

The role of mechanical and chemical cues on corneal cells function

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Declaration of work

I declare that this thesis is based on my own work and has not been submitted in any form for another degree at any university or any other tertiary education. Information derived from published and unpublished work of others have been acknowledged in the text and in the list of references given in the bibliography.

Abstract

This thesis describes the role of mechanical and chemical cues to preserve corneal cells function. Specifically, the use of decellularised cornea scaffolds is explored to look at the effects of mechanical stiffness in determining the fate of corneal limbal stem cells when they repopulate the acellular scaffolds. In addition, retinoic acid is introduced into the serum-free culture medium either in 2D or 3D environment to evaluate its potential in modulating the phenotype of both normal and diseased human corneal keratocytes.

The greatest challenge in developing a bio-engineered cornea has always been to recapitulate the corneal stromal biology within the artificial scaffold. The idea to use decellurised corneas is brought forward as it holds both the structure and composition of native cornea, which is what the bio-engineered cornea is lacking. Here, different methods of decellularisation are used to identify the best method which maintains the structural integrity of scaffolds as well as allowing recellularisation of limbal stem cells to model corneal epithelial growth. Further manipulation to the substrate stiffness via collagenase treatment is adapted to explore how stiffness affects limbal stem cell behaviour.

The quest to establish and promote the use of serum-free culture medium for corneal cell culture work is continued in this thesis. It is hypothesized that retinoic acid supplementation in culture medium is able to modulate specific chemical cues especially the matrix metalloproteases (MMPs) to retain quiescent properties of corneal keratocytes previously exposed to serum *in vitro*. Similar effect is also postulated onto fibroblast taken from keratoconic corneas, in which modulation of the MMPs enables the keratoconic fibroblast to de-differentiate into a more normal, quiescent keratocyte.

This work therefore demonstrates the influence of mechanical and chemical conditionings on corneal cells function and may provide a greater understanding in the study of corneal biology and tissue engineering application.

Acknowledgement

Alhamdulillah, all praises to Allah for His blessings and kindness that I finally reach the day where I am able to write this note of appreciation as the finishing touch on my thesis. This PhD has been a long and winding journey – but never once Allah makes me feel left alone nor does He leave my prayers unheard.

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Abbreviations

| | |
|--------------|----------------------------------------------|
| α SMA | Alpha smooth muscle actin |
| ABCG2 | ATP-binding cassette sub-family G member 2 |
| ACL | Anterior cruciate ligament |
| ALDH | Aldehyde dehydrogenase |
| AM | Acetoxymethyl |
| ANOVA | Analysis of variance |
| ASCs | Adipose-derived stem cells |
| bFGF | Basic fibroblast growth factor |
| bLESCs | Bovine limbal epithelial stem cells |
| cDNA | Complementary DNA |
| CHST6 | Carbohydrate sulfotransferase 6 |
| CK | Cytokeratin |
| CSSCs | Corneal stromal stem cells |
| CT | Threshold cycle |
| DMEM | Dulbecco's modified Minimal essential medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| ECM | Extracellular matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| ESCs | Embryonic stem cells |
| FAK | Focal adhesion kinase |
| FBS | Fetal bovine serum |

| | |
|--------------------|---------------------------------------------------------------|
| FGF | Fibroblast growth factor |
| HCSF | Human corneal stromal fibroblast |
| HGF | Hepatocyte growth factor |
| hLESCs | Human limbal epithelial stem cells |
| H&E | Hematoxylin & Eosin |
| IgG | Immunoglobulin G |
| IL-1 | Interleukin-1 |
| ITS | Insulin, transferrin and selenium |
| ITW | In-Tissue Western |
| KF | Keratoconic fibroblast |
| KGF | Keratinocyte growth factor |
| KSPGs | Keratan sulfate proteoglycans |
| LESCs | Limbal epithelial stem cells |
| LOX | Lysyl oxidase |
| LOXL | Lysyl oxidase-like |
| MCD | Macular corneal dystrophy |
| MEM | Minimal essential medium |
| MMP | Matrix metalloprotease |
| MSCs | Mesenchymal stem cells |
| MTT | (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| NH ₄ OH | Ammonium hydroxide |
| PBS | Phosphate buffer solution |
| PDGF | Platelet-derived growth factor |
| PV | Pallisades of Vogt |
| PVDF | Polyvinylidene difluoride |

| | |
|---------------|------------------------------------------------------------|
| qPCR | Quantitative polymerase chain reaction |
| RA | Retinoic acid |
| RARA | Retinoic acid receptor alpha |
| RIPA | Radioimmunoprecipitation assay |
| RNA | Ribonucleic acid |
| RON | Reactive nitric species |
| ROS | Reactive oxygen species |
| SCs | Stem cells |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SF | Serum-free culture medium |
| SLRPs | Small leucine-rich proteoglycans |
| SOD | Superoxide dismutase |
| STCF | Short-term chemical frozen |
| TACs | Transient amplifying cells |
| TGF- β | Transforming growth factor beta |
| TIMP | Tissue inhibitor of metalloprotease |
| TNF- α | Tumor necrosis factor alpha |
| UV | Ultraviolet |
| W_d | Dry weight |
| W_f | Final weight |
| W_i | Initial weight |

Chapter 1. Introduction

1.1 The cornea

Within the ocular system, cornea occupies the anterior one-sixth of the human eye, thereby constitutes the first boundary between the external environment and the eye (figure 1.1). Cornea plays an important role in controlling the entry of external light into the eye and focussing it (together with the lens) on to retina for optimal vision, as well as providing rigidity to the eyeball. Besides being the most sensitive tissue in the body, cornea is also a transparent, avascular and immune-privileged tissue (Jager *et al.*, 1995). Since it has no blood vessels to supply for nutrients and protects it against infection, cornea receives nourishment from tears and the aqueous humor (fluid that fills up the compartment behind the cornea at the anterior part of the eye).

The corneal surface has two main areas; the limbus at its edge, which is the niche area for corneal stem cells, and the central area which consists of a more differentiated type of corneal cells. Histologically, the cornea is made up of five discrete layers; the epithelium, Bowman's membrane, the stroma, Descemet's membrane and endothelium (figure 1.2). Every layer owns specific properties in order to provide optimal functionality for normal vision as well as protection for the delicate intraocular contents (Lu *et al.*, 2001).

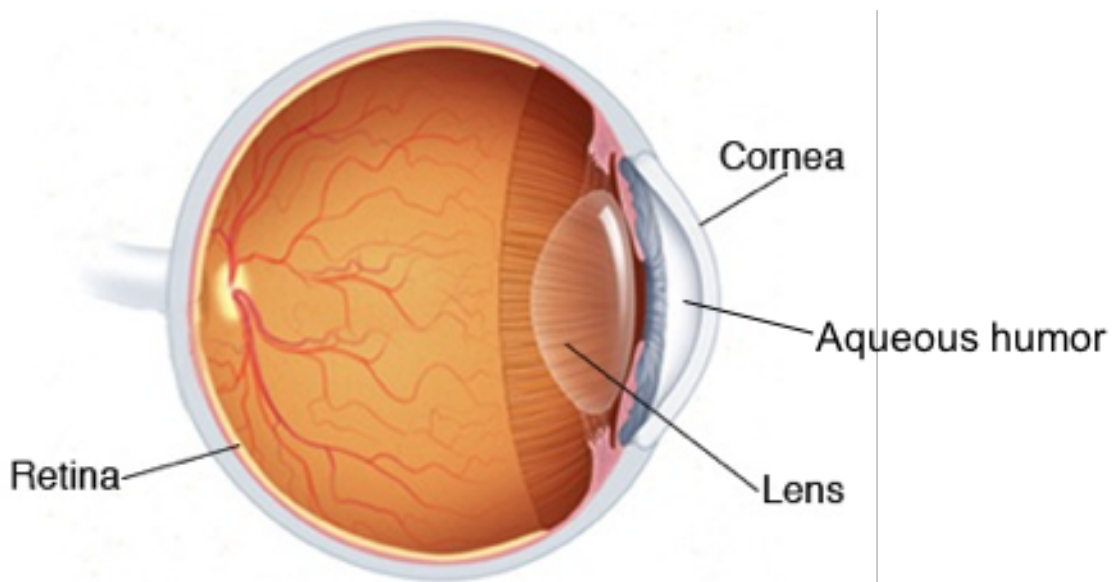


Figure 1.1- Diagram of human eye showing the location of cornea, lens and retina. The cornea refracts incoming light onto the lens, which will refocus the light onto the retina for translation into vision. Image taken with permission from <http://www.corneaconsultantstx.com>.

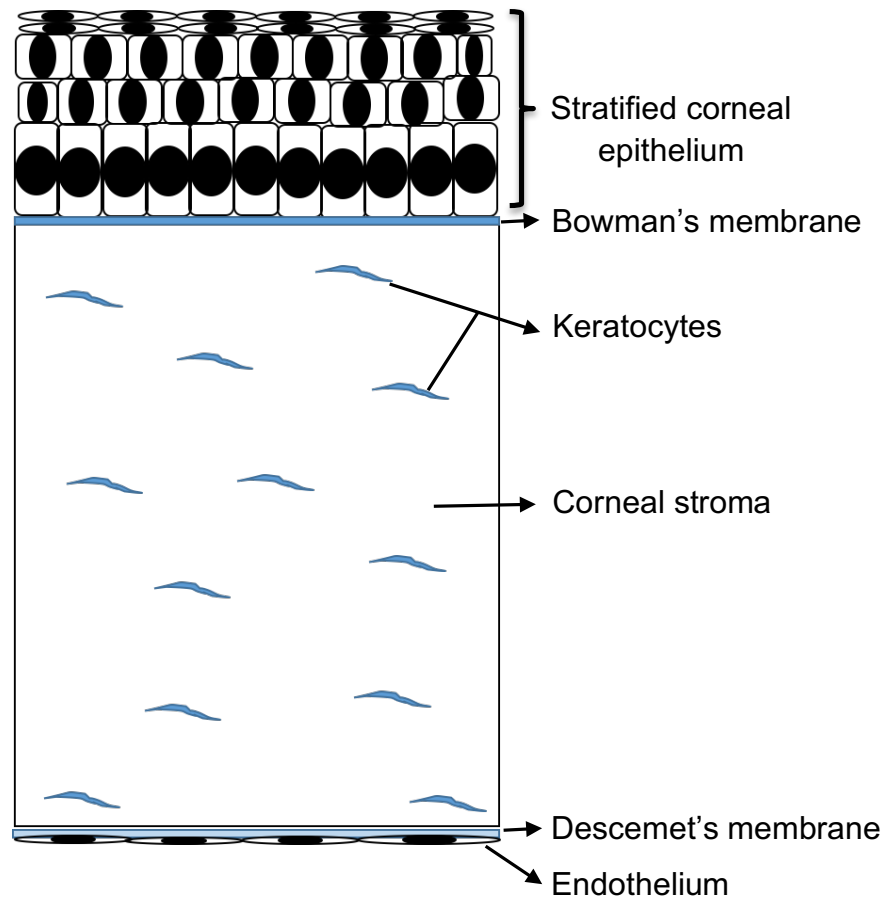


Figure 1.2 - Schematic diagram of histology of depicting the location of membranous layers sandwiched between the epithelium and stroma (Bowman's membrane) and between the stroma and endothelium (Descemet's membrane).

1.1.1 *Structure of the cornea*

Epithelium

The epithelium covers the outermost surface of the cornea, providing a smooth optical surface for optimum light refraction as well as an important protective barrier against infection or trauma to the eye (Dohlman, 1971). It is crucial to maintain the smoothness of epithelial surface as optimum light refraction is essential for optimal vision. The presence of tear film overlying the corneal epithelium helps to smooth out any micro-irregularities of the anterior surface in order to further maintain light refraction (DeMonte and Kim, 2011). The tear film, mainly composed of water, has three layers: mucous, aqueous and lipid – each layer contributed differently to the maintenance of tear stability (Linsen and Missoten, 1990). On top of that, tear film also offers an extra protection for the cornea against infection or trauma as well as

the natural way for the body to provide both immunological and growth factors that are required for corneal homeostasis (Cameron, 2005).

The epithelium is ectodermal-origin surface epithelium consists of stratified layers (between five to seven) of non-keratinized squamous cells with different morphologies and distinct characteristics (figure 1.2). The most superficial epithelial cells are flat polygonal cells, which are also the most differentiated type of epithelial cells. These superficial, flat epithelial cells possess extensive apical microvilli to further increase surface area for optimal contact between the tear film and the cell membrane to ensure a smooth and clear optical system (Pfister, 1973). At the same time, the cells form tight junctional complexes between their neighbours to prevent both beneficial tear film as well as harmful toxins and microbes from penetrating the deeper layers (Suzuki *et al.*, 2003). Further protection to internal layers are provided by tight lateral junctions (called desmosomes) between suprabasal or wing cells of the middle layers (made of two to three cell layer thick) (Rubinstein and Stanley, 1987; Kapprell *et al.*, 1988). The innermost basal layer mainly consists of columnar epithelium (Wiley *et al.*, 1991). Within this layer lies the stem cells, transient amplifying cells and the 'less differentiated' basal cells which all have the capability to undergo mitosis. Similar to the wing cells, the basal cells also have lateral gap junctions for tissue protection. In addition to that, they also possess hemidesmosomal system which anchor them to the underlying basement membrane to avoid detachment from the underlying corneal layers (Gipson *et al.*, 1988)

The average life span of corneal epithelial cells are between 5 to 7 days (Hanna *et al.*, 1961), after which they routinely undergo involution, apoptosis and desquamation (DelMonte and Kim, 2011). In this context, the stem cells, located exclusively at the limbal cornea, give rise to rapid proliferating cells known as transient amplifying cells (TACs). TACs are capable of proliferating and producing more differentiated epithelial cells which migrate towards the corneal centre (Davanger and Evensen, 1971; Thoft and Friend, 1983). In this manner, the stratified epithelia undergo rapid self-renewal, which requires constant recreation through differentiation as the cells move upward towards the surface (Wolosin *et al.*, 2000). A delicate balance between cell proliferation and differentiation is crucial for functional maintenance of the epithelium (Schlötzer-Schrehardt and Kruse, 2005).

Bowman's membrane

The Bowman's membrane is a smooth adhesion layer located in between the corneal epithelium and stroma. It is a thin, acellular layer made up of randomly arranged collagen fibers that adjoins the epithelial basement membrane anteriorly and becomes continuous with the anterior stroma posteriorly (Gordon *et al.*, 1994). Despite being a thin layer, Bowman's membrane is known to be significantly stiffer than the extracellular matrix beyond it (Last *et al.*, 2012), and is confined to the central part of the cornea only (Wiley *et al.*, 1991). It is speculated that the compliance state of corneal limbus is brought by the fact that it is devoid of Bowman's membrane, which may have implications on mechanotransduction as the elasticity gradient drive epithelial cell migration and differentiation across the ocular surface (Foster *et al.*, 2014). The precise function of Bowman's membrane is unknown, but it may act as protective barrier to the subepithelial nerve plexus as well as biological barrier to viral infection. This might be true as Bowman's membrane is an acellular layer and that viruses require cells for propagation and spread (Wilson and Hong, 2000). Its tough membrane may also provide further resistant to trauma, but lacks the ability to regenerate once destroyed and can form a scar (Lagali *et al.*, 2009; DelMonte and Kim, 2011).

Stroma

The shape of the cornea is maintained by the stroma, which makes 90% of the corneal volume. It is composed of cellular (keratocytes) and extracellular (collagen fibrils, ground matrix) components. Keratocytes are mesenchymal-derived, flattened cells which sparsely populate the corneal stroma, but mostly reside in the anterior stroma (Fini, 1999; He and Bazan, 2008). They are quiescent in nature and exhibit slow turn over (Müller *et al.*, 1995). Their primary function is to synthesize collagen molecules and maintain the extracellular matrix (ECM) component of the stroma which comprises the structural backbone of the cornea (West-Mays and Dwivedi, 2006). They also synthesize enzymes (matrix metalloproteases; MMPs) responsible for stromal remodelling and degradation especially during wound healing. More importantly, these keratocytes contain corneal 'crystallins', which maintain corneal transparency by reducing backscatter of light from the keratocytes (Jester *et al.*, 1999).

The extracellular matrix (ECM) is made up of fibrous proteins (mainly collagen type I and V) and glycosaminoglycans (keratan sulfate, chondroitin sulfate and dermatan sulfate). Both collagen types make heterodimeric complexes with each other to form collagen fibrils with narrow diameter, surrounded by glycosaminoglycans which regulate tissue hydration and maintain structural properties (Fini and Stramer, 2005). The precise organization of collagen fibrils within the stroma contributes to corneal transparency (Daxer *et al.*, 1998). Collagen fibrils (with uniform diameters of about 25 nm) are packed in parallel bundles, and these are packed in parallel arranged layers or lamellae (figure 1.3). Each lamella is arranged parallel to each other as well as to the central corneal surface (Smith, 1969; Boote *et al.*, 2003). However, the lamellae direction changes to run circumferentially as they approach the limbus to reduce forward light scattering and adds to the mechanical strength of the cornea (Meek and Boote, 2004).

The ECM serves not only as scaffold for structural integrity, but also influences cell activities including their differentiation, migration, proliferation, shape and metabolic functions (Gospodarowicz *et al.*, 1978; Blake *et al.*, 1997; Raghunathan *et al.*, 2013). The matrix undergoes constant controlled remodelling, degradation and re-synthesis during both development and wound healing (Gandhi and Jain, 2015). It is very important to ensure that both synthesis and degradation of ECM is well-regulated during growth and repair to maintain proper tissue architecture (Jain and Azar, 1994).

Descemet's membrane

The Descemet's membrane is constantly being produced by the endothelium to form an extraordinary thick basement membrane between the stroma and the endothelium (Gipson and Joyce, 2000). It is composed of collagen type IV and VIII (Gandhi and Jain, 2015). Unlike the Bowman's layer, this membrane is weakly attached to the stroma and therefore, can easily be dissected (Anwar and Teichmann, 2002; Price and Price, 2006). A recent study has claimed the existence of a pre-Descemet thin layer of corneal collagen between the corneal stroma and Descemet's membrane termed as 'Dua's layer' (Dua *et al.*, 2013). However, some researchers believe that the proposed Dua's layer is not a new corneal layer but is merely part of the stroma (McKee *et al.*, 2014)

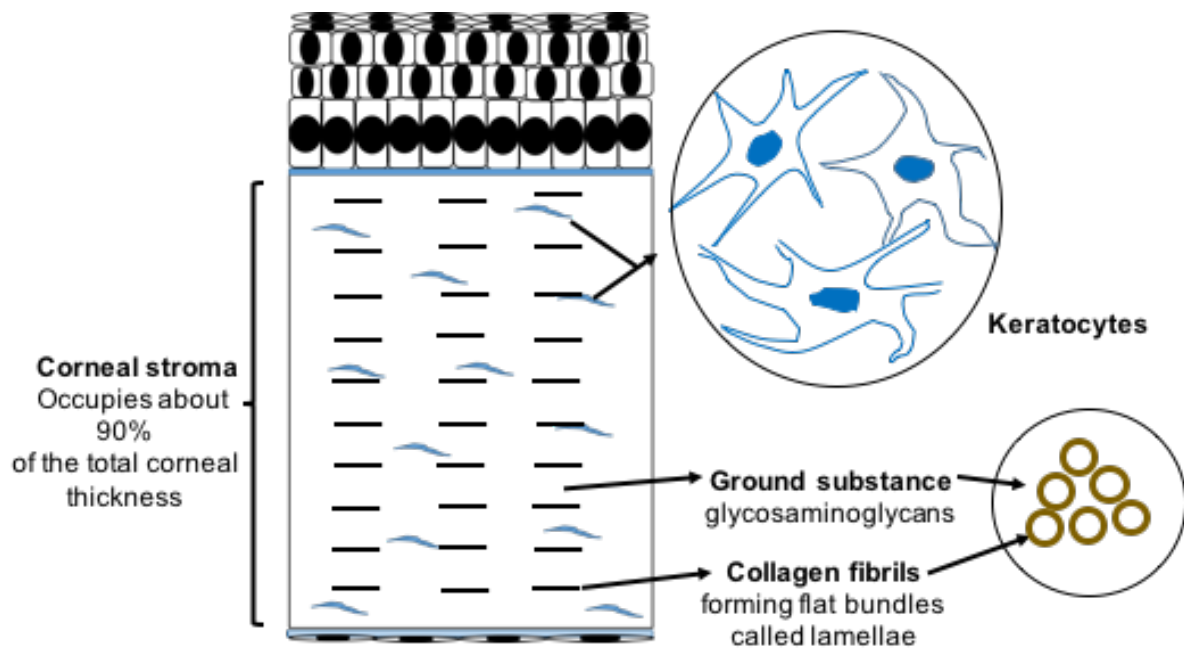


Figure 1.3 - Schematic diagram showing the composition of corneal stroma. Keratocytes are found interspersing in between collagen lamellae. Each lamella is arranged parallel to each other as well as to the central corneal surface.

Endothelium

The endothelium is made up of a single layer of polarised squamous epithelium. This layer maintains the hydration of the cornea by regulating the water content in the stroma through its ion transport system known as endothelium pump, which is important for corneal transparency. It also maintains the exchange of nutrients between the cornea and the rest of the eye (Waring *et al.*, 1982). The endothelial cells are not mitotically active in vivo, so nature has provided a significant reserve since birth. Any reduction in endothelial cell number due to age, trauma, inflammation or other diseases will lead to spreading of the remaining endothelial cells to maintain the overall function. However, this stretching will physically change the morphology of the endothelial cells, which in turn affect their ability to deturgesce the cornea (Stiemke *et al.*, 1991).

1.1.2 The limbus

Self-renewing tissues require a substantial amount of stem cells or progenitors to replenish themselves following normal wear and tear and injury throughout life. Therefore, nature has provided stem cells with a niche which is able to accommodate

all the factors required for continuous self-renewal and generation of epithelial cells. The limbus is an established corneal stem cell niche following evidences that identified the presence of stem cells there (Dua *et al.*, 2005; Shortt *et al.*, 2007), hence the name limbal epithelial stem cells (LESCs). Anatomically, the limbus circumscribes the cornea and act as a transition zone that separates the cornea from the conjunctiva (figure 1.4). It possesses striking architectural features that makes it suitable for stem cells to populate. Notably, it has undulated surface that resembles a wave-like structure with invaginations and projections into the cornea known as Pallisades of Vogt (PV) (Goldberg and Bron, 1982; Kobayashi and Sugiyama, 2005). This undulating region increases the limbal surface area and at the same time, provides LESCs with an environment that protects them from shearing forces (Gipson, 1989). The palisades are highly pigmented with melanocytes (Davanger and Evensen, 1971) which helps to protect against ultraviolet radiation and oxidative DNA damage (Bessou-Touya *et al.*, 1998; Echevarria and Di Girolamo, 2011). Therefore, PV is postulated to serve as a generative organ for corneal epithelial cells due to its many special features. Furthermore, the limbus also possesses a well-defined structure, known as limbal crypts, which are located deeper in the substantia propria of the limbus (Dua *et al.*, 2005). These limbal crypts predominantly occur in the superior and inferior cornea where the area is covered by eyelids, and this may be part of evolution towards protective environment for LESCs in human (Shortt *et al.*, 2007). Unlike the avascular central cornea, limbal niche is highly vascularized and highly innervated (Lawrenson and Ruskell, 1991) that potentially provides all the nutrients and growth factors for LESCs.

The idea that the limbus is the location of cells with regenerative capacity originates from a study that demonstrated the migration route of pigment from the limbal zone towards an epithelial defect following wounding of rabbit corneas (Mann, 1944). This has been supported by other research findings which have also observed centripetal migration of pigment and labelled stem cell progeny from limbus to central cornea (Davanger and Evensen, 1971; Cotsarelis *et al.*, 1989; Nagasaki and Zhao, 2003; Iannaccone *et al.*, 2012). Significant damage to the limbus may create a permanent corneal epithelial defect as evidenced in lab animals, and this supports the concept of limbal-based stem cells which is clearly important for vision (Srinivasan and Eakins, 1979; Chen and Tseng, 1991; Sitalakshmi *et al.*, 2008).

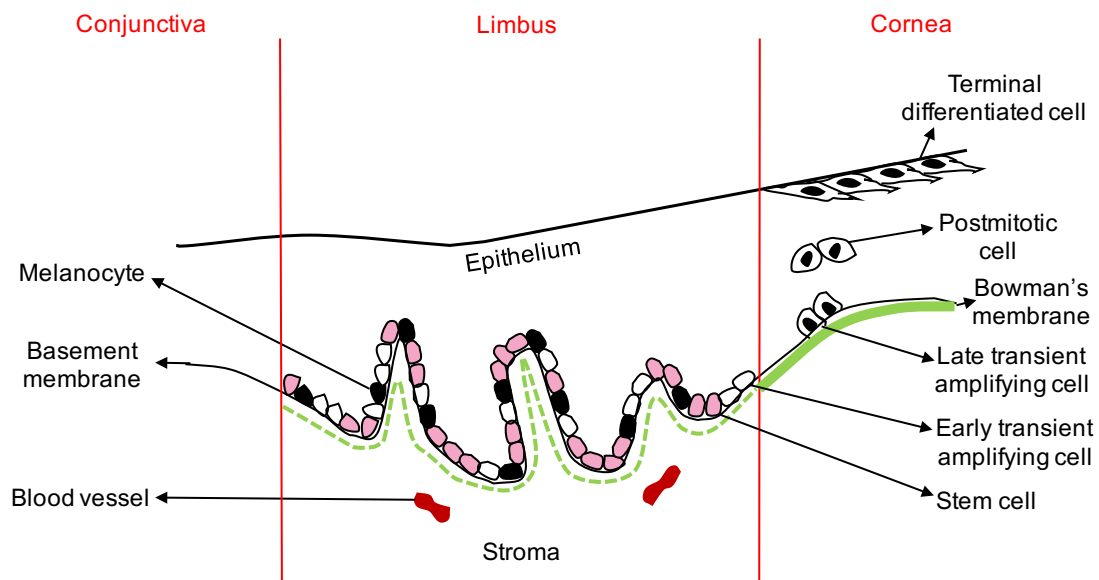


Figure 1.4 - Schematic diagram showing anatomical features of limbus.

