. One of the pathways which were implicated in this process was the Notch pathway. HIF-1α can directly interact with the intracellular domain of Notch preventing cleavage of the domain and its transport to the nucleus, whereby it can regulate subsequent gene expression.

Hypoxia has an effect on cellular proliferation, knockdown of HIF-1α increased mitotic activity of HSC's, shown using Ki67. The over expression of HIF-1α decreased the number of Ki67 positive cells (Takubo *et al.*, 2010). In the mouse cornea Ki67 positive cells within the basal cell layer were much more frequent in the central region, compared to no Ki67 positive cells being detected in the limbus (Fabiani *et al.*, 2009), HIF-1 may be the regulator for this difference in that case. Hypoxia is clearly an important parameter when culturing stem cells and should not be overlooked. It is still debated as to if hypoxia is beneficial or detrimental to the culture of LSCs.

1.1.4   Limbal stem cell controversies

One of the methods of assessing stemness of a culture *in vitro* is to use growth assays, such as colony forming assays, which score greater number of colonies and size as indicative of a higher proportion of stem cells. However *in vivo*, true stem cells are

considered to be in a state of quiescence. Cultures which can produce large numbers of cells quickly are beneficial in terms of generating cellular material for potential therapies. As cellular culture always stimulates proliferation of stem cells, it raises the question if true stem cells are the focus of many studies and transplantations or just the immediate progeny of these cells?

This highlights the second major controversy which is specific to the generation of cultures containing limbal stem cells. The lack of marker genes, means that one can only indirectly extrapolate and infer the generated results of any analysis resonate to potential stem cells within the tissue or culture, one reason for referring to these cells as limbal epithelial cells. One method of belaying this impediment is to use an array of genes, both positive and negative when assessing stem cell properties. It can be equally useful to show a reduction in negative marker genes as it is to show increases in positive genes. However there is no set consensus on the correct or optimal number of marker genes needed, which means comparing results between different groups can be difficult.

The third controversy is related to the lack of a specific stem cell marker, for determining the niche location (which has been covered previously). The majority of scientists argue that limbal stem cells are located primarily in the limbus (Cotsarelis *et al.*, 1989; Kenyon and Tseng, 1989; Bodnar *et al.*, 1998; Lehrer *et al.*, 1998), while others think that stem cells are distributed throughout the cornea (Majo *et al.*, 2008) (figure 1.6).

1.2 Ageing of stem cells

1.2.1 Ageing

Ageing is the process of growing old and is associated with a decline in function with an increased risk of mortality. Ageing can be characterised by physical changes, such as reduced weight, height, muscle mass and skin sagging. Internally ageing affects the ability to reproduce, hearing, sight, kidney function, liver function, immune responsiveness and some aspects of memory. Ageing also leads to an increased susceptibility to certain diseases.

Cellular ageing has common hallmarks, genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, dis-regulated nutrient sensing, mitochondrial dysfunction, senescence, stem cell dysfunction and altered cellular signalling.(Lopez-Otin

et al., 2013).

The major debate in ageing research is the cause of ageing, is it a deterministic process, i.e. does it follow a pre-determined program or is it a random process? Evolutionary, ageing is hard to rationalise, on the one hand the impairment of function, particularly fertility, would be a definite selection pressure. On the other hand however, in wild populations organisms hardly ever reach an age whereby the effects of age are present (Lack, 1954), be it through predation, disease or accidental death . This means that ageing is never subjected to selection pressure in the wild, so why does ageing occur?

1.2.1.1   DNA Damage

It is commonly accepted that DNA and protein damage builds up over time (Kirkwood, 2005; Vijg and Campisi, 2008; Moskalev *et al.*, 2013), through extrinsic chemical and biological damage. Intrinsic modifications can also occur, during the process of DNA replication which is prone to errors, despite DNA repair mechanisms. Damage is particularly detrimental when it occurs in stem cells, mutations which lead to either oncogene activation or senescence impact upon the maintenance of tissues (Rossi *et al.*, 2008; Jones and Rando, 2011). In humans deficient DNA repair mechanisms result in accelerated ageing, such as WRN protein in Werner syndrome (Goto *et al.*, 1992) and BLM in Bloom syndrome (Karow *et al.*, 1997) which both belong to the RecQ DNA helicase family of proteins. RecQ helicase's unwind DNA allowing for repair to take place, such as base excision repair. With age, the homeostasis of DNA damage to repair ratio may change resulting in a damage cascade. Whether this is a cause of ageing or symptom remains to be experimentally proven.

1.2.1.2   Telomeres

The telomeres erode with age and for some time were thought to be the key to cellular senescence and named the so called "cellular clock". In culture, telomere length governs the length of time a primary cell can remain mitotically active (Hayflick and Moorhead, 1961). Activation of telomerase often immortalises those cells (Bodnar *et al.*, 1998). Damage accumulates in these regions with age, due to the array of nucleoprotein complexes, such as Shelterin and t-loop elements which prevent the efficiency of repair mechanisms. These complexes normally hinder the DNA damage response machinery

from recognising the terminal sequences as double strand breaks (DSB). Accumulation of damage however in telomeres eventually leads to senescence and apoptosis (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). In some human diseases, where telomerase is impeded, an accelerated ageing phenotype is observed, an example being Dyskeratosis 25ongenital. Conversely the activation of telomerase in mice can delay the physiological effects of age (de Jesus *et al.*, 2011).

1.2.1.3 Epigenetic changes during ageing

Non genetic changes also occur with age, the methylation and acetylation status of many histones are altered (Fraga and Esteller, 2007). Methylation and acetylation of histones can govern the expression of large portions of genes through the shielding of sequences from transcription machinery. Sirtuins (SIRT) are a family of deacylases and ribosyltransferases. SIRT1 translocates to DNA damage sites to promote repair and alter gene expression profiles, through chromatin remodelling, over expression of SIRT1 increases genomic stability (Oberdoerffer *et al.*, 2008). SIRT6 regulates glycolytic genes through the deacetylation of histone H3 lysine 9 (H3K9) (Zhong *et al.*, 2010). SIRT3 can attenuate NF-κB signalling, by the deacetylation of H3K9 (Kawahara *et al.*, 2009), elevated NF-κB can lead to apoptosis and cellular senescence. Over expression of SIRT3 in stem cells improves the regenerative capacity of aged stem cells (HSCs), possibly through increased oxidative stress resistance shown by reductions in superoxide dismutase 2 (Brown *et al.*, 2013).

1.2.1.4 Mitochondria

Mitochondria are central to cellular energy production, producing ATP via oxidative phosphorylation (OXPHOS). Within the mitochondria, the tricarboxylic acid cycle generates molecules such as NADH which donate electrons to the inner membrane electron transport chain. The electron transport chain is a series of protein complexes which create a proton gradient between the cytoplasm and the mitochondrial matrix. This gradient is essential for ATP synthase, which requires protons to drive the rotary F_O and F_1 regions, causing a conformation change which leads to the conversion of ADP + Pi to ATP.

Mitochondrial dysfunction increases with age and the electron transport chain becomes less efficient. This has two effects, firstly it increases the amount of free

radicals in the cellular compartment and secondly it results in decreased ATP production. The by-products of aerobic respiration are reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl ion. These species can then go on to further damage the mitochondrial and nuclear DNA, expediting the reduction in ATP production and increasing free radicals (Harman, 1965). The impact of increased ROS with age however has recently been shown to have little effect on the longevity of an organism (Doonan *et al.*, 2008). In model organisms such as *Caenorhabditis elegans* it even increased lifespan (Van Raamsdonk and Hekimi, 2009). In mice, decreasing superoxide dismutase does not shorten lifespan despite increasing DNA damage and cancer formation (Van Remmen *et al.*, 2003). A new theory has been suggested which explains this phenomenon, based on a study which showed that ROS triggered proliferation and stem cell differentiation in MSCs (Tormos *et al.*, 2011) and HSCs (Owusu-Ansah and Banerjee, 2009). The theory states that as ROS levels increase with age, homeostatic responses also increase, however once a critical level is reached homeostatic responses cannot compensate for the quantity of ROS produced.

The effects of mitochondria on ageing however are not limited to ROS production. Deficiencies in the maintenance of mitochondrial DNA are associated with accelerated ageing. Mice which have deficient DNA polymerase γ, an enzyme responsible for mitochondria DNA proof reading, have an ageing like phenotype (Kujoth *et al.*, 2005; Edgar *et al.*, 2009). In humans mutations in this gene (*POLG*) lead to Alpers syndrome, which has some characteristics of ageing such as dementia, hearing loss and blindness (Hunter *et al.*, 2011). The mechanism by which mitochondrial mutations manifest themselves are thought to be through increased susceptibility to trigger apoptosis by decreasing the ability to maintain the mitochondrial membrane potential (Kroemer *et al.*, 2007). Mitochondrial function decreases with age (Sahin *et al.*, 2011) and has been shown to be ameliorated by telomerase activation shown by increased levels of Peroxisome proliferator-activated receptor γ co-activator 1-alpha (PGC-1α), ATP synthase and Estrogen-related receptor alpha (ERRα) (de Jesus *et al.*, 2012). PGC-1α interacts with transcription factors to increase the effectiveness of transcription, PGC-1α has a large number of transcription factor targets, namely those involved in mitochondrial biogenesis. One of the key transcription factors PGC-1α co-activates is the nuclear respiratory factor (NRF)1, which leads to increased mitochondria transcription

factor A (Wu *et al.*, 1999). Further to this evidence, overexpression of PGC-1α in *Drosophila* increases lifespan by 33%, the intestine was improved and maintained integrity with age (Rera *et al.*, 2011).

1.2.1.5 Stem cells and ageing

Stem cell ageing, may seem a contradiction as their ability to self-renew is a defining feature of stem cells. Self-renewal is described as the ability to produce progenitors which are identical to them self and can maintain a continuous steady stem cell population. The ability to replicate in this manner requires the exact replication of the genome. A theory emerged which could enable cells to maintain an "immortal strand" through the segregation of parental strands to preserve the "old" DNA (Cairns, 1975). In 2007 however, this theory was challenged when HSC were shown to segregate chromosomes randomly, thus not asymmetric (Kiel *et al.*, 2007).

Recently it has been shown that stem cells do acquire DNA mutations over time (Rossi *et al.*, 2007; Rube *et al.*, 2011). The origin for that is unknown, whether it comes from environmental stress or intrinsic replication errors. None the less, DNA alterations can lead to initiation of senescence or even apoptosis, or at least lead to a higher susceptibility to senescence and apoptosis in the resulting progenitors. Human mesenchymal stem cells are one type of adult stem cell which has been shown recently to undergo replicative senescence (Wagner *et al.*, 2008; Sahin and DePinho, 2010; Estrada *et al.*, 2013).

It was suggested, understandably that *hTERT* plays an important role in abrogating senescence (Estrada *et al.*, 2013), not only by telomere extension but also by managing mitochondrial oxidative stress. In addition to this telomeres have been shown to shorten in stem cell pools with age *in vivo*, in the small intestine, testis, brain and importantly in the corneal epithelium (Sharpless and Depinho, 2007; Flores *et al.*, 2008). In humans replicative senescence has been demonstrated in adipose tissue derived human multipotent stem cells *in vitro* conditions (Jung *et al.*, 2010). In their study, Sirtuins were implicated in the control of senescence as they were shown to down-regulate polycomb genes such as BMI1 and up-regulate jumonji domain containing 3 (JMJD3) which can govern p16[INK4A] driven senescence.

In mice, satellite cells have a reduced regenerative potential with age (Conboy *et al.*, 2005), which is now thought to be triggered by a switch from quiescence to

senescence in the stem cell population. This switch is proposed to be caused by de-repression of p16^{INK4a} (Sousa-Victor *et al.*, 2014). In mice exposed to thoracic irradiation, the alveolar epithelial cell progenitors isolated from these mice become sensitive to senescence (Citrin *et al.*, 2013). In addition to this, in the *BubR1*$^{-/-}$ mouse, tissue progenitors in muscle and fat were senescent leading to a progeroid like phenotype (Baker *et al.*, 2013b), implicating stem cells as the source of senescence accumulation within tissues.

Telomerase is an important age and stem cell related protein, as mentioned previously (1.1.2.4). Experiments to determine the functions of telomerase often use *Terc* or *Tert* deficient strains (Flores *et al.*, 2008). *Terc* or *Tert* alone are unable to extend telomeric DNA leading to critically short telomeres. F_3 (3rd generation) mice deficient in *Terc*, show an increase in the number of epidermal stem cells compared to F_1 mice (Flores *et al.*, 2005b). It has been suggested that the cause of this behaviour may be due to a "blunted proliferation" response, which means that the stem cell population can still divide however they are unable to differentiate and produce progeny. It may also be due to a compensatory reaction, similar to that seen in irradiated intestinal stem cells (Martin *et al.*, 1998). F_3 *Terc*$^{-/-}$ mice showed a decrease in their HSC proliferative capacity when serially transplanted into irradiated donors (Ju *et al.*, 2007). Colony-forming ability was also reduced, showing a reduction in the number of progenitors. Human haematopoietic stem cells show a loss in proliferative rate and decrease of telomeric DNA comparing a young bone marrow donor aged 16 years and an old, 56 year old donor (Vaziri *et al.*, 1994).

Lack of telomerase also affects highly proliferative tissues in patients with dyskeratosis 28ongenital (Lansdorp, 2009), including in the cornea (Aslan *et al.*, 2009). The disease primarily involves the genes *TERT, TERC, TRF1* and *DKC1*, all of which are related to telomere maintenance (Savage and Alter, 2009). Patients develop an ageing like phenotype with hair greying, dystrophy of the nails and anaemia.

It has been suggested that the non-canonical functions of TERT may prevent the addition of telomeric repeats in the nucleus by sequestration of TERT protein to other cellular compartments, such as the mitochondria (Ahmed *et al.*, 2008). So while telomerase levels may be high this might not translate into telomere extension which may have implications for stem cells.
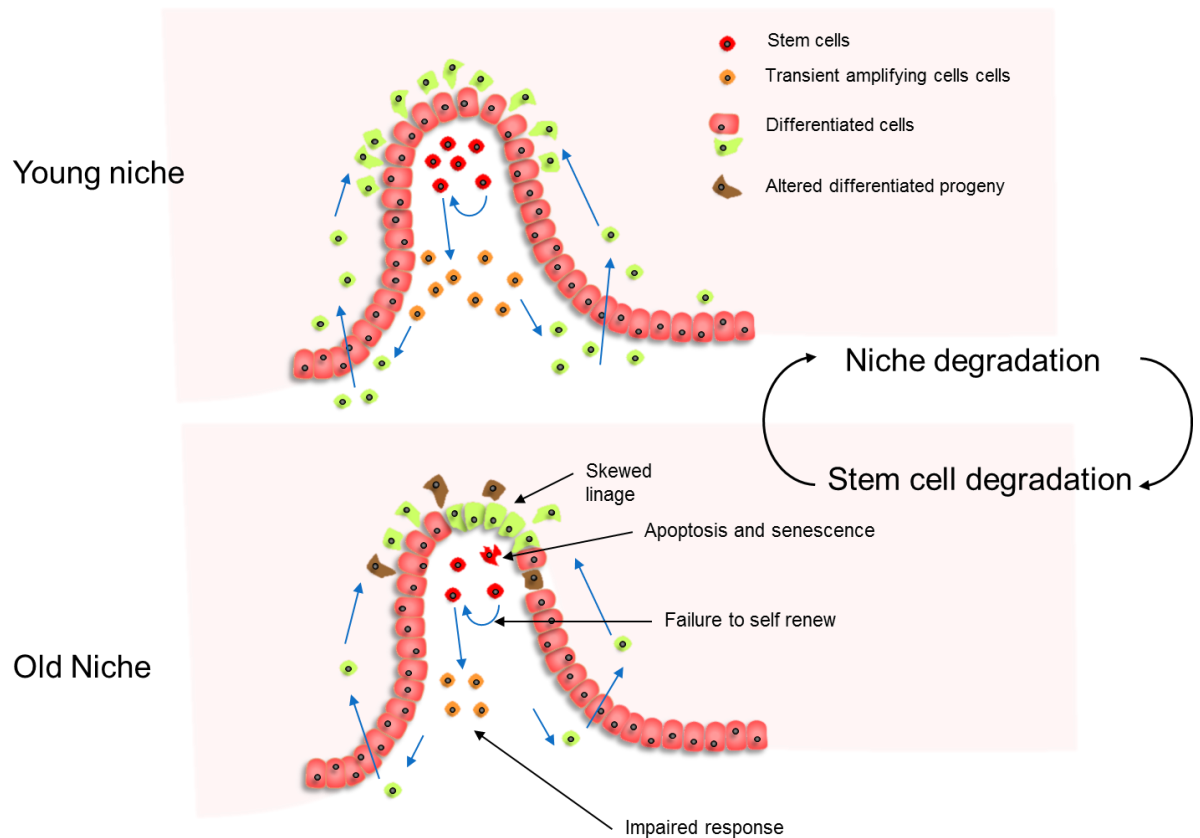
Figure 1.12 A combined theory of stem cell ageing involving stem cells and their niche
The niche and stem cells are implacably linked, dysregulation of one can lead to the other in a negative feedback loop, adapted from Jones and Rando (2011).

Stem cells are undoubtedly linked to their niche, in limbal stem cell deficiency, ablation of the niche (limbus) destroys the stem cell pool leading to disease progression. As stem cells produce cells which comprise the niche, anything which impacts upon the stem cell pool is likely to affect the niche (Ryu *et al.*, 2006; Boyle *et al.*, 2007) and *vice versa* (Chakkalakal *et al.*, 2012)(figure 1.12). The Notch signalling pathway is a key regulator of self-renewal and can react to extrinsic signals, such as those produced by the niche (Liu *et al.*, 2010a). Stem cells are suggested to undergo age related changes in a number of ways: failure to self-renew, impaired response, aberrant differentiation, senescence and apoptosis (Jones and Rando, 2011).

Self-renewal ability is one of the defining characteristics of stem cells. The skin is an organ which shows signs of a decline in self renewal ability with age. Linage skewing of stem cells may produce a lower number of melanocytes, which in turn increases the sensitivity of the skin with age to UV damage with age (Inomata *et al.*, 2009). Satellite cells become increasingly inefficient at regenerating skeletal muscle with age which leads to atrophy, perhaps due to an imbalance in Wnt/β-catenin signalling (Brack *et al.*, 2007).

Impaired response refers to a lack of a sufficient reaction by the stem cell pool to stimuli. Satellite cells suffer from a lack of responsiveness in terms of their decreased number of activated cells produced after environmental stimuli (Conboy *et al.*, 2003). This was then followed up with experiments to remediate these effects through exposure to a young environment (heterochronic parabiosis). The results showed an increase in Notch signalling via its ligand delta in satellite cells (Conboy *et al.*, 2005). The number of cells which are produced by the stem cell pool is important in maintaining tissue integrity. In the cornea, tight junctions between cells are important for protection against invading micro-organisms and maintenance of a smooth surface for its transparency. It is vital that enough cells are produced to maintain the cornea. In support of paradigm, it has been shown previously that the epithelium does lose some barrier function with age (Chang and Hu, 1993).

Another way in which stem cells are hypothesised to change with age is by producing aberrantly differentiated progeny. An example of this is in muscle regeneration, as we age muscle is replaced by fibrous and adipose tissues (Brack *et al.*, 2007), in addition to reduced numbers as stated previously. In the cornea, a change in the types of cells which are produced would have a detrimental effect on the transparency of the cornea and has not been documented. Limbal epithelial cells have however been noted as having a reduced ability to produce intracellular adhesion molecule 1, potentially due to the aberrant differentiation of progeny.

Stem cells have been documented to undergo apoptosis, under stress and ageing (Zhu *et al.*, 2006; Orelio and Dzierzak, 2007; Qin *et al.*, 2007; Roos *et al.*, 2007; Milyavsky *et al.*, 2010). In the skin, Sept4/ARTS were shown to regulate apoptosis within hair follicle stem cells. Sept4/ARTS are antagonists of the protein family inhibitor of apoptosis (IAP). Knockout of *Sept4*/ARTS in mice increased the resistance of hair follicle stem cells to apoptosis (Fuchs *et al.*, 2013). In addition to this, wound healing was also enhanced. Whist the dermis was extensively studied in this paper, the corneal epithelium was not analysed. A study which assessed the effect an embryonic stem cell microenvironment might have on human limbal stem cells, showed that ES conditioned medium could inhibit apoptosis compared to CnT-20 medium. This protection was reported as being telomerase mediated, however a direct mechanism was not elucidated (Liu *et al.*, 2013). Knockdown of *TERT* under this condition using siRNA

resulted in reductions in both p63 and ABCG2, whilst the negative marker KRT3 showed an increase.

1.2.1.6 Senescence

Cellular senescence is a natural process which safe guards the integrity of the genome. It ensures that possible detrimental mutations are not propagated to daughter cells (Campisi and di Fagagna, 2007). During senescence, cells enter a state of irreversible cell cycle arrest at the S phase checkpoint, so the cells remain at G_1 (Sherwood *et al.*, 1988) (figure 13).

Senescence is initiated through a response to stress, either intrinsic or extrinsic, there are four main causes: shortening (Harley *et al.*, 1990) or uncapping of telomeres (Karlseder *et al.*, 2002), oncogene induced senescence (Lowe, 1999), oxidative stress (Chen and Ames, 1994; von Zglinicki *et al.*, 1995) and irradiation (Passos *et al.*, 2010). Dependent on the method of induction, senescence can be coined "replicative senescence", in the case of telomere attrition, in the case of response to oxidative stress and irradiation it is called "stress induced premature senescence" (SIPS). Replicative senescence can occur after the random accumulation of DNA replication errors which are not repaired. Once DNA damage impacts upon genomic stability, the DNA damage response (DDR) triggers irreversible cell cycle arrest (senescence) (Remacle *et al.*, 1992). SIPS is a process of triggering an irreversible cell cycle arrest with high, acute stress, common inducers of senescence are ROS and H_2O_2.

The main induction pathway operates through the tumour suppressors p53 and pRb. When DNA becomes damaged, resulting in either double strand breaks (DSB) or single strand breaks (SSB), ataxia telangiectasia mutated (ATM) and ataxia telangiectasia Rad3 related (ATR) are activated. These proteins phosphorylate p53, allowing the activation of its transcriptional targets, one of these downstream targets is p21. P21 can activate the retinoblastoma protein (pRB) through the inhibition of Cyclin E and cdk2. pRB becomes hypophosphorylated and represses the family of E2F transcription factors. E2F normally activates or inactivates (Narita *et al.*, 2003) various genes associated with cell cycle progression, such as cyclins and cdks from G_1 to S phase (Gaubatz *et al.*, 2000). pRB can also be activated in a p53 independent manner through p16^{INK4a}. p16 is an inhibitor of cyclin D and cdk 4,6 and is not usually expressed in adult tissues (Zindy *et al.*, 1997). However, during senescence increased expression of p16^{INK4a} has been shown to

be induced independent of DNA damage, instead induced by oncogene activation (Ben-Porath and Weinberg, 2004). Cyclin D/cdk4,6 also hypophosphorylates pRB, inactivating E2F.

p38 is a mitogen-activated protein kinase (MAPK) which can also cause cell cycle arrest in a p53 and ATM independent manner (Reinhardt *et al.*, 2007). External stimuli such as ultra violet damage and genotoxic substances activate p38. MAPK-activated protein kinase-2 (MK2) is phosphorylated by p38, an active MK2 then phosphorylates hnRNPA0. hnRNPA0 is a heterogeneous nuclear ribonucleoprotein, which bind to pre-mRNA (Myer and Steitz, 1995) in particular stabilising Gadd45α (growth arrest and DNA-damage inducible gene 45 alpha) mRNA (Reinhardt *et al.*, 2010). Gadd45α causes demethlyation of CpG islands (Zhan *et al.*, 1994), demethylation allows the transcription machinery to bind to DNA, resulting in expression of numerous genes. MK2 also phosphorylates PARN (Poly(A)-specific ribonuclease) preventing its mRNA processing capabilities, which in turn inhibits the degradation of Gadd45α mRNA. This system is a positive feedback loop because Gadd45α activates the transcription of MK2. MK2's major role in senescence is the phosphorylation and sequestering of CDC25B (M-phase inducer phosphatase 2) into the cytoplasm (Manke *et al.*, 2005). Movement of CDC25B protein into the cytoplasm causes a halt in M to G_1 phase transition, but allows S to G_2 progression (Lammer *et al.*, 1998). It has also been shown that p38 can circumvent MK2 and directly bind to CDC25B, phosphorylating the protein which primes CDC25B for binding to 14-3-3 proteins (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) (Bulavin *et al.*, 2001). 14-3-3 proteins also sequester CDC25B into the cytoplasm (Dalal *et al.*, 1999).

ROS has also been shown to be part of a senescence induction feedback loop, where p53 activates p21, which in-turn activates Gadd45α, as previously stated this causes the demethylation of CpG islands resulting in p38 transcription. P38 in turn activates TGFβ resulting in release of ROS from the mitochondria (Passos *et al.*, 2010; Lawless *et al.*, 2012).

H2A.X is a histone protein which forms histone H2A and maintains chromatin structure. H2A.X is phosphorylated by ATM during DNA damage. This phosphorylation of H2A.X at serine 139 leads to the uncoiling of chromatin exposing the damaged DNA, these exposed regions allow proteins to accumulate a so called focal point or foci (di

Fagagna *et al.*, 2003). In senescence the number of foci is shown to increase compared to cells which are undergoing normal cycling (di Fagagna *et al.*, 2003). These foci occur throughout the genome and also accumulate at dysfunctional telomeres (di Fagagna *et al.*, 2003; Takai *et al.*, 2003).The DNA damage response (DDR) is triggered through ATM in response to this dysfunction (di Fagagna *et al.*, 2003; Herbig *et al.*, 2004). Recently it was shown that telomeres are associated with persistent DNA damage response, which increases in prevalence with age (Hewitt *et al.*, 2012). Telomere shortening in somatic cells causes replicative senescence, through the eventual cumulative DNA damage response signals from dysfunctional telomeres that activate p53 and result in a permanent cell cycle arrest.

1.2.2 Dietary Restriction

Dietary restriction (DR) refers to a reduction in the amount of the normal diet of an organism. Caloric restriction is a subtype of DR, whereby the total number of calories are reduced without inducing malnutrition. This means that caloric restriction maintains the amount of all components such as essential amino acids, vitamins, fat, carbohydrates and minerals.

In the context of ageing, DR is one of the only non-genetic interventions which has been shown to significantly increase lifespan. Reducing a rodent's calorie intake by around 30% increases lifespan by between 30-40% (McCay, 1934). In addition to longevity DR can also decrease age related diseases such as cancer, diabetes and immune deficiency (Messaoudi *et al.*, 2006; Colman *et al.*, 2009). Traditionally, the number of calories consumed was thought to determine longevity, however there is growing evidence that it is the composition of nutrients within food which is the determining factor. Autolysed yeast levels in the fruit fly appear to dictate lifespan (Mair *et al.*, 2005). Another group suggest that the levels of amino acids are implicated in lifespan extension (Grandison *et al.*, 2009). In *Saccharomyces cerevisiae* modulating the levels of amino acids can alter lifespan (Alvers *et al.*, 2009; Wu *et al.*, 2013). The non-essential amino acids asparagine and glutamate in particular have been implicated as dictating lifespan (Powers *et al.*, 2006). When looking at vertebrates, the lifespan of rats can also be ameliorated by the essential amino acid methionine, rather than the non-essential amino acids shown to be required in yeast (Malloy *et al.*, 2006; Elshorbagy *et al.*, 2010).

In mice, an animals genetic background appears to also play a substantial role in the outcome of DR. Liao and colleagues tested 41 mice strains, of which only 5% of male mice and 21% female showed any beneficial effects on lifespan extension. Conversely, DR decreased lifespan in 27% of mice strains. These differences were hypothesised to be due to the differing metabolism, resulting in DR of 30% being more severe on some strains while not sufficient for others (Liao *et al.*, 2010).

In primates the effects of DR are not as conclusive as in other organisms. Mattison and colleagues at the National Institute for Ageing (NIA) in the USA ran a 25 year long project on *Macaca mulatta*. Lifespan in these primates was not shown to be increased, however the health of the animals was improved, with lower cancer rates and diabetes (Mattison *et al.*, 2012). In another study, Colman and colleagues showed that DR improved survival rates, however the effects on lifespan are not significant yet, as this experiment is still ongoing. Similarly to the experiments conducted at the NIA on rhesus monkeys Colman showed a marked difference in the health of the DR treated animals, with significant decreases in cardiovascular disease and cancer by around 50%. Interestingly diabetes was completely absent from the DR group. The difference between these two studies is thought to lie in the experimental design, with the control animals used by Mattison have a healthy *ad libitum* (AL) diet, Colman's animals however had a more standard primate diet (Bourzac, 2012).

The evolutionary hypothesis or adaptive resource re-allocation hypothesis is one possible explanation for the physiological changes during DR. When an organism has a finite amount of energy self-preservation is favoured over reproduction (Kirkwood, 1977), in the hope that when nutrients become more plentiful reproduction can resume (Holliday, 1989). Adaptations which preserve an organism's vigour, so that it can later reproduce would be evolutionarily beneficial. A contrasting argument is that DR could trigger a response to reproduce. The reasoning behind this hypothesis lies in the mechanistic effect autophagy (Longo and Fontana, 2010; Rubinsztein *et al.*, 2011) and apoptosis have during DR, both are activated triggering a releases of nutrients and thus the possibility of reproduction.

1.2.2.1   Mechanisms of dietary restriction

Caloric restriction is a global process effecting multiple pathways with many molecular mechanisms which govern the response to DR. Nutrient sensing pathways, hormones,

growth factors and protein kinases, such as mTOR, Insulin/IGF-1, Sirtuins, cytokines, vascular endothelial growth factor, plasminogen activator inhibitor–1, nuclear factor light-chain-enhancer of activated B and phosphoinositide 3-kinase are all involved.

The first mechanism by which DR was thought to prolong lifespan was through the insulin and IGF-1/FOXO pathway, after mutant *daf-2* (IGF-1 homolog) in *C. elegans* were shown to live twice as long as wild type animals (Kenyon *et al.*, 1993). IGF-1 causes a phosphorylation cascade which deactivates the FOXO transcription factors. These transcription factors have been implicated in metabolism, proliferation and stress tolerance (Greer and Brunet, 2008). Hyperglycaemia triggers the release of the hormone insulin from the β cells of the pancreas. Consequently, high insulin levels up-regulate synthesis of IGF-1 in the liver. During DR or hypoglycaemia glucose levels are low, resulting in reduced insulin and thus IGF-1 levels. Normally, Insulin and IGF-1 bind to cell surface receptors resulting in a conformational change, the result of which is auto phosphorylation (Van Obberghen *et al.*, 2001). Phosphorylated residues are recognised by insulin receptor substrate proteins. The cascade continues with the phosphorylation of the insulin receptor substrates which lead to recognition by PI3K. PI3K generates the PtdIns (3,4,5) P_3 complex on the cell membrane, which leads to PDK1 phosphorylation of Akt. Akt interacts with a large number of proteins, including glycogen synthase kinase 3. Phosphorylation of glycogen synthase kinase protein renders it inactive, the result causes the activation of glycogen synthase (Lizcano and Alessi, 2002). DR has been shown to inhibit this pathway in humans skeletal muscle (Mercken *et al.*, 2013), in rhesus monkeys however, DR was shown to increase glycogen synthase (Hansen *et al.*, 1999). Glycogen synthase dynamics is the mechanism by which that the anti-cancer effects of DR are thought to act (Klement and Kammerer, 2011). To sustain a highly prolific nature cancer cells rely on a constant supply of glucose to perform cellular turnover. This was indicated when cancerous cells were more sensitive to the reduction of glucose in DR than non-cancerous cells (Hursting *et al.*, 2013).

In addition to the role of Akt in insulin/IGF-1 signalling pathways, Akt also regulates the mammalian target of rapamycin (mTOR) (Memmott and Dennis, 2009), via the phosphorylation of Tsc2 (tuberous sclerosis complex 2). After phosphorylation the Tsc2-Tsc1 complex is disrupted and its suppression of mammalian target of rapamycin complex 1 (mTORC1) is lifted (Manning *et al.*, 2002). Akt can also disrupt the

raptor/PRAS40 complex, which is a direct inhibitor of the kinase domain on mTORC1 (Wang *et al.*, 2007). mTORC1 activates the translation of proteins (Ma and Blenis, 2009), nucleotide biogenesis, glycolysis (Laplante and Sabatini, 2009) and autophagy. The tight control of mTORC1 ensures that a cell has enough resources to grow and proliferate. Amino acid availability seem to be required for mTORC1 function, even when stimulated with growth factors (Byfield *et al.*, 2005), amino acids are still a limiting factor for mTORC1. The direct way in which amino acids are detected by the complex are not yet well understood, however the nutrient-regulated lipid kinase hVPS34 (Class III PI 3-kinase) appears to be a strong target, as it also interacts with downstream mTORC1 S6 kinase (Byfield *et al.*, 2005). S6 kinase (S6K) is one of the main effectors of mTORC1. S6K has a number of important roles in transcription, translation, biosynthesis, growth and metabolism. Interestingly the knockout of S6K increased the lifespan in mice (Um *et al.*, 2004; Selman *et al.*, 2009), *S. cerevisiae* (Kaeberlein *et al.*, 2005), *C. elegans* (Vellai *et al.*, 2003) and *Drosophila* (Tatar *et al.*, 2001), indicating that S6K is an attenuator of lifespan extension. Besides S6K, another of the main attenuators of mTOR is eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), a repressor of mRNA translation.

mTORC2 is another complex formed from mTOR and associated with the regulation of cytoskeletal proteins, lipogenesis and glucose metabolism (Schmidt *et al.*, 1997; Jacinto *et al.*, 2004; Garcia-Martinez and Alessi, 2008) and phosphorylates Akt which in turn stimulates 3-phosphoinositide-dependent kinase 1 (PDK1) mediated phosphorylation, leading to the activation of a number of protein serine/threonine kinases including S6K. mTOR has a clear role in cellular dynamics during dietary restriction, which undoubtedly play a role in lifespan extension.

Other ways in which DR impacts upon cells is via fluctuations in levels of NAD^+ and AMP. These molecules are key to the control of mitochondrial function and biogenesis. SIRT1, AMPK (AMP-activated protein kinase) and PGC-1α detect fluctuations in these molecules (Canto and Auwerx, 2009). SIRT1 and AMPK react to starvation through increased AMP and NAD^+ (Vaziri *et al.*, 2001). These in turn activate, via deacetylation, PGC-1α which triggers mitochondrial biogenesis. An increases in mitochondrial mass is thought to play a protective role. Reactive oxygen species produced by the mitochondria can damage cellular components, including protein and

nucleic acids. DR has been shown to decrease mitochondrial ROS production in both the liver and muscle of mice (Mayhew *et al.*, 1998; Zainal *et al.*, 2000; Wang *et al.*, 2010). The effects of which are thought to be mediated through the increased expression of PGC-1α (Civitarese *et al.*, 2007). PGC-1α induces expression of transcription factors, nuclear respiratory factor 1 and 2, which have a number of downstream effectors, notably TFAM. TFAM orchestrates the expression of genes located on the mitochondrial genome (Puigserver and Spiegelman, 2003). In intestinal epithelial stem cells PGC-1α has been shown to control differentiation of enterocytes, through the mediation of ROS by increased mitochondrial biogenesis (D'Errico *et al.*, 2011).

An alternative method by which PGC-1α is activated is via the mitogen activated protein kinase p38, which is induced by high ROS levels. The MAPK induces a cascade which leads to the phosphorylation of PGC-1α, this event stabilises the protein and inhibits proteasomal targeting, increasing the half-life.

The production of ROS however has been implicated in enhancing the transcription of growth promoting transcription factors through MEK1/2-ERK1/2-c-Jun signalling (Aquilano *et al.*, 2009). This bidirectional paradigm could be due to the nature of certain ROS molecules as signalling molecules. Nitric oxide for example, is a reactive molecule which is also an essential neurotransmitter. Nitric oxide can trigger the shuttling of redox sensitive transcription factors into the nucleus (Lettieri Barbato *et al.*, 2012). Interestingly at a behavioural level decreased nitric oxide (NO) levels are linked to increased mating and aggression (Trainor *et al.*, 2007), via serotonin production (Chiavegatto *et al.*, 2001). During caloric restriction aggression is observed in, rats (Lore *et al.*, 1986), mice (Trainor *et al.*, 2007), dogs, cats (Houpt and Zicker, 2003) and monkeys (Ramsey *et al.*, 2000). The concentration of this molecule seems to be crucial to cellular homeostasis, as high concentrations of NO can activate p53 and trigger apoptosis (Baldelli *et al.*, 2008). In addition to this, high concentrations are also observed in the neurodegenerative disease, Parkinson's disease (Aquilano *et al.*, 2008). In summary, dietary restriction impacts upon a number of pathways and reacts to numerous stimuli, to significantly extend lifespan, more importantly decreasing age related diseases. There are a number of compounds which can mimic these effects, so called dietary restriction mimetics.

1.2.2.2 Dietary restriction mimetics

There are a number of dietary restriction mimetics which have similar phenotypic effects. These compounds interact with proteins such as mTOR, sirtuins and AMPK. Compounds which interact with sirtuins are often referred to as sirtuin activating compounds (STACs). Omega 3 (Jolly *et al.*, 2001; Wu *et al.*, 2007), ephedrine, caffeine, pioglitazone (Bogacka *et al.*, 2007) and melatonin (Gutierrez-Cuesta *et al.*, 2008) have shown beneficial effects, such as improved cardiovascular health and immune system function. All of these compounds result in an increase in SIRT-1, however the direct mechanism is unknown. Synthetic compounds such as SRT1720 show an increase in insulin sensitivity and increased mitochondrial biogenesis in muscle, liver and adipose tissues, by binding to the SIRT-1 enzyme-peptide substrate complex enhancing acetylation (Bemis *et al.*, 2009).

The polyphenol antioxidant resveratrol is a cardioprotective and chemopreventative which mimics many beneficial aspects of DR, including lifespan extension (Bass *et al.*, 2007) and mitochondrial biogenesis (Csiszar *et al.*, 2009). Resveratrol interacts with mTOR and DEPTOR to enhanced there association. The result is reduced downstream mTOR signalling. In addition to this, resveratrol also inhibits the insulin stimulation of Akt by phosphorylation (Liu *et al.*, 2010b; Brasnyo *et al.*, 2011). In endothelial cells the compound induces nitric oxide synthesis, which could have neuroprotective effects (Thirunavukkarasu *et al.*, 2007).

Rapamycin is a drug primarily used as an immunosuppressant, preventing the recognition of interleukin-2 by T and B cells. There is an increasing use of rapamycin as a tumour suppressor of Kaposi's sarcoma (Mohsin *et al.*, 2005) and liver cancer (Rizell *et al.*, 2005). Rapamycin as a dietary restriction mimetic that increases the lifespan of *S. cerevisiae* (Jiang *et al.*, 2000), *D. melanogaster* (Partridge *et al.*, 1987), *C. elegans* (Klass, 1977) and in mice (Weindruch *et al.*, 1986). Rapamycin binds to mTORC1 inhibiting downstream signalling. Rapamycin however, does not have a direct effect on mTORC2 (Jacinto *et al.*, 2004), however long term rapamycin treatments inhibit further synthesis of the mTOR protein leading to reduced activity (Zeng *et al.*, 2007). It is hypothesised that rapamycin may have a direct effect of a number of the downstream effectors of mTORC1, such as S6K, namely because *D. melanogaster* which overexpress S6K do not show any increase in lifespan extension (Partridge *et al.*, 1987). Rapamycin can also

interact with eukaryotic translation initiation factor 4E (eIF4E), leading to decreased activity and subsequently to decreased protein synthesis (Rubinsztein *et al.*, 2011). In *C.elegans* this down-regulation leads to increased lifespan (Syntichaki *et al.*, 2007). Additionally rapamycin up-regulates Atg, a serine/threonine-protein kinase (Mizushima, 2010) which can induce autophagy by phosphorylating Beclin-1, with Beclin-1 required for autophagy induction (Russell *et al.*, 2013).

Like all drugs however, rapamycin has a number of side effects, the best characterised being immune suppression. Rapamycin, however can also exacerbate type 2 diabetes (Fraenkel *et al.*, 2008), increase epithelial ulcers, anaemia and proteinuria. Mice also exhibit impaired wound healing response. In this instance diabetes is thought to be trigged due to a starvation response and can be reversible (Blagosklonny, 2011). Stem cells modulate there proliferation to maintain tissue integrity, this however is balanced against the risk of hyper-proliferation leading to cancer formation or depletion of the stem cell pool. This exhaustion has been reversed with the use of rapamycin in mouse spermatogonial stem cells, potentially thought the mediation of reactive oxygen species as superoxide dismutase 1 was upregulated (Kofman *et al.*, 2012). Conversely, embryonic stem cells are triggered to differentiate into an osteoblastic linage when treated with rapamycin, through the BMP/Smad pathway (Lee *et al.*, 2010b). It could be speculated that the effects of rapamycin treatment may be determined by the plasticity of the cells being applied to. Some of the side effects of rapamycin may be stem cell dependent, such as immune suppression (Geest *et al.*, 2009) and reduced wound healing capacity (Castilho *et al.*, 2009), which may require active mTOR activity.

1.2.3 Age related changes to the hypothetical limbal stem cell niche

Recently corneal scleral disks from young and old donors where assessed for microstructural changes with age, using scanning electron microscopy. The total surface area of the limbus decreases with age suggesting a reduction in the focal stromal projections and limbal epithelial crypts. Stem cells have been shown to be more prevalent in the superior and inferior regions of the eye (Pellegrini *et al.*, 1999), in these regions the surface area was especially reduced with age (Notara *et al.*, 2013) (figure 1.13).
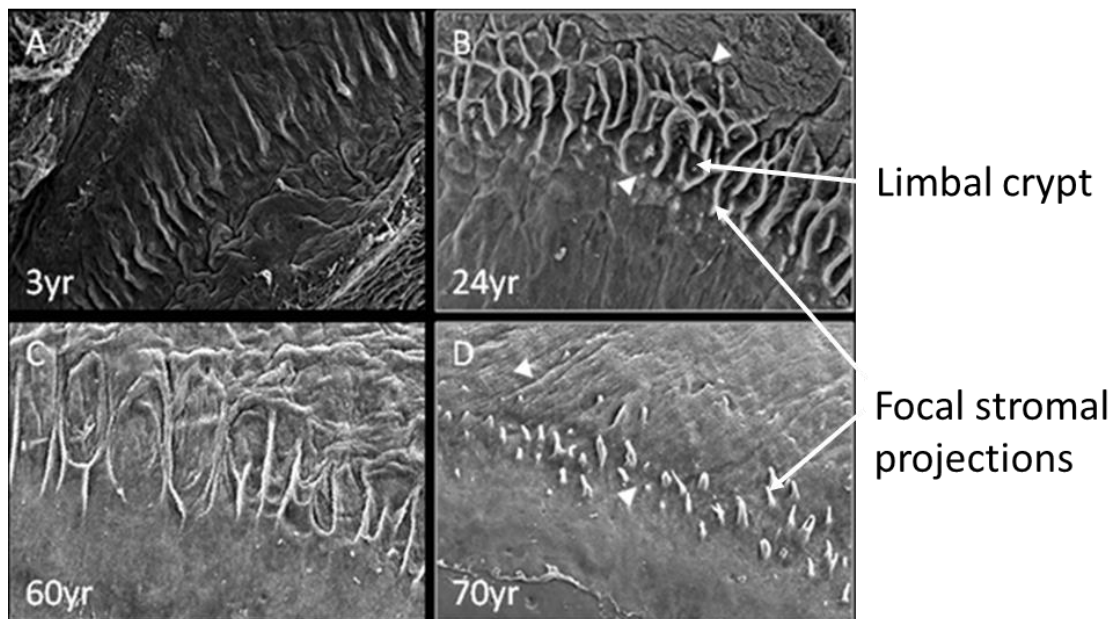
Figure 1.13 Changes to focal stroma projections and limbal crypts
Scanning electromicroscpy of the limbus. Focal stromal projections and limbal epithelial crypts decrease with age.Adpated from (Notara *et al.*, 2013).

1.3 The mouse cornea

1.3.1 Similarities and differences between mouse and human cornea

The mouse eye is anatomically similar to the human eye, with the exception of Bowman's layer. Bowman's layer is only present in primates. Although mice do have a thin layer of collagen fibres which reside between the basal epithelial layer and the stromal keratocytes (Smith, 2002). The epithelium of the mouse is documented as consisting of 13 layers in the central cornea and 3 layers in the peripheral cornea. The central corneal epithelium measured around 45µm while the peripheral cornea was around 25µm (Henriksson *et al.*, 2009) (isotonic solution used during fixation). Other studies put the central cornea at a thickness of 51µm (Zhang *et al.*, 1996) using cryostat

sections. Using *in vivo* confocal microscopy, the mouse epithelium was measured at around 49μm (Song *et al.*, 2003). The human corneal epithelium on the other hand is comprised of 5-7 cells with the total thickness around 50-52 μm (Reinstein *et al.*, 2008). The peripheral cornea is of a similar size to the central cornea in humans (Reinstein *et al.*, 2008), but in mice the periphery is much thinner than the central region (Dua *et al.*, 2009). The limbus is much less pronounced in mouse, morphologically only a slight indentation on the ocular surface. In sections this morphology is so slight that it is not always visible (Mort *et al.*, 2012), and can make identification of this region difficult. The mouse eye overall is circular as opposed to slightly elliptical as in human eyes (Henriksson *et al.*, 2009).

1.3.2   The ageing mouse cornea

Age related changes to the mouse cornea which have been documented have noted a build-up of extracellular calcinosis mineral deposits. In the stroma a decrease in the nerve plexus decreased significantly with age after 2 months old. (Wang *et al.*, 2012a). The corneal epithelium of mice is shown to have a reduction in the number of stem cells in the limbus. The ROSA26 *LacZ*$^{+/+}$ */LacZ*$^{-/-}$ chimera mouse uses random inactivation of the X chromosome to cause conditional expression of LacZ. When this occurred in a stem cell the trait was inherited by the daughter cells. This created radial stripes in the cornea, as the cell migrated from the limbus centripetally. With age these stripes decreased substantially, from around 100 stripes at 10 weeks of age to around 50 per eye at 39 weeks old. The same was seen in the *KRT5*$^{LacZ-/-}$ mouse, at 15 weeks around 1 stripe was observed per eye, at 30 weeks 0.5 stripes were observed on average per eye (Douvaras *et al.*, 2012). These results indicate that the stem cells in the limbus are undergoing asymmetric division with age. Alternatively the stem cells are entering into a state of mitotic inactivity, be it quiescence or senescence.

1.4   Aims

1. **To analyse the properties of limbal epithelial cells derived from donors of different ages, including the expression levels of positive and negative stem cell**

markers as well as telomerase activity. The amount of positive and negative markers reflects the number of progenitor cells within the culture of each donor defining the ability of each cultures' potential ability to replenish the cells of the corneal epithelium.

2. **To characterise the effect of ageing and anti-ageing interventions such as dietary restriction and rapamycin treatment on the mouse cornea.** My aim was to determine the effects of age on the mouse cornea, *in situ*, quantifying corneal morphology, progenitors, DNA damage and telomere associated damage with age.

3. **To assess the effects hypoxia on the relative number of limbal epithelial cell progenitor cells within each culture**. To address the contested benefits of hypoxic culture on limbal epithelial cells by assessing the amount of positive and negative markers, including metabolic and cell cycle status.

4. **To assess if different culture media have an effect on the number of limbal epithelial cell progenitors within each culture.** Addressing the question of the beneficial effects of serum free culture of limbal epithelial cells. The number of progenitors is indicative of the optimal culture parameters for limbal stem cell transplantations.

Chapter 2   Methods and Materials

2.0   Derivation of human limbal epithelial stem cell containing cultures

Human corneo/scleral tissue which had been consented for research was obtained from the UK eye bank (Bristol and/or Manchester). Tissue was stored at room temperature in Dextran organ culture medium until use (55 days ± 7 days). Tissue was normally processed within one week of receipt into the laboratory. Extraction of human limbal epithelial cells was performed by serial trypsinisation. Corneo/scleral rings were removed from organ culture medium and washed briefly with sterile Dulbecco's phosphate buffered saline (DPBS, *Invitrogen, UK*). The tissue was carefully placed cornea side up in a sterile Petri-dish. With a scalpel and forceps the tissue was cut into 28 triangular segments and placed into 5ml of pre-warmed trypsin (*Invitrogen, UK*) solution (0.05%) and incubated at 37°C for 10 minutes. After this time the tissue pieces were agitated with a 10ml serological pipette and incubated for a further 10 minutes. Tissue pieces were then agitated again before removal of trypsin solution, the solution was centrifuged for 3 minutes at 17.8*xg*. The resulting supernatant was removed and the cell pellet was re-suspended in 2ml of limbal medium (Dulbecco's modified® Eagle's medium (DMEM, *Invitrogen, UK*) containing 1g glucose, 33.3% Ham's F12 nutrient mixture (*Invitrogen, UK*) (Allen-Hoffmann and Rheinwald, 1984), 10% foetal bovine serum (FBS *Sigma, USA*) (Allman *et al.*, 1976; Maciag *et al.*, 1981a), 100U/ml penicillin and 100 µg/ml streptomycin (*Invitrogen, UK*), hydrocortisone (0.4µg/ml) (*Sigma, USA*), insulin (5µg/ml) (*Sigma, USA*), triiodothyronine (1.4ng/ml) (*Sigma, USA*), adenine (24mg/ml) (*Sigma, USA*), cholera toxin (8.4ng/ml) (*Sigma, USA*) and epidermal growth factor (10ng/ml) *Sigma, USA*). This process was repeated three more times to make a total of four rounds of extraction. To ascertain a general number of cells the re-suspended cells were counted using a haemocytometer, to determine the relative efficiency of the extraction. The cell suspension was then seeded together with mitotically inactivated NIH-3T3 feeder (Todaro and Green, 1963) cells (ATCC) into a the well of a 6-well plate. The culture was incubated in limbal medium at 37°C and 5% CO_2 (Series II Water Jacketed *Thermo, USA*) for two days without disturbance, on the third day the medium was replaced. When a well of a 6 well culture dish contained a monolayer of epithelial cells the well was washed with sterile PBS and then treated with a solution of 0.2M

EDTA in PBS for 30 seconds. The solution was then decanted with vigour repeatedly over the culture to remove any remaining 3T3 feeder cells. The culture was then treated with 2ml of pre-warmed trypsin and incubated for 3 minutes, the trypsin/cell solution was removed from the culture dish and 2ml of warm limbal medium was added. The cell suspension was centrifuged for 3 minutes at 17.8xg. The resulting cell pellet was re-suspended in 2ml of warm limbal medium. A cell count was performed using a haemocytometer.

2.0.1 Donor demographics

Due to the limited availabity of donor tissue no exclusion criteria were applied. The most frequent cause of death in the donors used in this study was infection, with 17 in total. 10 donors died of pneumomia, 3 donors had meningitis, 3 donors died from sepsis and one from a respiratory tract infection. The second most common cause of death was cancer, totaling 16 donors; other frequent causes of death incuded stroke (9 donors), chronic obstructive pulmonary disease (COPD) (7 donors) and cardiac arrest (7 donors).

2.1 Clonal analysis of human limbal epithelial cells

500 human limbal epithelial cells (as calculated in the previous section) were taken from the cell suspension and dispersed into 50ml of warm limbal medium. This solution gives an average of 1 cell per 100μl. One cell (suspended per 100μl) was then transferred into each well of five 96 well tissue culture (figure 2.1). Each well had a layer of 3T3 feeder cells pre-plated the previous day as described in section 1.6. Each well was monitored and cells were removed by trypsinisation when a monolayer had formed. The cells were counted using a haemocytometer and 500 cells were placed into a 90mm tissue culture dish for CFE analysis (also containing 3T3 feeder cells). 1000 cells were seeded onto 19mm coverslips inside a 12 well plate for p63 immunofluorescence staining (also pre-treated with 3T3 feeder cells).
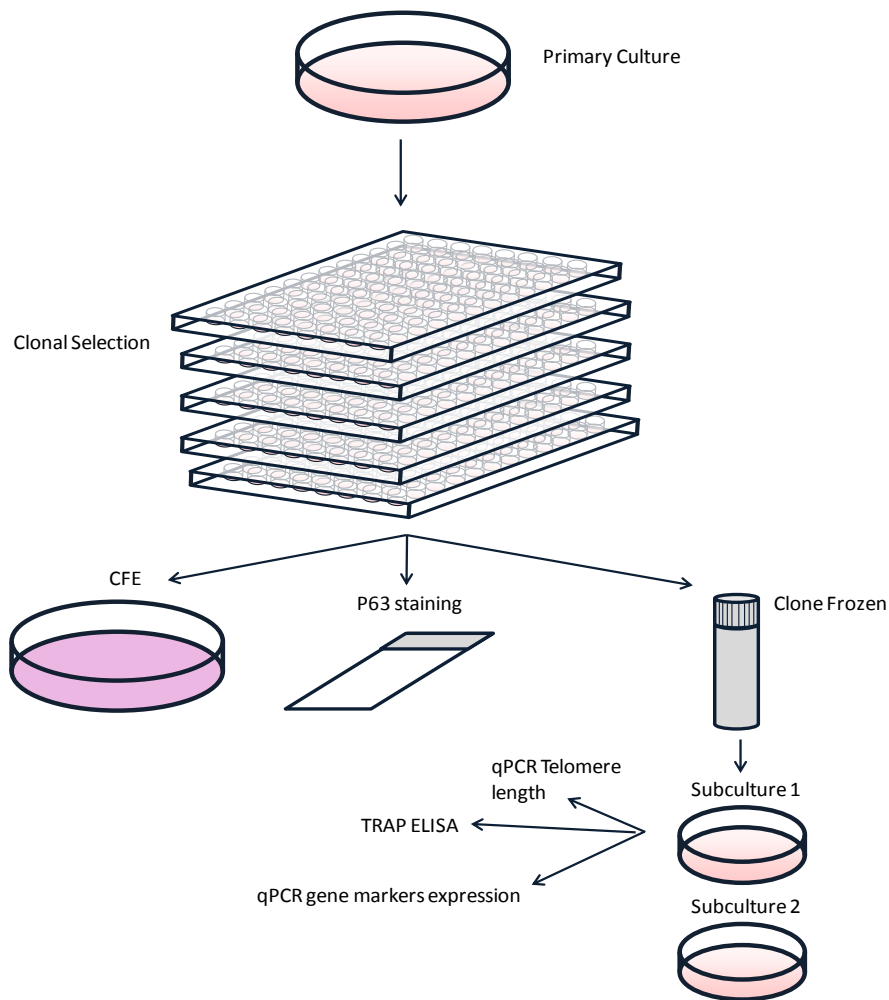
Figure 2.1 Experimental design for clonal analysis
Diagram showing the work flow for the clonal analysis study. Primary cultures were cultured as per the standard procedure (2.0), once confluencey was reached the cells were removed from the culture well with trypsin (0.05% Invitrogen, UK) and seeded into 5 96 well plates, so that one cell was seeded in to one well.

2.2 Culture of human epithelial stem cell cultures in serum free medium.

2.2.1 Selection of suitable culture medium

There are a number of serum free media available, including Epilife (*Invitrogen, UK*), supplemental hormonal epithelial medium (SHEM, *Invitrogen*) and defined keratinocyte serum free media (DKSFM, *Invitrogen*). DKSFM was chosen in this study as it has been shown to select cells which have the highest expression level of positive LSC markers ABCG2 and p63, whilst also having the lowest expression of differentiation markers, such as K3 (Loureiro *et al.*, 2013). These results were however obtained at the protein level from the analysis of immunofluorescent staining. When qPCR data for mRNA expression are analysed, using Epilife medium for culturing LSC's, the highest *p63* and

ABCG2 expression levels are obtained (Loureiro *et al.*, 2013). On the other hand, under these conditions an elevated *KRT3* expression profile, almost to the same fold change of *p63* expression was detected. DKSFM displayed the lowest of all *KRT3* values compared to SHEM and Epilife. This might indicate that there was a stable cellular population, more importantly, that using DKSFM is more reproducible (Loureiro *et al.*, 2013). SHEM was not selected because it contains a higher level of calcium than the other two media. Calcium has been shown to have a stimulatory effect on keratinocytes leading their differentiation (Boyce and Ham, 1983). Transforming growth factor-β (TGF-β) is also produced by keratinocytes in the presence of calcium, leading to further differentiation (Kruse and Tseng, 1992). The combination of the factors listed above lead to the decision to choose DKSFM over other serum free media. Unlike a previous study (Bath *et al.*, 2013), bovine pituitary extract (BPE) was not added to supplement DKSFM, making our system truly defined.

2.2.2   Culture of limbal epithelial cells in serum free medium

The primary cultures isolated from human cadaveric corneo-limbal tissue, Lot: 6066, 6084 and 6062, were kindly donated by Paul Knollman (Life Technologies research laboratories, Oregon). Cell lines were thawed upon arrival, and placed immediately into defined keratinocyte serum free medium (*Invitrogen, UK*) supplemented with epidermal growth factor (0.0397μg/μl) (DKSFM) (*Invitrogen, UK*). The cultures were then incubated at 37°C with 5% CO_2 with the medium being changed every two days.

Additional cultures grown in serum free conditions were isolated using the same method as those grown in LEM, however the resulting cells were seeded into collagen treated wells (Matrix coating kit, *Invitrogen UK*). The addition of 1% serum was used to inactivate any residual trypsin. The medium was changed after the second day to remove any on adherent cells and the serum. DKSFM was known to be comprised of 1624.4mg/l glucose and 984mg/l of L-glutamate.

A human telomerase (hTERT) immortalised corneal epithelial (HTEpi) cell line was utilised as an alternative to primary isolated cells and also as a positive control in some experiments. These cells were used with the permission of the creator James Jester (Robertson *et al.*, 2005). These cells were cultured under serum free conditions in DKSFM.

2.3 Cultivation of limbal epithelial cells in hypoxia.

Cells were extracted as per section 1.1.1, however rather than suspending extracted cells in LEM, cells were suspended in pre-warmed DKSFM. The cells were then cultured as per section 1.1.3 with the exception of cells grown in hypoxia. Hypoxic conditions were as follows: 37°C, 5% CO_2 and 3% O_2 in a nitrogen purging incubator (Hera cell 150 *Thermo, USA*).

2.4 Culture of mouse 3T3 fibroblast feeder cells

Cryopreserved 3T3 (strain J2) immortal mouse fibroblast cells were thawed in a water bath at 37°C and then dispersed into 3T3 medium containing Dulbecco's modified eagle medium containing 4.5g/l D-glucose and supplemented with 10% foetal calf serum and 1% Penicillin and Streptomycin. The cell suspension was centrifuged at 17.8xg for 3 minutes at room temperature, and the supernatant containing dimethyl sulfoxide (DMSO *Sigma, USA)* was removed. The cell pellet was suspended in fresh 3T3 medium and seeded into a $25cm^2$ tissue culture flask (*Corning, USA*) and incubated at 37°C in 5% CO_2. The medium was changed the day after seeding to remove any dead cells. 3T3 cultures were never allowed to become confluent to maintain the 3T3 cells in a proliferative state (log phase). For sub culturing the cells the medium was removed and the flask briefly washed with sterile DPBS, to remove any residual medium. The cells were then treated with trypsin (0.05% with 0.53mM EDTA *Invitrogen, UK*) for five minutes at 37°C chelating calcium, preventing the joining of cadherins between cells and cleaving proteins on the cell surface removing cell-cell contacts. The trypsin was then inactivated using 3T3 medium and the cells centrifuged at 17.8xg for 3 minutes. The supernatant was removed and the cell pellet was suspended in 3T3 medium. Cell viability was determined and 300,000 cells were seeded into a $75cm^2$ flask. The growth medium was changed every other day until the flask reached confluence.

2.5 Mitotic inactivation of mouse 3T3 cells

Cells were inactivated with 10 μg/ml mitomycin C (*Sigma, USA*) administered directly into the medium for 2 hours. Mitomycin C creates DNA crosslinks targeting guanine residues, this leads to particular interference in CpG islands which are frequently located either within promoter regions or upstream, damage to them results in reduced expression of affected genes as DNA damage limitation factors attempt to repair the

modifications. DNA repair normally takes place in a state of arrest, preventing further proliferation, the amount of damage inflicted incurs non reparable damage and thus permanent growth arrest. The medium/mitomycin C solution was removed and the flask washed three times with DPBS to remove any residual mitomycin C. The cells were then treated with trypsin (0.05% with 0.53mM EDTA) for five minutes at 37°C. The trypsin was then inactivated using 3T3 medium and the solution centrifuged at 1,000 rpm for 3 minutes. The supernatant was removed and the pellet was re-suspended in fresh medium. The 3T3 fibroblasts were then plated at a density of 24,000 cells per cm^2.

2.6 Colony forming efficiency assay

Colony forming efficiency is a measure of the proliferative potential of a cell culture, the more colonies a culture can produce from a defined number of cells indicates a higher number of these cells which are proliferating rapidly. Limbal epithelial cells were counted and approximately 2500 cells were seeded onto pre-prepared mitotically inactivated 3T3 feeder layers (as prepared in section 2.5) in triplicate at each successive subculture. The co-culture was then incubated at 37°C for 12 to 14 days, feeding the cultures with limbal epithelial medium every two days. At the end of the incubation period growth medium was removed and each well was washed with DPBS to remove any residual medium. Cultures were then fixed for 10 minutes using 1.5ml of a 3.8% solution of formaldehyde diluted in DPBS at room temperature. After 10 minutes the formaldehyde was removed and 1ml of 1% Rhodamine B (*Sigma, USA*) diluted in 100% methanol was added. After a further 10 minute incubation at room temperature the solution was removed and the stained visible cells were counted. The percentage of actively dividing cells was calculated from the colonies which were generated using the following formula: colonies formed/number of cells plated x 100. The cut off point for colony counting was ~75 cells, as this was roughly the size at which they became macroscopically visible.

2.7 RNA extraction

Cell pellets containing $1\times10^{6-7}$ cells were suspended in 1ml of TRIzol solution (as per the manufactures instructions) (*Invitrogen, UK*) and incubated at room temperature for 5 minutes. 200µl volume of chloroform was added to the solution and shaken vigorously for 15 seconds, the solution was allowed to incubate for 10 minutes at room

temperature. The solution was then centrifuged at 12,000g for 15 minutes at 4°C. This separated the solution into three phases, the top transparent phase, which contained the RNA, was transferred into a fresh tube. The aqueous solution (transparent phase) was carefully removed using a Gilson pipette, mixed with 500µl volume of isopropanol and incubated at room temperature for 10 minutes. The samples were then centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was then removed and discarded and the remaining pellet was washed with 75% ethanol and placed on a vortex for 30 seconds. When cell pellets where small, homogenisation of the cell lysates was performed using QIAshredder (*QIAGEN, Netherlands*) then RNA was extracted using an RNeasy Mini Kit (*QIAGEN, Netherlands*) as per manufacturer instructions.

2.8   Reverse transcription

Total RNA samples are quantified using a Nanodrop spectrophotometer (*Thermo Scientific, USA*) at 260nm and 280nm wavelengths. The 260:280 ratio was used to evaluate possible protein contamination. RNA was reverse transcribed if the 260:280 ratio was between 1.97-2.2. 1µg of RNA was used per each sample. 1 unit of DNase in reaction buffer (400mM Tris-HCL pH 8, 100mM MgSO4 and CaCl2) (*Promega, USA*) was added to each sample to remove any trace amounts DNA from the sample, the solution was incubated at 37°C for 30 minutes. After this time a 1µl of STOP solution (20mM EGTA pH 8) was added to inactivate the nuclease. 1µg of random oligonucleotide primers (*Promega, USA*) where added, and the solution was heated to 70°C for 5 minutes to denature the RNA and resolve any secondary structure, so that the primers can bind. Random oligo primers were used as RNA fragmentation may have occurred during TRIzol extraction (*Invitrogen, UK*), more importantly Telomerase RNA component (TERC) sequences would not be transcribed with polyA primers as this is a non-coding RNA gene. A solution consisting of dNTP's (0.5 mM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate, and deoxyguanosine triphosphate) 25 units of RNasin ribonuclease inhibitor, and 200 units of reverse transcriptase was added to the sample and incubated at 37°C for 1 hour, after this time the solution was heated to 99°C for 5minutes to inactivate the reverse transcriptase enzyme.

2.9 Quantitative polymerase chain reaction

qPCR was performed using an Applied Biosystems StepOne Plus thermocycler, with glyceraldehyde-3-phopshate dehydrogenase (GAPDH) used as the reference gene. SYBR Green (*Sigma, USA*) reaction technology was utilised. Each primer (primer details in the appendix 1) was used at a concentration of 10µM, and at a ratio of 50:50 for forward and reverse. The reaction parameters were as follows: 95°C for 15 minutes to denature the cDNA and primers, 50 cycles of 94°C for 15 seconds followed by primer specific annealing temperature for 30 seconds then 72°C for 20 seconds. A comparative Ct method was used to calculate the levels of relative expression, whereby the Ct was normalised to the endogenous control (GAPDH). This calculation gives the ΔCt value, which was then normalised to a reference sample (i.e. a positive control), giving the ΔΔCt. The fold change was calculated using the following formula: $2^{-\Delta\Delta Ct}$. A list of primers can be found in appendix A.

2.10 Bradford assay

Cell pellets were lysed with 10-50µl of CHAPS (3-[3-Cholamidopropyl dimethylammonio]-1-propanesulfonate) buffer (*Roche, Switzerland*). In order to determine the correct amount of protein a standard curve was generated using BSA protein standards of concentrations of 1.4µg, 2.8µg, 4.2µg, 5.6µg, 7µg, 8.4 µg and 9.8µg were added to 200µl of 1:5 diluted protein assay dye reagent (*Bio-Rad, USA*). 2µ of sample was added to 200µl of the same protein determination solution used for the preparation of protein standards. Samples were mixed by vigorous agitation with a pipette. 96 well plates containing the samples were placed in a Fluo-Star Omega microplate reader spectrophotometer (FLUOstar *Omega, BMG Labtech Germany*), absorbance was measured at 595 nm. The standard curve was normally with an r^2 of around 0.9. The obtained protein concentrations were averaged from the duplicate samples and divided by 2 in order to obtain the original sample concentration.

$$y = 0.5212x + 0.1579$$
$$R^2 = 0.9911$$
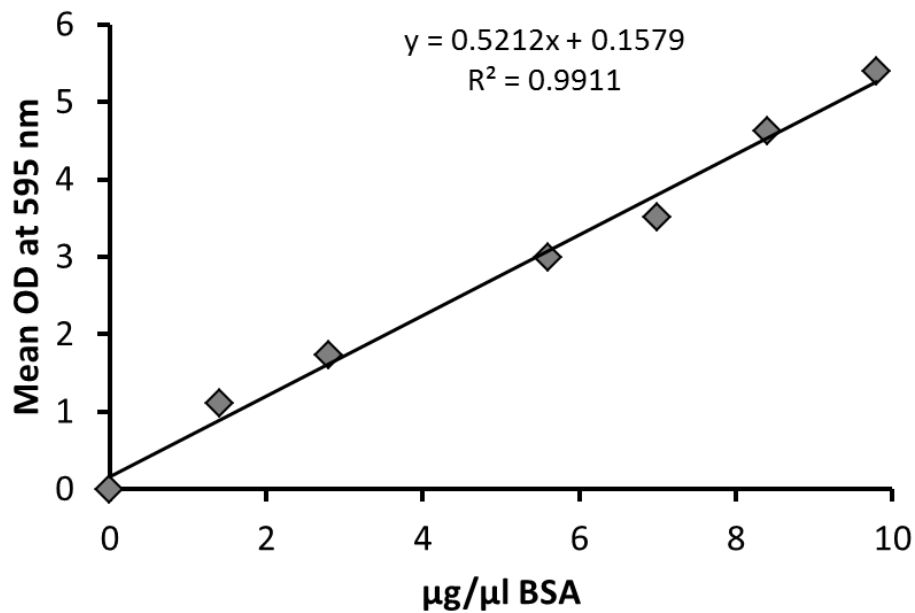
Figure 2.2 Protein determination standard curve
Known protein concentrations were used to establish a standard curve. The closer to 1 the R^2 value, the more accurate the interpolation/extrapolation will be to determine unknown sample amounts.

2.11 TeloTAGGG Telomerase PCR ELISA

Telomerase activity was measured by exploiting the reverse transcriptase function of the enzyme, using a protocol adapted from Kim and colleagues (Kim *et al.*, 1994). The process is comprised of two major steps, PCR and ELISA. Firstly, telomerase adds telomeric repeats to a synthetic biotin linked DNA primer via PCR. Secondly, the elongated primer is then hybridised and immobilised to a streptavidin coated microplate. Detection occurs by using an antibody specific to the telomerase amplification products, the more antibody which is able to bind is dependent on the telomeric extension of the primers.

Cell lysates containing 500ng of protein (determined as in section 2.10) was placed in thin walled 0.2ml PCR tubes, 25µl of 2x reaction mixture (*Roche, Switzerland*), was then added, a total volume of 50µl was made up using ultrapure DNAase/RNAase treated water.

	Time		Temperature		Cycles
Telomerase reaction	30 minutes		25°C		1
Telomerase inactivation	5 minutes		94°C		1
Amplification: Denaturation Annealing Polymerisation	30s	30s 90s	94°C	50°C 72°C	30
Polymerisation	10 minutes		72°C		1

Table 2.1 TRAP amplification parameters

5µl of amplification product was added to 20µl of denaturation reagent (Roche, *Switzerland*) and incubated for 10-15 minutes at room temperature. After which 225µl of hybridisation buffer (*Roche, Switzerland*) was added, the mixture within a tube was then placed on a vortex for 30 seconds. 100µl of this solution was transferred to a streptavidin percolated microplate well, the wells were then covered to prevent evaporation during incubation at 37°C for 2 hours with shaking at 250rpm. The hybridisation mixture was then removed and each well was washed 4 times for 30 seconds with 200µl of washing buffer (*Roche, Switzerland*). 100µl of Anti-DIG-POD (10mU/ml) solution (*Roche, Switzerland*) was added followed by incubation at room temperature shaken at 250rpm for 30minutes. This solution was then completely removed and each well was washed with 200µl of washing buffer (as stated previously) (Roche, *Switzerland*) 5 times for 30 seconds. Detection then followed, reaction of POD (peroxidase) by 100µl of TMB substrate (3,3',5,5' – tetramethylbenzidine) (Roche, *Switzerland*) reagent for 5-10 minutes, in order to form a coloured reaction product (blue). 100µl of stop reagent (Roche, *Switzerland*) was then added, this changed the substrate from blue to yellow, allowing for maximum sensitivity. Optical density was measured at 450nm using a microplate reader (FLUOstar *Omega, BMG Labtech Germany*).

2.12 General immunofluorescence staining

Immunofluorescence is used to identify the cellular location of proteins using fluorescent antibodies. Limbal epithelial cells were plated onto 19mm glass cover slips in 12 well plates at a density of 5×10^4 and incubated at 37°C overnight. The medium was

aspirated and the cover slip was washed twice with PBS for 2 minutes. Culture was then fixed with 1ml of a 4% paraformaldehyde solution (diluted in PBS) and incubated at room temperature for 10 minutes. This solution was then removed, and the coverslip was allowed to dry for 3 minutes. The coverslip was washed again with PBS twice for 5 minutes, after which 1ml of a PBG Triton (PBS, 0.5% BSA, 0.2% Fish skin gelatine, 0.5% Triton X-100 *Sigma, USA*) was added and the cover slip gently agitated for 45 minutes. Antibody was then added per the manufactures specifications (Table 1) diluted in PBG, and then incubated for 1 hour at room temperature. PBG was used to wash the cover slip twice for a duration of 10 minutes at room temperature, secondary fluorescently antibody was then applied (Alexa Fluor Anti- Goat 488/594 *Invitrogen, UK*) at a concentration of 1:2000. The coverslip and antibody were incubated for 1 hour with gentle agitation. The solution was then aspirated, the coverslip washed with PBS three times for 10 minutes at room temperature. For nuclear staining 400µl of DAPI (4',6-diamidino-2-phenylindole *Partec, Germany*) was incubated for 10 minutes. The DAPI solution was then removed and the cover slip was washed 3 times with PBS for 10 minutes at room temperature. The cover slip was mounted onto a microscope slide using anti-fade aqueous mounting medium (*Vector Laboratories, USA*) and sealed with nail polish. Slides were then examined using a fluorescence microscope (DM5500 B *Leica, Germany*).

Primary Antibodies	Company	Raised in	Dilution
Ki67	Abcam	Rabbit Polyclonal	1:500
ΔNp63 (4A4)	Santa Cruz	Mouse Monoclonal	1:100
TAp63	Abcam	Rabbit Monoclonal	1:100
Connexin 43	Sigma	Rabbit Polyclonal	1:1000
ɣH2A.X	Abcam	Rabbit Polyclonal	1:250 or 1:1000

Table 2.2 List if primary antibodies used

Ki67 and ɣH2A.X were optimised previously by former laboratory members, ΔNp63, TAp63 and Connexin 43 were optimised by titration before use in experiments.

2.13 p63 immunofluorescence staining

Cultures were grown on 19mm circular coverslips for 12 days or until confluent. The cells were fixed for 10 minutes in 2ml of pre-chilled methanol at -20°C, the methanol was removed and replaced with 2ml of pre-chilled acetone for 30 seconds also at -20°C. p63 4A4 primary antibody (*Santa Cruz, USA*)/p63 (EPR5701 *Abcam, UK*) was diluted with PBS^{+++} (10% BSA, 0.5% Triton X-100 and 0.5% Tween 20 in PBS) at a ratio of 1:100 (previously titrated by Reichelt laboratory) 300µl was added to each coverslip and then incubated for 1 hour at room temperature, these particular antibodies require the use of both Triton X and Tween 20 to produce specific staining. The coverslips were then washed 3 times for 5 minutes each with PBS before the secondary antibody was added. A secondary antibody (anti-Mouse IgG Alexa Fluor488/594 *Abcam, UK*) was diluted in PBS^{+++} at a ratio of 1:2000 and incubated in the dark for 40 minutes. After this incubation all steps were performed in a low light area to protect the fluorescent properties of the secondary antibody. Each well was washed 3 times for 5 minutes with PBS followed by a brief wash with Milli-Q water. Coverslips were then washed briefly with analytical grade Ethanol and allowed to dry before being mounted using Vector Shield (*Vector Laboratories, USA*) onto microscope slides. The slides were then sealed using nail polish and allowed to dry at room temperature, before being transferred to 4°C for short term storage.

2.14 Microscopy and Analysis

Microscopy was performed using *Leica DM5500B (Leica, Germany*) and *Zeiss observer 21 Quant EM51250* (*Zeiss, Germany*) using either x63 (human p63 quantification) 100x (mouse *in situ* staining) objectives. 3 randomised fields of view were using for the quantification of p63 in human cells, on average 11 cells were present in each FOV. Microscopy on mouse eyes was performed in peripheral cornea and central cornea, 5 FOV (field of view) for each section were analysed. Quantification of immuno-FISH for TAF (Telomere associated foci) recognition was performed by capturing z-stacks in the same regions. The basal cell layer was focused upon as this layer predominantly contains progenitor cells.

Analysis of p63 staining was performed as stated in (Gavet and Pines, 2010), briefly, total cellular fluorescence was calculated using the following formula:

$$CTCF = Whole\ Cell\ Signal - (Area\ \times Background)$$

Whole cell signal = Σ Intensity of pixels per single cell

Background = Average signal per pixel for a region beside cell

Area = Size in pixels of selected cell

2.15 Animal model *Mus musculus*

The mouse strain used in the animal study of corneal ageing was B6.129S-Tert, tm1Yjc/J (Chiang *et al.*, 2004) (*The Jackson Laboratory, USA*). The line had a GFP and neomycin cassette inserted, replacing the translation initiation site of exon 1 in the *Tert* gene, rendering the gene inactive. Wild type homozygous (*Tert*$^{+/+}$). As well as F_1 (first generation) *Tert*$^{-/-}$ mice were generated by crossing heterozygous mice with each other. (Chiang *et al.*, 2004). All animals had free access to water and those animal not in dietary restricted conditions had continuous access to standard rodent pelleted chow (*Special Diets Services, Witham*). The environmental conditions were kept constant throughout at 20 ± 2 °C with a day/night cycle of 12 hours, beginning and ending at 7am. At least 3 mice in each condition were tested in all experiments; this number was chosen after power calculations were performed in Minitab®. 2 sample and paired t-tests required 3 while ANOVA ideally required 4 mice.
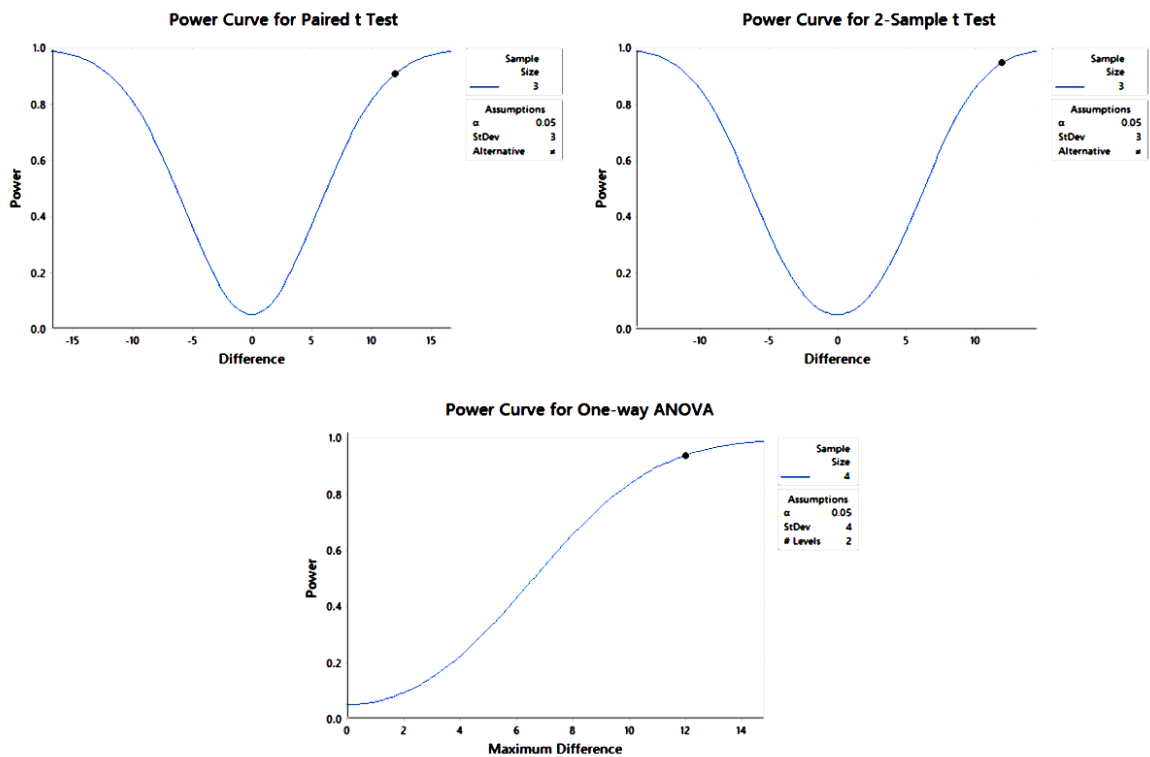
Figure 2.3 Power curves for paired, 2 sample t tests and ANOVA.
Overall the required number of mice required was between 3-4, in order to detect a meaningful significant change between groups.

2.16 Dietary restriction

Dietary restriction was implemented over 16 months, the initial age of the mice at the beginning of the intervention was 6 months old. Caloric value was reduced by 26% whilst retaining sufficient nutrition.

2.17 Rapamycin supplemented diet

12 month old mice were fed experimental chow containing 0.067mg of rapamycin per day/per mouse, gifted from John Strong, Texas USA (Miller *et al.*, 2011; Wilkinson *et al.*, 2012). Treatment lasted 4 months, at which point the mice were sacrificed.

2.18 Cryopreservation and cryosectioning of *Mus musculus* eyes

Mice were culled by cervical dislocation by an animal technician. Mouse eyes were extracted soon after death using forceps and placed into cryomoulds (Strain B6. 129s-Tert, tm1Yjc/J on a background of C57BL/6, *The Jackson Laboratory USA*). Optimum cutting temperature fluid (OCT) (*Cellpath, Wales*) was carefully poured over the tissue, which was then allowed to acclimatise for several minutes. The cryomould was then carefully placed into a bath of iso-pentane (*Fisher Scientific, USA*) pre-chilled by a

container of dry ice. The sample was allowed to freeze before transferring the mould into -80°C for long term storage. Upon retrieval from long term storage, samples were trimmed and mounted onto a metal chuck. Embedded tissue was then sectioned into 10µM sections on a Leica CM-1950 cryostat-microtome. The sections were transferred onto Aminopropyltriethoxysilane (APES) treated slides (*Sigma, USA*). Briefly, Slides were washed in acetone for 5 minutes, followed by treatment with 4% APES (Dissolved in acetone) for 5 minutes. The slides were then washed in deionised water for 5 minutes, slides were then placed in a 37°C oven overnight to dry the slides. APES creates a positive charge on the surface of the slides allowing for better attraction and attachment of tissue.

2.19 Combined Immunofluorescence and in situ hybridisation (Immuno FISH)

Cryosections from mouse eyes were allowed to acclimatise to room temperature for around 2 hours before being fixed using paraformaldehyde (PFA) for 10 minutes. PBG-triton was used to permeabilise the cells for 10 minutes. Primary antibodies ɣH2A.X mouse anti human (1:1000 *Cell signalling, UK)* and/or Ki67 rabbit anti human (1:1000 *Abcam, UK*) suspended in PBG were incubated for 45 minutes. Coverslips were then washed 3 times for 5 minutes in PBG. Following steps are performed in the dark, secondary antibodies goat anti mouse and goat anti rabbit (Alexa fluor 488 & 633 1:2000 & 1:1000, *Invitrogen, UK*) were suspended in PBG and incubated for 45 minutes. Samples were then washed with 1x PBS twice for 10 minutes. Additional fixation was performed using a mixture of methanol and acetone (3:1) for 30 minutes, to dissolve lipids allowing more efficient access to the nucleus. Coverslips were then dehydrated through ethanol washes 70%, 90% and finally 100% 2 minutes in each. Samples were then allowed to air dry. Warm PBS was added and the coverslips were incubated at 37°C for 5 minutes. 4% PFA was added to fix the chromosomes at 37°C for 2 minutes. Samples were briefly washed with 1x PBS. 10µl of Hybridisation mixture (1mM Tris pH 7.2, 70% deionised formamide, Magnesium chloride buffer [82 mM Disodium phosphate, 9 mM citric acid], 20 mM MgCl2, 25ug/ml Cy-3-labelled telomere specific (C_3TA_2) 3 peptide nucleic acid (PNA) probe (*Panagen, Korea*), 12.5µl of Vector laboratory Ltd Blocking reagent (2µlBR+18µl Maleic Acid) made up to 250 µl with distilled water) was placed

onto a clean glass slide, coverslips were then carefully placed onto the droplet. Slides were then placed onto a hot plate in an 80°C oven for 10 minutes to denature the DNA. Slides were removed from the oven and incubated in a humid chamber for 2 hours. Coverslips were very carefully removed from the glass slides in a wash buffer (70% formamide, 2% SSC) and transferred back into a fresh 12 well culture plate. The samples were then washed 3 times for 10 minutes in the wash buffer. 1ml of DAPI was added for 10 minutes followed by 3 washed of Tris Buffered Saline -Tween contents (0.05%) for 5 minutes each. Samples were then dehydrated with ethanol again and allowed to air dry before mounting in anti-fade mounting medium (Vectashield *Vector Laboratories, USA*) onto glass slides.