Limbal epithelial stem cells (LESCs)

The limbal epithelial stem cells (LESCs) are undifferentiated epithelial cells that are proposed to undergo asymmetric division giving rise to corneal epithelial cells (Castro-Muñozledo and Gómez-Flores, 2011). These adult stem cells appear small in size (Romano *et al.*, 2003), with high nuclear to cytoplasmic ratio (Barrandon and Green, 1987) and lack the expression of differentiation markers (cytokeratin 3 and 12) (Schermer *et al.*, 1986; Kurpakus *et al.*, 1990). The LESCs are slow cycling in nature (Cotsarelis *et al.*, 1989) but they become highly proliferative when corneal injury occurs (Cotsarelis *et al.*, 1989; Lehrer *et al.*, 1998; Lavker and Sun, 2003). Limbal stem cells also exhibit higher proliferative potential when compared to peripheral and central cornea (Ebato *et al.*, 1987; Ebato *et al.*, 1988; Lavker *et al.*, 1991).

The identification of limbal stem cells rely primarily on the presence or absence of general phenotype marker since there is no specific phenotypic marker for corneal limbal stem cells (Figueira *et al.*, 2007). At present, the limbal stem cell profile is defined as p63, Δ Np63 (especially Δ Np63 α), ABCG2, integrin- α 9, CK14, CK15 – positive and nestin, E-cadherin, connexin 43, involucrin, CK3, and CK12 – negative (Chen *et al.*, 2004b; Di Iorio *et al.*, 2005; Kawasaki *et al.*, 2006; Figueira *et al.*, 2007). Researchers are still attempting to discover robust markers for LESCs since that can

help to improve LESC's isolation and expansion strategies towards a more effective cell-based therapy.

The LESC's play a major role in the maintenance and regulation of the corneal surface (Cotsarelis *et al.*, 1989; Lehrer *et al.*, 1998). Any deficiency to the LESC's due to limbal damage or diseases may disrupt the normal vision causing corneal blindness via vascularisation or stromal opacity of the cornea (Samson *et al.*, 2002; Chan and Holland, 2013; Strungaru *et al.*, 2014). The *ex vivo* expansion of LESC's using feeder layer (Lindberg *et al.*, 1993; Pellegrini *et al.*, 1997) or biological/synthetic substrates (Grueterich *et al.*, 2003; Wright *et al.*, 2013) is currently the most commonly used method to treat this deficiency, collectively known as the cell-based therapy (Rama *et al.*, 2010; Fasolo *et al.*, 2016). Amongst the different substrates that have been used to expand the LESC's *in vitro* includes amniotic membrane (Chen *et al.*, 2010), contact lenses (Brown *et al.*, 2014), fibrin (Rama *et al.*, 2001) and collagen gels (Mi *et al.*, 2010b). Since corneal epithelium is an organised, stratified epithelium in nature, some LESC's expansion also include air-lifting, a procedure which adopts air-liquid interface treatment to promote epithelial stratification and differentiation. The idea of air-lifting was first introduced to cultured skin keratinocytes in order to produce transferable epithelial sheets (Prunieras *et al.*, 1983; Williams *et al.*, 1988). Some years later, similar technique was implemented to cornea research by Minami *et al.* (1993) in an attempt to reconstruct the cornea using 3D collagen gels. Although air-lifting induces proliferation and stratification of LESC's *in vitro* (Proulx *et al.*, 2010), it also causes an undesired differentiation of LESC's which show significant reduction in p63-positive stem cells following air-lifting (Ebrahimi *et al.*, 2010; Tseng *et al.*, 2010; Massie *et al.*, 2014). It is crucial for LESC's cell-based therapy to have sufficient number of stem cells prior to graft transplantation in order to ensure successful clinical outcomes (De Luca *et al.*, 2006). On the other hand, air-lifting should cautiously be used in researches which study factors affecting limbal stem cell differentiation *in vitro* as it may influence cell differentiation thereby creating false-positive results.

Once the LESC's have successfully been expanded, they will be transferred onto the corneal surface with or without the substrate (Basu *et al.*, 2012; Marchini *et al.*, 2012). Although its long-term success rate is still debatable, the use of LESC's cell-

based therapy have shown evidences of clinical success with probably some refinement of this procedure to further improve the clinical outcome.

Corneal stromal stem cells (CSSCs)

Small population of stem cells are believed to be present in many mesenchymal tissues, including corneal stroma (Du *et al.*, 2005; Yoshida *et al.*, 2006b). These cells share several key properties of mesenchymal stem cells (MSCs) including clonal growth, differentiation multi-potency and asymmetric cell division to produce both stem cell and differentiated progeny. In corneal stroma, these stem cells are termed as corneal stromal stem cells (CSSC) and are believed to be the descendants of the neural crest lineage as evidenced by the expression of PAX6, Six2, Six3 and Notch1 (Yoshida *et al.*, 2006b). The PAX6 expression is absent in corneal keratocytes, hence it can be used to differentiate between the two (Funderburgh *et al.*, 2005). On the other hand, CD34, a cell-surface marker of keratocyte phenotype can also be used to distinguish the CSSCs as this marker is not present on stem cells nor fibroblasts (Barbaro *et al.*, 2006).

The identification of CSSCs from human stroma was achieved by isolating 'side population' cells from the early passages that efflux the DNA-binding dye Hoechst 33342. These cells could then be expanded through exhaustive cumulative population doubling, and portray the ability to differentiate into multiple cell lineage depending on the biochemical cues. For example, the CSSCs express keratocyte-specific markers when cultured in serum-free medium supplemented with insulin and ascorbic acid. On the other hand, these cells were able to differentiate into cartilage and neural-cell lineage when exposed to chondrogenic and neurogenic induction culture medium respectively (Du *et al.*, 2005). However, CSSCs are believed to have a default lineage for keratocytes as they can steadily differentiate into keratocytes whenever they receive biochemical (Du *et al.*, 2005) or mechanical (Du *et al.*, 2007; Du *et al.*, 2009; Wu *et al.*, 2012a) cues from the culture environment.

Previous studies has demonstrated that LESC's expansion *in vitro* with the presence of limbal stroma showed a better outcome as compared to fibroblast feeder layer (Mariappan *et al.*, 2010; Ainscough *et al.*, 2011). Since the CSSCs are found in the anterior stroma of the limbus subjacent to the epithelial basement membrane near the Pallisades of Vogt (Pinnamaneni and Funderburgh, 2012), it might be possible

that such proximity of CSSCs and LESCes provide symbiotic support to each other for the maintenance of their stem cell phenotype. Espana *et al.* (2003) found that epithelial cell sheets removed from the limbus were maintained as undifferentiated phenotype only when they were recombined with limbal stroma, and not corneal stroma. Moreover, this epithelial-stromal interaction is also mediated by soluble factors, such as growth factors, cytokines and proteins. For example, limbal keratocytes help to inhibit limbal epithelial cell differentiation by producing soluble hepatocyte growth factor (HGF; Li and Tseng, 1995) as well as SPARC protein which contributes to LESCes adhesion (Shimmura *et al.*, 2006). This biological sustenance given by CSSCs to LESCes, together with their ability to replicate over high number of population doublings without invoking immune response even across different species (Du *et al.*, 2009) makes them the best candidate for generation of bioengineered corneal stromal constructs. Such constructs hold promises either for therapeutic application in the treatment of corneal scarring or in the field of biomolecular research to further understand the fundamentals of corneal biology.

1.1.3 Corneal diseases: causes and current treatments

According to the World Health Organization (WHO), corneal diseases are the second most important cause of blindness worldwide (Whitcher *et al.*, 2001). Diseases affecting the cornea may lead to clouding, corneal distortion, scarring and eventually a complete loss of vision. The causes can be due to: hereditary (corneal dystrophies), infection (microbial keratitis), injuries (penetrating or burn), or secondary to other eye diseases (e.g aniridia, Steven-Johnson syndrome, ocular cicatricial pemphigoid).

Corneal dystrophies are rare, progressive genetic diseases involving one or more layers of the cornea characterised by building up of materials that cloud the cornea thereby affecting its normal clarity. In most cases both eyes are usually involved, and it happens in otherwise healthy people. Examples of corneal dystrophies are epithelial basement membrane dystrophy (map-dot-fingerprint dystrophy), keratoconus and Fuchs's dystrophy (Mazzota *et al.*, 2014). In epithelial basement membrane dystrophy, the presence of by intraepithelial microcysts together with an abnormal protrusion of basement membrane into the epithelium is highly characteristic of the disease (Cogan *et al.*, 1974; Ehlers and Moller, 1988).

Phototherapeutic keratectomy (PTK) has been the accepted modality to treat this condition with good long-term outcome (Lee *et al.*, 2017). On the other hand, keratoconus is a bilateral, non-inflammatory, asymmetric condition characterized by focal thinning and protrusion resulting in conical shape of the cornea (Kennedy *et al.*, 1986; Zadnik *et al.*, 1996). This abnormal cone-shape cornea results in irregular astigmatism, high myopia, and in severe conditions, significant visual impairment (Krachmer *et al.*, 1984). At the moment, the treatment available for these patients ranges from glasses (for mild cases) to rigid contact lenses in moderate cases. However, these treatment plans are designed to address mainly the refractive issues found in keratoconus and not to curb the progress of the disease (McAnena *et al.*, 2016). Patients with severe keratoconus usually require corneal cross-linking as a standard method to help stabilize the progression of the disease (Wollensak, 2006; John, 2012), or ultimately corneal transplantation. Therefore, researchers are still looking into potential elements or treatment which may help to reduce or stop the disease progression which would greatly benefit these patients especially the young ones.

Microbial keratitis; be it bacterial, fungal or viral in origin, is a previously rare but serious complication especially amongst people who wear contact lens. However, the widespread use of soft contact lens for cosmetic, visual and therapeutic purposes has led to an increasing incidence of chronic epithelial defect, corneal ulceration and opacity among contact lens wearers (Remeijer *et al.*, 1990; Clinch *et al.*, 1992; Mela *et al.*, 2003) resulting from aberrant wound healing and stromal regeneration (Roszkowska *et al.*, 2013). On the other hand, chemical burn may cause serious visual disability secondary to major exposure to acid or alkali substances from cleaning agents, personal care products and industrial chemicals (Blackburn *et al.*, 2012; Sher *et al.*, 2012). Topical eyedrops using antimicrobial agents is currently the standard treatment method in most cases of microbial keratitis (Allan and Dart, 1995; Wong *et al.*, 2012). It is also being used in chemically-injured cornea to prevent secondary infection, along with topical steroids to control inflammation, which facilitates epithelial healing (Singh *et al.*, 2013).

Unfortunately, severe infections or injuries to the cornea, as well as certain eye diseases (e.g aniridia, Steven-Johnson syndrome, ocular cicatricial pemphigoid) can also damage the limbus, causing partial or full LESC's deficiency. This deficiency,

collectively known as limbal epithelial stem cell deficiency (LSCD), results in conjunctivalisation, neovascularization, chronic inflammation, ulceration and stromal opacity of the cornea which all lead to painful vision loss (Samson *et al.*, 2002; Chan and Holland, 2013; Strungaru *et al.*, 2014). Attempts to treat LSCD using 'cell-based therapy' through cultivation of LESC on amniotic membrane, followed by autologous transplantation onto corneal surface with defect have been performed (Kenyon and Tseng, 1989; Koizumi *et al.*, 2001; Nakamura *et al.*, 2006). Some studies showed successful regeneration of cornea surface epithelium with no epithelial defect for up to 19 months post-transplantation (Tsai *et al.*, 2000; Nakamura *et al.*, 2004; Nakamura *et al.*, 2006). However, there were concerns regarding the usage of amniotic membrane as carrier for limbal stem cell expansion, such as the risk of disease transmission from donor to recipient (Addis *et al.*, 2001), discrepancies in material quality between donors (Hopkinson *et al.*, 2006) and variation in amniotic membrane transparency (Connon *et al.*, 2009). In the meantime, other studies consider the use of tissue-engineered biocompatible substrates as a matrix to support tissue repair with eventual integration (Rama *et al.*, 2001; Di Girolamo *et al.*, 2009; Rama *et al.*, 2010). Since it is crucial for LESC cell-based therapy to have sufficient number of stem cells prior to graft transplantation in order to ensure successful clinical outcomes (De Luca *et al.*, 2006), studies have now focused on understanding a range of cues which may influence LESC to maintain their stemness, including mechanical, biochemical and metabolic cues from the surrounding niche (Watt and Hogan, 2000; Watt and Huck, 2013).

1.2 Chemical cues: the standard dogma in how corneal cells work

In vitro studies have been accepted as means to provide opportunities to study physiological, biological and pharmacological activities at both cellular and tissue levels (Van der Valk *et al.*, 2010). However, these isolated cells need an artificial environment that drives cellular growth, proliferation and differentiation. Needless to say, cell culture medium plays an important role to achieve good experimental reproducibility. Any modification made by the chemical cues present in the medium may significantly give impact to the cellular maintenance environment; hence alter the long-term research results. These chemical signals come from all the elements present in the culture medium, including basal medium formulation, presence of serum, growth factors and additional media supplements. They may influence the physiology of cells through optimization of cell function and morphology (Abdellatef *et al.*, 2014), enhancement of cell growth (Joshi *et al.*, 2014), cell migration (Ridley *et al.*, 2003), cell alignment (Miller *et al.*, 2002), and cell differentiation (Hagmann *et al.*, 2013).

1.2.1 Basal medium formulation

Basal medium is formulated to provide essential nutrients to support cell or tissue maintenance and growth *in vitro*. Ringer's solution (Ringer and Buxton, 1887) was identified as the simplest medium developed using optimal concentrations of different salts to preserve frog heart muscles. Since then, the evolution of nutrient medium have progressed from isotonic salt solution to additions of buffering components, more complex salt species, carbohydrates, amino acids and vitamins to ensure that the cells are bathed in artificial environments that are closely mimicking the native cellular environment (Barnes *et al.*, 1986). In 1955, Harry Eagle published his basal medium formulation known as Eagle's basal medium, which is an 'isotonic, pH-balanced mixture of salt, amino acids, sugars, vitamins and other necessary nutrients' (Harry, 1955). Following that, the Eagle's basal medium formulation has been used as the basis for most nutrient media prepared until today, with some modifications made to further improve its support to cell growth and viability.

Minimal Essential Medium (MEM) was the first modified version of Eagle's basal medium, in which it contains more amino acid compositions to increase the nutritional complex (Eagle, 1959). Further studies have found that cell proliferation could be

greatly enhanced by further increasing the amino acids and vitamins levels from the original Eagle's formulation. This has led to the formulation of Dulbecco's modified Minimal Essential Medium (DMEM) which contains 4x nutrients than that of MEM, and the results showed that it was able to augment viral plaque formation in cells that were cultured using this medium (Dulbecco and Freeman, 1959).

The evolution of basal medium formulation reached its peak when the idea to combine two independent media formulation was proposed and became widely accepted (Barnes and Sato, 1980). By combining DMEM with F-12, one of Ham's Nutrient Mixtures (Ham, 1965) most frequently used nutrient formulation to support low density growth and maintenance of different cultured cell types, the DMEM/F12 combo has become the most widely used basal synthetic medium (Jayme *et al.*, 1997). This combination produces a better DMEM medium with components of Ham's F-12 such as biotin, putrescine, lipoic acid, glycine, proline, copper and zinc that are not included in DMEM alone (Barnes and Sato, 1980; Chaudhry *et al.*, 2008). The DMEM/F12 medium has been known to work best with or without the presence of serum (Jayme *et al.*, 1997; Van der Valk *et al.*, 2010), making it one of the most versatile basal medium ever been formulated for *in vitro* culture work.

The DMEM/F12 basal medium has been extensively used for corneal cell culture works involving physiological (Park *et al.*, 2006), biochemical (Etheredge *et al.*, 2009), and pharmacological (Yoeruek *et al.*, 2007) studies. The medium is also suitable for use while constructing bioengineered corneas (Mi *et al.*, 2010b; Yoeruek *et al.*, 2012). Although the use of DMEM/F12 has been shown to support cell growth for all three types of corneal cells (epithelial cells, keratocytes and endothelial cells), newer formulations of culture medium have emerged which selectively show a better culture support for specific type of corneal cells. For example, the Epilife® medium comes as an alternative medium to DMEM/F12 to support corneal epithelial cell cultures (Tocce *et al.*, 2010). A low calcium serum-free medium, known as CnT-20 medium, has been shown to have supported the human limbal epithelial stem cell better than serum-supplemented DMEM/F12 medium (Lu *et al.*, 2011; Zakaria *et al.*, 2014).

1.2.2 Presence of serum

Almost all mammalian cell types require the presence of serum in the culture media to support their growth. Serum is an important supplement to basal medium either by

supplying the necessary nutrients which may not be present in the basal medium alone, or by increasing the amount of nutrients already present in the basal medium but is insufficient to sustain exponential growth (Ham and McKeehan, 1979). The most common type of serum used is fetal bovine serum (FBS), which is derived from fetal calf. The FBS contains a mixture of components: growth factors, proteins, vitamins, trace elements, hormones, etc., crucial for cellular growth and maintenance (Van der Valk *et al.*, 2010). Besides FBS, other types of serum that have been used for *in vitro* cultures (but to a much lesser extent) includes sheep serum (Thompson *et al.*, 1992), goat serum (Paranjape, 2004), horse serum (Franke *et al.*, 2014), rabbit serum (Crowley *et al.*, 2013) and human serum (Tel *et al.*, 2013).

In general, serum provides both growth boosters as well as protective elements to the cells in culture. Specifically, the presence of hormones in the serum helps to stimulate cell growth (Sato, 1975). Serum also possesses cell-attachment promoting proteins which assists the cells for proper attachment to the plastic culture plates (Hayman and Ruoslahti, 1979; Hayman *et al.*, 1985). In addition, serum contains proteins that bind to vitamins, lipids, or some hormones in order to help stabilize or modulate their activities (Vogt *et al.*, 1969; Chen *et al.*, 1992). These binding proteins may also bind to toxic metals to help detoxify the medium (Horie *et al.*, 2009). At the same time, serum promotes further protection to the growing cells by providing protease inhibitors to combat harmful proteases which are either released by the cells or added during cell passaging (Sato, 1975).

Despite all the benefits provided by the serum, motivation towards developing serum-free culture media has been driven by a number of reasons. Firstly, the collection of serum from the unborn calf are limited and costly, and the procedure itself has been considered as inhumane (Van der Valk *et al.*, 2004). Next, there is a significant lot-to-lot variation in the serum composition secondary to seasonal and continental differences which in turn, affects the reproducibility of the experimental results. More importantly, there is risk of viral contamination which may be carried by some of the components in the serum, and this type of contamination has been seen in up to 50% of commercial FBS (Wessman and Levings, 1998).

On the other hand, although less harmful, the presence of serum in culture medium may also induce phenotypical changes to cells like corneal keratocytes, which

changes from quiescent dendritic phenotype into spindle-shaped cells resembling fibroblasts and myofibroblasts found during corneal wound healing (Barry *et al.*, 1994; Jester *et al.*, 1996). This may be of significant importance when studying the biology of cornea *in vitro* as the activated phenotypes do not produce similar protein markers like the quiescent keratocytes (Beales *et al.*, 1999; Jester *et al.*, 1999). As far as corneal keratocytes is concerned, any attempt to omit the serum from the culture medium needs to include a proper substitute (i.e supplements) that are able to carry out all the critical functions previously played by serum (Gouveia and Connon, 2013; Foster *et al.*, 2015). Therefore, the focus of stromal cell culture works has been steered towards developing a serum-free culture medium with the most appropriate supplements that support both proliferation and quiescent state of the cells.

1.2.3 Additional medium supplements

The nutritional requirements amongst all mammalian cells may differ from one another, which the basal medium may not be able to fulfil on its own. This is particularly true in serum-free media, where most of the nutrients normally supplied by the serum is no longer present. Even in the presence of serum, some supplements will still need to be added into the basal medium. Therefore, a strategy for developing a good chemically-defined, serum-free medium rich with necessary nutrients from additional medium supplements is crucial and needs to be carefully laid down to ensure continuity on cell survival, growth and differentiation.

Growth factors encourage cell growth by increasing cell proliferation as well as stimulating specific cell functions. These factors can either be present in the medium through FBS or as additional supplements. For example, transforming growth factor beta (TGF- β) in FBS (Kropf *et al.*, 1997) has been known to induce myofibroblast differentiation in corneal keratocytes cell culture (Funderburgh, 2001; Jester and Ho-Chang, 2003). An addition of epidermal growth factor (EGF) into the culture medium has been the standard practice in culturing corneal epithelial cells since it has positive effects on epithelial cells' proliferation (Imanishi *et al.*, 2000; Park *et al.*, 2013). The use of EGF in enhancing corneal epithelial wound healing has also been studied extensively (Kitazawa *et al.*, 1990; Nakamura *et al.*, 2001; Hori *et al.*, 2007). Besides EGF, keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) also influence corneal epithelial cell growth *in vitro* by promoting epithelial cells

proliferation, motility and differentiation (Wilson *et al.*, 1994; Sotozono *et al.*, 1994). The addition of basic fibroblast growth factor (bFGF) into DMEM/F12 culture medium in the presence of serum and leukemia inhibitory factor has been shown to be the most appropriate medium to cultivate clinical-grade cornea stromal stem cells (CSSC) to be used in cell-based therapy (Sidney *et al.*, 2015).

At the same time, the basal medium needs to have the essential trio, namely the insulin, transferrin and selenium (ITS supplement). The success stories behind ITS supplementation has been focusing on the promotion of germ cell maturation and embryo development (Córdova *et al.*, 2010; Hu *et al.*, 2011; Hammami *et al.*, 2013), as well as chondrocytes proliferation and the formation of tissue engineered cartilage (Chua *et al.*, 2005; Yang and Barabino, 2011; Liu *et al.*, 2014). The supplementation of ITS in corneal keratocytes cell culture has been established (Gouveia and Connon, 2013; Abidin *et al.*, 2015). Individually, insulin supplementation has been tracked back to the 1920s (Gey and Thalhimer, 1924) and since then, becomes the most commonly used hormone in culture media. This is because insulin is able to influence cell development (Zhang and Armstrong, 1990), proliferation and differentiation (Yuksel *et al.*, 2000; zur Nieden *et al.*, 2005). Besides insulin, other hormones that has also been widely used as medium supplement are glucocorticoids (dexamethasone and hydrocortisone), triiodothyronine (T₃) and hormones that are able to increase intracellular cAMP levels. On the other hand, transferrin is an essential protein in culture medium which aids the transfer of iron into the cells. Cells require iron for a variety of metabolic processes, including electron transport and DNA synthesis (Ponka *et al.*, 1998). Meanwhile, selenium is an essential trace element which is an important element for cell protection against oxidative stress (Helmy *et al.*, 2000).

Another important medium supplement is glutamine, which is an essential precursor for protein and ribonucleotides syntheses that is extremely needed by rapidly dividing cells and cells that use glucose inefficiently (Glacken, 1988). However, glutamine supplementation carries hazardous side effects to the cells especially in serum-free and/or protein-free cultures, as accumulation of ammonium which is one of the end products for glutamine catabolism is toxic to cells (Schneider *et al.*, 1996). The use of glutamine was replaced by glutamate, and a more recent invention using glutamine-containing dipeptides under the trade name of GLUTAMAX™ are now commercially

available. The GLUTAMAX™ are more stable and heat resistant, and produces lower rates of ammonia to make it safer for cells in culture (Christie and Butler, 1994).

Majority of vitamins which are essential for cell growth and proliferation are available in the basal medium. Interestingly, the additions of ascorbic acid (vitamin C) into basal medium has been related to cell proliferation and stem cell differentiation (Takahashi *et al.*, 2003; Choi *et al.*, 2008; Huijskens *et al.*, 2015). In corneal cell cultures, supplementation of ascorbic acid particularly in corneal keratocytes culture medium induces the cells to produce more collagen and extracellular matrix (Saika *et al.*, 1992; Guo *et al.*, 2007b; Grobe and Reichl, 2013).

Another type of vitamin that has shown influential effects on cornea is vitamin A. Retinoic acid, the active form of vitamin A, plays a critical role in cell differentiation at the cornea, conjunctiva and the limbus. A deficiency in vitamin A or its metabolites (e.g. retinoic acid) causes epidermal keratinization and squamous metaplasia of the cornea and conjunctiva, corneal ulceration, night blindness and retinopathy (Smith and Steinemann, 2000). Supplementation of retinoic acid in culture medium has focussed on looking at its effect on limbal epithelial cells clonal growth (Kruse and Tseng, 1994) and differentiation (Kim *et al.*, 2012), DNA synthesis, cell morphology and collagen degradation by corneal fibroblasts (Kirschner *et al.*, 1990; Kimura *et al.*, 2016) as well as modulation of keratocyte-characteristic markers by corneal keratocytes (Gouveia and Connon, 2013). Not only that, retinoic acid has also recently been shown to aid in the differentiation of adipose-derived stem cells (ASCs) towards keratocytes (Lynch and Ahearne, 2017). Since retinoic acid has shown some great effects on corneal stromal cell culture, it is interesting to know what retinoic acid can do to engineered corneal stromal constructs as well as its effect on pathological keratocytes.

1.3 Mechanotransduction: its possible role in ocular surface homeostasis

The maintenance of corneal epithelium by limbal epithelial stem cells are through well-orchestrated proliferation followed by migration and maturation centripetally towards the centre of the cornea (Collinson *et al.*, 2002; Nagasaki and Zhao, 2003; Walczysko *et al.*, 2016) as shown in figure 1.5. However, the actual mechanism that drives the centripetal migration and differentiation of these cells during homeostasis process remains unclear. The possible role of mechanical environment in influencing epithelial cell migration and differentiation has been put into perspective based on the idea that cell movement can be guided by substrate rigidity, or elasticity of the substrate, termed as durotaxis (Pelham and Wang, 1997; Pelham and Wang, 1998; Lo *et al.*, 2000; Allioux-Guérin *et al.*, 2009). Sheetz *et al.* (1998) speculated that stiffness of the extracellular matrix might function as a mechanical cue to orient the direction of cell movement. This mechanical cue is then translated into intracellular biochemical signals which then enables the cells to respond to the extracellular environment accordingly (Vogel and Sheetz, 2006; Ross *et al.*, 2013).