2.19.1 DNA damage and telomere associated foci

Damage foci were visualised by the association of γH2A.X with these sites. An Anti-γH2A.X antibody was used (*Abcam*, *UK*). Z-stacks were taken and each individual focus was counted throughout the cross-section of the cell to give the total cellular foci number. Telomeres were visualised using a fluorescently labelled DNA probe which was complimentary to telomeric sequences (*Panagen, Korea*). At regions were fluorescent signals indicated γH2A.X foci co-localised to those of the telomere these were termed telomere associated foci (Hewitt *et al.*, 2012) (figure 2.4). The number of co-localisation events was recorded for each cell.
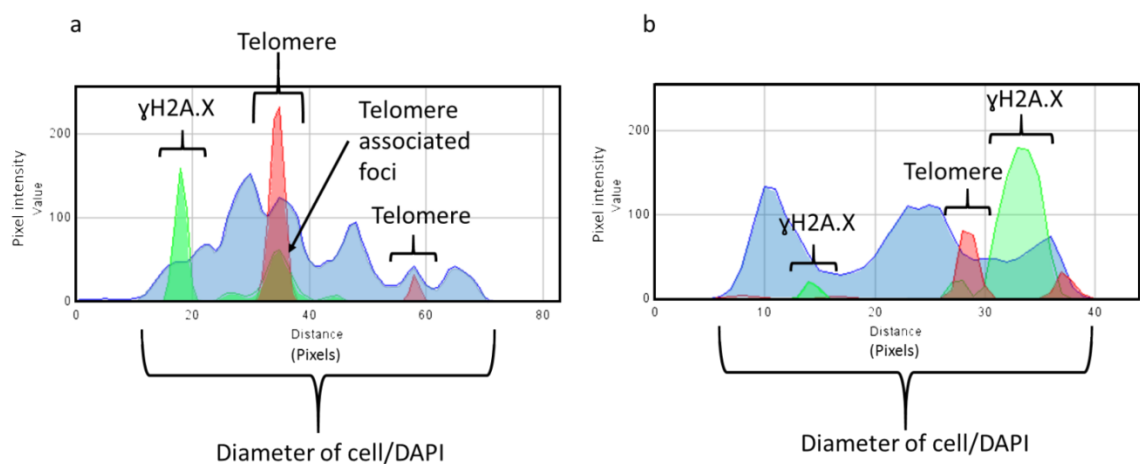
Figure 2.4 Red, green & blue fluorescence signal profiles.
A cross section of two cells was taken, panel a, shows a profile which contains a TAF, while panel b shows a profile without.

2.20 Paraffin embedding

After dissection, mouse eyes were fixed in 4% paraformaldehyde at 4°C overnight. Mouse eyes were then processed in an ASP300 tissue processor (*Leica, Germany*) which firstly dehydrated the tissue though 50%, 70%, 90%, 95% and 100% ethanol at room temperature. Samples were then washed with histoclear (*National Diagnostics, USA*) for 1 hour at room temperature. Half the histoclear was removed and replaced with paraffin wax (*Leica, Germany*) after which the tissue was incubated for 1 hour at 60°C. The histoclear:wax mixture was removed and the samples placed into 100% molten paraffin wax. Wax was replaced every hour for 6 hours. Tissue was then embedded in a dispomould, the wax was allowed to cool for several hours on a cold plate. Blocks were removed from the moulds and pre cooled at 4°C before trimming and sectioning using a microtome (*Finesse E+ Thermo Scientific, USA*) producing 10µM sections. Sections were placed into a 40°C water bath before transferring onto APES treated slides.

2.21 Haematoxylin and Eosin Staining

Paraffin embedded tissue sections mounted on slides were fixed using 4% PFA for 10 minutes. Alternatively alternately frozen OCT embedded sections were fixed using 100% methanol for 10 minutes temperature followed by acetone fixation for 30 seconds at -20°C. Sections were washed briefly in tap water then placed in Harris Haematoxylin for 30 seconds to stain the nuclei. Sections were then washed in tap water. Haematoxylin (*Sigma, USA*) was differentiated by briefly immersing the slides in acid alcohol (70% Ethanol and 10% HCl). In acidic conditions, haematin, the active compound in haematoxylin binds to lysine residues of histones. Acid alcohol leaches undesirable colouration. The slides were then washed again in water, before adding several droplets of ammonia solution to blue the haematin. Slides were washed in water before incubation in 1% eosin solution (*RAL Diagnostics, UK*) for 30 seconds. Eosin acts as a counter stain against haematoxylin, it stains basic parts of the cell such as the cytoplasm. The slides were then dehydrated through 70%, 95% and 100% ethanol. They were then transferred into Xylene for 10 minutes after which they were air dried for 15 minutes before placing cover glass over the sections and mounted with a mixture of distyrene, plasticizer and xylene (DPX) (*Sigma, USA*). This mount was used as it dries quickly and

preserves haematoxylin-Eosin stains. Visible light microscopy was used to view the samples (*Nikon ECLIPSE E800, Japan*).

2.22 Metabolic assay using Extracellular flux analyser XF24

The Extracellular FLUX Analyser XF24 (*Seahorse, USA*) is a real time proton flux and oxygen consumption assay, otherwise referred to as oxygen consumption rate and extracellular acidification rate (OCR and ECAR). Solid state probes detect H^+ ions and oxygen produced by a monolayer of cells. Up to four electron transport chain (ETC) altering compounds can be added into the system at timed intervals (3 minutes). For optimum measurements between 5,000-10,000 cells were plated onto XF24 culture plates (*Seahorse Bioscience, UK*) and allowed to grow for approximately 1 week. Once the wells became around 70% confluent (~25,000 cells) the culture medium (100 μl of D-KSFM) in the XF24 plate was replaced in a step wise process with XF Assay Medium containing 2mM L-glutamate (*Sigma, USA*) and 5mM glucose (*Sigma, USA*). 100 μl of Assay medium (*Seahorse bioscience, UK*) was added then 50 μl was removed, 300 μl was added then 300 μl removed, finally 450 μl of assay medium was added. The plate was then placed in a non CO_2 incubator at 37°C for 2 hours. To assess the respiratory profile of the cultures three compounds were added separately at intervals (3 minutes). Oligomycin A (*Sigma, USA*) (5μg/ml) was used as an inhibitor of F^0F^1 ATP synthase (Complex V), where it binds a peptide on the rotary motor which internally hinders oxidative phosphorylation (OXPHOS) and leads to a reduction in ATP production. This significantly reduces the amount of electrons in the electron transport chain, giving a baseline level of proton leak (proton diffusion into the mitochondrial matrix).

Trifluorocarbonylcyanide Phenylhydrazone (FCCP) (3μM) (*Sigma, USA*) can permeabilise the inner mitochondrial membrane, allowing the passage of protons through the lipid bilayer and into the intermembrane space. This has the effect of destroying the ΔpH and the Δψ uncoupling the ETC from OXPHOS and as a result no ATP is generated. In this state maximal mitochondrial respiration can be observed, so called state 3. A second smaller dose to FCCP (0.5μM) was added after a set interval of 3 minutes to ensure that this state has been achieved. Finally Antimycin A (*Sigma, USA*) (2.5μM) was added to the cultured cells. Antimycin binds to complex III inhibiting the transfer of electrons, by preventing the oxygenation of the Q_1 site. This keeps *b* haemes reduced and increases superoxide which ergo prevents a formation of a proton gradient.

(In a selected number of wells pyruvate (0.05M *Sigma, USA*) was added to allow maximum cellular respiration to be achieved through the citric acid cycle. After data collection, cells were fixed using 4% PFA for 15 minutes, after which DAPI was added for 10 minutes. The cells were then imaged using a spinning disk confocal microscope (CSU-X1) (*Zeiss, Germany*) and QuantEM camera (*Photometrics, USA*) (figure 2.5). Data was analysed using excel with the methods outlined by (Birket *et al.*, 2011).
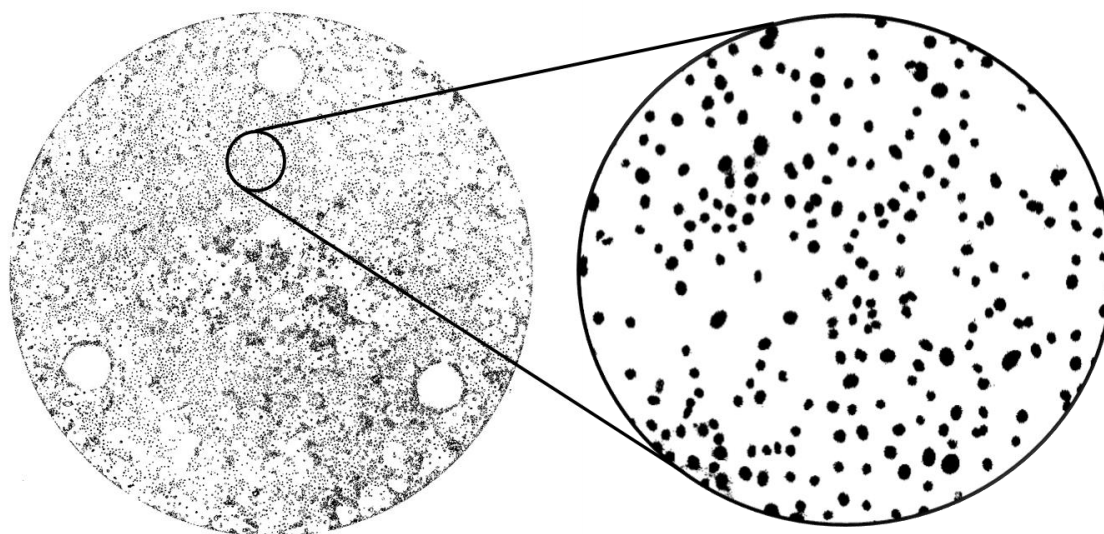
Figure 2.5 Cell counting methods for accurate normalisation of the extracellular metabolic flux assay
Cells in a well were crosslinked with paraformaldehyde and incubated with DAPI for 10 minutes. Images of the wells were captured on a spinning disk laser confocal microscope. The resulting images were thresholded with imageJ software, as shown above in the right circle. Each signal was then counted using the particle analyser tool.

2.23 Cell cycle analysis using flow cytometry

Cells were cultured as stated in 2.0, in both normoxia (21%) and hypoxia (3%) stated in section 1.15. Cells were dissociated from each well by an enzymatic treatment at 37°C for 15 minutes (TrypLE™ Express, *Invitrogen UK*). Cell cycle analysis was performed using a CyStain® DNA 2 step (*Partec, Germany*) kit. Nuclei were extracted using the supplied extraction buffer. 100μl of buffer was added to approximately $1x10^6$ cells and incubated at room temperature for 15 minutes. 500μl of supplied 4',6-diamidino-2-phenylindole (DAPI) was added to the nuclei suspension and incubated over night at 4°C. Immediately before processing the samples, the nuclei were passed through a 30 micron filter to separate aggregated nuclei. DAPI incorporates in A-T rich regions of DNA, and increases fluoresce by over 20 fold when incorporated. Fluorescence was measured using a

FACSCanto II (*Beckton Dickinson, USA*) at 405/450nD, data was analysed using Modfit software (*Verity software house, USA*). The principle for which includes the user defining the cells required for analysis by gating the relevant population. In cell cycle analysis two peaks are normally present, cells with a diploid number of chromosomes produce half the amount of fluorescence of those which are about to divide (G_1/M) . The software measures the number of cells in each peak to give a percentage over the total population.

2.24 Statistical analysis

The analysis performed on the data depended upon a number of factors which are summarised in figure 2.6. Minitab®, SPSS® and Sigma plot® were utilised to generate the statistical significances.
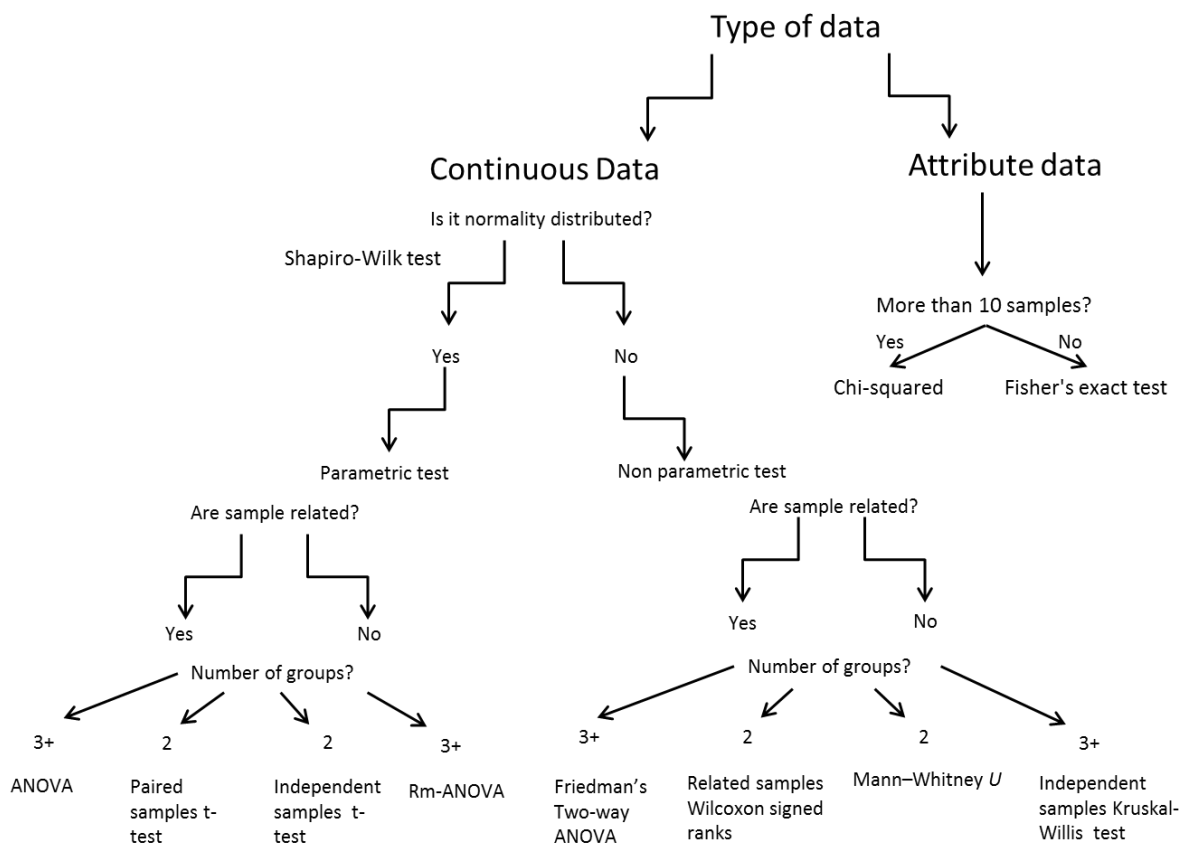
Figure 2.6 Statistical analysis decision trees
The analysis of statistical analysis of results was depended on the type of data and its distribution.

Probabilities which were less than 0.05 were considered to be statistically significant, allowing for acceptation of the alternative hypothesis (H_1). The alternative hypothesis states that there is a significant difference between the data sets which is not attributed

to chance. In contrast the null hypothesis (H_0) states that there is no significant difference between the means of data sets and that of those predicted to occur by chance. Data which was assessed using parametric tests was represented with bar charts, while generally data which was assessed using non-parametric tests are shown as box and whisker plots. Where there a small number of samples have been analysed the raw data has been plotted, often as scatter plots.

2.25 Ethical Considerations

The research described in this thesis was performed using corneal tissue donated for research, obtained from the UK eye bank. Ethical approval was obtained from the local research ethics committee (11/NE/0236). The Institute of Genetic Medicine (IGM) and Institute for Ageing and Health (IAH) hold licences from the Human tissue authority to store and use human tissues on site. The use of mice in this study was approved by the Animal and Welfare Ethical Review Board of Newcastle University under the project license from Prof. T. von Zglinicki (IAH) (PPL60/3864). Husbandry was performed by qualified staff in the CBC (Comparative Biology Centre) of Newcastle University.

Chapter 3 Investigating the effect of age on the growth of limbal epithelial cells

3.0 Introduction

3.0.1 The effects of age on human limbal epithelial cells

The effective growth of isolated and cultured limbal epithelial cells is important for the treatment of limbal stem cell deficiency (LSCD). The damaged corneal epithelium must be replaced in order to resolve the condition (Daniels *et al.*, 2001). A technique pioneered in 1989 used a section of healthy limbus and grafted it onto the afflicted eye (Kenyon and Tseng, 1989). This technique however does not come without risk with the potential of causing LSCD in the previously healthy eye. Allogeneic transplants from recently deceased donors are also used in cases were the risk of causing further damage is too high or when LSCD is bilateral. These transplantations however can face problems of immune-rejection (Williams *et al.*, 1995). Limbal epithelial cells were first reported as being successfully cultured from human donors in 1997 when techniques for cultivating primary keratinocyte were applied (Pelligrini *et al.,* 1997). Small sections of limbus could be excised and cultured on irradiated 3T3 fibroblasts to produce a stratified sheet of epithelial cells which could then be transferred onto an effected cornea. This technique reduced the risk of causing LSCD in a donor eye by reducing the quantity of tissue removed from the healthy eye.

The effect of age on progenitor limbal epithelial cells was thought to play an important role in the subsequent growth of these cells, in-turn this would have an effect on the successful culture of cells used for transplantation or their ability to regenerate the cornea once implanted (James *et al.*, 2001). It is known that senescent cells accumulate in tissues with age and lead to functional decline (Dimri *et al.*, 1995). Previous studies have shown no statistically significant change in the quality of cells harvested from older donors using 3T3 feeder layers (Shanmuganathan *et al.*, 2006) with the exception of CFE experiments (Notara *et al.*, 2013). In explanted cells grown on human amniotic membranes age was also shown to have no effect on the outgrowth size (Baylis *et al.*, 2013). Our aim was to replicate and expand on these contradictory results, employing additional techniques to ascertain the true effect of age on limbal epithelial cells using the 3T3 culture system.

3.1 Methods

CFE, quantitative polymerase chain reaction (qPCR), immunofluorescence and TRAP assays were utilised to analyse the number and quality of stem cells within each culture. A greater number of cells generated in CFE assays are generally indicative of a larger number of progenitor cells. Likewise the quantities of mRNA transcripts and protein accumulation of stem cell associated genes, positive or negative are indicative of the status of that cellular population. TRAP assays measure the quantity of active telomerase per a defined quantity of total protein from a cellular population. Activity of telomerase is indicative of stem cell populations.

The experimental techniques used to measure gene expression levels were qPCR and immunofluresence were chosen instead of western blotting and flow cytometry. The rational for performing qPCR was because we could measure the difference in mRNA transcripts with this method compared to westen blotting which shows protein quanties. I used immunofluresence to measure the protein amount in a particular cell type rather than in a whole crude extract of donor tissue (which is required for western blotting). Often the number of cells which could be isolated per donor was limited and therefore immunofluorescence seemed to be the best method to visualise and measure the difference in gene expression. I was concerned that the cell type that I wanted to measure could have been lost in a whole crude extract of tissue if I used western blotting as a technique. Similarly, for flow cytometry often the number of cells isolated and cultured was not sufficient to meet a sensible number of events. The TRAP assay was preferred over telomere length immunoFISH since it requires very few cells compared to other methods.

3.1.1 Colony forming efficiency assay of limbal epithelial cells

The assessment of the quantity of putative limbal epithelial stem cells within any given culture can be difficult to ascertain. The reason for this is a lack of stem cell specific markers, however colony forming efficiency assays can be used to identify donors which have a greater proportion of clonogenic cells indicative of stem cells. Colony forming efficiency assays are suited to cells derived from epithelia and highlight those progenitors which produce the highest number of TAC cells, which then go on to produce large colonies. Colony forming efficiency is widely considered to be an indirect measure of epithelial progenitors including those derived from the corneal limbus.

3.1.2 The use of clonal analysis to assess culture quality

The use of clonal analysis on limbal epithelial cells is not novel, previous studies have used clonal analysis to assess the number of progenitors at different anatomical regions, nasal, temporal, superior and inferior limbus (Pellegrini *et al.*, 1999). In HSCs clonal drift has been observed, from lymphoiesis to myelopoiesis, with age (Cho *et al.*, 2008; Dykstra *et al.*, 2011). To date clonal analysis has not been utilised to assess the effects of age on limbal epithelial cells. It was hypothesised that perhaps a clonal drift may occur in limbal epithelial cells, observable through the type of clones isolated.

Clonal analysis isolates single cells to assess their clonogenic ability. 480 cells from each primary culture were isolated using a dilution method and cultured in individual wells (described in chapter 2). Each cell would then go on to form a single colony. Epithelial cells form three colony types, holoclones, meroclones and paraclones (Barrandon and Green, 1985; Barrandon and Green, 1987). A greater percentage of progenitors are found in holoclones than in meroclones with paraclones containing the least number of progenitor cells (see 3.2.6.3). With this in mind it is possible to infer that donors containing a higher amount of holoclones had a larger pool of stem cells. It has been shown that holoclones express the highest quantities of *ΔNp63α*, thus as such this gene was used to assist in the assessment of clone type (Di Iorio *et al.*, 2005).

3.2 Aims

3.2.1.1 Aims of studying the influence of donor age on quality of limbal epithelial cells

The aim of this chapter was to determine if age correlates with the successful culture of human limbal epithelial cells using CFE, population doubling times, specific marker gene and clonal analysis.

3.3 Results

3.3.1 Does colony forming efficiency change with age?

An important fundamental property of stem cells is to produce daughter/transit amplifying cells to replenish or regenerate tissues. The measurement of the number of stem cells extracted from a single donor can be inferred from the number of colonies they can generate. Colonies can vary in size and shape (figure 3.1). To avoid any bias in the extraction

efficiency of the primary cells from the tissue pieces, cells taken for colony forming efficiency (CFE) were first cultured until 70% confluent (around 2 weeks). 1,000 cells were portioned from the primary cultures, co-cultured with 3T3 fibroblasts and incubated for 12 days. Each donor/subculture had 3 replicates. Even so one found that the percentage efficiency varied greatly from donor to donor as shown in figure 3.2. This variation seems to increase in older donors. If one takes subculture 1 as an example the standard error of the mean (SEM) was 0.9 (Standard deviation 1.8) in young donors (20-69) compared to 1.2 (Standard deviation 3.7) in the older category (70-90).
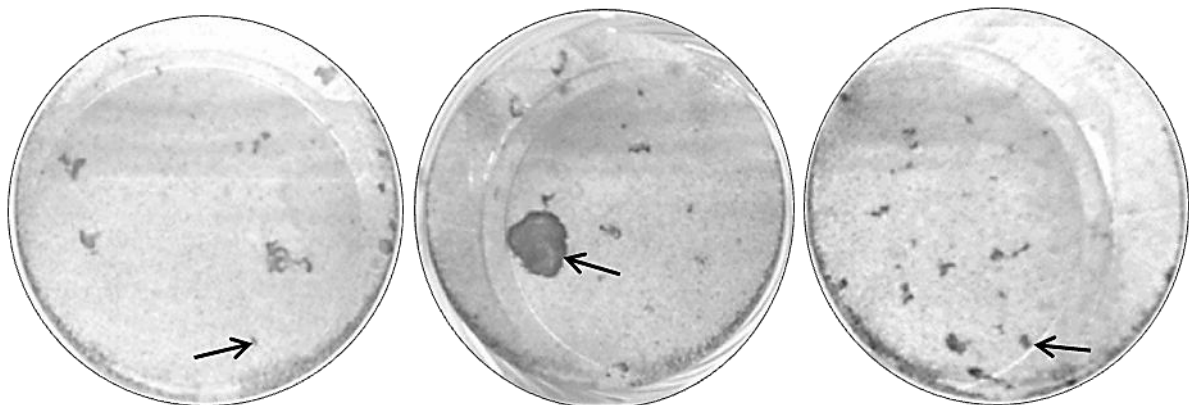
Figure 3.1 Examples of colony forming efficiency assays
Example colony forming efficiency plates, colonies are labelled with black arrows, colony size and number was recorded, with the minimum size around 75 cells, at which point they became visible to the naked eye. Colonies were stained with 1% Rhodamine (see methods) to give a high contrast to the colonies, allowing for ease of counting. The images have been converted to grey scale so that this contrast was not lost after digital conversion

A Shapiro-Wilk test was required to determine if the data was normally distributed. It showed that overall the data was not normally distributed ($p<0.05$) (figure 3.2). The Shapiro-Wilk test is a useful tool for assessing normality. As the data was not normally distributed a Mann-Whitney test was performed on independent samples for example comparing between age categories. Wilcoxon signed rank tests was used on related samples when observing between subcultures of the same category. The standard confidence level of 0.95 was applied to this and all subsequent statistical tests. The alternative hypothesis (H_1) states that the samples were not equally distributed, whereas the null hypothesis (H_0) stated the converse, that samples were equally distributed for this and all subsequent tests. If the data had been normally distributed and had equal variance, an analysis of variance (ANOVA) for 2 or more group comparisons would have been used. Related (paired) samples Students t-test would have been utilised for two related groups, for example between subculture 1 and 2 of

the young category. Between categories an independent (unpaired) samples t-test would have been used, this rationale was applied to this and all subsequent analysis. When 3 or more groups are to be tested in a non-parametric way and the samples are independent of one another, a Kruskal-Wallis one way analysis of variance on ranks was used (Lehmann, 1998). Comparing subculture 1 between young (20-69) and old groups (70-90), showed no significant difference ($p=0.57$). Subculture 2 was also not significantly different between age categories either ($p=0.46$). Subculture 3 showed a statistical difference between the two age categories with $p=0.016$. The data suggests that older donors are less likely to produce colonies after subculture 2. Confirming the data shown by Lindberg and colleagues, who demonstrated a decline in colony forming with successive sub-culturing (Lindberg *et al.*, 1993).

To ascertain the differences between subcultures within the same age category related samples Wilcoxon signed rank tests were performed. Subcultures 1 and 2 in the young (20-69 years) group $p=0.068$. Analysis on subcultures 1 and 3 showed a p value of *0.068*, subculture 2 and 3 also had a p value of *0.068*. This lack of significance could in part be due to a lack of sufficient sample size and high variation between donors.

In the subcultures of the 70-90 year old group the p value between subculture 1 and 2 was *0.012* indicating significance. Studying the data between subcultures 1 and 3 also showed significance, ($p=0.012$). There was not however a significant difference between subcultures 2 and 3 ($p=0.063$).
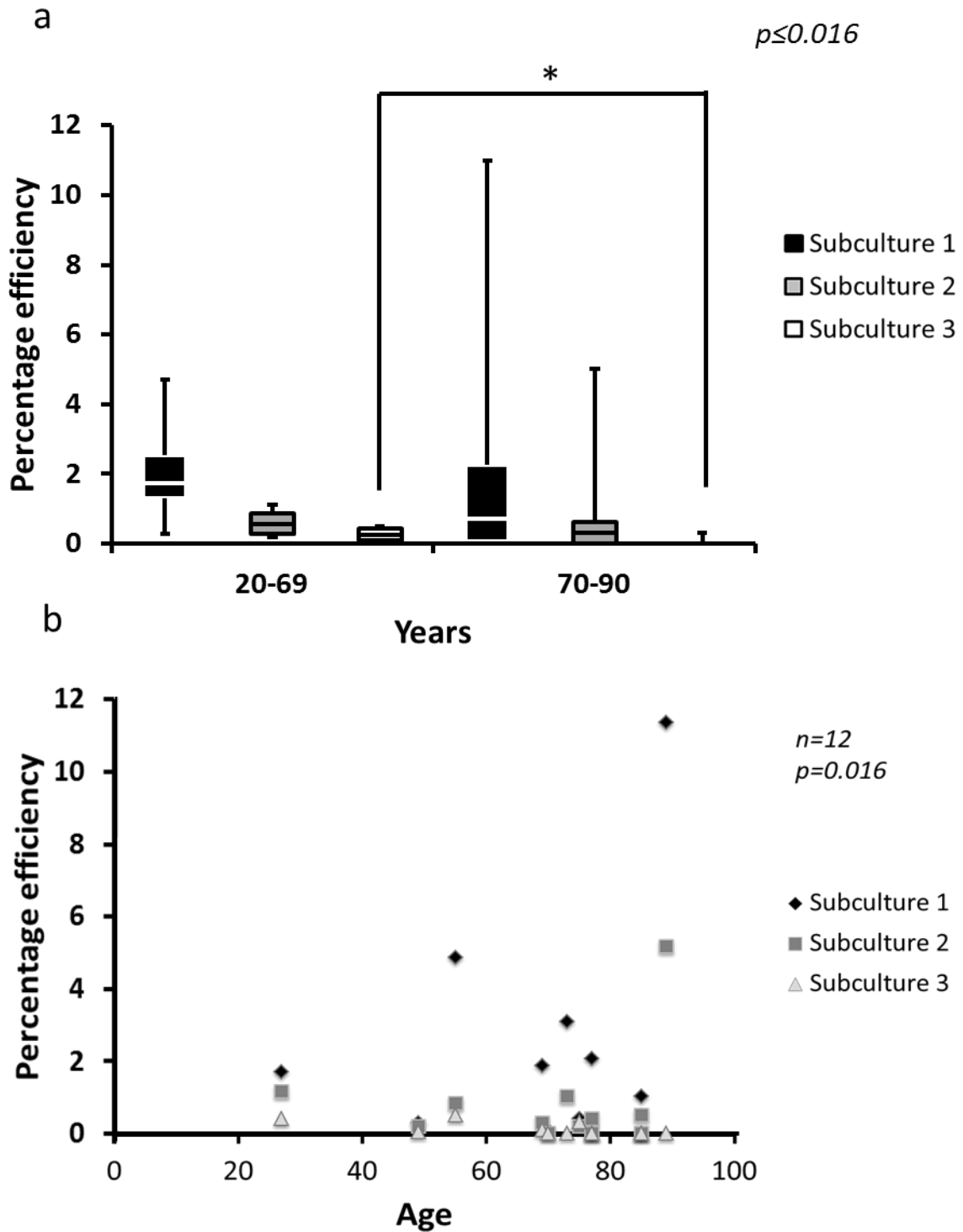
Figure 3.2 The effect of donor age on colony forming efficiency over multiple subcultures.
Box plots (a) and scatter plot (b) to show the colony forming efficiency between subcultures in all donors. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk. (N=4 for the group 20-69 years old and 8 in group 70-90 years old). (Subculture 3, 20-69 vs 70-90, p=0.016) (p=0.012, SC1 vs SC2 70-69) (p=0.012, SC1 vs SC3).

In addition to age, the time in which corneoscleral tissue is stored for might also have an

effect on the successfulness of limbal cultures. Figure 3.3 shows the effect of time in organ

storage medium on the subsequent CFE of each donors cells over multiple subcultures. A Shapiro-Wilk test revealed that overall the data was not normality distributed ($p=0.050$). A non-parametric test comparing subcultures between the two time categories showed that there was not a significant difference between either (Mann-Whitney on independent samples)($p=1$, 0.485 and 0.937, subcultures 1-3).To assess if there was a statistical difference between subcultures within each time group a Kruskal-Wallis One Way Analysis of Variance on Ranks was performed. The 30+ days in organ culture medium group showed no statistical difference between each subculture ($p=0.067$). The 0-29 days group did however show that there was a difference ($p=0.034$). Further pairwise analysis with Tukey's test, showed that between subculture 1 and 3 ($p<0.03$) there was statistical significance. This indicates that there is a greater reduction over subsequent subcultures compared to the 30+ days group, this might however be due to a slightly higher number of progenitors at subculture 1.

It was not possible to perform a three way analysis, comparing both age, time in organ culture medium and subcultures because there was only one donor which was both in the young category and had been in organ culture medium for longer than 30 days.
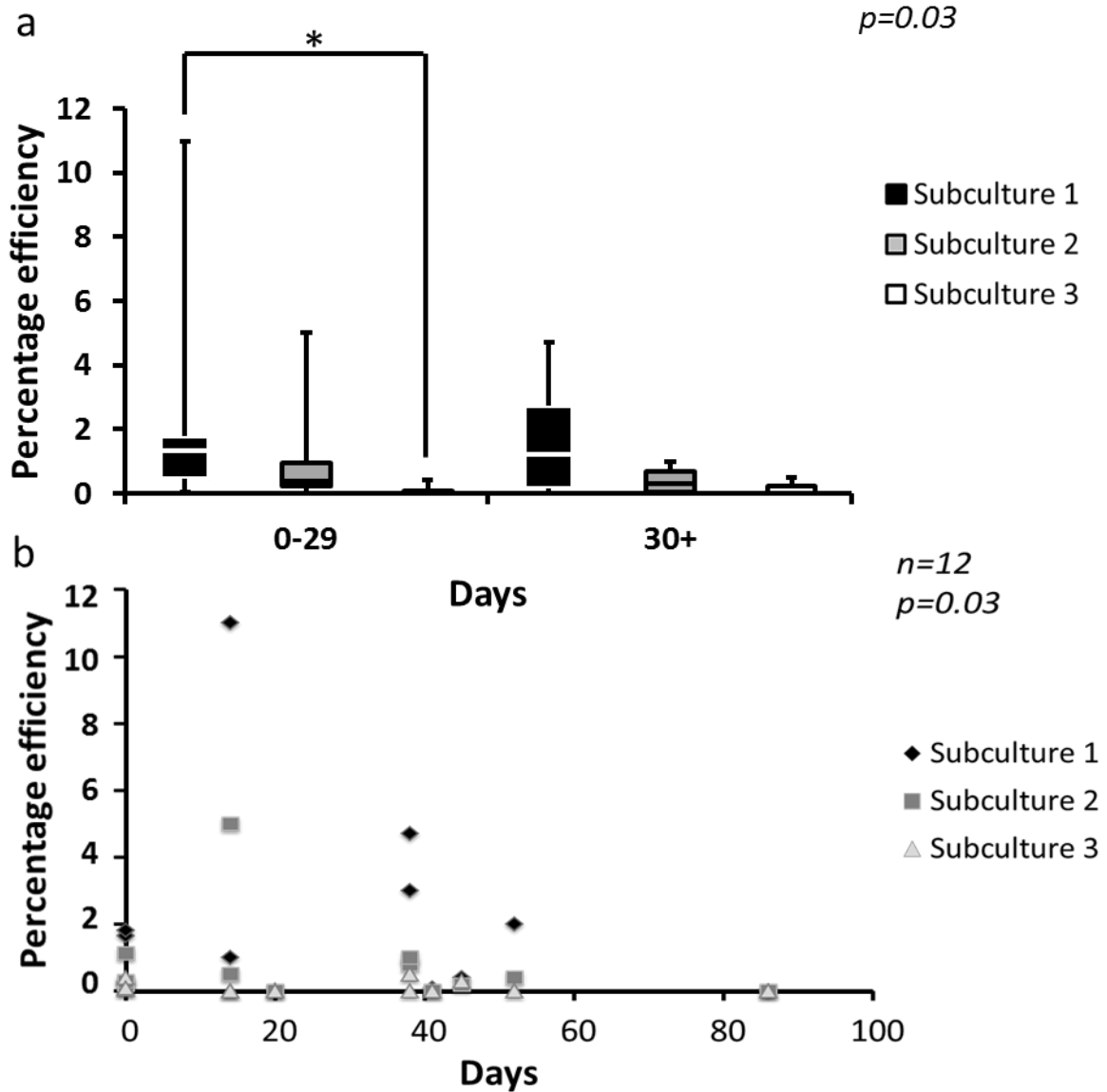
Figure 3.3 The effect of time in organ culture medium on colony forming efficiency over multiple subcultures.
Box plots (a) and scatter graph (b) shows colony forming efficiency compared against tissue storage time in organ culture medium for 0-29 days (N=6) and more than 30 days (N=6). The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk (*p<0.03,* SC1 –SC3 0-29 days).

3.3.1.1 Does colony size change over multiple subcultures?

The size of colonies can be indicative of the proportion of progenitor cells which gave rise to it, smaller colonies tend to originate from paraclones, whereas larger colonies tend to originate from holoclones (Barrandon and Green, 1987).
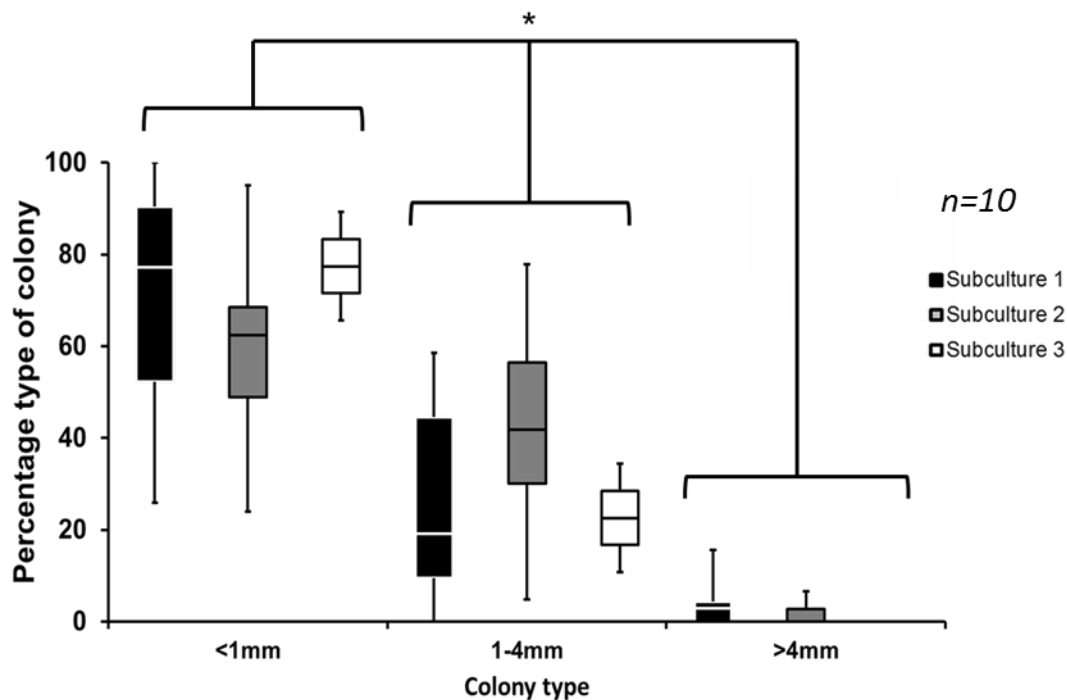
Figure 3.4 Box and whisker plots of colony size in subcultures 1, 2 and 3.
Colonies were categorised into 3 groups, <1mm, 1-4mm and >4mm. There were 10 donors in subculture 1 of those 7 survived to subculture 2. 2 cultures from this experiment survived until subculture 3. Panel a, The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk. (*p<0.001*, between overall colony sizes).

The Shapiro-Wilk test showed that the data was not normality distributed (*p<0.050*). Two way analysis of the data was not performed using a non-parametric test due to the large sample sizes needed to satisfy the criteria for a chi-squared test. The chi-squared test would have likely produced a type II error (failure to detect true differences). Mann Whitney tests and Wilcoxon signed ranks tests are more robust (as they analyse the sum of ranks, ergo outliers are less likely to influence the result) and the results from which are shown in panel b of figure 3.4.

Comparing the difference between size categories using Kruskal-Wallis One Way Analysis of Variance on Ranks showed that there was a statistical difference between them (all *p<0.001*). The result verifies that the defined categories are in fact distinct from one another. The results also suggest that the proportion of progenitors do not decline with serial culture.

3.3.1.2 Is colony size affected by donor age?

The relationship between donor age and resulting colony size is an important parameter to investigate as it can indicate a shift in the type of colonies produced. Any changes to the composition could indicate linage skewing. The data was first analysed for normality using the Shapiro-Wilk test, *p=0.131* showing that the data was normally distributed. Two way ANOVA indicated that there was a statistically significant interaction between age and colony size (*p=0.032*). Post hoc pairwise analysis was performed using the Holm-Sidak method, as the data also failed the equal variance test. Holm-Sidak is more powerful than Tukey's and Bonferroni tests and was thus selected over these tests. Overall comparisons of old vs young were not significant (*p=0.99*). Looking at colony size however significance was found between all groups (*p<0.001*) (figure 3.5).
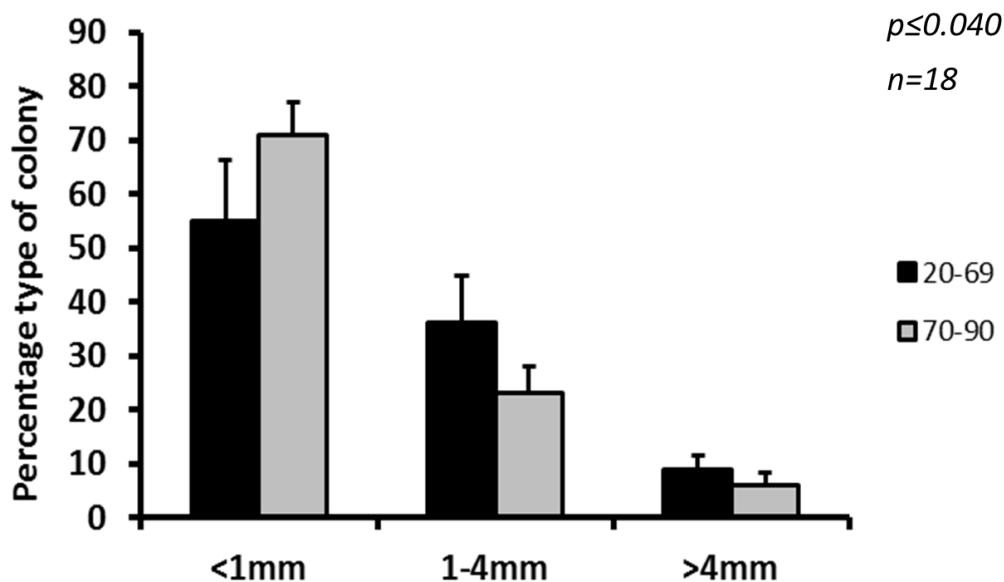
Figure 3.5 Colony sizes of cells isolated from young and old donors at subculture 1.
Colonies were categorised into 3 groups, <1mm, 1-4mm and >4mm. There were 14 donors in the 70-90 year old category, whereas there were only 4 donors in the young (20-69 years. Significance is indicated with an asterisk. 20-69 year old <1mm and >4mm (*p<0.001*), 1-4mm and >4mm (*p=0.014*), <1mm and 1-4mm (*p=0.084*).70-90 year olds donor cells, <1mm vs >4mm (*p<0.001*), <1mm vs 1-4mm (*p=0.001*) and 1-4mm vs >4mm (*p=0.002*). Standard error of the mean is shown.

Comparing colony size in the young donors cells showed that there was significance between <1mm and >4mm (*p<0.001*) as well as between 1-4mm and >4mm (*p=0.014*). However the <1mm and 1-4mm groups were not significantly different from one another (*p=0.084*). This indicates that a greater proportion of small colonies were present compared to the larger colonies. Given the rarity of stem cells these few large colonies are likely to

have originated from stem cells, while the numerous smaller colonies are likely to have been derived from transit amplifying cells.

Comparing colony size in the older donor's cells showed that there was significance between all groups, <1mm vs >4mm (*p<0.001*), <1mm vs 1-4mm (*p=0.001*) and 1-4mm vs >4mm (*p=0.002*). The greater number of <1mm sized colonies indicates that a higher number of differentiated and transit amplifying cells were isolated from older donors compared to other sized colonies. Indeed if we compare the <1mm group between young and old, there is a significant increase in the number of these smaller colonies (*p=0.040*). However the 1-4mm and >4mm groups were not statistically different, *p=0.104* & *p=0.657*). These results suggest that the total proportion of larger colonies does not change with age, thus the number of progenitors which give rise to these colonies.

3.3.1.3   Is colony size affected by number of days in organ culture medium?

As stated previously the number of days in organ culture medium is thought to effect the growth of limbal epithelial cells. The size of colonies was again measured, as previously stated. Analysis of the results of this experiment firstly began with a normality test which showed that the data was normality distributed (*p=0.276*). No statistical difference was detected between the number of days in organ culture medium and the sizes of colonies produced in each of these populations (*p=0.932*) (2 way ANOVA). However as previously found, there was statistical difference between size categories (all, *p<0.001*) (Post hoc Holm-Sidak). These results indicate that the time in organ culture medium does not impact upon the proportion of colonies which are able to form, inferring that the number of progenitors within the tissue do not decline whilst in storage.
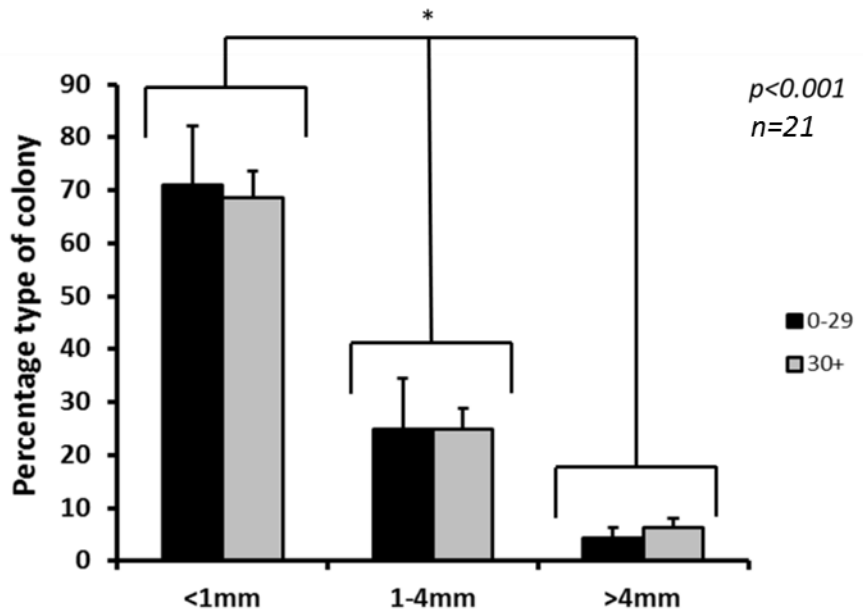
Figure 3.6 Colony sizes of donors cells with respect to length of time in organ culture medium at subculture 1. The histogram shows the length of time in days that tissue was stored in organ culture medium before culturing. 5 donors were in organ culture medium for 0-29 days whereas 16 donors were in the medium for 30+ days. A post hoc least significant difference test showed that *p<0.001*, between all size categories. Standard error of the mean is shown.

3.3.2 Is the population doubling time effected by age or storage conditions?

The time it takes a culture to become confluent from a defined number of cells is an indicator of the proliferative potential of the culture and can indicate a higher number of progenitors contained within the primary culture. One measured the time it took for cultures to become 70% confluent importantly from a defined number of seeding cells, between subculture 1 and 2 (ATCC, 2012). Both age and the time that tissue is stored in organ culture medium are thought to have an effect on the cells which can be isolated and subsequently cultured (Liu *et al.*, 2012). The limbal cultures processed for this study could not be cultured in the same time frame due to varying shipping time and the lead times associated with pathogen testing. As a result it was important to assess what effect this extended time had on the proliferative capacity of the isolated cells.

A Shapiro-Wilk test showed that the young donor data was normally distributed (*p=0.511*), data from the older donors however was not normally distributed (*p=0.015*). A non parametric test was performed (independent samples Mann Whitney) were *p=1*, thus one can accept the null hypothesis (figure 3.7). The null hypothesis in this instance stated that donor age does not have an effect on population doublings.
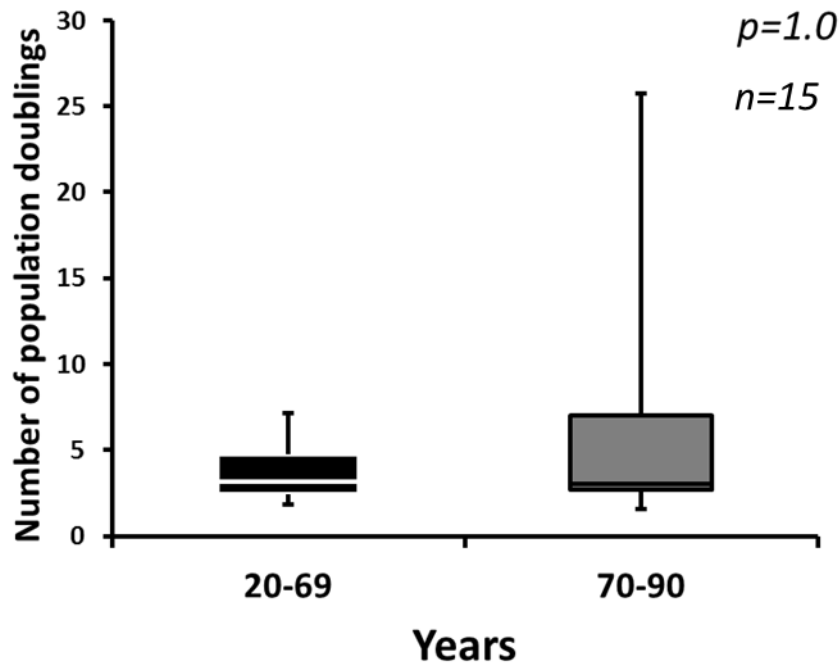
Figure 3.7 The effect of age on the population doubling time between subculture 1 and subculture 2
Population doubling time was calculated by the number of days it took cultures to achieve 70% confluence. The total number of younger donors (20-69) measured was 5, while there were 10 donors in the old category (70-90). The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values.
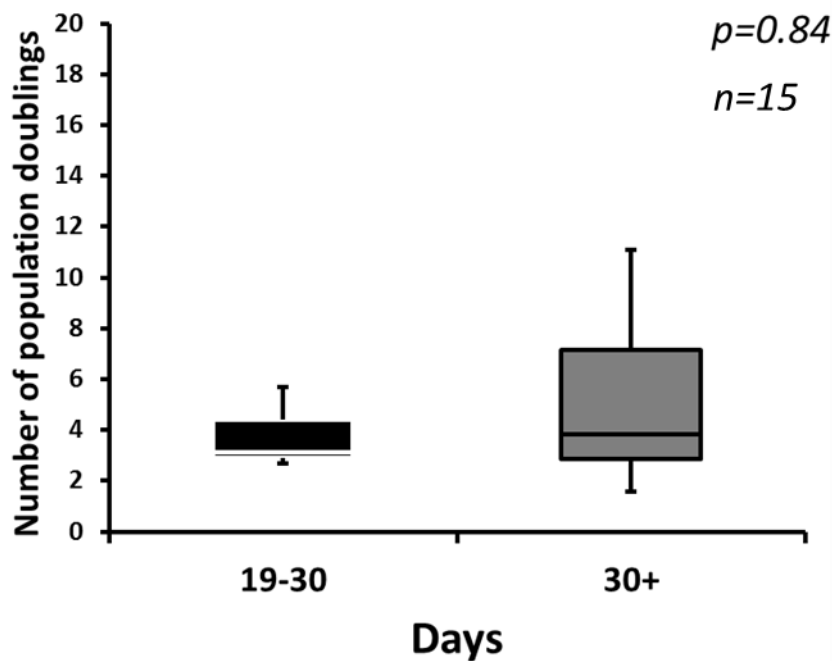
Figure 3.8 The effect of the number of days stored in organ culture medium on the population doublings between subculture 1 and subculture 2
The total number of donors in the 19-30 days group was 3, in the more than 30 days group there were 12 donors. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values.

Looking also at the effect of storage time in organ culture, firstly a Shapiro-Wilk test was performed, showing that the data from the +30 days group was not normally distributed ($p= 0.17$). A Mann Whitney test was then applied to the data, this resulted in a p value of *0.84*. Both figure 3.7 and 3.8 shows that there is no statistical difference between different donor ages or time in organ culture medium. The data did show that age has a greater effect on the variation of samples, which in part could have possibly led to a lack of significance between the groups. These results correspond to similar ones performed on limbal explanted tissue (Shanmuganathan *et al.*, 2006; Baylis *et al.*, 2013) and the 3T3 culture system (Notara *et al.*, 2013). Our data does seem to favour a slight suggestion that increased time in organ culture medium and increased age leads to more limited proliferation potential. Presumably though fewer progenitors s in the old and 30+days stored cultures, although not significantly in this study. A similar suggestion was made by Lui *et al.,* (2012), who grew the cells on denuded amniotic membranes and with observations taking place after only 4 days in organ culture medium.

3.3.3 Can the number of days that corneal disks are in organ culture medium affect the quality of a culture?

In addition to assessing population doubling the fate of each culture was tracked and its parameters logged. Each culture was assigned into a success group or a failure group, dependent on if cells could be isolated and cultured from that particular donor, defined by any growth in the initial isolate or absolutely no colony formation. The average age of donors was 71 years old, with the oldest donor being aged 90 years old, whist the youngest donor was 20 years old. Analysis of the data firstly began with a Shapiro-Wilk test, it showed that the failure group was not normally distributed (*p=0.01*). The success group did however have a normal distribution (*p=0.081*). An independent samples Mann Whitney test was performed which showed that there was no difference between the average age of those cultures which failed and those which were successful (*p=0.421*) (Figure 3.9). This allows us to accept the null hypothesis that donor age does not affect the successful culture of cells isolated from them.

The data was then analysed to assess if time in organ culture medium has an effect on the success of isolated limbal epithelial cells. Again, the normality of the data was tested with a Shapiro-Wilk test, it showed that the success category was normally distributed

(*p=0.060*) whereas the failure category was not normally distributed (*p=0.046*). Utilising the Mann Whitney test showed that there was no difference between the success and failure categories (*p=0.768*) (Figure 3.10). One can accept the null hypothesis in this instance, that time in organ culture medium has no effect on the success or failure of a limbal epithelial cell containing culture.
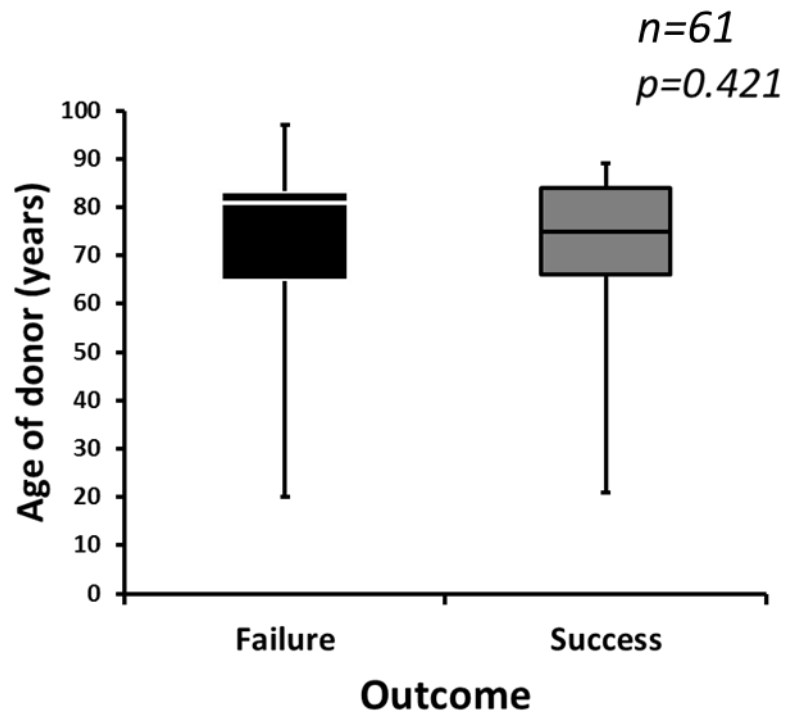
Figure 3.9 The effect of donor age on the successful growth of each culture
The total number of donors in the successful category was 45, whereas there were only 16 in the failure category. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values.
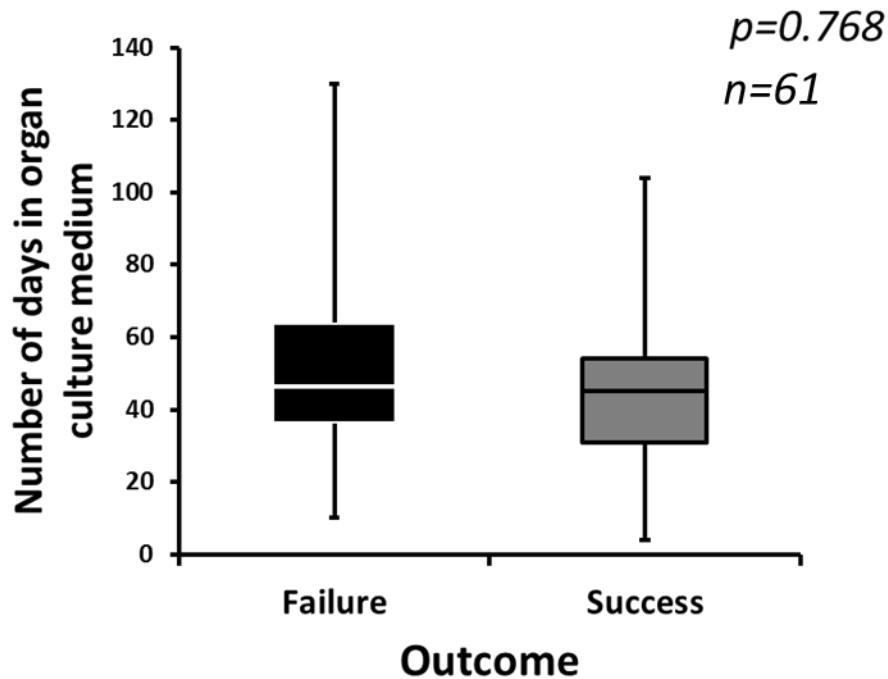
Figure 3.10 The effect of tissue storage time on the successful growth of each culture
The total number of donors in the successful category was 47, whereas the failure category has 22 donors. The number of donors differs because time in organ culture medium was not present for all donors. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values.

3.3.4 Does gene expression at mRNA and protein levels change with age or organ culture storage time?

Firstly, an appropriate "housekeeping" gene was selected in order to normalise the data for accurate analysis. GAPDH was chosen as it deviated the least between different donors and conditions out of all genes tested, see appendix A. *ΔNp63α* is a reliable marker of limbal epithelial cells, in both a quiescent state and activated state, hence it can detect stem cells and transit amplifying cells. Shapiro-Wilk analysis revealed that the data was not normally distributed (*p<0.001*), thus a Mann Whitney test was performed. The difference between the groups 20-69 and 70-90 was not significant (*p=0.33*) (Figure 3.11). The H_0 can be accepted and alternative hypothesis can be rejected (H_1). The conclusion is that donor age does not seem to have an effect on p63 expression in the samples tested.
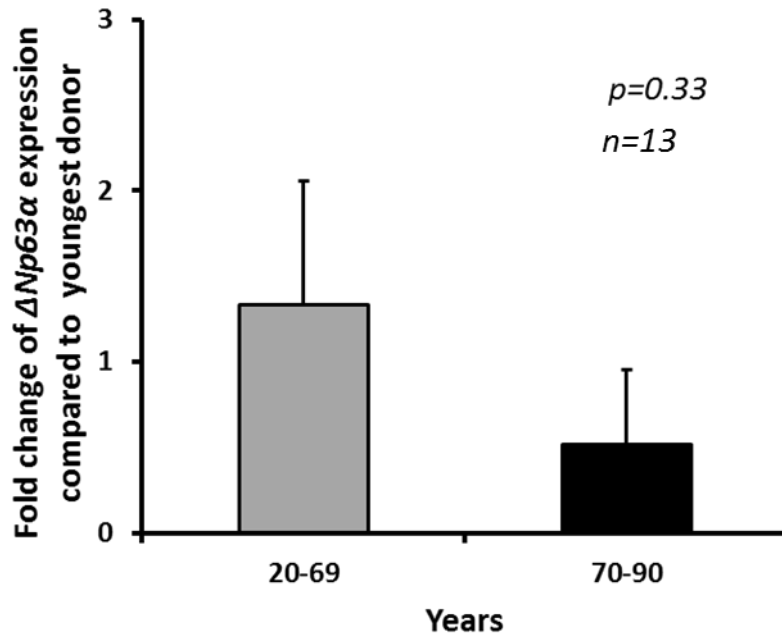
Figure 3.11 The effect of age on the expression of *ΔNp63α*
The number of donors in the young category (20-69) was 3, whereas the older (70-90) category was 10.
Standard error of the mean is shown.

Figures .12 shows an array of genes used to assess limbal epithelial cells. The genes *ABCG2*, *BMI1* and *TERT* are positive markers of limbal epithelial cells, while *KRT3* and *KRT12* are considered negative markers. The gene *p21* was used to assess any change in DNA damage and stress (see 1.1.2). Normality tests performed on the data showed that all but the *KRT3* (70-90 years) and *KRT12* (20-69 years) categories were normally distributed (Appendix B, table 7.5). As a result one performed paired sample t-tests on the data, with the exception of *KRT3* were a non-parametric test were used. All comparisons were not significant. *KRT12* (20-69 years) only had two donors in this category as a result a Shapiro-Wilk test or a significance test could not be performed.
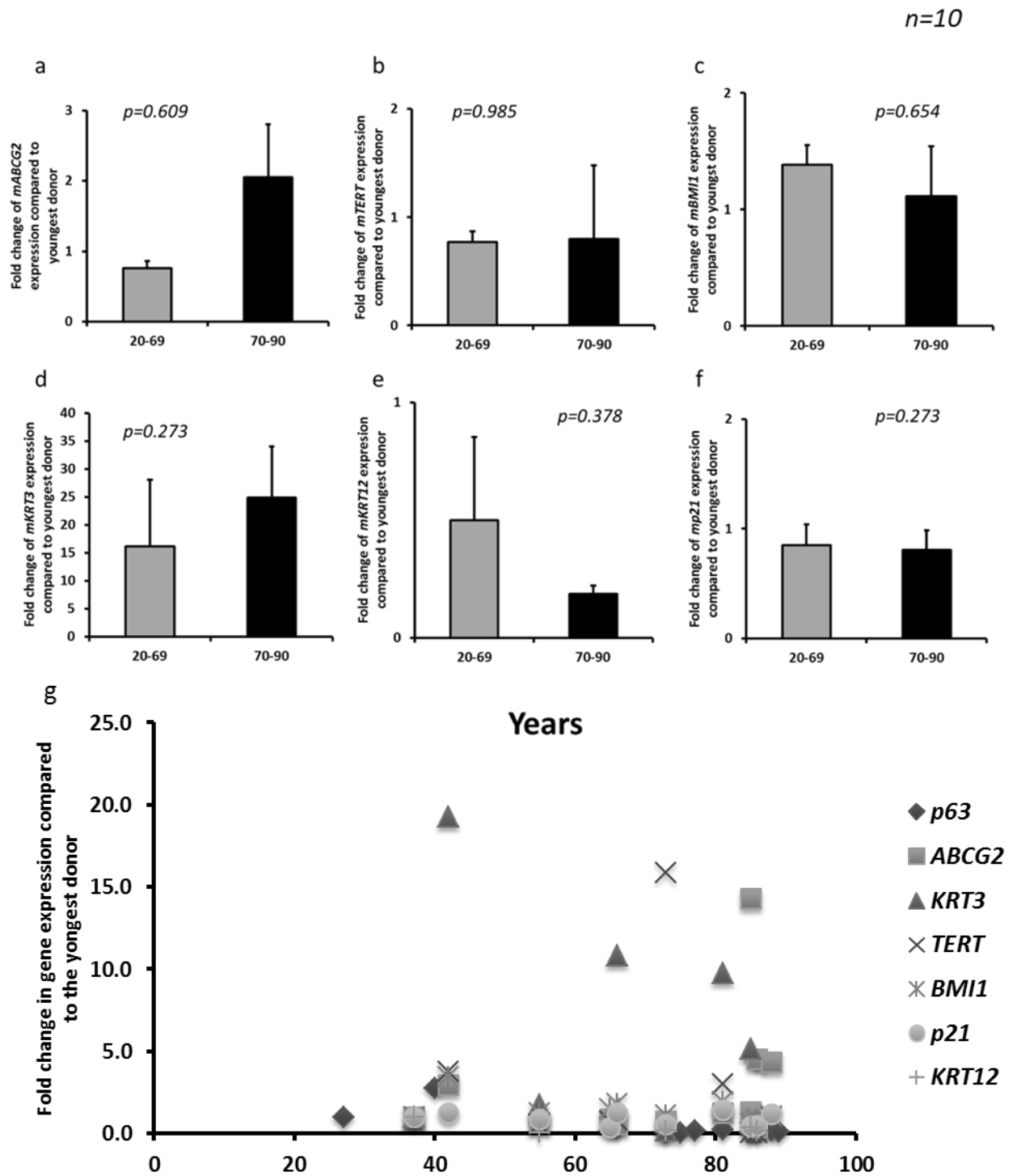
Figure 3.12 The effect of age on the gene expression profile of limbal epithelial cells
The number of donors in the young category was 4, whilest there were 6 in the old category. Panel a, shows ABGG2, b, TERT, c, BMI1, d, KRT3, e, KRT12, f, p21. Standard errors of the mean are shown. Panel g shows the spread of the raw data.

Figure 3.13 shows the effect of the number of days limbal epithelial cells were stored in organ culture medium before extraction. The data was first analysed for normality, a Shapiro-Wilk test showed that the data was not normally distributed (*p>0.001*). Ergo a non-parametric test was utilised. A Mann Whitney test on independent samples indicated that the presumed increase in *ΔNp63α* transcripts was in fact not a significant increase with *p=0.71*.
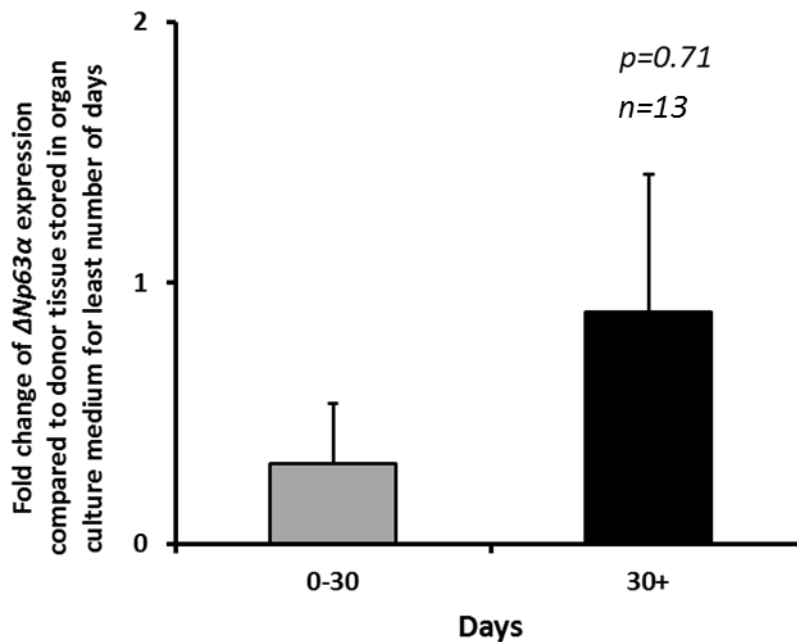
Figure 3.13 The effect of storage time on the expression of *ΔNp63α*
The number of donors in the least number of days category was 4, whereas there were 9 donors in the more than 30 days category. Standard errors of the mean are depicted.

Figure 3.14 shows the same array of genes as tested in figure 3.11, all but *TERT* (+30 days) was normally distributed (Appendix B, table 7.6). Performing unpaired t-tests on the data showed that there was no statistical difference in gene expression between the number of days in organ culture medium. Where the Shapiro Wilk test indicated a non-normally distributed population non-parametric test were performed (Mann-Whitney on independent samples), there was no statistical difference between the number of days in organ culture medium and any of the genes tested.
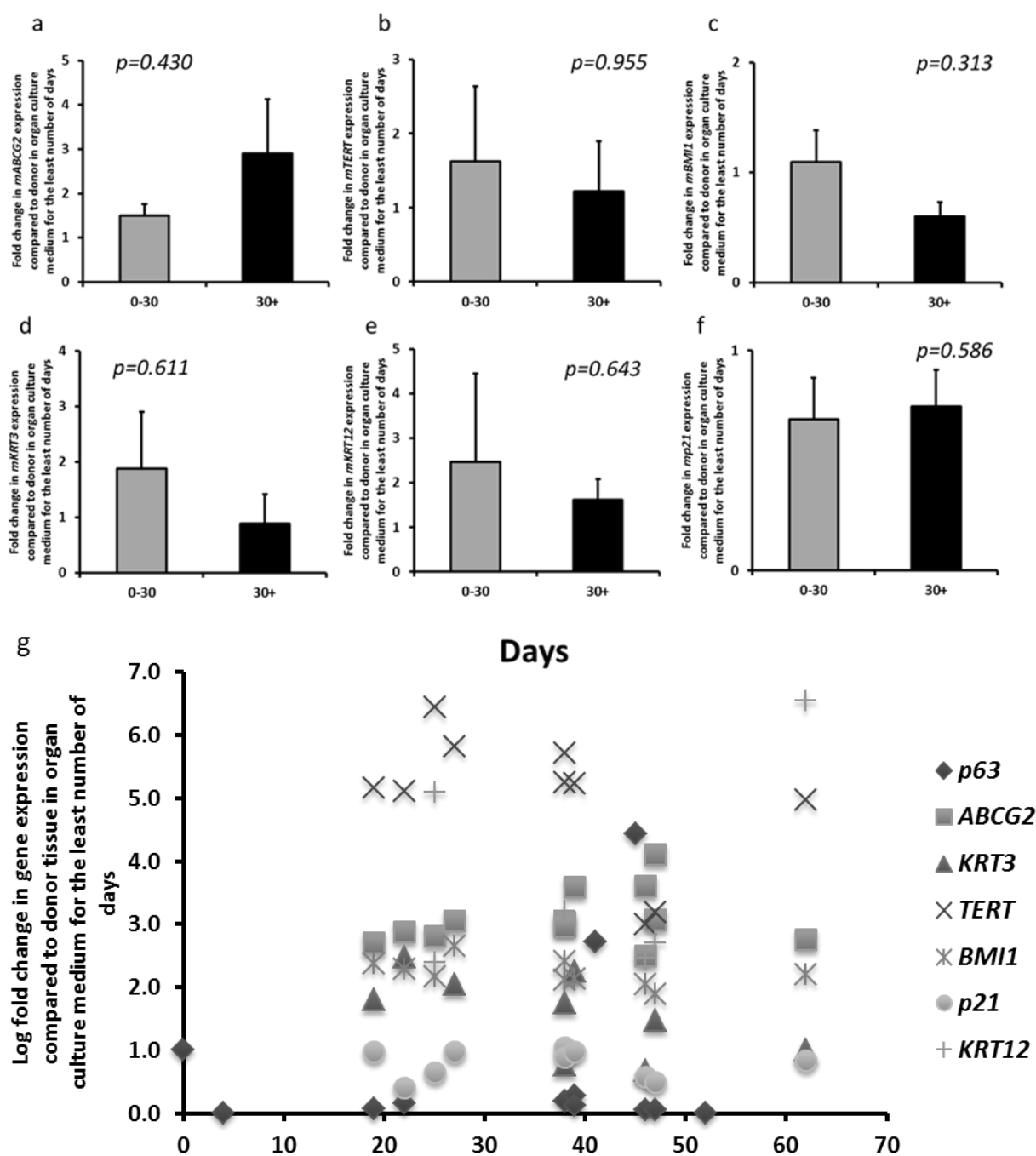
Figure 3.14 The effect of tissue storage time on the gene expression profile of limbal epithelial cells
The number of donors in the 0-30 days group was 4 while the number of donors in the 30+ days group was 5.
Standard errors of the mean are shown. . Panel a, shows *ABGG2*, b, *TERT*, c, *BMI1*, d, *KRT3*, e, *KRT12*, f, *p21*.
Panel g shows the spread of the raw data.
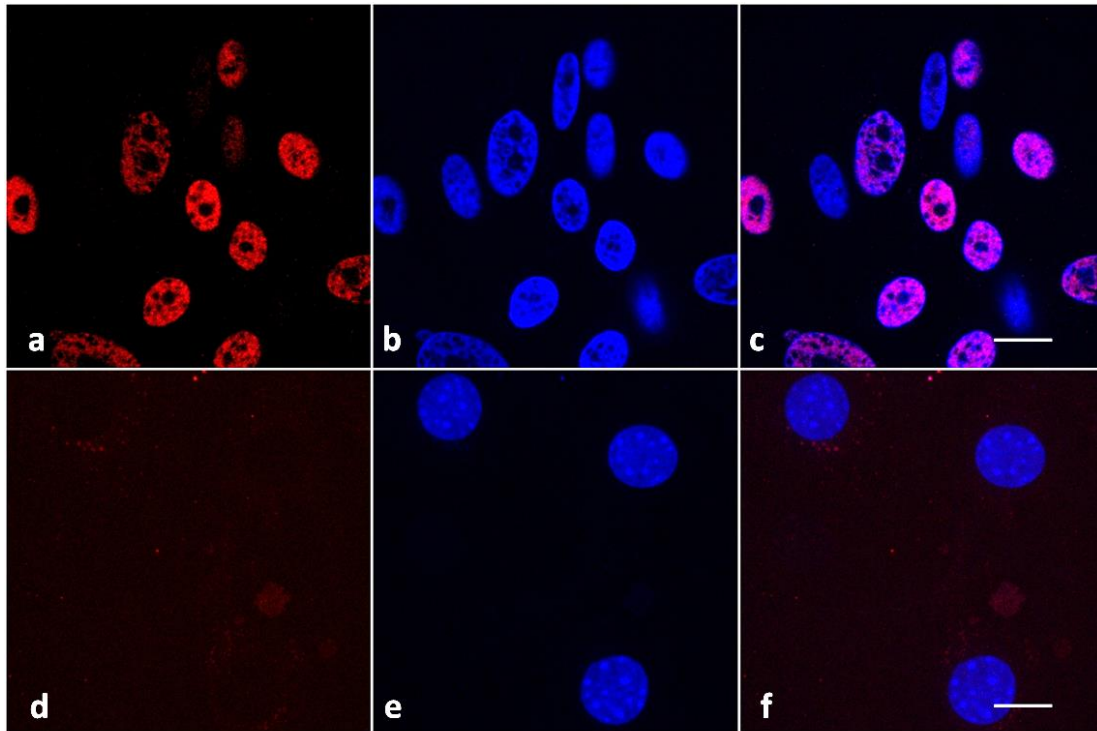
Figure 3.15 Representative images of immunofluorescence staining for ΔNp63 in a human limbal epithelial cell containing culture

Panel a shows p63 staining in a limbal epithelial cell containing colony, panel b shows DAPI incorporation into nuclear DNA in the same colony. Panel c is the merged image of a & b. 3T3's were also present in the cultures, these mouse cells had a distinct staining pattern, large nuclei, numerous nucleoli and very defuse p63 staining. Panel d shows the p63 in 3T3 fibroblasts, e DAPI and f is a combined image. The scale bar indicates 50μm, a magnification of x63 was used.

Figure 3.15 a & b show that limbal colonies were easily distinguishable from the co-cultured 3T3 fibroblasts, both in morphology and staining pattern. As a transcription factor p63 is often located within the nucleus, it was from this location that the intensity was measured. 3T3 fibroblast cells are much larger both in cytoplasmic size and nuclear size and have a more diffuse p63 staining pattern. Thus, both cell types were easily distinguishable and only the small limbal epithelial cells were analysed, as shown in figure 3.16.
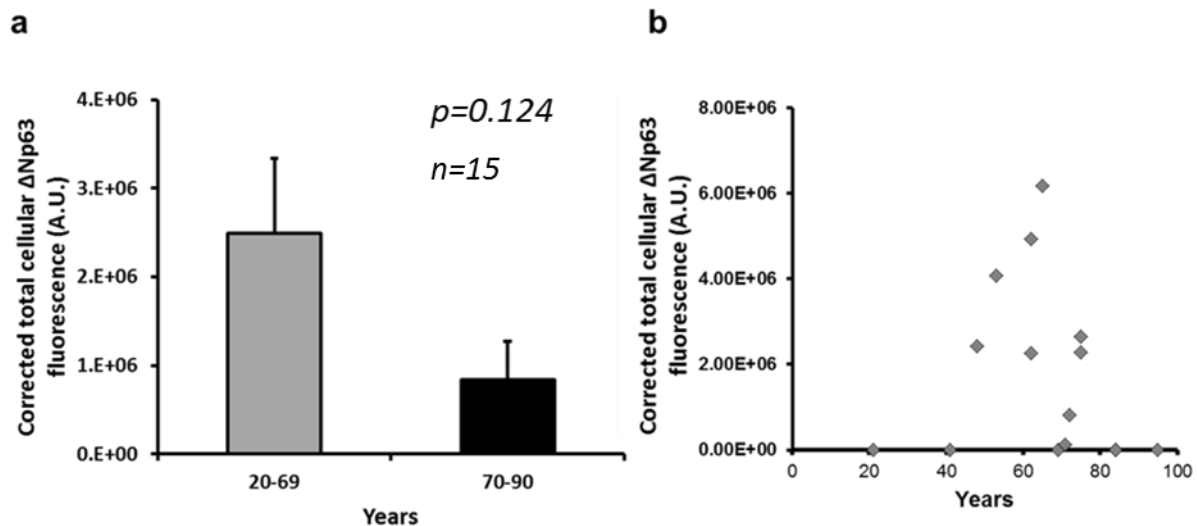
Figure 3.16 Quantification of the intensity of ΔNp63 immunofluorescence in human limbal epithelial cell containing cultures

The number of donors tested in the young category (20-69 years) was 8 and in the old category (70-90 years) was 7.(N=15). Total cellular fluorescence was calculated from 15 random fields of view in each donor, with 3T3's excluded from the analysis using the lasso tool in ImageJ (see methods section). Over 4,200 cells were analysed. Standard errors of the mean are shown.

A Shapiro-Wilk test indicated that the data was normally distributed (see appendices B).The data alludes to a decrease in ΔNp63 in older donors (Figure 3.16, a). This decrease however was not significant using a *t-test* with *p=0.124*. Figure 3.16, b indicates the possibility of a bimodal distribution, with an increase in intensity between 50 and 70 years. This distribution was similar to the qPCR data for *ΔNp63α* shown by Notara and colleagues (Notara *et al.*, 2013). In contrast their 30-60 years old age group had increased *ΔNp63α* expression.

3.3.5 Does the activity of telomerase change with age or time in culture?

Telomerase activity is an important parameter for determining the level of telomere maintenance. Stem cells are long lived and a required to elongate there telomeres to avoid entering into replicative senescence.

Shapiro-Wilk tests showed that the 70-90 year old group was not normally distributed. As a result a Mann Whitney test was performed between the two groups. There was no statistically significant difference between the two age groups (20-69 and 70-90) (*p=0.744*) (Figure 3.17). The cells which were sub-cultured appeared to decrease with time in culture. An independent samples Kruskal-Wallis test showed significance between subculture 1 and subculture 3 (*p=0.010*). Although no significance was detected in subculture 1, there was an indication of a decline at this time point (SC2-SC1 *p=0.498* and

SC1-PC *p=0.480*). The results obtained from the two age categories confirm those observed by Notara and colleagues (Notara *et al.*, 2013). These authors, using many more donors than our study, could not correlate telomerase activity with age in limbal epithelial cell containing cultures. The effect of subculturing on telomerase activity was shown to decrease in endothelial cells (Hsiao *et al.*, 1997). In mesenchymal stem cells however, subculturing does not affect telomerase activity (Otte *et al.*, 2013). Interestingly this was dependent on the microenvironment of the cells, removal of umbilical cord tissue resulted in a loss of stemness in these cells. If one infers from these results, the loss of telomerase activity in limbal epithelial cells could be due to environmental factors such as non-biologically relevant culture conditions.
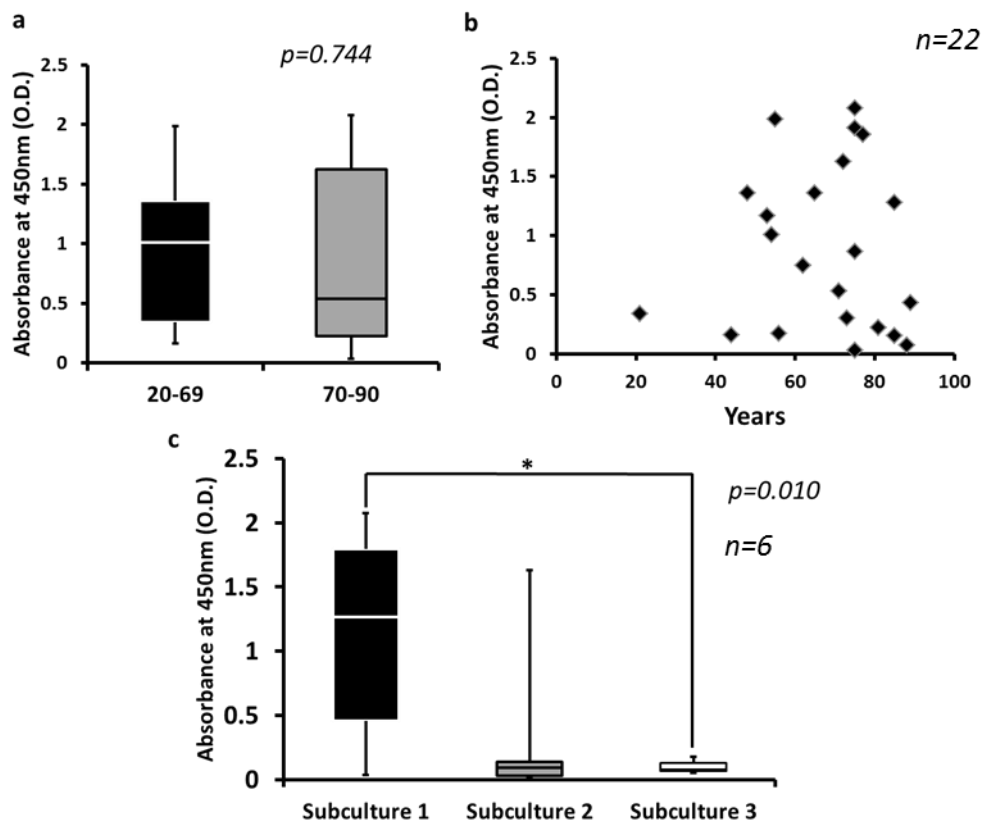
Figure 3.17 The effect of donor age and serial subculturing on telomerase activity
Telomerase activity was determined using a telomere repeat amplification protocol and detected with ELISA. Panels a and b show the effects of age on telomerase activity. Panel b is a scatterplot showing the distribution of activity with age. Panel c, shows the effect of subculturing on the activity of telomerase. The number of donors tested was 22 for panel a and b. 6 donors were used in the sub-culturing experiment (panel, c). No significance was detected between the age groups 20-69 and 70-90 (*p=0.744*). The subculture 1 had significantly more telomerase activity than sub-culture 3 (*p=0.010*).

3.3.6 Can clonal analysis be used to assess culture quality with regards to age?

Clonal analysis is a method of studying clones derived from the same single cell. Single cells were isolated by a dilution method and cultured in individual wells in a 96 well culture plate. Epithelial cells can form one of three types of colonies, holoclones, meroclones and paraclones, which can be indicative of the number of progenitors within the population (Barrandon and Green, 1987; Schlotzer-Schrehardt, 2013).

3.3.6.1 Aims of the clonal analysis study

An in depth study was performed on 3 donors, a 21 year old (meningitis), a 38 year old (septicaemia) and a 75 year old (heart failure). The main aim was to ascertain the clonal composition between donors of different ages, as previously shown in human keratinocyte stem cells (Barrandon and Green, 1987; Pellegrini *et al.*, 1999). Clonal composition can be an insightful marker of stem cell quantity and quality. The methods used below were also thought to be conducive to more accurate characterisation of each donor than analysing heterogeneous cultures. Morphological characterisation, CFE and immunofluorescence were used to define the clonal composition of each donor.