In general, cells migrate in a specific, defined direction during many physiological processes including morphogenesis, wound healing and tumor metastases (Ridley *et al.*, 2003). The directional signals may come from various environmental stimuli, including substrate chemical (chemotaxis, (Pettit and Fay, 1998), light (phototaxis, (Saranak and Foster, 1997), gravity (geotaxis, (Lowe, 1997) and electrostatic potential (galvanotaxis, (Erickson and Nuccitelli, 1984). Cell migration relies upon forces generated within the actin cytoskeleton and applies to the extracellular matrix via focal adhesions. In durotaxis, cells receive strong feedback when probed at stiff regions within the substrate using local protrusion, and remain stable and anchored to the substrate. In contrast, cells that landed on soft substrate regions receive weak feedback, have mobile anchorage and become unstable. In this manner; cells that adhere to stiffer substrates invest less energy to remain attached when compared to cells that adhere to softer substrates (Bischofs and Schwarz, 2003). Cells on stiff substrates will form numerous actin filament bundles, while cells on soft substrates will have fewer bundles with a smaller number of filaments (Walcott and Sun, 2010). This 'biasness' guides cell movement from softer towards stiffer substrate regions (Lo *et al.*, 2000; Lazopoulos and Stamenović, 2008).

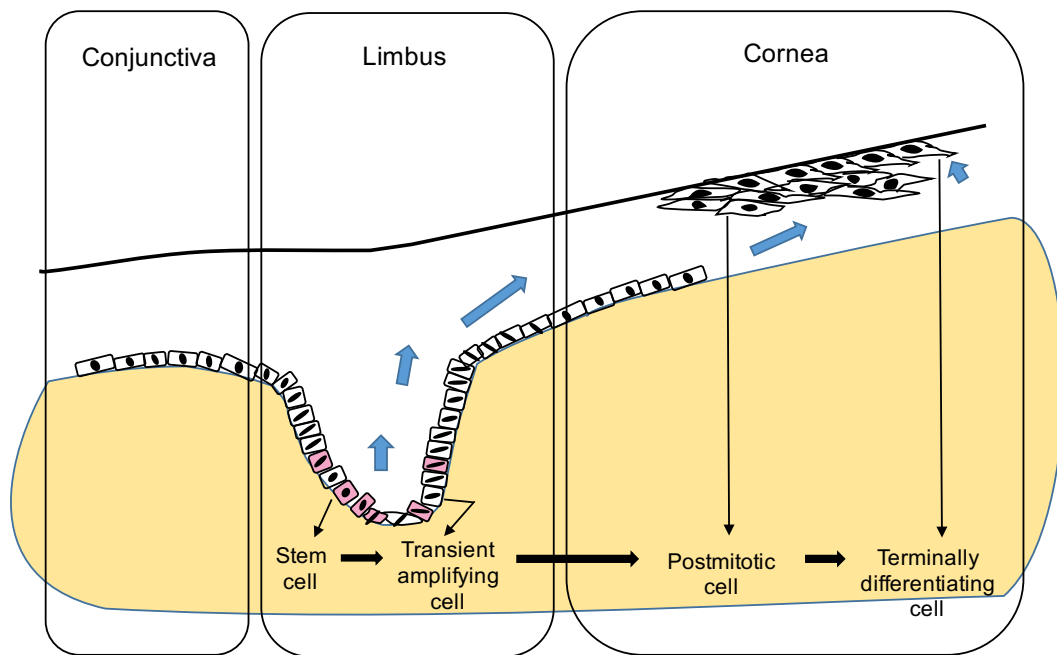


Figure 1.5 - Schematic diagram showing the location of corneal stem cells and its movement during corneal homeostasis. The stem cells are found at the basal layer of the limbus, which divides and replicates to become transient amplifying cells (TACs). The centripetal movement begins when the TACs undergo further mitosis and differentiation while moving towards central cornea to become terminally differentiated cells at superficial strata.

A study by Lo et al. (2000) using 3T3 fibroblasts on collagen-coated polyacrylamide substrate with a rigidity gradient indicated that cells were able to detect and respond to substrate stiffness. Interestingly, they proposed that durotaxis takes place only when there are no other cells in the vicinity in order to exclude the pulling or pushing force from neighbouring cells to guide such movement. However, Gray et al. in 2003 showed that cells preferentially migrated towards stiffer substrates regardless of whether or not they were in contact with another cell. This was shown on two dimensional surfaces where areas of stiff and compliant substrate were micro-patterned in order to measure preferential migration, and after 24 hours, NIH 3T3 fibroblasts accumulated in the stiffer regions in the gel.

Tensile testing and bulge testing are the two most common methods used to determine the bulk elastic modulus of the cornea. Other techniques, for example pressure loading (Hjortdal, 1996), x-ray scattering technique (Meek and Boote, 2009), high resolution digital cameras with digital image correlation software (Whitford *et al.*, 2016) and Brillouin spectroscopy (Lepert *et al.*, 2016), have also been applied to quantify stiffness between the limbus and central area of the cornea.

Structural and mechanical differences between the edge (limbus) and central corneal stroma have previously been reported (Meek and Boote, 2009; Lepert *et al.*, 2016), the latter having approximately 20% more stiffness of the former (limbus 7.6MPa, central cornea 9.4MPa) (Hjortdal, 1996). However, these measurements were taken using corneas with cells in situ, whereas no stiffness quantification has been done so far on acellular cornea (following decellularisation) to identify any changes in stiffness gradient between the two areas following exposure to decellularising agents. The need for special stiffness quantifying devices previously mentioned may be the main contributing factor as these instruments may not be available in all research settings.

The nuclear localisation of Yes-associated protein (Yap), a transcriptional co-activator that mediates cellular response and mechanical stimuli, was shown to increase in the central cornea whereas the Yap localisation was predominantly cytoplasmic within basal limbal epithelial cells (Foster *et al.*, 2014). More recently, a study by Bongiorno *et al.* (2016) demonstrated that even the stem cells found near the limbus were softer than the corneal epithelial cells in the central cornea, thereby proposing it as a 'biomechanical stemness marker' for stem cell identification purposes. Based on these results, it is hypothesized that the centripetal migration of corneal epithelial cells from the limbus towards the central cornea observed during corneal homeostasis and wound healing (Hanna, 1966; Davanger and Evensen, 1971a; Buck, 1979; Thoft and Friend, 1983) is driven by the difference in elasticity between the two areas.

Substrate stiffness can also influence the long-term fate of cellular development. Studies have shown that mesenchymal stem cells differentiated to more specialized cells under the influence of cellular substrate stiffness (Engler *et al.*, 2004; Georges and Janmey, 2005; Yeung *et al.*, 2005). Both limbal and corneal stroma are able to strongly influence epithelial proliferation and differentiation suggesting it is the underlying matrix which dictates the plasticity of epithelial differentiation (Espana *et al.*, 2003). Crucially, following on from a landmark study (Engler *et al.*, 2006a) we have recently shown how differences in substrate stiffness can radically affect the level of limbal stem cell differentiation using two different *in vitro* ocular surface models (Chen *et al.*, 2012; Jones *et al.*, 2012b), offering an elegant explanation to the previously observed epithelial plasticity (Espana *et al.*, 2003).

1.4 Hypothesis

The aim of this thesis is to investigate the effect of mechanical and chemical cues to further understand how normal corneal cell functions. The established therapy for limbal stem cell disease based on ex vivo expanded limbal stem cells is currently at a stage of refinement. However, little consideration is given to the preparation of the corneal surface prior to cell transplantation. Explicitly, loss of tissue compliance reduces the ability of the limbus to act as a stem cell niche contributing both to the initial loss of stem cells (resulting in blindness) and the subsequent limited success rate of limbal stem cell therapy. Furthermore, it is believed that the provision of a normal centripetal difference in substrate stiffness across the corneal surface also controls ocular surface homeostasis i.e. drives the previously observed centripetal migration and differentiation of epithelial cells across the corneal surface (from the compliant limbus to the stiffer central cornea).

Chemical cue is an important element in the control and maintenance of the corneal stromal cells (or keratocytes) phenotype in order to investigate its biology *in vitro*. Specifically, a good medium supplement is required in keratocytes culture medium that is able to promote cell growth and proliferation without encouraging fibroblastic differentiation. Therefore, the quest to establish and promote new supplement to be used in serum-free culture medium for corneal stromal cell culture work is continued in this thesis.

In this thesis, it is hypothesized that:

1. Decellularised cornea can be used as scaffold to study the effect of mechanical stiffness on limbal epithelial stem cells differentiation.
2. The limbal epithelial stem cells grown on decellularised limbal area maintain their stem cell phenotype as compared to cells cultured on central cornea.
3. The mechanical properties of bovine cornea can be controlled by enzymatic digestion (artificial softening) which can affect human limbal epithelial stem cells fate.
4. In 3D environment, chemical cues from retinoic acid supplementation is able to modulate keratocytes proliferation, the expression of keratocyte-characteristic markers as well as improving the overall hydration and weight of the 3D construct.

5. Retinoic acid supplementation is able to modulate the progress of keratoconus by reducing the matrix metalloproteases (MMPs) expressed by keratoconic fibroblasts while de-differentiating the fibroblasts into more native, quiescent keratocytes by regulating keratocyte-characteristic markers.

The first part of the thesis shall look into the effect of mechanical cues from decellularised cornea scaffolds on corneal epithelial stem cell differentiation. Three decellularisation protocols that were previously developed for bovine corneas are going to be tested and optimised, but only one protocol with the best overall outcome in terms of effectiveness in cellular removal, preservation of the ECM and its ability to support corneal cell growth post-decellularisation is chosen for further assessment. The effect of substrate stiffness from the remaining extracellular matrix (following decellularisation) is investigated by culturing bovine limbal epithelial stem cells on decellularised scaffolds for 21 days. Further to that, the effect of softening treatment on de-epithelised bovine corneas using collagenase is also performed which is later reseeded with human limbal epithelial stem cells to further elucidate that modulation of substrate stiffness can affect limbal epithelial stem cells fate. Here, the analysis covers from histological and immunofluorescence staining using stem cell/differentiation markers, stratification analysis as well as protein assay using the new In-Tissue Western assay technique.

The second part of the thesis will concentrate on looking at the effect of chemical cues on culturing both normal and diseased corneal keratocytes using retinoic acid as the main medium supplement in 2D/3D environment. For normal keratocytes, the cells are encapsulated in compressed collagen gels to create the 3D environment and cultured in serum-free medium supplemented with retinoic acid. The effect of retinoic acid supplementation on these cells is assessed by their proliferative potential using Alamar Blue® assay, and their ability to produce collagens and keratocyte-characteristic markers while modulating proteineases and fibroblastic markers both at transcription and protein levels using qPCR and western blotting, respectively. The study also looks into the 'side-effects' the cells give to the surrounding collagen gels following retinoic acid supplementation by measuring gel weight (both dry and wet), calculating gel hydration as well as performing gel contraction assay. For diseased keratocytes, cells from keratoconus cornea are cultured in monolayers in serum-free environment supplemented with a range of

retinoic acid concentrations. The effect of retinoic acid supplementation on these cells is assessed by looking at viability assay using both live-dead and MTT assays, cell morphology using microscopy imaging, and their ability to modulate proteinases and their inhibitor, keratocyte-characteristic markers as well as lysyl oxidase at transcription and protein levels using qPCR and western blotting, respectively.

Chapter 2. Optimisation of decellularisation techniques for bovine cornea

2.1 Introduction

The worldwide limited availability of suitable corneal donor tissue has led to the development of alternatives, including keratoprotheses and tissue engineered constructs (Wilson *et al.*, 2013). However, the main obstacle to successfully engineering corneal tissue has been the replication of the structural and biochemical composition of native cornea in a tissue engineered scaffold. Thus, decellularised corneal scaffolds have been suggested as an alternative to fabricated biomaterial as a method of generating scaffolds similar to native human cornea in structure and composition (Lynch and Ahearne, 2012).

Decellularisation, a process of cell and associated nucleic acid debris removal, has been used to create acellular scaffolds that are useful for a number of biomedical applications such as understanding the physicochemical properties of extracellular matrix (ECM) and providing a tissue specific scaffold for engineering functional tissues (Gillies *et al.*, 2010). Removal of cells from either animal or human tissue leaves the complex mixture of structural and functional proteins that constitute extracellular matrix (Gilbert *et al.*, 2006). While still in its infancy, decellularisation attempts to reuse, maintain and characterize the 3D tissue architecture in place (Traphagen and Yelick, 2009). Besides the cornea, decellularisation has been employed to a variety of tissues from organs such as the heart valves (Rieder *et al.*, 2004; Grauss *et al.*, 2005), blood vessels (Conklin *et al.*, 2002; Dahl *et al.*, 2003), skin (Chen *et al.*, 2004a), nerves (Kim *et al.*, 2004), skeletal muscle (Borschel *et al.*, 2004), tendons (Cartmell and Dunn, 2000), ligaments (Woods and Gratzer, 2005), small intestines submucosa (Badylak *et al.*, 1995), urinary bladder (Chen *et al.*, 1999), and liver (Lin *et al.*, 2004).

So far, three decellularisation protocols have been developed for bovine corneas, and all methods have been shown to be able to support cell growth upon repopulating the scaffolds with keratocytes (Ponce Márquez *et al.*, 2009; Dai *et al.*, 2012). However, no attempt was done (in those studies) to look at the capability of these scaffolds to accept epithelial cells, particularly epithelial stem cells. For this

reason, the development of corneal epithelial growth model derived from decellularised bovine corneas was investigated.

2.2 Materials and Methods

2.2.1 *Preparation of decellularised cornea scaffold*

Whole bovine eyes were obtained from abattoirs (Chitty abattoir, Guildford, UK and Lindens Food Burradon Ltd, Newcastle, UK) within 2 hours of death and transported to the laboratory on ice ready for dissection. Corneas were dissected under sterile condition and washed thoroughly with antibiotic-antimycotic solutions (Gibco Invitrogen, UK) in phosphate-buffered saline (PBS, Gibco Invitrogen, UK). The corneas were dissected into 8 equal pie-pieces and placed in 6 well plates (figure 2.1). These pie-pieces (from each animal) were then divided into 3 groups and decellularised as previously described by using either ethanol (Ponce Márquez *et al.*, 2009), sodium dodecyl sulphate (SDS, Sigma-Aldrich, UK) (Ponce Márquez *et al.*, 2009) or short-term chemical frozen (STCF) (Dai *et al.*, 2012) methods with slight modification to each.

Ethanol

In the ethanol method, corneal pieces were immersed in 75% ethanol to give a solvent/tissue mass ratio of 20:1 (vol/wt). The samples were incubated at room temperature on an orbital shaker for a total of 72 hours, with solvent replacement at 1, 3, 12, 24, 48, and 72-hour time points. The tissue was subsequently rinsed for 12 hours in distilled water, then incubated at room temperature for 96 hours with a solution of sterile 0.05% trypsin-EDTA (Gibco Invitrogen, UK) prepared in a trypsin/tissue mass ratio of 15:1 (vol/wt.).

Sodium dodecyl sulphate (SDS)

In the SDS method, corneal pieces were immersed in a 1% (wt./vol.) SDS solution with a solvent/tissue mass ratio of 25:1 (vol/wt.). The samples were placed on an orbital shaker for 12 hours and then rinsed 5 times in PBS for 2 hours. Samples were then washed for 12 hours in 75% ethanol with a solvent/tissue mass ratio of 25:1 ratio (vol./wt.). Finally, the samples were washed 3 x 20 minutes in PBS to remove residual substances.

Short-term chemical frozen (STCF)

In the final method, the corneal pieces were treated with 0.5% Triton X-100 (Acros Organics, US) and 20 mM NH₄OH (Sigma-Aldrich, UK) mixture using a solvent/tissue

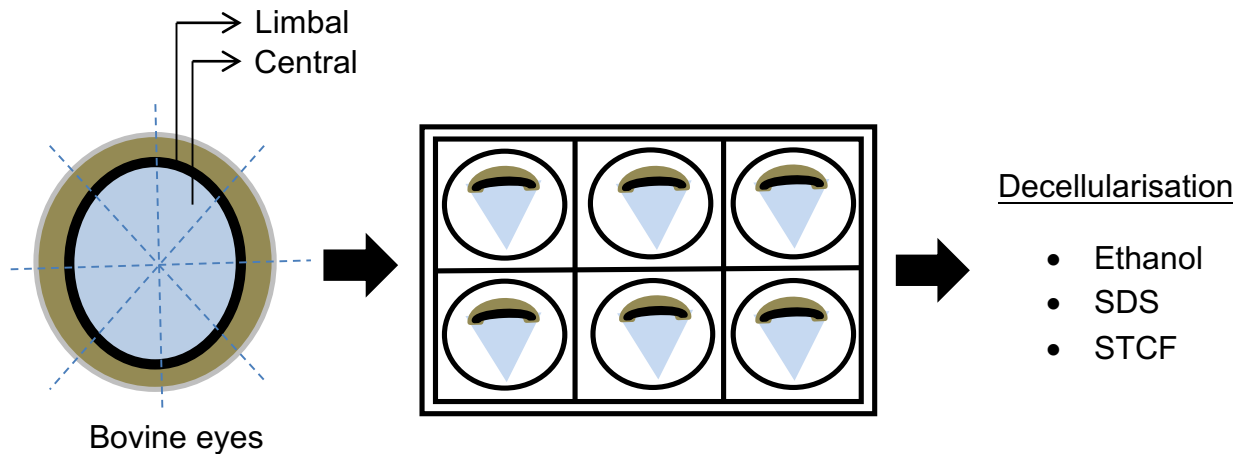


Figure 2.1 - Schematic diagram showing the preparation of bovine corneas prior to decellularisation. Fresh bovine corneas were cut into eight equal pie-pieces and placed in three different 6-well plates for decellularisation process using either ethanol, sodium dedocyl sulphate (SDS) or short-term chemical freeze (STCF) method.

mass ratio of 20:1 ratio (vol./wt.) for 10 minutes on an orbital shaker. The samples were then rinsed with PBS for 3 times followed by sample freezing in -80°C for 3 days and then preserved in 4 ml of 100% glycerol (Sigma-Aldrich, UK) at 4°C. Prior to use, the dehydrated corneas were quickly thawed and rehydrated in PBS for 20 minutes and a gentle scraping was performed with the aid of a dissecting microscope to remove the epithelium.

2.2.2 Isolation of fresh bovine limbal epithelial stem cells (bLESCs)

The fresh bovine corneas used to harvest limbal epithelial cells were dissected into six pieces and the central cornea region (beyond the pigmented limbus area) and the sclera were cut away and discarded. The remaining corneal limbal tissue was further cut into several small pieces approximately 25 mm² in area and incubated overnight in 2 mg/ml Dispase (1.10 units/mg, Gibco Invitrogen, UK) dissolved in DMEM/F-12 medium at 4°C. The loosened epithelial sheets were then removed gently from the stroma with the aid of a scalpel and dissecting microscope. The dissected epithelial sheets and cell clumps were aspirated through a 21-gauge syringe needle to

dissociate the tissue and isolate single limbal epithelial cells. This was followed by a second digestion with 0.05% trypsin-EDTA (Gibco Invitrogen, UK), incubated at 37°C with gentle shaking for 5 minutes. Cells were washed and suspended in the basal medium comprising DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), antibiotic solution (Gibco Invitrogen, UK), B27 supplement (Invitrogen, UK) and 10 ng/mL epithelial growth factor (Sigma-Aldrich, UK) prior to seeding onto decellularised bovine corneas. Another medium formulation (CnT-20) which is usually used to culture human limbal epithelial stem cells had also been tried onto these bLESCs but the cells did not survive when cultured in this medium.

2.2.3 Recellularisation of bovine corneas using bLESCs

Decellularised pie-pieces corneas (from all three methods) were placed in 12 well plates, and freshly isolated bLESCs were seeded onto the surface of each scaffold at 5×10^3 cells/mm² in 2 ml medium suspension. Specimens were fully submerged in the culture medium containing DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), antibiotic solution (Gibco Invitrogen, UK), B27 supplement (Invitrogen, UK) and 10 ng/mL epithelial growth factor (Sigma-Aldrich, UK), and incubated for 10 days at 37°C in a humidified 5% CO₂ incubator. The medium was changed every two days.

2.2.4 Histological study and immunofluorescence staining

Histological examination using Hematoxylin and Eosin (H&E) staining was performed on the normal bovine cornea, decellularised and recellularised bovine corneas. Frozen samples embedded in OCT matrix (Thermo Scientific, UK) were cut into 7 µm thick sections using cryostat machine, thaw-mounted onto poly-lysine coated slides (Thermo Scientific, UK) and air-dried for 2-3 hours at room temperature. Both limbal and central areas were marked on the slides (according to their respective locations on frozen samples) for future microscopy viewing reference. The staining was as follows: sectioned slides were washed in PBS for 2 minutes, followed by 7 minutes incubation in the hematoxylin solution (Sigma-Aldrich, UK) to stain the nucleic material. The hematoxylin was removed by a 2-minute wash in distilled water followed by 2 dips in acidic alcohol. The slides were submerged in running water for 5 minutes before being placed in 70% ethanol for 1 minute. The slides were then placed in 1% eosin for 5 minutes to stain the protein material and washed in a series

of ethanol steps (1 x 90%, 3 x 100%; 2 minutes for each step) to remove unbound eosin before a final rinse in xylene (Fisher Scientific, UK; 2 x 3 minutes).

Immunofluorescence staining was performed against basement membrane protein Collagen Type IV (on normal and decellularised corneas) as well as several key corneal epithelial markers (p63, ABCG2 and CK3). Cryostat sections of 7 µm thick were fixed in 4% paraformaldehyde for 20 minutes prior to incubation with 1% (wt./vol.) bovine serum albumin (Sigma-Aldrich, UK) at room temperature to block non-specific binding. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS (table 1). There were also sections that were incubated with PBS only (no primary antibody) for immunolabelling control purposes. Incubation with secondary antibodies (diluted in PBS) was carried out for 1 hour using Alexa Fluor® 488 goat anti-rabbit (1:100, Life Technologies, UK) or Alexa Fluor® 488 goat anti-mouse (1:100, Life Technologies, UK). Finally, the slides were counterstained with DAPI (Vector Laboratories) and analysed by fluorescence microscopy (Carl Zeiss Meditec).

| Antigen | Host | Antibody | Dilution | Source | Catalogue number |
|------------------|--------|-----------------------|----------|-----------------------|------------------|
| Collagen type IV | Rabbit | Anti-Collagen type IV | 1:50 | Abcam | ab6586 |
| p63 | Rabbit | Anti-p63 | 1:50 | Santa Cruz | sc-8343 |
| ABCG2 | Rabbit | Anti-ABCG2 | 1:50 | Aviva Systems Biology | ARP43649_T100 |
| Cytokeratin 3 | Mouse | Anti-CK3 | 1:50 | Santa Cruz | sc-80000 |

Table 1. Details of primary antibodies used for immunofluorescence staining.

2.3 Results

2.3.1 *Histological characterisation of decellularised bovine cornea*

Hematoxylin-eosin (H&E) staining showed all decellularisation methods were able to eliminate all cells from bovine corneas. The entire 10-15 layers of epithelial cells were completely removed from both the limbal and central areas of these corneas as compared to the normal, untreated bovine cornea (figure 2.2a,b). In addition to the

marked area on the slides to differentiate between limbal and central area, the limbal area was recognised by the undulating surface area, possibly representing the palisades of Vogt. On the other hand, the surface area of the central area appeared less undulating. At the same time, the stromal layer of the decellularised corneas was devoid of cells too, and pockets of 'empty spaces' appeared within the stroma especially in corneas treated with STCF method (figure 2.2c-h, shown by the asterisk). No cell fragments were observed within the matrix scaffolds in all decellularised corneas as shown by the lack of hematoxylin staining.

Successful recellularisation of decellularised bovine corneas with bLESCs taken from another cornea (and cultured for 10 days) was observed by histology on the surface of both ethanol- (figure 2.3a,b) and STCF-treated (figure 2.3c,d) corneas, but not from the SDS-treated cornea (figure 2.3e,f). H&E staining showed 1-2 layers of epithelial cells mostly in direct contact with each other on both limbal and central area. These cells were also seen to reside within the undulating surface in the limbal region, thereby filling in the area left from the decellularisation process.

Immunofluorescence revealed that Collagen type IV antibody (basement membrane protein) was stained better near the limbus and stained weakly, if not absent, near central cornea on both fresh and decellularised corneas (figure 2.4). As for stem cell marker expressions, the normal bovine cornea showed strong labelling of p63 (both in the cell nucleus and cytoplasm) throughout the basal epithelium on limbal area accompanied by weaker staining on the suprabasal layers (figure 2.5a). There was also additional but weak staining observed in central cornea as seen in figure 2.5b. The recellularised bLESCs attached on ethanol-treated cornea exhibited stronger staining for p63 on both limbal and central cornea (figure 2.5c,d) as compared to those attached on STCF-treated cornea (figure 2.5e,f). ABCG2, a member of the ATP binding cassette (ABC) transporters, which has been proposed as a universal stem cell marker, were stained across all epithelial layers in both limbal and central area of normal bovine cornea with maximum staining within the limbal compartment (figure 2.5g,h). This staining was also present in the bLESCs on both ethanol-treated (figure 2.5i,j) and STCF-treated corneas (figure 2.5k,l) with similar staining strength found on both limbal and central area. On a different note, CK3 (marker of differentiation) was highly expressed in suprabasal layers of epithelium in the limbal area, as well as across the corneal epithelium in the central area of normal bovine

cornea (figure 2.5m,n). The basal epithelial layer in the limbus was spared and no CK3 labelling was evident in this area. Similarly, only bLESCs on the suprabasal layer of ethanol-treated cornea were stained with CK3, however this observation was found on both limbal (figure 2.5o) and central area (figure 2.5p) of the cornea. bLESCs attached to STCF-treated cornea showed positive staining towards CK3 in all cell layers in both limbal and central area, with weaker staining found on cells at the basal layer compared to the suprabasal cells (figure 2.5q,r).

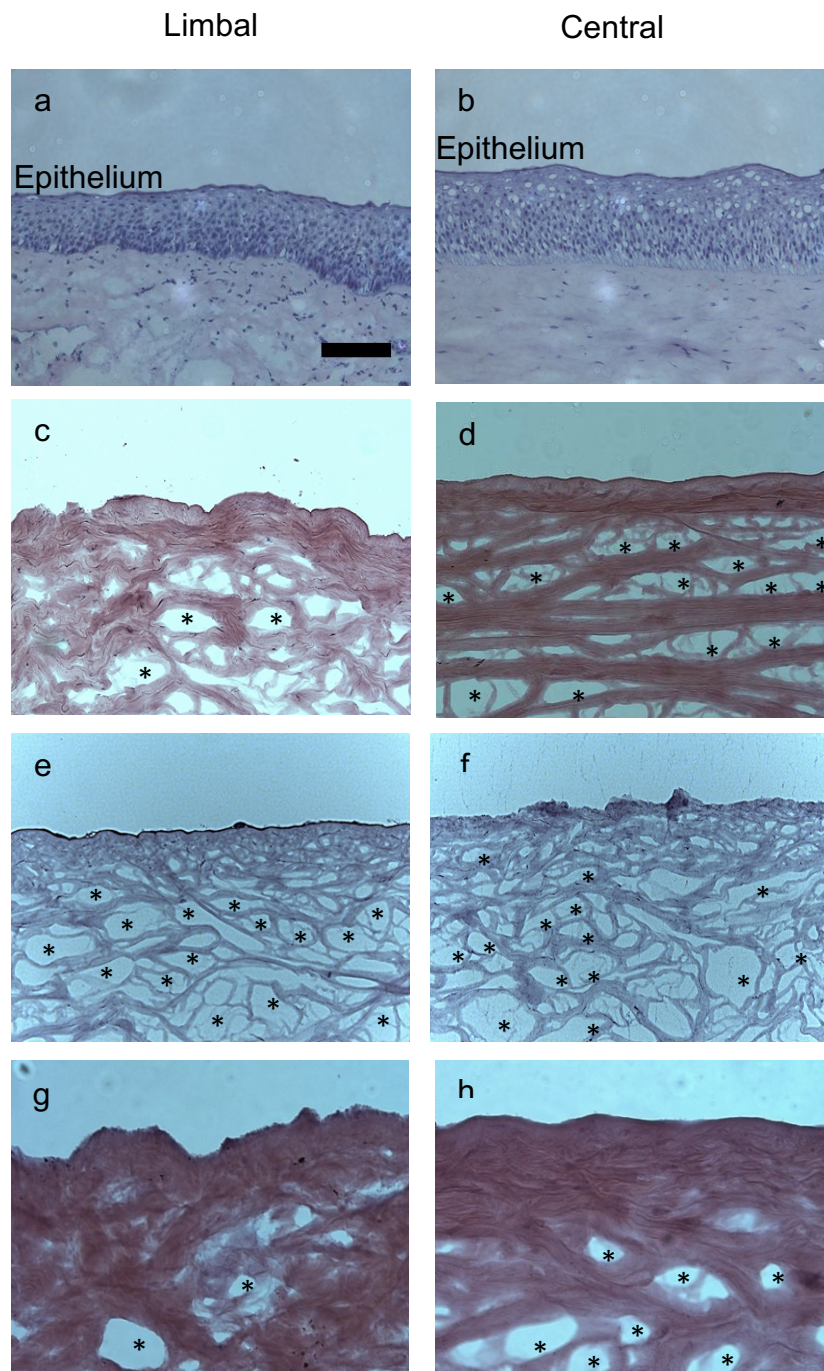


Figure 2.2 - Cross-sections of normal bovine corneas (a,b) and decellularised bovine corneas from three different methods: i) Ethanol treatment (c,d), ii) STCF treatment (e,f), and iii) SDS treatment (g,h), stained with hematoxylin and eosin (H&E). No cells were observed in decellularised corneas from all three methods. Gaps between the collagen fibers in the stroma (shown by the asterisk) were found in all three methods, most prominent in cornea decellurised using STCF method. All photographs were taken at x200 magnification. Scale bar = 50 μ m.

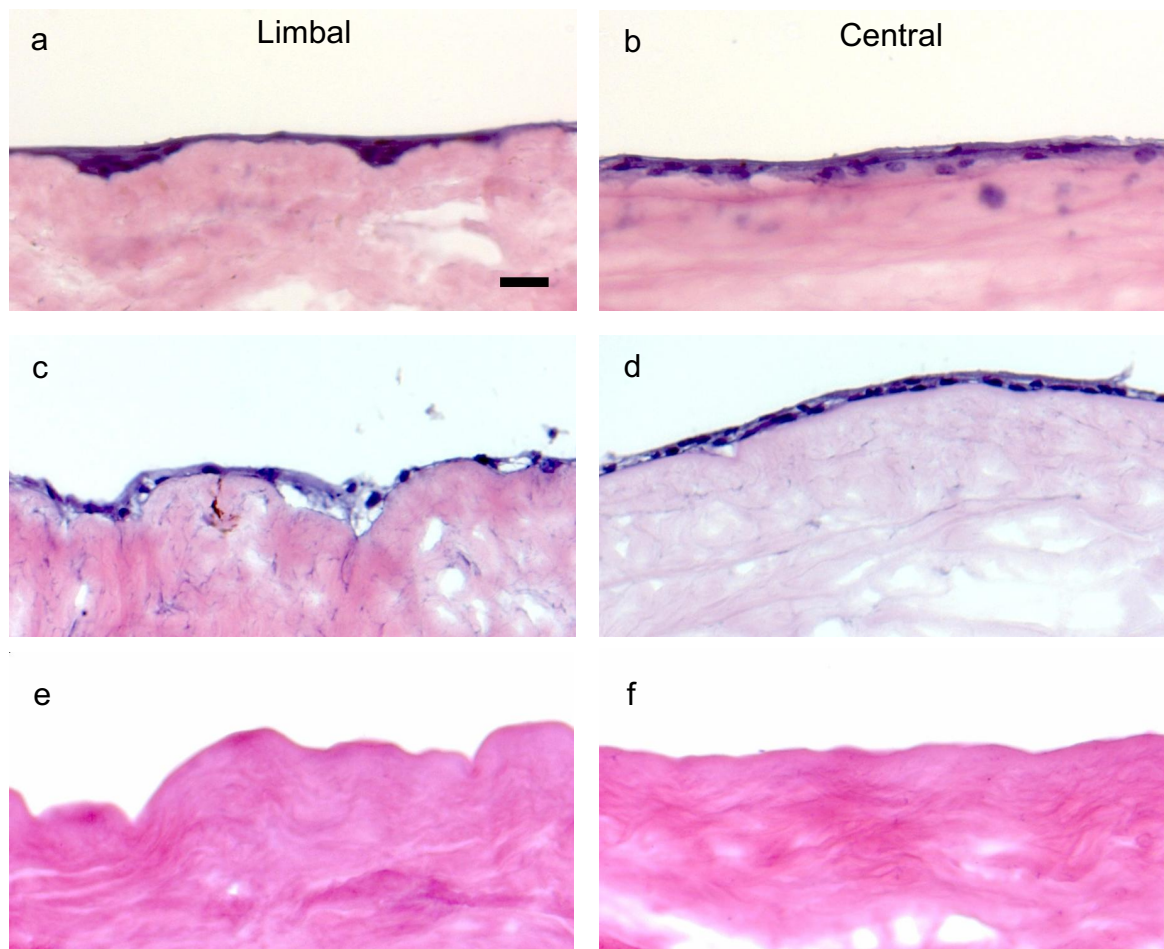


Figure 2.3 - Histological evaluation of recellularised bovine corneas on, 1) ethanol-treated cornea (a,b), 2) STCF-treated cornea (c,d) and 3) SDS-treated cornea (e,f) using hematoxylin and eosin (H&E) staining. After 10 days in culture, epithelial cells were seen attached on both ethanol-treated and STCF-treated bovine scaffolds, but not on SDS-treated bovine scaffold (n=3). All photographs were taken at x200 magnification. Scale Bar = 50 μ m.

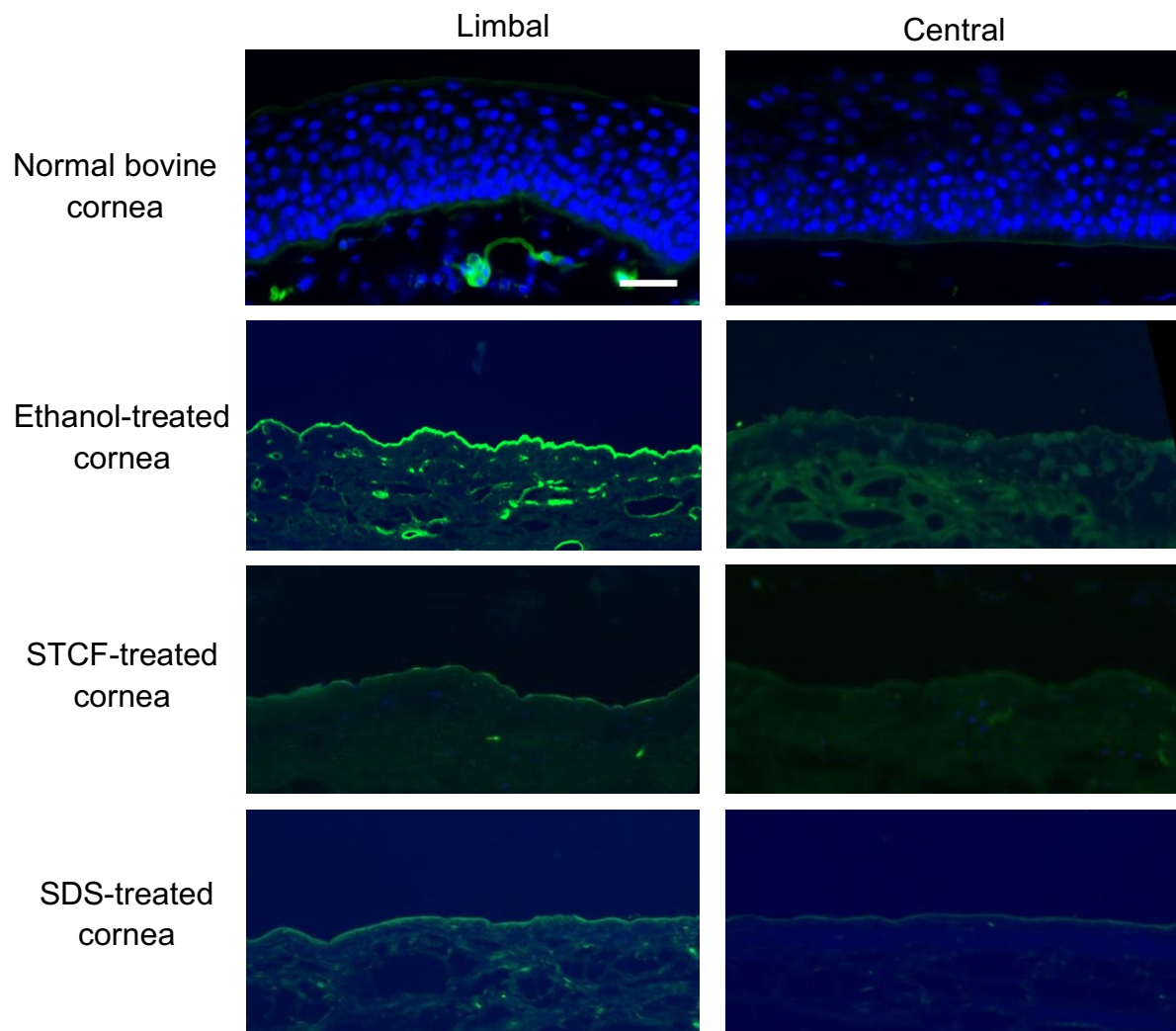


Figure 2.4 - Representative immunostaining of Collagen type IV (Col. IV) and cell nuclei; DAPI (blue) and Col. IV (green) on normal bovine corneas and decellularised bovine corneas from both limbal and central regions. All photographs were taken at x200 magnification. Scale bar = 50 μ m.

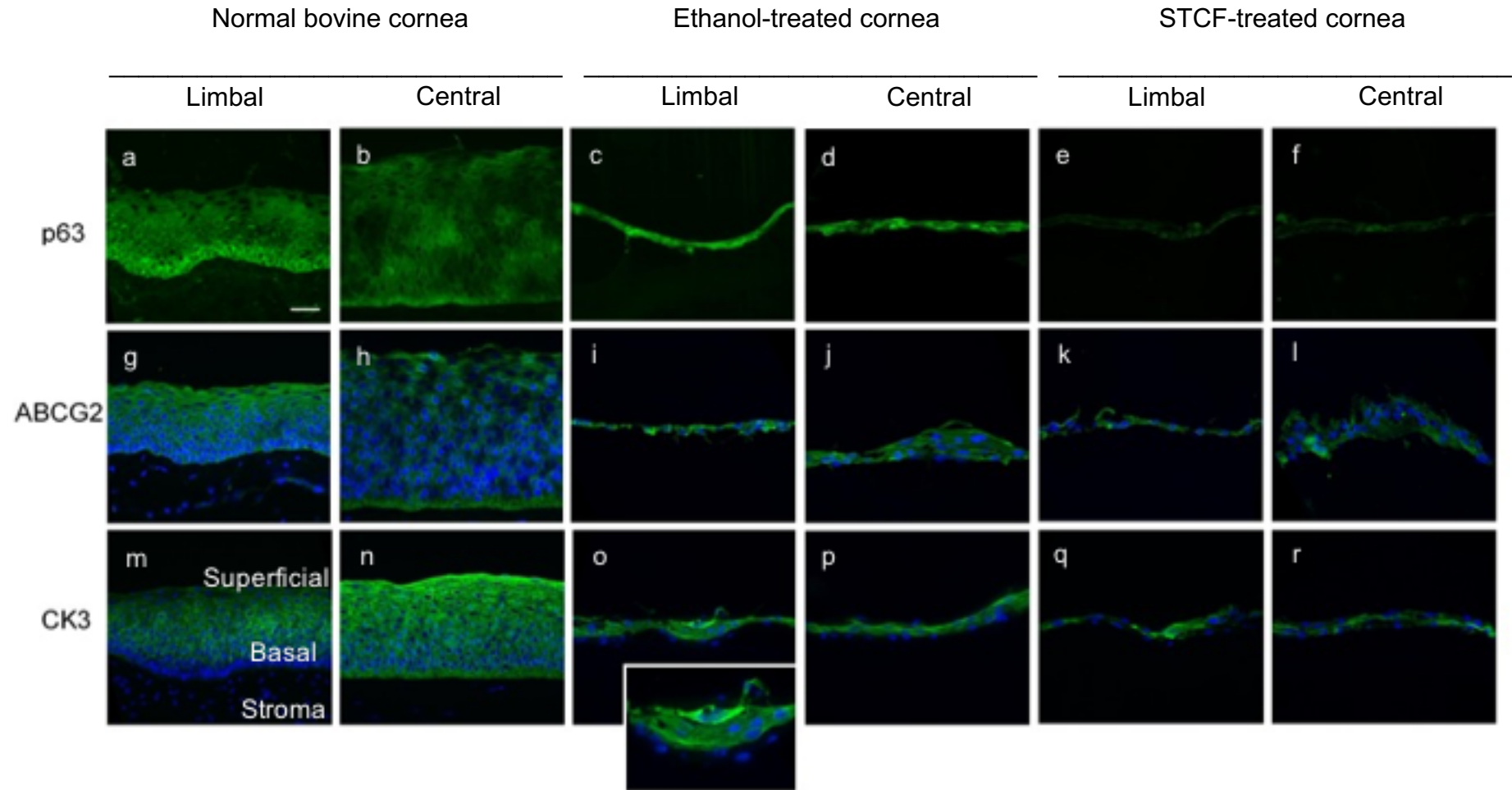


Figure 2.5 - Representative immunofluorescence staining showing the expressions of p63 (a-f), ABCG2 (g-l) and CK3 (m-r) of normal and recellularised bovine corneas. Both p63 and ABCG2 are putative stem cell markers, with CK3 as differentiation marker. Cell nuclei were stained with DAPI (blue). No DAPI staining was used for figures with p63 staining to highlight the nuclear staining within the cells. All photographs were taken at x200 magnification. Scale bar = 50 μ m.

2.4 Discussion

Classification of decellularisation techniques are made based on the chemicals used, such as acid or alkaline treatment, detergent treatment, or enzymatic digestion, and the physical methods used, such as snap freezing and mechanical agitation (Badylak, 2004; McFetridge *et al.*, 2004; Ketchedjian *et al.*, 2005; Sawada *et al.*, 2008). The most robust and effective decellularisation protocols include a combination of physical, chemical and enzymatic approaches (Gilbert *et al.*, 2006), which include several steps to promote lysis of the cell membrane, separate the cellular components, and remove the cellular debris from the tissue (Ponce Márquez *et al.*, 2009).

Our findings showed that all three decellularisation methods used (ethanol, SDS and STCF) were able to fully decellularise the bovine corneas i.e, the removal of the epithelial, stromal and endothelial cells. In particular, these methods used chemicals as their main agents to remove cells either through cell lysis via dehydration (ethanol), solubilisation of cell membranes (SDS, ammonium hydroxide) or disruption of lipid-lipid and lipid-protein interactions (Triton X-100). At the same time, they combined both the chemical and physical methods, by which the physical method (mechanical agitation) is commonly used to facilitate chemical exposure and removal of cellular materials. The addition of freezing step (also part of physical method) in STCF helped to further disrupt cell membranes through formation of intracellular ice crystals (Gilbert *et al.*, 2006; Crapo *et al.*, 2011).

Unfortunately, all methods used inevitably resulted in alteration to the extracellular matrix (ECM) compartment by the presence of large gaps between the collagen fibers, presumable due to subsequent swelling of the tissue. Although this effect was not reported in the original protocols, similar observations have been found in other previous decellularisation studies which involved the use of similar detergents (Du *et al.*, 2010; Shafiq *et al.*, 2011; Liu *et al.*, 2016). It is believed that the stromal gaps observed were not produced by freezing the sample for the cryostat as similar alterations were also seen in other studies which used paraffin wax to embed their samples (Du *et al.*, 2010; Liu *et al.*, 2016). In this study, these gaps were more prominent in scaffolds from the STCF method which can be caused by the usage of Triton X-100 as one of its decellularising agent. Similar effects to the ECM structure

were seen in other studies that used Triton X-100 for cell removal (Cartmell and Dunn, 2000; Funamoto *et al.*, 2010; Nara *et al.*, 2016). An excessive ice formation following the freezing step in STCF method may have also contributed to the worsening of this ECM disruption. This signifies that despite considerable efforts being given to ensure sustainability and maintenance to the overall structure of the tissue post-decellularisation, but in reality, each and every processing step involved will still affect the composition and ultrastructure of the tissue at the end.

Recellularisation helps to evaluate the capability of our acellular cornea scaffold to be repopulated by LESC for tissue engineering purposes. The ability of LESC to attach and maintain their stemness following recellularisation may indicate that the basic histoarchitecture of the decellularised corneas were maintained to support cell growth. Our results showed successful recellularisation of 1-2 layers of epithelial cells on decellularised bovine corneas treated with ethanol and STCF method after 10 days of fully submerged culture. Du *et al.* in 2010 observed 2 layers of rabbit corneal epithelial cells reseeded on decellularised porcine corneas following one-week incubation under static condition. A short period of culture duration, together with limited resources of nutrients and growth factors in the medium, may have contributed to this relatively small number of epithelial layers acquired following recellularisation. On the other hand, no attachment of bLESCs was observed on SDS-treated corneas. This observation supports previous findings which also found that SDS-treated cornea did not support epithelial cell growth (Shafiq *et al.*, 2011). SDS is a strong ionic detergent which is effective at removing cells but can be highly detrimental to ECM structure. The damage caused by SDS may include disorganization of collagen fibrils due to swelling, damage to basement membrane, or alterations of tissue matrix biochemistry including loss of GAG contents and growth factor elimination (Hattori *et al.*, 2009; Pang *et al.*, 2010; Reing *et al.*, 2010; Gonzalez-Andrades *et al.*, 2011). Both physical and biochemical changes in SDS decellularised porcine anterior cruciate ligament (ACL) were found responsible for the low cell-reseeding outcome using ACL fibroblast (Gratzer *et al.*, 2006), which may be the same reason for unsuccessful recellularisation of limbal epithelial stem cells in this study.

Epithelial basement membrane contains a complex network of ECM proteins that interact at an epithelial-stromal interface (Li *et al.*, 2006). Its major constituents

include collagen type IV, laminin, perlecan as well as type VII collagen (Timpl *et al.*, 1979; Ohji *et al.*, 1994). The interactions between basement membrane components and epithelial cells are crucial as they involved in the modulation of cell adhesion, migration, proliferation and differentiation (Jones *et al.*, 1995). Therefore, it is very important to ensure that the basement membrane is kept intact following decellularisation as disrupted basement membrane can lead to poor attachment of corneal epithelial cells to the underlying stroma.

Our immunofluorescence staining revealed that collagen type IV antibody was stained better near the limbus and stained poorly, if at all present, near central cornea on both fresh and decellularised corneas. More importantly, positive staining against collagen Type IV antibody on the limbus of decellularised corneas from all three methods suggests preservation of the basement membrane component following decellularisation. This difference in staining strength is in agreement with the other results shown by previous investigators whom did not find collagen type IV in the central basement membrane using immunostaining approach (Kolega *et al.*, 1989; Cleutjens *et al.*, 1990; Lauweryns *et al.*, 1993). More recent studies however demonstrated the presence of different collagen Type IV subchains between the limbus and central cornea (Ljubimov *et al.*, 1995; Wang *et al.*, 2011), which may have contributed to the different strength of staining observed between these two areas i.e higher intensity from limbal area. Also, it has been suggested that the difference in collagen type IV distribution across the entire cornea influenced the overlying corneal epithelial cell phenotype especially the epithelial stem cells (Kolega *et al.*, 1989; Schlötzer-Schrehardt and Kruse, 2005).

Corneal epithelial stem cells have been reported to be exclusively located within the limbal region (Tseng, 1989; Dua and Azuara-Blanco, 2000; Lavker and Sun, 2000), thus giving rise to the term limbal epithelial stem cells (LESCs). The transcription factor p63 has been proposed as one of the potential marker for LESCs (Pellegrini *et al.*, 2001) together with ABCG2 (Watanabe *et al.*, 2004a; Budak *et al.*, 2005), keratin 14 (Zhao *et al.*, 2008) and keratin 15 (Yoshida *et al.*, 2006a). Many studies have confirmed the presence of nuclear-staining p63 interspersed within the human limbal basal epithelial area (Pellegrini *et al.*, 2001; Dua *et al.*, 2003; Shortt *et al.*, 2007). Nevertheless, p63 is not exclusive to LESCs because it is also expressed in central corneal epithelium but to a significantly lesser extent (Arpitha *et al.*, 2005). This holds

true for bovine cornea as the immunofluorescence result showed presence of p63 immunolabelling in the cytoplasm of suprabasal and central epithelium but with no nuclei staining. ABCG2, the proposed universal marker for stem cells, is primarily immunodetected in the cell membrane and cytoplasm of LESC. Bovine cornea showed maximal strength of staining in both basal and suprabasal layers of epithelium near the limbus, with relatively low strength in the cytoplasm of central corneal epithelium. This is inconsistent with ABCG2 immunodetection in human cornea as positively-stained cells were found in the basal and suprabasal cells in limbus only and was not expressed in the central corneal epithelium (Watanabe *et al.*, 2004a; de Paiva *et al.*, 2005). The discrepancy observed between the staining of putative stem cell markers on bovine cornea cells in this study with the published literature suggest that they may not be sufficiently reliable to be used alone in identifying the stemness of the bLESCs attached to decellularised cornea. Therefore, a combination with differentiation marker such as cytokeratin 3 (CK3) might provide a better insight to the presence of these LESC. CK3-positive cells were found in the suprabasal epithelial cells in the limbus and central epithelium of bovine cornea, sparing the basal epithelial layer near the limbus which is the same area where p63 was positively stained. This is in conformity with the idea that the cytoplasm of LESC appear 'primitive' and contains few, if any, differentiation products (Tseng, 1989).

The bLESCs attached to central area of ethanol-treated cornea showed positive staining towards p63 and ABCG2. CK3 staining was only present in the suprabasal cells where two layers of cells were observed. This is somewhat peculiar as the cells were expected to lose their stemness following attachment to the central basement membrane, which can be the limitation of this method. On the other hand, the bLESCs attached to limbal area of STCF-treated cornea were only positive for ABCG2, and CK3 staining was present in all cell layers although the labelling strength was much weaker in the basal layer compared to the suprabasal layer. The use of alkaline base (ammonium hydroxide) may have degraded type IV collagen to some extent although the staining was still present following decellularisation. This is because alkaline bases are likely to degrade structural component of the matrix including collagen (Keane *et al.*, 2015).

To summarise, work was carried out to determine which decellularisation methods provide the best overall outcome in terms of effectiveness in cellular removal, preservation of the ECM and its ability to support corneal cell growth post-decellularisation. A combination of ethanol, EDTA plus agitation was able to reach the above-mentioned outcomes, and this protocol shall be used in the subsequent work. In addition, the protein expressions of p63, ABCG2 and CK3 in the bovine cornea will be used as reference in the following chapter to study the effect of stiffness on cellular fate and development.

Chapter 3. Effects of substrate stiffness on limbal epithelial stem cells fate

3.1 Introduction

Cell behaviour can be controlled by both biochemical and mechanical stimuli. Chemical from signalling molecules, growth factors and chemo attractants affect cell proliferation, migration and differentiation in a way that can similarly be induced by mechanical signals. The conversion of these mechanical signals from the environment into intracellular chemical signals by the cells are known as mechanotransduction (Huang *et al.*, 2004). Mechanotransduction, whether the stimulus comes from shear flow (Shiu *et al.*, 2004), substrate stiffness (Molladavoodi *et al.*, 2015) or mechanical loading of cells (Sadoshima and Izumo, 1997), has been shown to influence cell behaviour for different cell lines including proliferation and differentiation of stem cells (Hadjipanayi *et al.*, 2009; Cameron *et al.*, 2011; Sun *et al.*, 2012).

Several studies have explored the effect of substrate stiffness on differentiation capability of multipotent stem cells, particularly mesenchymal and embryonic stem cells. The differentiation of mesenchymal stem cells (MSCs) for example, are tunable according to the stiffness of the substrate they were plated on (Engler *et al.*, 2006b; Justin and Engler, 2011; Park *et al.*, 2011). Li *et al.* (2008) demonstrated that terminal differentiation in embryonic stem cells (ESCs) is affected by substrate compliance. The cellular fate of unipotent limbal epithelial stem cells (LESCs) can be influenced by varying stiffness of collagen gel scaffolds as previously described by our lab (Jones *et al.*, 2012a; Foster *et al.*, 2014).

Two studies which measured the stiffness level of different areas in the cornea have found that the limbus possesses lower stiffness value compared to central cornea (Hjortdal, 1996; Lepert *et al.*, 2016). It is hypothesized that the stiffness gradient found between the limbus and central cornea contributes greatly to the maintenance of LESCs in the stem cell niche (Foster *et al.*, 2014).