3.3.6.2 Colony forming assays of cultures from donors of 3 different ages

A defined number of cells (1,000) from each carefully isolated clone were seeded onto 35mm petri-dishes (effectively subculture 1). After 12 days the colonies were counted and measured. The average colony size (2.42mm +/- 2.72mm, approx. $4.5mm^2$) in the 38 year old (figure 3.16 & 3.18) donor was larger than that of the 21 (1.39mm +/- 1.39mm, approx. $1.49mm^2$) (figure 3.18 & 3.19) and 75 year olds (1.76mm +/- 1.13mm, approx. $2.4mm^2$) (figure 3.18 & 3.21). A Shapiro-Wilk test showed that the data was normally distributed, (*p>0.001*). One can be certain that this is not a false negative due to the high sample number (total colonies =1523). Kruskal-Wallis one way analysis of variance was used. This test produced a p value of *<0.001*. One can reject the null hypothesis that there is no difference in colony sizes between these donors. There was no difference between the 38 year old and the 75 year old donors (*p=0.425*) either. The 21 year old and 75 year old donors cells however did show a statistical difference in size (*p<0.001*). The average difference in colony size between 21 and 38 year old donors was also significant (*p<0.001*).
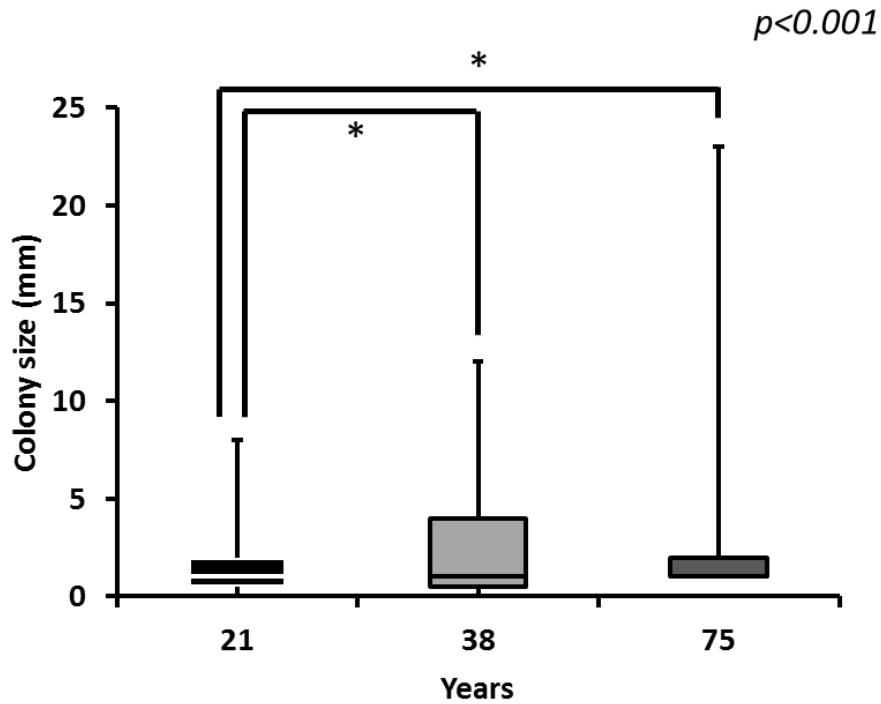
Figure 3.18 Average colony size of cells derived from a single clone isolated from cells of 3 different donors
The average size of the colonies generated by the 21 year old donor was 1.39mm from 177 colonies analysed. Whilst the size of the colonies from the 38 year old donor was 2.41mm from 72 colonies, the average colony size from the 75 year old donor was 1.75mm from a total of 1275 colonies. Units refer to the diameter of each colony. Significance is indicated with an asterisk.
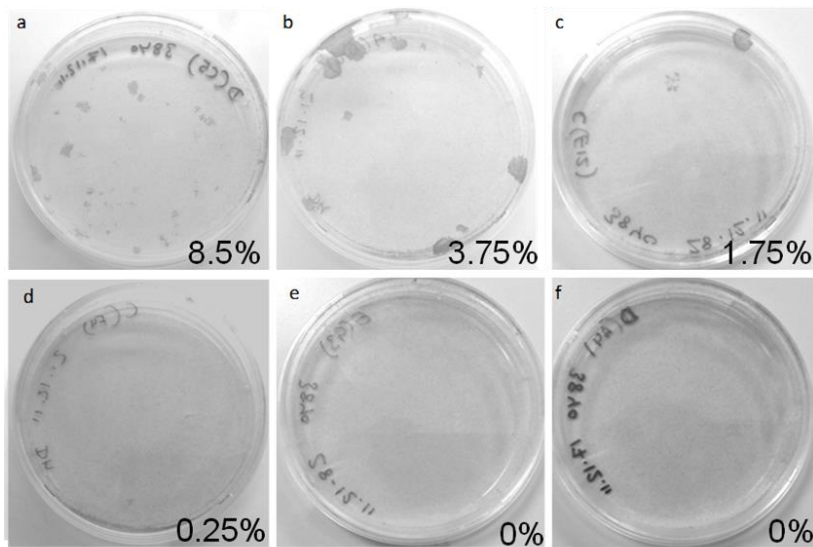
Figure 3.19 Examples of colony forming efficiency plates from individual isolated clones
6 CFE dishes taken at random from a total of 65 dishes analysed. A-C produced large smooth edged colonies consistent with holoclone and paraclones. D-F yielded few if any colonies, the edges of the colonies were crenated consistent with meroclone and paraclones.

3.3.6.3   Characterisation of colonies generated using the clonal isolation method.

Epithelial cells grown on a 3T3 feeder layer produce three subtly different colonies/clones, holoclones, meroclones and paraclones (Barrandon and Green, 1987) (Figure 3.20). The proportion of these different clones can be used to infer the greater or lesser number of initial stem cells isolated from that particular donor. Holoclones are characterised by circular smooth edged colonies which in epidermal cells are capable of up to 120 cell divisions with holoclones able to grow to macroscopic sizes. In the human epidermis and limbal epithelium these are considered to contain the highest proportion of stem cells (Barrandon, 1993; Mathor *et al.*, 1996; Dellambra *et al.*, 1998). Meroclones form more irregular shaped colonies possibly due to heterogeneity of the cellular composition. Meroclone proliferation is more limited compared to holoclones, and often produce smaller colonies (Barrandon and Green, 1987; Barrandon, 1993; Jones and Watt, 1993). Paraclones are thought to be constituted entirely from transit amplifying cells and produce highly irregular shaped colonies. During the growth of each clone/colony is it possible for clonal conversion to take place, holoclones to meroclone to paraclone, this process is irreversible. Clonal conversion is thought to occur under the either suboptimal growth conditions or in cells isolated from older donors (Lanza, 2004). A previous study has utilised the established technique of clonal analysis, to determine the location of progenitor cells on the ocular surface in humans (Pellegrini *et al.*, 1999), whereby, more holoclones were isolated from the superior and inferior regions than the nasal and temporal regions.

Holoclone Meroclone Paraclone
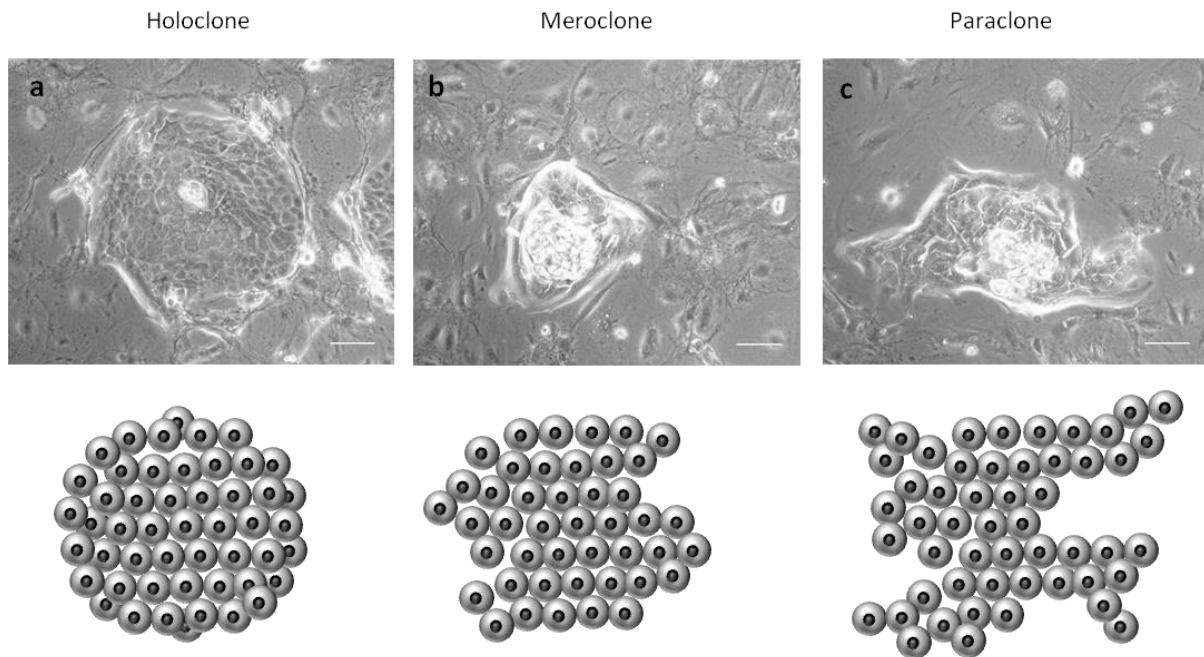
Figure 3.20 Brightfield images with diagrammatic representations of clonal types in epithelial cell culture
Three colonies form from cultured epithelial cells. They are the holoclone, meroclone and paraclone. Each one is comprised of cells at a different differentiated state. The holoclone (a) contains the highest proportion of progenitor cells and forms large regular shaped colonies. Meroclones (b) contain fewer progenitors than holoclones but more than paraclones. Meraoclones form more irregular shaped colonies than that of holoclones. Paraclones (c) contain the least number of progenitor cells of any clone type, forming highly irregular shaped colonies. Scale bar is 100µm.

The clonal composition determined by morphological analysis (using Barrandon and Green's parameters) of the 3 donors showed that the 38 year old donor had the largest proportion of holoclones compared to all other donors. In addition to this the 38 year old donor produced the least number of paraclones. Interestingly the 75 year old donor produced a larger proportion of paraclone colonies than that of the 21 year old. This observation suggests that older donors would produce colonies which contained less progenitor cells. As the data collected from this experiment was nominal and falls into three categories a Fishers exact test was used. Fishers exact tests are used to determine significance for categorical data, using contingency tables. A Chi-squared test would not work with data which has less than 10 observations. The p value for the 3x3 Fishers test was *>0.001*, analysing the 38 year old donor colony proportions proved to be problematic as there were very few numbers of paraclones compared to other donors. Performing a 2x2 test with only the 21 year old and the 75 year old, also showed significance (*P>0.01*) (figure 3.21).
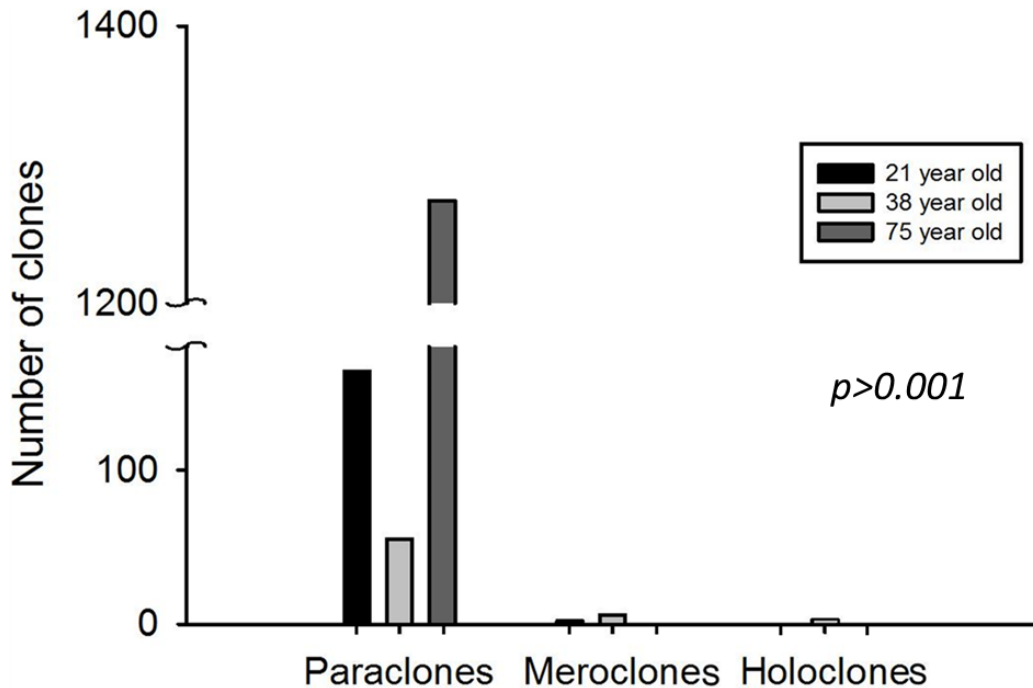
Figure 3.21 Morphological classification of different colony types generated from 3 different donors
Cells isolated from the 38 year old donor generated the highest number of holoclones and meroclones, compared to the other 2 donors. Their cells also produced the lowest number of paraclones. From the 21 year old donors cells more meroclones were produced and fewer paraclones, than from the 75 year old donors cells. Cells from the 75 year old donor did not produce any holoclones or meroclones, but did give rise to the largest number of paraclones out of all donors tested.

3.3.6.4   ΔNp63 immunofluorescence of clones from 3 different donors.

As stated previously ΔNp63 is a good marker of limbal progenitor cells (Pellegrini *et al.*, 2001). The relative quantity was measured in a selection of clones from each donor's cells. A Shapiro-Wilk test determined that the data was not normally distributed for figure 3.22. The data set also had unequal sample sizes, thus a non-parametric test was performed. A Kruskal-Wallis test on independent samples revealed that the difference between all donors was significant ($p<0.001$). The subsequent pairwise analysis also indicated significance between 75 year old vs 21 year old $p<0.001$, 75 year old vs 38 year old $p<0.001$ and 21 year old vs 38 year old $p<0.001$. This result allowed us to reject the null hypothesis that age has no effect on ΔNp63 aggregation in the context of clonal analysis. ΔNp63 was highest in the 38 year old donor which was statistically significant (Figure 3.23), this result correlates with the colony size and type of colonies observed in figures 3.18, 3.19 & 3.21. In the 75 year old donor there was little if any ΔNp63 immunofluorescence, which again correlates with an increase number of paraclones and decreased CFE compared to that of the 38 year old donor thus it seems that ΔNp63 protein level is the best marker which correlates with our

hypothesis that limbal epithelial cells derived from older donors have far less stem cells than younger donor ages (figure 3.22 & 3.23).
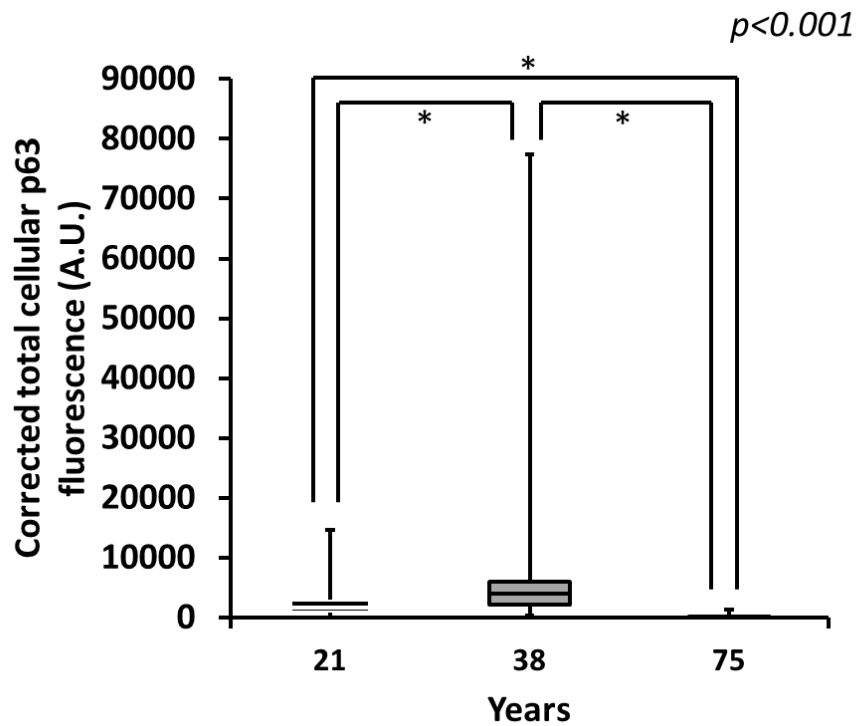
Figure 3.22 ΔNp63 immunofluorescence of colonies derived from 3 different donors quantified using total cellular fluorescence
The quantity of p63 as measured using an antibody which recognises ΔNp63. 107 nuclei were measured in cells from the 21 year old donor, 88 from the 75 year old and 98 from the 75 year old.
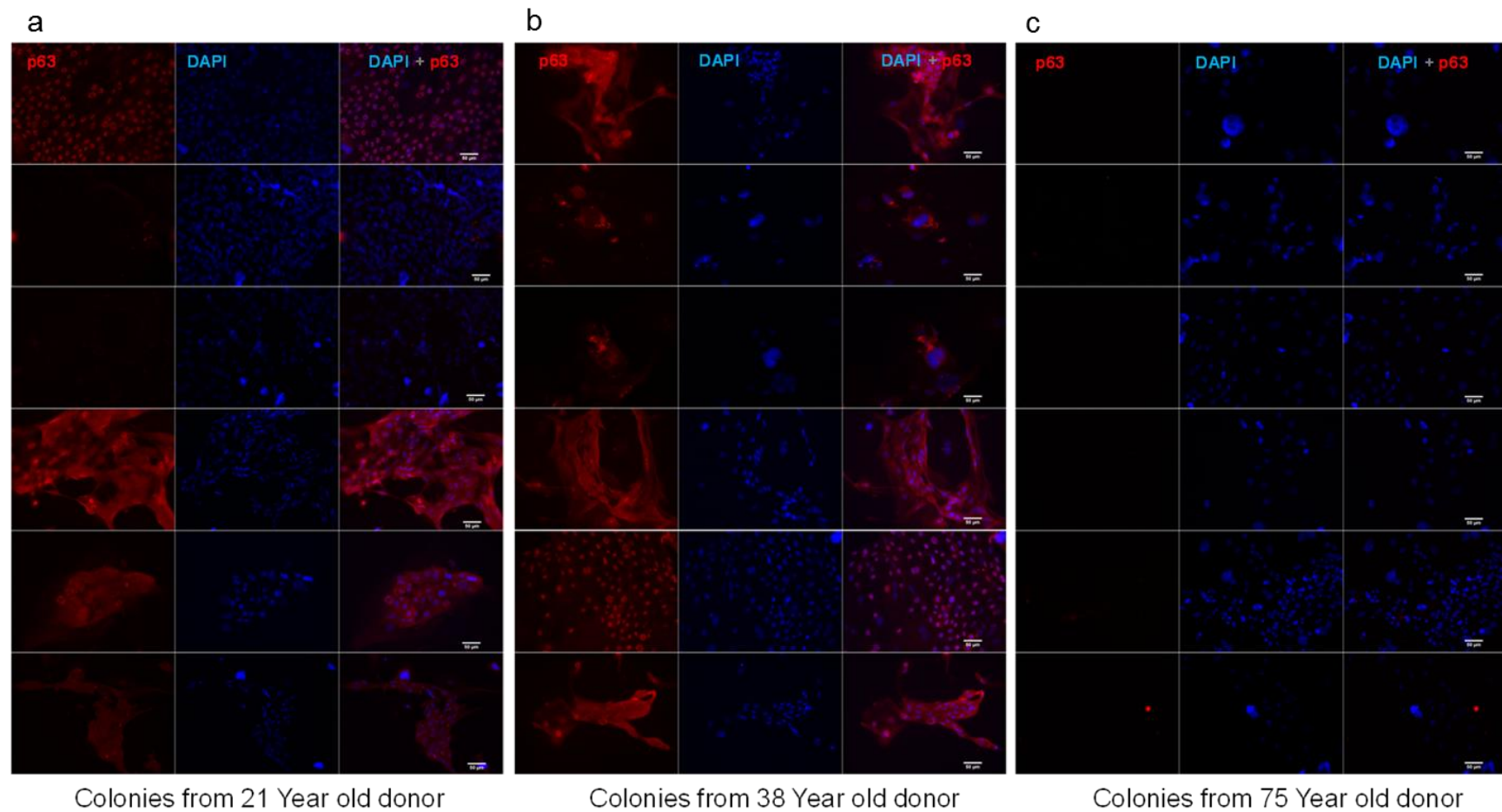
Figure 3.23 ΔNp63 immunofluorescence of cultured corneal clones
Panel a: p63 immunofluorescence in colonies derived from a 21 year old donor (201 nuclei, 6 example random field of view (FOV)). Panel b: colonies derived from the 38 year old donor (162 nuclei, 6 examples FOV). Panel c: colonies derived from a 75 year old donor (322 nuclei, 6 examples FOV). Staining intensity of p63 was more prominent in the 38 year old donor than that of the 21 and 75 year old donors when compared under identical staining and microscope exposure. Nuclear localisation was also particularly stronger in the 38 year old donor. Scale bar is 50 μm.

3.4   Discussion

3.4.1   Serial subculturing reduces the number of limbal epithelial cells.

Stem cell containing cultures were established from both young and old donors, using the serial trypsinisation method and cultivated on 3T3 fibroblasts. This study showed that stem cells could be isolated from donors regardless of their age. This is encouraging for the use of limbal epithelial cells as a cellular treatment for LSCD. The major implication of this observation is that more potential donor tissue can be utilised regardless of the age of the donor, whom might otherwise have been selected as unsuitable. In the light of research into the effects of age on other stem cells, such a hematopoietic, neural, muscle and melanocyte, which indicate a functional decline with age (Edwards *et al.*, 2002; Campisi, 2003; Pinto *et al.*, 2003; Keller, 2006; Flores *et al.*, 2008; Signer and Morrison, 2013), this information on limbal epithelial cells is especially important.

The serial cultivation of limbal epithelial cell containing cultures showed no statistically significant change in the amount of progenitor cells which could be derived from each culture, with the exception of subculture 3 in the older donors' category (Figure 3.3). One explanation for this decline after 2 subcultures would be that cells isolated from older donors are more likely to be closer to their replicative limit. *In vitro*, limbal epithelial cells are often grown in atmospheric oxygen conditions (21%) which could cause an oxidative stress response. Under oxidative stress telomeres have shown accelerated shortening (von Zglinicki *et al.*, 1995), partly because telomerase is shuttled to the mitochondria under stress and cannot maintain its canonical function of telomere maintenance (Ahmed *et al.*, 2008). This could explain the speed in which the decline in clonogenicity of the older donors falls away. As stated previously between the primary cultures one could not detect a difference in colony forming ability. These results contradict those stated by two another groups, whereby CFE analysis showed a decrease in colony number over the age of 60 years (Meyer-Blazejewska *et al.*, 2010; Notara *et al.*, 2013). As the sheer number of donors tested was significantly higher in these papers, one is inclined to favour the conclusion that age does have an effect on CFE.

It was also important to consider the time in which tissue pieces were stored in organ culture medium. This is something which the aforementioned papers did not

consider, it is not stated if there was any difference in the storage time of their primary tissue. One can only assume that the period was limited as they were locally sourced from Moorfields Eye Hospital Eye Bank. Our data however suggests that time in organ culture medium does not have any effect on the isolation of limbal epithelial cells, meaning that the trends observed by the previous papers are accurate.

Biologically it was also important to assess the type of colonies as well as sheer numbers, as one might assume that a high percentage of CFE has a direct proportionality to the number of progenitors. One way to characterise colony type is to measure its size, as smaller colonies are more indicative of terminating colonies such as paraclones, whereas holoclones tend to be larger. When comparing differences of both age, number of days in organ culture medium and subculture on the size of colonies, no statistical difference was found in either. This indicates that the type of colony does not change with age or serial subculturing. An interesting observation at subculture 3, was that only 2 donors survived to be processed for a third CFE experiment. The downside of this however is that it makes statistical analysis difficult to perform on such small sample sizes. In general there tended to be a significant difference between the size categories. This however only validates the test as one which can distinguish three types of colony. It would be sensible to include this data in addition to CFE data for future publications of the colony forming ability if limbal epithelial cells. In an ideal situation more donors may have aided in assuring that the trends which we observed were in-fact accurate representation of the overall population, in particular in the young (20-69) category. An age bias is always an issue when relying on donated tissue. It however can be argued that this test of quality indicates that highly prolific progenitors can still be isolated from tissue regardless of age.

As stated in the introduction stem cells and their progenitors are known to have a high proliferate potential (Boggs *et al.*, 1982). To assess this quality, population doubling time was measured between subcultures 1 and 2. Our study found that there was no effect on the time it takes for limbal epithelial cell containing cultures to undergo a doubling of a population. Additionally proliferation time was not affected by the length of time in storage medium. This seems to indicate that corneal cells do in-fact have a high proliferative potential which can be preserved in organ culture medium for long periods of time. This has an impact on the transportation methods which corneal tissue

is stored and transported in. One can conclude that existing methods are sufficient to maintain corneal stem cells within the corneoscleral disks.

The mRNA levels of the transcription factor *ΔNp63* were also studied. These levels indicated that *ΔNp63* did not change with age. These findings confirm those of Notara *et al* and the related study in skin (Carlson *et al.*, 2009; Notara *et al.*, 2013) as they showed no discernible difference with age. To confirm our qPCR data we observed ΔNp63 at the protein level using immunofluorescence. The results of which did in fact confirm our initial findings. One of the considerations however is that ΔNp63 is not confined to LSCs (Dua *et al.*, 2003), and more importantly antibodies available are unable to distinguish specific isoforms of p63, such as α, β & ɣ. The antibody used in our analysis recognises all three ΔNp63 isoforms, whereas the qPCR primers detect the more specific *ΔNp63α* isoform found in LSCs. It was the intention to also compare the time in which each donor was kept in organ culture medium, there were however too few donors in the 0-29 day category. Even with these differences the qPCR data and that of the IF tended to correlate with one another, both were unable to show any effect with age when cells are cultivated on 3T3 cells.

Another parameter which was assessed in donor cells of different ages was telomerase activity. No correlation with age could be found however, similar to the results shown by Notara and colleagues (Notara *et al.*, 2013). It was hypothesised that telomerase activity maybe a signature of limbal epithelial cells. In the gut, telomerase activity is most prevalent in the crypts, which contain the intestinal epithelial progenitor cells (Bachor *et al.*, 1999). The epidermis which has similar properties to the cornea, high telomerase expression in basal cell layer is observed, while more superficial and differentiated cells have less (Harle-Bachor and Boukamp, 1996). This study confirmed that telomerase is expressed in limbal epithelial cell containing cultures (Notara *et al.*, 2013), boding to the presence of progenitors within them.

The subculture of limbal epithelial cells did however show a decrease in telomerase activity. This suggests that the current culture conditions for limbal epithelial cells could be suboptimal for the maintenance of progenitors over the long term. The topic of which is covered in Chapter 5.

3.4.2 Clonal analysis is a good tool for assessing the proportion of progenitors in individual donors

An in-depth study, focusing on the clonal composition was performed on cells isolated from three human donor tissues. Using clonal analysis allows us to develop a more detailed understanding of the composition of each donors cells. The potential proportion of progenitors originating from those tissues was assessed, similar to the traditional analysis. However rather than analysing total cell populations, single cells were isolated and the individual clonal units were assessed. The advantages of clonal analysis over traditional methods are that it can aid in distinguishing the proportions of short lived cellular populations from longer lived stem cell containing colonies. Thus it allows for the selection of true stem cell populations.

A general trend was observed in the donors across multiple tests, which was that the 38 year old donor had the highest number of progenitors. The largest clones originated from the 38 year old donor, ΔNp63 protein also tended to accumulate more in the cells of this donor. In addition, it was observed that this donor had the highest proportion of holoclones and meroclones. Throughout the clonal analysis experiments generally the cells from the 75 year old donor performed the poorest, producing the greatest number of paraclones more than those of the other two donors combined. Looking carefully at Notara *et al.,* 2013 paper, it was noted that the data from these donors mirrors that of the groups which the authors penned (0-30, 30-60, 60-90). Our ΔNp63 immunofluorescence shown in figure 3.20, correlates with the observed qPCR data for *ΔNp63α* in figure 10 of that particular paper. The obvious difference being that the immunofluorescence data from this study was significant while the qPCR was not.

The size of the colonies in the 75 year old donor tended to be larger than those of the 21 year old donor. This contradicts a previous study which stated that colony size decreased with age from 8mm in diameter in young donors to 4mm in older donors (Pellegrini *et al.,* 1999; Meyer-Blazejewska *et al.,* 2010). One explanation of this data would be that clonal conversion might be occurring. It has been shown that holoclones can convert into meroclones, and that meroclones also convert to paraclones, in an irreversible manner. It is plausible that meroclones in the 75 year old donor were more sensitive to the stresses of culturing and after a short period, the remaining progenitor cells within these colonies underwent symmetric division into transit amplifying (TA) cells, producing the apparent increase in the number of colonies. This however was pure

speculation. Another theory would be that as one ages, epithelial cells become more sensitive to epithelial to mesenchymal transition (EMT). It has been shown that intracellular oxidative stress can trigger EMT in some epithelial cells (Smit and Peeper, 2010; Milara *et al.*, 2013). If one assumes that oxidative stress increases with age (Hamilton *et al.*, 2000; Donato *et al.*, 2007), one can infer that EMT might also increase. The result on epithelial cultures would be that the older donor was more probable to produce fibroblastic growths. These fibrotic growths could artificially increase the size of the colonies. This is why colony characterisation is an important parameter to consider when assessing human limbal epithelial cell containing cultures. Previously in this chapter, we showed that older donors are more sensitive to subculturing and rarely produce colonies after just 3 subcultures. Indicating a loss of clonogenicity. One then categorised the colonies according to their morphology. This painstaking process assigned each colony into the groups' holoclones, meroclones and paraclones. Tallying the colonies like this allowed us to verify the colony size data previously collected. As a result we observed that there were more holoclones and meroclones in the 38 year old donor which correlates to the colony size data. This is important because assigning labels to colonies can be quite subjective. Confirming that these assignments correlate to quantitative data was very important. Immunofluorescence was employed to probe a selection of clones from each donor with an antibody raised against the ΔNp63 isoforms (figure 3.22 & 3.23). It correlated to the two previous experiments showing that the 38 year old donor contained more progenitors/transit amplifying cells. It can be concluded that using these three techniques identified the 38 year old donor as containing the greatest number of progenitors, while the 75 year old contained the least. There are several paradigms which one can draw from these observations. Firstly, there does not seem to be a linear decline in stem cell function with age. Secondly, there is substance behind the theory that stem cells increase in number before declining. One definite conclusion that can be ascertained is that there is a difference in the stem cells isolated from different people, and that these behave differently when cultured in the laboratory. Ideally it would have been beneficial to have been able to apply the clonal analysis method to more donor tissues, to show if these observations are an accurate representation of the population. What causes these variations is multifactorial with

some examples being, genetic background, acquired diseases, cause of death and environment.

3.4.3   The effects of the cause of donor death on limbal epithelial cells

The 21 year old and the 38 year old donors studied in the clonal analysis experiments both died from systemic infections, which could have affected the eye. The 75 year old donor however died from heart failure which in no way affects the eye, apart from slight fluid retention. The severity of the infection could have had a detrimental effect on the 21 year old and 38 year old which could in turn have impacted upon their stem cells. For example the reduced p63 expression in the 21 year old may have arisen from severe meningitis compared to perhaps a less severe case of sepsis in the 38 year old donor. This is only speculation as the information regarding existing conditions and disease progression was not available.

In the main study, the main cause of death was infection, notably pneumonia, currently there is no evidence which suggest that this condition has any effects on limbal epithelial cells. Cancer was the second most probable cause of death, treatments for cancer such as chemotherapy target rapidly dividing cells, as limbal epithelial stem cells divide infrequently it is unlikely that these drugs would have a direct effect on limbal stem cells. TACs maybe targeted however which could impact the limbal niche indirectly effecting limbal epithelial cells. Systemic treatment with hydroxycarbamide has been shown to cause LSCD (Ding *et al.*, 2009). In this study the cells from 12 donor's cells were able to be cultured while only 4 were not. As these donors cells were divided between various different experiments it is difficult to say if cancer treatments had a detrimental effect.

3.5   Conclusions

3.5.1   Does age have an influence on the culture of limbal epithelial cells?

This study concluded that there was no overall difference between the two age groups defined using the parameters tested, with the exception of the clonal analysis results. These results however were not completely unexpected as others have found similar results in this specific tissue (Kim *et al.*, 2004; Meyer-Blazejewska *et al.*, 2010; Notara *et al.*, 2013). It was observed that colony forming decreases with successive subculturing, regardless of the age of the donor or the time the donor tissue was in organ culture

medium (Figure 3.2 & 3.3). This highlights one of two things. Firstly less stem cells survive over multiple passages, so serial propagation would be detrimental to corneal stem cell transplantations. Transplantations however, are currently only performed with primary cells grown from explanted tissues. Secondly it shows us that the cultivation methods are not yet fully optimised for the successive cultivation of limbal epithelial cells. Colony forming assays also indicated that older donors produced around the same number of colonies, but that these terminate past subculture 3. In terms of limbal epithelial cell transplantation this may mean that transplanted cells may only have a limited capacity to regenerate the cornea. Providing a short term benefit, but causing complications later on for patients, from the literature it is known that cellular stress can lead to the shortening of telomeres. Culture stress could then lead to premature reduction in telomere length, which could result in a more limited proliferative lifespan of those particular cells. In longitudinal studies patients were on average followed up for only 21 (+/-15) months after limbal stem cell transplantations (Pellegrini *et al.*, 1997; Schwab, 1999; Schwab *et al.*, 2000; Tsai *et al.*, 2000; Koizumi *et al.*, 2001; Rama *et al.*, 2001; Grueterich *et al.*, 2002; Nakamura *et al.*, 2003; Sangwan *et al.*, 2003; Nakamura *et al.*, 2004; Daya *et al.*, 2005; Sangwan *et al.*, 2005; Nakamura *et al.*, 2006; Sangwan *et al.*, 2006; Ang *et al.*, 2007; Fatima *et al.*, 2007; Kawashima *et al.*, 2007; Shimazaki *et al.*, 2007; Shortt *et al.*, 2008; Di Girolamo *et al.*, 2009; Meller *et al.*, 2009; Baradaran-Rafii *et al.*, 2010; Di Iorio *et al.*, 2010; Gisoldi *et al.*, 2010; Kolli *et al.*, 2010; Pauklin *et al.*, 2010; Rama *et al.*, 2010). It is not reported what occurs after this time. A counter argument which could be made is that donor cells are not detectable after 9 months (Daya *et al.*, 2005), implying that donor cells are possibly only required to restore the niche.

Figures 3.3-3.13 and 3.15 illustrate that variation between donors is large, high standard deviations can be seen in most of the graphs depicted in this chapter. This increase in biological heterogeneity with age poses a serious problem when performing statistical analysis. In order to achieve an accurate proportion of the population a large number of donors would be required, donor tissue for research is however limited. On average 3 donor tissues were available to this study to be processed per month. As mentioned previously, this study incorporated just under 100 donors for the 3 age groups while, in a recently published paper by Notara and colleagues, over 100 donors were available per each group, due the proximity to Moorfields eye hospital (Notara *et*

al., 2013). Even with these superior donor numbers their study still failed to detect a significant difference between young and old donors in the context of corneal markers. In studies where limbal explants were cultured on either a 3T3 feeder layer or on amniotic membranes, no discernible difference was detected with age (Kim *et al.*, 2004; Shanmuganathan *et al.*, 2006; Baylis *et al.*, 2013). In the Baylis study, however the youngest donor was only 42 years old, leaving a large proportion of the population omitted. In our study the youngest donor was 20 years old while the oldest was 90. The lack of a statistical trend in all of these independent studies indicates that natural variation far outweighs any possible change in the parameters measured in this study and studies by other research groups. Unlike inbred model organisms which nurtured in controlled conditions there are a number of factors which could impact on the quality of human limbal epithelial cells. First of all is the period between death and removal of the eye (enucleation), secondly the time that tissue pieces are stored in organ culture medium. Several other papers have shown that the delay between death and culturing can have detrimental effects on cellular function (Sorensen, 1975; Breslin and Ng, 1976), however techniques have advanced in the last 40 years. Focusing on the later parameter, the time in organ culture medium has the potential to be remediated. Time from death to enculeation is effected by many other factors, such as nurse availability and consultation to confirm consent. This study indicated, however that the time an corneoscleral tissue is in organ culture medium does generally not have a detrimental effect on the number of progenitors cells which can be extracted from it. This included their proliferative potential. Our results were confirmed by a similar study, culturing limbal explants on amniotic membranes (Baylis *et al.*, 2013). Baylis *et al* showed that time in organ culture medium had little, if no effect on the outgrowth rate of limbal explants. From this it was concluded that limbal epithelial cells are not affected by long periods in organ culture medium.

3.5.2 Clonal analysis could be used to assess the effects of age on limbal epithelial cells

It is difficult to make firm conclusions from the clonal analysis experiments. One can mostly only speculate biased on a continuing trend emerging. The primary culture derived from the 38 year old donor contained a higher proportion of holoclones than the younger and older donors tested. 21 and 75 year old donor tissue did yield a small

number of meroclones, with all cultures producing high numbers of paraclones. This means that most cultures contain a lot of already differentiating progenitor cells but only a minor fraction of "true", highly clonogenic stem cells. Interestingly these results were confirmed by another group (Notara *et al.*, 2013). Their results however were not significant, but they did show a tendency of the middle aged donors to have higher *ΔNp63α*, *TERT* and *ABCG2* expression. If more donors could have been processed into the clonal analysis experiments it may have discerned if this method was sensitive enough to detect any age related changes.

One theory which could explain the trends seen here, suggests that stem cell number increases with age (deHaan *et al.*, 1997; Brack and Rando, 2007), however the quality of these cells decreases, ergo quantity over quality (Cho *et al.*, 2008; Dykstra *et al.*, 2011), but then decline in number steadily with age. The effects of such changes are debated, it would be logical to assume that changes to the clonal composition decrease the functionality of the tissue. In intestinal stem cells however, clonal drift has no deleterious effect (Lopez-Garcia *et al.*, 2010; Snippert *et al.*, 2010).

One fact that the data did confirm was that human limbal epithelial cells can still be extracted from donors long after their death and be stored in organ culture medium for around 2 months, as also shown by another group under similar conditions (maximally 141 hours post mortem) (Slettedal *et al.*, 2007). This study shows that the cornea is a highly robust tissue and contains a tremendous regenerative capacity. To confirm and further the results attained in human donors, mouse corneas were used to assess the effects of age *in situ* forgoing tissue culture related artefacts.

Chapter 4 The effect of age, DR and rapamycin treatment on the mouse cornea

4.0 Introduction

There was a high degree of variation as well as an unavoidable age bias, preferential to older donors, when observing age parameters in human donor cells. A mouse model (*Tert* $^{+/+}$C57BL/6) was used in addition to human donor tissue, in order to identify if there was also age related changes in the mouse cornea and without intruding culturing related artefacts. One of the benefits of using a mouse model was their short life span and ability to initiate interventions. The mouse cornea is similar to the human cornea, with some subtle differences. The thickness of the central cornea is thinner in the mouse (100μm in C57BL/6J) (Lively *et al.*, 2010) than in humans (0.55mm). The proportion of epithelia to stroma is also slightly different, with 30% epithelial to 70% stroma in mice, humans on the other hand have a proportion of 10% to 90% (Henriksson *et al.*, 2009). Like in humans similarly in mice the origin of progenitor cells is debated. There are two main theories, one suggests that the cells are limbal in origin, (LESC) (Schermer *et al.*, 1986; Cotsarelis *et al.*, 1989; Lehrer *et al.*, 1998; Li *et al.*, 2007; Hayashi *et al.*, 2012). The alternative hypothesis suggests that the cells are corneal in origin, with the limbus involved only during wound healing (CESC) (Majo *et al.*, 2008).

The mouse is a good model of corneal ageing, as it has previously been shown that it is affected by age (Faragher *et al.*, 1997). Descemet's membrane (as described in the introduction) thickness increases with age (Jun *et al.*, 2006), its opacity increases (opacification) and corneal excrescences (guttae) increase (Hillenaar *et al.*, 2012). Most studies have focused on the corneal endothelium or stroma, very little is known about what occurs biochemically within the corneal epithelium with age. Even though there are many benefits of using mouse models as described briefly above there are also some disadvantages. The limbus is not as defined in mice as in humans, anatomically this region is a very slight indentation into the cornea and can be difficult to identify in some animals. For this reason, the region will be referred to as the peripheral cornea, as this is a more accurate description of the area which was measured.

4.0.1 Consequences of dietary restriction on epithelial cells in the cornea of mice

Dietary restriction (DR) is a non-genetic intervention which can increase life span (Mccay *et al.*, 1989). More accurately, caloric restriction was used in this study as micronutrients and vitamins were maintained at a normal level. Supplying adequate nutrition avoids causing malnutrition in the animals which is detrimental (Wiggins, 1995). In our study, total calorie intake was decreased by 26% in a cohort of mice (largely C57BL/6). This particular strain of mice (B6.129S) has been shown to live around 28 ±0.61 months when fed *ad libitum* (AL, Latin for "at one's pleasure). When calorie restricted however the maximum lifespan was greater than 37 months. The medium lifespan increased by 32% in the calorie restricted group (Cameron *et al.*, 2012). Various mechanisms such as down regulation of insulin/IGF-1, sirtuin and mTOR are thought to play an important role in DR.

4.0.2 The relationship between mTOR and dietary restriction

mTOR is mediated by nutrient levels, hormones and growth factors. mTOR complexes can activate many downstream genes including those to up regulate glycolysis (Schieke *et al.*, 2006) and suppress autophagy (Hands *et al.*, 2009). mTOR can form two protein complexes, mTORC1 and mTORC2. mTORC1 is comprised of mTOR, Raptor, MLST8, PRAS40 and DEPTOR. mTORC2 on the other hand is comprised of mTOR, RICTOR, GβL and mSIN1. mTORC1 controls protein synthesis and redox signalling, through the activation of S6 kinase and eIF4-BP (Caron *et al.*, 2010). The complex is mainly reactive to nutrient levels, growth factors and oxidative stress. In haematopoietic cells, up-regulation of the mTOR pathway leads to increase in the CDK genes *p16*, *p19* and *p21* (Chen *et al.*, 2009; Lee *et al.*, 2010a; Kalaitzidis *et al.*, 2012). Conversely it has also been shown that mTORC1 causes an increase in the proliferation of neural progenitors (Groszer *et al.*, 2001) and HSCs (Gan *et al.*, 2008). One of the roles of mTORC2's is to phosphorylate Akt/PKB (Guertin *et al.*, 2006), Akt can inhibit apoptosis, via subsequent phosphorylation of Bad (Kim *et al.*, 2001). Akt is also implicated in the glucose transport system (Sarbassov *et al.*, 2005) and downstream of PI3K mediated insulin signalling pathway (White, 2003). mTOR can be a major hub through which nutrient signals can impact upon cellular physiology.

4.0.3   Rapamycin vs dietary restriction

Rapamycin is an immunosuppressant drug isolated from streptomycete AY B-994 (Baker *et al.*, 1978) from Easter island . Rapamycin binds to FK-binding protein 12, the rapamycin/FKBP12 complex inhibits mTORC1. Inhibition of mTORC1 in this manner increases life span in mice (Harrison *et al.*, 2009). Although it is possible that dietary restriction acts through this pathway, some studies have shown a divergence in results when comparing rapamycin treatment to DR. Firstly, DR reduces body fat, while rapamycin treatment does not (Fok *et al.*, 2013). Second, DR increases insulin sensitivity, while rapamycin treatment decreases insulin sensitivity (Fraenkel *et al.*, 2008). Oxidative stress is reduced in DR (Gredilla and Barja, 2005), while in rapamycin treated mice no reduction was seen (Fok *et al.*, 2013). It has been suggested that DR may reduce oxidative stress via SIRT3 and in turn decrease the amount of insulin growth factor within the cell (Desbois-Mouthon *et al.*, 2001; Qiu *et al.*, 2010). The involvement of insulin growth factor and NAD-dependent deacetylase sirtuin-3 links both systems and offers a possible shared mechanism.

4.0.4   DR and ageing in stem cells

The ageing of stem cells can be slowed by DR demonstrated in neural progenitors (Lee *et al.*, 2000), HSCs (Chen *et al.*, 2003), satellite (Cerletti *et al.*, 2012) and germline cells (Mair *et al.*, 2010) measured via increased number and/or function. More efficient oxygen metabolism and increased mitochondrial number were found in satellite cells (Cerletti *et al.*, 2012). It is debated if DR is mediated solely by the mTOR pathway or if two separate mechanisms are at work. In favour of a multifaceted system was that several studies have demonstrated an equally significant extension to lifespan via different mechanisms (Kaeberlein *et al.*, 2005; Hansen *et al.*, 2007; Kaeberlein and Kennedy, 2009). DR is a global process which acts upon multiple pathways resulting in lifespan extension, potentially via improved survival of stem cells with age.

4.0.5   DNA damage and ageing

The cornea is almost continuously exposed to external stimuli which can cause DNA damage, such as UV radiation. DNA damage, particularly double strand breaks, can initiate a halt to the cycle, holding it in G_0 until the damage is resolved. The conversion from proliferative arrest to senescence can make this pause in the cell cycle permanent, causing the cell to enter into a senescent state. During this state the cell is still

metabolically active, however it can no longer divide. An accumulation of senescent cells within a tissue can impair its function and leads to reduced regenerative capacity. When DNA damage occurs, arrays of proteins are recruited to the damage site, such as ATM/ATR, which both phosphorylate H2A.X and then becomes ɣH2A.X. ɣH2A.X was used to assess DNA damage levels within the mouse system as it is an indicative marker of senescence. While many foci can be repaired, others can be more persistent (Nakamura *et al.*, 2010).

In pre senescent cells the prevention of the recognition of telomeres as DNA damage sites is performed by the telomeric structures and complexes such as the t-loop, D-loop and shelterin. Erosion of the telomeres can lead to recognition of the ends of chromosomes as DNA damage sites and are thought to lead to cell cycle arrest (di Fagagna *et al.*, 2003; Takai *et al.*, 2003). We used a fluorescently labelled antibody to localise ɣH2A.X to the telomeres to detect damage recognition in these areas. Telomeric sequences were detected using a fluorescently bound peptide nucleic acid probe.

4.1 Methods

Mouse eyes were enucleated immediately after death and placed either in 4% paraformaldehyde or OCT. Eyes were then sectioned to 10 μm slices and stored in applicable conditions. Haematoxylin and eosin staining was used to assess morphological parameters, while fluorescence microscopy was used to assess protein levels and localisation. Additionally, the used of a peptide nucleic acid probe was used to detect telomeric DNA.

4.2 Aims

The main aim of this chapter was to assess any age related changes to the corneal epithelium which included studying ɣH2A.X and TAp63 (see 1.1.2.1). A secondary aim was to examine if interventions such as dietary restriction and rapamycin treatment could benefit the corneal epithelium with age. The role of *Tert* in the cornea was also studied using *Tert*$^{-/-}$ mice, as its expression is present in stem cells and TACs while also being an important age related gene.

4.3 Results

4.3.1 Does the thickness of the corneal epithelium change with age and can dietary restriction or other interventions ameliorate this?

The thickness of Descemet's membrane has been shown to increase with age (Johnson *et al.*, 1982). This study measured corneal epithelial thickness to assess if there was any change with age, which may signify the rate of cell replenishment. DR and rapamycin treatments were also tested to assess if any changes which occurred with age could be remediated. Measurements were taken using imageJ on cryopreserved specimens to preserve their structure. The drying stages associated with the processing of paraffin embedded tissue can shrink the cornea, resulting in a thinner cornea. All measurements were taken along the same meridian of each individual cornea. The average size of the central cornea was 22μm ±4, while the peripheral cornea was thinner at 13μm ±1.6. This was much thinner than previously reported values, which puts the central corneal epithelium between 32-51μm and peripheral corneal epithelium at 25μm (Henriksson *et al.*, 2009; Lively *et al.*, 2010) in C57BL/6 mice. The ratio of central to peripheral is roughly half that previously reported. This is probably attributed to differences in fixation methods, Henriksson and colleagues used the fixative formula 2% glutaraldehyde in 80 mM sodium cacodylate on whole mouse globes. One of the methods which Lively and colleagues used was pachymetery (Lively *et al.*, 2010). Pachymetery is a method by which ultrasound is used to measure the thickness of the cornea *in vivo* based on the dispersion of sound waves. Our study used OCT for embedding of the eyes and cryosectioning to preserve their structure, fixation was only performed after transferring of the sections to glass slides. Another reason for the differences could be due to variation between the different mouse substrains.

4.3.1.1 Observing corneal thickness in old, young, DR, rapamycin and control mice

The central corneal region in young and DR and the peripheral cornea in old and DR mice were not normally distributed (Appendix B, table 7.32). As a result, non-parametric tests were used for comparing groups. Kruskal-Wallis tests on independent samples showed that the difference was significant between the categories of central corneal ($p<0.001$) (table 4.1). A separate test on the peripheral corneal showed that those categories were also significant ($p<0.001$). The results of the significance tests are summarised in table 4.2. The results are shown in figures 4.1 and 4.2. Comparing peripheral and central

regions within each group with related samples Wilcoxon signed rank test showed that the central cornea was significantly thicker than the peripheral region ($p<0.001$). This result was expected as previous studies have also noted this morphological trend (Henriksson *et al.*, 2009).

Sample /Condition	Wilcoxon signed ranks for independent samples (p values)
Rapamycin treated vs rapamycin control	0.002
DR vs old/AL	1.0
Old/AL vs young	1.0

Table 4.1 Statistical analysis of central corneal thickness, compared between old, young, dietary restricted, rapamycin treated mice and controls.

Sample /Condition	Kruskal-Wallis test for independent samples (p values)
Old/AL vs young	0.001
Rapamycin treated vs rapamycin control	0.003
Old/AL vs DR	1.0

Table 4.2 Statistical analysis of peripheral corneal thickness, between old, young, dietary restricted, mice rapamycin treated and controls.
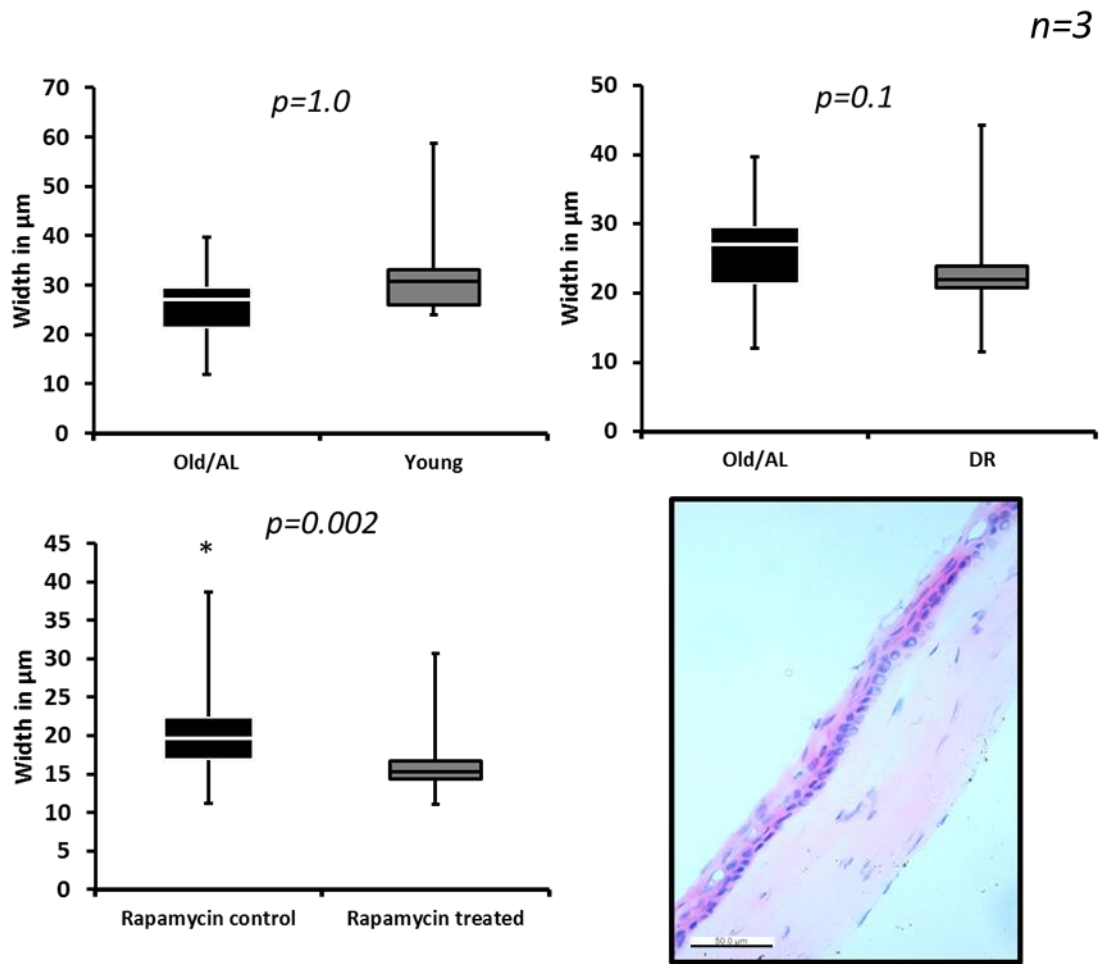
Figure 4.1 The width of the central mouse cornea at old age, under dietary restriction and rapamycin treatment. These box plots show the width of the central cornea in μm, comparing central regions .The width of the cornea was measured using ImageJ. Three measurements were taken on each image totalling 474 measurements. The cornea is dark pink in colour compared to the surrounding tissue (H&E staining). Five images were taken per mouse. At least three mice were tested in each condition. Old/AL and DR mice were 22 months old, young were 3 months old. Rapamycin treated mice were 16 months old and were treated for four months, starting at 12 months of age. Dietary restriction was implemented for a period of 16 months from 6 months of age. Independent samples Kruskal-Wallis tests for independent are shown in each graph. Significance is indicated with a asterisk**.** The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Scale bar is 50μm.
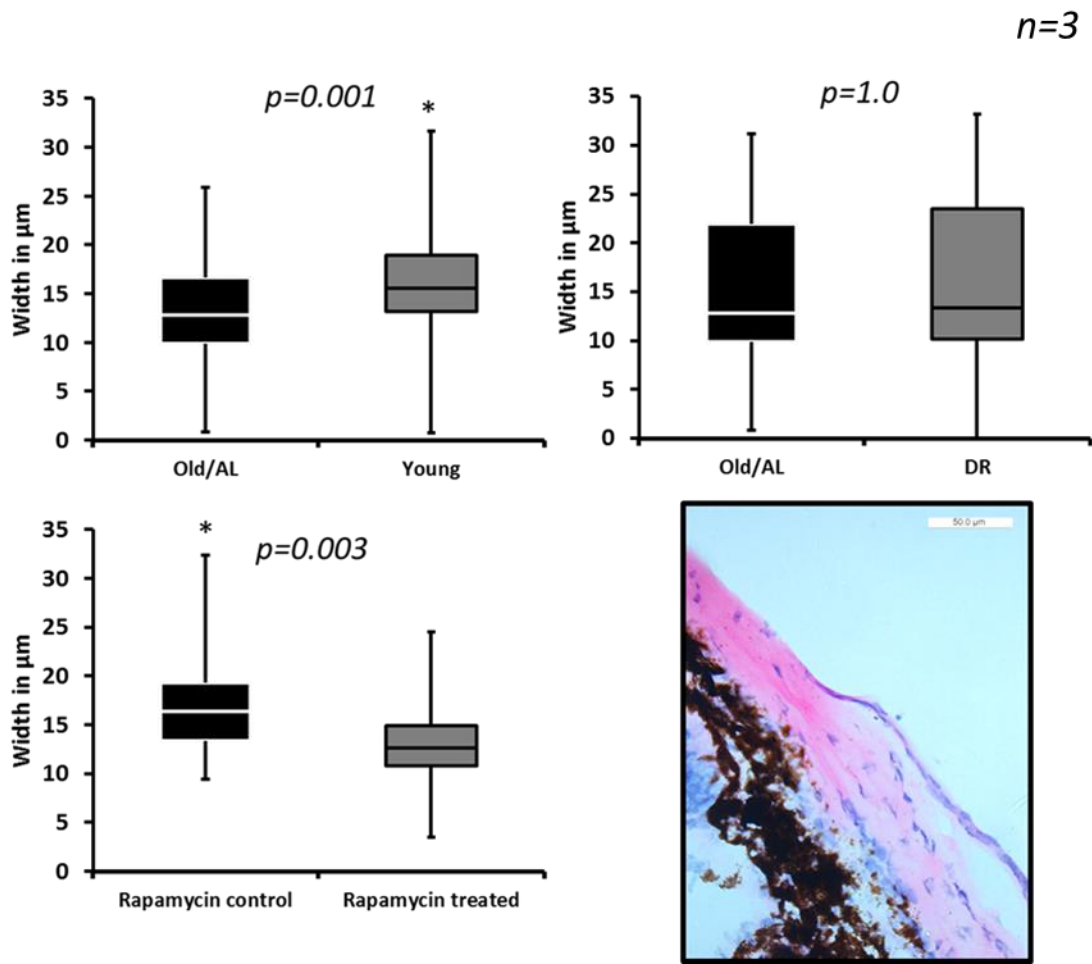
Figure 4.2 The width of the peripheral mouse cornea at old age, under dietary restriction and rapamycin treatment.

These box plots show the width of the peripheral cornea in μm, comparing peripheral regions .The width of the cornea was measured using ImageJ. The corneal epithelium is dark pink in colour compared to the surrounding tissue, nuclei are stained purple by haematoxylin. Three measurements were taken from each image. Ten images were taken per mouse, five for each peripheral region. At least three mice were tested per condition. P-values for Kruskal-Wallis test on independent samples are shown in each graph. Significance is indicated with an asterisk. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Scale bar is 50μm.

Sample	Wilcoxon signed ranks on related samples (p values)
Old central vs Old peripheral	<0.001
Young central vs Young peripheral	<0.001
Dietary restricted central vs Dietary restricted peripheral	<0.001
Rapamycin control central vs Rapamycin control peripheral	<0.001
Rapamycin treated central vs Rapamycin treated peripheral	0.001

Table 4.3 Statistical analysis for the comparison between central and peripheral corneal thickness in old, young, dietary restricted, and rapamycin treated mice and controls.

Rapamycin treatment seemed to reduce the thickness of the mouse cornea, both peripherally and centrally, suggesting that the mechanism for this morphological change could be controlled by mTOR (figure 4.1 & 2). The decrease in the thickness of the cornea under rapamycin treatment could be due to slowed cellular proliferation rate. Thus, rapamycin treatment might have an impact on the corneal replenishment rate. The corneal epithelium is comprised of several types of collagen, DR has been implicated in accelerating a decrease in the formation of collagen (Reiser, 1994). Although in this instance DR did not appear to alter the thickness of the corneal epithelium. It was expected that the thickness of the cornea might increase in older mice as previously shown (Lively *et al.*, 2010). Our results contradict the findings of Lively and colleagues', since we found that the corneal epithelium decreased in thickness in the central cornea, although not significantly. In the peripheral cornea there was however a significant decrease in the thickness of the cornea between young and old mice (table 4.3). This decrease could be due to a reduced number of progenitors in this region with age. The central region on the other hand may not show any change due other factors which result in the overall thickness increasing. Reasons for the increase could be due to decreased solute transport out of the cornea by the endothelial cells. An alternative hypothesis is that linage skewing could be producing cells which have larger cell size resulting in overall increases in the central region, this is most visible in the central

region as cells actively migrate to this region. These hypotheses are however speculative.

4.3.1.2 Corneal thickness of *Tert*^{-/-} and wild type mice fed *ad libitum* or dietary restricted

Telomerase plays a vital role in the maintenance of stem cells. The maintenance of the telomeres in these long lived cells is crucial to maintaining a normal pool of stem cells. Telomerase has numerous non-canonical functions which are also important for stem cell maintenance. For example, tissue specific over expression of *Tert* in the dermis of *Terc*^{-/-} mice caused hair follicles to be stimulated into anagen phase, a process which is controlled by stem cells in the hair bulge (Sarin *et al.*, 2005).

Our study used the *Tert*^{-/-} mice generated and used by Chiang and colleagues, to assess the involvement of *Tert* in the mouse cornea (Chiang *et al.*, 2004). In order to also measure the effect of DR on these mice, a subset of animals were kept on a restricted diet together with their wild type littermates.

As previously, the width of the cornea was measured using cryo sectioned eye sections through the median of each eye in wild type and *Tert*^{-/-} mice fed either *ad libitum* or restricted diets. Before deciding which statistical test to perform on the data a Shapiro Wilk test was performed, it showed that the majority of the data was not normally distributed (see appendices B). As a result, an independent samples Kruskal-Willis test was performed when comparing between sample groups. Within groups a Wilcoxon signed rank test showed that the central cornea was significantly thicker than the peripheral region (*p<0.001*). Biologically this is not surprising and was expected, as the peripheral regions are comprised of fewer cells, as shown by the literature (Henriksson *et al.*, 2009).
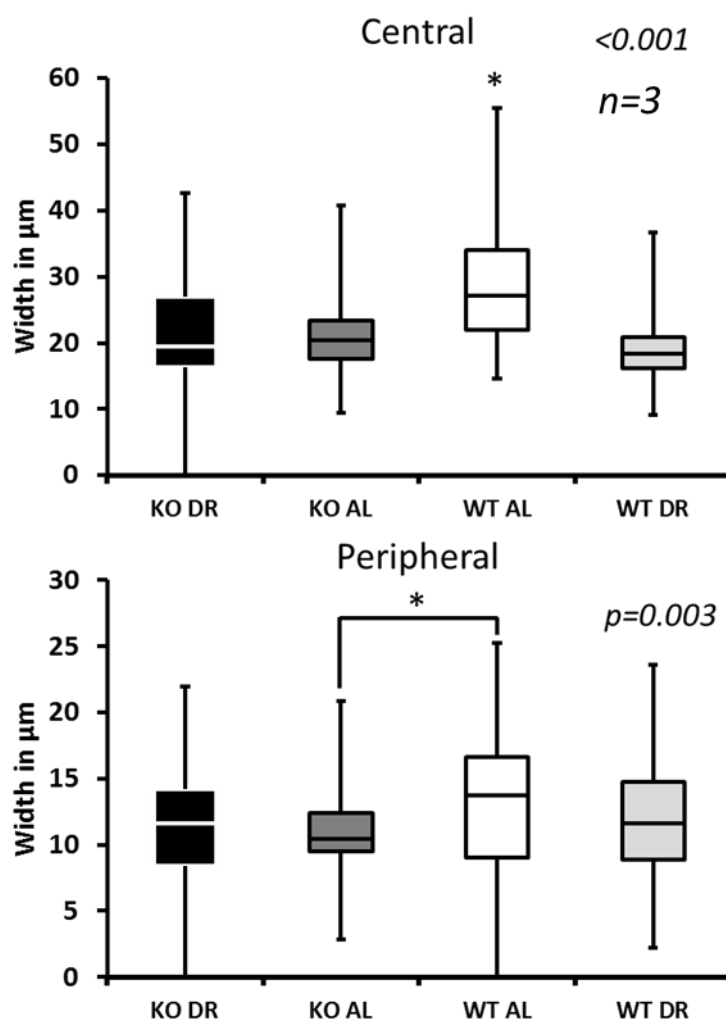
Figure 4.3 The width of the cornea in *Tert*^{-/-} and wild type mice under *ad libitum* and under dietary restriction. A total of 628 observations were made, 415 in the peripheral and 213 in the central region. A Kruskal-Wallis test on independent samples was performed and the p values are shown in each graph. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk.

In the central cornea, WT AL fed mice had a significantly thicker corneal epithelium than all other groups, in the central cornea (figure 4.3), and thicker than the KO AL mice in the peripheral region. This suggests that dietary restriction and *Tert*^{-/-} cause a thinning of the cornea. One explanation for this would be that the lack of *Tert* and DR both had a suppressive effect on cell proliferation. This effect however was not cumulative as the DR KO (dietary restricted *Tert*^{-/-}) group did not show any further corneal thinning, implying that dietary restriction is dependent on *Tert.* Previously however no change was detected in central corneal thickness between young and old mice.

There are a number of factors which may result in no overall change in the thickness of the cornea. *Tert* is normally expressed by stem cells of the corneal endothelium (Whikehart *et al.*, 2005), knockout of Tert may have functional effects on

the cells resulting in reduced transport of solutes from the cornea, increasing its size. Meanwhile limbal epithelial cells and TACs may produce fewer progeny resulting in a thinning of the cornea. The overall result is very little change in the cornea size.

4.3.2 Does corneal edema increase with age in the mouse cornea?

One of the fundamental properties of the cornea is to act as a barrier to the environment. With age however this function seems to decline. It has been shown previously that the cornea becomes permeable to fluorescein (Nzekwe and Maurice, 1994), indicating a reduction in barrier function. A reduction in the subunits of hemidesmosomes (α6 and β4 integrins) with age may play a role in this degradation (Trinkausrandall *et al.*, 1993). The endothelium helps to maintain the amount of water entering into the cornea from the anterior chamber. Endothelial cells do not readily regenerate in the cornea, a decrease in the density of these cells with age is well documented (Laing *et al.*, 1976; Laule *et al.*, 1978; Gwin *et al.*, 1982; Bourne *et al.*, 1997). With the loss of these cells, fluid transport cannot be maintained and results in both stromal and corneal edema. Breakdown of the hemidesmosomes and the Bowman's membrane result in the formation of bullae (Yanoff and Joesph, 2009). Bullae are fluid filled compartments within the cornea.
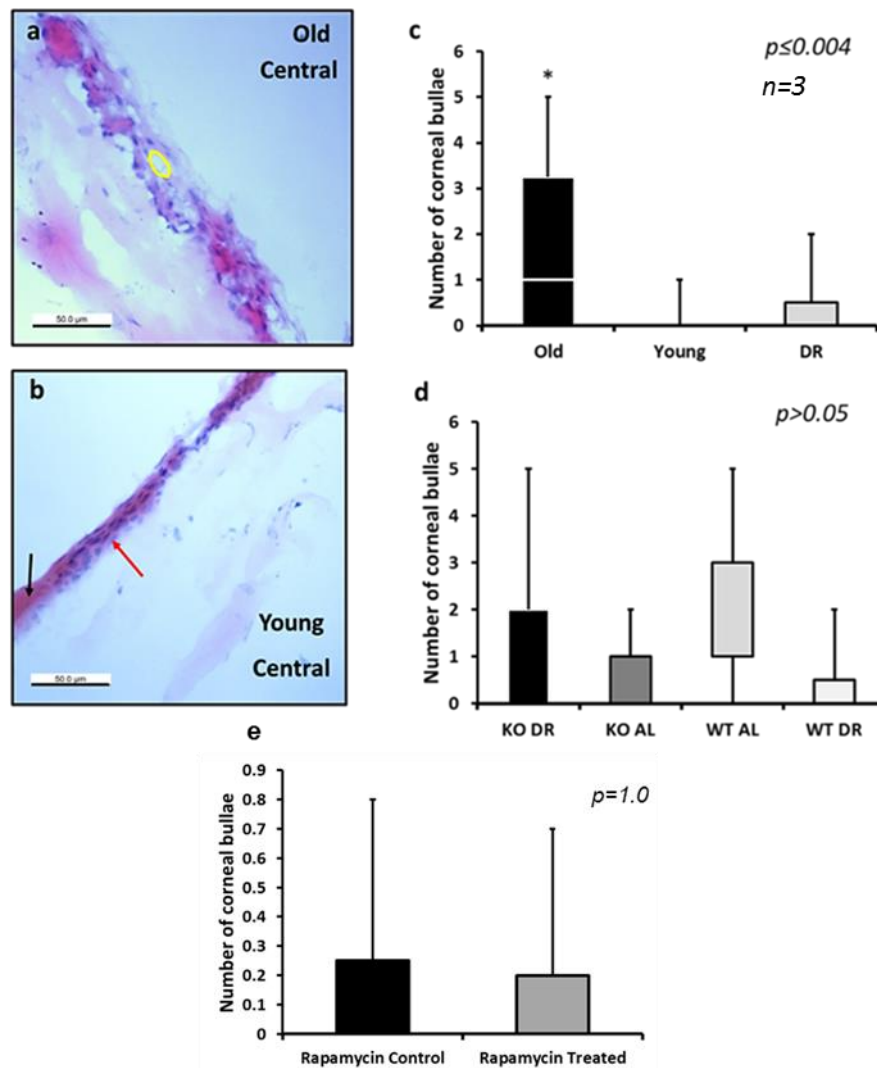
Figure 4.4 Corneal edema increase with age in the central cornea
The cornea was stained dark pink using eosin (black arrow panel b), while nuclei are stained purple using haematoxylin (red arrow panel b). An example of a bulla is highlighted in yellow, in panel a; these were most prevalent in the older mice. Old and DR treated mice were both 22 months old, while the young mice were 3 months old at time of sacrifice. Rapamycin treated mice were 16 months old and were treated for four months, starting at 12 months of age. *Tert*^{-/-} and wild type controls were 22 months old at time of sacrifice, dietary restriction was implemented for 16 months starting at 6 months of age. Results of a Kruskal-Willis test on independent samples are shown inlayed. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values, with the exception of rapamycin whereby histograms were used, standard deviations are displayed for these. Significance is indicated with an asterisk. Scale bars equal 50µm.

A Shapiro-Wilk test was performed on the numbers of corneal bullae, it showed that none of the groups were normally distributed. As a result, related samples Kruskal-Willis tests were performed on the data. There was a statistical increase between the old group and all other samples. Thus, in our study we observed that bullae in the central cornea increased with age, implying that the corneal endothelium decreased in either cell number or cell density (figure 4.4). DR prevented the age related increase in bullae in the mouse cornea, thus DR may be beneficial to the function of corneal endothelial

cells. Meanwhile there was no trend observed in the *Tert*$^{-/-}$ mice (*p>0.05*). Rapamycin treatment also had no effect on the incidence of bullae formation in the mouse cornea (*p=1.0*). Thus pathways associated with *Tert* and mTOR may not be responsible for the decline in endothelial cells.

4.3.3 Does p63 immunofluorescence decrease in the mouse cornea with age?

The putative positive stem cell marker p63 was used to assess the proportion of progenitor and transit amplifying cells. A rabbit monoclonal antibody raised against the recombinant protein fragment corresponding to amino acids 1-203 of human p63 was used for this analysis. The only reliable antibody which detects the ΔN isoforms was a mouse monoclonal antibody (as used in chapters 3 and 5). The amino acid sequences 1-203 confer the isoforms, TAp63 α,β & γ of the p63 protein. In humans, the TA isoform is associated with more differentiated cells such as TACs and terminally differentiated cells (TDCs) (Di Iorio *et al.*, 2005). In mouse however, TAp63 is implicated in the maintenance of epithelial progenitors, with a knockout of TAp63 resulting in an accelerated ageing phenotype (Keyes *et al.*, 2005). At a cellular and molecular level, an increase in senescent cells and an accumulation of γH2AX were observed in the TAp63 knockout mice, two important biomarkers of ageing (Su *et al.*, 2009). Therefore the use of the anti-TAp63 antibody was biologically relevant in the mouse system.

4.3.3.1 TAp63 immunofluorescence in cornea epithelium of old, young, dietary restricted and rapamycin treated mice

TAp63 was used to assess the relative amount of progenitors in the corneal epithelium for each afore mentioned conditions, in both the central and peripheral regions. Firstly, Shapiro-Wilk tests were performed on the data, all samples were normally distributed (see appendices B). A general ANOVA test showed that there was significance within the data set (*p<0.001*). Performing a post hoc LSD (least significant difference) test indicated between which conditions significance lay (see appendices B). When comparing data from the same group, between central and peripheral regions paired sample t-tests where used (figure 4.5). The results of the latter test are summarised in table 4.5.

Sample/Condition/Area	Paired samples t-tests (p values)
Old Central Cornea vs Old Peripheral Cornea	*0.055*
Young Central Cornea vs Young Peripheral Cornea	*<0.001*
DR Central Cornea vs DR Peripheral Cornea	*<0.001*
Rapamycin Treated Central Cornea vs Rapamycin Treated Peripheral Cornea	*<0.001*
Rapamycin control Central Cornea vs Rapamycin control Peripheral Cornea	*<0.001*

Table 4.4 Results of Student's *t-tests* for TAp63 immunofluorescence between central and peripheral regions of old, young, dietary restricted and rapamycin treated mice

In the central cornea, the levels of TAp63 were higher in the younger mice compared to the old (figure 4.5). This result confirmed our expectation that the level of TAp63 decreases with age in the mouse cornea. DR, on the other hand seemed to supress the accumulation of TAp63 in the central cornea. Thus, surprisingly, DR is further decreasing the amount of TAp63, which was rather unexpected. When comparing rapamycin treated samples with their age matched unrelated controls, there was a significant decrease in TAp63 in both the central and peripheral regions. Thus, rapamycin and DR both influence TAp63 levels in the same manner. One explanation for the observation of the TAp63 decrease in both DR and rapamycin treatment was that perhapsTAp63 might be regulated by an element of the mTOR pathway. TAp63 also seems to be affected by age, as there was a marked decrease in the protein with age in the central cornea.

The peripheral cornea had consistently lower quantities of TAp63 compared to the central region (figure 4.6) (table 4.4). If the same regional staining pattern was seen in humans, less TAp63 in the peripheral cornea would be consistent with the X, Y,Z hypothesis (Di Iorio *et al.*, 2005). In mice however, it suggests that perhaps more progenitors are present in the central region.
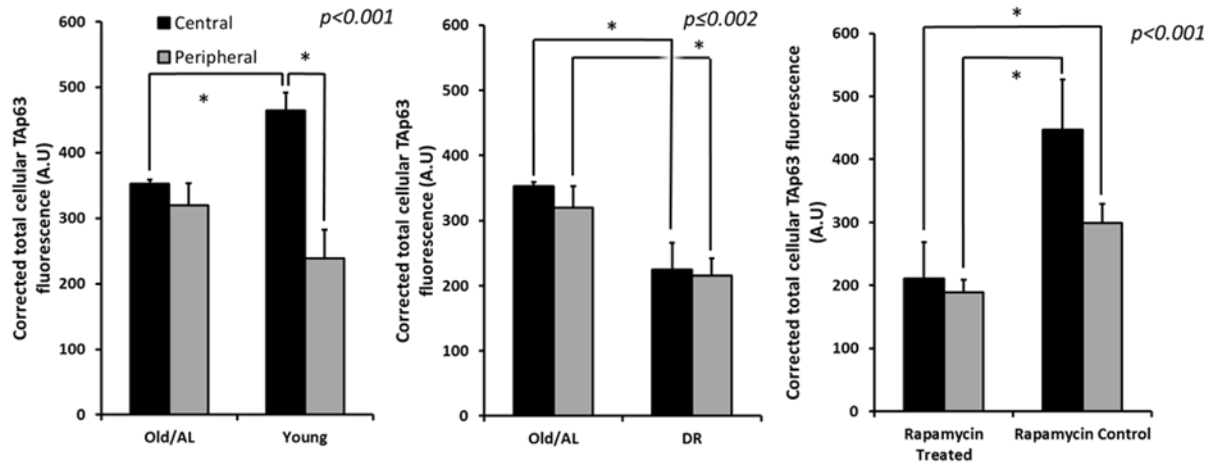
Figure 4.5 TAp63 immunofluorescence of old, young, dietary restricted, rapamycin treated and control mice Each group contained at least 3 individual mice. Cryosections through the meridian of the eye were used in this experiment. Five field of views (FOV) from the central cornea were taken, and five from each peripheral region. A total of 2,675 basal cells were measured. Results of ANOVA are inlayed into each graph. Significance is indicated with an asterisk Standard error of the mean is shown.

Examples of TAp63 immunofluorescence are shown in figure 4.6. Only the basal cells of the corneal epithelium were measured as this region is hypothesised as containing the potential progenitor cells.
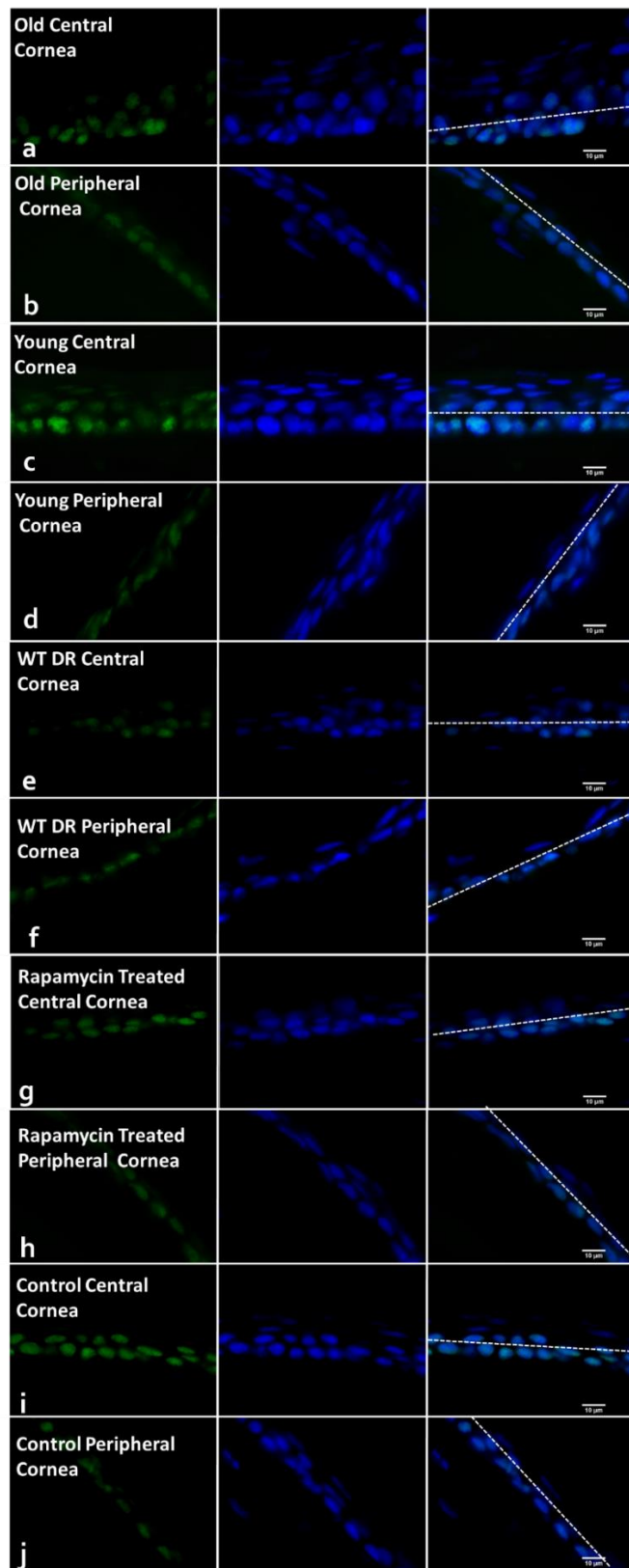
Figure 4.6 TAp63 immunofluorescence of young, old, dietary restricted, rapamycin treated and control corneal epithelium
Stem cells and transit amplifying cells are located in the basal cell layer of the mouse corneal epithelium. The basal layer can be observed under the white line as the immediate layer after the basement membrane, these cells are analysed for TAp63 intensity. The first column shows TAp63 protein in green, while the second column shows DAPI staining of DNA within the nucleus. The third column is a merged image. Scale bar is10μm.

4.3.3.2   TAp63 immunofluorescence in *Tert*^{-/-} and wild type mice fed *ad libitum* or dietary restricted

To investigate if the numbers of progenitor like cells differed dependent on diet and presence of the telomerase enzyme (TERT), TAp63 was measured in *Tert*^{-/-} mice fed both *ad libitum* and DR. The following staining was performed at a later time point to the initial staining (figure 4.6) and with a different set of mice, so that absolute intensities cannot be compared between the two experiments. *Tert* is expressed in limbal epithelial cells and transit amplifying cells, knockout of this gene may have an impact on the maintenance of stem cells in the cornea (Moore *et al.*, 2002). A subset of both wild type and *Tert* ^{-/-} mice were subjected to dietary restriction, to assess what role *Tert* might have on this treatment. A normality test was performed on the data, the results of which can be found in appendix B. All but the WT *ad libitum* peripheral cornea sample were not normally distributed.

A Kruskal-Willis test for independent samples was used to compare independent samples (see appendix B). The results showed that there was a significant decrease in TAp63 between wild type *ad libitum* and wild type DR in the central cornea. The same was true between wild type *ad libitum* and *Tert*^{-/-} *ad libitum* fed mice. In *Tert*^{-/-} dietary restricted mice however, TAp63 was maintained in the central cornea at the same level as the wild type *ad libitum* mice. In the peripheral cornea the wild type mice (both DR and AL) had a higher level of TAp63 than *Tert*^{-/-} mice (both DR and AL).

These observations confirm those previously shown in figure 4.6, regarding the wild type *ad libitum* and dietary restricted mice. Consistently the peripheral areas had lower TAp63 levels, than their central counterparts (figure 4.7) (table 4.5).

Sample/Condition	Wilcoxon signed ranks on related samples (p values)
WT AL	*<0.001*
WT DR	*<0.001*
KO AL	*<0.001*
KO DR	*<0.001*

Table 4.5 Statistical comparisons of p63 staining of central and peripheral corneal regions in the wild type and *Tert*^{-/-} mice fed either *ad libitum* or dietary restricted
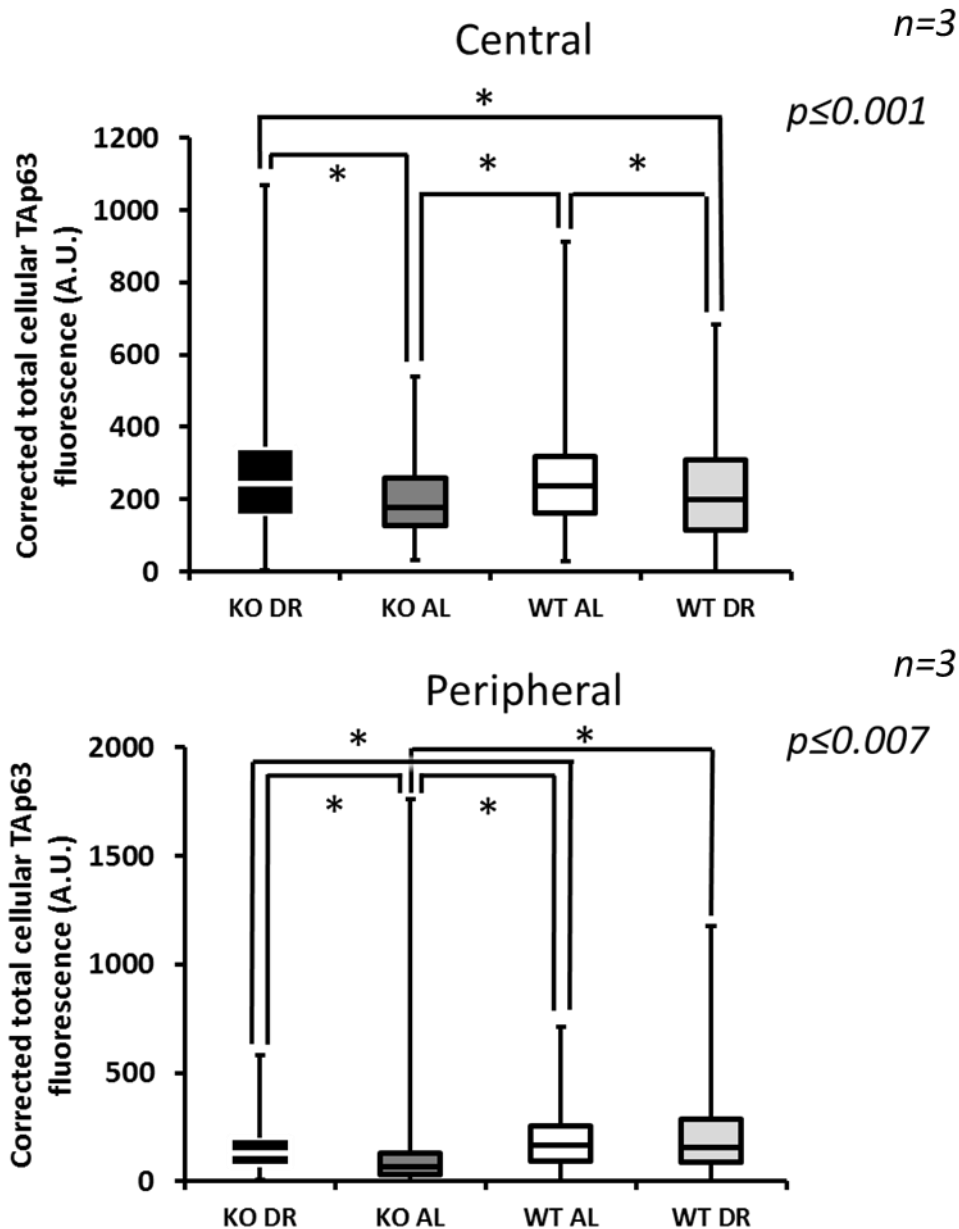
Figure 4.7 TAp63 immunofluorescence of *Tert*^{-/-} mice and wild type either dietary restricted or *ad libitum* fed

Each group contained at least 3 individual mice. Cryosections through the meridian of the eye were used in this experiment. Five FOV from the central cornea were taken, and five from each peripheral region. A total of 1,748 basal cells were analysed. The animals in this experiment were aged around 22 months. Dietary restriction was implemented for a period of 16 months at the age of 6 months. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk. Central cornea, WT AL vs KO AL *p=0.001*, WT AL vs WT DR *p=0.001*, KO DR vs KO AL *p<0.001*, KO DR vs WT DR *p<0.001*. Peripheral cornea, WT AL vs KO DR *p=0.007*, WT AL vs KO AL *p<0.001*, KO DR vs KO AL *p<0.001*, KO AL vs WT DR *p<0.001*.
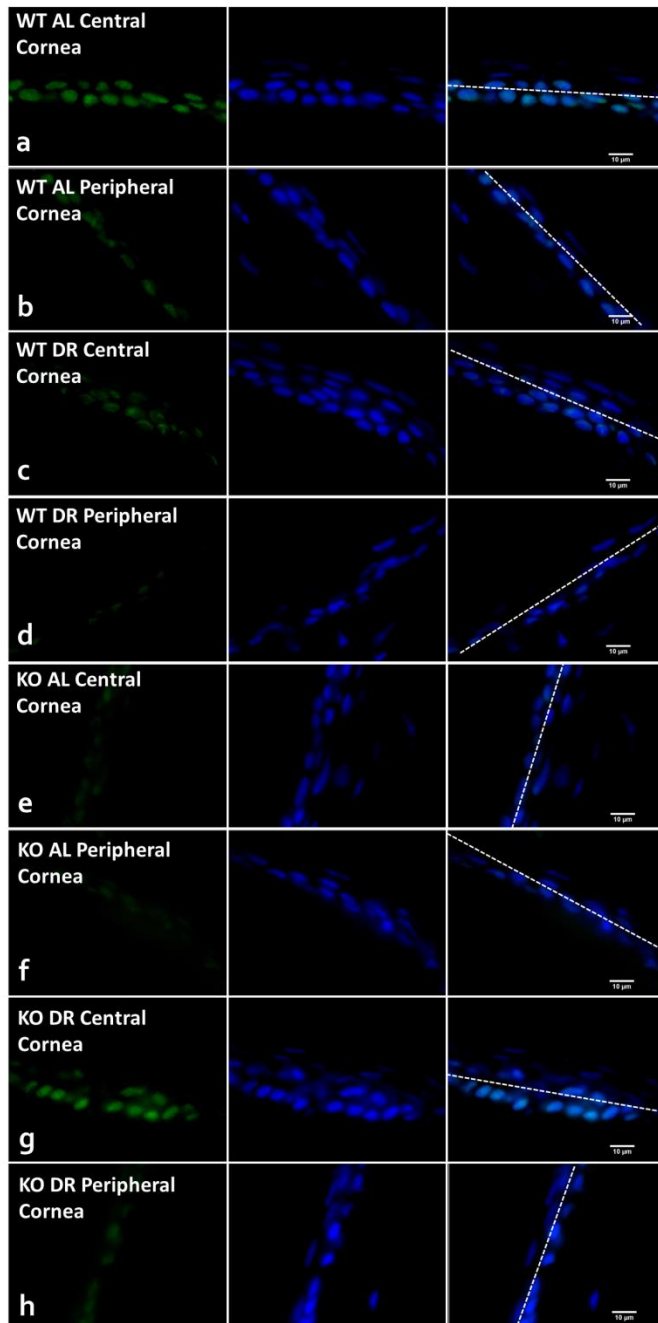
Figure 4.8 Images of TAp63 immunofluorescence in the corneal epithelium of *Tert*^{-/-} mice on dietary restriction or *ad libitum* fed

Stem cells and transit amplifying cells are located in the basal cell layer of the mouse corneal epithelium. The basal layer can be observed under the white line, these cells are analysed for TAp63 intensity. The first column shows TAp63 protein in green, while the second column shows DAPI staining of DNA within the nucleus. The third column is a merged image. Scale bar is10μm.