In the previous chapter, we have developed a model of corneal epithelial growth using decellularised bovine corneas prepared from chemical decellularisation. Chemical decellularisation is an often used method for scaffold preparation and makes possible a well-preserved three dimensional structure of extracellular matrix

(Hrebikova *et al.*, 2013). This scaffold provides initial support to the seeded cells, localises the cells in the appropriate spaces, provides physical and biological cues for adhesion, migration, proliferation and differentiation, and assembles the propagated cells and secreted matrices into functional tissues (Hoshiba *et al.*, 2010). In this chapter, we extend our previous experiments by increasing the culture duration up to three weeks, using only the ethanol-treated cornea as biological scaffold to explore the role of mechanical cue of the decellularised cornea on bovine LESC (bLESC) differentiation. By doing this, we would like to see if the bLESCs grown on decellularised limbal area maintain a more limbal phenotype when compared to cells cultured over the central cornea, and find similarities with the previous results using stiff and compliant collagen gels. Our idea is that differences in mechanical cue in the remaining extracellular matrix (following decellularisation) such as stiffness can affect LESC fate as to whether to maintain their stemness or to differentiate. Next, we performed softening treatment on deepithelised bovine corneas using collagenase and reseeded them with human LESC (hLESC) to further elucidate that modulation of substrate stiffness can affect LESC fate.

3.2 Materials and methods

3.2.1 *Preparation of recellularised bovine corneas using bLESCs*

The decellularised bovine scaffolds used for this experiment were only prepared using the ethanol-treated method as this gave the best results in terms of effectiveness in cellular removal, preservation of ECM and its ability to support corneal cell growth as described in the previous chapter. Recellularisation was performed using isolated bovine LESC as previously described. Recellularised corneas were fully submerged in the culture medium containing DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), antibiotic solution (Gibco Invitrogen, UK), B27 supplement (Invitrogen, UK) and 10 ng/mL epithelial growth factor (Sigma-Aldrich, UK) at 37°C in a humidified 5% CO₂ incubator. The medium was changed every two days and specimens were cultured for 21 days.

3.2.2 *Isolation of human limbal epithelial stem cells (hLESCs)*

Primary human limbal epithelial cells (hLESCs) were derived from the corneoscleral rim following removal of the central 7 mm for keratectomy (taken from 72 years old female donor with no prior history of corneal diseases or ocular trauma) and obtained

from Royal Victoria Infirmary, Newcastle, UK. Briefly, the corneoscleral tissue was cut into six small pieces and was grown in a 6-well plate containing CnT-07 medium (contains the same ingredients as CnT-20 medium which has been discontinued from the market) as explants cultures with the epithelial side directed downward for one week to isolate hLESCs. This method adopted technique used in other studies which applied human corneal limbal explant directly on denuded amniotic membrane with the epithelial side directed downward to facilitate limbal epithelial cells movement onto the amniotic membrane (Ban *et al.*, 2000; Kolli *et al.*, 2010; Pathak *et al.*, 2016). Following one week of culture, the explant tissue was removed and the isolated hLESCs were maintained using CnT-07 medium until reaching 70-80% confluence. The culture medium was changed every 2-3 days and hLESCS from passage 2 was used for the subsequent experiment.

3.2.3 Substrate softening treatment using collagenase and reseeding with hLESCs

Frozen whole bovine cornea was first immersed with PBS containing antibiotics to remove any dirt or potential source of contamination. Next, the epithelium was removed from the cornea by gentle scraping using sterile blade under the dissecting microscope. The deepithelised cornea was then washed with PBS 5 x 5 minutes before cutting it into quarters using sterile blade and transferred into a 6-well plate. These corneal pieces were then divided into 2 groups: control groups (untreated and negative control, in PBS) and treatment groups (with collagenase solution). The negative control group represents corneal scaffolds that neither underwent collagenase treatment nor re-seeded with hLESCs. The collagenase solution (Collagenase type-1, Invitrogen, 17018-029) isolated from *Clostridium histolyticum* which was used to soften the substrate was prepared by dissolving collagenase into phosphate-buffer solution (PBS) to make final concentration of 50 µg/ml. The treatment group was further divided into: i) superficial treatment (using filter paper) and ii) total treatment (submersion). The filter paper was first soaked in the collagenase solution for 5 minutes before placing it directly onto the epithelial side of the cornea, covering both limbal and central regions and ensuring a close contact between the soaked paper and the cornea. On the other hand, the submersion group placed the cornea piece into 10 ml of collagenase solution within the well. The cornea (from both untreated and treatment groups) was then incubated at 37°C in a

humidified 5% CO₂ incubator for one hour to activate the collagenase. This step was followed by vigorous washing with PBS using orbital shaker at 250 rpm 3 x 5 minutes. Then, all scaffolds were transferred onto transwell and were left under the hood for 1 hour to allow it to dry completely. Once dry, the hLESCs (from passage 2) were seeded onto the surface of each scaffold (excluding negative control group) at 5 x10⁵ cells/well in 6 ml of CnT-07 medium. The culture medium was changed every 2 days and the scaffolds were cultured for three weeks. Images were taken at the end of the experiment using inverted light microscope (Leica Microsystems, Wetzlar, Germany) to exhibit the presence of attached cells on each scaffold.

3.2.4 *In-Tissue Western assay*

In-Tissue Western (ITW) assay is a modified version of In-Cell Western™ assay, which is based on standard immunofluorescent methods. It is a quantitative immunofluorescence assay that incorporates both the specificity of western blotting with the reproducibility of ELISA. The detection of protein *in situ* is done by using target specific antibodies and IRDye® fluorescent secondary antibodies, and the target antibody signals are later viewed using Odyssey® Infrared Imager (Li-Cor Biosciences, Lincoln, NE, USA).

All tissues from collagenase treatment experiment (including controls) were fixed in 4% paraformaldehyde for 20 minutes, followed by tissue permeabilization using 0.1% Triton X-100 in PBS for 30 minutes. We used Odyssey PBS blocking buffer to block tissues for non-specific binding, and the tissues were incubated overnight with primary antibodies p63 and cytokeratin 3 (similar to antibodies in table 1) but at lower dilutions (1:500). Incubation with secondary antibodies was carried out for 1 hour using IRDye 800CW Goat-anti-rabbit-IgG (1:800, Li-Cor) and IRDye 680LT Goat-anti-mouse-IgG (1:800, Li-Cor). The detection of antibody targets in the 800 channels (green pseudocolour) and 700 channels (red pseudocolour) were performed using an Odyssey® Infrared Imager. For signal quantification, the antibodies signals were analysed using Image J v1.46 (National Institute of Health, Bethesda, MD). Statistical analysis was performed using ANOVA Tukey's multiple comparison post-hoc test for three independent experiments using Graphpad Prism 6. Results were expressed as relative expression (mean ± standard error of mean) of treated groups compared to untreated control.

3.2.5 *Histological study and immunofluorescence staining*

Histological examination using hematoxylin and eosin (H&E) and immunofluorescence staining were carried out as described in the previous chapter. The H&E staining was performed on decellularised bovine corneas following 21 days of recellularisation. Immunofluorescence staining was performed on cryo-sections taken from both decellularised corneas and corneas from collagenase-treatment at the end of their 21 days culture. Sections from decellularised corneas were incubated with primary antibodies against p63, ABCG2 and cytokeratin 3 (CK3), whereas sections from collagenase-treatment were incubated against p63, cytokeratin 14 (CK14, 1:50, Serotec) and CK3. Incubation with secondary antibodies was as previously described, with addition of Dylight 488 donkey anti-guinea pig (1:100, Jackson ImmunoResearch) against cytokeratin 14. The slides were finally counterstained with DAPI and analysed by fluorescence microscopy.

3.2.6 *Stratification Analysis*

The counting of cell layers from limbal epithelial stratification following recellularisation was performed using a double-blind method. Six images (from six different samples) of limbal epithelial cells on decellularised bovine corneas (at day 10 and day 21 of culture) taken from fluorescence microscopy at 20x magnification were used and analysed using Image J program. Each image was put on a grid and the number of cell layers was counted based on the number of cell nuclei stacked on top of each other. A two-tailed Student's t-test (unpaired) was performed to determine the statistical significance of these numbers (between cells at limbus and central cornea). This mode of analysis is used if the mean values of two normally distributed populations are equal. An unpaired test was carried out as each measurement and sample was independent i.e samples were not subjected to repeated measurements. Results were presented with a standard deviation of the mean where $p \leq 0.05$ was considered significant.

3.3 Results

3.3.1 *Bovine limbal epithelial stem cells differentiation on decellularised scaffold*

Hematoxylin-eosin (H&E) staining confirmed a better recellularisation result on ethanol-treated decellularised corneas using bovine LESC following a longer period of culture (figure 3.1). Over time, layers of bovine LESC were observed on the surface of the scaffolds, mostly in direct contact with neighbouring cells. Some areas within the new epithelium on the ethanol-treated bovine scaffold appeared detached from the underlying basement membrane as seen in figure 3, but this can happen as a result of staining procedure while handling the samples. Immunofluorescence staining revealed that bovine LESC attached near the basal layer of both limbus and central part of decellularised corneas were positively stained for p63 (figure 3.2a,b) but not CK3 (figure 3.2e,f), showing that these cells were still able to maintain their 'stemness'. The p63 staining was less apparent at the suprabasal layer of cells on both limbus and central cornea. The ABCG2 antibody staining was stronger near the basal layer of the limbus compared to the top-most suprabasal cell layer (figure 3.2c). This weak staining on the top-most suprabasal cell layer was noted to be similar to the ABCG2 staining near the central area of the cornea (figure 3.2d). Meanwhile, the recellularised corneas showed positive staining of CK3 (marker of differentiation) throughout the suprabasal cell layers in both the limbal and central area (figure 3.2e,f).

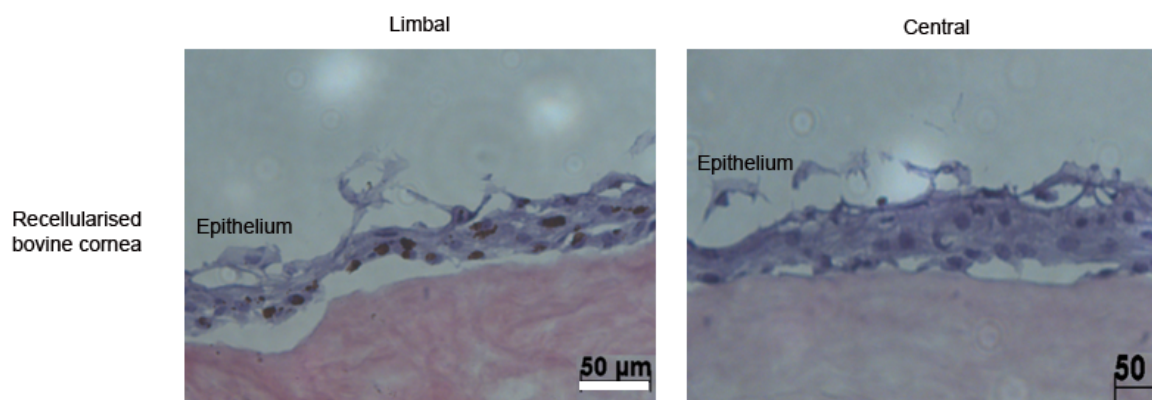


Figure 3.1 - Histological evaluation of recellularised bovine corneas using hematoxylin-eosin (H&E) staining at 3 weeks culture. All photographs were taken at x200 magnification. Scale bar = 50 µm.

3.3.2 Stratification analysis

Stratification analysis based on six different images taken from both the limbal and central cornea showed that the central cornea facilitated more cell layers compared to the ones near the limbus at day 10 and day 21 of culture (figure 3.3). At day 10, the central cornea possessed an average of 2 cell layers as compared to a single cell layer found on the limbus. After extending the tissue culture to three weeks, it is found that the number of cell layers in both areas has doubled, and that the difference in cell layers between the limbus and central cornea was consistent throughout the experiment.

3.3.3 Human limbal epithelial stem cells differentiation on collagenase-treated bovine corneas

Following 21 days of culture, we found that all bovine corneas were able to allow hLESCs attachment on both untreated and collagenase-treated groups (figure 3.4). All cells appeared small and rounded in shape and were in close contact with each other. At the same time, no significant difference in cell phenotype was observed under the inverted light microscope between the untreated and collagenase-treated corneas.

We extend the observation by assessing the level of p63 and CK3 proteins from all groups using In-Tissue Western (ITW) assay. Here, we focussed only at the central area of the corneas as the collagenase treatment was expected to modulate the stiffness of this area. We found that there was a striking increase in signal intensity of p63 antibodies from the total collagenase-treatment groups as compared to the others. On the other hand, the CK3 signals were very much similar in both untreated and collagenase-treated groups (figure 3.5). This was followed by quantification of signal intensity of targeted antibodies and normalised it against the untreated control group (figure 3.6). The p63 protein expression for superficial treatment group was almost similar to the untreated control group (1.067 ± 0.071), while the protein expression for total treatment group was increased three times compared to the untreated control (3.394 ± 2.616). Although the total treatment group exhibited a

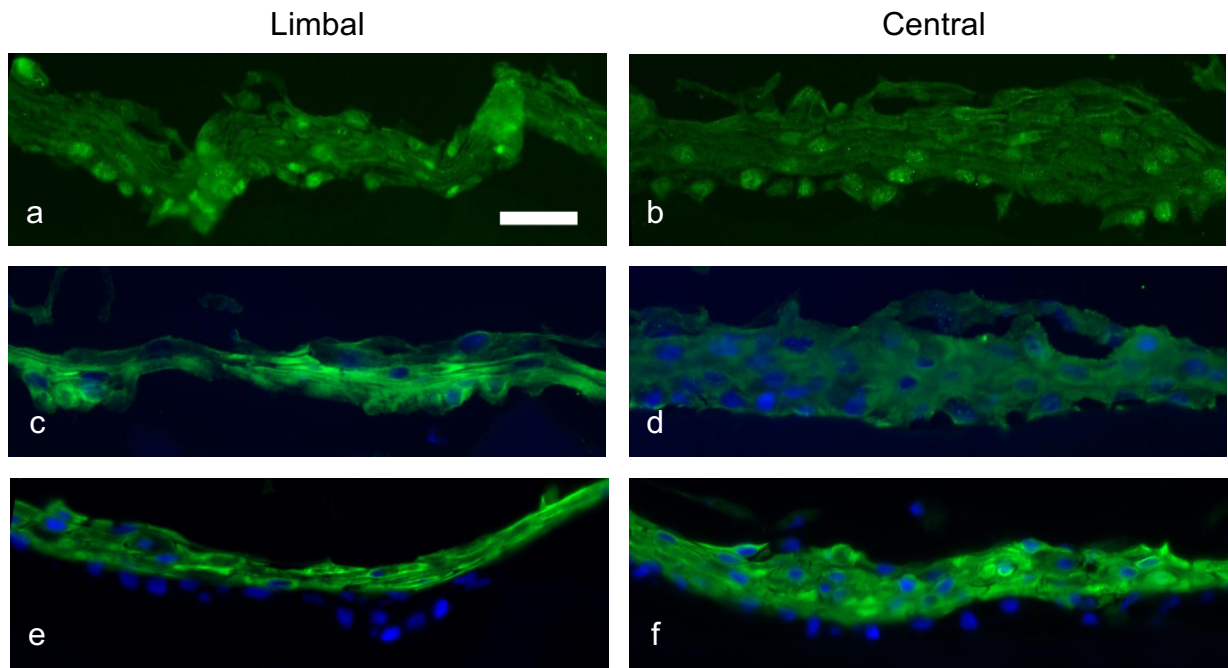


Figure 3.2 - Immunofluorescence staining of recellularised bovine corneas against p63 (a,b), ABCG2 (c,d) and K3 (e,f) antibodies; DAPI (blue) p63, ABCG2 and CK3 (green). All photographs were taken at x200 magnification. Scale bar = 50 μ m.

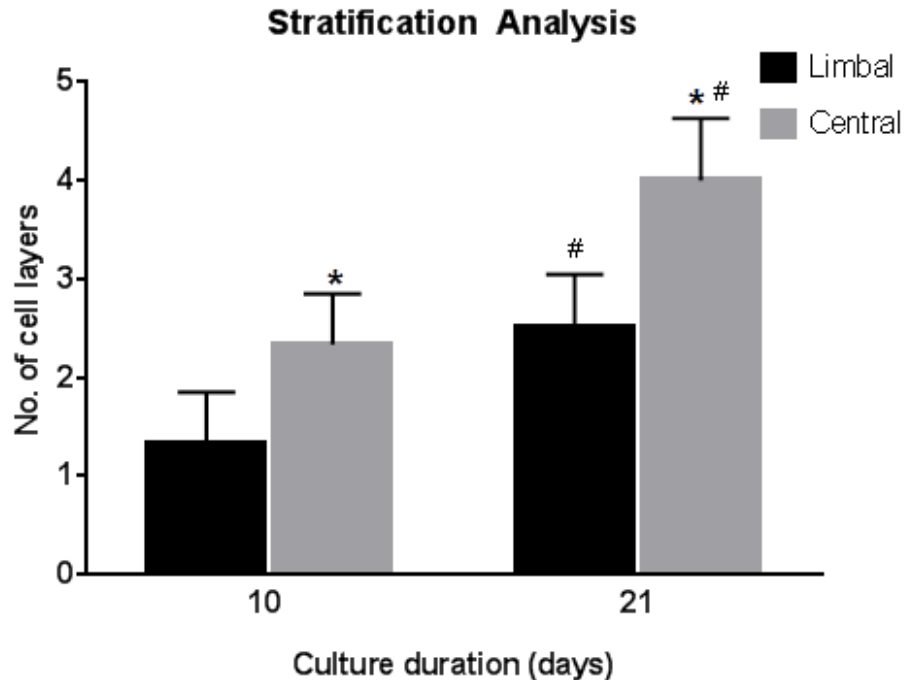


Figure 3.3 - Stratification analysis on recellularised bovine corneas (n = 6) following 10 days and 21 days of culture. The number of cell layers increased with time on both limbal and central cornea. * denotes significant difference between limbal and central cornea. # denotes significant difference of the same area between two time points (* corresponded to $p \leq 0.05$, # corresponded to $p \leq 0.05$).

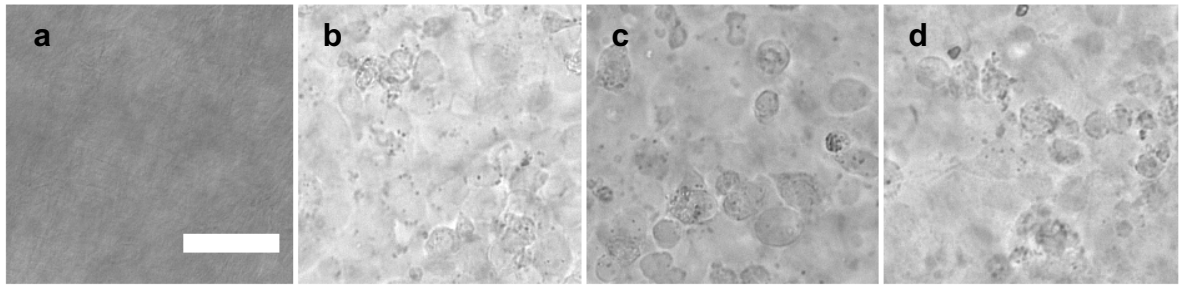


Figure 3.4 - Representative images of human LESC (hLESC) seeded on deepithelised bovine corneas with or without collagenase treatment on day 21 of culture using inverted light microscope. (a) Negative control (no cell), (b) untreated control, (c) superficial treatment, and (d) total treatment. The cells appeared small and rounded in shape and were in close contact with each other. All photographs were taken at x200 magnification. Scale bar = 50 μ m.

stronger p63 signals compared to other groups, these differences were not statistically significant. Meanwhile, the quantification for CK3 protein revealed a similar expression patterns between superficial treatment and total treatment groups as compared to the untreated control group (1.301 ± 0.737 and 0.913 ± 0.301 , respectively).

Immunofluorescence study revealed that hLESCs attached to both collagenase-treated group showed some positive staining of p63 in the cells nuclei (figure 3.7e, f, i, j). However, some weak p63 nuclei staining were also present amongst the cells attached to the untreated group (figure 3.7a, b). Interestingly, when the cells were tested with another putative LESC marker, CK14, there was a marked difference in staining between the collagenase-treated and untreated control groups. Cells from both superficial and total treatment groups showed cytoplasmic staining of CK14 in all cell layers (figure 3.7g, k), while no cytoplasmic staining was observed in all the cells found on the untreated group (figure 3.7c). This was a very significant finding as CK14 are usually not expressed by differentiated corneal epithelial cells. Meanwhile, the hLESCs on untreated group showed a very weak staining of CK3 (marker of differentiation) throughout the cell layers (figure 3.7d), whereas no CK3 staining was observed in both collagenase-treated groups (figure 3.7h, l).

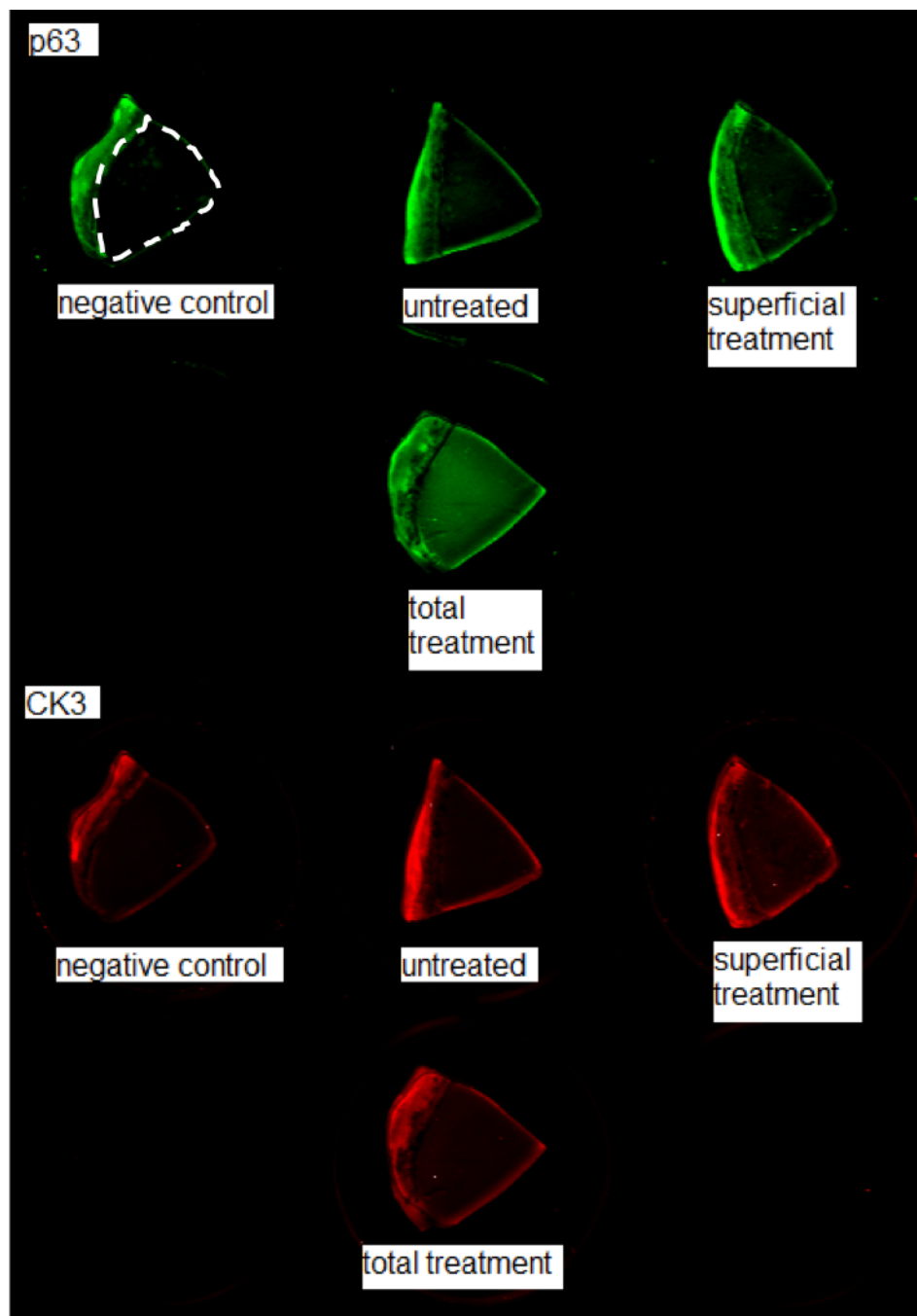


Figure 3.5 - Representative image of collagenase-treated bovine corneas (including controls) for In-Tissue Western assay. Bovine corneal pieces were placed in a 6 well plate in epithelial side-down position for signal viewing using Odyssey® IR scanner. Signals from anti-p63 appear as green fluorophores, while signals from anti-CK3 appear as red fluorophores. Only signals from central cornea area (outlined by the dotted lines) from each group were taken for quantification.

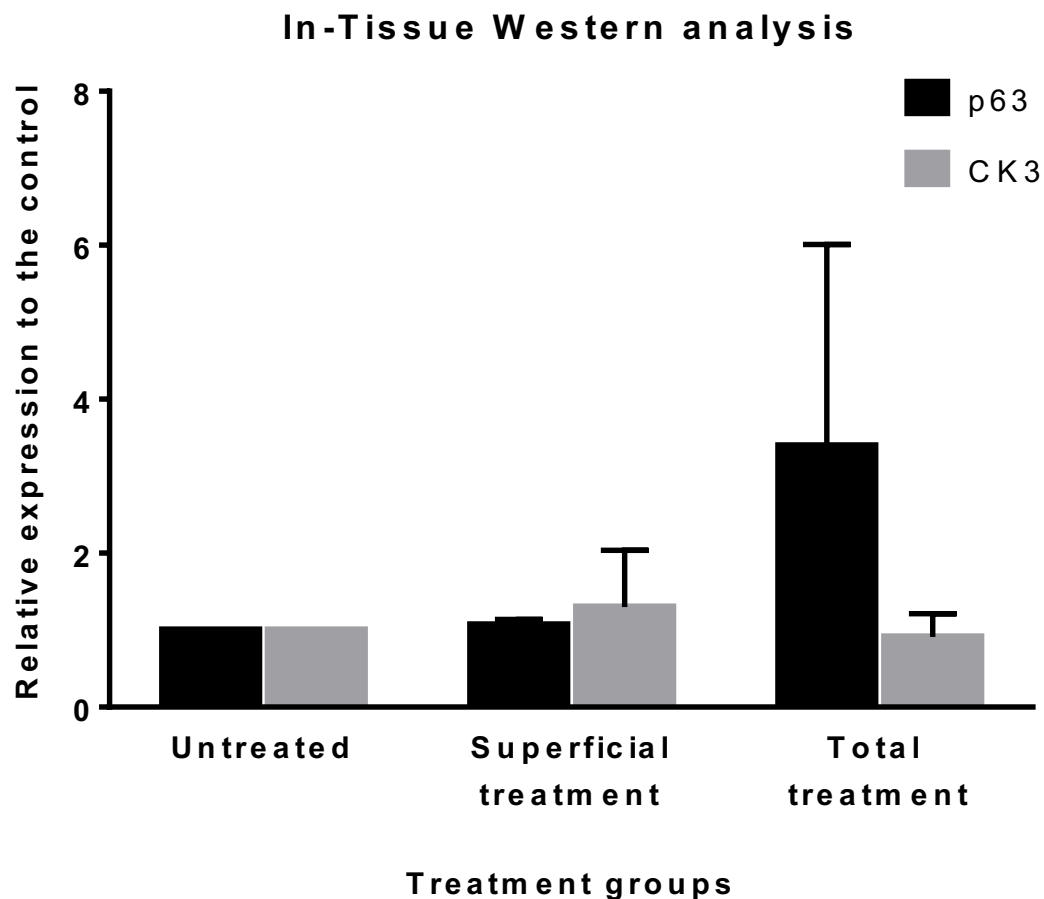


Figure 3.6 - Quantification of signals from In-Tissue Western assay. Signals from each group were calculated as an average from three independent readings. Normalised values are expressed as relative expression of each treated groups to the untreated control. Data (mean \pm SD) were obtained from three independent experiments (n=3) and compared using ANOVA with Tukey's post-hoc correction ($p < 0.05$).