In the central cornea there was no difference between the TAp63 levels of wild type dietary restricted and *Tert*^{-/-} *ad libitum* animals. This suggests that DR and the knockout of *Tert* have a similar effect on the expression of TAp63. In the peripheral cornea

however the two *Tert*$^{-/-}$ conditions have lower TAp63 than in the WT conditions. Suggesting that *Tert*$^{-/-}$ could be detrimental to the potential progenitors of this region.

4.3.4 Do γH2A.X foci increase with age?

A cells response to double strand breaks (DSB) is primarily to accumulate repair proteins around the site, the so called DNA damage response (DDR). These protein accumulations, when visualised using florescence microscopy are termed foci. H2A.X is one of the proteins which accumulate at DSB sites. At the DSB, H2A.X is phosphorylated, after which it is referred to as γH2A.X. γH2A.X foci are one of the most sensitive methods of detecting this type of DNA damage, it has been estimated that over 2,000 protein molecules form/aggregate per DSB (Ismail and Hendzel, 2008). In these experiments the numbers individual γH2A.X foci were counted to give an indication of the level of DNA damage within the cells.

4.3.4.1 Frequency of γH2A.X foci in old, young, dietary restricted, rapamycin and control mice.

The main aim of this experiment was to assess if DNA damage varies between different ages and conditions. We tested eye sections from young, old, DR, rapamycin treated and control mice for the number of γH2A.X foci in both the central and peripheral regions. Normality tests showed the data was not normally distributed (*p<0.001*), as such non parametric tests were performed.
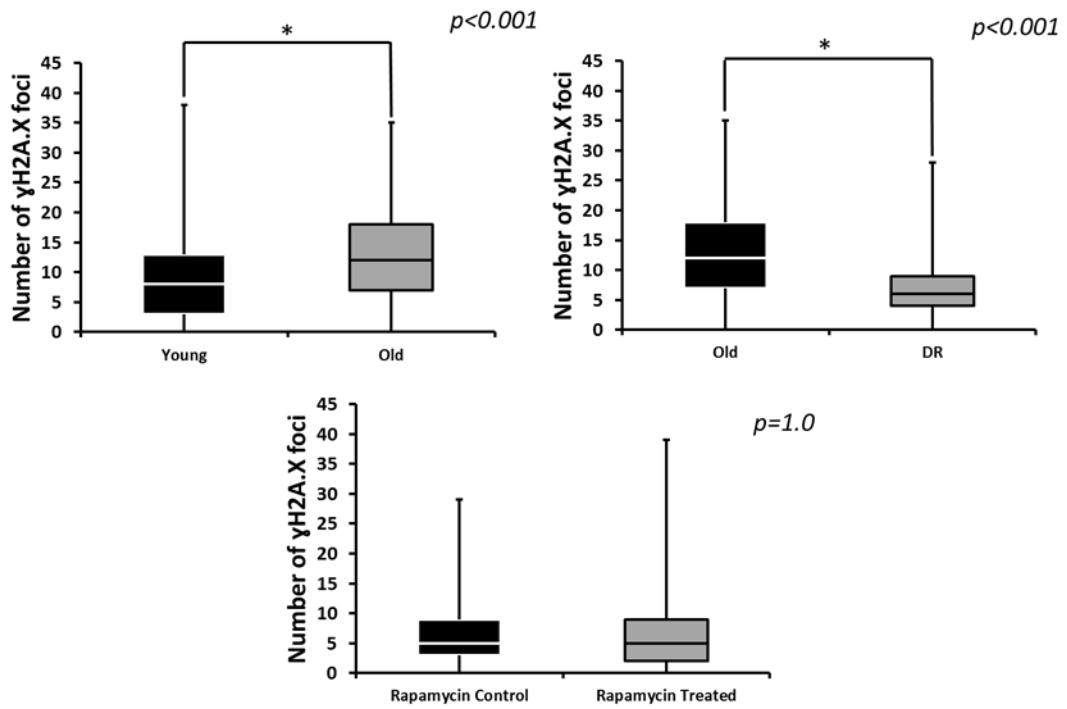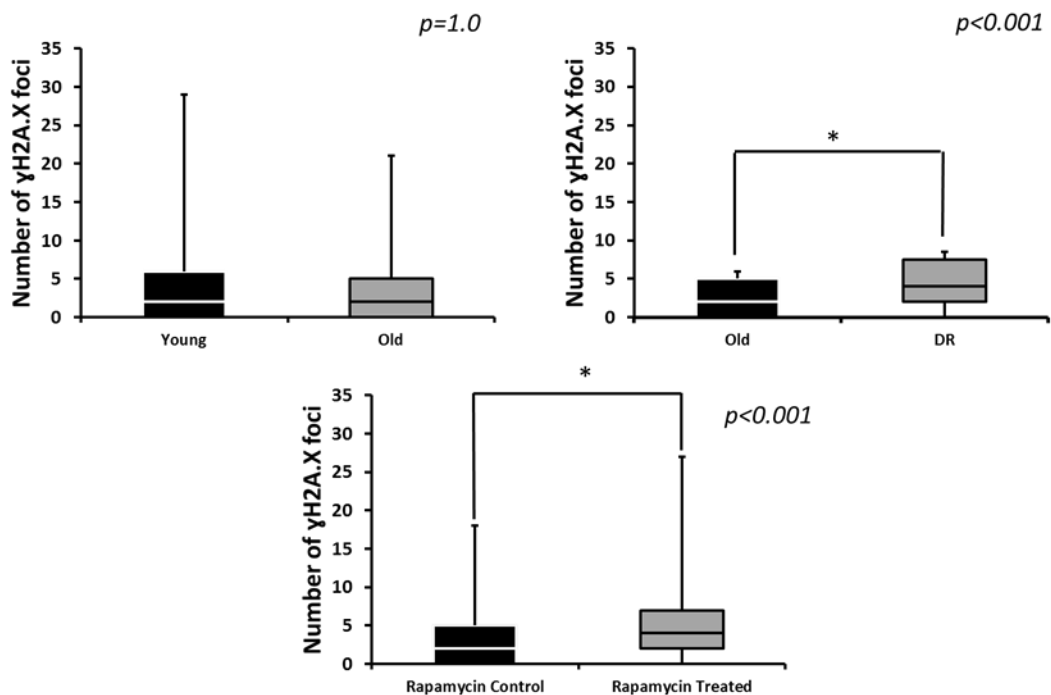
Figure 4.9 γH2A.X foci number in the cornea of young, old, dietary restricted, rapamycin treated mice
3 mice were analysed per condition, with five FOV from each peripheral cornea and 5 from the central region. 2,533 nuclei were analysed. 8,920 foci were counted in the central cornea, while 5,159 in the peripheral. Results of independent samples Kruskal-Willis test are shown as inlays. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk.

The number of γH2A.X foci increased significantly with age in the central cornea, while there was no change in the peripheral cornea. DR was able to remediate this accumulation in the central cornea. There was however, an increase in foci under DR compared to AL within the peripheral cornea. In the peripheral cornea more γH2A.X foci were observed within the rapamycin treated group compared to control mice. No change however was observed between rapamycin treatment and controls in the central cornea.

Dietary restriction can reduce the number of γH2A.X foci in the central cornea, but not in the peripheral cornea, indeed DR seemed to increase foci number in the periphery. Increases in the number of foci when both under DR and treated with rapamycin indicate that the mechanism for this increase in foci could be mTOR mediated. mTOR can cause a sensitisation of the DDR, which can in turn lead to accumulation of foci at damage sites, like those observed. In the central cornea however the relationship between DR and rapamycin was not the same, this may bode from the propensity of DR being a more global effector.

Sample	Wilcoxon signed ranks test on related samples (p values)
Young	*<0.001*
Old	*<0.001*
DR	*<0.001*
Rapamycin Treated	*0.002*
Rapamycin Control	*<0.001*

Table 4.6 Comparison between γH2A.X foci numbers between the central cornea and peripheral cornea of young, old, dietary restricted, rapamycin treated and control mice.

All conditions showed a significant difference between central and peripheral corneal regions, meaning that there are always more γH2A.X foci in the central cornea in these mice (table 4.6). Perhaps due to increased exposure to external stresses, while the periphery is somewhat protected by the eye lid.

4.3.4.2 Frequency of γH2A.X foci in *Tert*^{-/-} and wild type mice fed either *ad libitum* or dietary restricted.

The number of γH2A.X foci in *Tert*^{-/-} mice indicates the amount of DDR occurring in these mice. It was expected that the peripheral regions of these mice would harbour less γH2A.X foci as previous data shown in figure 5.13 demonstrated this trend. However a

Wilcoxon test on related samples did not detect a significant difference between these two anatomical regions (*p=0.220*). An average of 12 ±6.7 γH2A.X foci per cell in the central region and 11 ±7.3 in the periphery were measured overall. This could in part be due to the role *Tert* plays in stem cells in the peripheral region, absence of this protein may have triggered more damage to accumulate in par with the rate it occurs in the central region which has naturally less expression of *Tert*.
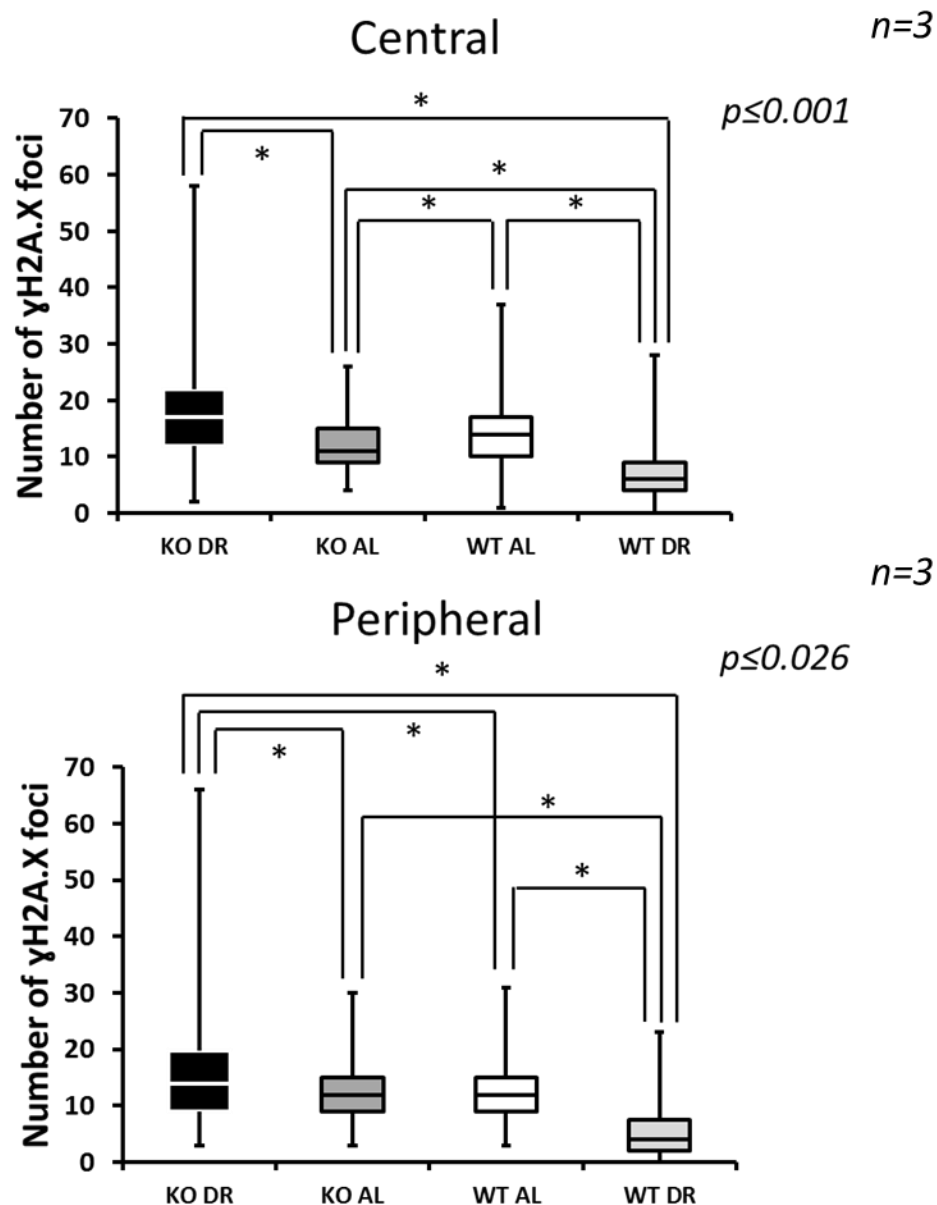
Figure 4.10 γH2A.X foci number in *Tert*^{-/-} and wild type mice fed *ad libitum* or dietary restricted
1,340 nuclei were analysed. 11,631 foci were counted in the central cornea, while 10,062 in the peripheral. p values for Kruskal-Willis test on independent samples are shown in each graph. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk.

Sample	Related samples Wilcoxon signed ranks (p values)
KO DR	*0.515*
KO AL	*0.090*
WT AL	*<0.001*
WT DR	*<0.001*

Table 4.7 Comparison between number of γH2A.X foci in the central cornea and peripheral cornea of *Tert*$^{-/-}$ and wild type mice fed *ad libitum* or dietary restricted.

Dietary restricted *Tert*$^{-/-}$ mice seemed to have more γH2A.X foci than their *ad libitum* fed counterparts (KO), in both central and peripheral corneal regions. Conversely, wild type *ad libitum* fed mice had more than there dietary restricted counterparts. The data seems to suggest that DR is not as beneficial when *Tert* is absent. Additionally, wild type *ad libitum* mice also had more foci than *Tert*$^{-/-}$ *ad libitum* mice in the central cornea. Thus, these *Tert*$^{-/-}$ mice seem to accumulate more foci (figure 4.10) (table 4.7). The mice used in these experiments were F$_1$ knockouts and should not have shorter telomeres than WT mice, omitting their involvement.

Tert$^{-/-}$ dietary restricted and *Tert*$^{-/-}$ *ad libitum* mice did not shown any difference between the number of foci in the central and peripheral cornea. This suggests that the loss of *Tert* does prevent DR from exerting its beneficial effect of decreasing DNA damage in the cornea. Wild type *ad libitum* and wild type DR mice both showed a difference between central and peripheral cornea, which was similar to the changes documented in the previous staining, where DR decreased γH2A.X foci number. Thus, the beneficial effect of DR in the WT mice on both central and peripheral cornea was reproduced with different mice from the same experiment (DR) while the lack of *Tert* seems to abolish the effects of DR.

4.3.5   Do telomere associated foci increase with age in the mouse cornea?

Telomere uncapping occurs when telomeres become critically short or the proteins which protect them become dysfunctional. Uncapped telomeres can lead to cell cycle arrest, senescence and apoptosis. γH2A.X foci which form within the telomeric regions are termed telomere associated foci (TAF) and can be an indication of cellular ageing. Increases in telomeric instability can lead to an increased chance of entering into senescence. TAF's were detected in a mouse corneal epithelium for the first time at an

average frequency of 2.15±1.6 foci per cell in the central cornea and 1.12±1.2 in the peripheral cornea. When comparing the two regions, firstly normality was analysed. A Shapiro-Wilk test indicated that the data was not normally distributed (*p<0.001*). Overall, there was a statistically significant increase in the numbers of TAFs in the central cornea over the peripheral cornea (*p<0.001* Wilcoxon test for related samples). As mentioned previously, this may be due to the protection which the eye lids provide to these regions. In humans more progenitor like cells are found in the areas which are better protected by the lids (12 o'clock and 6 o'clock positions) (Pellegrini *et al.*, 1999).

4.3.5.1   TAFs in old, young, DR, rapamycin treated and control mice

The main aim of the analysis was to assess if the frequency of TAFs changes with age, and if interventions have an impact in the mouse cornea. A Kruskal-Wallis test on independent samples was performed (summarised in appendix B). There was an increase in the number of TAF's within the basal cells of the corneal epithelium in the old mice compared to young. This suggests that TAF's do increase with age in the mouse cornea, indicating increased telomeric damage or an increase in exposed telomeric ends. There was also a difference noted between both DR and old/AL conditions. DR seemed to maintain the number of TAFs at a lower level than the young mice. Thus, DR might prevent telomeric damage and critically short telomeres, possibly through a slowing of metabolism, reduction of ROS and cellular turnover.

When comparing the levels of TAFs in the peripheral cornea of the older mice with that of the young, no decrease was observed (figure 4.12 & 4.13). This differed from the observation in the central region (figure 4.11 & 4.13), indicating that the peripheral regions might be protected from telomeric damage. Dietary restriction seemed to reduce this damage in the peripheral cornea when compared with old mice. The amount of damage was maintained to a similar level to the young mice. This suggests that DR reduces DNA damage over the lifetime of the animal. In the peripheral cornea rapamycin treatment appeared to increase the number of TAF's formed compared to the control group, indicating that rapamycin maybe enhancing the DDR in the cells of the peripheral cornea. Thus, there seems to be a pronounced difference between DR and rapamycin treatment.
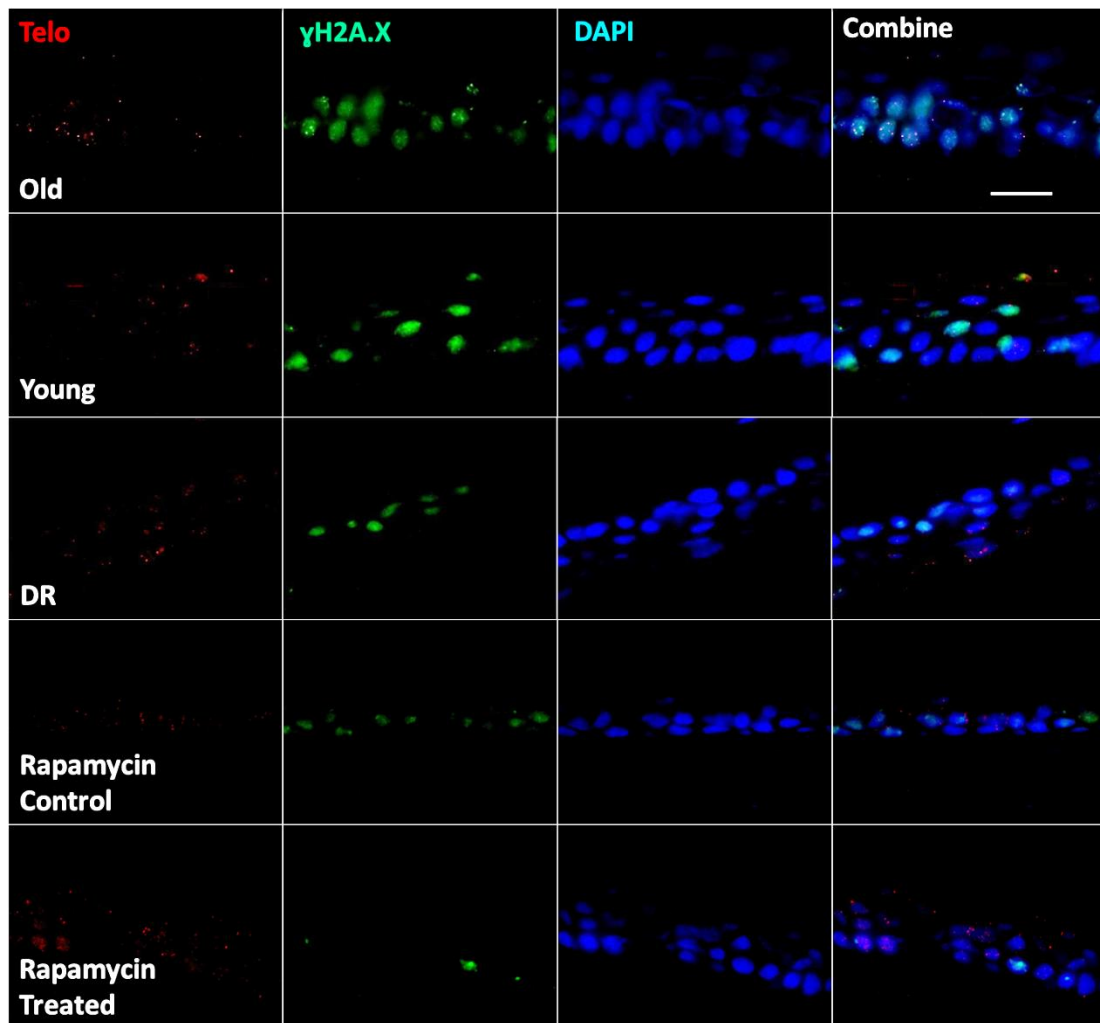
Figure 4.11 Telomere associated foci in the central cornea of old, young, dietary restricted, rapamycin treated and control mice

Representative images of co-localisations of the telomere probe (red) and ɣH2A.X foci (green). Nuclear DNA is stained blue with DAPI. Scale bar equals 20µm.
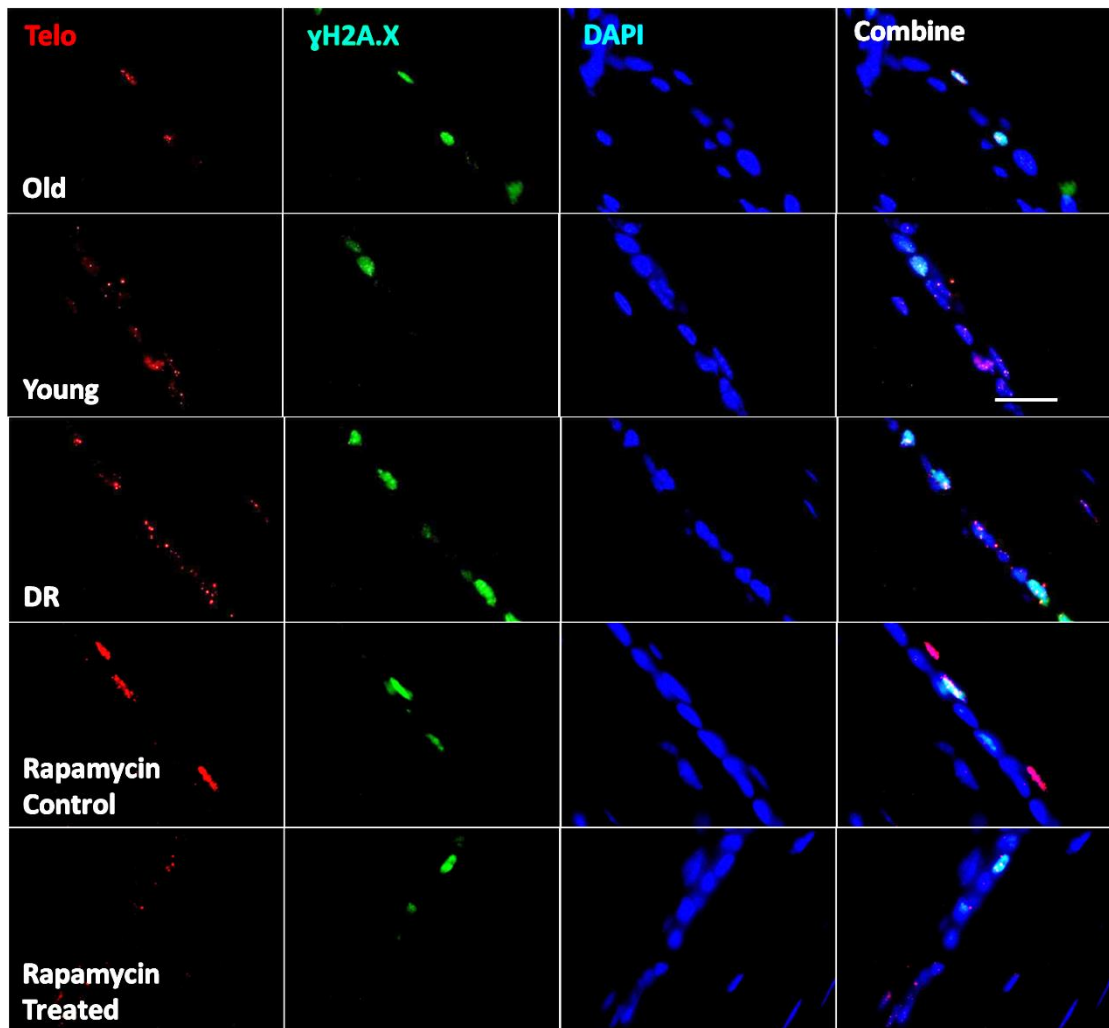
Figure 4.12 Telomere associated foci in the peripheral cornea of old, young, dietary restricted, rapamycin treated and control mice

Representative images of co-localisation of telomere probe (red) and ɣH2A.X foci (green). Nuclear DNA is stained blue with DAPI. Scale bar equals 20μm.
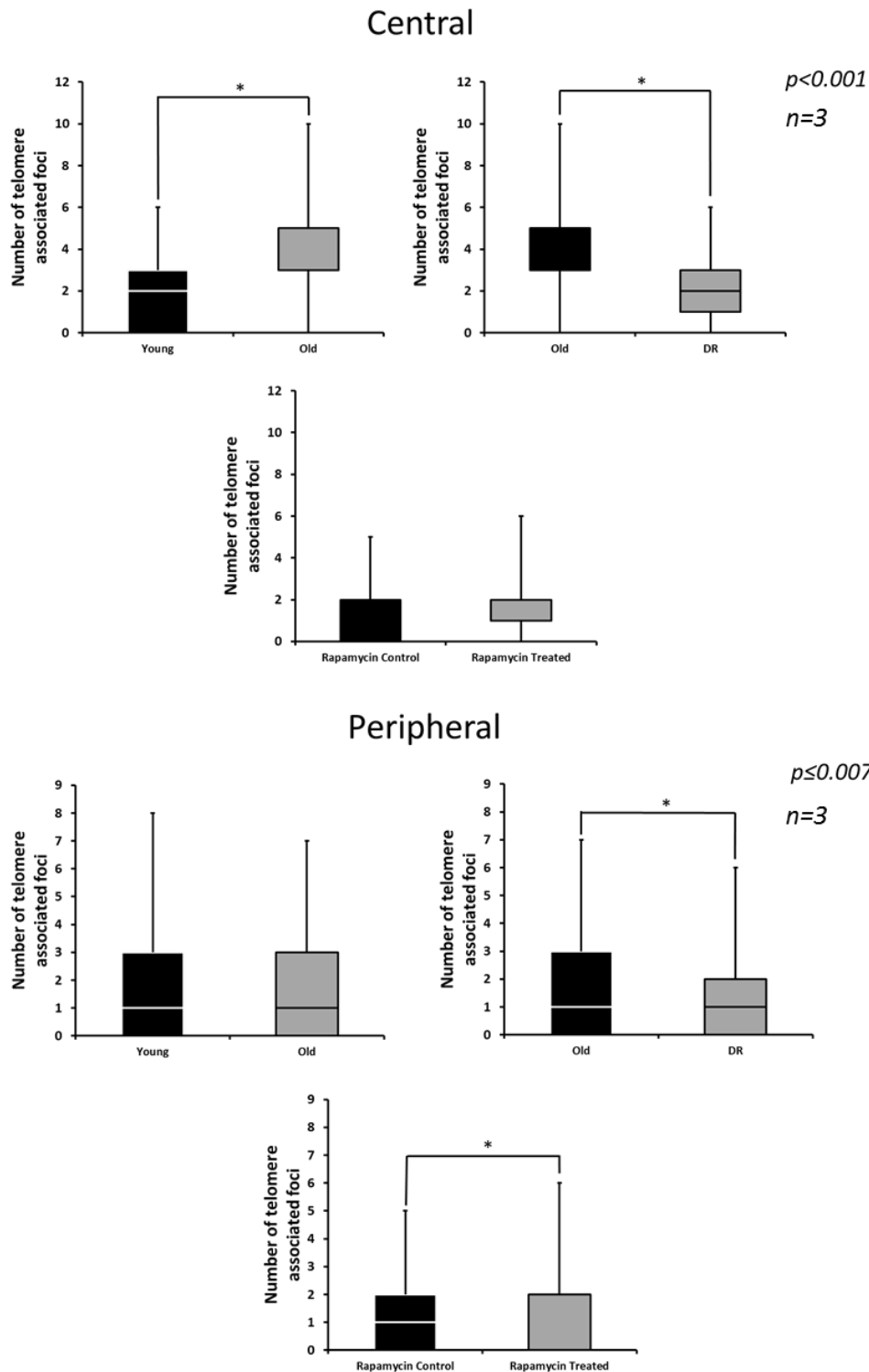
Figure 4.13 Comparisons of the number of TAFs in the mouse corneal epithelium under different conditions

A total of 1,659 basal cells were analysed in the central cornea, whilst 1,452 basal cells were analysed in the peripheral cornea. Result of Independent samples Kruskal-Wallis tests are shown as inlays. The box plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. Significance is indicated with an asterisk.

4.3.6 Can Ki67 and γH2A.X co-staining rule out the contribution of mitotic cells to DNA damage foci?

During the replication of DNA, exposed chromosomal sections can trigger the recruitment of γH2A.X (Ward and Chen, 2001) which cause false positive DNA damage γH2A.X foci data. To rule out this biological phenomenon from the previous data, the cell proliferation marker Ki67 was used to assess the proportion of proliferating basal cells and to assess whether this fraction changes under different conditions (figure 4.14).
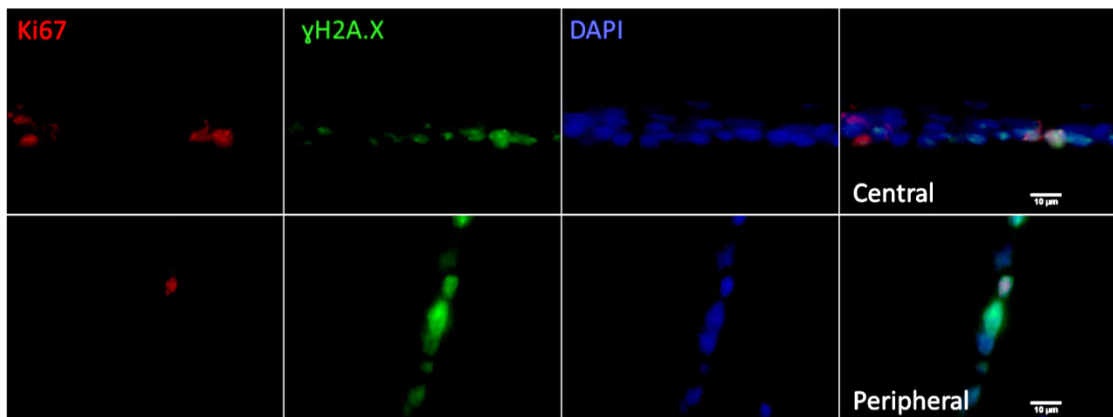
Figure 4.14 Example images of Ki67 and γH2A.X immunofluorescence in the peripheral and central cornea
The total number of cells per field were counted along with the number of Ki67 positive cells.

The percentage of Ki67 positive basal cells was counted in each field of view. Normality tests showed that about half of the data collected was not normally distributed (see appendices B). A Kruskal Wallis test on independent samples for central corneal values indicated that there was no difference between old, young, DR and rapamycin treated mice ($p=0.292$). The peripheral cornea also showed no difference ($p=0.415$). This was not expected since we hypothesised that different conditions might change the cellular proliferation rate. For example DR is known to decrease cell proliferation, however these results indicate that it may not apply to all tissue types (Ogura *et al.*, 1989). It did however mean that the TAF analysis and the γH2A.X foci data did not need to be adjusted for differences in proliferation between groups.

The average number of Ki67 positive cells in the central cornea was 13% ±9.1, while in the peripheral cornea it was 9% ±10. A Wilcoxon signed ranks on related samples test was performed which showed that there was no significant difference between the two anatomical regions in any of the samples ($p=0.086$) (figure 4.15).
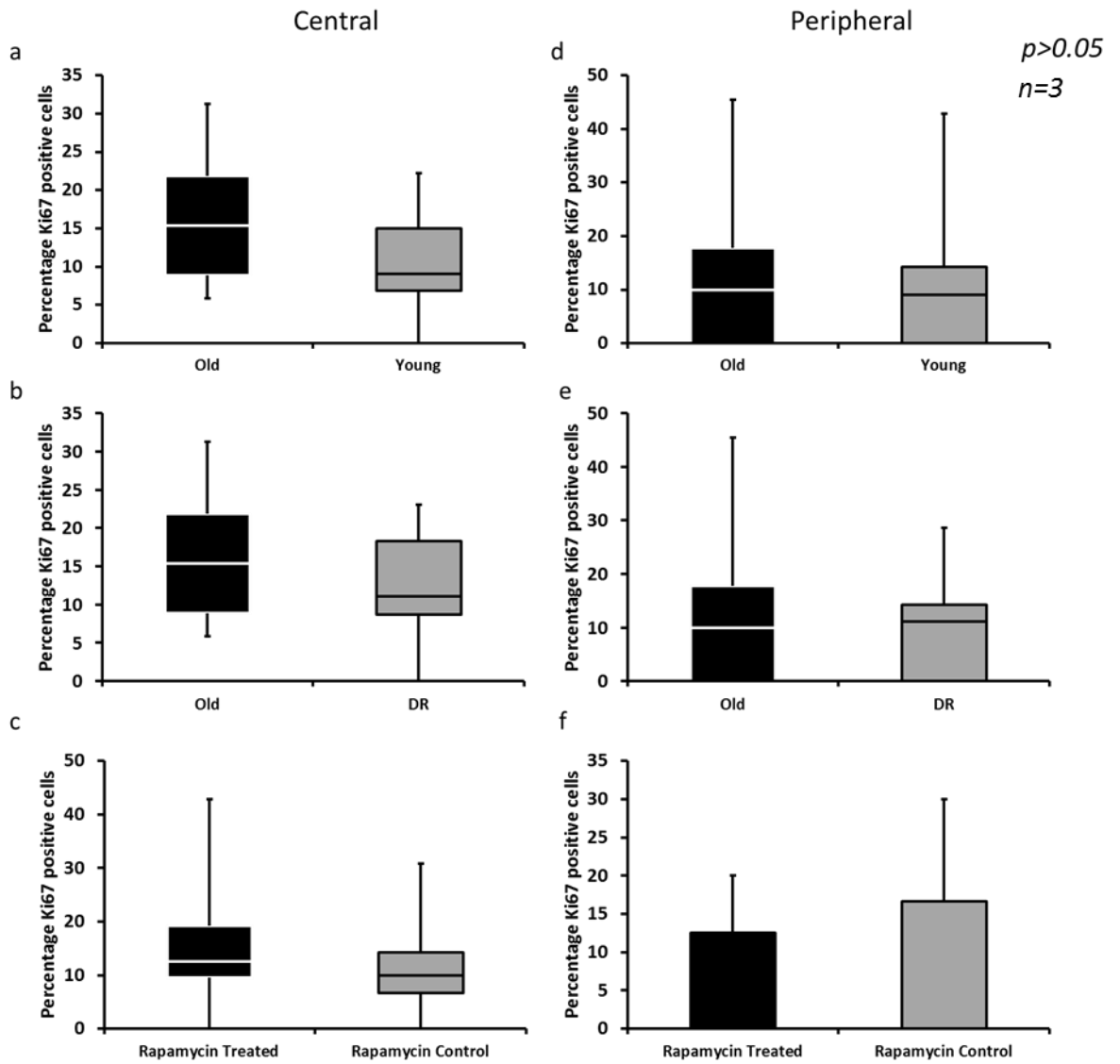
Figure 4.15 Percentage of Ki67 positive cells across the central and peripheral regions
Five fields of view were analysed for the central cornea (a-c). 10 fields of view were observed in the peripheral cornea (d-f), five from each. A total of 1,571 basal cells were analysed. The boxes plots show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values.

4.4 Executive summary

- Age decreased the thickness of the peripheral cornea, but not the central. Bullae formation increased in the cornea with age. The progenitor marker TAp63 decreased with age, whilst the DNA damage marker γH2A.X increased along with the numbers of TAFs in the central cornea. The peripheral cornea on the other hand, was unaffected by these parameters.

- DR reduced corneal thickness, bullae formation and TAp63. Thus, DR could not ameliorate these age-associated changes. However, it reduced the number of γH2A.X foci as well as TAFs in the central cornea, but not in the peripheral area. Consequently, DR was able to ameliorate general as well as telomere-associated DNA damage preferentially.

- Rapamycin treatment decreased the thickness of the cornea. It also decreased the levels of TAp63 in both central and peripheral regions while it increased the number of γH2A.X foci and TAFs in the peripheral cornea. Thus, it generated changes that were all in the same direction as ageing. This means, it does not have the same beneficial effects as DR and might even exacerbate age-related changes.

- Lack of TERT protein in $Tert^{-/-}$ mice and DR both caused a reduction in corneal size, however this was not additive. Lack of TERT also decreased the amount of TAp63 protein in the peripheral cornea, but not centrally. DR was able to recover TAp63 levels in the $Tert^{-/-}$ mice. This suggests that the DR effect on TAp63 levels were not dependent on the presence of TERT. In contrast, DR suppressed TAp63 protein level in WT mice. In $Tert^{-/-}$ AL mice, less γH2A.X foci formation was found than in WT AL mice. Importantly, γH2A.X foci number was higher in KO DR mice than in WT DR implicating that the effect of DR on DNA damage seems to depend on the presence of TERT protein. The reason why γH2A.X foci number was higher in WT AL than in KO AL mice is not known.

4.5   Discussion

4.5.1   Morphology of the ageing mouse cornea

The thickness of the cornea is an important parameter to measure, as it can indicate corneal disease or a loss of regeneration capacity. In humans it has been shown that the peripheral cornea as a whole decreases by 0.38μm per year (Jonsson *et al.*, 2006). It is debated whether the central corneal thickness changes with age, with the same study by Jonsson and colleagues showing no difference. A study which measured corneal thickness in an isolated Mongolian population noted a decrease in corneal thickness of around 0.5-0.6 μm per year. It was hypothesised that the lack of significant data for the central cornea may be down to variation between human donors. Conditions such as diabetes have been shown to increase corneal thickness, 6% of adults in England suffer from this condition (Diabetes-UK, 2014) and this prevalence may impact upon any human results (Pfeiffer *et al.*, 2007). In this study, mice were used in order to minimise variation. Even within the mouse model however, a change in the thickness of central cornea under different conditions and between different ages could not be detected. Interestingly, thickness of the peripheral cornea did decrease with age. If we assume a linear trend, then there was a loss of 0.12μm per month. Our study on mice differed from the human observations as we only measured the width of the corneal epithelium, compared to the total corneal thickness (Jonsson *et al.*, 2006).

Corneal integrity is crucial to its function as a barrier to the environment and movement of small molecules across its surface. As stated previously, it has been shown that permeability of the cornea decreases with age (Dekruijf *et al.*, 1987). This could be due to several reasons, for example, a reduction in the subunits which comprise hemidesmosomes (Trinkausrandall *et al.*, 1993). Hemidesmosomes anchor the basal cells to the basement membrane. Alternatively, a reduction in the endothelial layer causes a reduction of fluid movement out of the cornea. The accumulation of this fluid leads to the formation of bullae. Another theory is that the secretory function of the lacrimal gland decreases with age. The tear film is crucial in providing nutrients and supressing oxidative damage to the eye. A reduction in this function leads to dry eye in extreme cases (Baum, 1973). Dry eye is a condition which also increases with age (Schein *et al.*, 1997). Dietary restriction has been suggested as a mechanism by which lacrimal function can be maintained. The molecular mechanism however was not alluded to

(Kawashima and Tsubota, 2011). One hypothesis would be that DR prevents the increase in the proposed inflammatory response (Morgan *et al.*, 2007) of the lacrimal cells with age (Zoukhri, 2006). Thus restoring the secretory function required to maintain the corneal epithelium. Our study showed that the integrity of the cornea decreased with age while DR could maintain the integrity of the cornea to a level similar to that of young mice.

With age, the endothelium decreases in cell number (Laing *et al.*, 1976; Laule *et al.*, 1978; Gwin *et al.*, 1982; Bourne *et al.*, 1997), dietary restriction in rats was shown to ameliorate this change and maintain a healthy endothelial layer (Nadakavukaren *et al.*, 1987). It is possible that the same effect was present in the mice tested in our experiments, although not tested under the remit of this thesis.

4.5.2 The transcription factor TAp63 is a marker of limbal progenitors in the mouse cornea.

The detection of true progenitor limbal stem cells is a point of controversy in the limbal stem cell field. With no definitive stem cell marker gene, detection of progenitors is difficult. One of the more accurate marker genes to study is the transcription factor TP63. There are six main isoforms of p63: 3 ΔN and 3 TA. The antibody available for this experiment only detected the TA isoforms, which are associated mainly with more differentiated corneal cells in humans. TA isoforms however, play an important role in ageing and cancer formation (Flores *et al.*, 2005a). Mice which are deficient in TAp63 age prematurely. TAp63 has been shown to be essential for the maintenance of stem cells in a quiescent state in mouse epidermis (Su *et al.*, 2009). This study suggests that TAp63 is a valid marker for measuring progenitor cells in mice. The relatively high levels of this marker in young mice reinforce the statements made by Su and colleagues. Interestingly, both DR and rapamycin treatment appeared to repress the total amount of TAp63 in the central cornea, indicating a link between TAp63 and possibly the mTOR pathway. mTORC1 can up regulate p63, which is thought to be mediated through *Tsc2* or *Pten*. The repression of both the ΔN and TAp63 isoforms can be achieved by using rapamycin (Ma *et al.*, 2010). Rapamycin appears to also have a slight repressive effect on the expression level of TAp63 in a breast cancer cell line (Rosenbluth *et al.*, 2008). p73 and p53 are from the same protein family as p63 and both interact with mTOR (Feng *et*

al., 2005; Rosenbluth *et al.*, 2008). The evidence seems to suggest that p63 is also be involved in this pathway.

4.5.3 The accumulation of γH2A.X and telomere associated foci in the mouse cornea.

Increases in DNA damage can be indicative of reduced genomic stability. Irreparable DNA damage can trigger DDR and lead to an induction of senescence. Thus, increases in γH2A.X foci are indicators of an increased probability of senescence. The accumulation of senescent cells within a tissue leads to a decline in its functionality. The exact anatomical location of limbal stem cells in mice is unknown. For this reason, our study measured the number of DNA damage foci in the basal layer of the cornea in both central and peripheral regions. Overwhelmingly, our study showed an increase of γH2A.X foci in the central cornea suggesting that cells move centripetally, acquiring mutations, in post mitotic cells as they migrate across the cornea, before being lost to the tear film.

In *Tert*$^{-/-}$ mice the beneficial effects of DR, in terms of reduced DNA damage were negated. mTOR and telomerase are both controlled by the PI3K/Akt signalling cascade. In *Tert* $^{+/+}$ mice this is mediated through E2 estradiol which causes the nuclear translocation of *Tert* (Kimura *et al.*, 2004). This nuclear translocation may reduce the protective effect that the protein has in the mitochondria. Interestingly, Interleukin 2 up regulates *Tert* via mTORC1 in cancer cell lines (Zhou *et al.*, 2003; Yamada *et al.*, 2012). Similarly IGF-1 also stimulates telomerase activity (Wetterau *et al.*, 2003). During DR however, IGF-1 and mTOR are reduced/supressed indicating that an interaction is mediated through other means. SIRT1, which is up regulated during DR stimulates telomerase activity in yeast (Palacios *et al.*, 2010). We hypothesise that during DR the normal activation of Tert via SIRT1 is important for some of the effects of DR. This hypothesis is supported by Vera and colleagues who showed that WT DR mice had a reduction in telomere degradation compared to AL fed mice (Vera *et al.*, 2013).

Rapamycin treatment showed a different trend to that of DR, with increases in foci number in the peripheral cornea when treated. Previous studies have shown that rapamycin decreases DNA damage in mouse embryonic fibroblasts (Wang *et al.*, 2013) and human Werner syndrome ATP-dependent helicase-deficient fibroblasts (Saha *et al.*, 2013). The Werner syndrome ATP-dependent helicase (WRN) protein unwinds double

stranded DNA and is an essential part of DNA replication and repair (Monnat, 2010). Rapamycin can also increase the response to oxidative stress in adult mouse stem cells (Kofman *et al.*, 2012). The increase in response to DNA damage, seen in our results could be indicative of increased damage surveillance, however this is speculative.

Rapamycin is used as an immune suppressant to prevent allograft rejection of limbal stem cell and corneal transplantations (Penetrating keratoplasty) (Olsen *et al.*, 1994). It is thought that one of the ways in which rapamycin acts is through the inhibition of the neovascularisation element of rejection via the inhibition of TGF-β1 induced alpha-smooth muscle actin expression (α-SMA), α-SMA is important protein involved in angiogenesis (Shin *et al.*, 2013). The implication that rapamycin may increase DNA damage is important for the continued use of the drug on the eye, although the benefits may outweigh the risk in this instance.

Telomere associated foci were also more prevalent in the central cornea, indicating the presence of two distinct cellular populations. The progenitor cells in the limbal region express telomerase, resulting in increased telomere maintenance and a reduction in dysfunctional telomeres (Notara *et al.*, 2013). Consequently, our data favoured the LESC hypothesis.

ɣH2A.X foci and TAFs increased with age in the central cornea, showing that these were good markers of the ageing phenotype. Dietary restriction was able to maintain ɣH2A.X foci and TAF's at a more youthful level, possibly indicating less senescent cells within the tissue. In other mouse tissues, such as the small intestine and liver, TAF's have also been documented as accumulating with age. Overall, it was demonstrated that there was an approximate 25% increase in TAF's in both tissues with age (Hewitt *et al.*, 2012). It was expected then that the cornea may be similar to this value or even slightly lower since the gut is more proliferative than the cornea. The results of our study suggest that the increase was around 33% with age. This increase was however coupled to an increase in ɣH2A.X foci, so we cannot draw the conclusion that the telomeres are a preferential DNA damage site in this tissue.

Rapamycin treatment, again did not follow the same trends as DR, with regards to TAF formation. The results mirrored those of the overall ɣH2A.X foci number and indicate that the peripheral cornea is particularly susceptible to DNA damage. Rapamycin has been shown to inhibit telomerase expression in endometrial cancer cells

and indeed mTORC1 and TERT are proposed to complex via Hsp90 (Kawauchi *et al.*, 2005). The function of this complex however is unknown, our data would suggest that it has a protective effect on the genome, including the telomeres. It is important to note that up-regulation of both mTOR and TERT are hallmarks of cancer cells. We hypothesise that the up-regulation of the mTOR/Hsp90/TERT complex is beneficial for stem cell survival, however in differentiated cells could be oncogenic. In embryonic stem cells mTOR is required for maintaining cells in an undifferentiated state (Zhou *et al.*, 2012). We also propose then that the down regulation of TERT in the central cornea is indistinguishable between rapamycin treatment and control due to TERT already being down regulated in the central cornea.

TAF's cannot be resolved with a conventional photon driven microscopy system to the exact telomere region. This fact leaves the interpretation of these results open to debate, whether the DNA damage accumulated due to uncapped telomeres or just damage within the telomeric repeat units.

Random oxidative, UV damage and replicative errors contribute to the accumulation of DNA damage. These can persist in telomeric regions because of the nucleoprotein complexes which normally hinder non-homologous end joining. These complexes such as Shelterin, suppress the ability for a telomeric end to be recognised as DNA damage (Cesare *et al.*, 2009). Damage on the other hand has been shown to reduce the binding of two of the components of Shelterin, TRF1 and TRF2 (Opresko *et al.*, 2005). Recently it has been shown that removal of one component of the Shelterin complex, TRF2, from telomeric regions does not elicit a DNA damage response. The result further supports the theory that TAF's are not detecting exposed termini but sections of damaged telomere repeat units (Petrova *et al.*, 2014).

The nature of the telomeric repeat sequence makes it susceptible to oxidation. Being comprised primarily of the nucleic acid guanine, which can be oxidised to 7, 8-dihydro-8-oxoguanine, causing an increased chance of mismatch pairing and subsequent base substitutions (Simon and Vanvunakis, 1964; Ming *et al.*, 2014). If this was entirely true other areas of the genome, such as CpG islands would also be susceptible to these modifications with age. Mitomycin C which mitotically inactivates 3T3 mouse fibroblasts via CpG islands (Teng *et al.*, 1989) can also cause a shortening of telomeres (Wick and Gebhart, 2005). These convergent methods of base modification suggest that these

regions may share equal effects from base oxidation, which may have occurred due to ROS induced damage in the cornea. These experiments did not show a preferential telomeric localisation suggesting that these results support the non-preferential damage model.

Telomeres have been shown to be inefficiently repaired when damaged by UV radiation (Kruk *et al.*, 1995) or alkylating agents (Petersen *et al.*, 1998). However the mouse model which was used in this study was never exposed to UV radiation, ruling this out as a potential factor. UV damage may explain any major differences in foci number when human corneas are assessed.

4.6   Conclusion

4.6.1   Age has a detrimental effect on the mouse corneal epithelium.

The thickness of the corneal epithelium decreased with age in mice, this was just a trend in the central cornea, while in the peripheral region it was significant. We can perhaps attribute this change to a decrease in the ability to replenish the cells of the cornea from the peripheral regions. However if we assume that the cells move centripetally, a decrease in proliferation in the peripheral region would have an impact on the central cornea. Ki67 staining indicated that the cells of the peripheral cornea proliferated at a slower rate than those in the central regions, which could perhaps explain the difference in thickness of the corneal epithelium. Another factor which is worth discussing is that the limbus in mouse is difficult to observe, being only a slight indentation into the tissue. The fact that we see a decrease in thickness of the peripheral region could be the inadvertent measurement of a more recessed limbus with age.

In favour of a reduced number of progenitor cells within older mice, the amount of the positive marker TAp63 decreased in the central region, in the peripheral region however no decrease was observed. In addition to this TAp63 was expressed to a higher extent in the central region of the young donor compared to the periphery. These two factors make it difficult to draw a firm conclusion. The decrease of the TAp63 protein in the central cornea supports the hypothesis that age has a detrimental effect on progenitor populations, if we assume the CESC hypothesis to be true. However, the fact that the peripheral regions do not change with age suggests that they could be protected; perhaps the lid preserves the protein level of TAp63, and thus stem cells, in

this area.

The number of TAFs and general DNA damage foci both increased with age in the mouse cornea. This was unlike other mouse tissues were TAF's increased regardless of γH2A.X foci number (Hewitt *et al.*, 2012). The tissues which have previously been studied however were internal compared to the superficial corneal epithelium. It is possible that external tissues such as the epithelium face a higher burden of genomic attrition, and thus increased chance of accumulating DNA damage.

4.6.2   Bullae formation occurs with age in the mouse cornea.

One feature which was very striking in the cornea when comparing young and old mice was the formation of bullae. These fluid filled sacks were very prominent in all three of the older mice analysed, while in young mice very few bulla were observed. It was considered that these structures may be artefacts, from the freezing and cutting procedures. However all mouse eyes were processed in the exact same way, so these structures are unlikely to be artificial in nature. The proposed pathogenesis is via the ageing corneal endothelium causing a cascading effect on the corneal epithelium. The formation of bullae in the cornea could lead to increased opacity and increased risk of infection. The increased risk of infection is a factor which has been documented previously (Chang and Hu, 1993). Our study offers the conclusion that the increased susceptibility to infection could be in part related to the ageing corneal endothelium. This conclusion of course could be reversed, as the integrity of the corneal epithelium and increased susceptibility to infection could triggering to endothelial cell loss leading to a feedback crescendo (Faragher *et al.*, 1997).

4.6.3   DR mitigates age related changes in the mouse cornea.

Dietary restriction had very little effect on some parameters however on others it was quite dramatic, with the corneal thickness being one example. It was hypothesised that dietary restricted mice may have thinner corneas. This was based on the assumption that dietary restriction limits proliferation, via mTORC1 phosphorylation and inhibition of 4E-BP1 (Nojima *et al.*, 2003; Dowling *et al.*, 2010). However no statistical difference was found, only a slight trend in the central region. This leads to the conclusion that in the cornea, DR might not decrease cell proliferation.

The positive marker TAp63 however, was not shown to be up regulated in DR, in

either the peripheral or central regions. This result was interesting as TAp63 is thought to supress mTORC1 expression in the presence of DNA damage (Cam *et al.*, 2014). Conversely Ma and colleagues state that PI3K/Akt activates the p63/Jagged/notch pathway via mTOR, implying that mTOR regulates p63 (Ma *et al.*, 2010). Notch regulates cell differentiation, when activated the intra-membrane protein becomes cleaved and translocates to the nucleus whereby it interacts with DNA binding protein to form a transcription factor (Ehebauer *et al.*, 2006). What this means for limbal stem cells can only be speculated. A decrease in mTOR like that in the DR group could promote stemness in the stem cell pool, by driving down p63 expression and thus Notch pathway activation. This however is highly counter intuitive to existing data which promotes p63 as a positive LSC marker gene. The regulation of p63/Notch in keratinocytes at least appears to show that the interaction between these proteins is more complex. Nguyen and colleagues show that Notch1 activation supresses p63 in both humans and mouse, via Notch regulation of the interferon pathway. Also, that p63 modulates some of the effectors of Notch, such as Hairy and enhancer of split 1 (Hes-1) (Nguyen *et al.*, 2006) a suppressor of transcription (Kageyama *et al.*, 2007). So regulation works mutually between these proteins.

Dietary restriction appeared to have a suppressive effect on the build-up of ɣH2A.X foci in the central cornea. The decrease in ɣH2A.X foci has been documented previously in intestinal enterocytes and liver hepatocytes, 6% after 3 months DR (Wang *et al.*, 2010). The results cannot be directly compared however, as Wang's study counted the number of ɣH2A.X positive cells whereas we counted the number of individual foci using immunofluorescence.

In the peripheral cornea an increase in ɣH2A.X foci was noted between DR and AL matched controls, this was unusual and was not predicted. The number of telomere associated foci on the other hand, decreased in both the peripheral and central regions under DR compared to AL. A possible explanation could be that DR protects telomeres preferentially. mTOR inhibition has been shown to increase DDR through p53 and p21 causing phosphorylation of ATM (Bandhakavi *et al.*, 2010) or non-canonically through NF –κB/FANCD2 (Guo *et al.*, 2013). Wang and colleagues, suggested that mTOR decreases DNA damage through suppression of a cAMP response element-binding protein 1 (CREB1) (Wang *et al.*, 2013), which is known to be phosphorylated by ATM. The corneal

marker gene p63 also responds to DNA damage leading to the suppression of mTOR via REDD1/Tsc1 pathway (Cam *et al.*, 2014). It is important to consider that DR maybe promoting DNA repair rather than indicating a greater amount of damage, as we did not directly assess the levels of mTOR in our study.

4.6.4 Similarities and differences between rapamycin treatment and DR treatments.

Direct comparison between the effects of rapamycin and dietary restricted mice in this instance is not possible as the two sets of mice were not age matched to one another. In addition, treatment times were different, with 16 months of DR versus 4 months of rapamycin treatment. However, some of the parameters do show striking similarities, such as the reduction in TAp63 in both DR and rapamycin treatment and more γH2A.X foci in the peripheral cornea compared to their own experimental controls. These similarities provide evidence of a possible shared mechanism of both DR and mTOR in the effects on downstream genes. There were subtle differences however, while DR only reduced TAp63 in the central cornea, rapamycin reduced the amount of TAp63 in both corneal regions. Rapamycin caused no discernible change in the number of γH2A.X and TAFs in the central cornea unlike under DR where a decrease in both was observed. Surprisingly, rapamycin treatment increased the number of γH2A.X foci as well as TAF's in the peripheral cornea, which differed from DR whereby the number of TAF's decreased. In conclusion, rapamycin has some similar effects of DR, however, the differences between the two indicate that more is at play during DR than simply mTOR suppression.

4.6.5 TP63 is integral to mTOR and *Tert* regulation.

ΔNp63α has a duel role in regulation of *Tert*. Firstly, ΔNp63α can down regulate SIRT1 by targeting the molecule for proteasomal degradation. In the absence of SIRT1, p53 remains acetylated and does not inhibit RNA polymerase (Zhai and Comai, 2000), allowing for the transcription of *Tert*. In a contradictory paper, Palacious and colleagues state that SIRT1 increases telomerase activity (Palacios *et al.*, 2010), shown with telomere extension.

Secondly, the interaction of ΔNp63α with ABBP1, leads to the expression of alternative splice variants of *Tert* transcripts, α and β, which have a dominant negative effect on *Tert*, inhibiting its function (Vorovich and Ratovitski, 2009), however evidence

exists that these splice variants protect against apoptosis (Listerman *et al.*, 2013). Currently there is no information of the effect of the TAp63 isoforms on *Tert* expression. One could speculate that TAp63 might be up stream of *Tert* and mTOR signalling, as the knockout of *Tert* and suppression of mTOR did not change the accumulation rate of the TAp63 protein. Cam and colleagues present evidence for the interaction of TAp63 with mTORC1 (Cam *et al.*, 2014) as stated previously.

4.6.6 Foci formation is dependent on the presence of telomerase and diet.

When comparing ɣH2A.X levels it was expected that ɣH2A.X would be reduced in the WT DR mice, as telomerase has a protective function within mitochondria resulting in reduced ROS production (Indran *et al.*, 2011; Singhapol *et al.*, 2013). In combination with DR this should result in decreased levels of ROS, limiting DNA damage. Indeed, the cornea of WT DR mice had the fewest number of ɣH2A.X foci in the central cornea. Conversely, it was expected that if the presence of TERT contributes to the decrease of DNA damage during DR, then there should be no difference between KO DR and KO AL. However, considering a possible sensitisation of the DDR pathway by a decrease in mTOR, coupled with the lack of telomerase enzyme should increase foci numbers under DR and rapamycin treatment. In $Tert^{-/-}$ AL mice, a reduced number of foci were formed compared to $Tert^{-/-}$ under DR while in WT AL fed mice there was a higher number of foci formed compared to WT DR. Foci number was also higher in KO DR than in WT DR, This data seems to suggest that the beneficial effect of DR might be dependent on the presence of *Tert*. However when comparing foci number in the central cornea between KO AL and WT AL, there was a significantly higher amount of foci present in the wild type mice which cannot be explained easily. DR increased the foci number in both regions, central and peripheral with DR in $TERT^{-/-}$ mice. This seems to suggest that both, lack of TERT and DR exacerbate DNA damage in mouse cornea.

We can conclude that in the mouse cornea DR and telomerase both have important biological functions to safeguard the genome.

4.6.7 TAp63 is associated with the DDR

TAp63 is an important transcription factor, related to p53 and has similar roles in the cell as a tumour suppressor and in epidermal development (Candi *et al.*, 2007). p63 is a complicated gene, ΔNp63α can induce cell cycle arrest through the regulation of p53

(Dohn *et al.*, 2001). Conversely, ΔNp63α can also promote proliferation via interactions with β-catenin, a mediator of the Wnt pathway (Patturajan *et al.*, 2002).

Recently TAp63 has been suggested as being upstream of mTORC1, and is implicated in activation of the complex mediated via Akt. It is hypothesised that DDR increases Akt kinase activity, however Akt also activates mTORC1, via AMPK and TSC inhibition (Cam *et al.*, 2014). To prevent the cell from continuing to grow and respond to growth stimuli during DDR, REDD1 (regulated in development and DNA damage responses 1) is activated via phosphorylation by TAp63, which itself was phosphorylated by ATM (Ma *et al.*, 2010; Cam *et al.*, 2014). Phosphorylated REDD1 is able to inhibit mTORC1 by binding to 14-3-3/TSC complex, releasing TSC to inhibit mTORC1. In combination with REDD1, Sestrin-2 (p53 activated) act together to inhibit phosphorylation of 4E-BP1 (Cam *et al.*, 2014)(figure 4.19). Despite the activation of 4E-BP1, S6K is still active, potentially driving cell growth and proliferation.

In limbal epithelial cells p63 is up regulated, REDD1 may also be active, preventing excessive growth and proliferation which is essential for maintaining a stem cell pool, as shown in spermatogonial progenitors (Hobbs *et al.*, 2010). Perhaps p63 is up regulated in these cells to inhibit mTORC1 and hence prevent external stimuli, such as insulin and other growth factors, from stimulating cell growth. TAp63 is also connected to mTOR via NF- κB, mTOR can activate IKK (IκB kinase) which in turn regulates NF- κB. Repression of mTOR via rapamycin can lead to suppression of IKK (Dan *et al.*, 2008). Without IKK, IκB cannot phosphorylate IκB inhibitor (IκBα) and thus NF- κB is not released to translocate into the nucleus. NF- κB and p63 have an interesting effect on one another, both can interact with promoter regions of one another, creating a biological feedback loop (Sen *et al.*, 2011). The interaction with NF- κB is isoform dependent, with ΔNp63α down-regulated by NF- κB (Wu *et al.*, 2010), whilst TAp63α in combination with relA (transcription factor p65) can inactivate the NF- κB subunit p65 (Sen *et al.*, 2011), instigating cell death (Khandelwal *et al.*, 2011). This regulation controls a fine balance between cell cycle arrest and proliferation. Downstream of this interaction, NF- κB has been suggested as being supressed by mTOR in haematopoietic stem cells, leading to increased Fanconi anaemia group D2 protein (FANCD2) expression (Guo *et al.*, 2013). FANCD2 is part of the Fanconi anaemia DNA repair mechanism, which

localises to DNA breaks, sequestering the homology repair related proteins BRCA1 and BRCA2 (breast cancer 1-2, early onset) and γH2A.X (Bogliolo *et al.*, 2007).
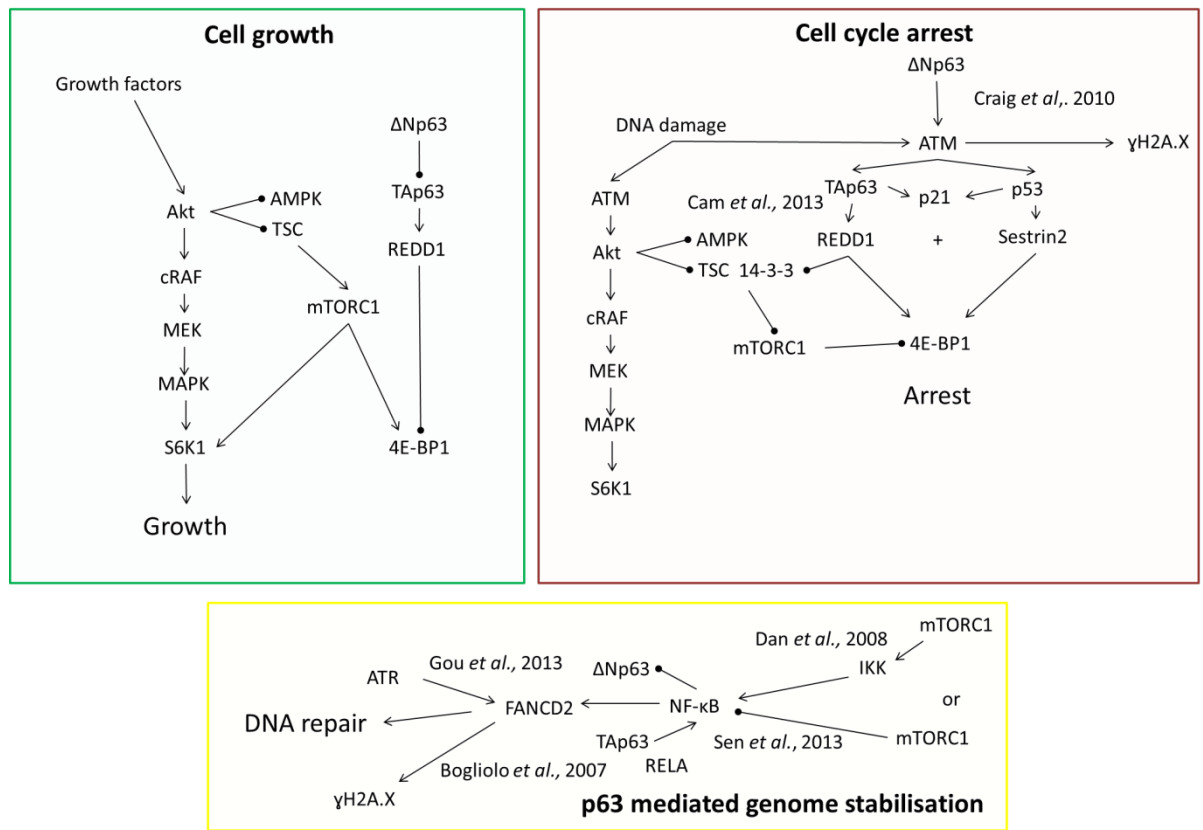
Figure 4.16 Scheme of the interactions of TAp63 with mTOR and DDR pathways
TAp63 plays an important role both up stream and down stream if mTOR, and is linked to the DDR pathway through NF-κB.

Drawing conclusions from these complex interactions is difficult, however what can be concluded is that p63 has a large effect on cellular growth and homeostasis. The fact that it is up regulated in limbal epithelial cells has implications on pathways such as mTOR. Figure 4.16 shows the interactions of TAp63, an important marker of limbal epithelial cells, in relation to the mTOR and DDR pathways. The up regulation of p63 in limbal epithelial cells could have an effect on these important nutrient sensing and DDR pathways.

4.6.1 The hypothesised TERT/Hsp90/mTORC1 complex could be required for genome stability in stem cells

Inhibition of both mTORC1 and TERT via independent mechanisms indicated an increase in genomic instability, shown by the increase in DNA damage during rapamycin treatment and nullification of the DNA damage amelioration during DR in TERT^{-/-} mice. Increases in Tert expression during DR are debated with some groups showing decreases

of *TERT* in the MDA-MB 231 breast cell line, however since the cells originated from a cancer cell line may influence the fundamental pathways associated with Tert expression (Hilal *et al.*, 2011). In wild type mice however DR has been shown to increase telomere length (Vera *et al.*, 2013). The later example has the implication that the up-regulation of *Tert* is Akt independent. Perhaps activated by an alternative pathway, potentially SIRT1, which is up-regulated during DR (Palacios *et al.*, 2010).

Direct sustained inhibition of mTOR and TERT leads to increased damage accumulation, as seen in the rapamycin group. Whereas DR does inhibit mTOR but TERT is potentially up regulated, as a result less DNA damage occurs. Where mTOR and TERT are both supressed (in DR Tert$^{-/-}$) damage was increased. We propose that stem cells have increased TERT expression and transient mTOR expression which culminates in less DNA damage and increased generation of progenitors, potentially via the TERT/Hsp90/mTORC1 complex.

In conclusion, the mouse central cornea is affected by age, it changes both morphologically and biochemically. We demonstrated that bullae increase with age while, the positive stem cell marker TAp63 decreases and the number of ɣH2A.X foci and TAFs increase. Interestingly, DR was able to remediate all of these changes, adding further weight to the benefits of this treatments anti-ageing effect. Rapamycin on the other hand, did not seem as beneficial for corneal tissue, with increased damage foci in the peripheral cornea. In light of the interactions with mTOR, perhaps this is due to the high expression of p63 isoforms within this tissue. The peripheral cornea often did not show the same patterns as the central cornea, indicating that this region seems functionally distinct from the central regions possibly harbouring the stem cell pool for this tissue.

Oxidative stress increases with age and has a big impact on stem cell function in HSC (Ito *et al.*, 2004) and MSCs (Brandl *et al.*, 2011). In addition to this the limbal niche like areas, which are suggested to shield stem cells for external stresses, plane with age (Notara *et al.*, 2013). We assessed if oxygen partial pressure had an effect on the culture of limbal epithelial cells.

Chapter 5 Hypoxia and limbal epithelial cells

5.0   Introduction

Since the first successful limbal stem cell transplantation was performed to treat LSCD, there have been numerous studies attempting to optimise the culture conditions of limbal cells. The goal being to increase the success rate of transplantations and reduce the amount of tissue needed. When optimising culture conditions one wishes to mimic the *in vivo* conditions as closely as possible. The most common oxygen concentration used in cell culture is 21% (160/760 mmHg), which is much higher than that present physiologically within major arteries (13%)(West, 2007). Once blood reaches distal regions oxygen concentration can dwindle to around 0.5-2.6% in capillaries (Brahimi-Horn and Pouyssegur, 2007). Epithelial cells of the cornea are, anatomically, uniquely positioned on the one hand close to atmospheric conditions but supplied by peripheral capillaries (Smith, 2002). The niche area however it can be argued is normally deeper within the epithelium. In 2013 Notara and colleagues showed that the limbal zone shows signs of degradation with age, in the form of eroded focal stromal projections and planed limbal crypts (Notara *et al.,* 2013).

5.0.1   Hypoxia, the niche and limbal epithelial cells

The niche helps to moderate the fate of the contained cells both regulating their differentiation and proliferation. In general the niche is often comprised of an extracellular matrix, nerves, neighbouring stem cells, and blood vessels (Jones and Watt, 1993; Gomez-Gaviro *et al.*, 2012; Ula, 2012). Some of these structures, such as the extracellular matrix, aid in allowing signalling molecules to penetrate into the niche, these can include juxtacrine and pararine molecules (Ordonez and Di Girolamo, 2012). The physical interaction with the niche has been shown to govern cellular fate of daughter cells, through the orientation of mitotic spindle during replication, triggering asymmetric division in sub-niche areas (Huttner and Kosodo, 2005; Lechler and Fuchs, 2005). In *Drosophila melanogaster*, the protein complexes in Inscuteable and Partner of Inscuteable (Pins) are implicated (Fuller and Spradling, 2007) as they are sorted in a polar manner and inherited by one daughter cell. In the human cornea, the exact location of the niche has yet to be defined, due mostly to inadequate positive markers.

The palisades of Vogt (POV) lie within the limbal region (Goldberg and Bron, 1982). Some scientists hypothesise that these fibrovascular ridges harbour limbal stem cells between each palisade (Townsend, 1991). Within the POV are microstructures which resemble crypt like structures these have been termed limbal crypts and focal stromal projections (Dua *et al.*, 2005; Shortt *et al.*, 2007; Molvaer *et al.*, 2013). Until truly accurate markers are discovered, the exact location of the limbal niche will remain unknown. Because of this uncertainty the exact oxygen concentration which limbal stem cells reside in physiologically will also remain hypothetical.

Limbal stem cells are ectodermal in origin, the same linage to which neural crest stem cells originate. Neural crest stem cells give rise to neurons and astrocytes among other cell types and are often routinely cultured under hypoxic conditions (Morrison *et al.*, 2000). Growth under hypoxic conditions can have many beneficial effects (Stacpoole *et al.*, 2013), the most notable being decreased exposure to reactive oxygen species (Prasad *et al.*, 2009; Yang *et al.*, 2012). It has been suggested that growth under hypoxia could be the result of early multicellular evolution, when the atmospheric oxygen conditions were low (Early Cambrian, 12.5% O_2) (Berner, 1999; Mills *et al.*, 2014). If this would be true however cells must also be adapted to high oxygen conditions, like those which occurred around 300 million years ago (Carboniferous, 32.5% O_2) (Goldblatt *et al.*, 2006).

Hypoxia is known to trigger the up regulation of key stem cell related signalling proteins such as Notch (Pistollato *et al.*, 2010), Oct-4 (Covello *et al.*, 2006) and TERT (Nishi *et al.*, 2004).

5.1 Methods

Epidermal cell culture was first achieved in 1975 (Rheinwald and Green, 1975a), quickly followed by corneal epithelium in 1977 (Sun and Green, 1977). The medium composition has varied very little over time and traditionally was comprised of basal DMEM supplemented with Ham's F12 at a 3:1 ratio (Ahmad *et al.*, 2006). Ham's F12 increases the proliferation rate of epithelial cells (Allen-Hoffmann and Rheinwald, 1984). The modern medium contains 6 major additives, plus antibiotics. These additives include hydrocortisone, insulin, tri-iodothyronine, adenine, cholera toxin and epidermal growth factor. These additives aid in producing the distinctive morphology of epithelial cells or reduce the serum requirements of the cultures (Rheinwald and Green, 1975b; Hayashi *et*

al., 1978). Morphology is improved by the promotion of motility of the cultured cells, as to inhibit the clumping of cells in the centre of colonies (Rheinwald and Green, 1977). Both insulin and tri-iodothyronine can reduce the requirements of serum from 20% to the commonly used 10% used in the majority of limbal epithelial media (LEM) today (Freshney *et al.*, 2002).

The first serum free medium developed for human keratinocytes was produced in 1981 (Maciag *et al.*, 1981b). Bovine pituitary extract (BPE) however was still included in this medium. BPE has a poorly defined variety of growth factors, hormones and other unknown substances (Kent and Bomser, 2003). Addition of these unknown animal products inhibits the use of subsequently cultured cells to be utilised for human transplantation purposes. The presence of unknown factors in the FCS allows for variability between batches. This can, in theory, alter the experimental outcomes, or render them null and void as one cannot distinguish between experimental and technical variability. In this study defined keratinocyte serum free medium (DKSFM, Invitrogen) was used without the addition of BPE. The full description of this medium choice is described in the introduction to this thesis. In brief, this medium has a low calcium content and has been shown to produce more consistent results. Positive corneal marker genes *ΔNp63* and *ABCG2* were shown to be increased in this medium, whilst the differentiation marker *KRT3* was reduced. Hence this medium seems to select for more progenitor like cells.

The growth of limbal epithelial cells in hypoxia is still a contentious area of study as there are contradictory results within the literature. Two different culture methods were used in these papers, Miyashita and colleagues used DKSFM, whilst O'Callaghan used LEM and the 3T3 fibroblast feeder system (Miyashita *et al.*, 2007; O'Callaghan *et al.*, 2011). To complicate comparisons even further, cell origins were also different. The feeder system was analysed in rabbit, whilst the human system was investigated using DKSFM. A recent paper however has confirmed some of the results stated in Miyashita's paper, showing an increase in CFE under hypoxia in LEM with 3T3 cells (Bath *et al.*, 2013). Again there is a disparity with the choice in media, with Bath and colleagues opting for Epilife medium. Since this medium has BPE as a supplement and also contains 60 μM of calcium, DKSFM was selected for this study. It is also important to note that Invitrogen recommends DKSFM for serum free growth of corneal cells and not Epilife

(Gibco, 2014). It was important then to further establish the biological trend of hypoxic growth of limbal epithelial cells with DKSFM.

5.2   Aims

The main aims of this chapter were to assess if two different limbal epithelial cell culture media (LEM and DKSFM) have an effect on the culture and growth of the stem cells and to investigate the effect of different oxygen tensions for both medium compositions on growth and stem cell parameters. Additionally, to characterise corneal epithelial cultures using colony forming assays, gene expression, cell cycle analysis and a metabolic assay.

5.3   Results

5.3.1   Can serum free hypoxic conditions increase the efficiency of limbal epithelial cell containing cultures?

Cells were extracted using methods outlined in chapter 2 and cultivated under hypoxic condition of 3% and normoxic condition of 21% oxygen. To ascertain the effect that hypoxic and normoxic conditions had upon limbal epithelial cells grown in two different media conditions, several experiments were designed. These included growth assays in the form of colony forming assay, gene expression profiles with qPCR and protein dynamics using immunofluorescence. In addition to this for the first time the metabolic state of these cells was assessed. One major difference when culturing limbal epithelial cells in serum free conditions is the difference in shape when forming colonies. When epithelial cells are grown on 3T3 cells, colonies form which have defined boundaries and cells appear small and compacted (figure 5.1). Serum free culture on the other hand produces no defined colonies, cells appear larger and flattened.
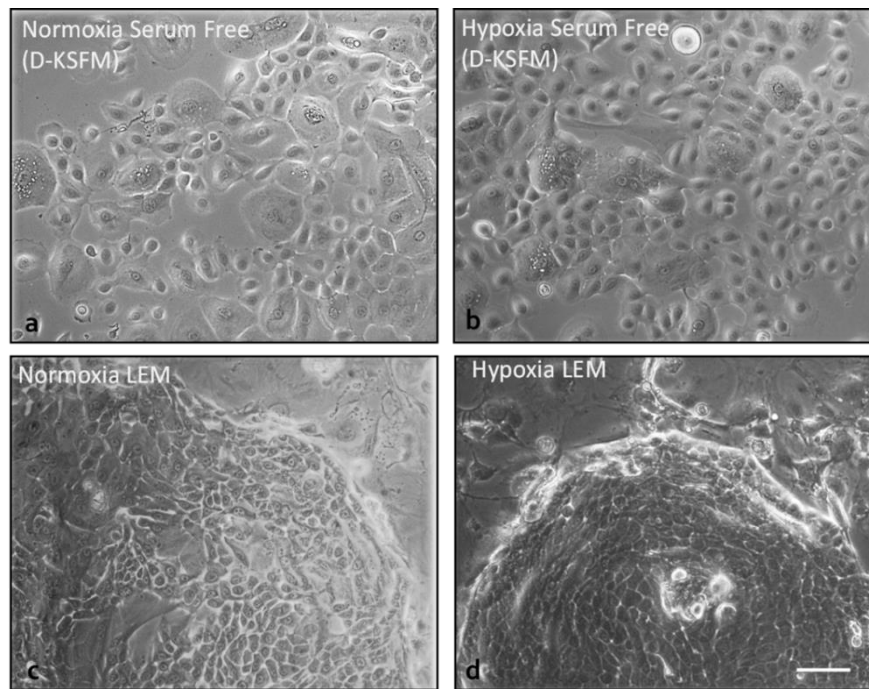
Figure 5.1 Phase contrast images of limbal epithelial cell containing cultures
The top two panels are limbal cells isolated into serum free conditions, whilst the bottom two panels are cells isolated onto 3T3 fibroblasts in limbal epithelial medium (LEM) (Scale bar equals 50μM).

5.3.2   Analysis of colony forming abilities of cultivated limbal epithelial cells.

Colony forming efficiency assay is a basic test which can be used to determine the quality of the culture (see chapter 2). The more numerous and larger size colonies produced are thought to be directly proportional to the approximate number of initial stem cells. For this reason this assay was chosen as a benchmark.
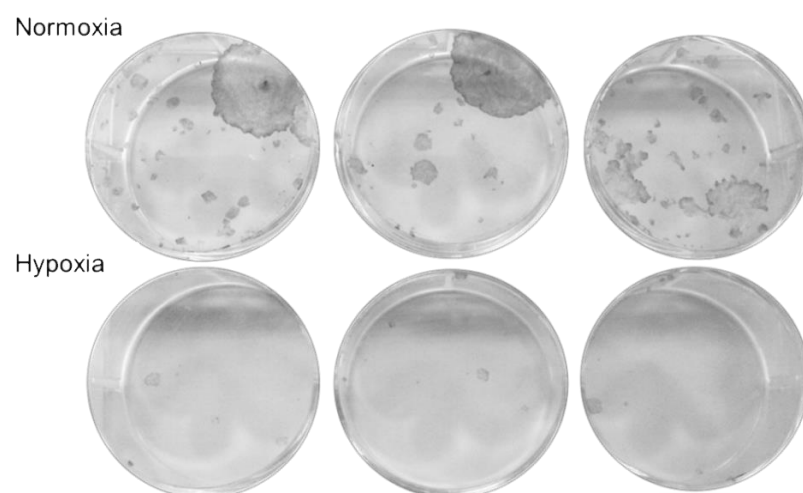
Figure 5.2 Example of a colony forming efficiency assay of a culture cultivated in LEM under normoxic and hypoxic conditions
A series of 3 repeats taken from the same donor primary culture. Normoxic conditions are depicted on the top panel and hypoxic conditions on the bottom panel (M18052B, 44 year old donor).
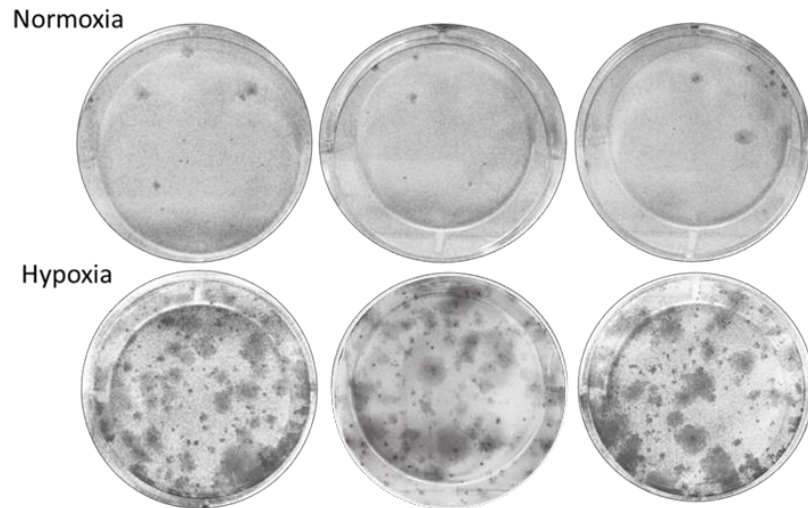
Figure 5.3 Example of a colony forming assay of cultures cultivated in DKSFM under normoxic and hypoxic conditions
In serum free conditions under normoxia (top panel) very few colonies formed compared to hypoxia (bottom panel) (M18362B, 85 year old donor).