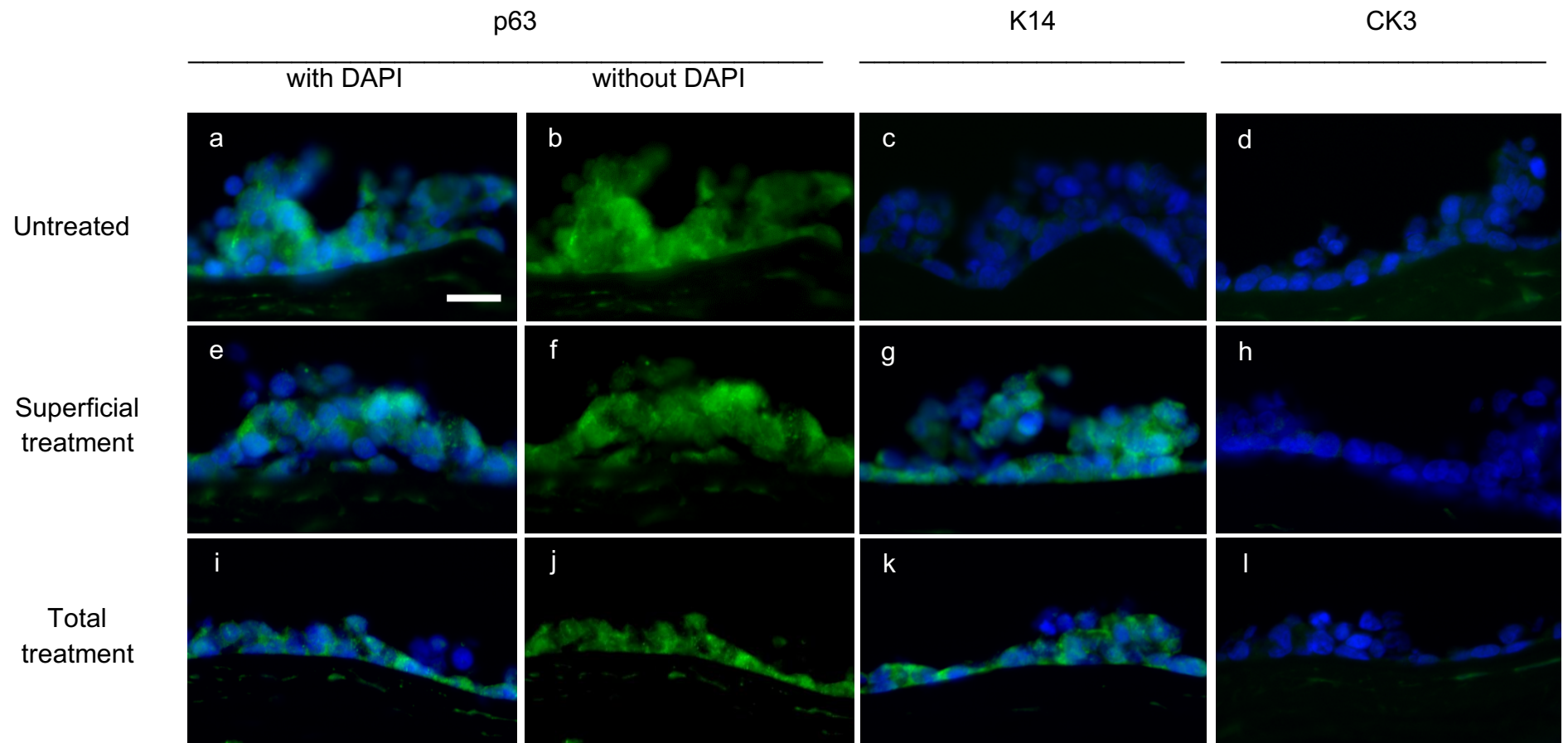


Figure 3.7 - Representative immunofluorescence staining showing the expressions of p63 (a-b, e-f, i-j), K14 (c,g,k) and CK3 (d,h,l) of untreated and collagenase-treated bovine corneas. Cell nuclei were stained with DAPI (blue). No DAPI staining was used for figures with p63 staining to highlight the nuclear staining within the cells. All photographs were taken at x200 magnification. Scale bar = 50 μ m.

3.4 Discussion

Ten years ago, Engler and his team opened up a whole new field of cell-matrix interaction by demonstrating the ability of cells, particularly stem cells (SCs), to sense their mechanical environment and to respond accordingly. Through their outstanding discovery, it is now known that mesenchymal stem cells (MSCs) are able to differentiate into different cell lineage by modulating the substrate stiffness they are plated on to mimic the elastic modulus of the tissue of interest. Since then, the effect of substrate stiffness on multipotent SCs expanded greatly; with more evidences pointing towards the success of stiffness manipulation in navigating the cellular fate of multipotent SCs (Evans *et al.*, 2009; Pek *et al.*, 2010; Park *et al.*, 2011; Kim *et al.*, 2015). Our cells of interest, the limbal epithelial stem cells (LESCs) are unipotent stem cells only fate to differentiate into corneal epithelial cells. Similar to multipotent SCs, LESCs behaviour (in terms of proliferative capability and committed differentiation) are influenced by the specialized niche within the native cornea (i.e the limbus) that surrounds the cells. This niche has been adapted by nature to accommodate these cells with all the necessary cues, including mechanical and biochemical cues that are unique for the appropriate functioning of limbal stem cells at all times (Li *et al.*, 2007).

The mechanical properties across the corneal regions have been investigated using a few different techniques. An earlier *in vitro* study by pressure loading which looked at regional elastic performance of the cornea involving eighteen human corneas has found that the elastic modulus of the central cornea is higher than the limbus in the meridional direction, but is vice versa when measured in circumferential direction (Hjortdal, 1996). This result is supported by another study on human cornea using Brillouin spectroscopy which also revealed a clear contrast between the stiffer centre and the softer limbus (Lepert *et al.*, 2016). However, this 'stiff-centre-compliant-limbus' notion has recently been challenged by Whitford *et al.* (2016) following their measurement of displacement across the surface of the eye using high-resolution digital cameras and digital image correlation software. Their results showed that the central cornea is stiffer than the peripheral cornea, and both were much more compliant than the limbus. Nevertheless, this study lacks sample size as they only tested on one human and one porcine eye, and further confirmation is needed involving larger number of test specimens. At the same time, this result may not be in

favour with several other studies which focussed on investigating the effect of stiffness on corneal epithelial cells using artificial substrates with varying stiffness.

A recent study by Moers *et al.* in 2013 using immortalized corneal keratinocytes showed cell differentiation when cultured on super-rigid substrate with non-physiological stiffness range as compared to physiologically-relevant elastic substrates. On the other hand, increasing the stiffness level of the underlying substrates showed obvious differentiation influence to the overlying limbal epithelial stem cells. These LESC lost their stemness over rigid substrates, as shown by strong K3 labelling (marker for corneal differentiation) on the superficial cell layer by immunofluorescent staining (Jones *et al.*, 2012) as well as nuclear localisation of Yap from both Western Blotting and immunofluorescent staining (Foster *et al.*, 2014). Interestingly, Foster and his team had also demonstrated similar Yap localisation in the cells' nuclei on epithelial cells residing in central cornea, while epithelial cells near the limbus showed cytoplasmic staining of Yap instead. An earlier study by Dupont *et al.* in 2011 has shown that cytoplasmic retention of Yap is involved in the cellular response to mechanical stimulation on soft, compliant substrates. Taken together, the relationship between Yap localisation and mechanical environment has put forward a hypothesis that the centripetal migration of corneal epithelial cells during corneal epithelium homeostasis is driven by the differences in the underlying stiffness between the limbus and central area of the cornea (Foster *et al.*, 2014). This is because the centripetal movement has always been unidirectional, but it is still not fully understood as to what extent this 'movement orientation' is influenced by the differences in stiffness underneath.

In our study, we moved a step forward by investigating the effect of substrate stiffness on LESC using decellularised cornea scaffold. We utilized the previously optimized decellularised bovine corneas and reseeded them with bovine LESC for three weeks. It has been shown previously that the three weeks' duration is enough for the cells to show any effect of differentiation and stratification even without air-lifting (Jones *et al.*, 2012). The remaining extracellular matrix (ECM) after decellularisation serves not only as structural support for the tissue, but also exerts both biochemical and mechanical cues to the newly recellularised cells, affecting the cells directly on cell proliferation, migration, differentiation and behaviour (Hoshiba *et al.*, 2010). Although decellularised matrices from various tissues have been used to

manipulate the direction of stem cell differentiation (Ross *et al.*, 2009; Flynn, 2010; Tedder *et al.*, 2010), to our knowledge this is the study that pioneers the use of decellularised cornea to elucidate the relationship between stiffness and LESC's fate.

Following the three weeks' culture, we found that some of the cells located at the basal layer at the limbus were still able to maintain their 'stemness', justified by their p63-positive and CK3-negative staining. Transcription factor p63 has been suggested as one of the possible stem cell marker for LESC's (Pellegrini *et al.*, 2001; Chen *et al.*, 2004b). Although the suprabasal cells attached to the limbal area showed negative staining of p63, and stained for CK3, this is very much similar to the normal bovine cornea as shown by the immunostaining results in the previous chapter. Furthermore, it has been shown that the nuclear p63 is abundantly expressed only in the basal layer of limbal epithelium (Pellegrini *et al.*, 2001), and CK3 is being expressed by suprabasal limbal epithelium and central corneal epithelial cells (Lauweryns *et al.*, 1993). On the other hand, the findings at basal cell layer of central cornea, which also stained positive for p63 and negative for CK3 similar to the ones near the limbus, may not support the hypothesis that the stiffness around the central cornea is the driving factor that induces LESC differentiation in this area. However, previous studies that used bLESC's and cultured them either on stiff collagen gels or amniotic membranes (intact or denuded) without the presence of keratocytes underneath for the same culture duration (three weeks) with or without airlifting had also reported similar negative staining of CK3 on the basal cell layer in their studies (Chen *et al.*, 2010; Mi *et al.*, 2010a; Jones *et al.*, 2012). Since our findings is contrary to the 'usual' basal layer of corneal epithelium which stains positive for K3 and negative for p63, this may suggest that the basal cells in normal central cornea are in a more advanced state of differentiation than those in our model.

Besides p63, another promising marker for LESC's that we tested was ABCG2. We found that both basal and suprabasal cell layers attached to the limbus stained strongly for ABCG2, but with markedly less staining on the top-most cell layer. This weak staining of ABCG2 was similarly found throughout the whole cell layers on central cornea. Previous studies have shown that ABCG2 is expressed by limbal basal and frequently also in suprabasal layers of limbal epithelium (Chen *et al.*, 2004b; Watanabe *et al.*, 2004b; de Paiva *et al.*, 2005; Dua *et al.*, 2005), but not in the central corneal and conjunctival epithelium (Schlötzer-Schrehardt and Kruse, 2005).

It is worth noting that the above studies were carried out on human corneas, and that our previous immunostaining on bovine cornea also showed weak staining of ABCG2 throughout the central epithelium. This may have suggested that the bLESCs attached to central cornea and the top-most suprabasal layer in the limbus have all differentiated and lost their stemness.

Cell stratification following recellularisation on our decellularised corneal model showed more cell layers found at the central cornea as compared to the ones at the limbus. Our lab has shown that more stratified epithelial cell layers were observed on stiffer substrates as compared to softer substrates (Chen *et al.*, 2012; Feng *et al.*, 2012; Jones *et al.*, 2012). However, no direct relation between mechanical stiffness and cell stratification has been linked together. Nevertheless, it has been suggested that an increase in cell proliferation can generate local stratification (Li *et al.*, 2016; Linde 1984). There have been extensive studies that found a strong link between cell proliferation and ECM rigidity, including on corneal epithelial cells (Jones *et al.*, 2012), fibroblast (Wang *et al.*, 2000; Hadjipanayi *et al.*, 2009), hepatocytes (Semler *et al.*, 2000), and neural stem cells (Saha *et al.*, 2008). An increase in matrix stiffness during breast tumor progression has been found to induce mammary epithelial cells to increase the expression of proliferative genes associated with cell proliferation (Provenzano *et al.*, 2009). It is possible that ECM rigidity might regulate cell proliferation by synergistically modulating mechanotransductive and mitogenic signalling pathways (Ulrich *et al.*, 2009). On the other hand, this may also support previous findings on durotaxis, in which cells are able to orient and migrate in response to gradients of elasticity (or stiffness), with preference for cell movement from softer towards stiffer substrate regions (Lo *et al.*, 2000). It is an interesting observation whereby the direction of cell migration is always towards the area of increasing stiffness and never in the opposite direction (Lo *et al.*, 2000; Wong *et al.*, 2003). ECM stiffness gradient may serve as a guide to stem cell migration to ensure that the cells can differentiate in the right place, possibly as part of the healing process (Engler *et al.*, 2006).

Taken together, it is important to highlight that newly-attached bovine LESC in the limbus were able to maintain their stemness as evidenced by their positive staining towards stem cell markers (p63 and ABCG2) and the absence of differentiation marker (CK3). This is a significant finding as it may indirectly prove that the limbus is

indeed a compliant compartment and that the newly attached bLESCs were able to maintain their stemness in the limbus due to the influence given by the underlying compliance substrate. The loss of ABCG2 signal strength of bLESCs attached on central cornea, together with a significant increase in cell layers in this area, may have suggested that the 'stiffer' central cornea is affecting the differentiation and proliferation capabilities of these LESC. However, since we failed to demonstrate the presence of differentiation marker in the basal cell layer near central cornea, it is still difficult to conclude whether the fate of LESC are directly affected by the underlying substrate stiffness.

Although alcohol may increase tissue stiffness via collagen crosslinking in the ECM, this effect was prominent when the alcohol was combined with acetone as shown by Lumpkins et al. in 2008. We postulate that if the stiffness of the scaffold has changed following exposure to ethanol during decellularisation, the shift of stiffness level would involve both the limbus and central regions as both were involved in this process. Therefore, the stiffness gradient between the limbus and central cornea would still maintain, but only to a higher value. We also believe that even if there was a change in the stiffness value in the limbal area following decellularisation, this change was not significant as it did not compromise the ability of the newly-attached bLESCs to behave as how they normally do in the normal cornea. However, since there was no measurement taken on the tissue stiffness before-and-after decellularisation, the effect of alcohol onto the stiffness level of our decellularised scaffolds remains inconclusive.

On a different note, results may also represent the idea that any changes to the underlying stiffness will only compromise the ability of the limbus to maintain the stemness of LESC if it reaches certain threshold which is high. Or, it takes more than just a shift in stiffness level of the basal cells in the central cornea to lose their 'stem cell' identity and become more like differentiated epithelial cells, for example crosstalk interactions between the basal epithelial cells and the underlying keratocytes. Therefore, we perform collagenase-treatment experiment to see if the LESC are able to maintain their stemness when they re-attach to central cornea that has been softened.

The use of collagenase in manipulating tissue stiffness has been considered in various *in vitro* studies including the heart (Majkut *et al.*, 2013), blood vessels (Dobrin and Canfield, 1984), cartilage (Lyyra *et al.*, 1999) and intervertebral disc (Barbir *et al.*, 2010). Even *in vitro* pathological studies like osteoarthritis (Grenier *et al.*, 2014) and uterine fibroid (Jayes *et al.*, 2016) used collagenase to soften the tissues of interest. Collagenase treatment has also been used in an attempt to soften the cornea (Wang *et al.*, 2014), and it has been proven that an increase in collagenase activity results in collagen degradation in rheumatoid corneal ulceration (Riley *et al.*, 1995). We believe the softening effect following collagenase treatment is directly due to degradation of collagen fibrils (collagen type I) within corneal stroma, and not due to loss of type IV collagen in the basement membrane. Levine *et al.* in 2014 set up an *in vitro* test to look at the activity of collagenase from *Clostridium histolyticum* on Peyronie's plaque explant, and found that the collagenase degrades collagen type I and III while sparing type IV collagen. This is because both types of collagen (type I and IV) possess different variation in the molecular structure, which in turn require different enzymes to degrade them (Harrington 1996). A preliminary study done by our group using collagenase-soaked filter papers which were placed in the middle of compressed collagen gels has found that the collagenase degraded collagen fibers underneath within the area of contact (unpublished data). We therefore extended the study by administering the collagenase using two different methods onto deepithelised bovine corneas and investigated its effect upon recellularisation with human limbal epithelial stem cells (hLESCs).

As mentioned previously, In-Tissue Western (ITW) assay is a modified version of In-Cell Western™ assay, which is based on standard immunofluorescent methods. It is a quantitative immunofluorescence assay that incorporates both the specificity of western blotting with the reproducibility of ELISA. The detection of protein *in situ* is done by using target specific antibodies and IRDye® fluorescent secondary antibodies, which are later viewed using Odyssey® Infrared Imager. This method provides a good alternative to conventional western blotting as the target protein can be tagged directly on tissues without the need for prior tissue processing. ITW is particularly useful for experiments with anticipated low number of cells attached to underlying tissue matrix as it can help to reduce false negative results secondary to cell loss during tissue handling and processing. The results from our In-Tissue Western assay and immunofluorescence staining showed that the hLESCs attached

to the central area of both collagenase-treated corneas were able to maintain their stemness as evidenced by the differences in p63 protein expressions as compared to the untreated control, as well as negative immunostaining staining of CK3. They also stained positive for p63 and cytokeratin 14 (CK14), which is another proposed limbal stem cell marker (Zhao *et al.*, 2008). CK14 has been used to identify epidermal stem cells (Bickenbach, 2005), and with respect to the limbal stem cells, the CK14 positive cells have been identified at both basal and suprabasal layers of limbal epithelium, and not in the central epithelium (Wang *et al.*, 2003; Zhao *et al.*, 2008). Taken together, these results suggested that the hLESCs did not undergo differentiation because the stiffness level of the central cornea has been modulated by the collagenase treatment. Unfortunately, the 'unforeseen' effect of corneal swelling has greatly influenced the results outcome of the untreated control group. Lepert *et al.* (2016) have recently demonstrated using Brillouin spectroscopy that bovine corneas undergo significant swelling following exposure to PBS over time, which also affect the tissue's overall elasticity. The study proved that as the swelling increases, the corneal tissue becomes softer which in turn, reduced the tissue stiffness. Both ITW assay and immunostaining results indicate that the hLESCs on the untreated control did not fully differentiate as how we expected. However, since they stained weakly for CK3, and negative for CK14, we believe that some degree of differentiation had still taken place along the way.

There are several ways that can be considered to overcome the corneal swelling problem to optimise the corneal condition for collagenase treatment study. Firstly, the cornea can be stiffened via collagen or photochemical cross-linking prior to collagenase treatment. The stiffening effect from collagen crosslinking has been shown to alter the swelling properties of porcine cornea by increasing the corneal resistance to swelling (Wollensak *et al.*, 2007). Nevertheless, the exposure time to enzymatic (collagenase) treatment may need to be increased as the cornea becomes more resistant to collagenase digestion (Spoerl *et al.*, 2004). Secondly, the swelling may also be controlled by adding dextran into the culture medium. Supplementation of 5% dextran during corneal decellularisation using 1% Triton-X 100, 0.5% sodium dodecyl sulfate (SDS) and nucleases resulted in significant reduction in corneal swelling compared to decellularised cornea without dextran (Lynch *et al.*, 2016). However, the use of dextran in cultures containing epithelial cells may require

extensive consideration as dextran seems to have a negative effect on the conditions of epithelial cells (Salla *et al.*, 1995).

In conclusion, it is feasible to use decellularised cornea as scaffold to study the mechanical cues which may influence the behaviour corneal epithelial cells, in particular the corneal stem cells. The LESC's appear to be more sensitive towards softer substrate as compared to stiffer substrate. Since the corneal stem cells source comes from the limbus alone, this sensitivity towards compliant environment might be one of the ways the nature provides to protect these cells from getting easily differentiated especially when stiffness shift occurs in the limbus. The relationship between substrate stiffness and corneal stem differentiation needs to be explored further as it can be a useful insight for controlling cell fate and generating regenerative therapies.

Chapter 4. Retinoic acid as a powerful serum-free medium supplement to maintain quiescent keratocytes in 3D stromal construct

4.1 Introduction

Cells are dependent on signals provided by the extracellular matrix which control their processes and ultimately dictate their fate (Badylak, 2007; Hynes, 2009). These signals, be it mechanical (stiffness, matrix porosity, topography, *etc.*) or biochemical (peptides, growth factors, cytokines, *etc.*) determine greatly the way cells respond during cell adhesion, proliferation, migration and differentiation (Genzer and Bhat, 2008; Seidi *et al.*, 2011; Wu *et al.*, 2012b). The effects of mechanical stiffness of decellularised scaffolds on corneal epithelial stem cells differentiation have been discussed in detail in the previous chapter. Chemical cue is an important element in the control and maintenance of the corneal stromal cells (or keratocytes) phenotype in order to investigate its biology *in vitro* (Berryhill *et al.*, 2002). Biochemical cues have been effective for the differentiation of various cell lines (Jaiswal *et al.*, 1997; Capo-chichi *et al.*, 2005; Cho *et al.*, 2010), but the optimal use of supplements and additives for corneal stromal cell culture in order to maintain them in their natural, quiescent phenotype remains a considerable challenge.

The addition of serum to stromal cell culture medium helps to accelerate cell proliferation, but at the same time results in the differentiation of keratocytes to fibroblasts with altered physiological and biochemical properties namely its phenotype, keratan sulfate production and the expressions of certain genes such as α -smooth muscle actin, aldehyde dehydrogenase (ALDH) and fibronectin (Jester *et al.*, 1996; Beales *et al.*, 1999; Fini, 1999). On the other hand, the use of serum-free medium has been shown to maintain the quiescence phenotype but does not support keratocytes proliferation (Barry *et al.*, 1994; Beales *et al.*, 1999; Berryhill *et al.*, 2002) thus making cultivation of large quantity of cells by subculturing a difficult task. Therefore, supplementation is required in keratocytes culture medium in order to promote cell growth and proliferation without encouraging fibroblastic differentiation (Wilson *et al.*, 2012). Previous studies have tested several media formulations that allow corneal stromal cells to proliferate and survive for extended period in culture while maintaining the expression of keratocyte-specific markers. In particular, fibroblast growth factor 1 (FGF-1) (Maltseva *et al.*, 2001), FGF-2 (Long *et al.*, 2000),

FGF-2 + heparin (Maltseva *et al.*, 2001), and transforming growth factor β 3 (TGF- β 3)(Karamichos *et al.*, 2014) have been used to reverse the serum-induced (myo)fibroblast transformation. Others focussed on medium supplements which includes ascorbic acid (Guo *et al.*, 2007a), insulin (Musselmann *et al.*, 2006), growth factors (Builles *et al.*, 2006), cytokines (Jester and Ho-Chang, 2003) and low glucose (Foster *et al.*, 2015). Our previous work too has demonstrated the capability of retinoic acid (RA) as an important supplement to culture keratocytes in serum-free conditions that support cell growth and proliferation in 2D environment (Gouveia and Connon 2013).

The human body produces retinoic acid (RA), an active form of vitamin A, through two sequential oxidation steps: from retinol (absorbed by intestinal mucosa cells following dietary ingestion) to retinaldehyde, and then to RA (Samarawickrama *et al.*, 2015). Retinoic acid plays a crucial role in vertebrate development, cellular differentiation and homeostasis (Hofmann and Eichele, 1994; Kastner *et al.*, 1994; Lohnes *et al.*, 1994) and is essential to ocular health. In cornea, both retinol and retinoic acid binding proteins have been located in corneal cells (Wiggert *et al.*, 1977; Wiggert *et al.*, 1978), and take up both retinol and retinoic acid (Wiggert *et al.*, 1982). Corneal epithelial cells require vitamin A for glycoprotein synthesis (Hassell *et al.*, 1980), by which a deficiency to vitamin A results in abnormal keratinization, epithelial squamous metaplasia and deficient conjunctival goblet cells (Hatchell and Sommer, 1984; Smith and Steinemann, 2000). Other *in vitro* studies have demonstrated that RA modulates proliferation and differentiation of limbal epithelial stem cells in dose-dependent manner (Kruse and Tseng, 1994; Kim *et al.*, 2012). At the same time, researchers have also found the potential use of RA in improving corneal wound healing through faster epithelialization (Mehra *et al.*, 1982; Johansen *et al.*, 1998; Hattori *et al.*, 2012).

With regard to corneal stromal cells, different studies have come out with different results to signify the effects that RA gave to these cells. For example, the biosynthesis of extracellular matrices by cultured rabbit corneal fibroblasts is altered in response to RA, but showed no changes in the cell morphology (Kenney *et al.*, 1986). A later study by Kirschner *et al.* in 1990 reported that RA caused morphological changes to rabbit stromal fibroblasts *in vitro* but has limited effects on cell proliferation. Interestingly, our previous study have found that by supplementing

keratocytes culture medium with RA at specific concentration helped to improve cell proliferation, increased proteoglycans secretion as well as significantly reduced the matrix metalloproteases (MMP) production (Gouveia and Connon, 2013).