The CFE plates shown in figure 5.2 and 5.3 are typical examples of colony forming efficiency plates. In figure 5.2 there are defined colonies with clear borders, typical of the traditional culture system (LEM). In contrast figure 5.3 shows examples of colonies derived from cells grown in DKSFM. These colonies do not have the same defined edges, expected in serum free growth of epithelial cells (Miyashita *et al.*, 2007).
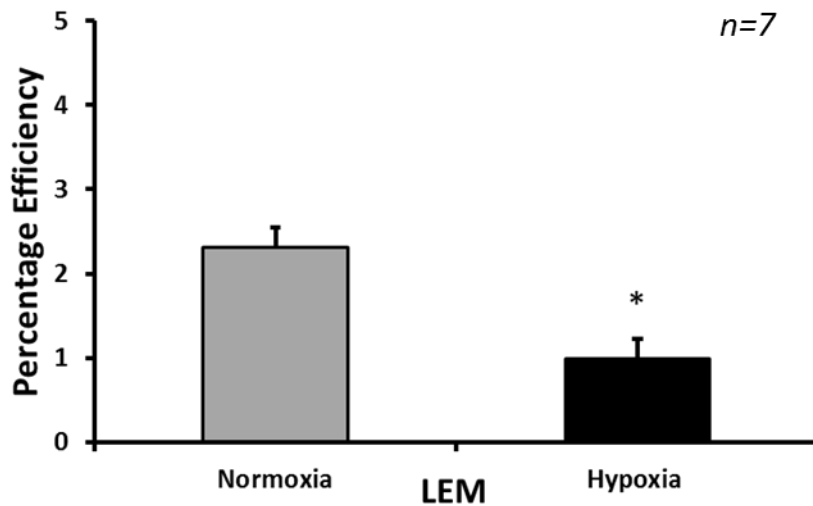
Figure 5.4 Colony forming efficiency of cultures grown in LEM
Cultures were cultivated for 12 days under normoxia and hypoxia. 7 donors were tested in a pairwise manner. The initial extracted cells were divided equally between normoxic conditions and hypoxic conditions. Standard error of the mean is shown, *p=0.003*.

The data in figure 5.4 was normally distributed, according to a Shapiro-Wilk test *p=0.880* and *0.145* for the normoxic and hypoxic groups respectively. A paired sample *t-test*

showed that the difference between groups was significant at *p=0.003* with the confidence interval set to 0.95. A total of seven donors were tested in this condition. This indicates that cultures grown in LEM favour the normoxic condition.
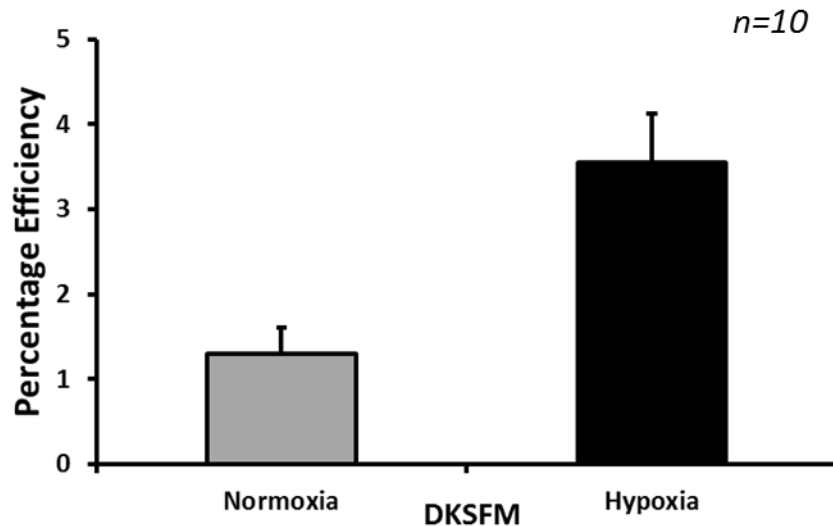
Figure 5.5 Colony forming efficiency of cultures grown in DKSFM
Cultures were cultivated for 12 days under normoxia and hypoxia 10 donors were analysed. Under serum free conditions limbal epithelial cells produced more colonies in hypoxia conditions than in normoxic conditions, however this was statistically not significant. Standard error of the mean is shown.

Colony forming efficiencies of another set of seven donors were analysed in DKSFM (Figure 5.5). A Shapiro-Wilk test was performed, it showed that the data was distributed normally with *p=0.998* and *0.174* respectively. A paired samples t-test showed that there was a no statistical difference between the two conditions (*p=0.067).*

Independent samples *t-tests* were used to compare between growth in LEM and DKSFM in normoxia, there was a statistical difference between the two medium (*p=0.025*). Indicating that LEM produces more clones in the traditional culture conditions. In hypoxia the reverse is true, with significantly more colonies being present in hypoxia when grown in DKSFM. To find out if the hypoxic growth in DKSFM produced more colonies than the normoxic growth in LEM another independent samples *t-test* was performed. There was no statistical difference between the two conditions (*p=0.261*), indicating that both conditions are equal in terms of the successful culture of LSCs.

This experiment showed a decrease in colony forming ability of potential LSC in LEM under hypoxia. This result is consistent with O'Callaghan and colleagues observations in rabbit epithelial cell cultures (O'Callaghan *et al.*, 2011). In serum free

medium however there appeared to be an increase in cells under hypoxia, this difference however was not significant. One can conclude that DKSFM is not as detrimental to the ability of LSC to form colonies in hypoxia compared to LEM.

5.3.3 Does colony size differ between cultures under different oxygen tensions and culture conditions?

Barradon and Green emphasised that colony size was an important parameter in determining the clonal composition of cultures. They stated that larger colonies were more likely to have originated from holoclones (Barrandon and Green, 1987). Holoclones are documented as containing the highest proportion of stem cells of any clonal group.

The data generated from measuring colony size included some samples which were not normality distributed (Shapiro-Wilk test), values for which are summarised in table 7.31.

The size of colonies was not dictated by the oxygen concentration used ($p=0.720$), neither were they affected by the two different medium ($p=0.167$). The assigned categories (<1mm, 1-4mm and >4mm) did indicate a significant difference between one another ($p<0.001$), regardless of media composition. Multiple pairwise analyses were performed using the Holm-Sidak method to determine were significance lay between sizes (table 5.1).

Size	Holm-Sidak test (p values)
1mm vs 4mm	*<0.001*
1mm vs 1-4mm	*<0.001*
1-4mm vs 4mm	*<0.001*

Table 5.1 Holm Sidak tests for colony size

These results mirror those of chapter 3 which also showed that the colony size categories were distinct from one another.
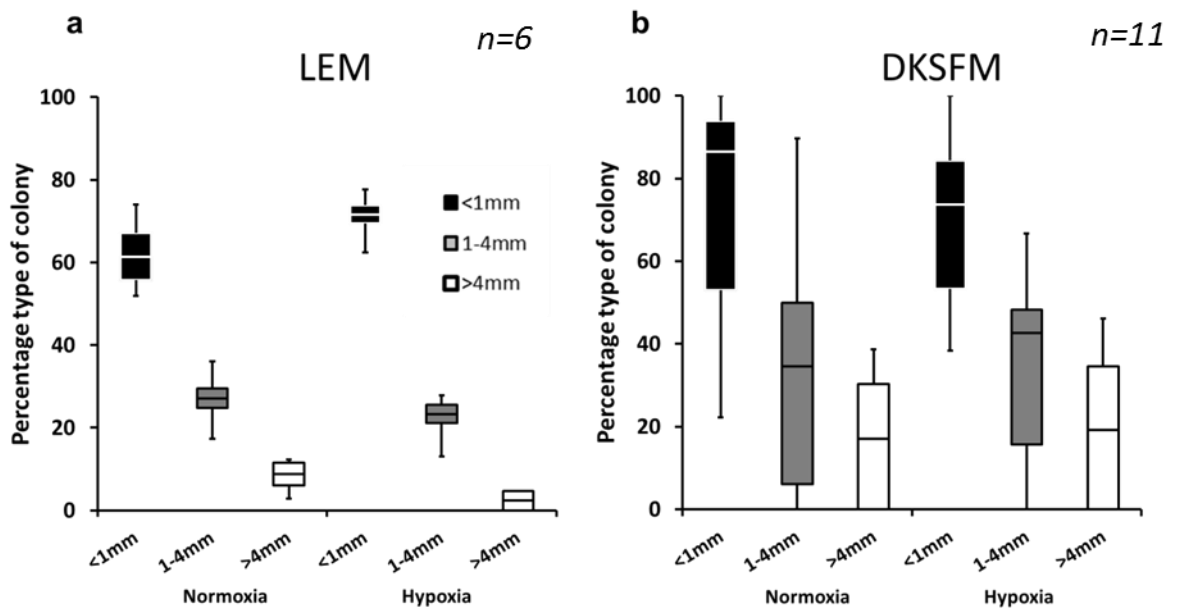
Figure.5.6 Box and whisker plots of colony size between cultures cultivated under normoxia and hypoxia in DKSFM and LEM
Panel a and b depict box plots which show the maximum, the 75th percentile, the median, the 25th percentile and the minimum values. 6 donors were tested for the LEM group, while 11 donors were in the DKSFM group.

In DKSFM colonies are more diffuse which made quantifying the size more difficult. This is probably the source of the variation in these samples (figure 5.6, b).

5.3.4 Gene expression analysis of cultures grown in LEM and DKSFM media under normoxia and hypoxia

Previous studies have focused on generating gene expression data in serum supplemented cultures. However the amount of data currently available for serum free cultures is limited, therefore the main focus was on producing new and novel gene expression data in the primary cells isolated into serum free conditions in both normoxia and hypoxia. *ABCG2*, *TERT* and *BMI1* were all used to determine stem cell quantities and are positive marker genes (Li *et al.*, 2004; Watanabe *et al.*, 2004; Barbaro *et al.*, 2007; Aslan *et al.*, 2012). *KRT3* and *KRT12* (Keratins 3 & 12) are negative markers for limbal epithelial cells and are associated with differentiated cells (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). *p21* is a gene which is associated with DNA damage and stress downstream of p53 (Insinga *et al.*, 2013).

Firstly the expression levels were assessed between the two medium in normoxia (figure 5.7) and then hypoxia (figure 5.8). In order to compare the data in this way a normalising sample was required. The LEM sample with the lowest C_t was chosen. In this

instance both the lowest or highest DKSFM and LEM C_t values would have been valid
normalisers and would consequently produce the same statistical result.
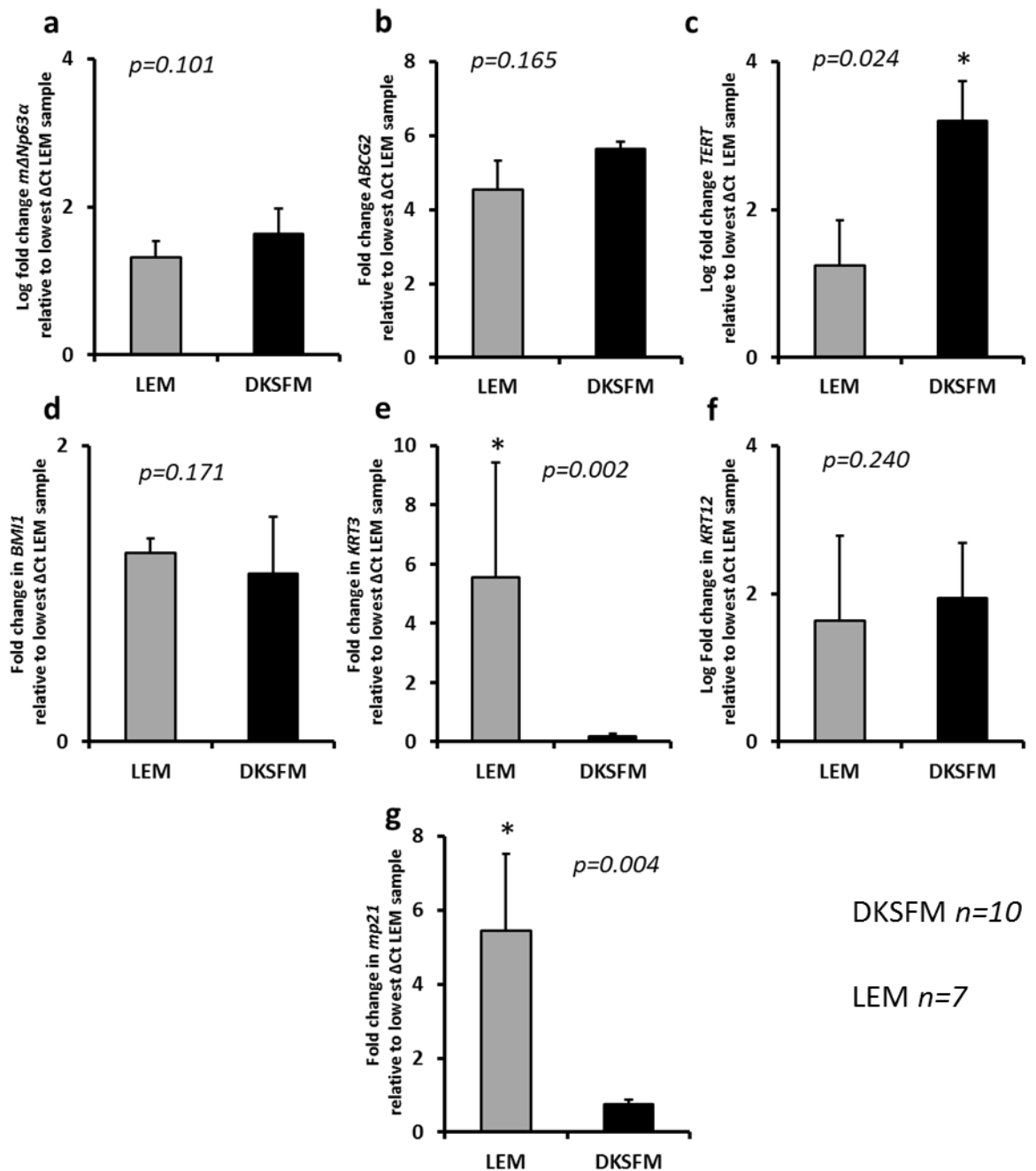
Figure 5.7 Expression of various genes compared to GAPDH between LEM and DKSFM in normoxia
Expression levels were normalised to the lowest ΔCt value of the LEM sample. DKSFM has a significant
amount more of the positive marker *TERT* than cultures in LEM. The negative marker *KRT3* was
significantly up regulated in LEM compared to DKSFM. The DNA damage marker *p21* was also significantly
up regulated in LEM. Standard error of the mean is shown.

Shapiro-Wilk tests showed that data from *ΔNp63α* and *TERT* were normally distributed
($p=0.534$ and 0.078), independent samples t-tests were performed on these samples. All
other data sets were not normally distributed, thus independent samples Mann Whitney

tests were performed on those data sets. DKSFM seems to increase the levels of *TERT* while suppressing *KRT3*, indicating that this medium could be beneficial for the culture of limbal epithelial cells (figure 5.7).
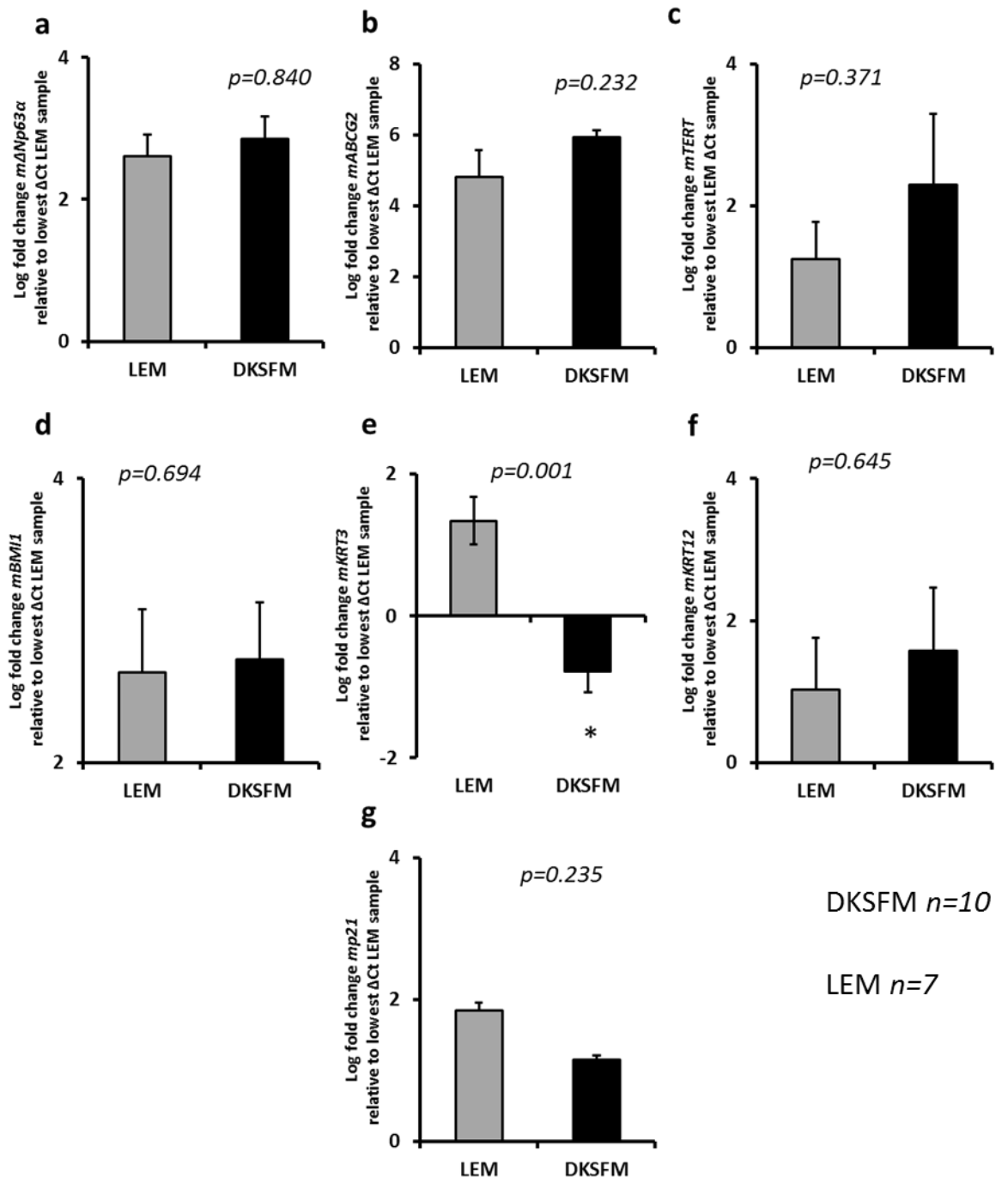
Figure 5.8 Expression of various genes compared between LEM and DKSFM in hypoxia
Expression levels were normalised to the lowest ΔCt value of the LEM sample. There was significantly reduced KRT3 expression in DKSFM under hypoxic conditions. Standard error of the mean is shown.

In hypoxic conditions the data for *ΔNp63α*, *ABCG2* and *BMI1* were not normality distributed (*p=0.035*, *p<0.001* and *p<0.001*). As a result independent samples Mann

Whitney tests were performed. All other genes were distributed normally and independent samples *t-tests* were performed on those. Only the negative marker *KRT3* showed a significant decrease in DKSFM (figure 5.8). This indicates that DKSFM could promote more progenitor-like cells in hypoxic conditions. The comparison of data in this way can often lead to false positives, due to the variation between each individual qPCR run. To rule this out a comparison between the C_t values of GAPDH was performed. The average deviation between each run was around 0.5 Ct (see appendices A), which equates to around 0.7 fold change (assuming 100% efficiency) which was much lower than any of the differences measured.

Interpretation of qPCR results is dependent on the normalisation of each dataset. An alternative but equally valid method of quantification is by using an experimental condition as the "normal" gene expression level. The following analysis was normalised against normoxia allowing assessment of the change between the two oxygen tensions directly. This differed from the analysis performed in figure 5.7 and 5.8, which compared media directly.
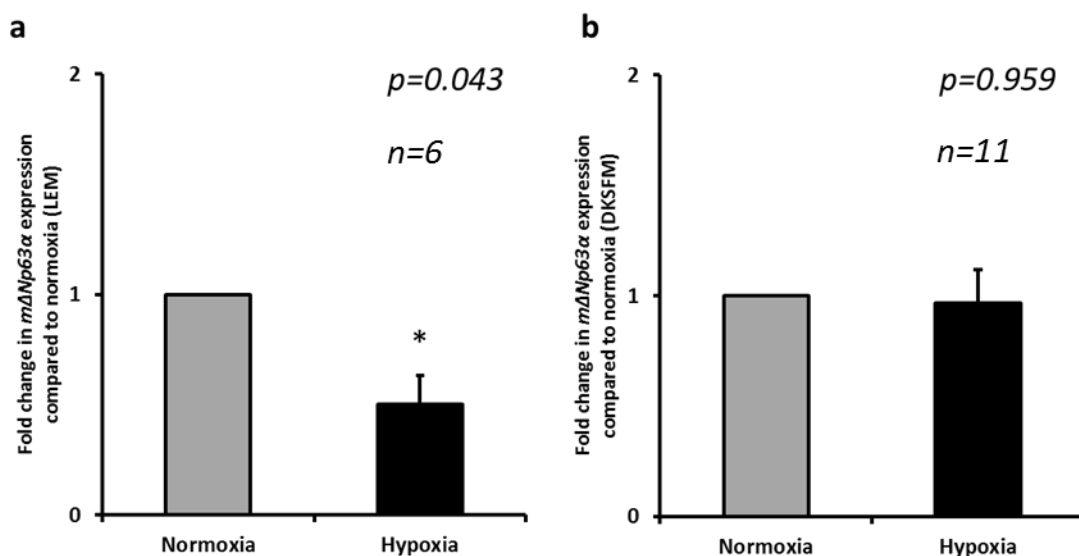
Figure 5.9. Gene expression of ΔNp63α in DKSFM and LEM under either normoxia or hypoxia
ΔNp63α expression of cells from each donor under hypoxia was normalised against the counterpart sample grown under normoxia. All values were normalised to the reference gene GAPDH. A total of 6 donors were tested in LEM and 11 donors were tested in DKSFM, error bars shown are standard error of the mean.

As previously stated, each independent sample was normalised to its normoxic counterpart, because of this there was a non-normal and a normally distributed data set. This required a non-parametric test to be performed. The non-parametric test

utilised was the related samples Wilcoxon signed rank test (Wilcoxon, 1946; Yuan *et al.*, 2006). A Shapiro-Wilk test showed that the hypoxic LEM data was normally distributed (*p=0.367*), however the DKSFM hypoxic data was not normally distributed (*p=0.005*) (table 7.33). A *t-test* showed that the difference in *ΔNp63α* expression between normoxia and hypoxia was significant when cells where cultured with LEM and co-cultured with 3T3 fibroblasts (Figure 5.9, a) (*p=0.043*). When limbal epithelial cells were grown in DKSFM under the two atmospheric conditions there was no significant difference between the amounts of mRNA transcripts of *ΔNp63α* (*p=0.959*) (figure 5.9, b).

The same array of genes as shown in figures 5.7 and 5.8 were then tested. Shapiro-Wilk tests showed that the data for the *ABCG2* expression was not normally distributed. A related samples Wilcoxon signed ranks test was used to determine significance, with the significance level set to 0.05. *BMI1*, *KRT3* and *p21* were significantly down regulated in hypoxia when compared to normoxic counterparts. *KRT3* codes for an intermediate filament protein keratin 3 found in the corneal epithelium, its expression is associated with more differentiated limbal cells (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). This down regulation suggests that hypoxia suppresses the differentiation of limbal cells. BMI1 is a polycomb complex protein which can regulate *CDKN2A* (*p16*) and is associated with cell cycle progression. Upon DNA damage, *BMI1* is recruited to these damaged sites, where it ubiquitylates the histone H2A (Ismail *et al.*, 2010; Ginjala *et al.*, 2011). In adult stem cell biology *BMI1* is required for the self-renewal of neural and haematopoietic stem cells (Lessard and Sauvageau, 2003; Molofsky *et al.*, 2005). More importantly, it has been associated with the renewal of the dermis (Claudinot *et al.*, 2005). The down regulation of *BMI1* is surprising. On one hand it can be inferred that there could be less DNA damage occurring. However a reduction in *BMI1* can also be indicative of a reduced number of stem cells or of a reduction of self-renewal capacity. One possible explanation would be that BMI1 was only required during cellular replication (Lessard and Sauvageau, 2003), hence hypoxic cultures might be less prolific.

Results on colony forming efficiency shown in figure 5.5 however contradict this assumption as there appears to be an increase in the number of cells which arise in hypoxia, although this was not significant. *CDKN1A* (p21) is a cyclin dependent kinase inhibitor, the subsequent protein this gene produces inhibits CDK1,2 & CDK4/6 (Gartel and Radhakrishnan, 2005). These are key proteins in the regulation of G_1 to S phase cell cycle progression. It has been shown that p21 can also initiate senescence (Chen *et al.*, 2005). Lower levels of p21 in hypoxia indicate that there could be less DNA damage events. This result confirms the *BMI1* expression and further solidifies the link between the two genes (Fasano *et al.*, 2007).
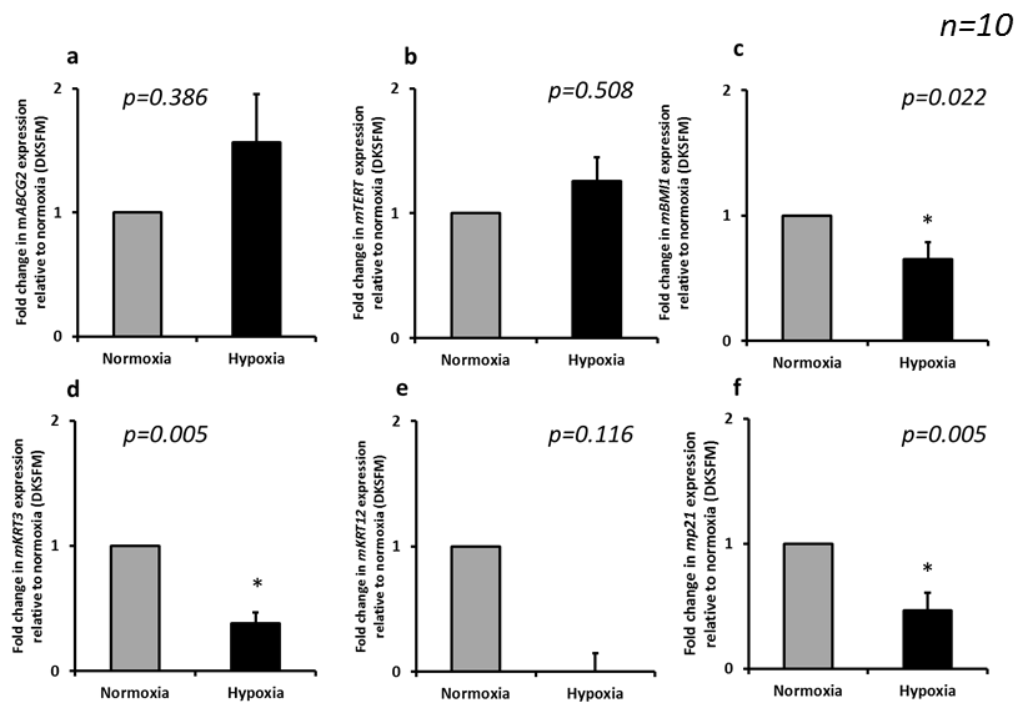
Figure 5.10 Gene expression of limbal epithelial cells derived from ten donors cultured in DKSFM under normoxia and hypoxia
GAPDH was always used as the reference gene. All results were normalised to normoxia which was set to 1 (100%). The total number of donors tested was 10 for each gene. Asterisks indicate significance Standard error of the mean is shown. (*BMI1, p=0.022, KRT3 p=0.005, p21 p=0.005*).

Shapiro-Wilk tests showed that the *BMI1*, *KRT12* and *p21* data was normally distributed (Table 7.34) for donors grown in LEM under the atmospheric conditions of normoxia and hypoxia. Non parametric tests were used as the normalised data was not normally distributed. Firstly related samples Wilcoxon signed ranks tests were performed on the data. Figure 5.11 shows that there was no significant difference between the gene expression of *ABCG2*, *BMI1* and *KRT12* under normoxia and hypoxia in the analysed samples. *KRT3*, *TERT* and *p21* were significantly different under hypoxia compared to

normoxia. *TERT* codes for the catalytic subunit of telomerase, a reverse transcriptase responsible of the addition of repeat telomeric units to the termini of each chromosome (Greider and Blackburn, 1985). Telomerase is expressed in adult stem cells (Hiyama *et al.*, 1995; Chiu *et al.*, 1996), including limbal epithelial cells (Umemoto *et al.*, 2006; Notara *et al.*, 2013). The up regulation of this gene in hypoxia was interesting as it could indicate more actively dividing progenitors in this condition. This was further affirmed by also observing a reduction of *KRT3*, a corneal differentiation marker, as described previously.

Interestingly, the expression of *KRT3* was down regulated in both DKSFM and LEM, under hypoxia. Suggesting that hypoxia regulates this gene and is regardless of the culture medium. Hypoxia can induce the degradation of keratin filaments 8 and18 in rat alveolar epithelial cells, via the mediation of oxidants in the cytosol (Na *et al.*, 2010). It seems then that hypoxia may also affect keratins at the transcriptional level, oxygen tension appears to be a key mediator of these intermediary filaments.
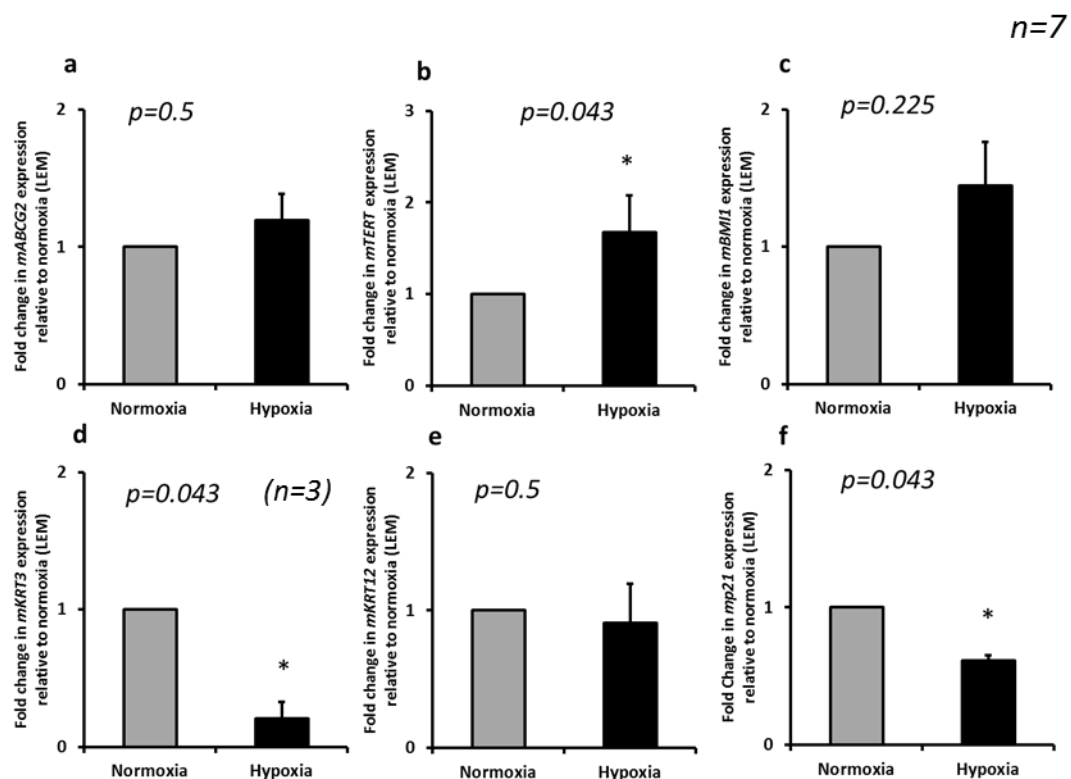
Figure 5.11 Gene expression of limbal epithelial cells from seven donors cultured in LEM under normoxia and hypoxia
Cells from seven donors were analysed for all genes, with the exception of *KRT12* where only three donors where used. Asterisks indicate significance. Standard error of the mean is shown.

5.3.5 Does ΔNp63 immunofluorescence intensity change with oxygen tension in cells cultured in DKSFM and LEM?

To study ΔNp63 at the protein level, immunofluorescence was carried out. The main reason for selecting immunofluorescence over western blotting was because the method requires fewer cells from each culture. At various points throughout this project donor tissue was scarce; to maximise each donor cultures they were often divided between multiple experiments. Statistical analysis showed that the data for the immunofluorescence of ΔNp63 was normally distributed (Shapiro-Wilk, *p=0.495* and *0.109*). A paired sample *t-test* was performed on the DKSFM data between normoxia and hypoxia which showed the value *p=0.167* (figure 5.12). The null hypothesis can be accepted, that there is no difference between the two oxygen tensions in DKSFM. This result may be due to the limited numbers of donors tested (n= 6). In LEM the data was also normally distributed, *p=0.728* normoxia and *p=0.165* hypoxia. A *t-test* revealed that there was also no statistically significant difference between normoxia and hypoxia *p=0.811*.
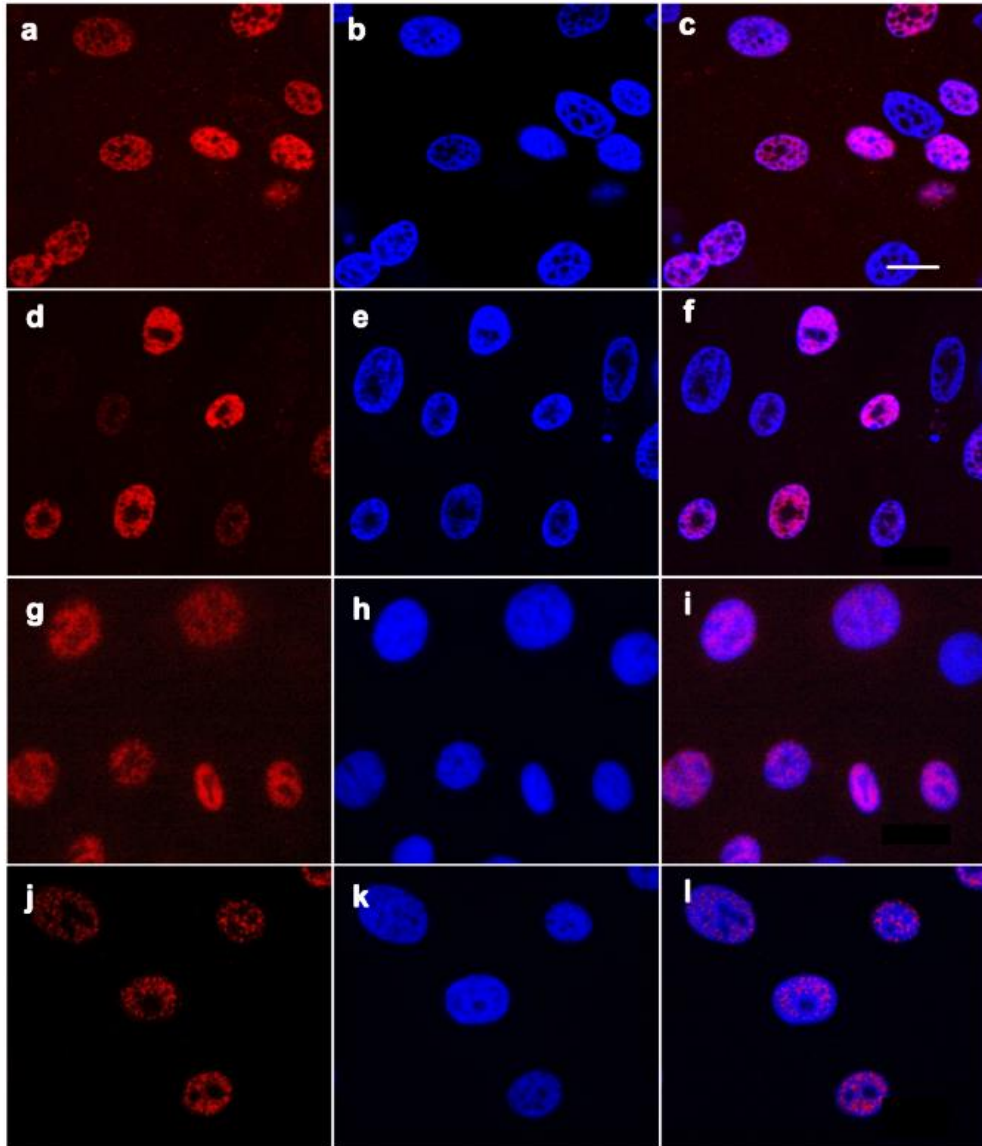
Figure 5.12 Immunofluorescence analysis of ΔNp63 in limbal epithelial cell containing cultures under normoxia and hypoxia in DKSFM or LEM

Cultures were measured at the same time and with same exposure conditions, x63 magnification was used. Standard error of the mean is shown. Panels a, d, g & j show the localisation of ΔNp63 in limbal epithelial cells (red), the nuclei were stained with DAPI (blue) (Panels b, e, h & k). Panels c, f, i & l show the two channels (red and blue) combined. Panels a c are cells cultured in LEM under normoxia. Panels d- f are LEM cultured cells in hypoxia. g-i are cells cultured in DKSFM under normoxia. j-l are cells cultured in DKSFM under hypoxia. There was no statistical difference between the amounts of ΔNp63 in cultures in hypoxia over those in normoxia cultured in DKSFM or LEM. Scale bar is 15μm.
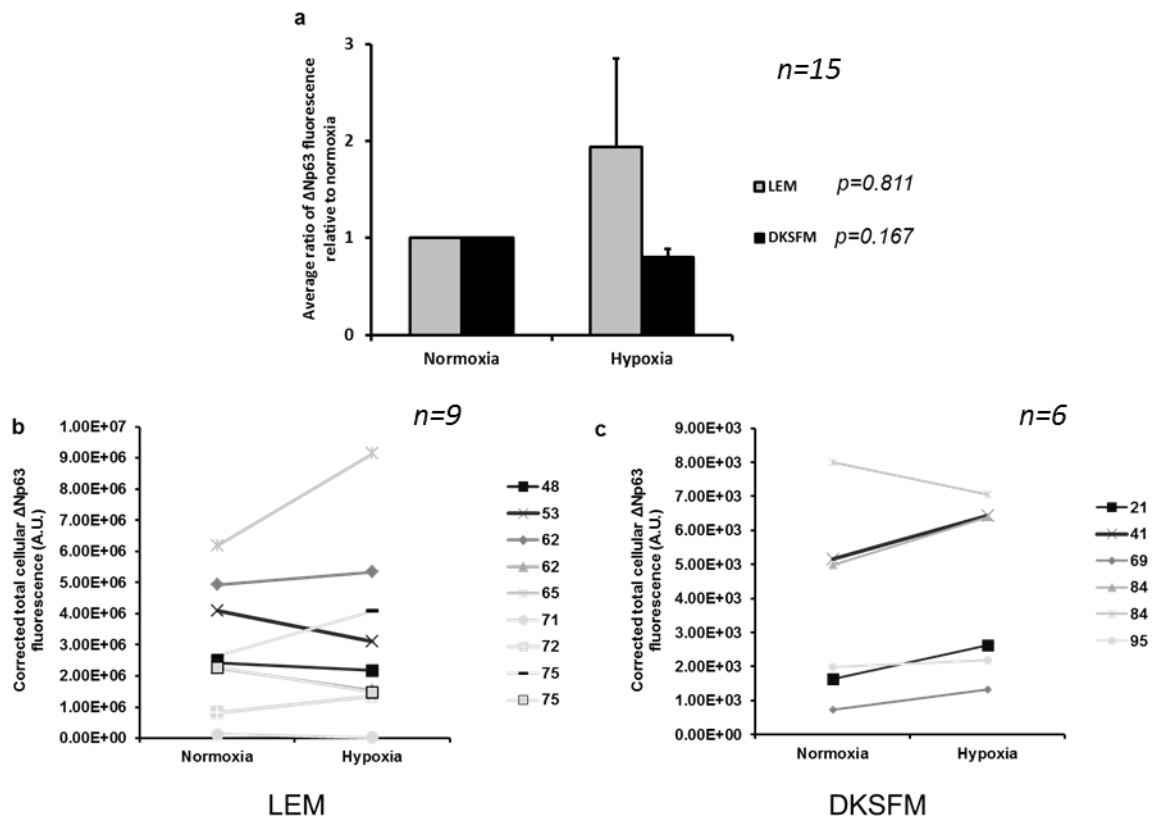
Figure 5.13 Comparison of ΔNp63 immunofluorescence of individual cultures between normoxia and hypoxia in DKSFM or LEM
There appeared to be no correlation between donor age and the quantity of ΔNp63 detected. Within each different medium all cultures were analysed on the same day with identical staining parameters. The ratio between normoxia and hypoxia was used to compare the data between DKSFM and LEM (a), to account for variation in staining due to temporal separation. Panels b and c show correlations with age. (*p=0.175*).

To test the difference between ΔNp63 in LEM and DKSFM, which had been performed on different days, the ratio between normoxia and hypoxia was calculated. Comparison between the ratios which each medium produced was not normally distributed, as a result a Mann Whitney test was performed. There was no statistical difference between LEM and DKSFM when normalised to normoxia (*p=0.175*) (Figure 5.13, a). The lack of significance is likely to be due to the high variation in donor cells cultured in LEM. Figure 5.13 also shows the correlation between ΔNp63 protein level and donor age (panel, b and c), indicating that age had little if no effect on the quantity of ΔNp63 protein. Generally however the amount of ΔNp63 cannot be affected by hypoxia, although there was a trend which indicated an increase of ΔNp63 in LEM under hypoxia. This challenges the results of Robertson and colleagues, who stated that *ΔNp63α* is down regulated in hypoxia (Robertson *et al.*, 2012). This paper however used a telomerase-immortalised human corneal epithelial cells (hTCEpi) cell line and not primary donor cells. In addition to the use of the cell line, the condition in which hypoxia was induced was different,

with $CoCl_2$ used as opposed to a nitrogen purged incubator as in this study. A nitrogen purged incubator has the benefit of a constant level of passive hypoxia. $CoCl_2$ on the other hand is oxidised and requires replenishment of the substance, this makes monitoring the exact level of hypoxia difficult. Nitrogen purging incubators also have there drawbacks, namely the needed removal of cultures from the incubator in order to feed. Our data does confirm that observed by Bath and colleagues, with hypoxia increasing ΔNp63 in LEM, although in DKSFM our study showed no difference. Bath on the other hand demonstrated an increase in serum free medium under hypoxia (Bath *et al.*, 2013). These experiments differed however, as Bath and colleagues used Epilife medium rather than DKSFM (Bath *et al.*, 2013).

5.3.6 Does the proportion of cells in S phase change in limbal epithelial cells cultured in DKSFM under normoxia and hypoxia

An important parameter of stem and TA cells is their ability to undergo replication. DNA replication occurs during synthesis (S) phase of the cell cycle, it is possible to detect the amount of DNA at this stage by observing the accumulation of double the normal quantity of DNA within the nuclei (4N) (figure 5.14, a). Stem cells should be especially sensitive to this test as they are thought to remain in this state for prolonged periods (Morris *et al.*, 1985; Kruse and Tseng, 1992).
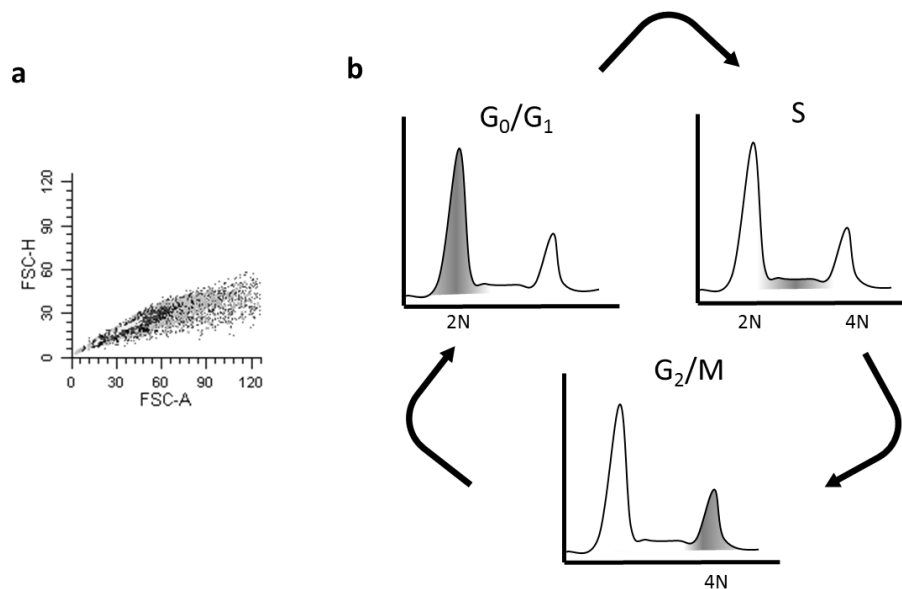
Figure 5.14 Scheme detailing the principles of cell cycle analysis
Data are normally collected for the fluorescence emitted from exposed nuclei and plotted accordingly. This was normally done after gating the events to exclude duplets and debris based on their side scatter (a). Nuclei with the normal diploid nucleic content (2N) were indicative of cells in G_0/G_1, between 2N and 4N indicated the S phase fraction. G_2/M phase had double the normal nucleic content at 4N. The grey area was measured for each cell cycle point(s) (b).
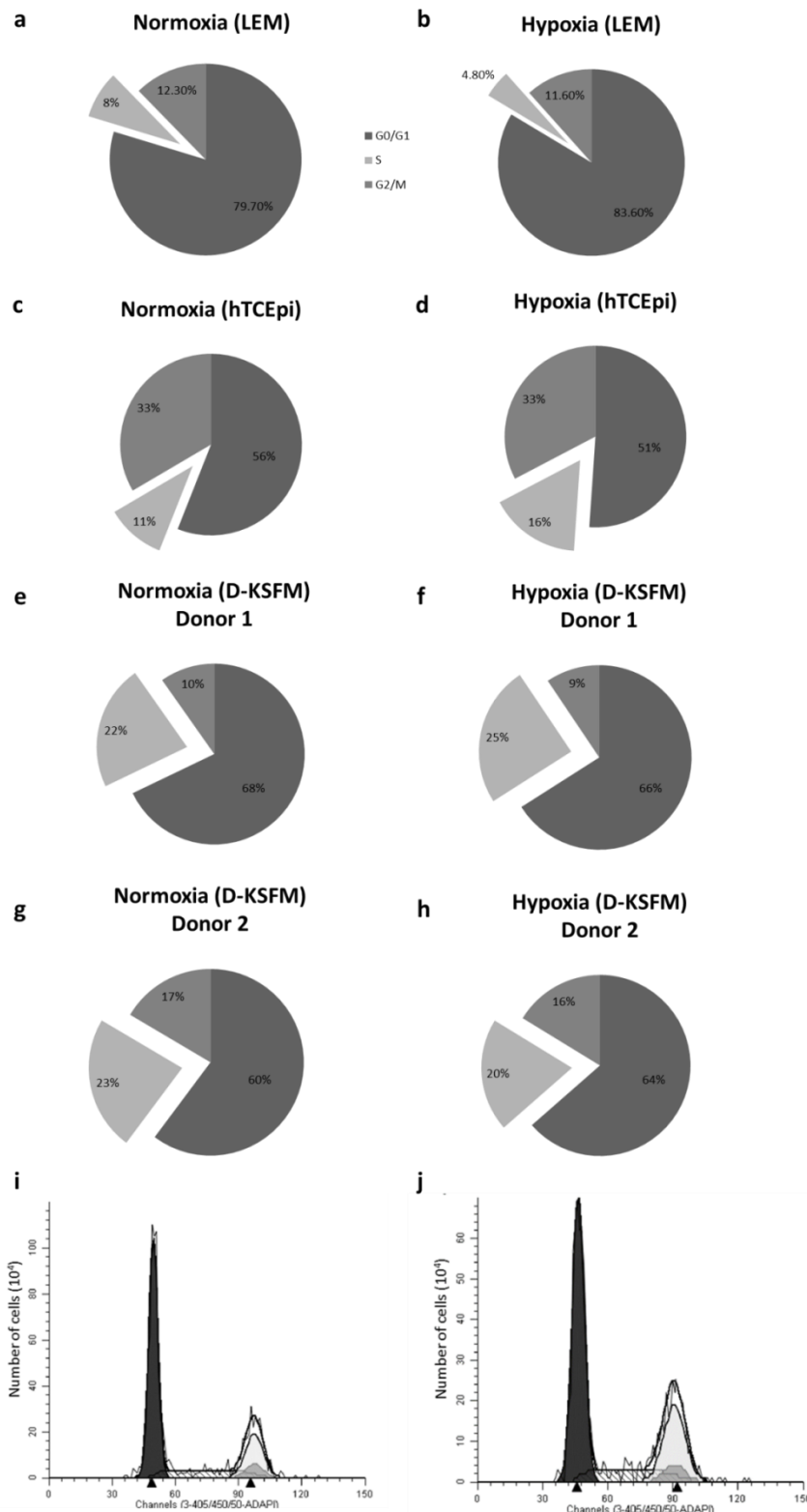
Figure 5.15 Percentage for different cell cycle phases/stages of human limbal epithelial cell containing cultures.

Cell cycle analysis was performed using DAPI to detect the quantity of DNA within each nucleus from a population. Cells were lysed and processed through a flow cytometer Canto II. On average 10^5 cells were assessed per sample. Panels, a-h show the proportions of cell in each part of the cell cycle, S phase is shown as a cut out slice in each pie chart. Panel I & j, fluorescence is depicted on the x-axis and the cell count on the y-axis. The solid arrows in the x axis show 2N and 4N. One donor was tested in LEM, while two were tested in DKSFM. The cell line HTCEpi was used to validate the experiment before primary samples were used.

Figure 5.15 shows nuclei in G_0/G_1 (Darker grey), S phase (Light grey) and G_2/M (Dark grey). G_0 and G_1 are grouped together because for this experiment the only intact cellular component tested was the nuclei. The rest of the cell was lysed, so no increase in cell size can be measured with this assay. Synthesis phase increases the amount of DNA from 2N to 4N, thus any quantity of DNA between these amounts would be S phase. This is represented as the hatched area on the histograms (figure 5.15, I & j). G_2 is the second gap phase before mitosis can begin, and allows the cell to check for DNA damage. It was not possible to distinguish this phase from mitosis (M phase) because both can contain the same quantity of DNA (4N).

In LEM the percentage of nuclei in S phase under normoxia was higher than that in hypoxia, however only 1 donor was tested. This suggests that there are less putative stem cells in hypoxia in this medium. In DKSFM, where 2 donors were tested, there was little change of S phase proportion between the two conditions, 22 & 23% of the total cellular population normoxia and 20 & 25% in hypoxia. As only 3 donors overall were tested, this means that one can only make vague assumptions based upon these initial results. Additional donors were analysed, however the number of events/cells registered by the FACSCanto was not sufficient enough to perform analysis on.

5.3.7 Does medium composition and hypoxia change telomerase activity in limbal epithelial cells?

To complement the *TERT* gene expression data, the activity of telomerase was measured, using the reverse transcriptase function of the enzyme.

A two way ANOVA was performed on the cultures grown in DKSFM and LEM which showed that the difference between the mean values of each medium was greater than would normally be assumed by chance, with *p<0.001* (figure 5.16). Oxygen concentration on the other hand did not prove to be statistically significant with *p=0.474*. There appeared to be a slight tendency however, which indicated that DKSFM may increase telomerase activity in hypoxia. The converse was true in LEM with less telomerase activity in hypoxia over normoxia. The combination of these results indicated that normoxic growth in LEM to be most favourable for telomerase activity and perhaps progenitors.
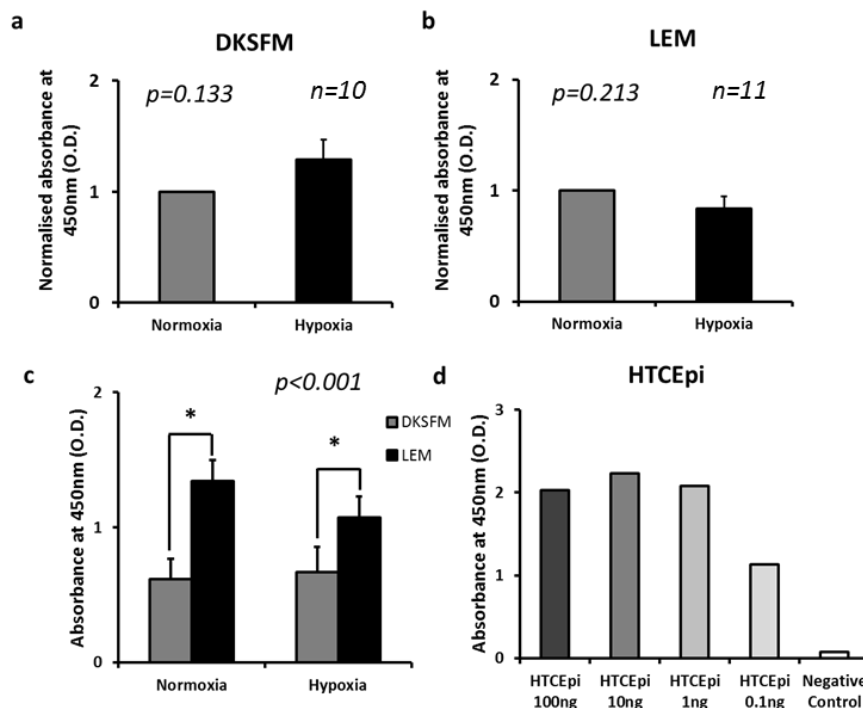
Figure 5.16 Telomerase activities of cells cultured in DKSFM and LEM under normoxia or hypoxia
Telomerase activity was determined using a telomere repeat amplification protocol (TRAP assay, *Roche*) and detected with ELISA. Cells from 10 donors were analysed in DKSFM, (Panel a). In LEM 11 donors were tested (Panel b). Panel c, highlights the difference between medium compositions on the activity of telomerase (p<0.001, DKSFM vs LEM, p=0.474, Normoxia vs Hypoxia). The corneal cell line HTCEpi was used as a positive control, since telomerase is constitutively expressed (Panel d), it shows that the assay losses sensitivity at 2 O.D. For each sample 2 technical replicates were performed. Each donor's cells were normalised to the normoxia sample (panel, a and b). Standard errors of the means are shown. 500ng of protein was used per donor. Water was used as a negative control.

The lack of change in telomerase activity between hypoxia and normoxia was recently shown in embryonic stem cells grown in 2% and 20% oxygen. These results were in spite of elevated nuclear *TERT* expression, which lead to increased pluripotency markers and cell survival (Radan *et al.*, 2014). These results suggest that the activity of telomerase is not necessarily indicative of stem cells. Elevated TERT expression on the other hand is still a good indicator.

5.3.8 Does the amount of ATP produced vary with different culture conditions?

The amount of ATP produced by each cell can be theoretically measured by observing the amount of protons released and consumption of oxygen from the medium. An increase in protons in the medium leads to acidification which can be monitored my measuring changes in pH. It was possible to calculate the amount of ATP produced because the net number of protons produced by glycolysis is proportional to the number of ATP molecules also produced. With the pH scale basically a measure of the concentration of hydrogen ions this is a good system for calculating the amount of ATP.

$$DGlucose + 2[NAD]^2 + 2[ATP] + 2[P]_i = Pyruvate + 2H^+ + 2[ATP] + 2H_2O$$
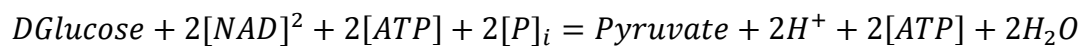
Calculating oxidative phosphorylation (OXPHOS) was more difficult, as the proportion of ATP is not directly related to the amount of hydrogen ions produced in this biochemical reaction. Instead the amount of ATP produced was calculated by measuring the consumption of oxygen rather than the acidification rate. The hypothetical phosphate/oxygen ratio (P:O) was utilised. The P:O is inferred from the amount of ATP produced by the movement of two electrons through the electron transport chain, which have previously been donated from the reduction of an atom of oxygen.

$$P:O = [ATP\ formed] : [oxygen\ consumed]$$

If a conservative P:O ratio of 2.3 (Hinkle and Yu, 1979) is assumed, and the P:O ratio equation is rearranged to calculate the quantity of ATP produced per molecules of oxygen consumed.

To calculate the amount of ATP produced per cell, an absolute cell count was performed by fixing cells with paraformaldehyde and staining each nucleus with DAPI. The wells were then imaged using a spinning disk laser confocal microscope and subsequent images were processed using imageJ, to obtain a highly accurate total cell

number per well (see Chapter 2).

Shapiro-Wilk tests were performed on the data, to determine if the results were normally distributed. The results of the test are shown in appendix B, table 7.30. In summary, values for all conditions and donors were normally distributed, this allowed parametric tests to be performed on the data. When samples were related, a paired sample *t-test* was used. For samples which were not related independent samples *t-test* were used. One of the criteria which govern the outcome of an independent samples *t-test* is the equality of variance. In this study a Levene's test was used to determine this parameter. If the resulting p value is less than *0.05* then sampled data is likely not to have originated from a population which has equal variances. If this was the case, a variant of the *t-test* which does not assume homogeneity of variance was used.

Comparison (Parameter, Medium & Oxygen condition)	Paired samples t-test (p values)	
Glycolysis DKSFM Normoxia Vs Glycolysis DKSFM Hypoxia	*0.405*	
Glycolysis LEM Normoxia Vs Glycolysis LEM Hypoxia	*0.386*	
Glycolysis HTCSC Normoxia Vs Glycolysis HTCEpi Hypoxia	*0.62*	
OXPHOS DKSFM Normoxia Vs OXPHOS DKSFM Hypoxia	*0.117*	
OXPHOS LEM Normoxia Vs OXPHOS LEM Hypoxia	*0.687*	
OXPHOS HTCSC Vs OXPHOS HTCEpi Hypoxia	*0.135*	
	Levene's Test (p values)	Independent samples t-test (p values)
Glycolysis Normoxia DKSFM Vs LEM	*0.959*	0.085
OXPHOS Normoxia DKSFM Vs LEM	*0.089*	*0.003*
Glycolysis Hypoxia DKSFM Vs LEM	*0.58*	*0.045*
OXPHOS Hypoxia DKSFM Vs LEM	*0.704*	*<0.001*

Table 5.2 Summary of significance tests for the metabolic assay

The majority of comparisons were not significant. In particular there was no statistical difference between normoxia and hypoxia in either media (table 5.2). With this in mind the data has been depicted below separately (figure 5.17 & 5.18). This lack of

significance could be due to the technical performance of this experiment. The experiment was performed in normoxic conditions including lengthy incubations which must be performed in a non-CO_2 incubator, because carbon dioxide can acidify the medium leading to inaccurate acidification rate readings. These factors may have affected the metabolism of the LSCs cultured under hypoxia (Shimmura *et al.*, 1998). Short of procuring a very large hypoxic chamber to sit the device within, this limitation was unavoidable. It is not a surprise then that significance as only detected when comparing medium and not the effects of oxygen concentration.

It was observed that there was an increase of OXPHOS in LEM under hypoxia compared to DKSFM. In the normoxic samples both OXPHOS and glycolysis are significantly increased. In general, the cultures kept in LEM were nearly twice as energetic. Embryonic and adult stem cells have been shown to be more glycolytic than somatic cells (Chung *et al.*, 2007; Varum *et al.*, 2011; Shyh-Chang *et al.*, 2013). Assuming one medium or oxygen condition was more beneficial to stem cells than another, it was expected that more glycolysis would be a signature of their presence. These experiments have never been performed on human limbal epithelial cells before and are therefore novel.

In addition to the primary donors, two cell lines were also tested. It has been postulated that the 3T3 feeder cells could also be affected by hypoxia. This in turn, might be causing a positive or negative effect on the cells. The difference in OXPHOS and glycolysis does not appear to change between oxygen concentrations, suggesting that 3T3 fibroblasts are not the cause of the differences between the conditions. It is important to note however that the vast majority of 3T3 fibroblasts within the co-cultures were removed by EDTA before the experiment, to avoid interference with the results. As the cells do not change with the two oxygen concentrations, the negligible amount of cells which may have remained in the culture were not likely to have affected the experiment.

The cell line HTCEpi was used as a control, to test the feasibility of the experiment. HTCEpi is a human telomerase immortalised corneal epithelial cell line which behaves similarly to primary corneal epithelial cells and expresses corneal markers, see 5.2.9 (Robertson *et al.*, 2005). Isolated side populations HTCEpi have been shown to express the markers *ABCG2*, *Sox2* and ΔNp63 (Shaharuddin *et al.*, 2014).

HTCEpi cells were then assessed as a viable replacement for primary human LSCs, similarities between metabolic states could indicate its suitability as an experimental replacement. No differences in HTCEpi cells were detected between the two oxygen conditions. This result is similar to the results of cells grown in DKSFM. This was encouraging as it indicated that this cell line might be a good control and could be used as a surrogate for rare primary donor cultures.
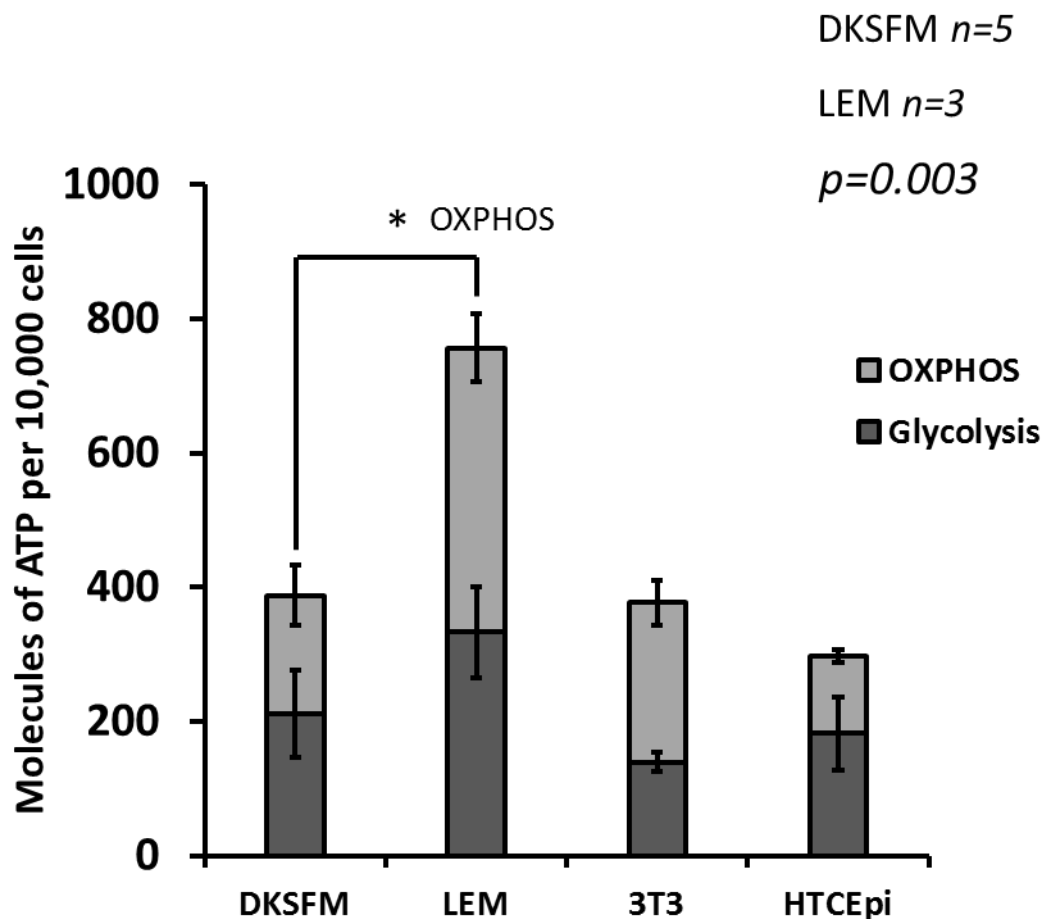
Figure 5.17 ATP produced by OXPHOS and glycolysis by limbal epithelial cells in LEM and DKSFM under normoxia
In LEM 3 donors were tested, with 5 technical replicates for each donor cells. In DKSFM 5 donors were tested with 5 technical replicates for each donor cells. Five technical replicates were performed for the cell lines 3T3 fibroblasts, while 12 technical replicates were used for HTCEpi cells. Standard error of the mean is shown.
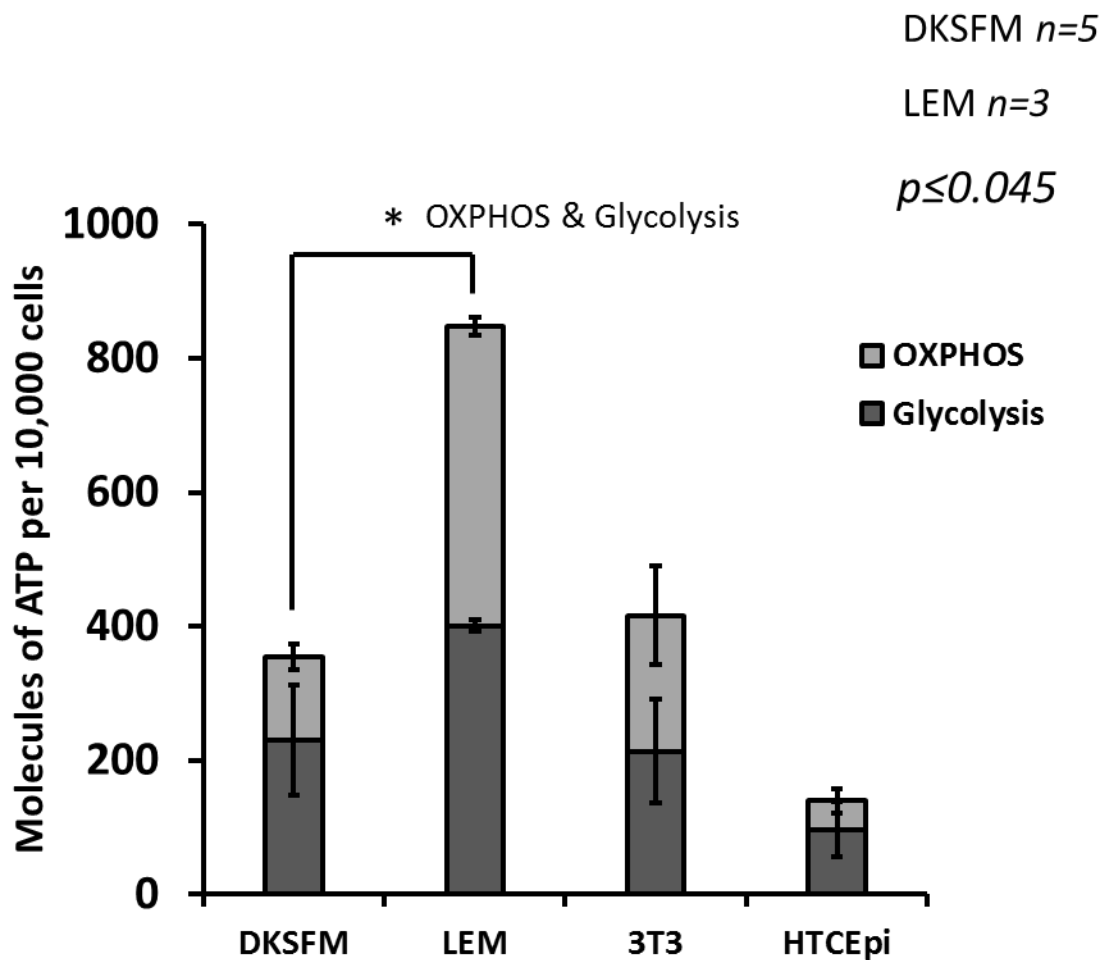
Figure 5.18 ATP produced by OXPHOS and glycolysis by limbal epithelial cells in LEM and DKSFM under hypoxia
In LEM 3 donors were tested, with 5 technical replicates for each donor cells. In DKSFM 5 donors were tested with 5 technical replicates for each donor cells. Five technical replicates were performed for the cell lines 3T3 fibroblasts, while 12 replicates were present for HTCEpi cells. Standard error of the mean is shown.

These results show that under normoxia the Glycolysis:OXPHOS ratio in the DKSFM group was on average 1:1 while in hypoxia it was 1.9:1. In LEM under normoxia the ratio of Glycolysis:OXPHOS was 0.8:1, similarly in hypoxia this value was 0.9:1. This, however was only on average, significance could not be attained. Though, under both oxygen conditions, cells cultivated in LEM had a higher ATP production compared to cells grown in DKFSM.

5.3.9 Assessing the suitability of human telomerase immortalised corneal cell line as a model in which to observe the effects of hypoxic growth conditions

The cell line generated by James Jester and colleagues, HTCEpi, has proven to be a useful control for many of the experiments in this chapter (Robertson *et al.*, 2005). As primary corneal tissue was at a premium during the course of this study, the feasibility of using the corneal cell line was assessed. Primarily to observe if it was biologically relevant for studying the effects of hypoxia, qPCR was performed on the cell line to quantify potential changes in gene expression (figure 5.19). Shapiro-Wilk tests were performed on the data, the results of which are summarised in Appendix B, table 7.29. All but the data from the *p21* gene expression were normally distributed. Paired sample *t-tests* were performed on the normally distributed samples, while a related samples Wilcoxon signed rank test was performed on the *p21* data. The normalised group however was for obvious reasons not normally distributed, thus a non-parametric test was also performed. The significance tests are summarised in figure 5.19.
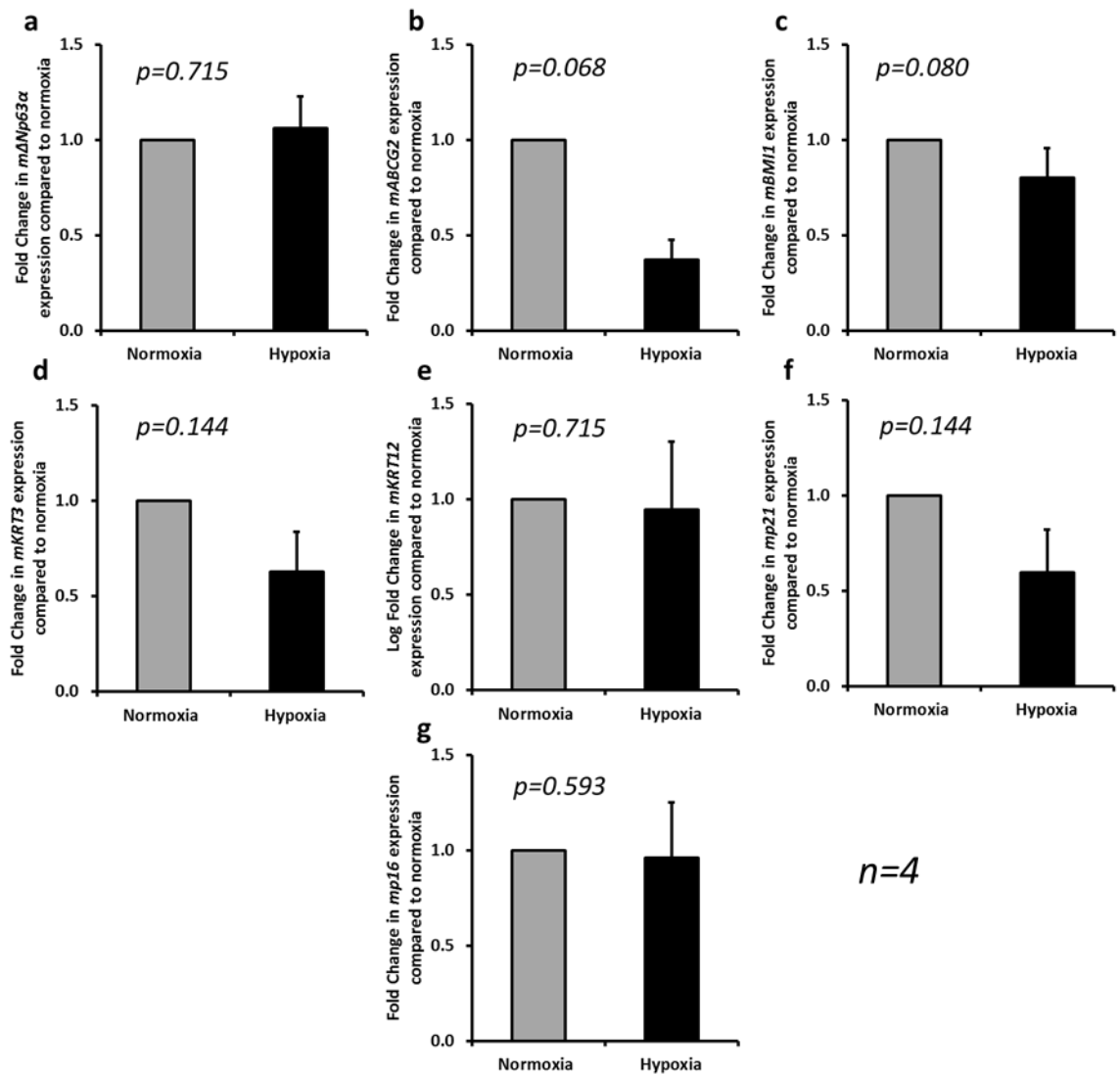
Figure 5.19 Gene expression profile of the immortalised corneal cell line HTCEpi under normoxia and hypoxia

qPCR for each gene was repeated at least four times. *KRT12* is shown as log of the original values. Standard error of the mean is shown.

There was no statistical difference between the two conditions, thus the null hypothesis has been accepted. In figures 5.10 & 5.11 it was shown that hypoxia does have an effect on the gene expression profile of primary corneal cells. We concluded that the cell line HTCEpi is not an applicable model for determining the effect of oxygen tension on corneal epithelium derived cells. It can however be assumed that over expression of *TERT* has a nullifying effect on possible gene expression changes in hypoxia of corneal cells. These results contradict those of the group who established the cell line, who stated that *ΔNp63α* gene expression was down regulated in hypoxia. That study differed from ours with regards to the length of time the cells were cultured in hypoxia (3hrs-24hrs) (Robertson *et al.*, 2012). The cells in this experiment were placed in prolonged

hypoxia of more than 3 months. In addition to this Robertson and colleagues used RT-PCR which is much less sensitive than qPCR, used in these experiments (Robertson *et al.*, 2012).

The protein levels of ΔNp63 and connexin 43 were assessed using immunofluorescence in the cell line (figure 5.20). Connexins are transmembrane proteins, which combine to form gap junctions. Gap junctions allow channels to form between neighbouring cells allowing for the transport of molecules between cells. Connexin 43 is a considered a negative marker of limbal progenitors as its expression is absent in the basal layer of the limbus, but present in the central corneal region.
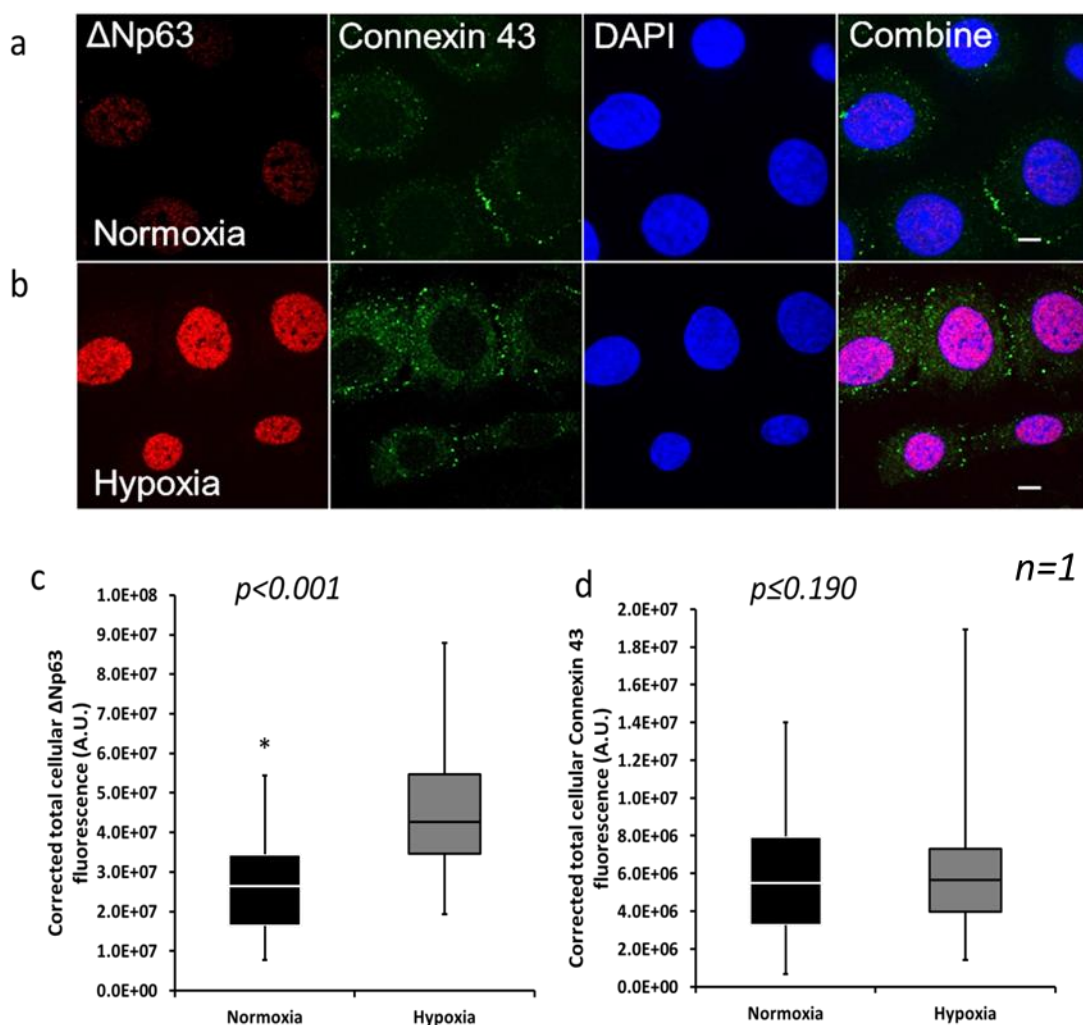
Figure 5.20 Immunofluorescence of ΔNp63 and Cx43 in the cell line HTCEpi
Cultures were stained and measured at the same time and with same exposure conditions. 15 random fields of view were taken at x100 magnification. 75 cells were analysed in total for each condition. Scale bar is 5μm.

A Shapiro-Wilk test showed that the hypoxic ΔNp63, connexin 43 and normoxic connexin 43 data was not normally distributed ($p=0.022$, <0.001 and <0.001). A related

samples Wilcoxon signed ranks tests was used to assess the data. ΔNp63 and connexin 43 tended to be up regulated at the protein level in hypoxia, however this was only significant in ΔNp63 (*p<0.001*) (Connexin 43, *p=0.190*). This result suggests that the expression of ΔNp63 is modified at the protein level in the immortalised corneal cell line. Again these results contradict those of Robertson and colleagues, whom however did not study ΔNp63 at this level in the cell line (Robertson *et al.*, 2012).

5.4   Discussion

It is important to continuously aim to optimise culture conditions, to mimic those found in the cells natural environment. The closer the cells are to *in vivo* conditions the more relevant any tests which are performed on those cells will be. It is especially important in the case of limbal epithelial cells, because of their use in treating LSCD. Potentially the closer the conditions to those found naturally the more progenitor cells can be maintained and subsequently transplanted onto the corneal surface. A higher number of progenitors have been shown to increase the success rate of LSCT (Pellegrini *et al.*, 1999; Rama *et al.*, 2010).

The use of DKSFM has only once before been attempted with human limbal epithelial cells in hypoxia. The benefits of using defined medium eliminates unknown factors, and is closer related to media used for cellular transplantations with the exception of homologous human serum supplemented medium. Additionally, the combination of the removal of mouse 3T3 cells and LEM should reduce variance, boding to more accurate results.

The concentration of oxygen chosen for these experiments was 3%, based upon extrapolations from the literature, which suggest that the limbal niche maybe subjected to around 2-5% oxygen (Brahimi-Horn and Pouyssegur, 2006). Although it could be argued that biochemically this range is still large enough to elicit a different response between 2% and 5%.

It was observed that under 3% oxygen in LEM there was a statistically significant reduction in the number of colonies produced, whilst in DKSFM there was a close to statistically significant difference to suggest the opposite (*p=0.06*). Bath and colleagues, who also studied a "serum free" medium, argued that CFE analysis cannot be performed in this system (Bath *et al.*, 2013). Mainly because defined colonies do not form, and it is more difficult to macroscopically visualise each individual colony. Miyashita's and our

study showed that with the initial seeding density of 1,000 cells it is possible to distinguish individual colonies (Miyashita *et al.*, 2007). It was also possible to measure colony size, a method which O'Callaghan performed on cultured rabbit limbal cells. O'Callaghan, however, studied cell size whereas our study observed colony size. As a result, this parameter is difficult to correlate with any other papers. The observation that fewer and smaller colonies formed in LEM under hypoxia, suggests that this condition is detrimental for limbal epithelial cell culture. In the three other studies where LEM was used, one showed no real trend with oxygen concentration (Bath *et al.*, 2013), another showed a reduction in CFE with decreasing O_2 (O'Callaghan *et al.*, 2011) and the third paper did not perform CFE as they used the explant method (Ma and Liu, 2011). The results from this study, agree with O'Callaghan and colleagues. However that study was performed on rabbits and not on human LSCs. In general CFE experiments normally assume that more colonies equal more stem cells. This assumption however does not take into consideration cells which are in a quiescent state. More quiescent cells would also contribute to a decrease in CFE. It is known, that culturing cells forces them out of quiescence. It is possible however to detect a change in the proportion of cells in quiescence by assessing the number of cells in G_0 using cell cycle analysis. Since this study showed there was no increase in the proportion of cells in G_0 compared to normoxia in the donor tested this event could potentially be ruled out. However, additional donor cells for this experiment would have aided in affirming this assumption.

5.4.1 Hypoxia can impact upon the gene expression profile of limbal epithelial cells.

This is the first study to also look at a number of genes in LSCs under hypoxia using qPCR, with other studies focusing mainly on immunofluorescence. Where PCR has been previously utilised it was often semi-quantitative RT-PCR (Miyashita *et al.*, 2007; O'Callaghan *et al.*, 2011). RT-PCR, suffers however from a lack of sensitivity. Our study quantitatively detected changes in the expression level of a number of genes in hypoxia. In LEM the down regulation of *ΔNp63α*, was consistent with those shown in the immortalised corneal cell line (Robertson *et al.*, 2012), although this study could not replicate these results. Interestingly, in the context of hypoxia *ΔNp63α* has been found to aid in the stabilisation of both exogenously and endogenous *HIF1α*. It has been suggested that *ΔNp63α* does so by up-regulating the transcription of *HIF1α*, and also

interacting with the transcription factor *p300/CBP* (Senoo *et al.*, 2002). This study did not analyse *HIF1α* as the main hypoxia mediated modifications, such as the lack of hydroxylation at proline residuals, do not affect mRNA levels. To try and confirm the mRNA results, ΔNp63 immunofluorescence was also performed on the cells from donors cells cultivated in DKSFM and LEM. There was no statistically significant change in ΔNp63 under hypoxia. However different media seemed to have an effect on ΔNp63, the LEM hypoxia combination showed a tendency to increase ΔNp63. This might suggest that soluble factors in the medium may affect the expression of basic limbal epithelial cell markers.

The adult stem cell marker *ABCG2*, is an ATP binding cassette (ABC) transporter. Stem cells can be isolated using the ability of cells to efflux Hoechst 33342 dye, primarily through the ABCG2 transport protein. Cells in the basal epithelium have been shown to express *ABCG2* (Watanabe *et al.*, 2004; Budak *et al.*, 2005; de Paiva *et al.*, 2005). The up-regulation of *HIF1α* in hypoxia has been shown to be related to the up-regulation of *ABCG2* expression in cochlear stem cells (Chen *et al.*, 2011). Thus, we expected that an increase in *ABCG2* would be observed under hypoxia. However no difference could be detect in the expression of this marker in any of the conditions tested.

qPCR analysis also indicated that *TERT* was up regulated in LEM under hypoxia. This was expected since *HIF1α* has been shown to up regulate *TERT* (Nishi *et al.*, 2004; Yatabe *et al.*, 2004; Anderson *et al.*, 2006). Although it has been suggested that hypoxia may decrease *TERT* expression in colon cancer (Koshiji *et al.*, 2004). Many stem cells, such as HSCs, are present under hypoxia *in vivo*, the up regulation could be related to telomere maintenance or the protective non-canonical functions of *TERT* in these cells (Ahmed *et al.*, 2008; Haendeler *et al.*, 2009; Singhapol *et al.*, 2013). *TERT* expression is especially important in stem cells as the cells are long lived within the tissue. Interestingly, an increase in telomerase activity or *TERT* expression was not seen between oxygen tensions in the DKSFM group. This was despite a greater number of donors tested in this group. We concluded that an additive within LEM or even the serum itself may up regulate *TERT* expression under hypoxia. An increase was not however observed regarding telomerase activity within the LEM cultured donor cells. The exact composition of serum is unknown, making it difficult to determine what factor or combination of factors are responsible for the increase in *TERT* gene expression but

not activity. Telomerase has a number of known non canonical functions which are important for genomic stability, such as reducing mitochondrial ROS production. When consulting the literature, another puzzling set of observations was revealed. In serum starvation experiments *HIF1α* was shown to be a key survival factor in prostate cancer cells (Thomas and Kim, 2008). If *HIF1α* is activated by serum starvation and hypoxia then stabilises the protein it was expected that telomerase activity may also increase. Unfortunately this could not be confirmed by a TRAP assay, in either DKSFM or LEM.

The down regulation of BMI1 under hypoxia in donor cells cultured in DKSFM would appear initially to be a detrimental property for establishing stemness. If one looks however at the role of BMI1 in hypoxia it is revealed that one of the regulators of BMI1 is controlled by HIF-1. HIF-1 binds to the hypoxia response element of twist basic helix-loop-helix transcription factor 1 (TWIST1), a master regulator of gastrulation and EMT (Yang *et al.*, 2008; Yang *et al.*, 2010; Wu and Yang, 2011) . The knockdown of *BMI1* expression has been shown to impact upon the progression of EMT (Yang *et al.*, 2010). EMT is most effective at points of injury/replenishment, in the cornea this is often located centrally. One could speculate that EMT may be delayed until closer to the target site, and that this may be controlled in part by the oxygen gradient mediated though TWIST1/HIF-1 and subsequently by BMI1. However, the down regulation of BMI1 was found only in DKSFM. Perhaps, soluble factors in LEM could be overriding this mechanism, resulting in a lack of significant change in expressions levels under this condition.

Keratin 3 (*KRT3*) was down regulated in hypoxia under both conditions. It has been documented with immunofluorescence that a decrease of *KRT3* was detected after reducing the amount of oxygen to 10% (Bath *et al.*, 2013). The literature on the process of inducing stratification within epithelial cultures (air-lifting) shows that exposing the culture to the medium/air barrier increases the accumulation of *KRT3* (Liu *et al.*, 2006; Ang *et al.*, 2010), suggesting that *KRT3*could be controlled by the oxygen gradient within the cornea. No direct experiment links however have been made between *HIF1α* and *KRT3*. The only known gene which is shared with this system is p300, a major transcription factor which interacts with many other genes. This interaction is only hypothetical based on the homology of p300's binding site.

Keratin 12 (KER12), like KRT3 is a structural protein of the corneal epithelium and

also considered a negative marker LSCs. Keratin's form heterodimers of type I and II filaments, with KRT3 being a type II and KRT12 a type I filament. The heterodimeric unit is the main type in the tissue. In this study no discernible difference was detected in expression level of *KRT12*. This was not expected as the two keratin proteins are linked in their function. It was expected that *KRT12* would mimic *KRT3*'s expression.

The cyclin-dependent kinase inhibitor 1 (*CDKN1A*) inhibits the activity of cyclin dependent kinases 1, 2 and 4/6 regulating the progression at G_1 and S phase of the cell cycle (Gartel and Radhakrishnan, 2005). The *CDKN1A* gene is an important downstream target of p53 which is activated upon DNA damage and is responsible for the initiation of senescence and apoptosis. Senescence is induced by p21, hypoxia can suppress this induction process favouring a quiescent state (Leontieva *et al.*, 2012). The promoter of p21 can be transactivated by HIF-1, (Salnikow *et al.*, 2000), it has been demonstrated in endothelial cells that an up regulation of *HIF-1* resulted in the subsequent up regulation of *p21* (Iida *et al.*, 2002). This contradicts the findings in our study where in LEM, *p21* decreased. One explanation would be that in limbal epithelial cells, there are other mediating proteins which help to override the HIF-1 interaction with the promoter region of *p21*.

5.4.2 Telomerase activity is not dependent on oxygen tensions but is dependent of medium composition.

Telomerase activity was shown in chapter 3 to decrease with successive culture of LSCs, suggesting that culture conditions are suboptimal. LEM appeared to be the most favourable medium increasing activity over DKSFM. Comparisons of telomerase activity between different medium have been tested previously in (*Callithrix jacchus* embryonic stem cells) cESCs either grown on MEF's or without in a defined medium. The results showed no difference in the telomerase activity (Trettner *et al.*, 2014). *Callithrix jacchus*, however could be biologically different from human ESC and adult stem cells. The microenvironment has been shown to impact upon telomerase activity, placing limbal epithelial cells in embryonic stem cell conditioned medium increased telomerase activity (Liu *et al.*, 2013). The extent of the increase however was probably masked as the absorbance levels were higher than the tolerance of the test (absorbance at 450nm of over 2.18). We maintained protein levels at 500ng in limbal samples, to reduce the chance of over saturation of the assay. The positive controls, of HTCEpi highlight the

effect of oversaturation. Overall medium can affect telomerase activity, whether this increase in indicative of more progenitors or just the up-regulation of telomerase remains to be seen. It was hypothesised that hypoxia would increase telomerase expression; previous studies demonstrated that *TERT* is up regulated, as stated previously. In this study cultures grown in LEM under hypoxia showed an increase in *TERT* mRNA, it is interesting then that an increase in telomerase activity was not detected. This result could indicate that more telomerase is being produced for non-canonical functions such as protection of mitochondrial DNA integrity.

5.4.3 Limbal epithelial cells cultured in LEM produce more ATP than those in DKSFM.

This study also analysed the dynamics of cellular respiration in limbal epithelial cells for the first time using an extracellular flux analyser (Seahorse XFS). We found that medium conditions could shift the bivalency of the metabolism, although not significantly, due to the low number of donors analysed. Interestingly, while the quantity of ATP produced by each individual respiration method was different, this was only true between medium conditions. Oxygen concentration however, did not alter ATP production. This was not expected, as hypoxia is known to up regulate glycolytic genes such as glucose transporter 1 (GLUT1), 6-phospho-2-kinase/fructose 2, 6-biphosphatase (PFKFB1-4) along with lactate dehydrogenase A (LDHA) (Robin *et al.*, 1984; Iyer *et al.*, 1998; Maher *et al.*, 2007). In addition to this HIF-1 can also activate genes which reduce OXPHOS, such as pyruvate dehydrogenase kinase 1 (PDK1). It was also expected that cultures with more stem cells within them would be more glycolytic, as it was already demonstrated in the adult stem cells of the haematopoietic and cardiac systems (Kimura and Sadek, 2012). The lack of a detectable difference between oxygen conditions was in part perhaps due to experimental short comings, whereby the hypoxic samples could not be maintained in hypoxia for the duration of the experiment.

As stated previously, LEM caused cells to produce more ATP per 10,000 cells than DKSFM. This observation indicates that a component of the serum could artificially increase the respiratory capacity of limbal cells. The two media did also contain differing quantities of glucose (DKSFM, 1624.4mg/l; LEM, 667.5mg/l) and L-glutamate (DKSFM, 984mg/l; LEM, 862mg/l), which could impact upon the total energy produced. For the respiration experiment, however, the glucose levels were normalised by replacing

medium with specific assay medium. Metabolic studies on hybridoma cells have shown that the rate of glycolysis was reduced in cultures supplemented with FCS compared to serum free cultures (Smith and Greenfield, 1992).The undefined nature of foetal calf serum makes comparing this experiment with those of others more difficult. Variation within sera from batch to batch and country to country has been shown to be large (Honn *et al.*, 1975). The same batch of FCS was used for these experiments respiration in order to minimise variability within experiments.

Other additives to LEM such as insulin have been shown to increase the coupling efficiency of OXPHOS and the glucose sensitivity of anaerobic glycolysis both of which would show an increase in total ATP produced (Nisr and Affourtit, 2014). The entire formula for DKSFM is not published, hence one cannot conclude that insulin is the factor which is causing the dramatic increase in ATP production in LEM.

5.4.4 Attaining hypoxia

In an ideal situation an isolator unit, such as the biospherix unit used by Bath and colleagues would have been used at all times to minimise re-oxygenation, which occurs during feeding and in various experiments. The passive nitrogen purging system used in these experiments was preferable to the use of cobalt chloride as the fluctuations in oxygen tension could be monitored more easily. An oxygen sensor with which could be used *in vitro* was not available ergo the dosage of cobalt chloride could not be optimised accurately.

5.5 Conclusion

The findings of this study have shown novel trends in parameters which have never before been shown in limbal epithelial cell containing cultures. Hypoxia has a definite effect on limbal epithelial cells. The results here could add to the growing belief that *in vitro* culture conditions for maintaining precursor cells should reflect physiologically relevant O_2 saturations (Simon and Keith, 2008). The results from our study highlight the need to re-evaluate tissue culture techniques in order to be biologically relevant. The summary diagram of relevant interactions in figure 5.21 highlights the lack of information for the negative markers *KRT12* and *KRT3* as annotated sequence data was the only information available. This study did show a definite effect of hypoxia on one of

the negative markers, *KRT3*. In light of the effect that airlifting has upon this gene it seems highly likely that oxygen tensions effect its expression.
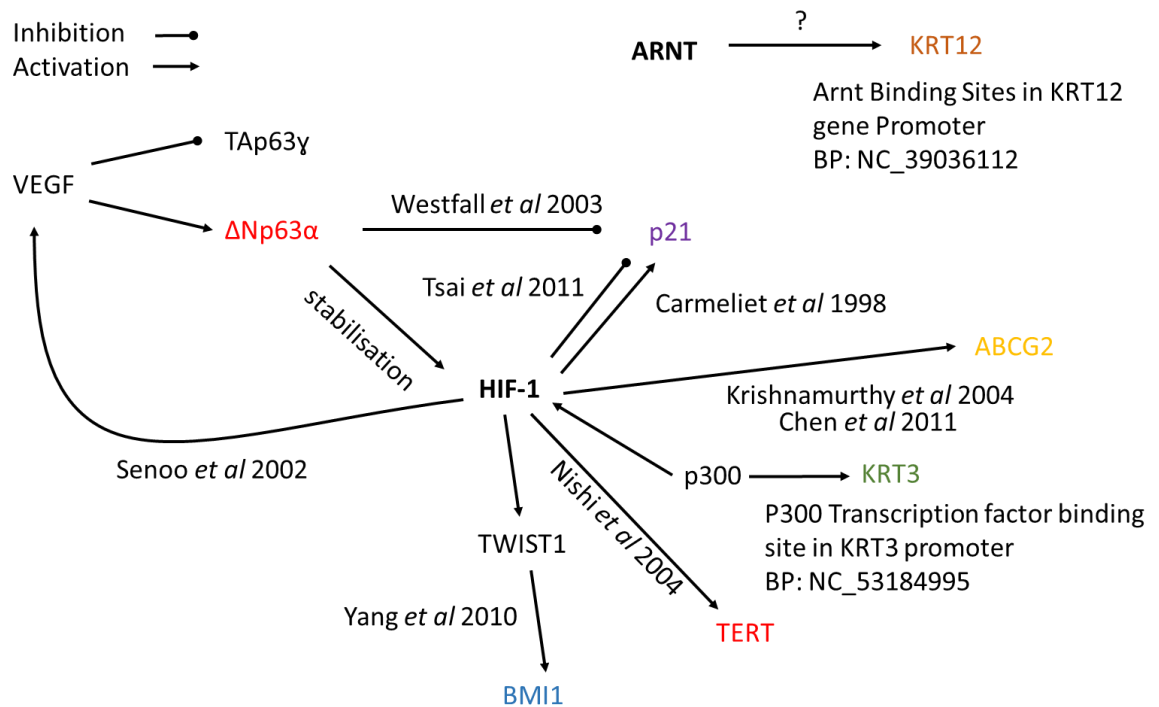
Figure 5.21 Summary of relevant corneal marker gene interactions with HIF-1
Binding sites were extracted from SABiosciences' Text Mining Application and the UCSC Genome Browser. Other data was sourced as follows: (Carmeliet *et al.*, 1998; Senoo *et al.*, 2002; Westfall *et al.*, 2003; Krishnamurthy *et al.*, 2004; Yang *et al.*, 2010; Chen *et al.*, 2011; Tsai *et al.*, 2011).

Importantly, a decrease was observed in *p21*, which should have increased under hypoxia, according to Carmeliet and colleagues (Carmeliet *et al.*, 1998). A recent paper studying the effects of hypoxia in mesenchymal stem cells however supports our experimental findings, suggesting that hypoxia down regulates p21 through the formation of HIF-TWIST complex (Tsai *et al.*, 2011). Interestingly, the LSC marker *ΔNp63α* has an antagonistic effect on *p21* repressing its expression (Westfall *et al.*, 2003), but it is thought to be through VEGF, which is up-regulated by the HIF-1 protein. Many of the genes involved in the biological response to hypoxia are major signalling genes, and by no means function independently. Other interactions which have not currently been defined are likely to be responsible for the expression patterns which are shown in this study.

Assessing the metabolic status of limbal epithelial cells proved to be difficult when comparing normoxic growth and hypoxic growth. It further reinforces the effect re-oxygenation has on cellular cultures, soon after removal from that environment. We

could however show that culture medium does have a dramatic effect on ATP production. One can thus conclude that choice of medium is important when assessing metabolomics in limbal stem cell containing cultures. Which media however would be most biologically relevant remains to be identified.

It can be concluded that the oxygen tension is a parameter which should not be overlooked when culturing adult stem cells, with limbal epithelial cells being no exception. If the knowledge from the existing papers studying hypoxia in human limbal epithelial cells are combined with the results shown here (Miyashita *et al.*, 2007; Bath *et al.*, 2013), hypoxia seems generally to be beneficial to the preservation of progenitor like cells in these cultures.

Chapter 6 Final discussion

The human ageing population is expanding, in the next 40 years the number of people over 60 is predicted to increase from around 524 million to around 1.5 billion (Kowal *et al.*, 2012). As we age, our susceptibility to degenerative diseases and oncogenesis increases (Ruzankina and Brown, 2007). The generation of cells *ex vivo* for use in regenerative cellular therapies has increased over the past decade. Limbal stem cells are used to restore sight in patents with LSCD. However, currently around 24% of limbal stem cell transplantations fail, the reasons for which are largely unknown (Baylis *et al.*, 2011). It has been suggested previously that donor age may play a role in poorer clinical outcomes (James *et al.*, 2001). The effects of age on the culture of limbal epithelial cells, which can be used for transplantations, have to date been largely inconclusive with no real trends occurring with age (Shanmuganathan *et al.*, 2006; Baylis *et al.*, 2013; Notara *et al.*, 2013). Our study sought to address the question of the quality of limbal epithelial cells correlating to donor age using multiple techniques to assess progenitor properties of cultured limbal epithelial cells. Understanding the basic biology of the cornea with age is crucial to the implementation of future cellular therapies and may aid in increasing the efficiency of limbal stem cell transplantations.

Oxidative stress and cell damage increase with age. Understanding the way in which limbal epithelial cells respond to different oxygen tensions can allude to the basic biological properties of these cells. Many stem cells are cultured in hypoxia, but previous studies on limbal epithelial cell growth in this condition have been contradictory (Miyashita *et al.*, 2007; Li *et al.*, 2011; Ma and Liu, 2011; O'Callaghan *et al.*, 2011; Bath *et al.*, 2013). To address this, limbal epithelial cells were cultured using both traditional methods and those more akin to clinical practices i.e. xenobiotic free defined medium.

A better understanding of the biological processes occurring with age and improved culture techniques both benefit the implementation of cellular therapies. In turn, greater understanding results in the restoration of sight in more people with limbal stem cell deficiency than ever before.

6.0.1 The human cornea has remarkable post mortem regenerative capacity and is seemingly resistant to age related changes.

In this study corneal cells from over 100 human donors were used to investigate the effects of age on limbal epithelial cells, in the hope of better understanding the influence of donor age on the quality of derived LSCs for cell culture in order to improve the efficiency of cellular therapies.

The ability of human LSCs to be subcultured and the percentage of colony forming ability decreased with donor age. The activity of telomerase also decreased over multiple passages in human LSCs. These results suggest that perhaps a proportion of the isolated cells lose their stem cell like qualities sooner when isolated from older donors. All other parameters of human LSCs however were not significantly influenced by age, such as gene expression, population doubling time and p63 expression. There are numerous reasons why one might not detect a change with age. Specific to limbal epithelial cell biology however the reason may in part be due to the high level of expression of p63 in the cornea. TP63 is part of the tumour suppressor family to which p53 belongs and shares similar roles to p53 (Yang *et al.*, 1998). p53, the "guardian" of the genome, regulates the transcription of many genes, such as *p21* and *PUMA* (p53 up-regulated modulator of apoptosis) it also controls cell cycle arrest and apoptosis. Isoforms of TP63 which confer the protein TAp63 can bind to p53 response elements (RE) which lay within regulatory regions of many genes. ΔNp63 on the other hand can also bind to p53RE inhibiting the binding of TA isoforms through either completion or a direct mechanisms (Benard *et al.*, 2003). The response of p63 to DNA damage can vary, ΔNp63 has been shown to decrease when cells are exposed to UV radiation and cisplatin (Liefer *et al.*, 2000), but not in response to doxorubicin (Harmes *et al.*, 2003). Expression of TA isoforms seems to increase during DNA damage caused by bleomycin, doxorubicin and cisplatin (Gressner *et al.*, 2005). In limbal epithelial cells, increased levels of ΔNp63, which is a dominant negative regulator of TAp63, may then interfere not only with TAp63 but also with the protective effects of p53. Whilst potentially making the cells more sensitive to the effects of DNA damage, maybe in favour of increased proliferative capacity. Alternatively, the concert of TAp63 and ΔNp63 in progenitor cells could be masking aberrations in the stem cell populations with age. Higher levels of TAp63 in more differentiated corneal epithelial cells in humans could function to repress aberrant growth and proliferation of these cells. In mice, it is suggested that TAp63 is associated with progenitor cells, this could indicate that

maintenance of genomic stability might be prioritised over enhanced proliferation. Dysregulation of TAp63 might however occur with age, as we showed that this marker decreased in the mouse cornea.

As mentioned previously the TAp63γ isoform can induce apoptosis in response to DNA damage (Yang *et al.*, 1998), in addition MEFs deficient in TAp63 are resistant to apoptosis (Flores *et al.*, 2002), implying that TAp63 might be required to remove damaged cells from the cornea. It could be argued however, that p53 still has overall control over ΔNp63, as p53 controls the caspase-dependent degradation of ΔNp63 (Ratovitski *et al.*, 2001). There is a clear interplay between p53 and p63, governing development and tissue homeostasis, with their interactions being highly intricate.

In other tissues/compartments increased donor age on stem cell populations has been shown to negatively influence the expansion and differentiation ability of HSCs both *in vivo*, most recently by Holstege and colleagues (Holstege *et al.*, 2014) and *in vitro* (Vaziri *et al.*, 1994). Specifically age decreased the proportion of B-lymphoid progenitors with age (Kuranda *et al.*, 2011), known as lineage skewing. Human MSCs derived from adipose tissue formed fewer colonies with age and expressed a greater proportion of *p21* and *p16*, markers of cellular senescence and stress. Their chondrogenic differentiation properties also decreased as shown by alcian blue staining (Choudhery *et al.*, 2014). Many scientists hypothesise that a decrease in the functionality of stem cells leads to the deterioration of tissues over time. The accumulation of DNA damage is thought to be a major factor involved in this decline with age. This is because stem cells are one of the longest lived cell types in the body (Behrens *et al.*, 2014). The results of our study showed that DNA damage increased with age *in vivo* within the mouse corneal epithelium. The main factors which can cause this increase are short or dysfunctional telomeres and the failure of DNA repair mechanisms (Hadshiew *et al.*, 1999). However, mice have very long telomeres, so length would be less likely a reason. In stem cells the effect of DNA damage can alter gene function, potentially causing dysregulation of self renewal (Wang *et al.*, 2012b), differentiation of progenitors (Brack *et al.*, 2007) and even activation of oncogenes (Snippert *et al.*, 2014). DNA repair triggered by the damage response however provides a way of limiting these detrimental effects, through halting the cell cycle until the damage is repaired. If however repair mechanisms are dysfunctional or

unsuccessful the cell can initiate senescence or apoptosis. These safety measures however, come at a cost, reducing the number and function of the stem cell pool.

6.0.2 Hypoxia and medium composition both affect the culture of human limbal epithelial cells

Ageing causes a functional decline in stem cell niches, such as haematopoietic (Pearce *et al.*, 2007; Oakley and Van Zant, 2010), dermal (Gago *et al.*, 2009), germline (Zahidov *et al.*, 2010), muscle (Carlson *et al.*, 2009) and, as recently shown, in the limbus (Notara *et al.*, 2013). Stem cell niches are often hypoxic (Parmar *et al.*, 2007), and a degeneration of these structures can cause the oxygen equilibrium to change. Higher oxygen concentrations can lead to increased ROS generation and thus, DNA damage. The oxygen sensitive transcription factor *HIF1- α* can regulate stem cell function during ageing, as reduced *HIF1-α* protein causes a loss of HSCs with age (Takubo *et al.*, 2010). The reaction of limbal epithelial cells to different oxygen conditions was hypothesised to indicate under which condition these cells might reside *in vivo*. *KRT3* and *p21* were consistently down regulated in hypoxia in our study on human LSCs. This down regulation of *p21* could possibly inhibit senescence under hypoxia similar to the effects observed in MSCs (Tsai *et al.*, 2011). This result combined with the down regulation of the negative stem cell marker *KRT3* lead us to suggest that limbal epithelial cells could be under hypoxia *in vivo*. Notara and colleagues showed that the focal stromal projections and limbal crypts decreased with age in humans, a possible sign of the dysregulation of the niche (Notara *et al.*, 2013). The loss of these compartments could lead to exposure of the stem cells to a higher oxygen tension.

In addition to oxygen tension, another parameter of successful stem cell growth was the culture medium. What was of particularly interest was the use of serum in the medium which leads to increased variation between medium batches. Removing this component and using a more defined medium should reduce variation and expose more subtle changes in the proportions of progenitors within each population. We observed that standard deviations for ΔNp63 staining were reduced by culturing cells in DKSFM (figure 5.11), compared to traditional medium (LEM).

The amount of respiration and production of ATP can indicate the quality of a culture. We showed for the first time the *in vitro* levels of ATP produced by limbal epithelial cells. Interestingly, there was less ATP produced by cells in DKSFM compared to cultures in LEM. It has been suggested that one of the major components of FCS,

BSA, modulates succinate dehydrogenase (SDH) (Complex II) via oxaloacetate removal. Oxaloacetate can activate SDH and cause free radical production which occurs during succinate oxidation (Panov *et al.*, 2010). BSA has however, also been shown to increase the reduction level of Cytochrome α, increasing potential ATP production (Sharpe *et al.*, 1996). The literature to date is clearly divided on the effects of BSA on mitochondrial respiration, making it difficult to surmise the effects of this component. DKSFM may have fewer growth factors than LEM as a result of the removal of FCS. Growth factor withdrawal has been shown to decrease the rate of respiration and mitochondrial membrane potential (Chandel and Schumacker, 1999) in p^0 depleted cells. Additionally, Interleukin-3 withdrawal can inhibit the membrane exchange of adenine nucleotides (Vander Heiden *et al.*, 1999), having the effect of reducing ADP and thus the production of ATP in mouse pro-B cell lines. We suggest then that a reduction in the number of growth factors within DKSFM may be the reason behind the difference in ATP levels seen between the two media.

6.0.3   The mouse corneal epithelium shows signs of decline with age

We used the thickness of the cornea as a biological indicator of cell renewal in the mouse cornea. Inferring that a decline in the thickness was proportional to the cells and the matrices they produced to replenish the tissue. Age did seem to have a detrimental effect on the overall thickness of the cornea. The difference in thickness could be due to either the number of cells, reduced cell size or a reduction in extracellular matrices and connective filaments, such as collagen. The integrity of the cornea also appeared to become compromised with age, with the formation of fluid filled compartments, called bullae. It is likely that these structures could compromise the barrier function of the cornea, perpetuating age related corneal diseases. This morphological change however was thought to be due to a decline with age in the number of endothelial cells in the cornea.

The stem cell marker gene TAp63 decreased with age in the central region of the mouse cornea. The peripheral cornea on the other hand remained unchanged with age. The peripheral cornea is often protected by the eye lid, providing shielding from external stresses which could have a detrimental effect on the stem cell pool.

6.0.3.1 Mouse limbal epithelial cells accumulate non-telomeric and telomeric DNA damage with age

Breaks the DNA helix can result in a stalled replication fork, which can trigger a response to repair the damage. A number of proteins are recruited to these sites one of which is H2A.X, which becomes phosphorylated on serine 139 by proteins of the PI3 kinase family. Overall DNA damage and that which occurred within the telomeres increased the central cornea, while the peripheral cornea remained unchanged with age, again indicating a more sheltered population. These results lead one to speculate that the peripheral corneal region is home to a distinct cellular population or that this population is protected by anatomical features. In conclusion, these two areas are distinct in their accruement of DNA damage with age.

It has been suggested that stem cells repair DNA damage in different ways, dependent on if the cells reside mainly in a quiescent state or divide frequently (Behrens *et al.*, 2014). HSCs for example, are mostly quiescent (a mechanism for reducing replicative damage) and use non homologous end joining as a repair mechanism for DNA damage. Intestinal stem cells on the other hand, proliferate rapidly, existing in S phase for extended periods which allows the cells to use homologous recombination to repair their DNA (Mohrin *et al.*, 2010). Limbal epithelial cells do not proliferate as quickly as intestinal progenitors (Loureiro *et al.*, 2013). Evidence suggests that true limbal stem cells are only active after wounding (Majo *et al.*, 2008), implying that they mainly reside in a state of quiescence. Our study saw a marked increase in γH2A.X, a double strand break marker during ageing. NHEJ or HEJ may be activated to repair double strand DNA damage in limbal epithelial cells. Further studies would need to be conducted to assess which repair mechanism might be used.

The increase in DNA damage could be indicative of decreased tumour suppressor proteins, damaged repair mechanisms or higher genomic attrition rate, perhaps from increased ROS production from aged mitochondria. The same trend of increased damage with age however, was not observed in the peripheral cornea suggesting that the population of cells in this region are shielded. Perhaps to protect the stem cell population which may reside in this region. Stem cells can avoid increased DNA damage rates from extrinsic factors in part due to the protective function of the niche, in addition to slower proliferation rates which reduce DNA replication errors. The protective effect of telomerase could aid in maintaining

mitochondrial integrity with age reducing the attrition of ROS on the genome.

The DNA damage response can also be triggered by dysfunctional or short telomeres. In the mouse skin, stem cells have been shown to have the longest telomeres within the tissue (Flores *et al.*, 2008). Stem cells can potentially up regulate telomerase activity, upon activation, despite this telomeres still shorten with age (Flores *et al.*, 2008; Wang *et al.*, 2009). Short telomeres can lead to augmented differentiation of embryonic stem cells (Pucci *et al.*, 2013). Implying that telomere shortening could lead to aberrant differentiation of stem cells with age and may be another mechanism by which tissue functionally declines with age. The number of TAFs increased with age throughout the cornea. We propose that this increase is likely due to increases in dysfunctional telomeres as opposed to eroded telomeres, since mice have very long telomeres (Prowse and Greider, 1995). It takes several generations of telomerase knockout before major detrimental effects of telomere erosion are observed (Chang, 2005).

6.0.4   Dietary restriction has an anti-ageing effect on the mouse cornea

Detection of DNA damage is important in maintaining the genome, as it reduces the chance of propagating deleterious mutations and to ensure proper functioning of cells and tissues (Polo and Jackson, 2011). Accumulation of DNA damage can lead to oncogene activation or senescence and apoptosis. In HSCs DR prevents the age related decline of these cells and maintains their functionality (Chen *et al.*, 2003). MSCs derived from adipose tissues in mice have been shown to increase in number during DR, coupled with an increase in telomerase activity, however their proliferative capacity was reduced (Schmuck *et al.*, 2011). In the eye, DR has been shown to have a beneficial effect on the lens epithelial cells of C57BL/6 mice, in terms of proliferative capacity (Li *et al.*, 1997). In the Emory mouse, a model of cataract, DR reduced the prevalence and severity of cataracts (Taylor *et al.*, 1995). To date the effects of DR on the mouse corneal epithelium had not been investigated.

Our study showed that double strand breaks remained at a more youthful level and that telomere damage was also attenuated under DR. DR however is a global process and its effects reduced the limbal epithelial cell marker TAp63. TAp63 is related to p53 and is involved in multiple pathways associated with cell growth and the DNA damage response (Levrero *et al.*, 2000; Cam *et al.*, 2014). DR might activate ΔNp63 which in turn can aid in the recruitment of DNA damage proteins to DSB via

ATM (Craig *et al.*, 2010), increases in ΔNp63 could then be inhibiting TAp63. The dominant negative binding of ΔNp63 however would not prevent detection of TAp63 via immunofluorescence. Although TAp63 has also been implicated in enhancing DNA damage response (Cam *et al.*, 2014). TAp63 can, via REDD1, inhibit mTORC1, perhaps the down regulation of TAp63 reduces redundancy as mTOR is down regulated via other means during DR.

The increase in the formation of bullae with age, suggests indirectly that a loss of a functional endothelium occurs with age. In contrast, DR was able to remediate the formation of bullae indicating that the endothelium remains intact under this condition. The corneal endothelium is a monolayer of cells which resides between the stroma and the anterior chamber. One of the main functions of these mitochondrial rich cells is to pump solutes to and from the cornea. The maintenance of a slightly dehydrated cornea is essential for the optical properties of the tissue. The corneal endothelium is post mitotic, as a result it is not readily regenerated and cells of the endothelium are lost with age (Wilson and Roper-Hall, 1982). A loss of these cells with age can lead to ineffective transport of solutes from the cornea, leading to the formation of fluid filled pockets called bullae. Our results infer that DR appears to ameliorate bullae formation and maintains a functional endothelial layer with age, corresponding to previously findings in rats (Nadakavukaren *et al.*, 1987). In other endothelial cell populations, the role of NO is proposed to mediate the beneficial effects of dietary restriction, potentially through reduced inflammatory response. NO synthase is deacetylated and thus activated by SIRT1 (Mattagajasingh *et al.*, 2007). These findings are associated with vascular endothelium and may behave differently to the avascular corneal endothelium. It is clear that more investigation is required to determine the exact molecular mechanism by which the corneal endothelium is beneficially affected by DR.

6.0.4.1 The number of DNA damage foci formed within the mouse corneal epithelium is dependent on telomerase and diet

WT DR treated mice had a lower number of γH2A.X foci, in comparison to *Tert*$^{-/-}$ DR mice. This observation suggested that the beneficial effects of DR could be mediated by *Tert* in the mouse cornea. In addition, when *Tert*$^{-/-}$ AL mice were compared to *Tert*$^{-/-}$ DR mice an increase in the number of foci formed was observed, demonstrating a greater amount of damage occurring in the absence of telomerase, despite DR. This

shows that both *Tert*^{-/-} and DR can influence the DNA damage dynamics in the mouse cornea. Telomerase is known to be shuttled to the mitochondria (Santos *et al.*, 2004), whereby it provides a protective function reducing the amount of ROS produced (Ahmed *et al.*, 2008; Haendeler *et al.*, 2009; Singhapol *et al.*, 2013), thus reducing DNA damage. In stem cells, high expression of telomerase may not just be to extend telomeres but also to safeguard the genome.

6.0.5 Rapamycin treatment is detrimental to the mouse cornea

The DR mimetic and immunosuppressant rapamycin, targets the mTOR pathway by binding to mTORC1 inhibiting its activity. mTORC1 responds to signals from the insulin/IGF pathway and through sensitivity to amino acid levels. In turn mTORC1 regulates a number of cellular processes notably cell growth, proliferation and protein synthesis. In the mouse corneal epithelium rapamycin treatment did not show the same beneficial effects as DR. Treatment with rapamycin, similar to DR, reduced the amount of TAp63 in both corneal regions, unlike DR however, rapamycin increased the number of γH2A.X foci and TAFs in the peripheral cornea. Currently it is suggested that TAp63 is upstream of mTOR (Cam *et al.*, 2014), however our data suggests that either mTOR suppression feeds back into TAp63 down regulation, or that rapamycin suppresses TAp63 either directly or indirectly (Ma *et al.*, 2010). The role of TAp63 in DNA damage repair (via NF-κB and Akt, figure 4.19) and genomic stability mean that the rapamycin mediated down regulation could be increasing DNA damage accumulation, as we observed with increased γH2A.X foci number. Rapamycin treatment also reduced the thickness of the cornea, no difference however was detected in the amount of cellular proliferation rate and thus amount of cell turnover in the cornea which could mediate the number of cells in the cornea. Perhaps instead rapamycin inhibits the production of structural proteins which leads to a reduction in the thickness in the cornea. In conclusion, rapamycin treatment did not have the same beneficial effects that were observed when assessing DR. We speculate that rapamycin may exacerbate the effects of ageing in the mouse cornea.

6.1   Implications for the culture of limbal epithelial cells

Neither donor age nor the number of days corneal scleral disks were stored in organ culture medium were found to have any definite detrimental effect on the culture of these cells. Prolonged culture of limbal epithelial cells leads to a loss of stem cell

characteristics, shown through a decrease of telomerase activity and reduced CFE. Thus it would be recommended that only primary cultures are used for transplantations. Intriguingly, donor age does not seem to have a definite detrimental effect on the culture of these cells, neither does the number of days corneoscleral tissue is stored in organ culture medium. We suggest therefore, that the selection parameters for allogeneic transplantations could be extended to include donors of all ages. This would substantially increase the likelihood of finding immune-matched donors.

The culture parameters for cultivating human limbal epithelial cells have been modified over many years, an important parameter shown in this thesis was that hypoxia has a definite effect on the growth and quality of limbal epithelial cells. Its suppression of negative stem cell marker *KRT3* and the important stress marker *p21*, suggests that it might be a preferable culture condition to preserve native stem cell characteristics. In addition, it might also indicate that the LSC niche might be hypoxic *in vivo* for these cells, similar to HSCs for example (Harrison *et al.*, 2002; Jang and Sharkis, 2007). Our research might also affect recommendations for the cultivation of other types of adult stem cells which are used in cellular therapies, such as tolerogenic dendritic cells (Stoop *et al.*, 2010) used in arthritis treatments and haematopoietic stem cells, used for treating various blood related disorders (Burt *et al.*, 2008). For embryonic SC this condition has been described as beneficial before, showing increased Oct4, NANOG and telomerase activity (Zachar *et al.*, 2010). Confirming the oxygen status of limbal epithelial cells *in vivo* however, is no trivial matter due to the lack of specific stem cell markers.

6.1.1 Implications for the understanding of the basic biology of the mouse corneal epithelium

Our data in the mouse cornea alludes to the possibility of an interaction between TAp63 and mTOR which has not previously been described in the literature. Currently it is believed that TAp63 inhibits mTORC1 in the presence of DNA damage (Cam *et al.*, 2014). We showed a reduction in TAp63 under DR, which is a known suppressor of mTOR. We hypothesise that there might be a molecular feedback between mTORC1 and TAp63; when less mTORC1 is present in the cell TAp63 is consequently down regulated.

We have shed light onto numerous aspects of limbal epithelial cell biology in both human and mouse which are notable in the field (Figure 6.1). Firstly, confirming that age does not have a detrimental effect on the primary culture of human limbal epithelial cells, conversely, novel *in vivo* data showed that age does detrimentally affect the mouse cornea. We have also demonstrated that the corneas of mice respond negatively to treatment with rapamycin. This has the implication that the use of topical rapamycin on the surface of the cornea after allogeneic transplantations (Shin *et al.*, 2008), which could be detrimental to corneal epithelial cells. The future culture of limbal epithelial cells for research and transplantation might also benefit from being cultured in hypoxic conditions, as these may be closer to physiological levels.
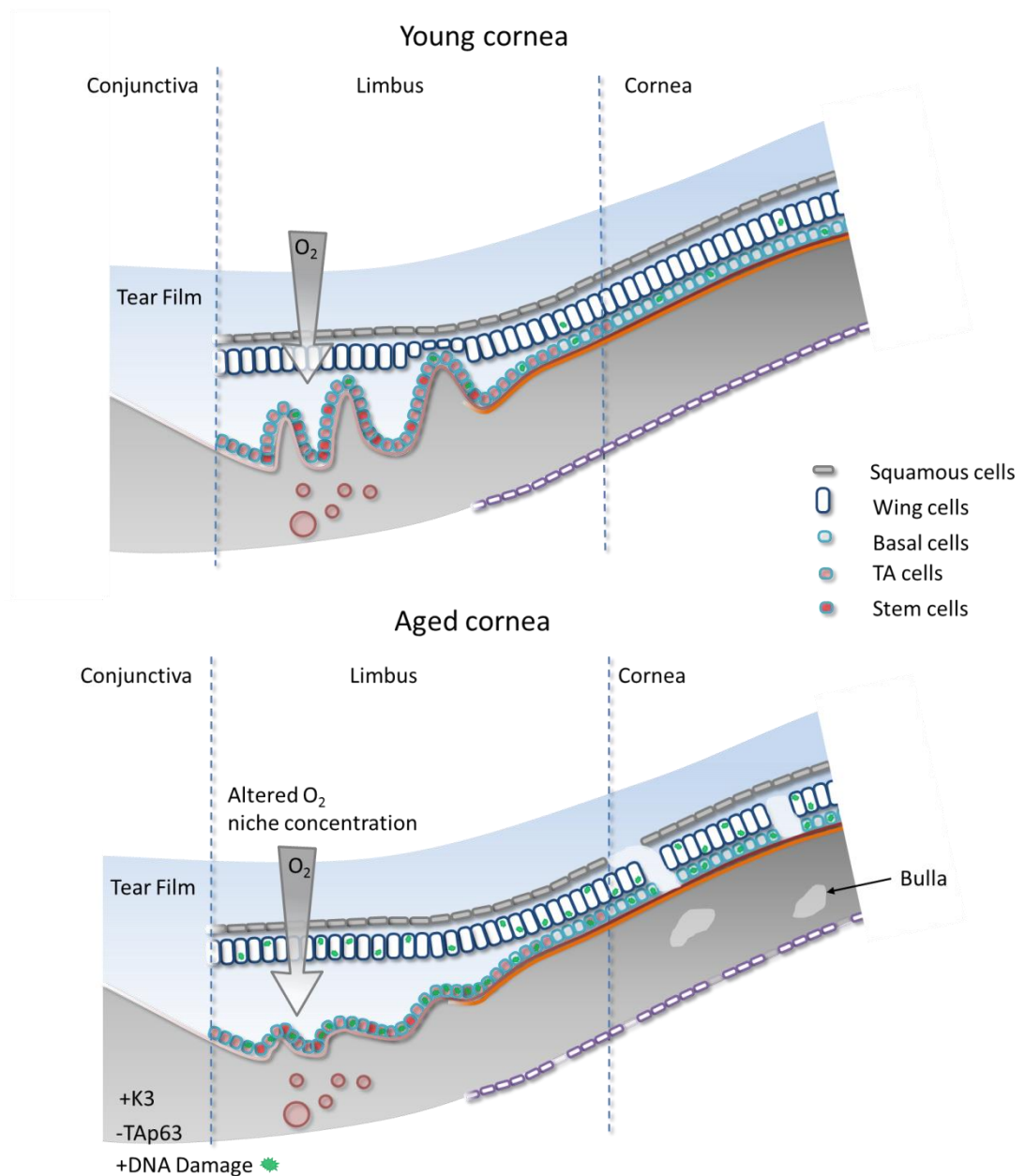
Figure 6.1 The aged corneal epithelium
We showed that DNA damage increased and TAp63 protein levels decreased with age in the mouse cornea. In human donors the peripheral corneal structures plane with age, possibly exposing these regions to an increased amount of oxygen. It was also found that under normoxic conditions the expression of the differentiation marker *KRT3* increased in this condition.

6.2 Limitations of the study

The overall aim of this project was to elucidate the biological significance of ageing on limbal epithelial cells, which led to the study of the growth parameters of human LSC cultures. In humans the effects of older donors on the growth rate and number of progenitors have been mostly inconclusive (Kim *et al.*, 2004; Shanmuganathan *et al.*, 2006; Meyer-Blazejewska *et al.*, 2010; Baylis *et al.*, 2013; Notara *et al.*, 2013). The reasons behind this observation are proposed to be three fold; firstly, there is an age

bias in donated tissue, with most donors aged around 70 years old (figure 6.2). This means that fewer young donors were available to test leading to the broad assignment of age ranges. Notara and colleagues, however who had more donors and age ranges 0-30, 30-60 and 60-90 came to the same conclusion that age does not seem to affect the culture of human limbal epithelial cells (Notara *et al.*, 2013). Secondly, variation between individuals can mask true trends in the data collected and this heterogeneity increases with age. Finally, the time from death to enucleation (post mortem decay) were thought to impede the number of progenitors which could be isolated from the mentioned tissue. Data regarding the time from death to enucleation was not always obtainable.
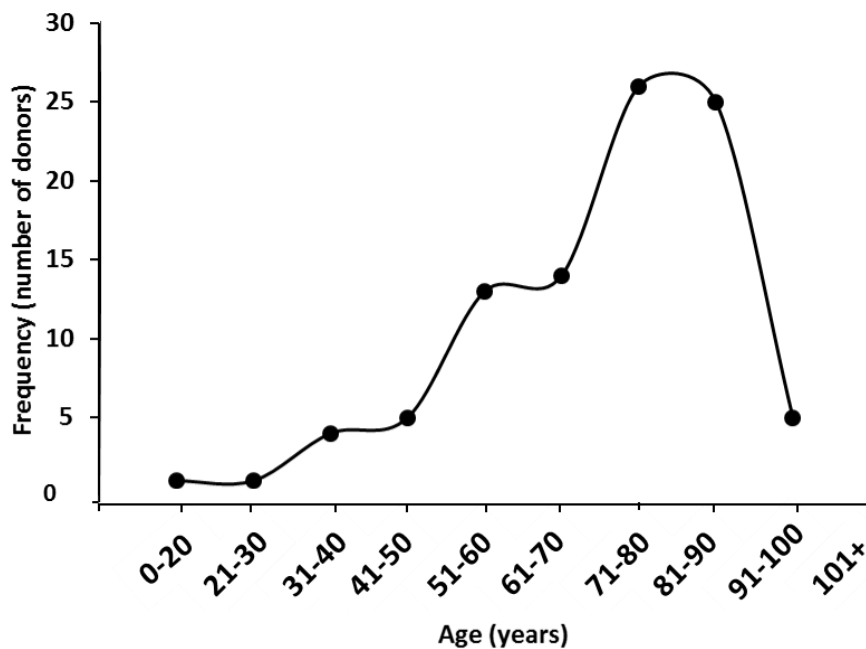
Figure 6.2 Distribution of age in the donors tested in this study
The number of donors was plotted against age categories which spanned 9 years, with exception of the 0-20 group. Most of the donors tested were around 85 years old with the average age of around 71±14 years.
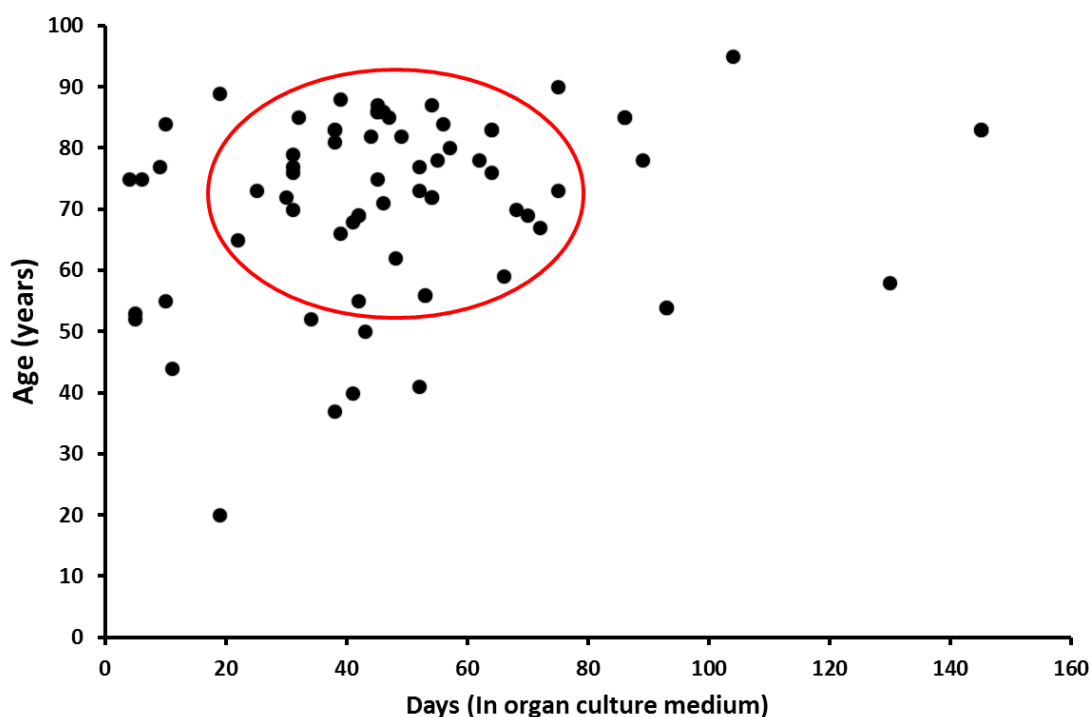
Figure 6.3 Age versus time in organ culture medium
No linear trend was detected between number of days in organ culture medium and age. The red circle highlights that the major proportion of donated tissue was around 50 days in organ culture medium. Mostly the tissue was stored for 50 days±29 in organ culture medium and the donor age around 71 years old ±14.

Figure 6.3 shows that the majority of human donors whose cells were used in this study originated from a similar group. As stated previously the average age was around 71±14. Plotting time in organ culture medium against age also showed that the majority of tissue was stored between 30-60 days.

The culture of limbal epithelial cells is a well-defined process, however there are limitations associated with the extraction process. It is difficult to assess the efficiency of each extraction and the proportion of progenitors. The number of initial progenitors can influence growth rates and CFEs. It was noted in some donors that more calcification had occurred, which could hinder enzymatic digestion of the tissue. The use of mouse eyes allowed us to circumvent these problems, observing the cells *in situ* removed the effects of extraction associated with *in vitro* culture. The time to extraction and time in storage medium of corneal scleral disks was thought to affect the extraction efficiency, however recently it has been shown that these parameters do not exert a significant effect on the culture of explanted limbal tissues (Baylis *et al.*, 2013), merely increasing the lag phase of the cells but not overall yield. These were parameters which this study was very much aware of and it has shown in parallel that the number of days in organ culture medium nor age did not have any statistical effect

on the results.

The cause of a donor's death and their pre existing conditions such as diabetes has an impact on the quality of cells which can be harvested from donated tissue. Stem cells isolated from diabetic donors have been shown to have a reduced ability to generate muscle from adipose derived mesenchymal stem cells (Krawiec *et al.*, 2014). The limbus is also affected by diabetes, with reduced progenitor markers such as ABCG2 and ΔNp63α (Saghizadeh *et al.*, 2011). Regarding the cause of death, pneumonia and meningitis for example can have an effect on stem cell populations. Streptococcus pneumonia can opportunistically infect the corneal epithelium causing lysis and transport of molecules into the cells, via the release of pneumolysin (Taylor *et al.*, 2013). Cancer was the second common cause of death in the donors. Treatments for cancer can be very aggressive and systemic chemotherapy has been shown to actually induce LSCD (Ellies *et al.*, 2001), implying that limbal stem cells are ablated by this treatment. Even habits and lifestyle can affect the number of stem cells for example, smoking has been shown to cause the dysfunction of endothelial progenitor cells (Paschalaki *et al.*, 2013).

The growth of limbal epithelial cells in hypoxia was performed using a nitrogen purging incubator to maintain an oxygen level of 3%, access was also limited to this incubator to reduce the time the door was open. When changing medium however, the cultures were exposed to atmospheric oxygen as an isolator tissue culture cabinet such as a Bio-Spherix unit like that used by Bath and colleagues (Bath *et al.*, 2013) was not available. The exposure time was, however, limited to 3-4 minutes, which is less than the half-life of HIF-1α in normoxia which is 5-8 minutes (Berra *et al.*, 2001). In conclusion, while ultra-specialised equipment was not used, all precautions were taken to ensure that the experiments were performed to the highest stringency possible.

RNA yields were often low and impacted on the quantity of reverse transcription products (cDNA), causing some donors cells to be rejected on the grounds of failing to meet the strict guidelines outlined in MIQE (Minimum information for publication of quantitative real-time PCR experiments) (Bustin *et al.*, 2009). The reason for low RNA yields could be attributed to low cell numbers.

The culture of mouse corneal epithelial cells was attempted on a number of occasions, using the methods outlined by Ma and Liu (Ma and Liu, 2011). We were not able to replicate the successful culture of mLSCs shown in this paper. The failures we

encountered were thought to be caused by bacterial contamination from the natural microorganisms on the surface of the cornea and surrounding fur, in conjunction with poorly defined isolation and cultivation methods. For example, one cannot use 3T3's to co-culture the cells as remnants of these cells would contaminate downstream experiments. Human fibroblasts could be used however they can react poorly to mitotic inactivation. There is a growing trend to culture mouse corneal epithelial cells in serum free conditions, which was the attempted method.

We also tried to obtain *in situ* human limbal sections from stored tissues in approximately five donors, frustratingly however we found that even after careful cryopreservation or paraffin embedding no intact corneal epithelium could visualised using haematoxylin and eosin staining. We concluded that perhaps the organ culture medium or post mortem delay lead to a poorly preserved epithelial layer which limbal stem cells are protected from as they reside deeper within the cornea.

6.3 Future directions

The next logical step would be to assess the number of ɣH2A.X foci and TAFs in human cornea of different donor ages both in cultured cells and in tissue pieces. Comparing *in vitro* and *in vivo* changes in the same donors would bridge the gap between the *in vivo* results on the mouse cornea shown in this study and that of the cultured human cells. Assessing the mechanisms behind which genomic instability increases with age has been studied thoroughly; the downstream effect of how this translates into reduced functionality however remains to be elucidated.

Reducing genomic stress and increasing DNA damage repair in mice has shown to prolong lifespan (Haley-Zitlin and Richardson, 1993; Baker *et al.*, 2013a) and reduce the number of senescent cells in the liver, whether this would work in humans however remains to be seen.

We showed that TAp63 decreased in DR, but it would be interesting to see which mechanism or interactions caused this effect. REDD1 has been shown as a possible mediator between mTOR and p63, inhibiting the activity of REDD1 could shed light on whether p63 is up stream or downstream of mTOR. This could be carried out by one of two ways, by generating a conditional REDD1 knockout mouse and observing p63 expression. If p63 is downstream of the mTOR/14-3-3/REDD1 interaction or completely independent, then knockout of REDD1 would result in no mitigation of p63

on mTOR. Alternatively, by cultivating mouse or human limbal epithelial cells and applying a siRNA REDD1 silencer may yield similar results.

The elusive marker gene for limbal stem cells has evaded many researchers. Identification of a specific marker is a key goal for the field, and would have benefited the investigations carried out in this thesis. The use of "telomapping" for measuring telomere length in different mouse tissues including the cornea (Flores *et al.*, 2008), could be used to isolate this population for further studies such as microarray analysis; as stem cells have longer telomeres than other cells from the same tissue.

Further studies are required to establish the effects of hypoxia on limbal epithelial cells and whether this condition is beneficial to the cultures. It would have been interesting to grow cultures using the full clinical practices, xenobiotic free on human amniotic membranes, and observe the rate of outgrowth from the cultures. The cost of commercial amniotic membrane and troublesome in-house processing of human amniotic membranes inhibited this line of investigation in this study. In an ideal situation, a longitudinal assessment of human LSCs, applying the same tests as in this study and sampling the stem cell population over time would have given us a clearer indication of the effects of age. The efficiency of limbal epithelial cell extractions and outgrowths from small limbal biopsies could be tracked in individual donors over a number of decades, providing an insightful view of ageing dynamics.

In humans, donor age does not have an effect on primary isolated cells. However continuous culture is less viable from older donor's cells. Hypoxia seems to reduce expression of the negative marker gene *KRT3* and stress indicator p21 regardless of the use of DKSFM or LEM. In the mouse cornea *in situ* observations showed that age has a detrimental impact upon the corneal epithelium, however reduced caloric intake can remediate these changes.

Chapter 7 Appendices

Appendix A

Gene and direction	Primers	Primer specificity	Position	Product length (bp)	Tm (°C)	Biological significance
ΔNp63α Sense	GAAACGTACAGGCAACAGCA	Tumour protein 63, (TP63), transcript variant 4, ΔNp63α	1,156	161	55	Positive LSC
ΔNp63α Antisense	GCTGCTGAGGGTTGATAAGC		1,318			
ABCG2 Sense	TCTCTTCTTCCTGACGACCAA	ATP-binding cassette, sub-family G, member 2 (ABCG2), transcript variant 1	1,780	109	60	Positive LSC
ABCG2 Antisense	TCTGTAGTATCCGCTGATGTATTC		1,888			
BMI1 Sense	ACGAGAATCAAGATCACTGAGC	BMI1 polycomb ring finger oncogene (BMI1)	519	122	60	Positive LSC
BMI1 Antisense	CGAACAATACACGTTTTACAGAAG		640			
TERT Sense	CATCCTCTCCACGCTGCTC	Telomerase reverse transcriptase (TERT), transcript variant 1	2,563	114	60	Positive LSC
TERT Antisense	CCAACAAGAAATCATCCACCAAA		2,675			
p16 Sense	ATGTCCTGCTTTTAACGTAGATA	Cyclin-dependent kinase inhibitor 2A, (p16, CDKN2A), transcript variant 1	913	146	60	Arrest
p16 Antisense	CTCACTCCAGAAAACTCCAACA		1,057			
p21 Sense	GCGGCAGACCAGCATGA	Cyclin-dependent kinase inhibitor 1A, (CDKN1A), transcript variant 1	551	80	60	Arrest
p21 Antisense	GTGGGCGGATTAGGGCTTC		629			
KRT3 Sense	TTCCATCTCAGGCACAACAA	Keratin 3, (KRT3)	783	130	60	Negative LSC
KRT3 Antisense	CAGGTCCTCCATGTTCTTAG		912			
KRT12 Sense	GAAGAAGAACCACGAGGATG	Keratin 12, (KRT12)	813	144	53	Negative LSC
KRT12 Antisense	TCTGCTCAGCGATGGTTTCA		958			

Table 7.1 Human cDNA primers list

Genorm

qPCR is a powerful mRNA level quantification tool. Before performing any analysis however, it was important to assess which reference gene was best suited. In this experiment the expression stability of six common reference (housekeeping) genes was analysed: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), actin β (ACTB), 18s ribosomal RNA (18s), ATP synthase subunit β (ATP5B), 60S ribosomal protein L13a (RPL13A) and succinate dehydrogenase complex (SDHA). Cells from four different donors (75, 73, 89 and one 85 year old) were used for this analysis, cultured in hypoxia and normoxia (see chapter 4). All genes were tested in all samples, the subsequent C_t values were firstly subtracted from the highest C_t value generated. This generated the ΔC_t, the $2^{(-\Delta\Delta C_t)}$ equation was then applied. This subsequently generated the relative expression compared to that of the least expressed gene. To calculate the variation, multiple pairwise analyses of the standard deviations were performed. The average of each genes standard deviation was calculated and compared (Vandesompele *et al.*, 2002) (figure 3.11).
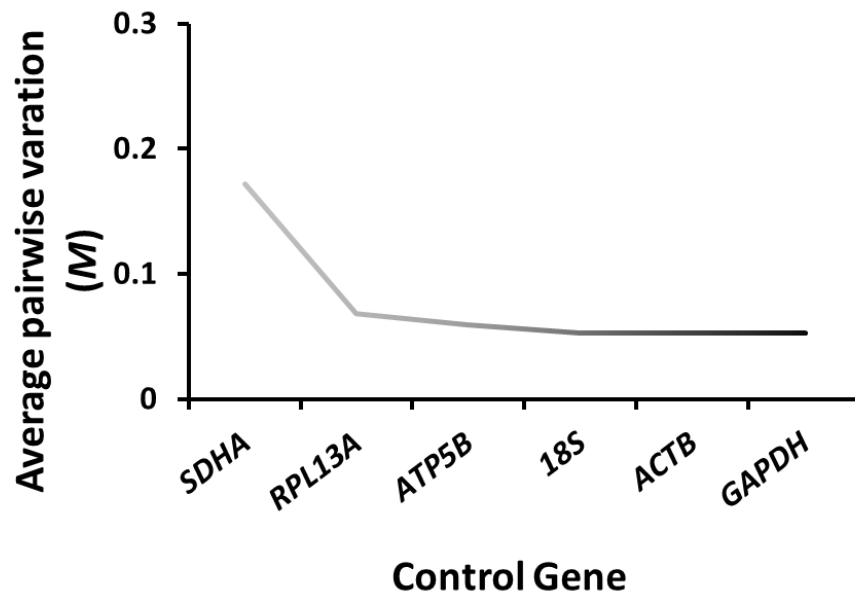
Figure 7.1 GeNorm analysis of 6 common reference genes in cultures containing limbal epithelial cells
GAPDH and Actin β were shown to the most stable reference genes over the other 4 genes tested. The average pairwise variation (*M*) was calculated from multiple pairwise comparisons of each gene in the different donor and conditions stated (see appendices).

As GAPDH had the least amount of variation between different donors and conditions this gene was used as the housekeeping gene in all qPCR analysis in the following experiments.

GeNorm	Primer specificity	Anchor nucleotide	Context sequence	Tm (°C)	Biological significance
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 1	235	99	60	Control
RLP13A	Ribosomal protein L13a, transcript variant 1	727	223	60	Control
ATP5B	ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide	1200	150	60	Control
ACTB	Actin, beta	1195	106	60	Control
SDHA	Succinate dehydrogenase complex, subunit A	1032	154	60	Control

Table 7.2 Human geNorm primers list

Donor/Condition	GAPDH	18s	SD
444 PC	6.46E-06	3.26E-07	4.33969E-06
444 SC2	0.002616	0.000234	0.001684215
431 PC	0.000316	7.59E-06	0.000217826
431 SC2	0.005995	0.001434	0.003224779
423 SC1	7.81E-06	1.55E-07	5.41027E-06
Normoxia	5.14E-10	2.88E-06	2.03831E-06
Hypoxia	0.00016	2.8E-07	0.000113075

	18s	RPL13A	SD
444 PC	3.26E-07	0.000192	0.000136
444 SC2	0.000234	0.069796	0.049188
431 PC	7.59E-06	0.007074	0.004996
431 SC2	0.001434	0.416446	0.293458
423 SC1	1.55E-07	0.000218	0.000154
Normoxia	2.88E-06	1.42E-05	7.98E-06
Hypoxia	2.8E-07	0.000202	0.000142

	RPL13A	ATP5B	SD
444 PC	0.000192	0.000139	3.73E-05
444 SC2	0.069796	0.202613	0.093916
431 PC	0.007074	0.004298	0.001963
431 SC2	0.416446	0.065418	0.248214
423 SC1	0.000218	0.000187	2.2E-05
Normoxia	1.42E-05	9.32E-07	9.36E-06
Hypoxia	0.000202	0.001787	0.001121

	ATP5B	ACTB	SD
444 PC	0.000139	1.62E-06	9.74E-05
444 SC2	0.202613	0.001948	0.141892
431 PC	0.004298	0.000142	0.002939
431 SC2	0.065418	0.004408	0.043141
423 SC1	0.000187	6.18E-06	0.000128
Normoxia	9.32E-07	0.000157	0.00011
Hypoxia	0.001787	6.58E-05	0.001217

	ACTB	SDHA	SD
444 PC	1.62E-06	0.001192	0.000842
444 SC2	0.001948	0.215152	0.150758
431 PC	0.000142	1	0.707007
431 SC2	0.004408	0.621461	0.436322
423 SC1	6.18E-06	0.002909	0.002053
Normoxia	0.000157	0.00016	2.34E-06
Hypoxia	6.58E-05	0.020874	0.014714

	GAPDH	RPL13A	SD
444 PC	6.46E-06	0.000192	0.000131286
444 SC2	0.002616	0.069796	0.047503659
431 PC	0.000316	0.007074	0.004778644
431 SC2	0.005995	0.416446	0.290232943
423 SC1	7.81E-06	0.000218	0.000148351
Normoxia	5.14E-10	1.42E-05	1.00196E-05
Hypoxia	0.00016	0.000202	2.93205E-05

	18s	ATP5B	SD
444 PC	3.26E-07	0.000139	9.83E-05
444 SC2	0.000234	0.202613	0.143103
431 PC	7.59E-06	0.004298	0.003034
431 SC2	0.001434	0.065418	0.045243
423 SC1	1.55E-07	0.000187	0.000132
Normoxia	2.88E-06	9.32E-07	1.38E-06
Hypoxia	2.8E-07	0.001787	0.001263

	RPL13A	ACTB	SD
444 PC	0.000192	1.62E-06	0.000135
444 SC2	0.069796	0.001948	0.047976
431 PC	0.007074	0.000142	0.004902
431 SC2	0.416446	0.004408	0.291355
423 SC1	0.000218	6.18E-06	0.00015
Normoxia	1.42E-05	0.000157	0.000101
Hypoxia	0.000202	6.58E-05	9.61E-05

	ATP5B	SDHA	SD
444 PC	0.000139	0.001192	0.000745
444 SC2	0.202613	0.215152	0.008866
431 PC	0.004298	1	0.704068
431 SC2	0.065418	0.621461	0.393182
423 SC1	0.000187	0.002909	0.001925
Normoxia	9.32E-07	0.00016	0.000113
Hypoxia	0.001787	0.020874	0.013497

	GAPDH	ATP5B	SD
444 PC	6.46E-06	0.000139	9.40002E-05
444 SC2	0.002616	0.202613	0.141419283
431 PC	0.000316	0.004298	0.002815687
431 SC2	0.005995	0.065418	0.042018563
423 SC1	7.81E-06	0.000187	0.000126358
Normoxia	5.14E-10	9.32E-07	6.58683E-07
Hypoxia	0.00016	0.001787	0.001150267

	18s	ACTB	SD
444 PC	3.26E-07	1.62E-06	9.13E-07
444 SC2	0.000234	0.001948	0.001212
431 PC	7.59E-06	0.000142	9.47E-05
431 SC2	0.001434	0.004408	0.002103
423 SC1	1.55E-07	6.18E-06	4.26E-06
Normoxia	2.88E-06	0.000157	0.000109
Hypoxia	2.8E-07	6.58E-05	4.63E-05

	RPL13A	SDHA	SD
444 PC	0.000192	0.001192	0.000707
444 SC2	0.069796	0.215152	0.102782
431 PC	0.007074	1	0.702105
431 SC2	0.416446	0.621461	0.144967
423 SC1	0.000218	0.002909	0.001903
Normoxia	1.42E-05	0.00016	0.000103
Hypoxia	0.000202	0.020874	0.014618

	GAPDH	ACTB	SD
444 PC	6.46E-06	1.62E-06	3.42646E-06
444 SC2	0.002616	0.001948	0.000472516
431 PC	0.000316	0.000142	0.000123087
431 SC2	0.005995	0.004408	0.001122148
423 SC1	7.81E-06	6.18E-06	1.14857E-06
Normoxia	5.14E-10	0.000157	0.000110928
Hypoxia	0.00016	6.58E-05	6.67505E-05

	18s	SDHA	SD
444 PC	3.26E-07	0.001192	0.000843
444 SC2	0.000234	0.215152	0.15197
431 PC	7.59E-06	1	0.707101
431 SC2	0.001434	0.621461	0.438425
423 SC1	1.55E-07	0.002909	0.002057
Normoxia	2.88E-06	0.00016	0.000111
Hypoxia	2.8E-07	0.020874	0.01476

	GAPDH	SDHA	SD
444 PC	6.46E-06	0.001192	0.000838548
444 SC2	0.002616	0.215152	0.150285542
431 PC	0.000316	1	0.706883587
431 SC2	0.005995	0.621461	0.435200317
423 SC1	7.81E-06	0.002909	0.00205167
Normoxia	5.14E-10	0.00016	0.000113273
Hypoxia	0.00016	0.020874	0.014647065

Table 7.3 GeNorm multiple pairwise analysis of housekeeping genes

Run 1

Sample	Gene	Average C_t	Standard deviation
Donor 1 Normoxia	GAPDH	16.33550262	0.061323881
Donor 1 Hypoxia	GAPDH	15.09079742	0.412290126
Donor 2 Normoxia	GAPDH	15.4974699	0.138759106
Donor 2 Hypoxia	GAPDH	15.37895203	0.159935817
Donor 3 Normoxia	GAPDH	15.19962025	0.122729786
Donor 3 Hypoxia	GAPDH	14.63101196	0.111248538
Donor 4 Normoxia	GAPDH	16.29699326	0.11499203
Donor 4 Hypoxia	GAPDH	14.99137592	0.058257744

Combined deviation	Average deviation
0.694247606	0.50957959
0.196807572	
0.543225671	
0.823408483	
0.601235249	
0.500156986	
0.539566655	
0.177988499	

Run 2

Sample	Gene	Average C_t	Standard deviation
Donor 1 Normoxia	GAPDH	15.35368824	0.541330099
Donor 1 Hypoxia	GAPDH	14.81246948	0.148650974
Donor 2 Normoxia	GAPDH	14.72923279	0.14248392
Donor 2 Hypoxia	GAPDH	14.21447659	0.243733421
Donor 3 Normoxia	GAPDH	14.34934521	0.117583677
Donor 3 Hypoxia	GAPDH	13.92368317	0.032306761
Donor 4 Normoxia	GAPDH	15.53393078	0.157003269
Donor 4 Hypoxia	GAPDH	14.73966217	0.30863592

Table 7.4 Deviations between qPCR runs

Appendix B

Gene (Category)	Shapiro-Wilk test (p values)
ABCG2 (20-69 years)	*0.723*
ABCG2 (70-90 years)	*0.052*
TERT (20-69 years)	*0.978*
TERT (70-90 years)	*0.051*
BMI1 (20-69 years)	*0.973*
BMI1 (70-90 years)	*0.102*
KRT3 (20-69 years)	*0.053*
KRT3 (70-90 years)	*0.039*
KRT12 (20-69 years)	*N/A*
KRT12 (70-90 years)	*0.866*
p21 (20-69 years)	*0.542*
p21 (70-90 years)	*0.168*

Table 7.5. Normality tests for the effect of donor age on gene expression in limbal epithelial cells

Gene (Category)	Shapiro-Wilk test (p values)
ABCG2 (0-30 days)	*0.852*
ABCG2 (30+ days)	*0.109*
TERT (0-30 days)	*0.167*
TERT (30+ days)	*0.027*
BMI1 (0-30 days)	*0.894*
BMI1 (30+ days)	*0.076*
KRT3 (0-30 days)	*0.381*
KRT3 (30+ days)	*0.540*
KRT12 (0-30 days)	*0.278*
KRT12 (30+ days)	*0.872*
p21 (0-30 days)	*0.497*
p21 (30+ days)	*0.879*

Table 7.6 Normality tests for the effect of number of days in organ culture medium on the gene expression of limbal epithelial cells

Sample/Condition/Area	Shapiro-Wilk test (p values)
KO DR Central Cornea	*0.001*
KO DR Peripheral Cornea	*<0.000*
KO AL Central Cornea	*0.172*
KO AL Peripheral Cornea	*0.582*
WT AL Central Cornea	*0.033*
WT AL peripheral Cornea	*0.069*
WT DR Central Cornea	*0.017*
WT DR Peripheral Cornea	*0.946*

Table 7.7 Normality tests for corneal thickness of wild type and *Tert*^{-/-} fed either *ad libitum* dietary restricted.

Sample/Conditions	Kruskal-Willis test on independent samples (p values)
WT DR vs WT AL	*<0.001*
KO AL vs WT AL	*<0.001*
KO DR vs WT AL	*<0.001*
WT DR vs WT AL	*0.46*
KO AL vs KO DR	*1.0*
WT DR vs KO AL	*1.0*

Table 7.8 Summary of significance between corneal thickness in wild type, *Tert*^{-/-} fed either *ad libitum* or dietary restriction in the central cornea

Sample/Conditions	Kruskal-Willis test on independent samples (p values)
KO AL vs WT AL	0.003
KO DR vs WT AL	0.179
WT DR vs WT AL	0.866
KO DR vs WT DR	1.0
KO AL vs WT DR	1.0
KO AL vs KO DR	1.0

Table 7.9 Summary of significance between corneal thicknesses in wild type, *Tert^{-/-}* fed either *ad libitum* or dietary restriction in the peripheral cornea.

Sample	Kruskal-Willis test on independent samples (p values)
Young vs Old	0.004
Dietary restricted vs Old (AL)	0.027
Rapamycin treated vs Rapamycin control	1.0
KO DR vs WT AL	1.0
KO AL vs WT AL	1.0
WT DR vs KO DR	1.0
WT DR vs KO AL	1.0
KO AL vs KO DR	1.0

Table 7.10 Summary of significance data for the frequency of corneal bullae

Sample/Condition/Area	Shapiro-Wilk test (p values)
Old Central Cornea	*0.884*
Old Peripheral Cornea	*0.564*
Young Central Cornea	*0.542*
Young Peripheral Cornea	*0.449*
DR Central Cornea	*0.817*
DR Peripheral Cornea	*0.145*
Rapamycin Treated Central Cornea	*0.314*
Rapamycin Treated Peripheral Cornea	*0.906*
Rapamycin control Central Cornea	*0.819*
Rapamycin control Peripheral Cornea	*0.218*

Table 7.11 Summary of normality tests for TAp63 immunofluorescence in peripheral and central regions of old, young, dietary restriction, rapamycin treated and controls

Sample/Condition/Area	ANOVA (p values)
Old vs Young	*<0.001*
Old (AL) vs DR	*0.002*
Rapamycin treated vs rapamycin control	*<0.001*

Table 7.12 Summary of significance results TAp63 immunofluorescence in the central cornea of old, young, dietary restriction, rapamycin treated and controls

Sample/Condition/Area	ANOVA (p values)
Old vs Young	*<0.001*
Old (AL) vs DR	*<0.001*
Rapamycin treated vs rapamycin control	*<0.001*

Table 7.13 Summary of significance results TAp63 immunofluorescence in the peripheral cornea of old, young, dietary restriction, rapamycin treated and controls

Sample/Condition	Shapiro-Wilk test
WT AL Central Cornea	*<0.001*
WT AL Peripheral Cornea	*0.418*
KO DR Central Cornea	*0.002*
KO DR Peripheral Cornea	*<0.001*
KO AL Central Cornea	*0.002*
KO AL Peripheral Cornea	*0.008*
WT DR Central Cornea	*0.002*
WT DR Peripheral Cornea	*<0.001*

Table 7.14 Summary of normality test of between TAp63 immunofluorescence in wild type, *Tert*^-/- mice fed either *ad libitum* or dietary restricted

Sample/Condition (Central Cornea)	Kruskal-Willis test on independent samples (p values)
WT AL vs KO DR	*1.0*
WT AL vs KO AL	*0.001*
WT AL vs WT DR	*0.001*
KO DR vs KO AL	*<0.0001*
KO DR vs WT DR	*<0.0001*
KO AL vs WT DR	*1.0*

Table 7.15 Pairwise comparison between TAp63 immunofluorescence in the central cornea of wild type, *Tert*^-/- mice fed either *ad libitum* or dietary restricted

Sample/Condition (Peripheral Cornea)	Kruskal-Willis test on independent samples (p values)
WT AL vs KO DR	0.007
WT AL vs KO AL	<0.001
WT AL vs WT DR	0.679
KO DR vs KO AL	<0.001
KO DR vs WT DR	0.677
KO AL vs WT DR	<0.001

Table 7.16 Pairwise comparison between TAp63 immunofluorescence in the peripheral cornea of wild type, *Tert*^{-/-} mice fed either *ad libitum* or dietary restricted

Sample	Shapiro-Wilk test (p values)
Young	<0.001
Old	0.002
Dietary restriction	<0.001
Rapamycin Treated	<0.001
Rapamycin Control	<0.001

Table 7.17 Normality tests for central cornea of γH2A.X foci data from the central cornea of young, old, dietary restriction, rapamycin and control mice

Sample	Shapiro-Wilk test (p values)
Young	<0.001
Old	<0.001
Dietary restriction	<0.001
Rapamycin Treated	<0.001
Rapamycin Control	<0.001

Table 7.18 Normality tests for central cornea of γH2A.X foci data from the peripheral cornea of young, old, dietary restriction, rapamycin and control mice

Sample	Kruskal-Willis test on Independent samples (p values)
Rapamycin Treated vs Rapamycin Control	1.0
DR vs Old (AL)	<0.001
Young vs Old	<0.001

Table 7.19 Summary of statistical analysis of the difference in the number of γH2A.X foci in the central cornea of young, old, dietary restriction, rapamycin and control mice

Sample	Kruskal-Willis test on independent samples (p values)
Old vs Young	1.0
(AL) Old vs DR	<0.001
Rapamycin Control vs Rapamycin Treated	<0.001

Table 7.20 Summary of statistical analysis of the difference in the number of γH2A.X foci in the peripheral cornea of young, old, dietary restriction, rapamycin treated and control mice

Sample	Kruskal-Willis test on independent samples (p values)
WT DR vs KO AL	<0.001
WT DR vs WT AL	<0.001
WT DR vs KO DR	<0.001
KO AL vs WT AL	0.001
KO AL vs KO DR	<0.001
WT AL vs KO DR	0.057

Table 7.21 Summary of statistical analysis of the difference in the number of γH2A.X foci in the central cornea of _Tert_[-/-] and wild type mice fed _ad libitum_ or dietary restricted

Sample	Kruskal-Willis test on independent samples (p values)
WT DR vs WT AL	*<0.001*
WT DR vs KO AL	*<0.001*
WT DR vs KO DR	*<0.001*
WT AL vs KO AL	*1.0*
WT AL vs KO DR	*0.026*
KO AL vs KO DR	*0.019*

Table 7.22 Summary of statistical analysis of the difference in the number of ɣH2A.X foci in the peripheral cornea of *Tert⁻ᐟ⁻* and wild type mice fed ad libitum or dietary restricted

Sample/Conditions	Kruskal-Wallis tests on independent samples (p values)
Young vs Old	*<0.001*
DR vs Old (AL)	*<0.001*
Rapamycin Control vs Rapamycin Treated	*0.438*

Table 7.23 Summary of statistical analysis of the differences in the number of TAF's in the central cornea of old, young, dietary restriction, rapamycin and control mice

Sample/Conditions	Kruskal-Wallis tests on independent samples (p values)
Young vs Old	*1.0*
DR vs Old (AL)	*0.007*
Rapamycin Control vs Rapamycin Treated	*<0.001*

Table 7.24 Summary of statistical analysis of the difference in the number of TAF's in the peripheral cornea, between old, young, dietary restriction, rapamycin and control mice

Sample/Condition	Shapiro-Wilk test (p values)
WT AL Peripheral cornea	<0.001
WT DR Peripheral cornea	<0.001
KO AL Peripheral cornea	<0.001
KO DR Peripheral cornea	<0.001
WT AL Central cornea	<0.001
WT DR Central cornea	<0.001
KO AL Central cornea	<0.001
KO DR Central cornea	<0.001

Table 7.25 Normality test for *Tert*$^{-/-}$ and wildtype

Sample/Condition	Kruskal-Wallis tests on independent samples (p values)
WT AL vs WR DR	<0.001
KO DR vs WT DR	<0.001
KO AL vs WT DR	<0.001
KO DR vs WT AL	1.0
KO AL vs WT AL	1.0
KO DR vs KO AL	1.0

Table 7.26 TAF's in the central cornea of *Tert*$^{-/-}$ and wild type mice fed either *ad libitum* or dietary restricted

Sample/Condition	Kruskal-Wallis tests on independent samples (p values)
WTAL vs WR DR	<0.001
KO DR vs WT DR	<0.001
KO AL vs WT DR	<0.001
KO DR vs WT AL	0.077
KO AL vs WT AL	1.0
KO DR vs KO AL	1.0

Table 7.27 TAF's in the peripheral cornea of *Tert*$^{-/-}$ and wild type mice fed either ad libitum or dietary restricted

Sample/Condition	Shapiro-Wilk test (p values)
Old Central Cornea	*0.292*
Old Peripheral Cornea	*0.037*
Young Central Cornea	*0.491*
Young Peripheral Cornea	*0.042*
DR Central Cornea	*0.490*
DR Peripheral Cornea	*0.110*
Rapamycin Treated Central Cornea	*0.070*
Rapamycin Treated Peripheral Cornea	*0.015*
Rapamycin Control Central Cornea	*0.023*
Rapamycin Control Peripheral Cornea	*<0.001*

Table 7.28 Normality tests for the percentage of Ki67 positive cells.

Gene	Shapiro-Wilk test (p values)
ΔNp63α	*0.133*
ABCG2	*0.254*
BMI1	*0.424*
CK3	*0.873*
CK12	*0.629*
p21	*0.023*
p16	*0.889*

Table 7.29 Summary of normality data for the gene expression analysis of HTCEpi cells

Sample	Shapiro-Wilk test (p values)
Glycolysis 3T3 Normoxia	0.412
Glycolysis HTCEpi Normoxia	0.021
OXPHOS 3T3 Normoxia	0.98
OXPHOS HTCSC	0.503
Glycolysis 3T3 Hypoxia	0.627
Glycolysis HTCEpi Hypoxia	0.543
OXPHOS 3T3 Hypoxia	0.787
OXPHOS HTCEpi Hypoxia	0.154
Glycolysis DKSFM Normoxia	0.285
Glycolysis LEM Normoxia	0.126
OXPHOS DKSFM Normoxia	0.33
OXPHOS LEM Normoxia	0.702
Glycolysis DKSFM Hypoxia	0.046
Glycolysis LEM Hypoxia	0.403
OXPHOS DKSFM Hypoxia	0.917
OXPHOS LEM Hypoxia	0.175

Table 7.30 Summary of Shapiro-Wilk tests for the metabolic assay

Size/Condition	Shapiro-Wilk test (p values)
<1mm (DKSFM) Normoxia	0.303
1-4mm (DKSFM) Normoxia	0.532
>4mm (DKSFM) Normoxia	0.001
<1mm (LEM) Normoxia	0.893
1-4mm (LEM) Normoxia	0.902
>4mm (LEM) Normoxia	0.178
<1mm (DKSFM) Hypoxia	0.5
1-4mm (DKSFM) Hypoxia	0.750
>4mm (DKSFM) Hypoxia	0.097
<1mm (LEM) Hypoxia	0.895
1-4mm (LEM) Hypoxia	0.499
>4mm (LEM) Hypoxia	0.042

Table 7.31 Shapiro-Wilk normality tests for colony size cultures in LEM and DKSFM under normoxia and hypoxia

Sample/Condition/Area	Shapiro-Wilk test (p values)
Old Central Cornea	0.046
Old Peripheral Cornea	0.001
Young Central Cornea	0.013
Young Peripheral Cornea	0.739
DR Central Cornea	0.015
DR Peripheral Cornea	0.024
Rapamycin Treated Central Cornea	0.166
Rapamycin Treated Peripheral Cornea	0.097
Rapamycin control Central Cornea	0.080
Rapamycin control Peripheral Cornea	0.176

Table 7.32 Normality tests on data for corneal thickness of old, young, dietary restricted, rapamycin treated and control mice.