In this study, we extended the corneal keratocytes experiment into a 3D compressed collagen construct to investigate the use of RA supplementation in the generation of potential biologically relevant tissue-engineered corneal stroma. Specifically, we were interested to know if RA supplementation can help to improve the quality of our compressed collagen gels by ‘manipulating’ keratocytes embedded within these gels. We considered its effect on cell viability, collagen production, contraction assay and hydration of tissue constructs, as well as the expression of keratocyte-characteristic markers at both transcriptional and protein level.

4.2 Materials and methods

4.2.1 *Preparation of human keratocyte*

Human corneal keratocytes were isolated from human corneas ($n = 3$) obtained from Royal Berkshire Hospital, Reading, UK and Royal Victoria Infirmary, Newcastle, UK (donors’ age between 39 and 76; average \pm S.D. = 61 ± 12 years, male-female donor ratio 2:3 with no prior history of corneal diseases or ocular trauma). Briefly, corneal stroma from epithelia-depleted human limbal tissue were minced using a scalpel, solubilised using collagenase digestion [DMEM:F12 medium (Invitrogen), 5% fetal bovine serum (FBS; Gibco Invitroge), 2 g/L (450 units/ml) collagenase type-1 (Invitrogen)] for 5 hours under continuous rotation at 37°C , followed by incubation with 0.25% trypsin-EDTA (Gibco Invitrogen) for 10 minutes. The isolated keratocyte were initially cultured in tissue culture flasks (Greiner Bio-One) with serum-containing culture medium [DMEM:F12 medium, 5% FBS, 1×10^{-3} M ascorbic acid (Sigma-Aldrich), 1 x ITS liquid media supplement (Sigma-Aldrich), 1% penicillin/streptomycin (Invitrogen)] until reaching 70-80% confluence. Medium was then replaced with serum-free culture medium (SF) to induce quiescence as previously described (Gouveia *et al.*, 2014; Foster *et al.*, 2015), and passaged after three days. Each subsequent experiment was performed three independent times using keratocytes from passages three to five from specific donors.

4.2.2 Formation of compressed collagen gels with embedded keratocytes

Collagen gels were prepared as previously described (Foster *et al.*, 2014) by mixing 2 ml sterile rat-tail collagen type I (2.2 mg/ml in 0.6% acetic acid, First Link Ltd.) and 0.5 ml modified Eagle's minimum essential medium (MEM; Fisher Scientific) in a centrifuge tube which was placed on ice. Sodium hydroxide, 1 M (Fisher Scientific) was added drop-wise to neutralise the pH of the mixture, along with intermittent, gentle mixing of the solution until it changed its colour from yellow to light pink. The mixture was then cast into 12-well plate moulds (Greiner Bio-One), along with 2.5×10^5 serum-deprived keratocytes in each well prior to polymerization (gelling), which subsequently took place for 30 minutes at 37°C in a humidified 5% CO₂ incubator. The polymerised collagen gels were placed in between layers of nylon mesh, and compressed at room temperature using a 134g piece of thick glass for 5 minutes. The resulting compressed collagen gels, embedded with keratocytes were then transferred into 6-well plates (Greiner Bio-One) and cultured using SF medium supplemented with either 1×10^{-7} M retinoic acid dissolved in dimethyl sulfoxide (DMSO) (RA, Sigma-Aldrich) or equivalent volume of DMSO vehicle (control; Fisher Scientific) for 30 days. The culture medium was changed every 2-3 days.

4.2.3 Viability assay

The alamarBlue® assay was used to assess keratocytes' viability within the collagen gels with or without supplementation of RA in SF medium for 30 days. The gels were incubated with resazurin reagent (Sigma-Aldrich; prepared in 1:10 dilution using fresh culture medium) for 2 hours at 37°C, after which 100 µl of culture supernatants (in triplicate) were sampled for fluorescence emission analysis at 590nm using Fluoroskan Ascent fluorescent spectrophotometer (Thermo Labsystems, Franklin, MA). The gels were washed extensively with phosphate buffer solution (PBS) before replenishing them with fresh media. Cell viability was calculated by interpolation using a standard curve for fluorescence values of 1, 5, 10, 20, 50, and 100 x 10⁴ cells. All values correspond to average ± SD of 3 independent experiments based on assays done on days 3, 7, 14, 21 and 30 of culture.

4.2.4 Hydration study

The weight of all cell-encapsulated collagen gels from RA-supplemented and control groups were measured before starting the experiment to find the initial weight (W_i), and similarly after the 30-day culture period has ended for the final weight (W_f). The ratio of weight increment (wet weight) was then calculated using the following equation (Equation 1).

$$\text{Equation 1: Wet weight (ratio)} = \text{final weight } (W_f) / \text{initial weight } (W_i)$$

Next, we used a freeze-drying method to sublimate frozen water from the solid to gas phase under controlled pressure in order to determine the percentage of water bound to these gels. The weight of all freeze-dried gels was again measured to find the dry weight (W_d), which was later used to find the hydration percentage of all gels using the following equation (Equation 2).

$$\text{Equation 2: Hydration (\%)} = (W_f - W_d / W_f) \times 100$$

This experiment was performed three independent times, using five replicate gels.

4.2.5 Gel contraction assay

Digital photography was used to image the compressed collagen gels at the beginning of the experiment and on day 30 of culture. The images were then analysed using Image J v1.46 (National Institute of Health, Bethesda, MD). For each experiment, three compressed collagen gels containing keratocytes cultured in serum-free medium (with RA or DMSO control) were evaluated and the average contraction was determined by calculating the percent decrease in surface area compared to the original gel surface area.

4.2.6 qPCR on keratocyte markers

DNase-treated RNA isolated from constructs using standard Trizol (Invitrogen) extraction was performed following 30 days of culture. The assessment of RNA quality was performed using Nanodrop 2000 spectrophotometer (Thermo Scientific, UK) to ensure the 260/280 ratio was within the range of 1.7 to 2.0. The isolated total RNA (1 μ g) was reverse-transcribed to cDNA using the RT2 First Strand kit (Qiagen)

according to the manufacturer's protocol, in a TcPlus thermocycler (Techne, Staffordshire, UK). Quantitative polymerase chain reaction (qPCR) of cDNA was carried out with direct dye binding (SYBR Green, Thermo Fisher) using the default thermal profile of the Eco Real-Time System (Illumina, San Diego, CA), with the following 40 x three-step cycles: 10-second denaturation, 95°C; 30-second annealing, 60°C; 15-second elongation, 72°C. The relative expression of keratocytes genes coding (summarized in table 2) were calculated by the comparative threshold cycle (CT) (Eco Software v3.1; Illumina) and normalized to the expression of the POLR2A housekeeping gene using the following equation:

$$\Delta Cq \text{ Normalize} = Cq (\text{gene of interest}) - Cq (\text{housekeeping gene})$$

$$\Delta Cq \text{ Expression} = 2^{-\Delta Cq}$$

Results from three independent experiments from three different donors were normalized relative to the expression from compressed collagen gels embedded with keratocytes cultured using DMSO control medium.

4.2.7 Western Blotting

The expression of keratocan, lumican and decorin proteoglycans, ALDH1 and ALDH3 crystallins, carbohydrate sulfotransferase 6 (CHST6), Collagen Type I and V, MMP1 and MMP9 proteases, fibronectin and α -smooth muscle actin (α SMA) were analysed from day 30 compressed collagen gels embedded with keratocytes in both RA-treated and control groups lysates using ice-cold RIPA lysis buffer supplemented with 1x Proteases Inhibitors Cocktail (Roche) for 10 minutes. Both human donor cornea (CS) and keratocytes grown for 7 days in serum-containing medium (FBS) were used as controls. After precipitation with 4x volumes of ethanol and pellet resuspension in sample buffer, lysates were run by reducing SDS-PAGE using 10% Mini-Protean precast gels (Bio-Rad) and blotted onto polyvinylidene difluoride (PVDF) membranes (Thermo Scientific). Membranes were then blocked in Tris-buffered saline (Fisher Scientific) supplemented with 5% bovine serum albumin (First-Link) and 0.1% Tween 20 (Fisher Scientific), and incubated with primary antibodies listed in table 3. This was followed by corresponding horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat secondary antibodies (Vector Labs, PI-1000, PI-2000 and PI-9500, respectively) diluted 1: 1000 in PBS. Anti-tubulin was used for

| Gene | Primers 5'→3' |
|----------------------------------------|----------------------------------|
| <i>POLR2A</i> | F: cat cat cga aga caa tgg tg |
| (housekeeping gene) | R: aac aat gtc ccc atc aca ca |
| <i>Keratocan</i> | F: tat tcc tgg aag gca agg tg |
| (<i>KERA</i>) | R: acc tgc ctc aca ctt cta gac c |
| <i>Lumican</i> | F: cct ggt tga gct gga tct gt |
| (<i>LUM</i>) | R: tag gat ggc ccc agg a |
| <i>Decorin</i> | F: aat tga aaa tgg ggc ttt cc |
| (<i>DCN</i>) | R: ctg ctg att ttg ttg cca tc |
| <i>Aldehyde dehydrogenase1A1</i> | F: ctc tca ctg ctc tcc acg tg |
| (<i>ALDH1A1</i>) | R: gag aag aaa tgg ctg ccc ct |
| <i>Aldehyde dehydrogenase3A1</i> | F: ccc ctt caa cct cac cat cc |
| (<i>ALDH3A1</i>) | R: gtt ctc act cag ctc cga gg |
| <i>Carbohydrate sulfotransferase 6</i> | F: acg acg ttt ggg agc ctt t |
| (<i>CHST6</i>) | R: tag agg ttc ctc agc acc cca |
| <i>Collagen Type V</i> | F: atc ttc caa agg ccc gga tg |
| (<i>COL5A</i>) | R: aaa tgc aga cgc agg gta ca |
| <i>Matrix metalloproteases 1</i> | F: agg tct ctg agg gtc aag ca |
| (<i>MMP1</i>) | R: ctg gtt gaa aag cat gag ca |
| <i>Matrix metalloproteases 3</i> | F: tgc ttt gtc ctt tga tgc tg |
| (<i>MMP3</i>) | R: aag ctt cct gag gga ttt gc |
| <i>α-smooth muscle actin</i> | F: ctg agc gtg gct att cct tc |
| (<i>ACTA2</i>) | R: ttc tca agg gag gat gag ga |

Table 2. Description of primers used in qPCR for keratocyte's gene expression analysis.

protein loading normalization. Quantification was performed by densitometry analysis of imaged bands using Image J v1.46 (National Institute of Health, Bethesda, MD).

| Antigen | Host | Antibody | Dilution | Source | Catalogue number |
|-------------------|--------|-------------------------|----------|------------------------------------------------------------------|------------------|
| Keratocan | Rabbit | Anti-keratocan | 1:500 | Santa Cruz | sc66941 |
| Lumican | Mouse | Anti-lumican | 1:500 | Kindly given by Dr Bruce Caterson, Cardiff School of Biosciences | - |
| Decorin | Goat | Anti-decorin | 1:500 | Calbiochem | PC673 |
| ALDH1A1 | Rabbit | Anti-ALDH1A1 | 1:500 | Abcam | ab23375 |
| ALDH3A1 | Rabbit | Anti-ALDH3A1 | 1:500 | Thermo Scientific | 10304480 |
| CHST6 | Rabbit | Anti-CHST6 | 1:500 | Abcam | ab154332 |
| MMP1 | Rabbit | Anti-MMP1 | 1:500 | Abcam | EP1247y |
| MMP9 | Rabbit | Anti-MMP9 | 1:500 | Abcam | EP1254 |
| α SMA | Mouse | Anti- α SMA | 1:500 | Vector Lab | VPS281 |
| Fibronectin | Mouse | Anti-fibronectin | 1:500 | Vector Lab | VFP705 |
| α -tubulin | Rabbit | Anti- α -tubulin | 1:500 | Abcam | ab4074 |

Table 3. Details of primary antibodies used for western blotting.

4.2.8 Immunofluorescence staining

The immunofluorescence staining was performed by sectioning the corneal constructs in OCT using the cryotome and collected them on adhesion glass slides as previously described in chapter 2. Sections were then incubated overnight at 4°C with primary antibodies against keratocan, lumican, ALDH1A1, Collagen type I and V (same as used for Western blotting). Incubation with secondary antibodies was carried out for 1 hour using Alexa Fluor® 488-conjugated goat anti-mouse (A11001) or anti-rabbit (A11008) diluted in PBS (1:200, Life Technologies). Finally, the slides were imaged by fluorescence microscopy (Carl Zeiss Meditec, Germany).

4.3 Results

4.3.1 *Effects of RA on keratocytes viability*

The alamarBlue® assay showed a steady increase in cell number in both RA-supplemented and control groups from day 3 to day 21 (figure 4.1), and showed comparable values between both groups. However, only the cells in RA-supplemented group remained viable significantly until day 30, while keratocytes in the control group showed a decline in cell number. Hence, day 30 was taken as the day for further assessment on the expression of keratocytes markers as well as other parameters.

4.3.2 *Contraction assay analysis*

We used digital images of the engineered stromal constructs to calculate the percentage change in surface area relative to their initial surface area following 30 days of culture (figure 4.2a). The engineered stromal constructs cultured in RA-supplemented medium successfully maintained more than 95% of their surface area at the end of the experiment (figure 4.2b). On the other hand, the surface area of the constructs cultured in serum-free medium with DMSO (control) were reduced by more than 10% of their original surface area. The differences in gel size within the control group was significant for the duration of the experiment ($p < 0.01$).

4.3.3 *Hydration studies*

Interestingly, there was a significant increase in the wet weight of collagen gels containing keratocytes supplemented with RA as compared to the control after 30 days (figure 4.3, left panel). Not only that, the results also showed that collagen gels in the control groups reduced their initial weight following the 30-day culture period. The dry weight of freeze-dried gels from the RA-supplemented group was also found to be significantly higher by 18% than the controls (figure 4.3, central panel). At the same time, the hydration percentage within RA-supplemented collagen gels showed to be higher compared to the collagen gels from the control group (84 ± 1 and $80 \pm 2\%$, respectively; figure 4.3, right panel).

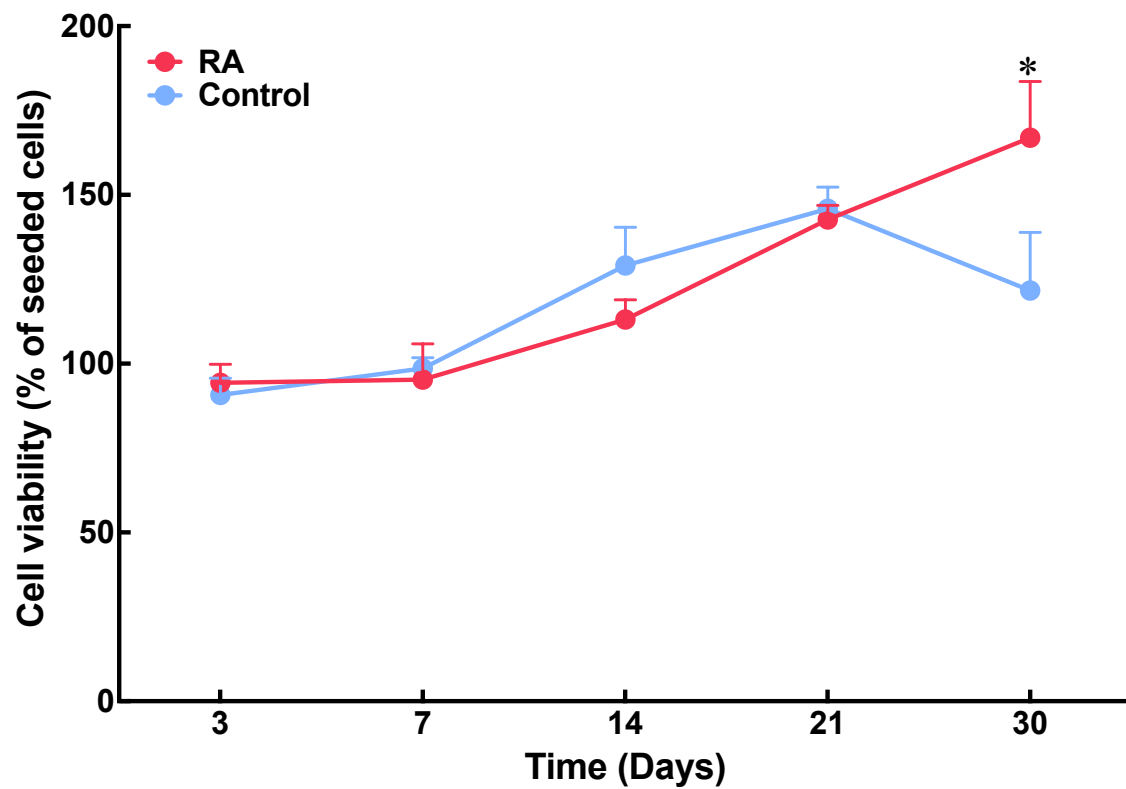


Figure 4.1 - Cell viability as quantified by alamarBlue® assay. The number of viable cells were normalized as a percentage of cell initially seeded. Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared using t-test (* corresponds to $p < 0.05$).

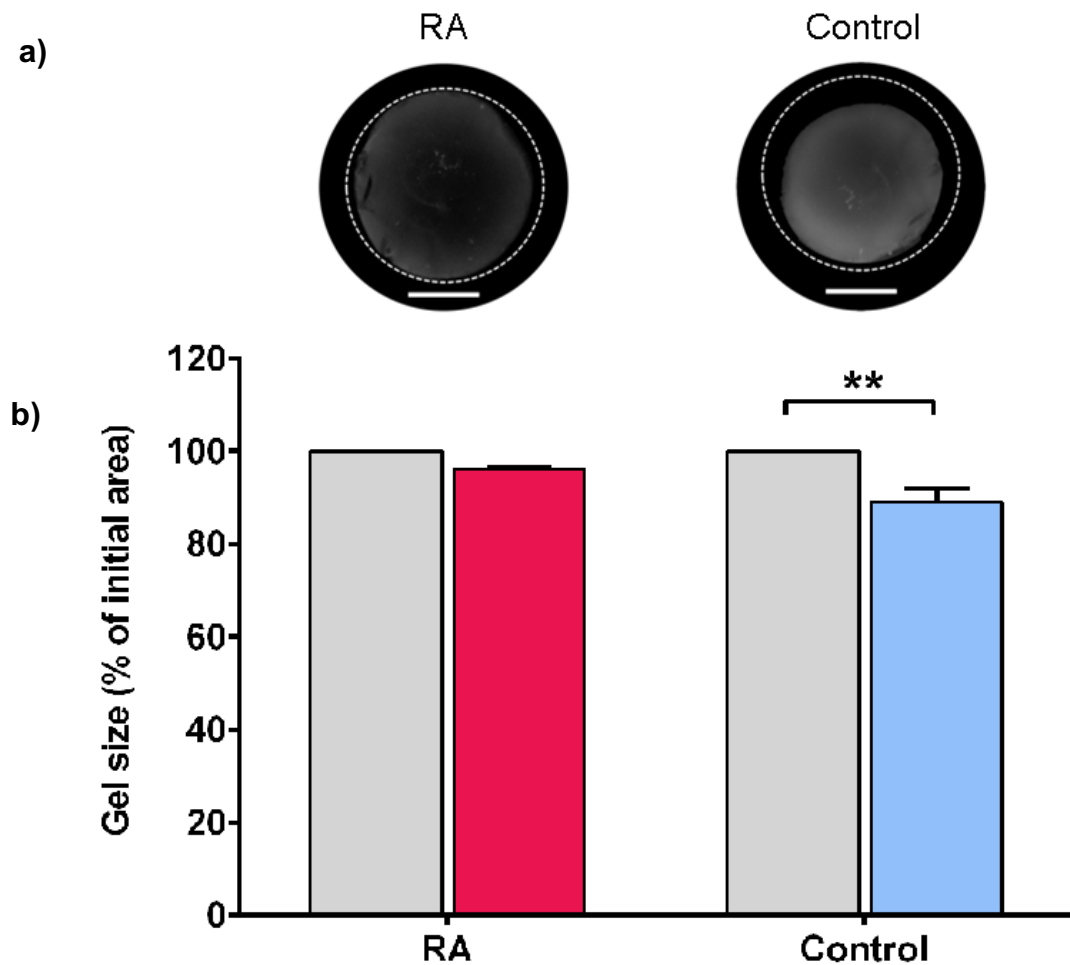


Figure 4.2 - Contraction assay. **(a)** Contraction assay of collagen constructs embedded with corneal keratocytes and cultured in serum-free medium (with RA supplementation or DMSO control). The outer dotted line represents the size of the gel at the beginning of the experiment. Scale bar = 50 μ m. **(b)** The gel size (in percentage) was determined by calculating the difference in constructs' surface area between the initial day of experiment (grey bar) and day 30 (coloured bar). Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared using t-test (** correspond to $p < 0.01$).

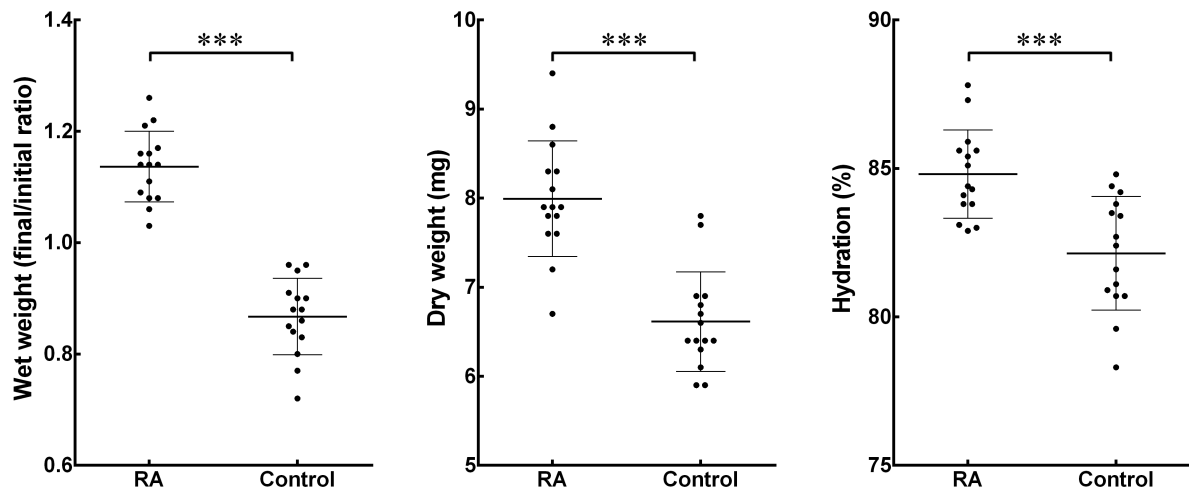


Figure 4.3 - Effects of RA supplementation on the hydration study of cell-encapsulating compressed collagen gels. The weight of all gels was measured before and after 30 days culture period to find the differences in the wet weight. The dry weight was quantified following freeze drying of the gels. The percentage of hydration denotes the loss of bound water from the collagen gels with or without supplementation of RA. Data (mean \pm SD) were obtained from three independent experiments ($n = 5$) and compared using t-test (***) correspond to $p < 0.001$.

4.3.4 Expressions of keratocyte markers

Several important keratocytes and fibroblastic markers were analyzed at the gene transcript level and compared with the control. Results showed that RA treatment significantly increased transcription of genes coding for key corneal proteoglycans (keratocytes markers - *keratocan*, *lumican* and *decorin*) by, 1436 ± 12 -, 6410 ± 37 -, and 1510 ± 5 - percent of the control, respectively (figure 4.4). In addition, transcription levels of genes coding for corneal crystallins (*ALDH1A1* and *ALDH3A1*), *CHST6* and collagen type V (*COL5A*) were also studied and shown to increase by 1181 ± 7 -, 3826 ± 38 -, 596 ± 1 - and 941 ± 8 - percent of the control, respectively. On the other hand, genes coding for two fibroblastic markers for matrix metalloproteases, *MMP1* and *MMP3* were significantly down regulated to 6.6 ± 0.04 - and 5.2 ± 0.04 -percent respectively when compared to the control (figure 4.4). No difference was observed for transcripts of *ACTA2*, the gene coding for α SMA (α -smooth muscle *actin*) which is another fibroblastic marker.

Furthermore, these data were supported by protein expression levels analyzed by immunoblotting and quantified by densitometry. RA-supplemented keratocytes

showed significant percentage-increased expression for keratocan (197.4 ± 0.5), lumican (251.7 ± 0.5), decorin (321.2 ± 0.5), collagen type I (210.4 ± 0.8), collagen type V (198.5 ± 0.3), ALDH1A1 (575.9 ± 2.9), ALDH3A1 (689.0 ± 3.5), CHST6 (301.2 ± 0.6) relative to the control (figure 4.5). Similar to gene transcript levels, the expressions of some fibroblastic markers including MMP1 and MMP9 in RA supplemented group were significantly reduced to 7.4 ± 0.08 - and 10.1 ± 0.07 -percent of the control levels, respectively (figure 4.5). In addition, α SMA and fibronectin levels (another fibroblastic marker) were not affected by RA supplementation, and remained residual compared with the heightened levels normally seen in FBS-activated fibroblasts.

The localizations of these proteins, shown by immunofluorescence study, were not substantially modified by RA supplementation despite their stronger detections. However, localization of collagen type V was shown to be similar throughout the depth of collagen gels in both groups despite its stronger detection in the RA-supplemented group at both transcriptional and protein levels (figure 4.6).