Gene	Shapiro-Wilk test (p values)
ABCG2	0.004
TERT	0.079
BMI1	0.722
KRT3	0.403
KRT12	0.471
p21	0.222

Table 7.33 p-values of a Shapiro-Wilk test for qPCR data from DKSFM cultured donor cells

Gene	Shapiro-Wilk test (p values)
ABCG2	0.015
TERT	0.041
BMI1	0.732
KRT3	0.004
KRT12	0.574
p21	0.894

Table 7.34 Results of the Shapiro-Wilk tests on qPCR data from LEM cultured donor cells

Appendix C
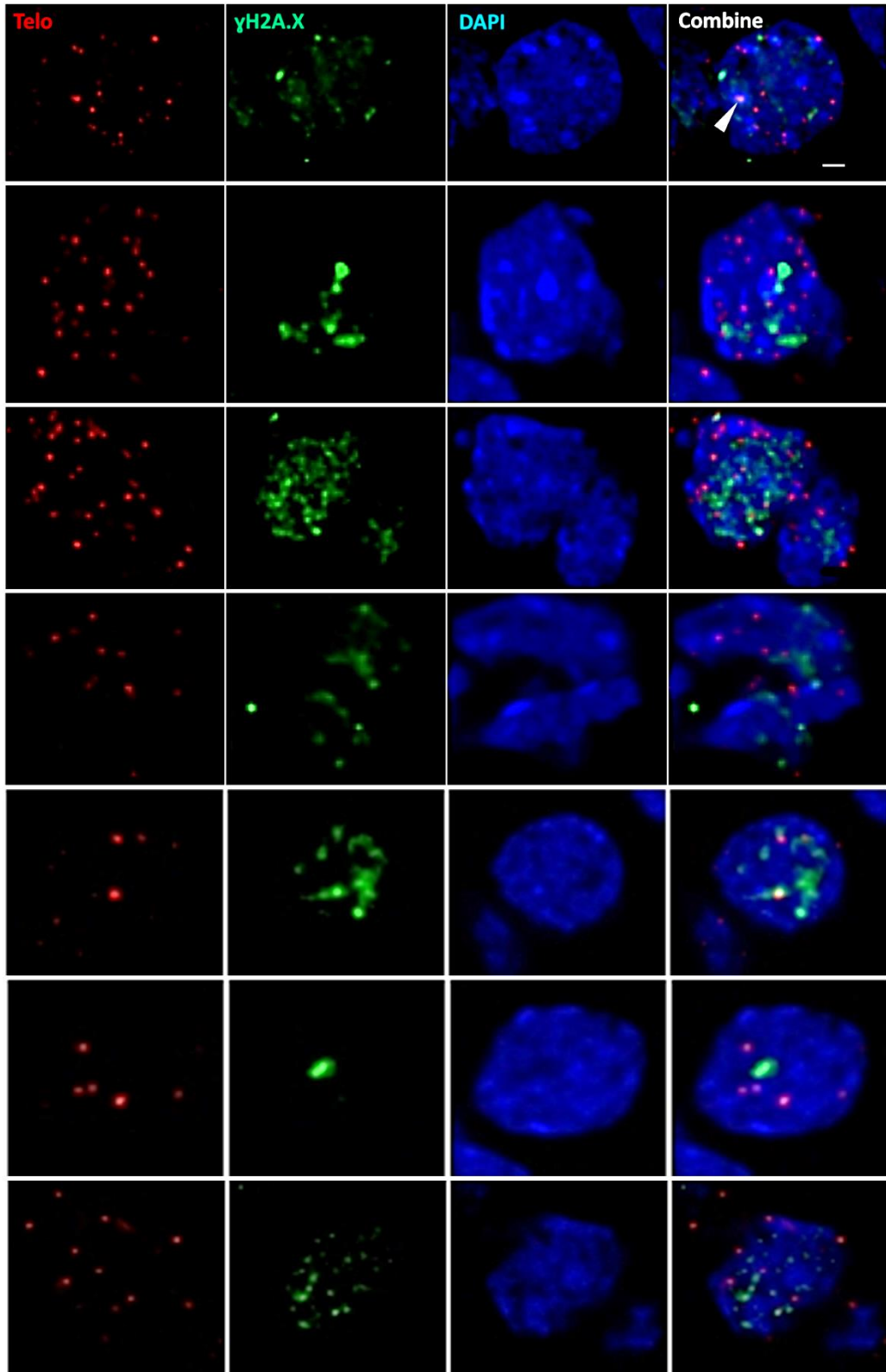
Figure 7.2 Representative deconvoluted images of γH2A.X, telomere and nuclear staining from a selection of samples
Telomere probe (red), γH2A.X foci (green) and nuclear DNA is stained blue with DAPI. An example of a telomere associated foci is indicated with a white arrow. Images were deconvoluted with Huygens (SVI) software, X100 magnification, scale bar equals 5μm
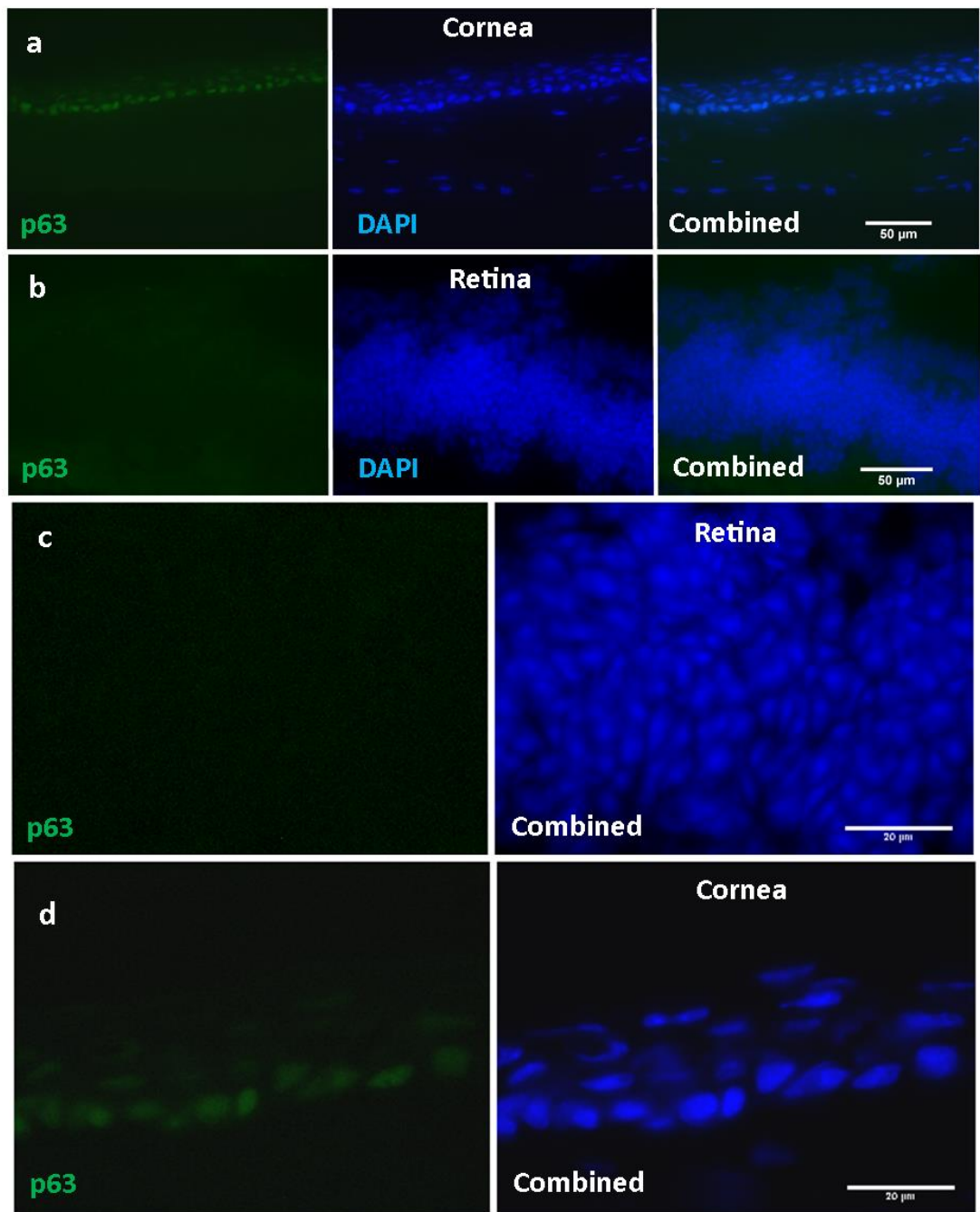
Figure 7.3 TAp63 antibody specificity controls
To test the specificity of the directly conjugated TAp63 antibody, anatomical regions which do not express p63 were used to validate the antibody. The retina in panel b and c was negative for non specific staining while in the same conditons the cornea (panel a, and d) shows positive staining.

Appendix D

Author	Year	Medium	Species	Oxygen (%)	CFE (%) Approx	qPCR/RT PCR p63	ABCG2	Immunofluorescence p63	ABCG2	CK3	Cell cycle			Donors	Incubator/Condition	Notes
Bath *et al*	2013	LEM	Human	21	10	N/A	N/A	No Difference	No Difference	No Difference	Less S/G2 in 2%			6 to 9 pooled	Biospherix Xvivo	
				15	7	N/A	N/A	Decrease	Decrease	Increase						
				10	12	N/A	N/A	Increase	No Difference	Decrease						
				5	14	N/A	N/A	No Difference	No Difference	Decrease						
				2	14	N/A	N/A	No Difference	No Difference	Decrease						
		Epilife	Human	21	Not done	N/A	N/A	Decrease	No Difference	No Difference	No Significance			6 to 9 pooled	Biospherix Xvivo	Plus bovine pituitary extract, bovine insulin, bovine transferrin, human corneal growth supplement (HCGS) solution and mouse EG
				15	Not done	N/A	N/A	Increase	Decrease	Increase						
				10	Not done	N/A	N/A	Increase	Decrease	Increase						
				5	Not done	N/A	N/A	Increase	No Difference	Decrease						
				2	Not done	N/A	N/A	Increase	Increase	Decrease						
						RT PCR		**K15**								
Miyashita *et al*	2007	DKSFM	Human	21	1	More KRT3 in 21%	N/A	No Difference	N/A	N/A	G1 increase in hypoxia			6	Nitorgen purging	Plus 100 ng/mL cholera toxin & 10 ng/mL human recombinant EGF
				5	3		N/A	No Difference	N/A	N/A				6		
				2			N/A	No Difference	N/A	N/A						
						RT PCR p63	**ABCG2**									
O'Callaghan *et al*	2011	LEM	Rabbit	21	9	No Significance	No Significance	N/A	N/A	N/A				12	Nitorgen purging	
				14	12	No Significance	No Significance	N/A	N/A	N/A	More S Phase	Less G0/G1	More G2/M	12		
				8	7	No Significance	No Significance	N/A	N/A	N/A	More S Phase	Less G0/G1	More G2/M	12		
				2	0	No Significance	No Significance	N/A	N/A	N/A	Not done	Not done	Not done	12		
						QPCR		**Western blots** p63	**KRT14**							
Li *et al*	2011	LEM	Human	21	N/A Explant method	N/A	N/A	Increase	Increase		N/A	N/A	N/A	Not stated	Nitorgen purging	
				2		N/A	N/A	Decrease	Decrease		N/A	N/A	N/A			
						K10	**K12**		**K10**	**K12**						
		LEM	Rabbit	21	N/A Explant method	Decrease in normoxia	No Significance		Increase	No Significance	N/A	N/A	N/A			
				2					Decrease	No Significance	N/A	N/A	N/A			
								Immunofluorescence p63	**K19**							
Ma *et al*	2011	DKSFM	Mouse	21	11.13±1.86	N/A	N/A	No Significance	No Significance		N/A	N/A	N/A	Not stated	Nitorgen purging	
				2	12.50±1.50	N/A	N/A	No Significance	No Significance		N/A	N/A	N/A			

Table 7.35 Summary table of previous papers studying the effects of hypoxia on limbal epithelial cells.

Appendix E

Donor	Age of Donor	Cause of death
B27339A	97	?
B27374A	70	?
B28252B	91	Pneumonia
B28262B	?	Multiorgan failure
B28280a	72	Pneumonia
B28613B	91	Cancer
B29062A	69	COPD
B29063A	82	?
B29063B	82	MI
B29394B	?	Cancer
B29439B	?	IHD
B29442B	?	Pneumonia
B29944B	71	CVA
B29947A	86	End-stage heart failure
B29947B	86	?
B30479A	?	?
M14581A	73	ovarian cancer
M14716A	39	?
M14747A	67	intracranial haem
M15030B	77	COPD
M15273B	75	?
M15312A	54	Sepsis
M15312A	54	Sepsis
M15332B	85	multi organ failure
M15332B	85	multi organ failure
M15423B	89	stroke
M15431A	73	pneumonia
M15444B	75	heart disease
M15669A	53	intracranial haem
M15761A	83	?
M15761A	83	?
M15773A	63	cerebral infarct
M15837A	59	heart disease
M15851A	78	respiratory failure
M15890A	78	cardiac arrest
M15902B	77	stroke
M15921B	87	stroke
M15922B	62	cardiac arrest
M16058B	62	pancreatitis
M16093A	51	intracranial haem
M16174B	75	heart disease
M16225B	21	meningitis

M16225B	21	meningitis
M16260A	90	?
M16272A	58	multi organ failure
M16321A	47	ventricular failure
M16330A	38	septicaemia
M16718A	80	intracranial haem
M16920A	26	multi organ failure
M16942B	55	suicide - hanging
M16952B	68	intracranial haem
M17093A	64	meningitis
M17117B	83	pneumonia
M17662B	83	COPD
M17760B	50	hypoxic brain injury
M17904A	59	intracranial haem
M18013A	55	liver disease
M18052B	44	intracranial haem
M18158B	86	pneumonia
M18168A	40	intracranial haem
M18187A	93	pneumonia
M18321A	87	renal failure
M18362B	85	intracranial haem
M18363A	86	stroke
M18373A	95	myocardial fibrosis
M18373B	95	myocardial fibrosis
M18432B	84	cardiac arrest
M18452B	41	liver disease
M18488B	90	cancer
M18512A	84	pneumonia
M18524B	70	renal failure
M18590A	78	COPD
M18717A	69	multi organ failure
M18743B	85	cardiac failure
M18746B	79	COPD
M18749B	77	resp tract infection
M18752B	76	alcoholic liver disease
M18878B	65	bowel cancer
M18930A	51	lung cancer
M19016A	72	perforated gastric cancer
M19022B	88	multi organ failure
M19023A	66	cardiac arrest
M19030B	81	cardiac failure
M19051A	55	cardiac arrest
M19076B	76	bowel cancer
M19081A	37	cystic fibrosis

M19081B	37	cystic fibrosis
M19153A	63	liver failure - overdose
M19196A	78	gastro intestinal haem
M19196B	78	gastro intestinal haem
M19213A	72	?
M19213B	72	?
M19261A	69	?
M19261B	69	?
M19290A	83	?
M19290B	83	?
M19297A	47	intracranial haem
M19302B	81	cancer
M19308B	?	?
M19309A	83	pneumonia
M19309B	83	pneumonia
M19314A	76	unascertained
M19377A	73	cancer
M19379A	42	intracranial haem
M19396B	82	COPD
M19408A	58	breast cancer
M19569A	56	breast cancer
M19569B	56	breast cancer
M19643A	66	cancer
M15698B	?	?
M19771A	52	lung cancer
M19971B	62	?
M19999B	72	COPD
M20132A	48	intracranial haem
M20095A	53	cardiac arrest
M20050B	71	stroke
M20103A	65	?
M20077A	72	multi organ failure
M20107A	75	intracranial haem
M20107B	75	intracranial haem
M20156A	62	hypoxic brain injury
M20098B	75	gastric cancer

Table 7.36 List of donors age and cause of death

Chapter 8 References

Abercrombie, M. (1970) 'Contact inhibition in tissue culture', *In Vitro*, 6(2), pp. 128-42.

Ahmad, S., Figueiredo, F. and Lako, M. (2006) 'Corneal epithelial stem cells: characterization, culture and transplantation', *Regen Med*, 1(1), pp. 29-44.

Ahmed, S., Passos, J.F., Birket, M.J., Beckmann, T., Brings, S., Peters, H., Birch-Machin, M.A., von Zglinicki, T. and Saretzki, G. (2008) 'Telomerase does not counteract telomere shortening but protects mitochondrial function under oxidative stress', *J Cell Sci*, 121(Pt 7), pp. 1046-53.

Alitalo, K., Kuismanen, E., Myllyla, R., Kiistala, U., Asko-Seljavaara, S. and Vaheri, A. (1982) 'Extracellular matrix proteins of human epidermal keratinocytes and feeder 3T3 cells', *J Cell Biol*, 94(3), pp. 497-505.

Allen-Hoffmann, B.L. and Rheinwald, J.G. (1984) 'Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture', *Proc Natl Acad Sci U S A*, 81(24), pp. 7802-6.

Allman, M.I., Harper, R.A., Yanoff, M., Curfman, L.J., Cameron, J.D. and Flaxman, B.A. (1976) 'Rabbit corneal epithelial cells grown in vitro without serum', *Invest Ophthalmol*, 15(8), pp. 666-8.

Alvers, A.L., Fishwick, L.K., Wood, M.S., Hu, D., Chung, H.S., Dunn, W.A., Jr. and Aris, J.P. (2009) 'Autophagy and amino acid homeostasis are required for chronological longevity in Saccharomyces cerevisiae', *Aging cell*, 8(4), pp. 353-69.

Anderson, C.J., Hoare, S.F., Ashcroft, M., Bilsland, A.E. and Keith, W.N. (2006) 'Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms', *Oncogene*, 25(1), pp. 61-9.

Ang, L.P., Tanioka, H., Kawasaki, S., Ang, L.P., Yamasaki, K., Do, T.P., Thein, Z.M., Koizumi, N., Nakamura, T., Yokoi, N., Komuro, A., Inatomi, T., Nakatsukasa, M. and Kinoshita, S. (2010) 'Cultivated human conjunctival epithelial transplantation for total limbal stem cell deficiency', *Invest Ophthalmol Vis Sci*, 51(2), pp. 758-64.

Ang, L.P.K., Sotozono, C., Koizumi, N., Suzuki, T., Inatomi, T. and Kinoshita, S. (2007) 'A comparison between cultivated and conventional limbal stem cell transplantation for Stevens-Johnson syndrome', *American Journal of Ophthalmology*, 143(1), pp. 178-180.

Aquilano, K., Baldelli, S., Rotilio, G. and Ciriolo, M.R. (2008) 'Role of Nitric Oxide Synthases in Parkinson's Disease: A Review on the Antioxidant and Anti-inflammatory Activity of Polyphenols', *Neurochemical Research*, 33(12), pp. 2416-2426.

Aquilano, K., Baldelli, S., Rotilio, G. and Ciriolo, M.R. (2009) 'trans-Resveratrol inhibits H2O2-induced adenocarcinoma gastric cells proliferation via inactivation of MEK1/2-ERK1/2-c-Jun signalling axis', *Biochemical Pharmacology*, 77(3), pp. 337-347.

Arpitha, P., Prajna, N.V., Srinivasan, M. and Muthukkaruppan, V. (2005) 'High expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: implications on epithelial stem cells', *Invest Ophthalmol Vis Sci*, 46(10), pp. 3631-6.

Aslan, D., Akata, R.F., Holme, H., Vulliamy, T. and Dokal, I. (2012) 'Limbal stem cell deficiency in patients with inherited stem cell disorder of dyskeratosis congenita', *Int Ophthalmol*, 32(6), pp. 615-22.

Aslan, D., Ozdek, S., Camurdan, O., Bideci, A. and Cinaz, P. (2009) 'Dyskeratosis Congenita With Corneal Limbal Insufficiency', *Pediatric Blood & Cancer*, 53(1), pp. 95-97.

ATCC (2012) *ATCC ANIMAL CELL CULTURE GUIDE - tips and techniques for continuous cell lines*. 2012 American Type Culture Collection

(Accessed: 17 March 2012).

Bachor, C., Bachor, O.A. and Boukamp, P. (1999) 'Telomerase is active in normal gastrointestinal mucosa and not up-regulated in precancerous lesions', *J Cancer Res Clin Oncol*, 125(8-9), pp. 453-60.

Baker, D.J., Dawlaty, M.M., Wijshake, T., Jeganathan, K.B., Malureanu, L., van Ree, J.H., Crespo-Diaz, R., Reyes, S., Seaburg, L., Shapiro, V., Behfar, A., Terzic, A., van de Sluis, B. and van Deursen, J.M. (2013a) 'Increased expression of BubR1 protects against aneuploidy and cancer and extends healthy lifespan', *Nat Cell Biol*, 15(1), pp. 96-102.

Baker, D.J., Weaver, R.L. and van Deursen, J.M. (2013b) 'p21 Both Attenuates and Drives Senescence and Aging in BubR1 Progeroid Mice', *Cell Reports*, 3(4), pp. 1164-1174.

Baker, H., Sidorowicz, A., Sehgal, S.N. and Vezina, C. (1978) 'Rapamycin (Ay-22,989), a New Antifungal Antibiotic .3. Invitro and Invivo Evaluation', *Journal of Antibiotics*, 31(6), pp. 539-545.

Baldelli, S., Aquilano, K., Rotilio, G. and Ciriolo, M.R. (2008) 'Glutathione and copper, zinc superoxide dismutase are modulated by overexpression of neuronal nitric oxide synthase', *International Journal of Biochemistry & Cell Biology*, 40(11), pp. 2660-2670.

Bandhakavi, S., Kim, Y.M., Ro, S.H., Xie, H.W., Onsongo, G., Jun, C.B., Kim, D.H. and Griffin, T.J. (2010) 'Quantitative Nuclear Proteomics Identifies mTOR Regulation of DNA Damage Response', *Molecular & Cellular Proteomics*, 9(2), pp. 403-414.

Baradaran-Rafii, A., Ebrahimi, M., Kanavi, M.R., Taghi-Abadi, E., Aghdami, N., Eslani, M., Bakhtiari, P., Einollahi, B., Baharvand, H. and Javadi, M.A. (2010) 'Midterm Outcomes of Autologous Cultivated Limbal Stem Cell Transplantation With or Without Penetrating Keratoplasty', *Cornea*, 29(5), pp. 502-509.

Barbaro, V., Testa, A., Di Iorio, E., Mavilio, F., Pellegrini, G. and De Luca, M. (2007) 'C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells', *J Cell Biol*, 177(6), pp. 1037-49.

Barrandon, Y. (1993) 'The epidermal stem cell: an overview', *Seminars in Developmental Biology*, 4(4), pp. 209-215.

Barrandon, Y. and Green, H. (1985) 'Cell size as a determinant of the clone-forming ability of human keratinocytes', *Proc Natl Acad Sci U S A*, 82(16), pp. 5390-4.

Barrandon, Y. and Green, H. (1987) 'Three clonal types of keratinocyte with different capacities for multiplication', *Proc Natl Acad Sci U S A*, 84(8), pp. 2302-6.

Bass, T.M., Weinkove, D., Houthoofd, K., Gems, D. and Partridge, L. (2007) 'Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans', *Mech Ageing Dev*, 128(10), pp. 546-52.

Bath, C., Yang, S.F., Muttuvelu, D., Fink, T., Emmersen, J., Vorum, H., Hjortdal, J. and Zachar, V. (2013) 'Hypoxia is a key regulator of limbal epithelial stem cell growth and differentiation', *Stem Cell Research*, 10(3), pp. 349-360.

Baum, J.L. (1973) 'Systemic disease associated with tear deficiencies', *Int Ophthalmol Clin*, 13(1), pp. 157-84.

Baylis, O., Figueiredo, F., Henein, C., Lako, M. and Ahmad, S. (2011) '13 years of cultured limbal epithelial cell therapy: a review of the outcomes', *J Cell Biochem*, 112(4), pp. 993-1002.

Baylis, O., Rooney, P., Figueiredo, F., Lako, M. and Ahmad, S. (2013) 'An investigation of donor and culture parameters which influence epithelial outgrowths from cultured human cadaveric limbal explants', *Journal of Cellular Physiology*, 228(5), pp. 1025-1030.

Behrens, A., van Deursen, J.M., Rudolph, K.L. and Schumacher, B. (2014) 'Impact of genomic damage and ageing on stem cell function', *Nat Cell Biol*, 16(3), pp. 201-7.

Bemis, J.E., Vu, C.B., Xie, R., Nunes, J.J., Ng, P.Y., Disch, J.S., Milne, J.C., Carney, D.P., Lynch, A.V., Jin, L., Smith, J.J., Lavu, S., Iffland, A., Jirousek, M.R. and Perni, R.B. (2009) 'Discovery of oxazolo[4,5-b]pyridines and related heterocyclic analogs as novel SIRT1 activators', *Bioorganic & Medicinal Chemistry Letters*, 19(8), pp. 2350-2353.

Ben-Porath, I. and Weinberg, R.A. (2004) 'When cells get stressed: an integrative view of cellular senescence', *Journal of Clinical Investigation*, 113(1), pp. 8-13.

Benard, J., Douc-Rasy, S. and Ahomadegbe, J.C. (2003) 'TP53 family members and human cancers', *Human Mutation*, 21(3), pp. 182-191.

Berner, R.A. (1999) 'Atmospheric oxygen over Phanerozoic time', *Proc Natl Acad Sci U S A*, 96(20), pp. 10955-7.

Berra, E., Roux, D., Richard, D.E. and Pouyssegur, J. (2001) 'Hypoxia-inducible factor-1 alpha (HIF-1 alpha) escapes O(2)-driven proteasomal degradation irrespective of its subcellular localization: nucleus or cytoplasm', *EMBO Rep*, 2(7), pp. 615-20.

Birket, M.J., Orr, A.L., Gerencser, A.A., Madden, D.T., Vitelli, C., Swistowski, A., Brand, M.D. and Zeng, X. (2011) 'A reduction in ATP demand and mitochondrial activity with neural differentiation of human embryonic stem cells', *J Cell Sci*, 124(Pt 3), pp. 348-58.

Blagosklonny, M.V. (2011) 'Rapamycin-induced glucose intolerance Hunger or starvation diabetes', *Cell Cycle*, 10(24), pp. 4217-4224.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S. and Wright, W.E. (1998) 'Extension of life-span by introduction of telomerase into normal human cells', *Science*, 279(5349), pp. 349-352.

Bogacka, I., Gettys, T.W., de Jonge, L., Nguyen, T., Smith, J.M., Xie, H., Greenway, F. and Smith, S.R. (2007) 'The effect of beta-adrenergic and peroxisome proliferator-activated receptor-gamma stimulation on target genes related to lipid metabolism in human subcutaneous adipose tissue', *Diabetes Care*, 30(5), pp. 1179-86.

Boggs, D.R., Boggs, S.S., Saxe, D.F., Gress, L.A. and Canfield, D.R. (1982) 'Hematopoietic stem cells with high proliferative potential. Assay of their concentration in marrow by the frequency and duration of cure of W/Wv mice', *J Clin Invest*, 70(2), pp. 242-53.

Bogliolo, M., Lyakhovich, A., Callen, E., Castella, M., Cappelli, E., Ramirez, M.J., Creus, A., Marcos, R., Kalb, R., Neveling, K., Schindler, D. and Surralles, J. (2007) 'Histone H2AX and Fanconi anemia FANCD2 function in the same pathway to maintain chromosome stability', *Embo Journal*, 26(5), pp. 1340-1351.

Bourne, W.M., Nelson, L.R. and Hodge, D.O. (1997) 'Central corneal endothelial cell changes over a ten-year period', *Invest Ophthalmol Vis Sci*, 38(3), pp. 779-82.

Bourzac, K. (2012) 'Interventions: Live long and prosper', *Nature*, 492(7427), pp. S18-20.

Boyce, S.T. and Ham, R.G. (1983) 'Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture', *J Invest Dermatol*, 81(1 Suppl), pp. 33s-40s.

Boyle, M., Wong, C., Rocha, M. and Jones, D.L. (2007) 'Decline in self-renewal factors contributes to aging of the stem cell niche in the Drosophila testis', *Cell Stem Cell*, 1(4), pp. 470-478.

Brack, A.S., Conboy, M.J., Roy, S., Lee, M., Kuo, C.J., Keller, C. and Rando, T.A. (2007) 'Increased Wnt signaling during aging alters muscle stem cell fate and increases fibrosis', *Science*, 317(5839), pp. 807-810.

Brack, A.S. and Rando, T.A. (2007) 'Intrinsic changes and extrinsic influences of myogenic stem cell function during aging', *Stem Cell Reviews*, 3(3), pp. 226-237.

Brahimi-Horn, C. and Pouyssegur, J. (2006) 'The role of the hypoxia-inducible factor in tumor metabolism growth and invasion', *Bull Cancer*, 93(8), pp. E73-80.

Brahimi-Horn, M.C. and Pouyssegur, J. (2007) 'Oxygen, a source of life and stress', *FEBS Lett*, 581(19), pp. 3582-91.

Brandl, A., Meyer, M., Bechmann, V., Nerlich, M. and Angele, P. (2011) 'Oxidative stress induces senescence in human mesenchymal stem cells', *Exp Cell Res*, 317(11), pp. 1541-7.

Brasnyo, P., Molnar, G.A., Mohas, M., Marko, L., Laczy, B., Cseh, J., Mikolas, E., Szijarto, I.A., Merei, A., Halmai, R., Meszaros, L.G., Sumegi, B. and Wittmann, I. (2011) 'Resveratrol improves insulin sensitivity, reduces oxidative stress and activates the Akt pathway in type 2 diabetic patients', *British Journal of Nutrition*, 106(3), pp. 383-389.

Brennan, N.A.E., N. (2005) 'Corneal oxygen consumption and hypoxia', in Bennett, E., S. Weissman, B. A. (ed.) Philadelphia: Clinical Contact Lens Practice, pp. 41-65.

Breslin, C.W. and Ng, W. (1976) 'The endothelial function of donor corneas: effects of delayed enucleation and refrigeration', *Invest Ophthalmol Vis Sci*, 15(9), pp. 732-9.

Bron, A.J. (2001) 'The architecture of the corneal stroma', *Br J Ophthalmol*, 85(4), pp. 379-81.

Brown, K., Xie, S., Qiu, X., Mohrin, M., Shin, J., Liu, Y., Zhang, D., Scadden, D.T. and Chen, D. (2013) 'SIRT3 reverses aging-associated degeneration', *Cell Reports*, 3(2), pp. 319-27.

Budak, M.T., Alpdogan, O.S., Zhou, M., Lavker, R.M., Akinci, M.A. and Wolosin, J.M. (2005) 'Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells', *J Cell Sci*, 118(Pt 8), pp. 1715-24.

Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E. and Fornace, A.J., Jr. (2001) 'Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase', *Nature*, 411(6833), pp. 102-7.

Burt, R.K., Loh, Y., Pearce, W., Beohar, N., Barr, W.G., Craig, R., Wen, Y., Rapp, J.A. and Kessler, J. (2008) 'Clinical applications of blood-derived and marrow-derived stem cells for nonmalignant diseases', *JAMA*, 299(8), pp. 925-36.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. and Wittwer, C.T. (2009) 'The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments', *Clin Chem*, 55(4), pp. 611-22.

Byfield, M.P., Murray, J.T. and Backer, J.M. (2005) 'hVps34 is a nutrient-regulated lipid kinase required for activation of p70 S6 kinase', *Journal of Biological Chemistry*, 280(38), pp. 33076-33082.

Cairns, J. (1975) 'Mutation selection and the natural history of cancer', *Nature*, 255(5505), pp. 197-200.

Cam, M., Bid, H.K., Xiao, L., Zambetti, G.P., Houghton, P.J. and Cam, H. (2014) 'p53/TAp63 and AKT Regulate Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling through Two Independent Parallel Pathways in the Presence of DNA Damage', *J Biol Chem*, 289(7), pp. 4083-94.

Cameron, K.M., Miwa, S., Walker, C. and von Zglinicki, T. (2012) 'Male mice retain a metabolic memory of improved glucose tolerance induced during adult onset, short-term dietary restriction', *Longev Healthspan*, 1, p. 3.

Campisi, J. (2003) 'Cancer and ageing: rival demons?', *Nat Rev Cancer*, 3(5), pp. 339-49.

Campisi, J. and di Fagagna, F.D. (2007) 'Cellular senescence: when bad things happen to good cells', *Nature Reviews Molecular Cell Biology*, 8(9), pp. 729-740.

Candi, E., Dinsdale, D., Rufini, A., Salomoni, P., Knight, R.A., Mueller, M., Krammer, P.H. and Melino, G. (2007) 'TAp63 and DeltaNp63 in cancer and epidermal development', *Cell Cycle*, 6(3), pp. 274-85.

Canto, C. and Auwerx, J. (2009) 'PGC-1 alpha, SIRT1 and AMPK, an energy sensing network that controls energy expenditure', *Current Opinion in Lipidology*, 20(2), pp. 98-105.

Carlson, M.E., Conboy, M.J., Hsu, M., Barchas, L., Jeong, J., Agrawal, A., Mikels, A.J., Agrawal, S., Schaffer, D.V. and Conboy, I.M. (2009) 'Relative roles of TGF-beta1 and Wnt in the systemic regulation and aging of satellite cell responses', *Aging cell*, 8(6), pp. 676-89.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C.J., Ratcliffe, P., Moons, L., Jain, R.K., Collen, D. and Keshert, E. (1998) 'Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis', *Nature*, 394(6692), pp. 485-90.

Caron, E., Ghosh, S., Matsuoka, Y., Ashton-Beaucage, D., Therrien, M., Lemieux, S., Perreault, C., Roux, P.P. and Kitano, H. (2010) 'A comprehensive map of the mTOR signaling network', *Molecular Systems Biology*, 6.

Castilho, R.M., Squarize, C.H., Chodosh, L.A., Williams, B.O. and Gutkind, J.S. (2009) 'mTOR Mediates Wnt-Induced Epidermal Stem Cell Exhaustion and Aging', *Cell Stem Cell*, 5(3), pp. 279-289.

Cerletti, M., Jang, Y.C., Finley, L.W.S., Haigis, M.C. and Wagers, A.J. (2012) 'Short-Term Calorie Restriction Enhances Skeletal Muscle Stem Cell Function', *Cell Stem Cell*, 10(5), pp. 515-519.

Cesare, A.J., Kaul, Z., Cohen, S.B., Napier, C.E., Pickett, H.A., Neumann, A.A. and Reddel, R.R. (2009) 'Spontaneous occurrence of telomeric DNA damage response in the absence of chromosome fusions', *Nature Structural & Molecular Biology*, 16(12), pp. 1244-U59.

Chakkalakal, J.V., Jones, K.M., Basson, M.A. and Brack, A.S. (2012) 'The aged niche disrupts muscle stem cell quiescence', *Nature*, 490(7420), pp. 355-+.

Chandel, N.S. and Schumacker, P.T. (1999) 'Cells depleted of mitochondrial DNA (rho(0)) yield insight into physiological mechanisms', *FEBS letters*, 454(3), pp. 173-176.

Chang, C.Y., Green, C.R., McGhee, C.N.J. and Sherwin, T. (2008) 'Acute Wound Healing in the Human Central Corneal Epithelium Appears to Be Independent of Limbal Stem Cell Influence', *Investigative Ophthalmology & Visual Science*, 49(12), pp. 5279-5286.

Chang, C.Y.A., McGhee, J.J., Green, C.R. and Sherwin, T. (2011) 'Comparison of Stem Cell Properties in Cell Populations Isolated From Human Central and Limbal Corneal Epithelium', *Cornea*, 30(10), pp. 1155-1162.

Chang, S. (2005) 'Modeling aging and cancer in the telomerase knockout mouse', *Mutat Res*, 576(1-2), pp. 39-53.

Chang, S.W. and Hu, F.R. (1993) 'Changes in corneal autofluorescence and corneal epithelial barrier function with aging', *Cornea*, 12(6), pp. 493-9.

Chen, C., Liu, Y. and Zheng, P. (2009) 'mTOR regulation and therapeutic rejuvenation of aging hematopoietic stem cells', *Sci Signal*, 2(98), p. ra75.

Chen, H.C., Sytwu, H.K., Chang, J.L., Wang, H.W., Chen, H.K., Kang, B.H., Liu, D.W., Chen, C.H., Chao, T.T. and Wang, C.H. (2011) 'Hypoxia enhances the stemness markers of cochlear stem/progenitor cells and expands sphere formation through activation of hypoxia-inducible factor-1alpha', *Hearing Research*, 275(1-2), pp. 43-52.

Chen, J.C., Astle, C.M. and Harrison, D.E. (2003) 'Hematopoietic senescence is postponed and hematopoietic stem cell function is enhanced by dietary restriction', *Experimental Hematology*, 31(11), pp. 1097-1103.

Chen, J.J. and Tseng, S.C. (1991) 'Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium', *Invest Ophthalmol Vis Sci*, 32(8), pp. 2219-33.

Chen, Q. and Ames, B.N. (1994) 'Senescence-Like Growth Arrest Induced by Hydrogen-Peroxide in Human-Diploid Fibroblast F65 Cells', *Proceedings of the National Academy of Sciences of the United States of America*, 91(10), pp. 4130-4134.

Chen, Z., Trotman, L.C., Shaffer, D., Lin, H.K., Dotan, Z.A., Niki, M., Koutcher, J.A., Scher, H.I., Ludwig, T., Gerald, W., Cordon-Cardo, C. and Pandolfi, P.P. (2005) 'Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis', *Nature*, 436(7051), pp. 725-30.

Chiang, Y.J., Hemann, M.T., Hathcock, K.S., Tessarollo, L., Feigenbaum, L., Hahn, W.C. and Hodes, R.J. (2004) 'Expression of telomerase RNA template, but not telomerase reverse transcriptase, is limiting for telomere length maintenance in vivo', *Mol Cell Biol*, 24(16), pp. 7024-31.

Chiavegatto, S., Dawson, V.L., Mamounas, L.A., Koliatsos, V.E., Dawson, T.M. and Nelson, R.J. (2001) 'Brain serotonin dysfunction accounts for aggression in male mice lacking neuronal nitric oxide synthase', *Proc Natl Acad Sci U S A*, 98(3), pp. 1277-81.

Chiu, C.P., Dragowska, W., Kim, N.W., Vaziri, H., Yui, J., Thomas, T.E., Harley, C.B. and Lansdorp, P.M. (1996) 'Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow', *Stem Cells*, 14(2), pp. 239-48.

Cho, R.H., Sieburg, H.B. and Muller-Sieburg, C.E. (2008) 'A new mechanism for the aging of hematopoietic stem cells: aging changes the clonal composition of the stem cell compartment but not individual stem cells', *Blood*, 111(12), pp. 5553-61.

Choudhery, M.S., Badowski, M., Muise, A., Pierce, J. and Harris, D.T. (2014) 'Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation', *J Transl Med*, 12, p. 8.

Chung, S., Dzeja, P.P., Faustino, R.S., Perez-Terzic, C., Behfar, A. and Terzic, A. (2007) 'Mitochondrial oxidative metabolism is required for the cardiac differentiation of stem cells', *Nature Clinical Practice Cardiovascular Medicine*, 4 Suppl 1, pp. S60-7.

Citrin, D.E., Shankavaram, U., Horton, J.A., Shield, W., Zhao, S.P., Asano, H., White, A., Sowers, A., Thetford, A. and Chung, E.J. (2013) 'Role of Type II Pneumocyte Senescence in Radiation-Induced Lung Fibrosis', *Jnci-Journal of the National Cancer Institute*, 105(19), pp. 1474-1484.

Civitarese, A.E., Carling, S., Heilbronn, L.K., Hulver, M.H., Ukropcova, B., Deutsch, W.A., Smith, S.R. and Ravussin, E. (2007) 'Calorie restriction increases muscle mitochondrial biogenesis in healthy humans', *Plos Medicine*, 4(3), pp. 485-494.

Claudinot, S., Nicolas, M., Oshima, H., Rochat, A. and Barrandon, Y. (2005) 'Long-term renewal of hair follicles from clonogenic multipotent stem cells', *Proceedings of the National Academy of Sciences of the United States of America*, 102(41), pp. 14677-14682.

Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W. and Weindruch, R. (2009) 'Caloric Restriction Delays Disease Onset and Mortality in Rhesus Monkeys', *Science*, 325(5937), pp. 201-204.

Conboy, I.M., Conboy, M.J., Smythe, G.M. and Rando, T.A. (2003) 'Notch-mediated restoration of regenerative potential to aged muscle', *Science*, 302(5650), pp. 1575-1577.

Conboy, I.M., Conboy, M.J., Wagers, A.J., Girma, E.R., Weissman, I.L. and Rando, T.A. (2005) 'Rejuvenation of aged progenitor cells by exposure to a young systemic environment', *Nature*, 433(7027), pp. 760-764.

Coster, D.J. (2002) *Cornea*. London: BMJ Books.

Cotsarelis, G., Cheng, S.Z., Dong, G., Sun, T.T. and Lavker, R.M. (1989) 'Existence of Slow-Cycling Limbal Epithelial Basal Cells That Can Be Preferentially Stimulated to Proliferate - Implications on Epithelial Stem-Cells', *Cell*, 57(2), pp. 201-209.

Coussens, M., Davy, P., Brown, L., Foster, C., Andrews, W.H., Nagata, M. and Allsopp, R. (2010) 'RNAi screen for telomerase reverse transcriptase transcriptional regulators identifies HIF1alpha as critical for telomerase function in murine embryonic stem cells', *Proc Natl Acad Sci U S A*, 107(31), pp. 13842-7.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C. and Keith, B. (2006) 'HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth', *Genes Dev*, 20(5), pp. 557-70.

Craig, A.L., Holcakova, J., Finlan, L.E., Nekulova, M., Hrstka, R., Gueven, N., DiRenzo, J., Smith, G., Hupp, T.R. and Vojtesek, B. (2010) 'DeltaNp63 transcriptionally regulates ATM to control p53 Serine-15 phosphorylation', *Mol Cancer*, 9, p. 195.

Csete, M. (2005) 'Oxygen in the cultivation of stem cells', *Ann N Y Acad Sci*, 1049, pp. 1-8.

Csiszar, A., Labinskyy, N., Pinto, J.T., Ballabh, P., Zhang, H.R., Losonczy, G., Pearson, K., de Cabo, R., Pacher, P., Zhang, C.H. and Ungvari, Z. (2009) 'Resveratrol induces

mitochondrial biogenesis in endothelial cells', *American Journal of Physiology-Heart and Circulatory Physiology*, 297(1), pp. H13-H20.

D'Errico, I., Salvatore, L., Murzilli, S., Lo Sasso, G., Latorre, D., Martelli, N., Egorova, A.V., Polishuck, R., Madeyski-Bengtson, K., Lelliott, C., Vidal-Puig, A.J., Seibel, P., Villani, G. and Moschetta, A. (2011) 'Peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC1 alpha) is a metabolic regulator of intestinal epithelial cell fate', *Proceedings of the National Academy of Sciences of the United States of America*, 108(16), pp. 6603-6608.

Dalal, S.N., Schweitzer, C.M., Gan, J. and DeCaprio, J.A. (1999) 'Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site', *Mol Cell Biol*, 19(6), pp. 4465-79.

Dan, H.C., Cooper, M.J., Cogswell, P.C., Duncan, J.A., Ting, J.P.Y. and Baldwin, A.S. (2008) 'Akt-dependent regulation of NF-kappa B is controlled by mTOR and Raptor in association with IKK', *Genes & Development*, 22(11), pp. 1490-1500.

Danet, G.H., Pan, Y., Luongo, J.L., Bonnet, D.A. and Simon, M.C. (2003) 'Expansion of human SCID-repopulating cells under hypoxic conditions', *Journal of Clinical Investigation*, 112(1), pp. 126-135.

Daniels, J.T., Dart, J.K., Tuft, S.J. and Khaw, P.T. (2001) 'Corneal stem cells in review', *Wound Repair Regen*, 9(6), pp. 483-94.

Daya, S.M., Watson, A., Sharpe, J.R., Giledi, S., Rowe, A., Martin, R. and James, S.E. (2005) 'Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction', *Ophthalmology*, 112(3), pp. 470-477.

de Jesus, B.B., Schneeberger, K., Vera, E., Tejera, A., Harley, C.B. and Blasco, M.A. (2011) 'The telomerase activator TA-65 elongates short telomeres and increases health span of adult/old mice without increasing cancer incidence', *Aging cell*, 10(4), pp. 604-621.

de Jesus, B.B., Vera, E., Schneeberger, K., Tejera, A.M., Ayuso, E., Bosch, F. and Blasco, M.A. (2012) 'Telomerase gene therapy in adult and old mice delays aging and increases longevity without increasing cancer', *EMBO molecular medicine*, 4(8), pp. 691-704.

de Paiva, C.S., Chen, Z., Corrales, R.M., Pflugfelder, S.C. and Li, D.Q. (2005) 'ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells', *Stem Cells*, 23(1), pp. 63-73.

deHaan, G., Nijhof, W. and VanZant, G. (1997) 'Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: Correlation between lifespan and cycling activity', *Blood*, 89(5), pp. 1543-1550.

Dekruijf, E.J.F.M., Boot, J.P., Laterveer, L., Vanbest, J.A., Ramselaar, J.A.M. and Oosterhuis, J.A. (1987) 'A Simple Method for Determination of Corneal Epithelial Permeability in Humans', *Current Eye Research*, 6(11), pp. 1327-1334.

Dellambra, E., Vailly, J., Pellegrini, G., Bondanza, S., Golisano, O., Macchia, C., Zambruno, G., Meneguzzi, G. and De Luca, M. (1998) 'Corrective transduction of human epidermal

stem cells in laminin-5-dependent junctional epidermolysis bullosa', *Hum Gene Ther*, 9(9), pp. 1359-70.

Desbois-Mouthon, C., Cadoret, A., Blivet-Van Eggelpoel, M.J., Bertrand, F., Cherqui, G., Perret, C. and Capeau, J. (2001) 'Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3 beta inhibition and Ras activation', *Oncogene*, 20(2), pp. 252-259.

di Fagagna, F.D., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., von Zglinicki, T., Saretzki, G., Carter, N.P. and Jackson, S.P. (2003) 'A DNA damage checkpoint response in telomere-initiated senescence', *Nature*, 426(6963), pp. 194-198.

Di Girolamo, N., Bosch, M., Zamora, K., Coroneo, M.T., Wakefield, D. and Watson, S.L. (2009) 'A Contact Lens-Based Technique for Expansion and Transplantation of Autologous Epithelial Progenitors for Ocular Surface Reconstruction', *Transplantation*, 87(10), pp. 1571-1578.

Di Iorio, E., Barbaro, V., Ruzza, A., Ponzin, D., Pellegrini, G. and De Luca, M. (2005) 'Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration', *Proc Natl Acad Sci U S A*, 102(27), pp. 9523-8.

Di Iorio, E., Ferrari, S., Fasolo, A., Bohm, E., Ponzin, D. and Barbaro, V. (2010) 'Techniques for Culture and Assessment of Limbal Stem Cell Grafts', *Ocular Surface*, 8(3), pp. 146-153.

Diabetes-UK (2014) *Diabetes prevalence 2013*. Available at: http://www.diabetes.org.uk/About_us/What-we-say/Statistics/Diabetes-prevalence-2013/ (Accessed: 18 March).

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O. and et al. (1995) 'A biomarker that identifies senescent human cells in culture and in aging skin in vivo', *Proc Natl Acad Sci U S A*, 92(20), pp. 9363-7.

Dimri, G.P., Martinez, J.L., Jacobs, J.J., Keblusek, P., Itahana, K., Van Lohuizen, M., Campisi, J., Wazer, D.E. and Band, V. (2002) 'The Bmi-1 oncogene induces telomerase activity and immortalizes human mammary epithelial cells', *Cancer Res*, 62(16), pp. 4736-45.

Ding, X., Bishop, R.J., Herzlich, A.A., Patel, M. and Chan, C.C. (2009) 'Limbal stem cell deficiency arising from systemic chemotherapy with hydroxycarbamide', *Cornea*, 28(2), pp. 221-3.

Dohn, M., Zhang, S.Z. and Chen, X.B. (2001) 'p63 alpha and Delta Np63 alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes', *Oncogene*, 20(25), pp. 3193-3205.

Donato, A.J., Eskurza, I., Silver, A.E., Levy, A.S., Pierce, G.L., Gates, P.E. and Seals, D.R. (2007) 'Direct evidence of endothelial oxidative stress with aging in humans - Relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappa B', *Circulation Research*, 100(11), pp. 1659-1666.

Doonan, R., McElwee, J.J., Matthijssens, F., Walker, G.A., Houthoofd, K., Back, P., Matscheski, A., Vanfleteren, J.R. and Gems, D. (2008) 'Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in Caenorhabditis elegans', *Genes & Development*, 22(23), pp. 3236-3241.

Douvaras, P., Webb, S., Whitaker, D.A., Dora, N., Hill, R.E., Dorin, J.R. and West, J.D. (2012) 'Rare corneal clones in mice suggest an age-related decrease of stem cell activity and support the limbal epithelial stem cell hypothesis', *Stem Cell Research*, 8(1), pp. 109-119.

Dowling, R.J.O., Topisirovic, I., Alain, T., Bidinosti, M., Fonseca, B.D., Petroulakis, E., Wang, X.S., Larsson, O., Selvaraj, A., Liu, Y., Kozma, S.C., Thomas, G. and Sonenberg, N. (2010) 'mTORC1-Mediated Cell Proliferation, But Not Cell Growth, Controlled by the 4E-BPs', *Science*, 328(5982), pp. 1172-1176.

Dua, H.S., Joseph, A., Shanmuganathan, V.A. and Jones, R.E. (2003) 'Stem cell differentiation and the effects of deficiency', *Eye (Lond)*, 17(8), pp. 877-85.

Dua, H.S., Miri, A., Alomar, T., Yeung, A.M. and Said, D.G. (2009) 'The Role of Limbal Stem Cells in Corneal Epithelial Maintenance Testing the Dogma', *Ophthalmology*, 116(5), pp. 856-863.

Dua, H.S., Miri, A. and Said, D.G. (2010) 'Contemporary limbal stem cell transplantation - a review', *Clin Experiment Ophthalmol*, 38(2), pp. 104-17.

Dua, H.S., Shanmuganathan, V.A., Powell-Richards, A.O., Tighe, P.J. and Joseph, A. (2005) 'Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche', *Br J Ophthalmol*, 89(5), pp. 529-32.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J. and Reed, S.I. (1994) 'P53-Dependent Inhibition of Cyclin-Dependent Kinase-Activities in Human Fibroblasts during Radiation-Induced G1 Arrest', *Cell*, 76(6), pp. 1013-1023.

Dykstra, B., Olthof, S., Schreuder, J., Ritsema, M. and de Haan, G. (2011) 'Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells', *J Exp Med*, 208(13), pp. 2691-703.

Ebato, B., Friend, J. and Thoft, R.A. (1988) 'Comparison of limbal and peripheral human corneal epithelium in tissue culture', *Invest Ophthalmol Vis Sci*, 29(10), pp. 1533-7.

Edgar, D., Shabalina, I., Camara, Y., Wredenberg, A., Calvaruso, M.A., Nijtmans, L., Nedergaard, J., Cannon, B., Larsson, N.G. and Trifunovic, A. (2009) 'Random point mutations with major effects on protein-coding genes are the driving force behind premature aging in mtDNA mutator mice', *Cell Metabolism*, 10(2), pp. 131-8.

Edwards, B.K., Howe, H.L., Ries, L.A., Thun, M.J., Rosenberg, H.M., Yancik, R., Wingo, P.A., Jemal, A. and Feigal, E.G. (2002) 'Annual report to the nation on the status of cancer, 1973-1999, featuring implications of age and aging on U.S. cancer burden', *Cancer*, 94(10), pp. 2766-92.

Ehebauer, M., Hayward, P. and Arias, A.M. (2006) 'Notch, a universal arbiter of cell fate decisions', *Science*, 314(5804), pp. 1414-5.

Eldeiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W. and Vogelstein, B. (1993) 'Waf1, a Potential Mediator of P53 Tumor Suppression', *Cell*, 75(4), pp. 817-825.

Ellies, P., Anderson, D.F., Topuhami, A. and Tseng, S.C. (2001) 'Limbal stem cell deficiency arising from systemic chemotherapy', *Br J Ophthalmol*, 85(3), pp. 373-4.

Elshorbagy, A.K., Valdivia-Garcia, M., Refsum, H., Smith, A.D., Mattocks, D.A. and Perrone, C.E. (2010) 'Sulfur amino acids in methionine-restricted rats: hyperhomocysteinemia', *Nutrition*, 26(11-12), pp. 1201-4.

Estrada, J.C., Albo, C., Benguria, A., Dopazo, A., Lopez-Romero, P., Carrera-Quintanar, L., Roche, E., Clemente, E.P., Enriquez, J.A., Bernad, A. and Samper, E. (2012) 'Culture of human mesenchymal stem cells at low oxygen tension improves growth and genetic stability by activating glycolysis', *Cell Death and Differentiation*, 19(5), pp. 743-755.

Estrada, J.C., Torres, Y., Benguria, A., Dopazo, A., Roche, E., Carrera-Quintanar, L., Perez, R.A., Enriquez, J.A., Torres, R., Ramirez, J.C., Samper, E. and Bernad, A. (2013) 'Human mesenchymal stem cell-replicative senescence and oxidative stress are closely linked to aneuploidy', *Cell Death & Disease*, 4.

Ezashi, T., Das, P. and Roberts, R.M. (2005) 'Low O-2 tensions and the prevention of differentiation of human embryonic stem cells.', *Biology of Reproduction*, pp. 232-233.

Fabiani, C., Barabino, S., Rashid, S. and Dana, M.R. (2009) 'Corneal epithelial proliferation and thickness in a mouse model of dry eye', *Exp Eye Res*, 89(2), pp. 166-71.

Faragher, R.G.A., Mulholland, B., Tuft, S.J., Sandeman, S. and Khaw, P.T. (1997) 'Aging and the cornea', *British Journal of Ophthalmology*, 81(10), pp. 814-817.

Fasano, C.A., Dimos, J.T., Ivanova, N.B., Lowry, N., Lemischka, I.R. and Temple, S. (2007) 'shRNA knockdown of Bmi-1 reveals a critical role for p21-Rb pathway in NSC self-renewal during development', *Cell Stem Cell*, 1(1), pp. 87-99.

Fatima, A., Vemuganti, G.K., Iftekhar, G., Rao, G.N. and Sangwan, V.S. (2007) 'In vivo survival and stratification of cultured limbal epithelium', *Clinical and Experimental Ophthalmology*, 35(1), pp. 96-98.

Feng, Z., Zhang, H., Levine, A.J. and Jin, S. (2005) 'The coordinate regulation of the p53 and mTOR pathways in cells', *Proc Natl Acad Sci U S A*, 102(23), pp. 8204-9.

Flaxman, B.A., Lutzner, M.A. and Van Scott, E.J. (1967) 'Cell maturation and tissue organization in epithelial outgrowths from skin and buccal mucosa in vitro', *J Invest Dermatol*, 49(3), pp. 322-32.

Flores, E.R., Sengupta, S., Miller, J.B., Newman, J.J., Bronson, R., Crowley, D., Yang, A., McKeon, F. and Jacks, T. (2005a) 'Tumor predisposition in mice mutant for p63 and p73: Evidence for broader tumor suppressor functions for the p53 family', *Cancer Cell*, 7(4), pp. 363-373.

Flores, E.R., Tsai, K.Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F. and Jacks, T. (2002) 'p63 and p73 are required for p53-dependent apoptosis in response to DNA damage', *Nature*, 416(6880), pp. 560-564.

Flores, I., Canela, A., Vera, E., Tejera, A., Cotsarelis, G. and Blasco, M.A. (2008) 'The longest telomeres: a general signature of adult stem cell compartments', *Genes Dev*, 22(5), pp. 654-67.

Flores, I., Cayuela, M.L. and Blasco, M.A. (2005b) 'Effects of telomerase and telomere length on epidermal stem cell behavior', *Science*, 309(5738), pp. 1253-6.

Fok, W.C., Zhang, Y.Q., Salmon, A.B., Bhattacharya, A., Gunda, R., Jones, D., Ward, W., Fisher, K., Richardson, A. and Perez, V.I. (2013) 'Short-Term Treatment With Rapamycin and Dietary Restriction Have Overlapping and Distinctive Effects in Young Mice', *Journals of Gerontology Series a-Biological Sciences and Medical Sciences*, 68(2), pp. 108-116.

Fraenkel, M., Ketzinel-Gilad, M., Ariav, Y., Pappo, O., Karaca, M., Castel, J., Berthault, M.F., Magnan, C., Cerasi, E., Kaiser, N. and Leibowitz, G. (2008) 'mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes', *Diabetes*, 57(4), pp. 945-957.

Fraga, M.F. and Esteller, M. (2007) 'Epigenetics and aging: the targets and the marks', *Trends in Genetics*, 23(8), pp. 413-418.

Freshney, M.G., Freshney, R.I. and John Wiley & Sons. (2002) *Culture of epithelial cells*. Wiley-Liss, [Electronic text (pdf)]. Available at: http://dx.doi.org/10.1002/0471221201.

Frucht-Pery, J., Siganos, C.S., Solomon, A., Scheman, L., Brautbar, C. and Zauberman, H. (1998) 'Limbal cell autograft transplantation for severe ocular surface disorders', *Graefes Archive for Clinical and Experimental Ophthalmology*, 236(8), pp. 582-587.

Fuchs, Y., Brown, S., Gorenc, T., Rodriguez, J., Fuchs, E. and Steller, H. (2013) 'Sept4/ARTS Regulates Stem Cell Apoptosis and Skin Regeneration', *Science*, 341(6143), pp. 286-289.

Fuller, M.T. and Spradling, A.C. (2007) 'Male and female Drosophila germline stem cells: two versions of immortality', *Science*, 316(5823), pp. 402-4.

Fumagalli, M., Rossiello, F., Clerici, M., Barozzi, S., Cittaro, D., Kaplunov, J.M., Bucci, G., Dobreva, M., Matti, V., Beausejour, C.M., Herbig, U., Longhese, M.P. and di Fagagna, F.D. (2012) 'Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation (vol 14, pg 355, 2012)', *Nature cell biology*, 14(5), pp. 555-555.

Fusenig, N.E. and Worst, P.K. (1974) 'Mouse epidermal cell cultures. I. Isolation and cultivation of epidermal cells from adult mouse skin', *J Invest Dermatol*, 63(2), pp. 187-93.

Gago, N., Perez-Lopez, V., Sanz-Jaka, J.P., Cormenzana, P., Eizaguirre, I., Bernad, A. and Izeta, A. (2009) 'Age-Dependent Depletion of Human Skin-Derived Progenitor Cells', *Stem Cells*, 27(5), pp. 1164-1172.

Gan, B., Sahin, E., Jiang, S., Sanchez-Aguilera, A., Scott, K.L., Chin, L., Williams, D.A., Kwiatkowski, D.J. and DePinho, R.A. (2008) 'mTORC1-dependent and -independent regulation of stem cell renewal, differentiation, and mobilization', *Proceedings of the National Academy of Sciences of the United States of America*, 105(49), pp. 19384-19389.

Garcia-Martinez, J.M. and Alessi, D.R. (2008) 'mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1)', *Biochem J*, 416(3), pp. 375-85.

Gartel, A.L. and Radhakrishnan, S.K. (2005) 'Lost in transcription: p21 repression, mechanisms, and consequences', *Cancer Res*, 65(10), pp. 3980-5.

Gaubatz, S., Lindeman, G.J., Ishida, S., Jakoi, L., Nevins, J.R., Livingston, D.M. and Rempel, R.E. (2000) 'E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control', *Mol Cell*, 6(3), pp. 729-35.

Gavet, O. and Pines, J. (2010) 'Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis', *Dev Cell*, 18(4), pp. 533-43.

Geest, C.R., Zwartkruis, F.J., Vellenga, E., Coffer, P.J. and Buitenhuis, M. (2009) 'Mammalian target of rapamycin activity is required for expansion of CD34(+) hematopoietic progenitor cells', *Haematologica-the Hematology Journal*, 94(7), pp. 901-910.

Gibco (2014) *Skin and eye sourcebook*.(Accessed: 13/08/13).

Ginjala, V., Nacerddine, K., Kulkarni, A., Oza, J., Hill, S.J., Yao, M., Citterio, E., van Lohuizen, M. and Ganesan, S. (2011) 'BMI1 is recruited to DNA breaks and contributes to DNA damage-induced H2A ubiquitination and repair', *Mol Cell Biol*, 31(10), pp. 1972-82.

Gipson, I.K. (1989) 'The epithelial basement membrane zone of the limbus', *Eye*, 3 (Pt 2), pp. 132-40.

Gisoldi, R.A.M.C., Pocobelli, A., Villani, C.M., Amato, D. and Pellegrini, G. (2010) 'Evaluation of Molecular Markers in Corneal Regeneration by Means of Autologous Cultures of Limbal Cells and Keratoplasty', *Cornea*, 29(7), pp. 715-722.

Goldberg, M.F. and Bron, A.J. (1982) 'Limbal palisades of Vogt', *Trans Am Ophthalmol Soc*, 80, pp. 155-71.

Goldblatt, C., Lenton, T.M. and Watson, A.J. (2006) 'Bistability of atmospheric oxygen and the Great Oxidation', *Nature*, 443(7112), pp. 683-6.

Gomez-Gaviro, M.V., Lovell-Badge, R., Fernandez-Aviles, F. and Lara-Pezzi, E. (2012) 'The vascular stem cell niche', *J Cardiovasc Transl Res*, 5(5), pp. 618-30.

Goto, M., Rubenstein, M., Weber, J., Woods, K. and Drayna, D. (1992) 'Genetic linkage of Werner's syndrome to five markers on chromosome 8', *Nature*, 355(6362), pp. 735-8.

Grandison, R.C., Piper, M.D. and Partridge, L. (2009) 'Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila', *Nature*, 462(7276), pp. 1061-4.

Gredilla, R. and Barja, G. (2005) 'Minireview: The role of oxidative stress in relation to caloric restriction and longevity', *Endocrinology*, 146(9), pp. 3713-3717.

Greer, E.L. and Brunet, A. (2008) 'FOXO transcription factors in ageing and cancer', *Acta Physiologica*, 192(1), pp. 19-28.

Greider, C.W. and Blackburn, E.H. (1985) 'Identification of a specific telomere terminal transferase activity in Tetrahymena extracts', *Cell*, 43(2 Pt 1), pp. 405-13.

Gressner, O., Schilling, T., Lorenz, K., Schulze Schleithoff, E., Koch, A., Schulze-Bergkamen, H., Lena, A.M., Candi, E., Terrinoni, A., Catani, M.V., Oren, M., Melino, G., Krammer, P.H., Stremmel, W. and Muller, M. (2005) 'TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria', *EMBO J*, 24(13), pp. 2458-71.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X. and Wu, H. (2001) 'Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo', *Science*, 294(5549), pp. 2186-2189.

Grueterich, M., Espana, E.M., Touhami, A., Ti, S.E. and Tseng, S.C.G. (2002) 'Phenotypic study of a case with successful transplantation of ex vivo expanded human limbal epithelium for unilateral total limbal stem cell deficiency', *Ophthalmology*, 109(8), pp. 1547-1552.

Grueterich, M., Espana, E.M. and Tseng, S.C. (2003) 'Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche', *Surv Ophthalmol*, 48(6), pp. 631-46.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J. and Sabatini, D.M. (2006) 'Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha but not S6K1', *Developmental Cell*, 11(6), pp. 859-871.

Guo, F., Li, J., Du, W., Zhang, S., O'Connor, M., Thomas, G., Kozma, S., Zingarelli, B., Pang, Q. and Zheng, Y. (2013) 'mTOR regulates DNA damage response through NF-kappa B-mediated FANCD2 pathway in hematopoietic cells', *Leukemia*, 27(10), pp. 2040-2046.

Gutierrez-Cuesta, J., Tajes, M., Jimenez, A., Coto-Montes, A., Camins, A. and Pallas, M. (2008) 'Evaluation of potential pro-survival pathways regulated by melatonin in a murine senescence model', *J Pineal Res*, 45(4), pp. 497-505.

Gwin, R.M., Lerner, I., Warren, J.K. and Gum, G. (1982) 'Decrease in Canine Corneal Endothelial Cell-Density and Increase in Corneal Thickness as Functions of Age', *Investigative Ophthalmology & Visual Science*, 22(2), pp. 267-271.

Hadshiew, I.M., Eller, M.S. and Gilchrest, B.A. (1999) 'Age-associated decreases in human DNA repair capacity: Implications for the skin', *Age (Omaha)*, 22(2), pp. 45-57.

Haendeler, J., Drose, S., Buchner, N., Jakob, S., Altschmied, J., Goy, C., Spyridopoulos, I., Zeiher, A.M., Brandt, U. and Dimmeler, S. (2009) 'Mitochondrial Telomerase Reverse Transcriptase Binds to and Protects Mitochondrial DNA and Function From Damage', *Arteriosclerosis Thrombosis and Vascular Biology*, 29(6), pp. 929-U400.

Haley-Zitlin, V. and Richardson, A. (1993) 'Effect of dietary restriction on DNA repair and DNA damage', *Mutat Res*, 295(4-6), pp. 237-45.

Hamilton, C.A., McIntyre, M., Brosnan, M.J., Graham, D. and Dominiczak, A.F. (2000) 'Superoxide excess in hypertension and aging: A common cause of endothelial dysfunction?', *Hypertension*, 36(4), pp. 697-697.

Hands, S.L., Proud, C.G. and Wyttenbach, A. (2009) 'mTOR's role in ageing: protein synthesis or autophagy?', *Aging-Us*, 1(7), pp. 586-597.

Hanna, C., Bicknell, D.S. and O'Brien, J.E. (1961) 'Cell turnover in the adult human eye', *Arch Ophthalmol*, 65, pp. 695-8.

Hanna, C. and O'Brien, J.E. (1960) 'Cell production and migration in the epithelial layer of the cornea', *Arch Ophthalmol*, 64, pp. 536-9.

Hansen, B.C., Bodkin, N.L. and Ortmeyer, H.K. (1999) 'Calorie restriction in nonhuman primates: Mechanisms of reduced morbidity and mortality', *Toxicological Sciences*, 52(2), pp. 56-60.

Hansen, M., Taubert, S., Crawford, D., Libina, N., Lee, S.J. and Kenyon, C. (2007) 'Lifespan extension by conditions that inhibit translation in Caenorhabditis elegans', *Aging cell*, 6(1), pp. 95-110.

Harle-Bachor, C. and Boukamp, P. (1996) 'Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes', *Proc Natl Acad Sci U S A*, 93(13), pp. 6476-81.

Harley, C.B., Futcher, A.B. and Greider, C.W. (1990) 'Telomeres shorten during ageing of human fibroblasts', *Nature*, 345(6274), pp. 458-460.

Harman, D. (1965) 'The Free Radical Theory of Aging: Effect of Age on Serum Copper Levels', *J Gerontol*, 20, pp. 151-3.

Harmes, D.C., Bresnick, E., Lubin, E.A., Watson, J.K., Heim, K.E., Curtin, J.C., Suskind, A.M., Lamb, J. and DiRenzo, J. (2003) 'Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes', *Oncogene*, 22(48), pp. 7607-16.

Harrison, D.E., Strong, R., Sharp, Z.D., Nelson, J.F., Astle, C.M., Flurkey, K., Nadon, N.L., Wilkinson, J.E., Frenkel, K., Carter, C.S., Pahor, M., Javors, M.A., Fernandez, E. and Miller, R.A. (2009) 'Rapamycin fed late in life extends lifespan in genetically heterogeneous mice', *Nature*, 460(7253), pp. 392-U108.

Harrison, J.S., Rameshwar, P., Chang, V. and Bandari, P. (2002) 'Oxygen saturation in the bone marrow of healthy volunteers', *Blood*, 99(1), p. 394.

Hashmani, K., Branch, M.J., Sidney, L.E., Dhillon, P.S., Verma, M., McIntosh, O.D., Hopkinson, A. and Dua, H.S. (2013) 'Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation', *Stem Cell Res Ther*, 4(3), p. 75.

Hayashi, I., Larner, J. and Sato, G. (1978) 'Hormonal growth control of cells in culture', *In Vitro*, 14(1), pp. 23-30.

Hayashi, Y., Watanabe, N. and Ohashi, Y. (2012) 'The "replacement hypothesis": corneal stem cell origin epithelia are replaced by limbal stem cell origin epithelia in mouse cornea during maturation', *Cornea*, 31 Suppl 1, pp. S68-73.

Hayflick, L. and Moorhead, P.S. (1961) 'The serial cultivation of human diploid cell strains', *Exp Cell Res*, 25, pp. 585-621.

Henriksson, J.T., McDermott, A.M. and Bergmanson, J.P.G. (2009) 'Dimensions and Morphology of the Cornea in Three Strains of Mice', *Investigative Ophthalmology & Visual Science*, 50(8), pp. 3648-3654.

Herbig, U., Jobling, W.A., Chen, B.P.C., Chen, D.J. and Sedivy, J.M. (2004) 'Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)', *Molecular Cell*, 14(4), pp. 501-513.

Hewitt, G., Jurk, D., Marques, F.D.M., Correia-Melo, C., Hardy, T., Gackowska, A., Anderson, R., Taschuk, M., Mann, J. and Passos, J.F. (2012) 'Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence', *Nat Commun*, 3.

Hilal, G.Y., Alaaeddine, N., Wardi, L. and Assad, C. (2011) 'Caloric Restriction Decreases Telomerase Activity and Enhances BIBR 1532 Inhibition Response on MDA-MB 231 Cell Line', *Faseb Journal*, 25.

Hillenaar, T., van Cleynenbreugel, H. and Remeijer, L. (2012) 'How Normal Is the Transparent Cornea? Effects of Aging on Corneal Morphology', *Ophthalmology*, 119(2), pp. 241-248.

Hinkle, P.C. and Yu, M.L. (1979) 'The phosphorus/oxygen ratio of mitochondrial oxidative phosphorylation', *J Biol Chem*, 254(7), pp. 2450-5.

Hiyama, E. and Hiyama, K. (2007) 'Telomere and telomerase in stem cells', *Br J Cancer*, 96(7), pp. 1020-4.

Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M.A., Shay, J.W., Ishioka, S. and Yamakido, M. (1995) 'Activation of telomerase in human lymphocytes and hematopoietic progenitor cells', *J Immunol*, 155(8), pp. 3711-5.

Hobbs, R.M., Seandel, M., Falciatori, I., Rafii, S. and Pandolfi, P.P. (2010) 'Plzf Regulates Germline Progenitor Self-Renewal by Opposing mTORC1', *Cell*, 142(3), pp. 468-479.

Holliday, R. (1989) 'Food, Reproduction and Longevity - Is the Extended Lifespan of Calorie-Restricted Animals an Evolutionary Adaptation', *Bioessays*, 10(4), pp. 125-127.

Holstege, H., Pfeiffer, W., Sie, D., Hulsman, M., Nicholas, T.J., Lee, C.C., Ross, T., Lin, J., Miller, M.A., Ylstra, B., Meijers-Heijboer, H., Brugman, M.H., Staal, F.J., Holstege, G., Reinders, M.J., Harkins, T.T., Levy, S. and Sistermans, E.A. (2014) 'Somatic mutations found in the healthy blood compartment of a 115-yr-old woman demonstrate oligoclonal hematopoiesis', *Genome Res*.

Honn, K.V., Singley, J.A. and Chavin, W. (1975) 'Fetal bovine serum: a multivariate standard', *Proc Soc Exp Biol Med*, 149(2), pp. 344-7.

Houpt, K.A. and Zicker, S. (2003) 'Dietary effects on canine and feline behavior', *Veterinary Clinics of North America-Small Animal Practice*, 33(2), pp. 405-+.

Hsiao, R., Sharma, H.W., Ramakrishnan, S., Keith, E. and Narayanan, R. (1997) 'Telomerase activity in normal human endothelial cells', *Anticancer Res*, 17(2A), pp. 827-32.

Huang, A.J. and Tseng, S.C. (1991) 'Corneal epithelial wound healing in the absence of limbal epithelium', *Invest Ophthalmol Vis Sci*, 32(1), pp. 96-105.

Huang, L.E., Arany, Z., Livingston, D.M. and Bunn, H.F. (1996) 'Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit', *Journal of Biological Chemistry*, 271(50), pp. 32253-32259.

Hunter, M.F., Peters, H., Salemi, R., Thorburn, D. and Mackay, M.T. (2011) 'Alpers syndrome with mutations in POLG: clinical and investigative features', *Pediatr Neurol*, 45(5), pp. 311-8.

Hursting, S.D., Dunlap, S.M., Ford, N.A., Hursting, M.J. and Lashinger, L.M. (2013) 'Calorie restriction and cancer prevention: a mechanistic perspective', *Cancer Metab*, 1(1), p. 10.

Huttner, W.B. and Kosodo, Y. (2005) 'Symmetric versus asymmetric cell division during neurogenesis in the developing vertebrate central nervous system', *Current Opinion in Cell Biology*, 17(6), pp. 648-57.

Iida, T., Mine, S., Fujimoto, H., Suzuki, K., Minami, Y. and Tanaka, Y. (2002) 'Hypoxia-inducible factor-1alpha induces cell cycle arrest of endothelial cells', *Genes Cells*, 7(2), pp. 143-9.

Indran, I.R., Hande, M.P. and Pervaiz, S. (2011) 'hTERT overexpression alleviates intracellular ROS production, improves mitochondrial function, and inhibits ROS-mediated apoptosis in cancer cells', *Cancer Res*, 71(1), pp. 266-76.

Inomata, K., Aoto, T., Binh, N.T., Okamoto, N., Tanimura, S., Wakayama, T., Iseki, S., Hara, E., Masunaga, T., Shimizu, H. and Nishimura, E.K. (2009) 'Genotoxic Stress Abrogates Renewal of Melanocyte Stem Cells by Triggering Their Differentiation', *Cell*, 137(6), pp. 1088-1099.

Insinga, A., Cicalese, A., Faretta, M., Gallo, B., Albano, L., Ronzoni, S., Furia, L., Viale, A. and Pelicci, P.G. (2013) 'DNA damage in stem cells activates p21, inhibits p53, and induces symmetric self-renewing divisions', *Proc Natl Acad Sci U S A*, 110(10), pp. 3931-6.

Ismail, I.H., Andrin, C., McDonald, D. and Hendzel, M.J. (2010) 'BMI1-mediated histone ubiquitylation promotes DNA double-strand break repair', *J Cell Biol*, 191(1), pp. 45-60.

Ismail, I.H. and Hendzel, M.J. (2008) 'The gamma-H2A.X: Is it just a surrogate marker of double-strand breaks or much more?', *Environmental and Molecular Mutagenesis*, 49(1), pp. 73-82.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., Ikeda, Y., Mak, T.W. and Suda, T. (2004) 'Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells', *Nature*, 431(7011), pp. 997-1002.

Iwama, A., Oguro, H., Negishi, M., Kato, Y., Morita, Y., Tsukui, H., Ema, H., Kamijo, T., Katoh-Fukui, Y., Koseki, H., van Lohuizen, M. and Nakauchi, H. (2004) 'Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1', *Immunity*, 21(6), pp. 843-851.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y. and Semenza, G.L. (1998) 'Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha', *Genes Dev*, 12(2), pp. 149-62.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A. and Hall, M.N. (2004) 'Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive', *Nature cell biology*, 6(11), pp. 1122-U30.

James, S.E., Rowe, A., Ilari, L., Daya, S. and Martin, R. (2001) 'The potential for eye bank limbal rings to generate cultured corneal epithelial allografts', *Cornea*, 20(5), pp. 488-94.

Jang, Y.Y. and Sharkis, S.J. (2007) 'A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche', *Blood*, 110(8), pp. 3056-3063.

Jeong, C.H., Lee, H.J., Cha, J.H., Kim, J.H., Kim, K.R., Yoon, D.K. and Kim, K.W. (2007) 'Hypoxia-inducible factor-1 alpha inhibits self-renewal of mouse embryonic stem cells in Vitro via negative regulation of the leukemia inhibitory factor-STAT3 pathway', *J Biol Chem*, 282(18), pp. 13672-9.

Jeong, J.W., Bae, M.K., Ahn, M.Y., Kim, S.H., Sohn, T.K., Bae, M.H., Yoo, M.A., Song, E.J., Lee, K.J. and Kim, K.W. (2002) 'Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation', *Cell*, 111(5), pp. 709-20.

Jiang, J.C., Jaruga, E., Repnevskaya, M.V. and Jazwinski, S.M. (2000) 'An intervention resembling caloric restriction prolongs life span and retards aging in yeast', *FASEB J*, 14(14), pp. 2135-7.

Johnson, D.H., Bourne, W.M. and Campbell, R.J. (1982) 'The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas', *Arch Ophthalmol*, 100(12), pp. 1942-7.

Jolly, C.A., Muthukumar, A., Avula, C.P.R., Troyer, D. and Fernandes, G. (2001) 'Life span is prolonged in food-restricted autoimmune-prone (NZB x NZW)F(1) mice fed a diet enriched with (n-3) fatty acids', *Journal of Nutrition*, 131(10), pp. 2753-2760.

Jones, D.L. and Rando, T.A. (2011) 'Emerging models and paradigms for stem cell ageing', *Nature cell biology*, 13(5), pp. 506-12.

Jones, P.H. and Watt, F.M. (1993) 'Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression', *Cell*, 73(4), pp. 713-24.

Jonsson, M., Markstrom, K. and Behndig, A. (2006) 'Slit-scan tomography evaluation of the anterior chamber and corneal configurations at different ages', *Acta Ophthalmol Scand*, 84(1), pp. 116-20.

Ju, Z., Jiang, H., Jaworski, M., Rathinam, C., Gompf, A., Klein, C., Trumpp, A. and Rudolph, K.L. (2007) 'Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment', *Nat Med*, 13(6), pp. 742-7.

Jun, A.S., Chakravarti, S., Edelhauser, H.F. and Kimos, M. (2006) 'Aging changes of mouse corneal endothelium and Descemet's membrane', *Exp Eye Res*, 83(4), pp. 890-6.

Jung, J.W., Lee, S., Seo, M.S., Park, S.B., Kurtz, A., Kang, S.K. and Kang, K.S. (2010) 'Histone deacetylase controls adult stem cell aging by balancing the expression of polycomb genes and jumonji domain containing 3', *Cell Mol Life Sci*, 67(7), pp. 1165-76.

Kaeberlein, M. and Kennedy, B.K. (2009) 'Ageing: A midlife longevity drug?', *Nature*, 460(7253), pp. 331-2.

Kaeberlein, M., Powers, R.W., Steffen, K.K., Westman, E.A., Hu, D., Dang, N., Kerr, E.O., Kirkland, K.T., Fields, S. and Kennedy, B.K. (2005) 'Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients', *Science*, 310(5751), pp. 1193-1196.

Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007) 'The Hes gene family: repressors and oscillators that orchestrate embryogenesis', *Development*, 134(7), pp. 1243-1251.

Kalaitzidis, D., Sykes, S.M., Wang, Z., Punt, N., Tang, Y.F., Ragu, C., Sinha, A.U., Lane, S.W., Souza, A.L., Clish, C.B., Anastasiou, D., Gilliland, D.G., Scadden, D.T., Guertin, D.A. and Armstrong, S.A. (2012) 'mTOR Complex 1 Plays Critical Roles in Hematopoiesis and Pten-Loss-Evoked Leukemogenesis', *Cell Stem Cell*, 11(3), pp. 429-439.

Kallio, P.J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L. (1997) 'Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor', *Proc Natl Acad Sci U S A*, 94(11), pp. 5667-72.

Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R.C. and Conaway, J.W. (2000) 'Activation of HIF1alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex', *Proc Natl Acad Sci U S A*, 97(19), pp. 10430-5.

Karlseder, J., Smogorzewska, A. and de Lange, T. (2002) 'Senescence induced by altered telomere state, not telomere loss', *Science*, 295(5564), pp. 2446-9.

Karow, J.K., Chakraverty, R.K. and Hickson, I.D. (1997) 'The Bloom's syndrome gene product is a 3 '-5 ' DNA helicase', *Journal of Biological Chemistry*, 272(49), pp. 30611-30614.

Katikireddy, K.R., Dana, R. and Jurkunas, U.V. (2014) 'Differentiation potential of limbal fibroblasts and bone marrow mesenchymal stem cells to corneal epithelial cells', *Stem Cells*, 32(3), pp. 717-29.

Kaufman, H.E., Barron, B.A. and McDonald, M.B. (1998) *The cornea*. 2nd edn. Boston: Butterworth-Heinemann.

Kawahara, T.L., Michishita, E., Adler, A.S., Damian, M., Berber, E., Lin, M., McCord, R.A., Ongaigui, K.C., Boxer, L.D., Chang, H.Y. and Chua, K.F. (2009) 'SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span', *Cell*, 136(1), pp. 62-74.

Kawashima, M., Kawakita, T., Satake, Y., Higa, K. and Shimazaki, J. (2007) 'Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency', *Archives of Ophthalmology*, 125(10), pp. 1337-1344.

Kawashima, M. and Tsubota, K. (2011) 'Effect of calorie restriction on change in lacrimal gland with age', *Cornea*, 30 Suppl 1, pp. S29-33.

Kawauchi, K., Ihjima, K. and Yamada, O. (2005) 'IL-2 increases human telomerase reverse transcriptase activity transcriptionally and posttranslationally through phosphatidylinositol 3'-kinase/Akt, heat shock protein 90, and mammalian target of rapamycin in transformed NK cells', *J Immunol*, 174(9), pp. 5261-9.

Keller, J.N. (2006) 'Age-related neuropathology, cognitive decline, and Alzheimer's disease', *Ageing Res Rev*, 5(1), pp. 1-13.

Kent, K.D. and Bomser, J.A. (2003) 'Bovine pituitary extract provides remarkable protection against oxidative stress in human prostate epithelial cells', *In Vitro Cell Dev Biol Anim*, 39(8-9), pp. 388-94.

Kenyon, C., Chang, J., Gensch, E., Rudner, A. and Tabtiang, R. (1993) 'A C-Elegans Mutant That Lives Twice as Long as Wild-Type', *Nature*, 366(6454), pp. 461-464.

Kenyon, K.R. and Tseng, S.C. (1989) 'Limbal autograft transplantation for ocular surface disorders', *Ophthalmology*, 96(5), pp. 709-22; discussion 722-3.

Keyes, W.M., Wu, Y., Vogel, H., Guo, X., Lowe, S.W. and Mills, A.A. (2005) 'p63 deficiency activates a program of cellular senescence and leads to accelerated aging', *Genes Dev*, 19(17), pp. 1986-99.

Khandelwal, N., Simpson, J., Taylor, G., Rafique, S., Whitehouse, A., Hiscox, J. and Stark, L.A. (2011) 'Nucleolar NF-kappaB/RelA mediates apoptosis by causing cytoplasmic relocalization of nucleophosmin', *Cell Death Differ*, 18(12), pp. 1889-903.

Kiel, M.J., He, S.H., Ashkenazi, R., Gentry, S.N., Teta, M., Kushner, J.A., Jackson, T.L. and Morrison, S.J. (2007) 'Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU', *Nature*, 449(7159), pp. 238-U10.

Kim, A.H., Khursigara, G., Sun, X., Franke, T.F. and Chao, M.V. (2001) 'Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1', *Mol Cell Biol*, 21(3), pp. 893-901.

Kim, H.S., Song, X.J., de Paiva, C., Chen, Z., Pflugfelder, S.C. and Li, D.Q. (2004) 'Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures', *Experimental Eye Research*, 79(1), pp. 41-49.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) 'Specific association of human telomerase activity with immortal cells and cancer', *Science*, 266(5193), pp. 2011-5.

Kimura, A., Ohmichi, M., Kawagoe, J., Kyo, S., Mabuchi, S., Takahashi, T., Ohshima, C., Arimoto-Ishida, E., Nishio, Y., Inoue, M., Kurachi, H., Tasaka, K. and Murata, Y. (2004) 'Induction of hTERT expression and phosphorylation by estrogen via Akt cascade in human ovarian cancer cell lines', *Oncogene*, 23(26), pp. 4505-15.

Kimura, W. and Sadek, H.A. (2012) 'The cardiac hypoxic niche: emerging role of hypoxic microenvironment in cardiac progenitors', *Cardiovasc Diagn Ther*, 2(4), pp. 278-89.

Kinoshita, S., Friend, J. and Thoft, R.A. (1981) 'Sex chromatin of donor corneal epithelium in rabbits', *Invest Ophthalmol Vis Sci*, 21(3), pp. 434-41.

Kinoshita, S., Koizumi, N. and Nakamura, T. (2004) 'Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction', *Experimental eye research*, 78(3), pp. 483-491.

Kirkwood, T.B. (1977) 'Evolution of ageing', *Nature*, 270(5635), pp. 301-4.

Kirkwood, T.B. (2005) 'Understanding the odd science of aging', *Cell*, 120(4), pp. 437-47.

Klass, M.R. (1977) 'Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span', *Mech Ageing Dev*, 6(6), pp. 413-29.

Klement, R.J. and Kammerer, U. (2011) 'Is there a role for carbohydrate restriction in the treatment and prevention of cancer?', *Nutrition & Metabolism*, 8.

Kofman, A.E., McGraw, M.R. and Payne, C.J. (2012) 'Rapamycin increases oxidative stress response gene expression in adult stem cells', *Aging-Us*, 4(4), pp. 279-289.

Koizumi, N., Inatomi, T., Suzuki, T., Sotozono, C. and Kinoshita, S. (2001) 'Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome', *Archives of Ophthalmology*, 119(2), pp. 298-300.

Koizumi, N.J., Inatomi, T.J., Sotozono, C.J., Fullwood, N.J., Quantock, A.J. and Kinoshita, S. (2000) 'Growth factor mRNA and protein in preserved human amniotic membrane', *Curr Eye Res*, 20(3), pp. 173-7.

Kolli, S., Ahmad, S., Lako, M. and Figueiredo, F. (2010) 'Successful Clinical Implementation of Corneal Epithelial Stem Cell Therapy for Treatment of Unilateral Limbal Stem Cell Deficiency', *Stem Cells*, 28(3), pp. 597-610.

Koshiji, M., Kageyama, Y., Pete, E.A., Horikawa, I., Barrett, J.C. and Huang, L.E. (2004) 'HIF-1alpha induces cell cycle arrest by functionally counteracting Myc', *EMBO J*, 23(9), pp. 1949-56.

Koster, M.I., Kim, S., Mills, A.A., DeMayo, F.J. and Roop, D.R. (2004) 'p63 is the molecular switch for initiation of an epithelial stratification program', *Genes Dev*, 18(2), pp. 126-31.

Kowal, P., Chatterji, S., Naidoo, N., Biritwum, R., Fan, W., Lopez Ridaura, R., Maximova, T., Arokiasamy, P., Phaswana-Mafuya, N., Williams, S., Snodgrass, J.J., Minicuci, N., D'Este, C., Peltzer, K. and Boerma, J.T. (2012) 'Data resource profile: the World Health Organization Study on global AGEing and adult health (SAGE)', *International Journal of Epidemiology*, 41(6), pp. 1639-49.

Krachmer, J.H., Mannis, M.J. and Holland, E.J. (2005) *Cornea*. 2nd edn. Philadelphia: Elsevier/Mosby.

Krawiec, J.T., Weinbaum, J.S., St Croix, C.M., Phillippi, J.A., Watkins, S.C., Rubin, J.P. and Vorp, D.A. (2014) 'A Cautionary Tale for Autologous Vascular Tissue Engineering: Impact of Human Demographics on the Ability of Adipose-Derived Mesenchymal Stem Cells to Recruit and Differentiate into Smooth Muscle Cells', *Tissue Eng Part A*.

Krishnamurthy, P., Ross, D.D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K.E., Sarkadi, B., Sorrentino, B.P. and Schuetz, J.D. (2004) 'The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme', *Journal of Biological Chemistry*, 279(23), pp. 24218-24225.

Krishnamurthy, P. and Schuetz, J.D. (2006) 'Role of ABCG2/BCRP in biology and medicine', *Annu Rev Pharmacol Toxicol*, 46, pp. 381-410.

Kroemer, G., Galluzzi, L. and Brenner, C. (2007) 'Mitochondrial membrane permeabilization in cell death', *Physiol Rev*, 87(1), pp. 99-163.

Kruk, P.A., Rampino, N.J. and Bohr, V.A. (1995) 'DNA damage and repair in telomeres: relation to aging', *Proc Natl Acad Sci U S A*, 92(1), pp. 258-62.

Kruse, F.E., Chen, J.J., Tsai, R.J. and Tseng, S.C. (1990) 'Conjunctival transdifferentiation is due to the incomplete removal of limbal basal epithelium', *Invest Ophthalmol Vis Sci*, 31(9), pp. 1903-13.

Kruse, F.E. and Tseng, S.C. (1992) 'Proliferative and differentiative response of corneal and limbal epithelium to extracellular calcium in serum-free clonal cultures', *J Cell Physiol*, 151(2), pp. 347-60.

Ksander, B.R., Kolovou, P.E., Wilson, B.J., Saab, K.R., Guo, Q., Ma, J., McGuire, S.P., Gregory, M.S., Vincent, W.J.B., Perez, V.L., Cruz-Guilloty, F., Kao, W.W.Y., Call, M.K., Tucker, B.A., Zhan, Q., Murphy, G.F., Lathrop, K.L., Alt, C., Mortensen, L.J., Lin, C.P., Zieske, J.D., Frank, M.H. and Frank, N.Y. (2014) 'ABCB5 is a limbal stem cell gene required for corneal development and repair', *Nature*, 511(7509), pp. 353-+.

Kubota, Y., Takubo, K. and Suda, T. (2008) 'Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche', *Biochem Biophys Res Commun*, 366(2), pp. 335-9.

Kujoth, G.C., Hiona, A., Pugh, T.D., Someya, S., Panzer, K., Wohlgemuth, S.E., Hofer, T., Seo, A.Y., Sullivan, R., Jobling, W.A., Morrow, J.D., Van Remmen, H., Sedivy, J.M., Yamasoba, T., Tanokura, M., Weindruch, R., Leeuwenburgh, C. and Prolla, T.A. (2005)

'Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging', *Science*, 309(5733), pp. 481-4.

Kuranda, K., Vargaftig, J., de la Rochere, P., Dosquet, C., Charron, D., Bardin, F., Tonnelle, C., Bonnet, D. and Goodhardt, M. (2011) 'Age-related changes in human hematopoietic stem/progenitor cells', *Aging cell*, 10(3), pp. 542-6.

Kurpakus, M.A., Stock, E.L. and Jones, J.C. (1990) 'Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium', *Invest Ophthalmol Vis Sci*, 31(3), pp. 448-56.

Kwan, M., Niinikoski, J. and Hunt, T.K. (1972) 'In vivo measurements of oxygen tension in the cornea, aqueous humor, and anterior lens of the open eye', *Invest Ophthalmol*, 11(2), pp. 108-14.

Lack, D.L. (1954) *The natural regulation of animal numbers*. Oxford,: Clarendon Press.

Laing, R.A., Sandstrom, M.M., Berrospi, A.R. and Leibowitz, H.M. (1976) 'Changes in Corneal Endothelium as a Function of Age', *Experimental eye research*, 22(6), pp. 587-594.

Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W. and Hoffmann, I. (1998) 'The cdc25B phosphatase is essential for the G2/M phase transition in human cells', *Journal of cell science*, 111, pp. 2445-2453.

Lansdorp, P.M. (2009) 'Telomeres and disease', *Embo Journal*, 28(17), pp. 2532-2540.

Lanza, R.P. (2004) *Handbook of stem cells*. (2 vols). Amsterdam: Elsevier Academic.

Laplante, M. and Sabatini, D.M. (2009) 'An Emerging Role of mTOR in Lipid Biosynthesis', *Current Biology*, 19(22), pp. R1046-R1052.

Laule, A., Cable, M.K., Hoffman, C.E. and Hanna, C. (1978) 'Endothelial cell population changes of human cornea during life', *Arch Ophthalmol*, 96(11), pp. 2031-5.

Lavker, R.M., Dong, G., Cheng, S.Z., Kudoh, K., Cotsarelis, G. and Sun, T.T. (1991) 'Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes', *Invest Ophthalmol Vis Sci*, 32(6), pp. 1864-75.

Lawless, C., Jurk, D., Gillespie, C.S., Shanley, D., Saretzki, G., von Zglinicki, T. and Passos, J.F. (2012) 'A stochastic step model of replicative senescence explains ROS production rate in ageing cell populations', *PLoS One*, 7(2), p. e32117.

Lechler, T. and Fuchs, E. (2005) 'Asymmetric cell divisions promote stratification and differentiation of mammalian skin', *Nature*, 437(7056), pp. 275-80.

Lee, J., Duan, W.Z., Long, J.M., Ingram, D.K. and Mattson, M.P. (2000) 'Dietary restriction increases the number of newly generated neural cells, and induces BDNF expression, in the dentate gyrus of rats', *Journal of Molecular Neuroscience*, 15(2), pp. 99-108.

Lee, J.Y., Nakada, D., Yilmaz, O.H., Tothova, Z., Joseph, N.M., Lim, M.S., Gilliland, D.G. and Morrison, S.J. (2010a) 'mTOR Activation Induces Tumor Suppressors that Inhibit

Leukemogenesis and Deplete Hematopoietic Stem Cells after Pten Deletion', *Cell Stem Cell*, 7(5), pp. 593-605.

Lee, K.W., Yook, J.Y., Son, M.Y., Kim, M.J., Koo, D.B., Han, Y.M. and Cho, Y.S. (2010b) 'Rapamycin Promotes the Osteoblastic Differentiation of Human Embryonic Stem Cells by Blocking the mTOR Pathway and Stimulating the BMP/Smad Pathway', *Stem Cells and Development*, 19(4), pp. 557-568.

Lehmann, E.L. (1998) *Nonparametrics : Statistical Methods Based on Ranks, Revised,*.

Lehrer, M.S., Sun, T.T. and Lavker, R.M. (1998) 'Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation', *J Cell Sci*, 111 (Pt 19), pp. 2867-75.

Leontieva, O.V., Natarajan, V., Demidenko, Z.N., Burdelya, L.G., Gudkov, A.V. and Blagosklonny, M.V. (2012) 'Hypoxia suppresses conversion from proliferative arrest to cellular senescence', *Proc Natl Acad Sci U S A*, 109(33), pp. 13314-8.

Lessard, J. and Sauvageau, G. (2003) 'Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells', *Nature*, 423(6937), pp. 255-60.

Lettieri Barbato, D., Baldelli, S., Pagliei, B., Aquilano, K. and Ciriolo, M.R. (2012) 'Caloric Restriction and the Nutrient-Sensing PGC-1alpha in Mitochondrial Homeostasis: New Perspectives in Neurodegeneration', *Int J Cell Biol*, 2012, p. 759583.

Levrero, M., De Laurenzi, V., Costanzo, A., Gong, J., Melino, G. and Wang, J.Y. (1999) 'Structure, function and regulation of p63 and p73', *Cell Death Differ*, 6(12), pp. 1146-53.

Levrero, M., De Laurenzi, V., Costanzo, A., Sabatini, S., Gong, J., Wang, J.Y.J. and Melino, G. (2000) 'The p53/p63/p73 family of transcription factors: overlapping and distinct functions', *Journal of cell science*, 113(10), pp. 1661-1670.

Li, C., Yin, T., Dong, N., Dong, F., Fang, X., Qu, Y.L., Tan, Y., Wu, H., Liu, Z. and Li, W. (2011) 'Oxygen tension affects terminal differentiation of corneal limbal epithelial cells', *J Cell Physiol*, 226(9), pp. 2429-37.

Li, D.Q., Sharpe, L.C.Z., de Paiva, C.S., Luo, L.H. and Pfugfelder, S.C. (2004) 'Characterization of putative stem cell phenotype in human limbal epithelia', *Investigative Ophthalmology & Visual Science*, 45, pp. U124-U124.

Li, W., Hayashida, Y., Chen, Y.T. and Tseng, S.C. (2007) 'Niche regulation of corneal epithelial stem cells at the limbus', *Cell Res*, 17(1), pp. 26-36.

Li, Y., Yan, Q. and Wolf, N.S. (1997) 'Long-term caloric restriction delays age-related decline in proliferation capacity of murine lens epithelial cells in vitro and in vivo', *Invest Ophthalmol Vis Sci*, 38(1), pp. 100-7.

Liao, C.Y., Rikke, B.A., Johnson, T.E., Diaz, V. and Nelson, J.F. (2010) 'Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening', *Aging cell*, 9(1), pp. 92-95.

Liefer, K.M., Koster, M.I., Wang, X.J., Yang, A., McKeon, F. and Roop, D.R. (2000) 'Down-regulation of p63 is required for epidermal UV-B-induced apoptosis', *Cancer research*, 60(15), pp. 4016-4020.

Lin, S.H. (1976) 'Oxygen tension in the in vivo cornea', *Bull Math Biol*, 38(3), pp. 269-75.

Lindberg, K., Brown, M.E., Chaves, H.V., Kenyon, K.R. and Rheinwald, J.G. (1993) 'In vitro propagation of human ocular surface epithelial cells for transplantation', *Invest Ophthalmol Vis Sci*, 34(9), pp. 2672-9.

Listerman, I., Sun, J., Gazzaniga, F.S., Lukas, J.L. and Blackburn, E.H. (2013) 'The major reverse transcriptase-incompetent splice variant of the human telomerase protein inhibits telomerase activity but protects from apoptosis', *Cancer Res*, 73(9), pp. 2817-28.

Liu, J.N., Sato, C., Cerletti, M. and Wagers, A. (2010a) 'Notch Signaling in the Regulation of Stem Cell Self-Renewal and Differentiation', *Notch Signaling*, 92, pp. 367-409.

Liu, M.L., Wilk, S.A., Wang, A.P., Zhou, L.J., Wang, R.H., Ogawa, W., Deng, C.X., Dong, L.Q. and Liu, F. (2010b) 'Resveratrol Inhibits mTOR Signaling by Promoting the Interaction between mTOR and DEPTOR', *Journal of Biological Chemistry*, 285(47), pp. 36387-36394.

Liu, S., Li, J., Wang, C., Tan, D. and Beuerman, R. (2006) 'Human limbal progenitor cell characteristics are maintained in tissue culture', *Ann Acad Med Singapore*, 35(2), pp. 80-6.

Liu, T., Wang, Y., Duan, H.Y., Qu, M.L., Yang, L.L., Xu, Y.Y., Zang, X.J. and Zhou, Q.J. (2012) 'Effects of preservation time on proliferative potential of human limbal stem/progenitor cells', *Int J Ophthalmol*, 5(5), pp. 549-54.

Liu, Z., Wan, P., Duan, H., Zhou, J., Tan, B., Liu, Y., Zhou, Q., Zhou, C., Huang, Z., Tian, B., Li, C. and Wang, Z. (2013) 'ES micro-environment enhances stemness and inhibits apoptosis in human limbal stem cells via the maintenance of telomerase activity', *PLoS One*, 8(1), p. e53576.

Lively, G.D., Jiang, B., Hedberg-Buenz, A., Chang, B., Petersen, G.E., Wang, K., Kuehn, M.H. and Anderson, M.G. (2010) 'Genetic Dependence of Central Corneal Thickness among Inbred Strains of Mice', *Investigative Ophthalmology & Visual Science*, 51(1), pp. 160-171.

Lizcano, J.M. and Alessi, D.R. (2002) 'The insulin signalling pathway', *Current Biology*, 12(7), pp. R236-R238.

Longo, V.D. and Fontana, L. (2010) 'Calorie restriction and cancer prevention: metabolic and molecular mechanisms', *Trends in Pharmacological Sciences*, 31(2), pp. 89-98.

Lopez-Garcia, C., Klein, A.M., Simons, B.D. and Winton, D.J. (2010) 'Intestinal stem cell replacement follows a pattern of neutral drift', *Science*, 330(6005), pp. 822-5.

Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M. and Kroemer, G. (2013) 'The hallmarks of aging', *Cell*, 153(6), pp. 1194-217.

Lore, R., Gottdiener, C. and Delahunty, M.J. (1986) 'Lean and Mean Rats - Some Effects of Acute Changes in the Food-Supply Upon Territorial Aggression', *Aggressive Behavior*, 12(6), pp. 409-415.

Loureiro, R.R., Cristovam, P.C., Martins, C.M., Covre, J.L., Sobrinho, J.A., Ricardo, J.R., Hazarbassanov, R.M., Hofling-Lima, A.L., Belfort, R., Jr., Nishi, M. and Gomes, J.A. (2013) 'Comparison of culture media for ex vivo cultivation of limbal epithelial progenitor cells', *Mol Vis*, 19, pp. 69-77.

Lowe, S.W. (1999) 'Hot papers - Cancer - Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a by M. Serrano, A.W. Lin, M.E. McCurrach, D. Beach, S.W. Lowe - Comments', *Scientist*, 13(22), pp. 15-15.

Lu, L.L., Liu, Y.J., Yang, S.G., Zhao, Q.J., Wang, X., Gong, W., Han, Z.B., Xu, Z.S., Lu, Y.X., Liu, D., Chen, Z.Z. and Han, Z.C. (2006) 'Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials', *Haematologica*, 91(8), pp. 1017-26.

Ma, J.H., Meng, Y., Kwiatkowski, D.J., Chen, X.X., Feng, H.Y., Sun, Q., Zha, X.J., Wang, F., Wang, Y., Jing, Y.L., Zhang, S., Chen, R.R., Wang, L.M., Wu, E., Cai, G.F., Malinowska-Kolodziej, I., Liao, Q., Liu, Y.Q., Zhao, Y., Sun, Q., Xu, K.F., Dai, J.W., Han, J.H., Wu, L.Z., Zhao, R.C., Shen, H.X. and Zhang, H.B. (2010) 'Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade', *Journal of Clinical Investigation*, 120(1), pp. 103-114.

Ma, X.J.M. and Blenis, J. (2009) 'Molecular mechanisms of mTOR-mediated translational control', *Nature Reviews Molecular Cell Biology*, 10(5), pp. 307-318.

Ma, X.L. and Liu, H.Q. (2011) 'Effect of hypoxia on the proliferation of murine cornea limbal epithelial progenitor cells in vitro', *Int J Ophthalmol*, 4(2), pp. 147-9.

Maciag, T., Hoover, G.A., Stemerman, M.B. and Weinstein, R. (1981a) 'Serial propagation of human endothelial cells in vitro', *J Cell Biol*, 91(2 Pt 1), pp. 420-6.

Maciag, T., Nemore, R.E., Weinstein, R. and Gilchrest, B.A. (1981b) 'An endocrine approach to the control of epidermal growth: serum-free cultivation of human keratinocytes', *Science*, 211(4489), pp. 1452-4.

Maher, J.C., Wangpaichitr, M., Savaraj, N., Kurtoglu, M. and Lampidis, T.J. (2007) 'Hypoxia-inducible factor-1 confers resistance to the glycolytic inhibitor 2-deoxy-D-glucose', *Mol Cancer Ther*, 6(2), pp. 732-41.

Mair, W., McLeod, C.J., Wang, L. and Jones, D.L. (2010) 'Dietary restriction enhances germline stem cell maintenance', *Aging cell*, 9(5), pp. 916-918.

Mair, W., Piper, M.D. and Partridge, L. (2005) 'Calories do not explain extension of life span by dietary restriction in Drosophila', *PLoS Biol*, 3(7), p. e223.

Majo, F., Rochat, A., Nicolas, M., Jaoude, G.A. and Barrandon, Y. (2008) 'Oligopotent stem cells are distributed throughout the mammalian ocular surface', *Nature*, 456(7219), pp. 250-4.

Malloy, V.L., Krajcik, R.A., Bailey, S.J., Hristopoulos, G., Plummer, J.D. and Orentreich, N. (2006) 'Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction', *Aging cell*, 5(4), pp. 305-14.

Manke, I.A., Nguyen, A., Lim, D., Stewart, M.Q., Elia, A.E.H. and Yaffe, M.B. (2005) 'MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G(2)/M transition and S phase progression in response to UV irradiation', *Molecular Cell*, 17(1), pp. 37-48.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J. and Cantley, L.C. (2002) 'Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-Kinase/Akt pathway', *Molecular Cell*, 10(1), pp. 151-162.

Martin, K., Potten, C.S., Roberts, S.A. and Kirkwood, T.B. (1998) 'Altered stem cell regeneration in irradiated intestinal crypts of senescent mice', *J Cell Sci*, 111 (Pt 16), pp. 2297-303.

Masters, B.R. and Thaer, A.A. (1994) 'Real-time scanning slit confocal microscopy of the in vivo human cornea', *Appl Opt*, 33(4), pp. 695-701.

Mathor, M.B., Ferrari, G., Dellambra, E., Cilli, M., Mavilio, F., Cancedda, R. and De Luca, M. (1996) 'Clonal analysis of stably transduced human epidermal stem cells in culture', *Proc Natl Acad Sci U S A*, 93(19), pp. 10371-6.

Mattagajasingh, I., Kim, C.S., Naqvi, A., Yamamori, T., Hoffman, T.A., Jung, S.B., DeRicco, J., Kasuno, K. and Irani, K. (2007) 'SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase', *Proc Natl Acad Sci U S A*, 104(37), pp. 14855-60.

Mattison, J.A., Roth, G.S., Beasley, T.M., Tilmont, E.M., Handy, A.M., Herbert, R.L., Longo, D.L., Allison, D.B., Young, J.E., Bryant, M., Barnard, D., Ward, W.F., Qi, W.B., Ingram, D.K. and de Cabo, R. (2012) 'Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study', *Nature*, 489(7415), pp. 318-+.

Mayhew, M., Renganathan, M. and Delbono, O. (1998) 'Effectiveness of caloric restriction in preventing age-related changes in rat skeletal muscle', *Biochemical and biophysical research communications*, 251(1), pp. 95-99.

Mccay, C.M., Crowell, M.F. and Maynard, L.A. (1989) 'Nutrition Metabolism Classic - the Effect of Retarded Growth Upon the Length of Life-Span and Upon the Ultimate Body Size (Reprinted from Journal of Nutrition, Vol 10, Pg 63-79, 1935)', *Nutrition*, 5(3), pp. 155-171.

McCay, C.M.C., Mary F. (1934) 'Prolonging the Life Span', *The Scientific Monthly*, 39(5), pp. 405-414.

Meller, D., Fuchsluger, T., Pauklin, M. and Steuhl, K.P. (2009) 'Ocular surface reconstruction in graft-versus-host disease with HLA-identical living-related allogeneic cultivated limbal epithelium after hematopoietic stem cell transplantation from the same donor', *Cornea*, 28(2), pp. 233-6.

Memmott, R.M. and Dennis, P.A. (2009) 'Akt-dependent and -independent mechanisms of mTOR regulation in cancer', *Cellular Signalling*, 21(5), pp. 656-64.

Mercken, E.M., Crosby, S.D., Lamming, D.W., JeBailey, L., Krzysik-Walker, S., Villareal, D.T., Capri, M., Franceschi, C., Zhang, Y.Q., Becker, K., Sabatini, D.M., de Cabo, R. and Fontana, L. (2013) 'Calorie restriction in humans inhibits the PI3K/AKT pathway and induces a younger transcription profile', *Aging cell*, 12(4), pp. 645-651.

Messaoudi, I., Warner, J., Fischer, M., Park, B., Hill, B., Mattison, J., Lane, M.A., Roth, G.S., Ingram, D.K., Picker, L.J., Douek, D.C., Mori, M. and Nikolich-Zugich, J. (2006) 'Delay of T cell senescence by caloric restriction in aged long-lived nonhuman primates', *Proc Natl Acad Sci U S A*, 103(51), pp. 19448-53.

Meyer-Blazejewska, E.A., Kruse, F.E., Bitterer, K., Meyer, C., Hofmann-Rummelt, C., Wunsch, P.H. and Schlotzer-Schrehardt, U. (2010) 'Preservation of the limbal stem cell phenotype by appropriate culture techniques', *Invest Ophthalmol Vis Sci*, 51(2), pp. 765-74.

Mezey, E. (2011) 'Adult Stem Cell Plasticity Revisited', in Phinney, D.G. (ed.) *Adult Stem Cells*. Humana Press, pp. 113-131.

Milara, J., Peiro, T., Serrano, A. and Cortijo, J. (2013) 'Epithelial to mesenchymal transition is increased in patients with COPD and induced by cigarette smoke', *Thorax*, 68(5), pp. 410-420.

Miller, R.A., Harrison, D.E., Astle, C.M., Baur, J.A., Boyd, A.R., de Cabo, R., Fernandez, E., Flurkey, K., Javors, M.A., Nelson, J.F., Orihuela, C.J., Pletcher, S., Sharp, Z.D., Sinclair, D., Starnes, J.W., Wilkinson, J.E., Nadon, N.L. and Strong, R. (2011) 'Rapamycin, but not resveratrol or simvastatin, extends life span of genetically heterogeneous mice', *J Gerontol A Biol Sci Med Sci*, 66(2), pp. 191-201.

Mills, A.A., Zheng, B., Wang, X.J., Vogel, H., Roop, D.R. and Bradley, A. (1999) 'p63 is a p53 homologue required for limb and epidermal morphogenesis', *Nature*, 398(6729), pp. 708-13.

Mills, D.B., Ward, L.M., Jones, C., Sweeten, B., Forth, M., Treusch, A.H. and Canfield, D.E. (2014) 'Oxygen requirements of the earliest animals', *Proc Natl Acad Sci U S A*, 111(11), pp. 4168-72.

Milyavsky, M., Gan, O.I., Trottier, M., Komosa, M., Tabach, O., Notta, F., Lechman, E., Hermans, K.G., Eppert, K., Konovalova, Z., Ornatsky, O., Domany, E., Meyn, M.S. and Dick, J.E. (2010) 'A Distinctive DNA Damage Response in Human Hematopoietic Stem Cells Reveals an Apoptosis-Independent Role for p53 in Self-Renewal', *Cell Stem Cell*, 7(2), pp. 186-197.

Ming, X., Matter, B.A., Song, M., Veliath, E., Shanley, R., Jones, R.A. and Tretyakova, N.Y. (2014) 'Mapping Structurally Defined Guanine Oxidation Products along DNA Duplexes: Influence of Local Sequence Context and Endogenous Cytosine Methylation', *J Am Chem Soc*.

Miyashita, H., Higa, K., Kato, N., Kawakita, T., Yoshida, S., Tsubota, K. and Shimmura, S. (2007) 'Hypoxia enhances the expansion of human limbal epithelial progenitor cells in vitro', *Invest Ophthalmol Vis Sci*, 48(8), pp. 3586-93.

Mizushima, N. (2010) 'The role of the Atg1/ULK1 complex in autophagy regulation', *Current Opinion in Cell Biology*, 22(2), pp. 132-9.

Mohrin, M., Bourke, E., Alexander, D., Warr, M.R., Barry-Holson, K., Le Beau, M.M., Morrison, C.G. and Passegue, E. (2010) 'Hematopoietic stem cell quiescence promotes error-prone DNA repair and mutagenesis', *Cell Stem Cell*, 7(2), pp. 174-85.

Mohsin, N., Budruddin, M., Pakkyara, A., Darweesh, A., Nayyer, M., Amitabh, J. and Daar, A.S. (2005) 'Complete regression of visceral Kaposi's sarcoma after conversion to sirolimus', *Exp Clin Transplant*, 3(2), pp. 366-9.

Moll, R., Franke, W.W., Schiller, D.L., Geiger, B. and Krepler, R. (1982) 'The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells', *Cell*, 31(1), pp. 11-24.

Molofsky, A.V., He, S.H., Bydon, M., Morrison, S.J. and Pardal, R. (2005) 'Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16(Ink4a) and P19(Arf) senescence pathways', *Genes & Development*, 19(12), pp. 1432-1437.

Molofsky, A.V., Pardal, R., Iwashita, T., Park, I.K., Clarke, M.F. and Morrison, S.J. (2003) 'Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation', *Nature*, 425(6961), pp. 962-7.

Molvaer, R.K., Andreasen, A., Heegaard, S., Thomsen, J.S., Hjortdal, J., Urbak, S.F. and Nielsen, K. (2013) 'Interactive 3D computer model of the human corneolimbal region: crypts, projections and stem cells', *Acta Ophthalmol*, 91(5), pp. 457-62.

Monnat, R.J., Jr. (2010) 'Human RECQ helicases: roles in DNA metabolism, mutagenesis and cancer biology', *Semin Cancer Biol*, 20(5), pp. 329-39.

Moore, J.E., McMullen, C.B., Mahon, G. and Adamis, A.P. (2002) 'The corneal epithelial stem cell', *DNA Cell Biol*, 21(5-6), pp. 443-51.

Morgan, T.E., Wong, A.M. and Finch, C.E. (2007) 'Anti-inflammatory mechanisms of dietary restriction in slowing aging processes', *Interdiscip Top Gerontol*, 35, pp. 83-97.

Morris, R.J., Fischer, S.M. and Slaga, T.J. (1985) 'Evidence that the centrally and peripherally located cells in the murine epidermal proliferative unit are two distinct cell populations', *J Invest Dermatol*, 84(4), pp. 277-81.

Morrison, S.J., Csete, M., Groves, A.K., Melega, W., Wold, B. and Anderson, D.J. (2000) 'Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells', *J Neurosci*, 20(19), pp. 7370-6.

Mort, R.L., Douvaras, P., Morley, S.D., Dora, N., Hill, R.E., Collinson, J.M. and West, J.D. (2012) 'Stem cells and corneal epithelial maintenance: insights from the mouse and other animal models', *Results Probl Cell Differ*, 55, pp. 357-94.

Moskalev, A.A., Shaposhnikov, M.V., Plyusnina, E.N., Zhavoronkov, A., Budovsky, A., Yanai, H. and Fraifeld, V.E. (2013) 'The role of DNA damage and repair in aging through the prism of Koch-like criteria', *Ageing research reviews*, 12(2), pp. 661-84.

Myer, V.E. and Steitz, J.A. (1995) 'Isolation and characterization of a novel, low abundance hnRNP protein: A0', *RNA*, 1(2), pp. 171-82.

Na, N., Chandel, N.S., Litvan, J. and Ridge, K.M. (2010) 'Mitochondrial reactive oxygen species are required for hypoxia-induced degradation of keratin intermediate filaments', *Faseb Journal*, 24(3), pp. 799-809.

Nadakavukaren, M.J., Fitch, K.L. and Richardson, A. (1987) 'Dietary restriction retards age-related decrease in cell population of rat corneal endothelium', *Proc Soc Exp Biol Med*, 184(1), pp. 98-101.

Nakamura, A.J., Rao, V.A., Pommier, Y. and Bonner, W.M. (2010) 'The complexity of phosphorylated H2AX foci formation and DNA repair assembly at DNA double-strand breaks', *Cell Cycle*, 9(2), pp. 389-397.

Nakamura, T., Inatomi, T., Sotozono, C., Ang, L.P.K., Koizumi, N., Yokoi, N. and Kinoshita, S. (2006) 'Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease', *Ophthalmology*, 113(10), pp. 1765-1772.

Nakamura, T., Inatomi, T., Sotozono, C., Koizumi, N. and Kinoshita, S. (2004) 'Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease', *Acta Ophthalmologica Scandinavica*, 82(4), pp. 468-471.

Nakamura, T., Koizumi, N., Tsuzuki, M., Inoki, K., Sano, Y., Sotozono, C. and Kinoshita, S. (2003) 'Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease', *Cornea*, 22(1), pp. 70-71.

Narita, M., Nunez, S., Heard, E., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J. and Lowe, S.W. (2003) 'Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence', *Cell*, 113(6), pp. 703-16.

Newsome, D.A., Takasugi, M., Kenyon, K.R., Stark, W.F. and Opelz, G. (1974) 'Human corneal cells in vitro: morphology and histocompatibility (HL-A) antigens of pure cell populations', *Invest Ophthalmol*, 13(1), pp. 23-32.

Nguyen, B.C., Lefort, K., Mandinova, A., Antonini, D., Devgan, V., Della Gatta, G., Koster, M.I., Zhang, Z., Wang, J., di Vignano, A.T., Kitajewski, J., Chiorino, G., Roop, D.R., Missero, C. and Dotto, G.P. (2006) 'Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation', *Genes & Development*, 20(8), pp. 1028-1042.

Niida, H., Matsumoto, T., Satoh, H., Shiwa, M., Tokutake, Y., Furuichi, Y. and Shinkai, Y. (1998) 'Severe growth defect in mouse cells lacking the telomerase RNA component', *Nat Genet*, 19(2), pp. 203-6.

Nishi, H., Nakada, T., Kyo, S., Inoue, M., Shay, J.W. and Isaka, K. (2004) 'Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT)', *Mol Cell Biol*, 24(13), pp. 6076-83.

Nisr, R.B. and Affourtit, C. (2014) 'Insulin acutely improves mitochondrial function of rat and human skeletal muscle by increasing coupling efficiency of oxidative phosphorylation', *Biochim Biophys Acta*, 1837(2), pp. 270-6.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J. and Yonezawa, K. (2003) 'The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif', *J Biol Chem*, 278(18), pp. 15461-4.

Notara, M., Shortt, A.J., O'Callaghan, A.R. and Daniels, J.T. (2013) 'The impact of age on the physical and cellular properties of the human limbal stem cell niche', *Age*, 35(2), pp. 289-300.

Nzekwe, E.U. and Maurice, D.M. (1994) 'The Effect of Age on the Penetration of Fluorescein into the Human Eye', *Journal of Ocular Pharmacology*, 10(3), pp. 521-523.

O'Callaghan, A.R., Daniels, J.T. and Mason, C. (2011) 'Effect of sub-atmospheric oxygen on the culture of rabbit limbal epithelial cells', *Curr Eye Res*, 36(8), pp. 691-8.

Oakley, E.J. and Van Zant, G. (2010) 'Age-Related Changes in Niche Cells Influence Hematopoietic Stem Cell Function', *Cell Stem Cell*, 6(2), pp. 93-94.

Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S.K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P., Wright, S.M., Mills, K.D., Bonni, A., Yankner, B.A., Scully, R., Prolla, T.A., Alt, F.W. and Sinclair, D.A. (2008) 'SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging', *Cell*, 135(5), pp. 907-18.

Ogura, M., Ogura, H., Ikehara, S., Dao, M.L. and Good, R.A. (1989) 'Decrease by Chronic Energy-Intake Restriction of Cellular Proliferation in the Intestinal Epithelium and Lymphoid Organs in Autoimmunity-Prone Mice', *Proceedings of the National Academy of Sciences of the United States of America*, 86(15), pp. 5918-5922.

Okazaki, T. and Okazaki, R. (1969) 'Mechanism of DNA chain growth. IV. Direction of synthesis of T4 short DNA chains as revealed by exonucleolytic degradation', *Proceedings of the National Academy of Sciences of the United States of America*, 64(4), pp. 1242-1248.

Olsen, T.W., Benegas, N.M., Joplin, A.C., Evangelista, T., Mindrup, E.A. and Holland, E.J. (1994) 'Rapamycin inhibits corneal allograft rejection and neovascularization', *Arch Ophthalmol*, 112(11), pp. 1471-5.

Opresko, P.L., Fan, J.H., Danzy, S., Wilson, D.M. and Bohr, V.A. (2005) 'Oxidative damage in telomeric DNA disrupts recognition by TRF1 and TRF2', *Nucleic acids research*, 33(4), pp. 1230-1239.

Ordonez, P. and Di Girolamo, N. (2012) 'Limbal epithelial stem cells: role of the niche microenvironment', *Stem Cells*, 30(2), pp. 100-7.

Orelio, C. and Dzierzak, E. (2007) 'Bcl-2 expression and apoptosis in the regulation of hematopoietic stem cells', *Leukemia & lymphoma*, 48(1), pp. 16-24.

Osei-Bempong, C., Henein, C. and Ahmad, S. (2009) 'Culture conditions for primary human limbal epithelial cells', *Regenerative Medicine*, 4(3), pp. 461-470.

Otte, A., Bucan, V., Reimers, K. and Hass, R. (2013) 'Mesenchymal stem cells maintain long-term in vitro stemness during explant culture', *Tissue Eng Part C Methods*, 19(12), pp. 937-48.

Owusu-Ansah, E. and Banerjee, U. (2009) 'Reactive oxygen species prime Drosophila haematopoietic progenitors for differentiation', *Nature*, 461(7263), pp. 537-41.

Palacios, J.A., Herranz, D., De Bonis, M.L., Velasco, S., Serrano, M. and Blasco, M.A. (2010) 'SIRT1 contributes to telomere maintenance and augments global homologous recombination', *J Cell Biol*, 191(7), pp. 1299-313.

Panov, A.V., Vavilin, V.A., Lyakhovich, V.V., Brooks, B.R. and Bonkovsky, H.L. (2010) 'Effect of bovine serum albumin on mitochondrial respiration in the brain and liver of mice and rats', *Bull Exp Biol Med*, 149(2), pp. 187-90.

Park, I.K., Morrison, S.J. and Clarke, M.F. (2004) 'Bmi1, stem cells, and senescence regulation', *J Clin Invest*, 113(2), pp. 175-9.

Park, J.I., Venteicher, A.S., Hong, J.Y., Choi, J., Jun, S., Shkreli, M., Chang, W., Meng, Z., Cheung, P., Ji, H., McLaughlin, M., Veenstra, T.D., Nusse, R., McCrea, P.D. and Artandi, S.E. (2009) 'Telomerase modulates Wnt signalling by association with target gene chromatin', *Nature*, 460(7251), pp. 66-72.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R. and Down, J.D. (2007) 'Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia', *Proceedings of the National Academy of Sciences of the United States of America*, 104(13), pp. 5431-5436.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S. and Campisi, J. (2003) 'Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts', *Nat Cell Biol*, 5(8), pp. 741-7.

Partridge, L., Green, A. and Fowler, K. (1987) 'Effects of Egg-Production and of Exposure to Males on Female Survival in Drosophila-Melanogaster', *Journal of Insect Physiology*, 33(10), pp. 745-749.

Paschalaki, K.E., Starke, R.D., Hu, Y., Mercado, N., Margariti, A., Gorgoulis, V.G., Randi, A.M. and Barnes, P.J. (2013) 'Dysfunction of endothelial progenitor cells from smokers and chronic obstructive pulmonary disease patients due to increased DNA damage and senescence', *Stem Cells*, 31(12), pp. 2813-26.

Passos, J.F., Nelson, G., Wang, C.F., Richter, T., Simillion, C., Proctor, C.J., Miwa, S., Olijslagers, S., Hallinan, J., Wipat, A., Saretzki, G., Rudolph, K.L., Kirkwood, T.B.L. and von Zglinicki, T. (2010) 'Feedback between p21 and reactive oxygen production is necessary for cell senescence', *Molecular Systems Biology*, 6, pp. -.

Patturajan, M., Nomoto, S., Sommer, M., Fomenkov, A., Hibi, K., Zangen, R., Poliak, N., Califano, J., Trink, B., Ratovitski, E. and Sidransky, D. (2002) 'DeltaNp63 induces beta-catenin nuclear accumulation and signaling', *Cancer Cell*, 1(4), pp. 369-79.

Pauklin, M., Fuchsluger, T.A., Westekemper, H., Steuhl, K.P. and Meller, D. (2010) 'Midterm results of cultivated autologous and allogeneic limbal epithelial transplantation in limbal stem cell deficiency', *Dev Ophthalmol*, 45, pp. 57-70.

Pearce, D.J., Anjos-Afonso, F., Ridler, C.M., Eddaoudi, A. and Bonnet, D. (2007) 'Age-dependent increase in side population distribution within hematopoiesis: Implications for our understanding of the mechanism of aging (vol 25, pg 828, 2007)', *Stem Cells*, 25(7), pp. 1852-1852.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F. and De Luca, M. (2001) 'p63 identifies keratinocyte stem cells', *Proc Natl Acad Sci U S A*, 98(6), pp. 3156-61.

Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P. and De Luca, M. (1999) 'Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface', *J Cell Biol*, 145(4), pp. 769-82.

Pellegrini, G., Traverso, C.E., Franzi, A.T., Zingirian, M., Cancedda, R. and De Luca, M. (1997) 'Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium', *Lancet*, 349(9057), pp. 990-3.

Petersen, S., Saretzki, G. and von Zglinicki, T. (1998) 'Preferential accumulation of single-stranded regions in telomeres of human fibroblasts', *Exp Cell Res*, 239(1), pp. 152-60.

Peterson, A.J., Kyba, M., Bornemann, D., Morgan, K., Brock, H.W. and Simon, J. (1997) 'A domain shared by the Polycomb group proteins Scm and ph mediates heterotypic and homotypic interactions', *Mol Cell Biol*, 17(11), pp. 6683-92.

Petrova, N.V., Velichko, A.K., Kantidze, O.L. and Razin, S.V. (2014) 'Heat shock-induced dissociation of TRF2 from telomeres does not initiate a telomere-dependent DNA damage response', *Cell Biol Int*.

Pfeiffer, N., Torri, V., Miglior, S., Zeyen, T., Adamsons, I. and Cunha-Vaz, J. (2007) 'Central corneal thickness in the European Glaucoma Prevention Study', *Ophthalmology*, 114(3), pp. 454-9.

Pfister, R.R. (1975) 'The normal surface of conjunctiva epithelium. A scanning electron microscopic study', *Invest Ophthalmol*, 14(4), pp. 267-79.

Pietersen, A.M., Evers, B., Prasad, A.A., Tanger, E., Cornelissen-Steijger, P., Jonkers, J. and van Lohuizen, M. (2008) 'Bmi1 regulates stem cells and proliferation and differentiation of committed cells in mammary epithelium', *Curr Biol*, 18(14), pp. 1094-9.

Pinto, A., De Filippi, R., Frigeri, F., Corazzelli, G. and Normanno, N. (2003) 'Aging and the hemopoietic system', *Crit Rev Oncol Hematol*, 48(Suppl), pp. S3-S12.

Pipe, D.M.R., L. J (1984) *Ocular Anatomy and Histology*. London: Association of Dispensing Opticians.

Pistollato, F., Rampazzo, E., Persano, L., Abbadi, S., Frasson, C., Denaro, L., D'Avella, D., Panchision, D.M., Della Puppa, A., Scienza, R. and Basso, G. (2010) 'Interaction of hypoxia-inducible factor-1alpha and Notch signaling regulates medulloblastoma precursor proliferation and fate', *Stem Cells*, 28(11), pp. 1918-29.

Polo, S.E. and Jackson, S.P. (2011) 'Dynamics of DNA damage response proteins at DNA breaks: a focus on protein modifications', *Genes & Development*, 25(5), pp. 409-433.

Potten, C.S. and Loeffler, M. (1987) 'Epidermal cell proliferation. I. Changes with time in the proportion of isolated, paired and clustered labelled cells in sheets of murine epidermis', *Virchows Arch B Cell Pathol Incl Mol Pathol*, 53(5), pp. 279-85.

Powers, R.W., 3rd, Kaeberlein, M., Caldwell, S.D., Kennedy, B.K. and Fields, S. (2006) 'Extension of chronological life span in yeast by decreased TOR pathway signaling', *Genes Dev*, 20(2), pp. 174-84.

Prasad, S.M., Czepiel, M., Cetinkaya, C., Smigielska, K., Weli, S.C., Lysdahl, H., Gabrielsen, A., Petersen, K., Ehlers, N., Fink, T., Minger, S.L. and Zachar, V. (2009) 'Continuous hypoxic culturing maintains activation of Notch and allows long-term propagation of human embryonic stem cells without spontaneous differentiation', *Cell Prolif*, 42(1), pp. 63-74.

Prowse, K.R. and Greider, C.W. (1995) 'Developmental and Tissue-Specific Regulation of Mouse Telomerase and Telomere Length', *Proceedings of the National Academy of Sciences of the United States of America*, 92(11), pp. 4818-4822.

Puangsricharern, V. and Tseng, S.C. (1995) 'Cytologic evidence of corneal diseases with limbal stem cell deficiency', *Ophthalmology*, 102(10), pp. 1476-85.

Pucci, F., Gardano, L. and Harrington, L. (2013) 'Short telomeres in ESCs lead to unstable differentiation', *Cell Stem Cell*, 12(4), pp. 479-86.

Puigserver, P. and Spiegelman, B.M. (2003) 'Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): Transcriptional coactivator and metabolic regulator', *Endocrine Reviews*, 24(1), pp. 78-90.

Qin, H., Yu, T.X., Qing, T.T., Liu, Y.X., Zhao, Y., Cai, J., Li, J., Song, Z.H., Qu, X.X., Zhou, P., Wu, J., Ding, M.X. and Deng, H.K. (2007) 'Regulation of apoptosis and differentiation by p53 in human embryonic stem cells', *Journal of Biological Chemistry*, 282(8), pp. 5842-5852.

Qiu, X.L., Brown, K., Hirschey, M.D., Verdin, E. and Chen, D. (2010) 'Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation', *Cell Metabolism*, 12(6), pp. 662-667.

Radan, L., Hughes, C.S., Teichroeb, J.H., Vieira Zamora, F.M., Jewer, M., Postovit, L.M. and Betts, D.H. (2014) 'Microenvironmental Regulation of Telomerase Isoforms in Human Embryonic Stem Cells', *Stem Cells Dev*.

Rama, P., Bonini, S., Lambiase, A., Golisano, O., Paterna, P., De Luca, M. and Pellegrini, G. (2001) 'Autologous fibrin-cultured limbal stem cells permanently restore the corneal

surface of patients with total limbal stem cell deficiency', *Transplantation*, 72(9), pp. 1478-85.

Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M. and Pellegrini, G. (2010) 'Limbal stem-cell therapy and long-term corneal regeneration', *N Engl J Med*, 363(2), pp. 147-55.

Ramsey, J.J., Colman, R.J., Binkley, N.C., Christensen, J.D., Gresl, T.A., Kemnitz, J.W. and Weindruch, R. (2000) 'Dietary restriction and aging in rhesus monkeys: the University of Wisconsin study', *Experimental gerontology*, 35(9-10), pp. 1131-1149.

Ratovitski, E.A., Patturajan, M., Hibi, K., Trink, B., Yamaguchi, K. and Sidransky, D. (2001) 'p53 associates with and targets Delta Np63 into a protein degradation pathway', *Proc Natl Acad Sci U S A*, 98(4), pp. 1817-22.

Reinhardt, H.C., Aslanian, A.S., Lees, J.A. and Yaffe, M.B. (2007) 'p53-deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage', *Cancer Cell*, 11(2), pp. 175-89.

Reinhardt, H.C., Hasskamp, P., Schmedding, I., Morandell, S., van Vugt, M.A., Wang, X., Linding, R., Ong, S.E., Weaver, D., Carr, S.A. and Yaffe, M.B. (2010) 'DNA damage activates a spatially distinct late cytoplasmic cell-cycle checkpoint network controlled by MK2-mediated RNA stabilization', *Mol Cell*, 40(1), pp. 34-49.

Reinstein, D.Z., Archer, T.J., Gobbe, M., Silverman, R.H. and Coleman, D.J. (2008) 'Epithelial thickness in the normal cornea: three-dimensional display with Artemis very high-frequency digital ultrasound', *J Refract Surg*, 24(6), pp. 571-81.

Reiser, K.M. (1994) 'Influence of age and long-term dietary restriction on enzymatically mediated crosslinks and nonenzymatic glycation of collagen in mice', *J Gerontol*, 49(2), pp. B71-9.

Remacle, J., Lambert, D., Raes, M., Pigeolet, E., Michiels, C. and Toussaint, O. (1992) 'Importance of Various Antioxidant Enzymes for Cell Stability - Confrontation between Theoretical and Experimental-Data', *Biochemical Journal*, 286, pp. 41-46.

Rera, M., Bahadorani, S., Cho, J., Koehler, C.L., Ulgherait, M., Hur, J.H., Ansari, W.S., Lo, T., Jones, D.L. and Walker, D.W. (2011) 'Modulation of Longevity and Tissue Homeostasis by the Drosophila PGC-1 Homolog', *Cell Metabolism*, 14(5), pp. 623-634.

Rheinwald, J.G. (1980) 'Serial cultivation of normal human epidermal keratinocytes', *Methods Cell Biol*, 21A, pp. 229-54.

Rheinwald, J.G. and Green, H. (1975a) 'Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma', *Cell*, 6(3), pp. 317-30.

Rheinwald, J.G. and Green, H. (1975b) 'Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells', *Cell*, 6(3), pp. 331-43.

Rheinwald, J.G. and Green, H. (1977) 'Epidermal growth factor and the multiplication of cultured human epidermal keratinocytes', *Nature*, 265(5593), pp. 421-4.

Rizell, M., Cahlin, C., Friman, S., Hafstrom, L., Lonn, L., Olausson, M. and Lindner, P. (2005) 'Impressive regression of primary liver cancer after treatment with sirolimus', *Acta Oncol*, 44(5), p. 496.

RNIB (2013) *Key information and statistics*. Available at: http://www.rnib.org.uk/aboutus/Research/statistics/Pages/statistics.aspx (Accessed: 26 March).

Robertson, D.M., Li, L., Fisher, S., Pearce, V.P., Shay, J.W., Wright, W.E., Cavanagh, H.D. and Jester, J.V. (2005) 'Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line', *Invest Ophthalmol Vis Sci*, 46(2), pp. 470-8.

Robertson, D.M., Zhu, M., Wu, Y.C. and Cavanagh, H.D. (2012) 'Hypoxia-induced downregulation of DeltaNp63alpha in the corneal epithelium', *Eye Contact Lens*, 38(4), pp. 214-21.

Robin, E.D., Murphy, B.J. and Theodore, J. (1984) 'Coordinate regulation of glycolysis by hypoxia in mammalian cells', *J Cell Physiol*, 118(3), pp. 287-90.

Roos, W.P., Christmann, M., Fraser, S.T. and Kaina, B. (2007) 'Mouse embryonic stem cells are hypersensitive to apoptosis triggered by the DNA damage O-6-methylguanine due to high E2F1 regulated mismatch repair', *Cell Death and Differentiation*, 14(8), pp. 1422-1432.

Rosenbluth, J.M., Mays, D.J., Pino, M.F., Tang, L.J. and Pietenpol, J.A. (2008) 'A gene signature-based approach identifies mTOR as a regulator of p73', *Molecular and cellular biology*, 28(19), pp. 5951-5964.

Rossi, D.J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J. and Weissman, I.L. (2007) 'Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age', *Nature*, 447(7145), pp. 725-U15.

Rossi, D.J., Jamieson, C.H. and Weissman, I.L. (2008) 'Stems cells and the pathways to aging and cancer', *Cell*, 132(4), pp. 681-96.

Rube, C.E., Fricke, A., Widmann, T.A., Furst, T., Madry, H., Pfreundschuh, M. and Rube, C. (2011) 'Accumulation of DNA damage in hematopoietic stem and progenitor cells during human aging', *Strahlentherapie Und Onkologie*, 187, pp. 130-130.

Rubinsztein, D.C., Marino, G. and Kroemer, G. (2011) 'Autophagy and Aging', *Cell*, 146(5), pp. 682-695.

Russell, R.C., Tian, Y., Yuan, H., Park, H.W., Chang, Y.Y., Kim, J., Kim, H., Neufeld, T.P., Dillin, A. and Guan, K.L. (2013) 'ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase', *Nat Cell Biol*, 15(7), pp. 741-50.

Ruzankina, Y. and Brown, E.J. (2007) 'Relationships between stem cell exhaustion, tumour suppression and ageing', *British journal of cancer*, 97(9), pp. 1189-1193.

Ryu, B.Y., Orwig, K.E., Oatley, J.M., Avarbock, M.R. and Brinster, R.L. (2006) 'Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal', *Stem Cells*, 24(6), pp. 1505-1511.

Saghizadeh, M., Soleymani, S., Harounian, A., Bhakta, B., Troyanovsky, S.M., Brunken, W.J., Pellegrini, G. and Ljubimov, A.V. (2011) 'Alterations of epithelial stem cell marker patterns in human diabetic corneas and effects of c-met gene therapy', *Mol Vis*, 17, pp. 2177-90.

Saha, B., Cypro, A., Martin, G.M. and Oshima, J. (2013) 'Rapamycin decreases DNA damage accumulation and enhances cell growth of WRN-deficient human fibroblasts', *Aging cell*.

Sahin, E., Colla, S., Liesa, M., Moslehi, J., Muller, F.L., Guo, M., Cooper, M., Kotton, D., Fabian, A.J., Walkey, C., Maser, R.S., Tonon, G., Foerster, F., Xiong, R., Wang, Y.A., Shukla, S.A., Jaskelioff, M., Martin, E.S., Heffernan, T.P., Protopopov, A., Ivanova, E., Mahoney, J.E., Kost-Alimova, M., Perry, S.R., Bronson, R., Liao, R., Mulligan, R., Shirihai, O.S., Chin, L. and DePinho, R.A. (2011) 'Telomere dysfunction induces metabolic and mitochondrial compromise', *Nature*, 470(7334), pp. 359-65.

Sahin, E. and DePinho, R.A. (2010) 'Linking functional decline of telomeres, mitochondria and stem cells during ageing', *Nature*, 464(7288), pp. 520-528.

Salnikow, K., Costa, M., Figg, W.D. and Blagosklonny, M.V. (2000) 'Hyperinducibility of hypoxia-responsive genes without p53/p21-dependent checkpoint in aggressive prostate cancer', *Cancer Res*, 60(20), pp. 5630-4.

Saltzman, D.J., Toth, A., Tsai, A.G., Intaglietta, M. and Johnson, P.C. (2003) 'Oxygen tension distribution in postcapillary venules in resting skeletal muscle', *Am J Physiol Heart Circ Physiol*, 285(5), pp. H1980-5.

Sangiorgi, E. and Capecchi, M.R. (2008) 'Bmi1 is expressed in vivo in intestinal stem cells', *Nature genetics*, 40(7), pp. 915-920.

Sangwan, V.S., Basu, S., Vemuganti, G.K., Sejpal, K., Subramaniam, S.V., Bandyopadhyay, S., Krishnaiah, S., Gaddipati, S., Tiwari, S. and Balasubramanian, D. (2011) 'Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study', *Br J Ophthalmol*, 95(11), pp. 1525-9.

Sangwan, V.S., Matalia, H.P., Vemuganti, G.K., Fatima, A., Ifthekar, G., Singh, S., Nutheti, R. and Rao, G.N. (2006) 'Clinical outcome of autologous cultivated limbal epithelium transplantation', *Indian J Ophthalmol*, 54(1), pp. 29-34.

Sangwan, V.S., Matalia, H.P., Vemuganti, G.K., Ifthekar, G., Fatima, A., Singh, S. and Rao, G.N. (2005) 'Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation', *Archives of Ophthalmology*, 123(3), pp. 334-340.

Sangwan, V.S., Vemuganti, G.K., Iftekhar, G., Bansal, A.K. and Rao, G.N. (2003) 'Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury - A case report of unique application', *Cornea*, 22(5), pp. 478-481.

Santos, J.H., Meyer, J.N., Skorvaga, M., Annab, L.A. and Van Houten, B. (2004) 'Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage', *Aging cell*, 3(6), pp. 399-411.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. and Sabatini, D.M. (2005) 'Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex', *Science*, 307(5712), pp. 1098-101.

Saretzki, G. (2011) 'Telomerase and Oxidative Stress in Embryonic Stem Cells', in Atwood, C. (ed.) *Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis*. InTech, pp. 337-362.

Sarin, K.Y., Cheung, P., Gilison, D., Lee, E., Tennen, R.I., Wang, E., Artandi, M.K., Oro, A.E. and Artandi, S.E. (2005) 'Conditional telomerase induction causes proliferation of hair follicle stem cells', *Nature*, 436(7053), pp. 1048-52.

Savage, S.A. and Alter, B.P. (2009) 'Dyskeratosis congenita', *Hematol Oncol Clin North Am*, 23(2), pp. 215-31.

Schein, O.D., Munoz, B., Tielsch, J.M., BandeenRoche, K. and West, S. (1997) 'Prevalence of dry eye among the elderly', *American Journal of Ophthalmology*, 124(6), pp. 723-728.

Schermer, A., Galvin, S. and Sun, T.T. (1986) 'Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells', *J Cell Biol*, 103(1), pp. 49-62.

Schieke, S.M., Phillips, D., McCoy, J.P., Jr., Aponte, A.M., Shen, R.F., Balaban, R.S. and Finkel, T. (2006) 'The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity', *J Biol Chem*, 281(37), pp. 27643-52.

Schlotzer-Schrehardt, U. (2013) 'Clonal analysis of limbal epithelial stem cell populations', *Methods Mol Biol*, 1014, pp. 55-64.

Schmidt, A., Bickle, M., Beck, T. and Hall, M.N. (1997) 'The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2', *Cell*, 88(4), pp. 531-542.

Schmuck, E.G., Mulligan, J.D. and Saupe, K.W. (2011) 'Caloric restriction attenuates the age-associated increase of adipose-derived stem cells but further reduces their proliferative capacity', *Age*, 33(2), pp. 107-118.

Schwab, I.R. (1999) 'Cultured corneal epithelia for ocular surface disease', *Trans Am Ophthalmol Soc*, 97, pp. 891-986.

Schwab, I.R., Reyes, M. and Isseroff, R.R. (2000) 'Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease', *Cornea*, 19(4), pp. 421-6.

Selman, C., Tullet, J.M.A., Wieser, D., Irvine, E., Lingard, S.J., Choudhury, A.I., Claret, M., Al-Qassab, H., Carmignac, D., Ramadani, F., Woods, A., Robinson, I.C.A., Schuster, E., Batterham, R.L., Kozma, S.C., Thomas, G., Carling, D., Okkenhaug, K., Thornton, J.M.,

Partridge, L., Gems, D. and Withers, D.J. (2009) 'Ribosomal Protein S6 Kinase 1 Signaling Regulates Mammalian Life Span', *Science*, 326(5949), pp. 140-144.

Sen, T., Sen, N., Huang, Y., Sinha, D., Luo, Z.G., Ratovitski, E.A. and Sidransky, D. (2011) 'Tumor protein p63/nuclear factor kappaB feedback loop in regulation of cell death', *J Biol Chem*, 286(50), pp. 43204-13.

Senoo, M., Matsumura, Y. and Habu, S. (2002) 'TAp63gamma (p51A) and dNp63alpha (p73L), two major isoforms of the p63 gene, exert opposite effects on the vascular endothelial growth factor (VEGF) gene expression', *Oncogene*, 21(16), pp. 2455-65.

Senoo, M., Pinto, F., Crum, C.P. and McKeon, F. (2007) 'p63 is essential for the proliferative potential of stem cells in stratified epithelia', *Cell*, 129(3), pp. 523-536.

Serber, Z., Lai, H.C., Yang, A., Ou, H.D., Sigal, M.S., Kelly, A.E., Darimont, B.D., Duijf, P.H., Van Bokhoven, H., McKeon, F. and Dotsch, V. (2002) 'A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism', *Mol Cell Biol*, 22(24), pp. 8601-11.

Shaharuddin, B., Harvey, I., Ahmad, S., Ali, S. and Meeson, A. (2014) 'Characterisation of Human Limbal Side Population Cells Isolated Using an Optimised Protocol From an Immortalised Epithelial Cell Line and Primary Limbal Cultures', *Stem Cell Reviews and Reports*, 10(2), pp. 240-250.

Shanmuganathan, V.A., Rotchford, A.P., Tullo, A.B., Joseph, A., Zambrano, I. and Dua, H.S. (2006) 'Epithelial proliferative potential of organ cultured corneoscleral rims; implications for allo-limbal transplantation and eye banking', *Br J Ophthalmol*, 90(1), pp. 55-8.

Sharma, G.G., Gupta, A., Wang, H., Scherthan, H., Dhar, S., Gandhi, V., Iliakis, G., Shay, J.W., Young, C.S.H. and Pandita, T.K. (2003) 'HTERT associates with human telomeres and enhances genomic stability and DNA repair', *Oncogene*, 22(1), pp. 131-146.

Sharpe, M., Perin, I. and Nicholls, P. (1996) 'Action of bovine serum albumin on cytochrome c oxidase activity and proton pumping: a role for fatty acids in enzyme function?', *FEBS Lett*, 391(1-2), pp. 134-8.

Sharpless, N.E. and Depinho, R.A. (2007) 'How stem cells age and why this makes us grow old', *Nature Reviews Molecular Cell Biology*, 8(9), pp. 703-713.

Sherwood, S.W., Rush, D., Ellsworth, J.L. and Schimke, R.T. (1988) 'Defining cellular senescence in IMR-90 cells: a flow cytometric analysis', *Proc Natl Acad Sci U S A*, 85(23), pp. 9086-90.

Shimazaki, J., Higa, K., Morito, F., Dogru, M., Kawakita, T., Satake, Y., Shimmura, S. and Tsubota, K. (2007) 'Factors influencing outcomes in cultivated limbal epithelial transplantation for chronic cicatricial ocular surface disorders', *Am J Ophthalmol*, 143(6), pp. 945-53.

Shimmura, S., Shimoyama, M., Hojo, M., Urayama, K. and Tsubota, K. (1998) 'Reoxygenation injury in a cultured corneal epithelial cell line protected by the uptake of lactoferrin', *Invest Ophthalmol Vis Sci*, 39(8), pp. 1346-51.

Shin, Y.J., Hyon, J.Y., Choi, W.S., Yi, K., Chung, E.S., Chung, T.Y. and Wee, W.R. (2013) 'Chemical injury-induced corneal opacity and neovascularization reduced by rapamycin via TGF-beta1/ERK pathways regulation', *Invest Ophthalmol Vis Sci*, 54(7), pp. 4452-8.

Shin, Y.J., Kim, M.K., Oh, J.Y., Wee, W.R., Lee, J.H., Ko, J.H., Lee, H.J., Lee, J.L., Min, B.M. and Sohn, Y.S. (2008) 'Short-term Efficacy of Topical Immunosuppressive Agents on the Survival of Cultivated Allo-Conjunctival Equivalents', *Korean J Ophthalmol*, 22(2), pp. 123-9.

Shortt, A.J., Secker, G.A., Munro, P.M., Khaw, P.T., Tuft, S.J. and Daniels, J.T. (2007) 'Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells', *Stem Cells*, 25(6), pp. 1402-9.

Shortt, A.J., Secker, G.A., Rajan, M.S., Meligonis, G., Dart, J.K., Tuft, S.J. and Daniels, J.T. (2008) 'Ex vivo expansion and transplantation of limbal epithelial stem cells', *Ophthalmology*, 115(11), pp. 1989-97.

Shortt, A.J., Tuft, S.J. and Daniels, J.T. (2011) 'Corneal stem cells in the eye clinic', *Br Med Bull*, 100, pp. 209-25.

Shyh-Chang, N., Daley, G.Q. and Cantley, L.C. (2013) 'Stem cell metabolism in tissue development and aging', *Development*, 140(12), pp. 2535-47.

Signer, R.A. and Morrison, S.J. (2013) 'Mechanisms that regulate stem cell aging and life span', *Cell Stem Cell*, 12(2), pp. 152-65.

Simon, M.C. and Keith, B. (2008) 'The role of oxygen availability in embryonic development and stem cell function', *Nat Rev Mol Cell Biol*, 9(4), pp. 285-96.

Simon, M.I. and Vanvunakis, H. (1964) 'The Dye-Sensitized Photooxidation of Purine and Pyrimidine Derivatives', *Arch Biochem Biophys*, 105, pp. 197-206.

Singhapol, C., Pal, D., Czapiewski, R., Porika, M., Nelson, G. and Saretzki, G.C. (2013) 'Mitochondrial telomerase protects cancer cells from nuclear DNA damage and apoptosis', *PLoS One*, 8(1), p. e52989.

Slettedal, J.K., Lyberg, T., Ramstad, H., Beraki, K. and Nicolaissen, B. (2007) 'Regeneration of the epithelium in organ-cultured donor corneas with extended post-mortem time', *Acta Ophthalmol Scand*, 85(4), pp. 371-6.

Smit, M.A. and Peeper, D.S. (2010) 'Epithelial-mesenchymal transition and senescence: two cancer-related processes are crossing paths', *Aging-Us*, 2(10), pp. 735-741.

Smith, C.G. and Greenfield, P.F. (1992) 'Mechanical agitation of hybridoma suspension cultures: Metabolic effects of serum, pluronic F68, and albumin supplements', *Biotechnol Bioeng*, 40(9), pp. 1045-55.

Smith, R.S. (2002) *Systematic evaluation of the mouse eye : anatomy, pathology, and biomethods*. Boca Raton: CRC Press.

Snippert, H.J., Schepers, A.G., van Es, J.H., Simons, B.D. and Clevers, H. (2014) 'Biased competition between Lgr5 intestinal stem cells driven by oncogenic mutation induces clonal expansion', *Embo Reports*, 15(1), pp. 62-69.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D. and Clevers, H. (2010) 'Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells', *Cell*, 143(1), pp. 134-44.

Song, J., Lee, Y.G., Houston, J., Petroll, W.M., Chakravarti, S., Cavanagh, H.D. and Jester, J.V. (2003) 'Neonatal corneal stromal development in the normal and lumican-deficient mouse', *Invest Ophthalmol Vis Sci*, 44(2), pp. 548-57.

Sorensen, F.H. (1975) 'Quantitative studies of the renal corpuscles iii: the influence of post mortem delay before taking renal tissue samples and of the duration of tissue fixation', *Acta Pathol Microbiol Scand A*, 83(2), pp. 251-8.

Sousa-Victor, P., Gutarra, S., Garcia-Prat, L., Rodriguez-Ubreva, J., Ortet, L., Ruiz-Bonilla, V., Jardi, M., Ballestar, E., Gonzalez, S., Serrano, A.L., Perdiguero, E. and Munoz-Canoves, P. (2014) 'Geriatric muscle stem cells switch reversible quiescence into senescence', *Nature*, 506(7488), pp. 316-321.

Sparmann, A. and van Lohuizen, M. (2006) 'Polycomb silencers control cell fate, development and cancer', *Nat Rev Cancer*, 6(11), pp. 846-56.

Srinivas, V., Zhang, L.P., Zhu, X.H. and Caro, J. (1999) 'Characterization of an oxygen/redox-dependent degradation domain of hypoxia-inducible factor alpha (HIF-alpha) proteins', *Biochemical and biophysical research communications*, 260(2), pp. 557-561.

Stacpoole, S.R., Webber, D.J., Bilican, B., Compston, A., Chandran, S. and Franklin, R.J. (2013) 'Neural precursor cells cultured at physiologically relevant oxygen tensions have a survival advantage following transplantation', *Stem Cells Transl Med*, 2(6), pp. 464-72.

Stoop, J.N., Harry, R.A., von Delwig, A., Isaacs, J.D., Robinson, J.H. and Hilkens, C.M. (2010) 'Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses', *Arthritis Rheum*, 62(12), pp. 3656-65.

Stucker, M., Struk, A., Altmeyer, P., Herde, M., Baumgartl, H. and Lubbers, D.W. (2002) 'The cutaneous uptake of atmospheric oxygen contributes significantly to the oxygen supply of human dermis and epidermis', *Journal of Physiology-London*, 538(3), pp. 985-994.

Su, X. and Flores, E.R. (2009) 'TAp63: The fountain of youth', *Aging (Albany NY)*, 1(10), pp. 866-9.

Su, X., Paris, M., Gi, Y.J., Tsai, K.Y., Cho, M.S., Lin, Y.L., Biernaskie, J.A., Sinha, S., Prives, C., Pevny, L.H., Miller, F.D. and Flores, E.R. (2009) 'TAp63 prevents premature aging by promoting adult stem cell maintenance', *Cell Stem Cell*, 5(1), pp. 64-75.

Sun, T.T. and Green, H. (1977) 'Cultured epithelial cells of cornea, conjunctiva and skin: absence of marked intrinsic divergence of their differentiated states', *Nature*, 269(5628), pp. 489-93.

Syntichaki, P., Troulinaki, K. and Tavernarakis, N. (2007) 'eIF4E function in somatic cells modulates ageing in Caenorhabditis elegans', *Nature*, 445(7130), pp. 922-6.

Takai, H., Smogorzewska, A. and de Lange, T. (2003) 'DNA damage foci at dysfunctional telomeres', *Current Biology*, 13(17), pp. 1549-1556.

Takatori, S.C., de la Jara, P.L., Holden, B., Ehrmann, K., Ho, A. and Radke, C.J. (2012) 'In Vivo Oxygen Uptake into the Human Cornea', *Investigative Ophthalmology & Visual Science*, 53(10), pp. 6331-6337.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M. and Suda, T. (2010) 'Regulation of the HIF-1 alpha Level Is Essential for Hematopoietic Stem Cells', *Cell Stem Cell*, 7(3), pp. 391-402.

Tarnok, A., Ulrich, H. and Bocsi, J. (2010) 'Phenotypes of stem cells from diverse origin', *Cytometry A*, 77(1), pp. 6-10.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M. and Garofalo, R.S. (2001) 'A mutant Drosophila insulin receptor homolog that extends life-span and impairs neuroendocrine function', *Science*, 292(5514), pp. 107-110.

Taylor, A., Lipman, R.D., Jahngen-Hodge, J., Palmer, V., Smith, D., Padhye, N., Dallal, G.E., Cyr, D.E., Laxman, E., Shepard, D. and et al. (1995) 'Dietary calorie restriction in the Emory mouse: effects on lifespan, eye lens cataract prevalence and progression, levels of ascorbate, glutathione, glucose, and glycohemoglobin, tail collagen breaktime, DNA and RNA oxidation, skin integrity, fecundity, and cancer', *Mech Ageing Dev*, 79(1), pp. 33-57.

Taylor, S.D., Sanders, M.E., Tullos, N.A., Stray, S.J., Norcross, E.W., McDaniel, L.S. and Marquart, M.E. (2013) 'The cholesterol-dependent cytolysin pneumolysin from Streptococcus pneumoniae binds to lipid raft microdomains in human corneal epithelial cells', *PLoS One*, 8(4), p. e61300.

Teng, S.P., Woodson, S.A. and Crothers, D.M. (1989) 'DNA-Sequence Specificity of Mitomycin Cross-Linking', *Biochemistry*, 28(9), pp. 3901-3907.

Thirunavukkarasu, M., Penumathsa, S.V., Koneru, S., Juhasz, B., Zhan, L., Otani, H., Bagchi, D., Das, D.K. and Maulik, N. (2007) 'Resveratrol alleviates cardiac dysfunction in streptozotocin-induced diabetes: Role of nitric oxide, thioredoxin, and heme oxygenase', *Free Radical Biology and Medicine*, 43(5), pp. 720-729.

Thoft, R.A. and Friend, J. (1983) 'The X, Y, Z hypothesis of corneal epithelial maintenance', *Invest Ophthalmol Vis Sci*, 24(10), pp. 1442-3.

Thomas, R. and Kim, M.H. (2008) 'HIF-1 alpha: A key survival factor for serum-deprived prostate cancer cells', *Prostate*, 68(13), pp. 1405-1415.

Todaro, G.J. and Green, H. (1963) 'Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines', *J Cell Biol*, 17, pp. 299-313.

Tondevold, E., Eriksen, J. and Jansen, E. (1979) 'Observations on long bone medullary pressures in relation to arterial PO2, PCO2 and pH in the anaesthetized dog', *Acta Orthop Scand*, 50(6 Pt 1), pp. 645-51.

Tormos, K.V., Anso, E., Hamanaka, R.B., Eisenbart, J., Joseph, J., Kalyanaraman, B. and Chandel, N.S. (2011) 'Mitochondrial complex III ROS regulate adipocyte differentiation', *Cell Metabolism*, 14(4), pp. 537-44.

Townsend, W.M. (1991) 'The limbal palisades of Vogt', *Trans Am Ophthalmol Soc*, 89, pp. 721-56.

Trainor, B.C., Workman, J.L., Jessen, R. and Nelson, R.J. (2007) 'Impaired nitric oxide synthase signaling dissociates social investigation and aggression', *Behav Neurosci*, 121(2), pp. 362-9.

Trettner, S., Findeisen, A., Taube, S., Horn, P.A., Sasaki, E. and Zur Nieden, N.I. (2014) 'Osteogenic induction from marmoset embryonic stem cells cultured in feeder-dependent and feeder-independent conditions', *Osteoporos Int*.

Trinkausrandall, V., Tong, M., Thomas, P. and Cornellbell, A. (1993) 'Confocal Imaging of the Alpha-6 and Beta-4 Integrin Subunits in the Human Cornea with Aging', *Investigative Ophthalmology & Visual Science*, 34(11), pp. 3103-3109.

Tsai, C.C., Chen, Y.J., Yew, T.L., Chen, L.L., Wang, J.Y., Chiu, C.H. and Hung, S.C. (2011) 'Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A-p21 by HIF-TWIST', *Blood*, 117(2), pp. 459-69.

Tsai, R.J., Li, L.M. and Chen, J.K. (2000) 'Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells', *N Engl J Med*, 343(2), pp. 86-93.

Tseng, S.C. (1989) 'Concept and application of limbal stem cells', *Eye (Lond)*, 3 (Pt 2), pp. 141-57.

Uccelli, A., Moretta, L. and Pistoia, V. (2008) 'Mesenchymal stem cells in health and disease', *Nat Rev Immunol*, 8(9), pp. 726-36.

Ugarte, F. and Forsberg, E.C. (2013) 'Haematopoietic stem cell niches: new insights inspire new questions', *EMBO J*, 32(19), pp. 2535-47.

Ula, K.R.K.a.J.V. (2012) 'Limbal Stem Cell Transplantation and Corneal Neovascularization', in Demirer, T. (ed.) *New Advances in Stem Cell Transplantation*. Intech, pp. 444-451.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J. and Thomas, G. (2004) 'Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity (vol 431, pg 200, 2004)', *Nature*, 431(7007), pp. 485-485.

Umemoto, T., Yamato, M., Nishida, K., Yang, J., Tano, Y. and Okano, T. (2006) 'Limbal epithelial side-population cells have stem cell-like properties, including quiescent state', *Stem Cells*, 24(1), pp. 86-94.

Van Obberghen, E., Baron, V., Delahaye, L., Emanuelli, B., Filippa, N., Giorgetti-Peraldi, S., Lebrun, P., Mothe-Satney, I., Peraldi, P., Rocchi, S., Sawka-Verhelle, D., Tartare-Deckert, S. and Giudicelli, J. (2001) 'Surfing the insulin signaling web', *Eur J Clin Invest*, 31(11), pp. 966-977.

Van Raamsdonk, J.M. and Hekimi, S. (2009) 'Deletion of the mitochondrial superoxide dismutase sod-2 extends lifespan in Caenorhabditis elegans', *PLoS Genet*, 5(2), p. e1000361.

Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S.R., Alderson, N.L., Baynes, J.W., Epstein, C.J., Huang, T.T., Nelson, J., Strong, R. and Richardson, A. (2003) 'Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging', *Physiol Genomics*, 16(1), pp. 29-37.

Vander Heiden, M.G., Chandel, N.S., Schumacker, P.T. and Thompson, C.B. (1999) 'Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange', *Mol Cell*, 3(2), pp. 159-67.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes', *Genome Biol*, 3(7), p. RESEARCH0034.

Varum, S., Rodrigues, A.S., Moura, M.B., Momcilovic, O., Easley, C.A., Ramalho-Santos, J., Van Houten, B. and Schatten, G. (2011) 'Energy Metabolism in Human Pluripotent Stem Cells and Their Differentiated Counterparts', *PLoS One*, 6(6).

Vaziri, H., Dessain, S.K., Eagon, E.N., Imai, S.I., Frye, R.A., Pandita, T.K., Guarente, L. and Weinberg, R.A. (2001) 'hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase', *Cell*, 107(2), pp. 149-159.

Vaziri, H., Dragowska, W., Allsopp, R.C., Thomas, T.E., Harley, C.B. and Lansdorp, P.M. (1994) 'Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age', *Proc Natl Acad Sci U S A*, 91(21), pp. 9857-60.

Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A.L., Orosz, L. and Muller, F. (2003) 'Genetics - Influence of TOR kinase on lifespan in C-elegans', *Nature*, 426(6967), pp. 620-620.

Vera, E., Bernardes de Jesus, B., Foronda, M., Flores, J.M. and Blasco, M.A. (2013) 'Telomerase reverse transcriptase synergizes with calorie restriction to increase health span and extend mouse longevity', *PLoS One*, 8(1), p. e53760.

Vijg, J. and Campisi, J. (2008) 'Puzzles, promises and a cure for ageing', *Nature*, 454(7208), pp. 1065-71.

von Zglinicki, T. (2000) 'Role of oxidative stress in telomere length regulation and replicative senescence', *Ann N Y Acad Sci*, 908, pp. 99-110.

von Zglinicki, T., Saretzki, G., Docke, W. and Lotze, C. (1995) 'Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence?', *Exp Cell Res*, 220(1), pp. 186-93.

Vorovich, E. and Ratovitski, E.A. (2009) 'Dual regulation of TERT activity through transcription and splicing by Delta NP63 alpha', *Aging-Us*, 1(1), pp. 58-67.

Wagner, W., Horn, P., Castoldi, M., Diehlmann, A., Bork, S., Saffrich, R., Benes, V., Blake, J., Pfister, S., Eckstein, V. and Ho, A.D. (2008) 'Replicative Senescence of Mesenchymal Stem Cells: A Continuous and Organized Process', *PLoS One*, 3(5).

Wang, C., Fu, T., Xia, C. and Li, Z. (2012a) 'Changes in mouse corneal epithelial innervation with age', *Invest Ophthalmol Vis Sci*, 53(8), pp. 5077-84.

Wang, C.F., Jurk, D., Maddick, M., Nelson, G., Martin-Ruiz, C. and von Zglinicki, T. (2009) 'DNA damage response and cellular senescence in tissues of aging mice', *Aging cell*, 8(3), pp. 311-323.

Wang, C.F., Maddick, M., Miwa, S., Jurk, D., Czapiewski, R., Saretzki, G., Langie, S.A.S., Godschalk, R.W.L., Cameron, K. and von Zglinicki, T. (2010) 'Adult-onset, short-term dietary restriction reduces cell senescence in mice', *Aging-Us*, 2(9), pp. 555-566.

Wang, J., Sun, Q., Morita, Y., Jiang, H., Gross, A., Lechel, A., Hildner, K., Guachalla, L.M., Gompf, A., Hartmann, D., Schambach, A., Wuestefeld, T., Dauch, D., Schrezenmeier, H., Hofmann, W.K., Nakauchi, H., Ju, Z., Kestler, H.A., Zender, L. and Rudolph, K.L. (2012b) 'A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage', *Cell*, 148(5), pp. 1001-14.

Wang, L.F., Harris, T.E., Roth, R.A. and Lawrence, J.C. (2007) 'PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding', *Journal of Biological Chemistry*, 282(27), pp. 20036-20044.

Wang, Y., Hu, Z.D., Liu, Z.B., Chen, R.R., Peng, H.Y., Guo, J., Chen, X.X. and Zhang, H.B. (2013) 'MTOR inhibition attenuates DNA damage and apoptosis through autophagy-mediated suppression of CREB1', *Autophagy*, 9(12), pp. 2069-2086.

Ward, I.M. and Chen, J.J. (2001) 'Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress.', *Journal of Biological Chemistry*, 276(51), pp. 47759-47762.

Watanabe, K., Nishida, K., Yamato, M., Umemoto, T., Sumide, T., Yamamoto, K., Maeda, N., Watanabe, H., Okano, T. and Tano, Y. (2004) 'Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2', *FEBS Lett*, 565(1-3), pp. 6-10.

Weindruch, R., Walford, R.L., Fligiel, S. and Guthrie, D. (1986) 'The Retardation of Aging in Mice by Dietary Restriction - Longevity, Cancer, Immunity and Lifetime Energy-Intake', *Journal of Nutrition*, 116(4), pp. 641-654.

West, J.B. (2007) *Pulmonary physiology and pathophysiology : an integrated, case-based approach*. 2nd edn. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Westfall, M.D., Mays, D.J., Sniezek, J.C. and Pietenpol, J.A. (2003) 'The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations', *Molecular and Cellular Biology*, 23(7), pp. 2264-2276.

Wetterau, L.A., Francis, M.J., Ma, L. and Cohen, P. (2003) 'Insulin-like growth factor I stimulates telomerase activity in prostate cancer cells', *J Clin Endocrinol Metab*, 88(7), pp. 3354-9.

Whikehart, D.R., Parikh, C.H., Vaughn, A.V., Mishler, K. and Edelhauser, H.F. (2005) 'Evidence suggesting the existence of stem cells for the human corneal endothelium', *Mol Vis*, 11, pp. 816-24.

White, M.F. (2003) 'Insulin signaling in health and disease', *Science*, 302(5651), pp. 1710-1.

Wick, U. and Gebhart, E. (2005) 'Studies on the action of mitomycin C and bleomycin on telomere lengths of human lymphocyte chromosomes', *Int J Mol Med*, 16(3), pp. 463-469.

Wiggins, S.A. (1995) 'Modern Nutrition in Health and Disease - Shils,Me, Olson,Ja, Shike,M', *Patient Educ Couns*, 25(3), pp. 335-336.

Wilcoxon, F. (1946) 'Individual comparisons of grouped data by ranking methods', *J Econ Entomol*, 39, p. 269.

Wilkinson, J.E., Burmeister, L., Brooks, S.V., Chan, C.C., Friedline, S., Harrison, D.E., Hejtmancik, J.F., Nadon, N., Strong, R., Wood, L.K., Woodward, M.A. and Miller, R.A. (2012) 'Rapamycin slows aging in mice', *Aging cell*, 11(4), pp. 675-82.

Williams, K.A., Brereton, H.M., Aggarwal, R., Sykes, P.J., Turner, D.R., Russ, G.R. and Coster, D.J. (1995) 'Use of DNA polymorphisms and the polymerase chain reaction to examine the survival of a human limbal stem cell allograft', *Am J Ophthalmol*, 120(3), pp. 342-50.

Wilson, R.S. and Roper-Hall, M.J. (1982) 'Effect of age on the endothelial cell count in the normal eye', *Br J Ophthalmol*, 66(8), pp. 513-5.

Wilson, S.E. and Hong, J.W. (2000) 'Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis', *Cornea*, 19(4), pp. 417-20.

Wu, A.G., Ying, Z. and Gomez-Pinilla, F. (2007) 'Omega-3 fatty acids supplementation restores mechanisms that maintain brain Homeostasis in traumatic brain injury', *Journal of Neurotrauma*, 24(10), pp. 1587-1595.

Wu, J., Bergholz, J., Lu, J.I., Sonenshein, G.E. and Xiao, Z.X.J. (2010) 'TAp63 Is a Transcriptional Target of NF-kappa B', *Journal of Cellular Biochemistry*, 109(4), pp. 702-710.

Wu, K.J. and Yang, M.H. (2011) 'Epithelial-mesenchymal transition and cancer stemness: the Twist1-Bmi1 connection', *Biosci Rep*, 31(6), pp. 449-55.

Wu, Z., Liu, S.Q. and Huang, D. (2013) 'Dietary restriction depends on nutrient composition to extend chronological lifespan in budding yeast Saccharomyces cerevisiae', *PLoS One*, 8(5), p. e64448.

Wu, Z.D., Puigserver, P., Andersson, U., Zhang, C.Y., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C. and Spiegelman, B.M. (1999) 'Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1', *Cell*, 98(1), pp. 115-124.

Yamada, O., Ozaki, K., Akiyama, M. and Kawauchi, K. (2012) 'JAK-STAT and JAK-PI3K-mTORC1 pathways regulate telomerase transcriptionally and posttranslationally in ATL cells', *Mol Cancer Ther*, 11(5), pp. 1112-21.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M.D., Dotsch, V., Andrews, N.C., Caput, D. and McKeon, F. (1998) 'p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities', *Mol Cell*, 2(3), pp. 305-16.

Yang, M.H., Hsu, D.S., Wang, H.W., Wang, H.J., Lan, H.Y., Yang, W.H., Huang, C.H., Kao, S.Y., Tzeng, C.H., Tai, S.K., Chang, S.Y., Lee, O.K. and Wu, K.J. (2010) 'Bmi1 is essential in Twist1-induced epithelial-mesenchymal transition', *Nat Cell Biol*, 12(10), pp. 982-92.

Yang, M.H., Wu, M.Z., Chiou, S.H., Chen, P.M., Chang, S.Y., Liu, C.J., Teng, S.C. and Wu, K.J. (2008) 'Direct regulation of TWIST by HIF-1alpha promotes metastasis', *Nat Cell Biol*, 10(3), pp. 295-305.

Yang, S., Pilgaard, L., Chase, L.G., Boucher, S., Vemuri, M.C., Fink, T. and Zachar, V. (2012) 'Defined xenogeneic-free and hypoxic environment provides superior conditions for long-term expansion of human adipose-derived stem cells', *Tissue Eng Part C Methods*, 18(8), pp. 593-602.

Yanoff, M. and Joesph, W.S. (2009) *Ocular pathology*. Edinburgh: Mosby/Elsevier.

Yatabe, N., Kyo, S., Maida, Y., Nishi, H., Nakamura, M., Kanaya, T., Tanaka, M., Isaka, K., Ogawa, S. and Inoue, M. (2004) 'HIF-1-mediated activation of telomerase in cervical cancer cells', *Oncogene*, 23(20), pp. 3708-3715.

Yuan, J.S., Reed, A., Chen, F. and Stewart, C.N., Jr. (2006) 'Statistical analysis of real-time PCR data', *BMC Bioinformatics*, 7, p. 85.

Zachar, V., Prasad, S.M., Weli, S.C., Gabrielsen, A., Petersen, K., Petersen, M.B. and Fink, T. (2010) 'The effect of human embryonic stem cells (hESCs) long-term normoxic and hypoxic cultures on the maintenance of pluripotency', *In Vitro Cellular & Developmental Biology-Animal*, 46(3-4), pp. 276-283.

Zahidov, S.T., Hohlov, A.N., Malolina, E.A., Kulibin, A.Y. and Marshak, T.L. (2010) 'Ageing of the spermatogenesis system', *Biology Bulletin*, 37(1), pp. 10-17.

Zainal, T.A., Oberley, T.D., Allison, D.B., Szweda, L.I. and Weindruch, R. (2000) 'Caloric restriction of rhesus monkeys lowers oxidative damage in skeletal muscle', *Faseb Journal*, 14(12), pp. 1825-1836.

Zeng, Z.H., Sarbassov, D.D., Samudio, I.J., Yee, K.W.L., Munsell, M.F., Jackson, C.E., Giles, F.J., Sabatini, D.M., Andreeff, M. and Konopleva, M. (2007) 'Rapamycin derivatives reduce mTORC2 signaling and inhibit AKT activation in AML', *Blood*, 109(8), pp. 3509-3512.

Zhai, W. and Comai, L. (2000) 'Repression of RNA polymerase I transcription by the tumor suppressor p53', *Mol Cell Biol*, 20(16), pp. 5930-8.

Zhan, Q., Lord, K.A., Alamo, I., Jr., Hollander, M.C., Carrier, F., Ron, D., Kohn, K.W., Hoffman, B., Liebermann, D.A. and Fornace, A.J., Jr. (1994) 'The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth', *Mol Cell Biol*, 14(4), pp. 2361-71.

Zhang, E.P., Schrunder, S. and Hoffmann, F. (1996) 'Orthotopic corneal transplantation in the mouse--a new surgical technique with minimal endothelial cell loss', *Graefes Arch Clin Exp Ophthalmol*, 234(11), pp. 714-9.

Zhao, B., Allinson, S.L., Ma, A., Bentley, A.J., Martin, F.L. and Fullwood, N.J. (2008) 'Targeted cornea limbal stem/progenitor cell transfection in an organ culture model', *Invest Ophthalmol Vis Sci*, 49(8), pp. 3395-401.

Zhong, L., D'Urso, A., Toiber, D., Sebastian, C., Henry, R.E., Vadysirisack, D.D., Guimaraes, A., Marinelli, B., Wikstrom, J.D., Nir, T., Clish, C.B., Vaitheesvaran, B., Iliopoulos, O., Kurland, I., Dor, Y., Weissleder, R., Shirihai, O.S., Ellisen, L.W., Espinosa, J.M. and Mostoslavsky, R. (2010) 'The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha', *Cell*, 140(2), pp. 280-93.

Zhou, C., Gehrig, P.A., Whang, Y.E. and Boggess, J.F. (2003) 'Rapamycin inhibits telomerase activity by decreasing the hTERT mRNA level in endometrial cancer cells', *Mol Cancer Ther*, 2(8), pp. 789-95.

Zhou, J., Li, D. and Wang, F. (2012) 'Assessing the function of mTOR in human embryonic stem cells', *Methods Mol Biol*, 821, pp. 361-72.

Zhu, W.Q., Chen, J.H., Cong, X.F., Hu, S.S. and Chen, X. (2006) 'Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells', *Stem Cells*, 24(2), pp. 416-425.

Zindy, F., Quelle, D.E., Roussel, M.F. and Sherr, C.J. (1997) 'Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging', *Oncogene*, 15(2), pp. 203-11.

Zoukhri, D. (2006) 'Effect of inflammation on lacrimal gland function', *Exp Eye Res*, 82(5), pp. 885-98.