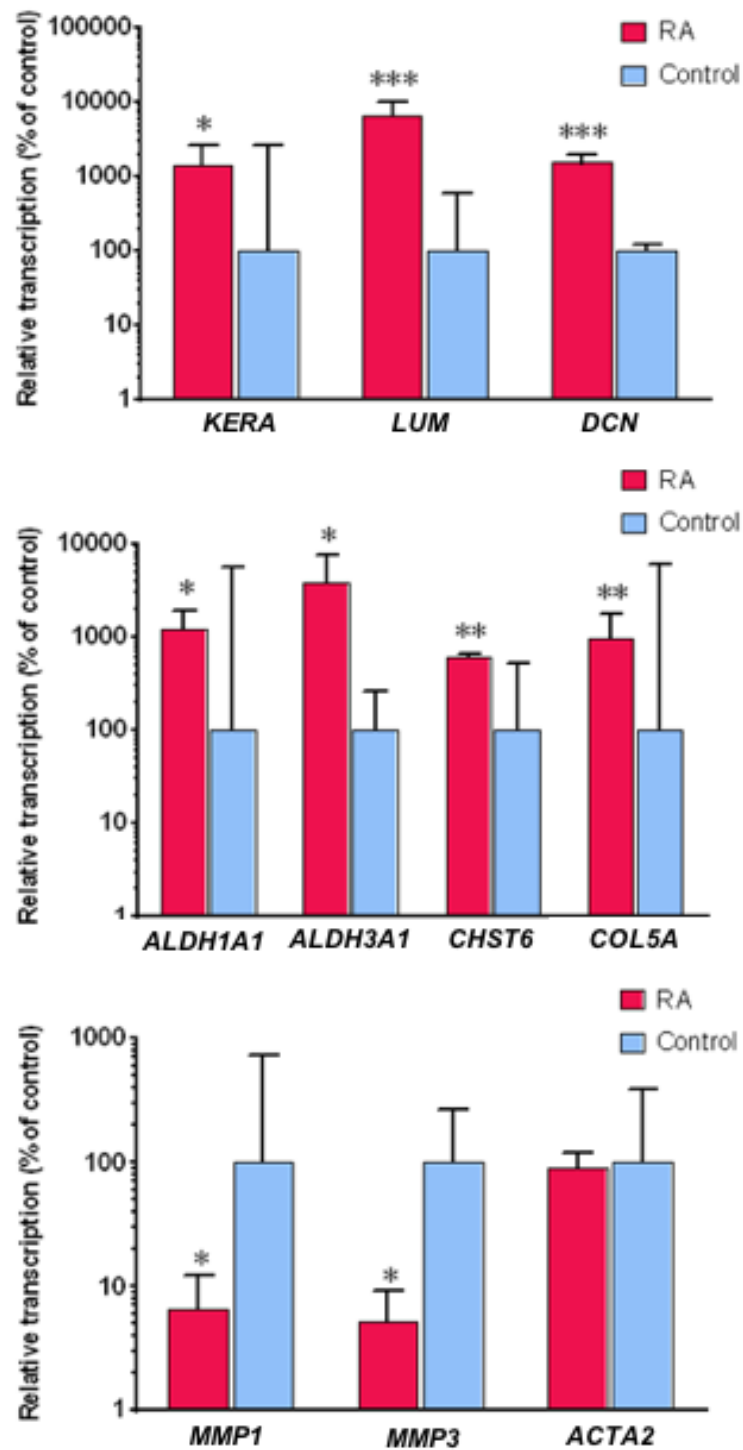


Figure 4.4 - Comparison of gene expression profiles for keratocytes embedded in compressed collagen gels cultured for 30 days in control (blue) and RA (red) supplemented groups analysed by qPCR. Gene expression was normalized relative to that of control group. Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared using t-test (*, ** and *** correspond to $p < 0.05$, <0.01 and <0.001 , respectively).

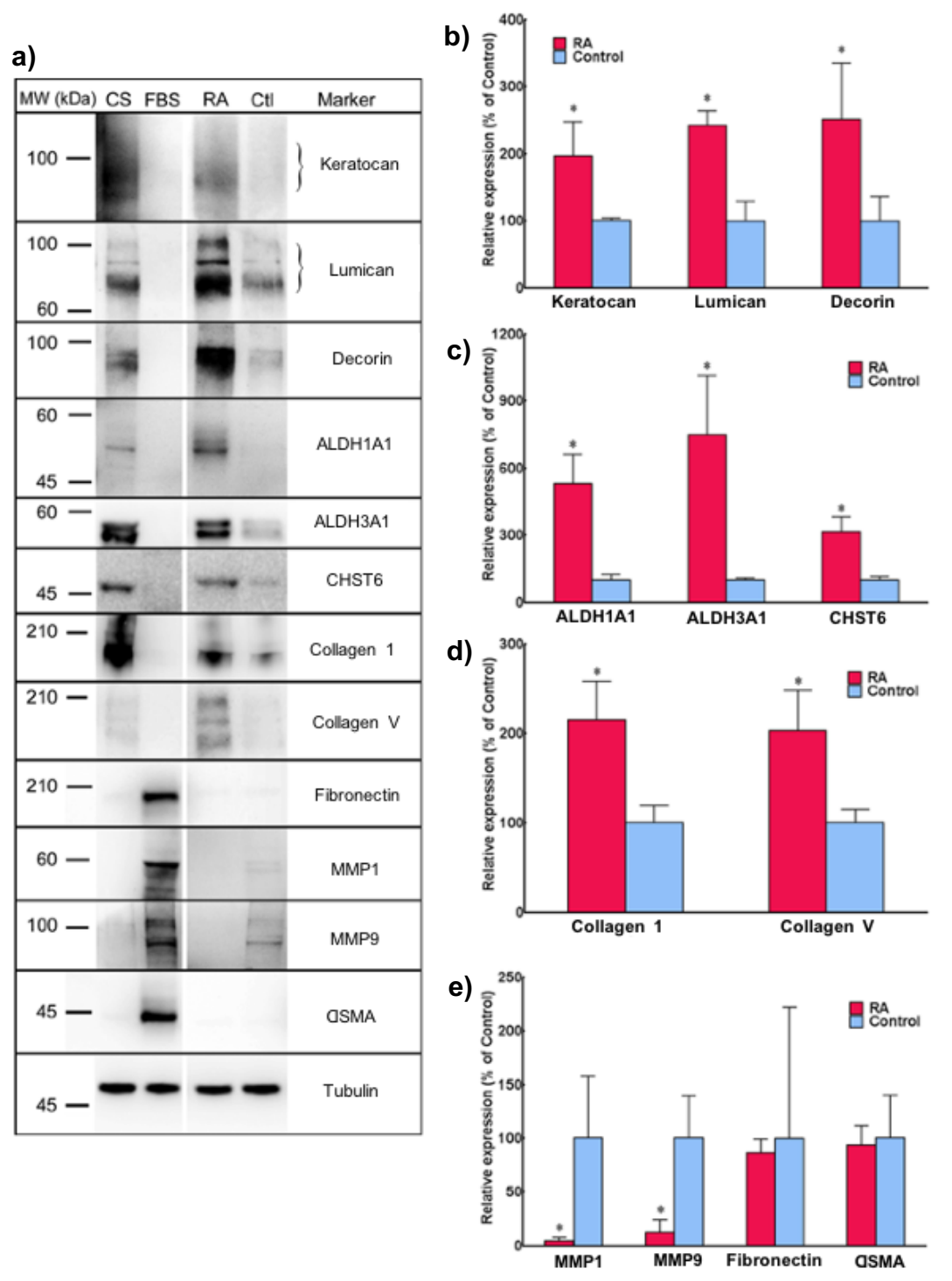


Figure 4.5 - Expression of keratocytes markers at the protein. **(a)** Lysates from corneal stroma (CS), keratocytes cultured in FBS (FBS), or 3D collagen constructs supplemented with RA (RA) or control (Ctl) were extracted and analyzed by reducing SDS-PAGE followed by immunoblotting. **(b-e)** Quantification of protein expression from control (blue) and RA (red) was performed by immunoblot densitometry for all markers. Protein expression was normalized relatively to that of control group. Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared using t-test (* correspond to $p < 0.05$ respectively).

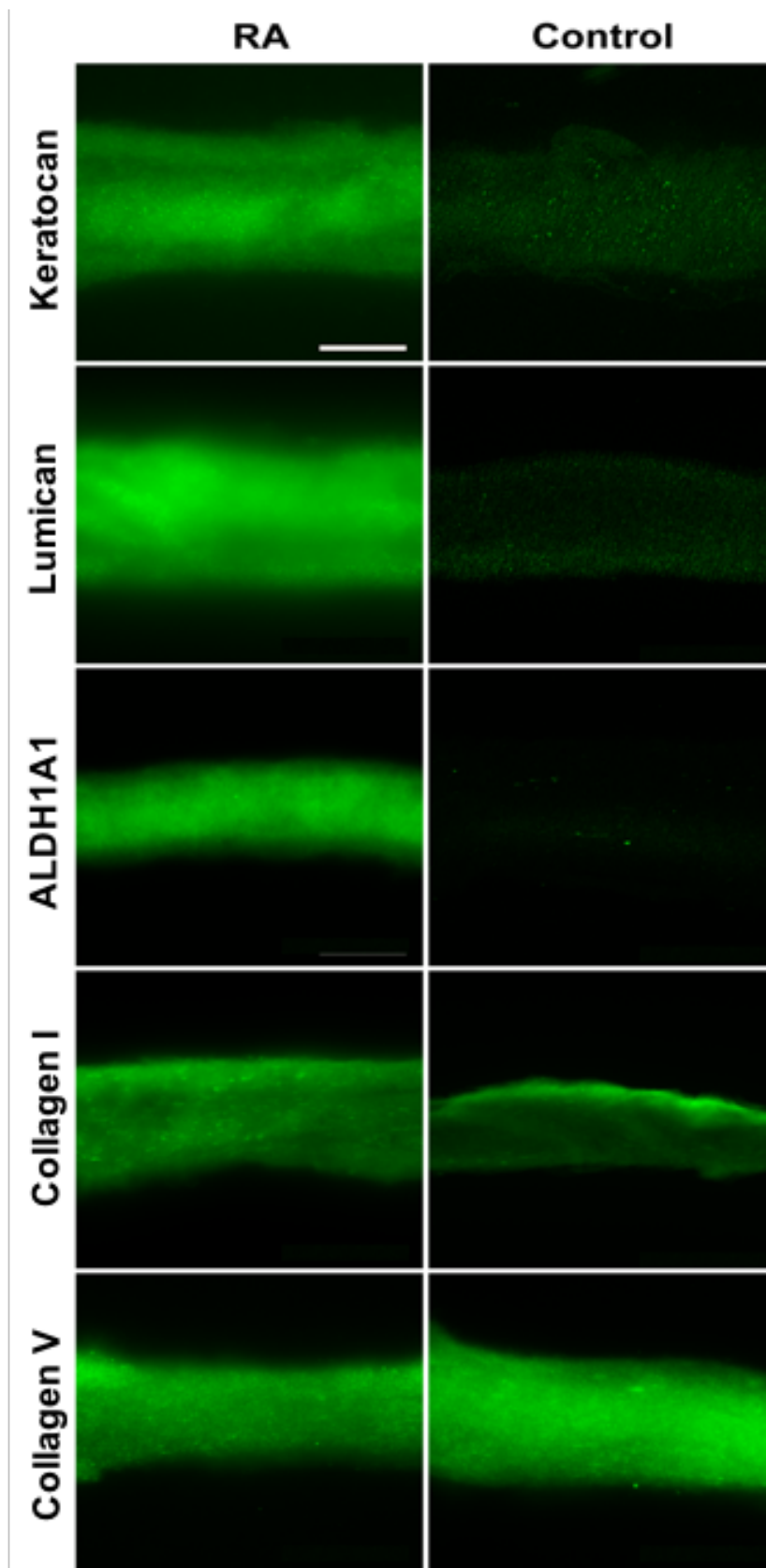


Figure 4.6 - Immunofluorescence staining of common corneal extracellular matrix proteins expressed by encapsulated keratocytes within compressed collagen gels with or without RA supplementation. All photographs were taken at x200 magnification.

4.4 Discussion

In the present study, we demonstrated that supplementation of RA in serum-free medium to culture corneal fibroblasts in a 3D collagen construct helped to revert the cells from being fibroblastic to become more keratocyte-like which was evidenced by an increasing production of keratocyte-markers and very low expressions on some of the fibroblastic-markers. Although the stromal cells were initially cultured in serum-containing medium, these corneal stromal fibroblasts maintained in serum-containing medium have been previously shown to revert to a more keratocyte-characteristic phenotype, expressing higher keratocyte-specific markers (Foster *et al.*, 2015) while showing reduced cell migration and contractility (Gouveia *et al.*, 2014; Foster *et al.*, 2015) after the three-day period of serum starvation prior to encapsulation.

Among the biggest challenge in stromal cell culture is to encourage the keratocytes to remain viable over time while maintaining the keratocyte phenotype in order to continue producing the ECM proteins essential for optical transparency. Our previous work in 2D culture condition has shown RA encouraged the keratocytes without the need for serum in the medium (Gouveia and Connon, 2013). In our 3D environment, the mitogenic effect from RA became apparent after one week of culture, and the effect sustained up to four weeks as compared to DMSO control which only supported cell growth until the third week of culture. We confirmed that similar number of keratocytes remained viable with or without RA supplementation for at least one week of culture based on our alamarBlue® assay result (figure 4.1). Kenney *et al.* (1986) reported a reduction in cultured rabbit corneal keratocytes as evidenced by a decrease in [*methyl*-³H] thymidine incorporation following exposure to RA (1µM) for 96 hours. Although the RA concentration used in that study was lower than ours, it does show similar trend which may indicate that the initial 'kick-start' to an increase in cell number made by RA can be slightly delayed. However, it has been reported that corneal keratocytes could be 'transiently stimulated' by RA as evidenced by a significant increase in thymidine uptake following 24 hours exposure, but the increase did not sustain when checked at 48 hours (Kirschner *et al.*, 1990).

At the same time, we found that RA significantly modulated the expressions of many keratocyte-characteristic ECM components (keratocan, lumican, decorin), the corneal crystallins (ALDH1A1), (ALDH3A1), carbohydrate sulfotransferase 6 (CHST6) as well

as increased expressions of both collagen type I and V. Keratocytes secrete collagen type I and V, which are the predominant fibrillar collagens in the corneal stroma. Keratocan, lumican and decorin are all belong to the family of small leucine-rich proteoglycans (SLRPs) which serve as regulators of tissue hydration and collagen fibrillogenesis (Fullwood *et al.*, 1996; Iozzo, 1997). These SLRPs bind to fibrillar collagens and affect the collagen matrix assembly needed for corneal transparency (Svensson *et al.*, 2000). Besides that, significant increases in ALDH1A1 and ALDH3A1 expression within the RA-supplemented group were also important findings. Prominent ALDH isoenzymes in cornea, such as ALDH1A1 and ALDH3A1, exert protective effect from the harmful effects of UV-induced lipid peroxidation (Downes *et al.*, 1993; Pappa *et al.*, 2003; Estey *et al.*, 2007) as well as maintaining corneal transparency (Jester *et al.*, 1999). ALDH is produced in greater amounts by quiescent keratocytes compared to their activated phenotype, which has been shown in both *in vitro* (following exposure to serum) (Jester *et al.*, 1994; Pei *et al.*, 2006) and *in vivo* experiments (Feng *et al.*, 2004). As such a decrease in corneal crystallins is posited to increase light scattering by the corneal fibroblast, and decrease corneal transparency (Jester *et al.*, 2005).

Interestingly, RA significantly modulated the expression of carbohydrate sulfotransferase 6 (CHST6), an enzyme that catalyzes the transfer of sulfate group to keratan sulfate proteoglycans (KSPGs) to help maintain corneal transparency. CHST6 is mainly expressed by the keratocytes both *in vivo* and *in vitro* (Di Iorio *et al.*, 2010). Defects in this gene are associated with macular corneal dystrophy (MCD), a rare autosomal recessive inherited disorder characterized by abnormal deposits of unsulfated KSPGs which causes progressive stromal haziness leading to visual impairment (Jones and Zimmerman, 1961; Klintworth, 1980). An increase synthesis of CHST6 following RA supplementation suggests that RA is safe for corneal keratocytes. At the same time, this result implies the huge considerable potential for RA to be used as a possible therapy for MCD, as the aim in curing this rare genetic disorder must involve and target corneal keratocytes (Di Iorio *et al.*, 2010).

The expression of MMP1 (i.e interstitial collagenase) and MMP3 (i.e stromelysin-1) were greatly reduced following RA supplementation, which is similar to previous findings (Lateef *et al.*, 2004) on diabetic human skin which showed improvement in collagen synthesis and reduction in MMPs in the presence of RA. Under normal

circumstances, MMP1 and MMP3 are not synthesized by quiescent keratocytes. However, the synthesis is upregulated when the keratocyte's phenotype changes into repair fibroblast (Fini *et al.*, 1992; Girard *et al.*, 1993). We also evaluated the expressions of α -SMA and fibronectin and found that they appear to be unaffected by the presence of RA in the culture i.e. low, as one would expect from serum-free conditions.

The increase in ECM production following RA-supplementation may also contribute to the measured increase in both wet and dry weight of the 3D collagen-keratocyte constructs. The wet weight of gels significantly increased after the 30-days period in culture in RA conditions compared to their initial wet weight, but decreased in control gels, indicating that RA induced the resident keratocytes to 1) produce larger amounts of newly-synthesized material, 2) enhanced the gel's ability to accumulate water, and/or 3) inhibited degradation of the initial collagen gel scaffold compared to control conditions. These conclusions are supported by the significant increase in dry weight of RA-treated gels (figure 4.3, central panel), as well as by the RA-induced enhancement of keratocyte-characteristic ECM components and the abrogation of MMP expression observed at the gene and protein levels (figure 4.4 and 4.5, respectively). Moreover, the higher content of keratocyte-characteristic proteoglycans (keratocan, lumican and decorin) in the RA-treated group might account for the higher capacity of these gels to retain water (figure 4.3, right panel). As mentioned previously, the SLRPs serve as regulators for tissue hydration. Since there were more SLRPs in collagen constructs supplemented with RA, it was not surprising that the water content inside these gels was found to be higher when compared to the control. Therefore, both wet and dry weight values were important to understand the dynamic changes in the properties in this gel-keratocyte system, and were necessary to determine the construct's hydration, an important property of the corneal stroma and its transparency.

Importantly, the contraction assay confirmed that the addition of RA showed minimal contraction of engineered constructs compared to the control gels. This is another positive finding showing that RA was not only mitogenic, but also maintained keratocytes in a non-contractile phenotype. However, the potentially limiting effects of increased collagen (as shown in the presence of RA) on gel contraction cannot be disregarded. The use of fetal bovine serum in the culture medium to promote cell

proliferation results in contraction of the engineered matrix (Borderie *et al.*, 1999) that often mimic scarred native tissue due to activation of stromal fibroblasts into myofibroblasts (Jester *et al.*, 1995). Whilst removal of serum can abrogate this phenomenon, we have demonstrated that further significant loss of contraction can be achieved by the simple addition of RA.

In conclusion, the use of RA for *in vitro* keratocytes studies holds great promise, since we have shown that its supplementation successfully induced proliferative potential of keratocytes when used in serum-free medium over an extended period in culture. This is important to tissue engineering as engineered constructs, such as the cornea, typically require many days in culture. RA also significantly reduced the expression of MMPs while not increasing the fibroblastic-markers. Furthermore, these effects were also correlated with the ability of RA to significantly inhibit the contractility of keratocytes while allowing the build-up of corneal stromal extracellular matrix within the 3D constructs. Thus, RA supplementation represents a promising strategy to improve the phenotype of 3D-cultured keratocytes, and their usefulness as a model of corneal stroma for corneal biology and regenerative medicine applications. These results, especially the reduction in MMPs expression, also enlightens for potential use of RA in understanding certain pathological conditions which involve the MMPs in the disease pathophysiology, for example in keratoconus.

Chapter 5: The effects of retinoic acid on human keratoconic fibroblasts *in vitro* under serum-free condition

5.1 Introduction

In the previous chapter, we have successfully shown the potential use of retinoic acid (RA) supplement on normal human corneal keratocytes in modulating cell proliferation and keratocyte-characteristic proteoglycans production *in vitro*. More importantly, RA supplementation is also capable of significantly reducing the expression of matrix metalloproteinase (MMP) by corneal keratocytes in both 2D and 3D cultures. Due to its success, this has sparked some interest to further explore the potential benefit of RA on diseased keratocytes taken from diseased cornea with known MMP involvement as one of its proposed pathophysiology. An example of disease is keratoconus.

Keratoconus is one of the most common causes for corneal transplant. It is a bilateral, non-inflammatory, asymmetric condition characterized by focal thinning and protrusion resulting in conical shape of the cornea (Kennedy *et al.*, 1986; Zadnik *et al.*, 1996). In other words, keratoconus is a pathological condition arising from mechanical weakness of the cornea. This abnormal cone-shape cornea results in irregular astigmatism, high myopia, and in severe conditions, significant visual impairment (Krachmer *et al.*, 1984). About 80% of keratoconic patients are young, adolescent patients with disease progression to third and fourth decade of life before it arrests. The remaining 20% starts later in life which may either progress or arrest at any age (Rabinowitz, 1998). Keratoconus is a genetic disease with relative of keratoconus patients having 15-67 times (Wang *et al.*, 2000) higher chances of acquiring it in their life than the general population (Millodot *et al.*, 2011). However, this genetic predisposition does not determine the severity of the disease as majority of patients were also triggered by environmental factors like contact lens wearing (Kenney and Brown, 2003), frequent eye rubbing (Weed *et al.*, 2008; Mashor *et al.*, 2011) and atopy (Bawazeer *et al.*, 2000; Kaya *et al.*, 2007).

Although the disease has been described for more than 160 years (Nottingham, 1854), the etiopathogenesis of keratoconus still remains unclear (Kenney and Brown, 2003). Many studies suggested biochemical involvement as the main factor that leads to all these structural changes. This includes imbalance between ECM

degradation enzymes and their inhibitors (Kao *et al.*, 1982; Smith *et al.*, 2006), imbalance between pro-inflammatory and anti-inflammatory molecules (Lema *et al.*, 2009; Balasubramanian *et al.*, 2012), the effects of oxidative stress (Arnal *et al.*, 2011), and ineffective stromal wound healing cascade (Cheung *et al.*, 2014). However, it remains unclear which event starts first and which is necessary for the disease to progress. There is higher possibility that all these events occur simultaneously and that they may cause positive feedback to one another.

At the moment, the treatment available for these patients ranges from glasses (for mild cases) to rigid contact lenses in moderate cases. However, these treatment plans are designed to address mainly the refractive issues found in keratoconus and not to curb the progress of the disease (McAnena *et al.*, 2016). Patients with severe keratoconus usually require corneal cross-linking as a standard method to help stabilize the progression of the disease (Wollensak, 2006; John, 2012), or ultimately corneal transplantation. Cross-linking treatment is an easy-to-perform procedure which uses a combination of riboflavin and ultraviolet-A radiation that helps to strengthen and stabilize the corneal tissue through the formation of photochemically triggered cross-links within the collagen network (Wollensak *et al.*, 2003). More importantly, cross-linking treatment offers possibility to reduce the need for corneal transplant. Follow-up studies on patients who received cross-linking treatment showed statistically significant improvement in the maximum simulated keratometry value (K_{\max}) and corrected visual distance acuity (CDVA) in crosslinked eyes as compared to the controls between three to ten years post treatment (Christine *et al.*, 2013; Raiskup *et al.*, 2015). At the same time, researchers are still looking into other potential elements or treatment which may help to reduce or stop the disease progression which would greatly benefit these patients especially the young ones.

This study is conducted to investigate the effects of RA on keratoconic fibroblast (KF) maintained under serum free condition *in vitro* for a maximum of two weeks. We look into its effect on KF morphology and cell viability following exposure to different concentrations of RA. At the same time, the expression of keratocyte-characteristic markers, as well as MMPs (MMP-1 and MMP-2) and their inhibitor (TIMP-1) following RA supplementation are also explored. We hypothesize that retinoic acid is able to influence the activity of MMPs in KF by directly reducing their expressions, or by stimulating the production of their inhibitors, or both. In addition, we also investigate

the expression of copper-dependent enzyme lysyl oxidase (LOX) and LOX-like proteins (LOXL1, LOXL2, LOXL3) by KF following RA supplementation. These enzymes are critical for connective tissue stability as they are required for collagen and elastin cross-linking in the extracellular matrix (Mizobe *et al.*, 2008). Previous studies have shown that there is a decrease of total LOX activity in cultured keratoconic fibroblasts, which possibly explains for the inadequacy in collagen cross-linking noticeable in this disease (Dudakova *et al.*, 2012).

5.2 Materials and methods

5.2.1 *Keratoconic and human corneal stromal fibroblasts cell culture*

Keratoconic fibroblasts (KF), isolated from human keratoconic corneas ($n = 5$; courtesy from Dr Dimitrios Karamichos) were maintained using culture medium containing DMEM:F12 medium, 10% FBS and 1% penicillin/streptomycin. The culture medium was changed every two to three days. Each subsequent experiment was performed three independent times using KF from passages four to six from specific donors.

At the same time, human corneal keratocytes isolated from human corneas ($n = 3$) were also used and the procedure was as described in the previous chapter. The isolated keratocytes were then termed as human corneal stromal fibroblasts (HCSF) as they were maintained in serum-containing medium (DMEM:F12 medium, 5% FBS, 1×10^{-3} M ascorbic acid, 1 x ITS liquid media supplement, 1% penicillin/streptomycin) until they reached 70-80% confluency before they were passaged. The HCSF was only used for qPCR experiment to look for any differences in gene expression compared to KF. The experiment was performed three independent times using HCSF from passages four to six from specific donors.

5.2.2 *Assessment of viable cell*

The keratoconic fibroblasts ($n = 2$) were seeded overnight in 6-well cell culture plate (Cellstar, Greiner Bio-one, Germany) with the seeding density of 1×10^4 cells cm^{-2} . The cells then underwent serum-starvation for three days, and later divided into two groups: 1) serum-free medium with 10 μM retinoic acid supplement, or 2) serum-free medium with equivalent amount of DMSO vehicle (control). Following supplementation, the cells were cultured for another ten days, and the culture

medium was changed every two days. The number of viable cells was enumerated daily by incubating the cells with 1 μ M calcein-AM for 15 minutes at 37°C. Live cells contain esterase enzymes which convert the cell-permeating calcein acetoxymethyl (AM, non-fluorescent) to the intensely fluorescent calcein that is well-retained within live cells. The counting of viable (stained) cells were done using a Countess® II FL automated cell counter (Invitrogen, UK).

5.2.3 MTT assay

The 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess the effects of different concentrations of retinoic acid on keratoconic fibroblasts' viability. The cells were seeded overnight in 96-well cell culture plate (Cellstar, Greiner Bio-one, Germany) with the seeding density of 3.2×10^4 cells/cm². The cells then underwent serum-starvation for three days, and later divided into different groups: 1) serum-free medium with DMSO control, and 2) serum-free medium supplemented with RA (1, 5, 10, 15 and 25 μ M). Following supplementation, the cells were cultured for another fourteen (14) days. In addition to the standard culture medium change (every two days) to replenish the nutrient in the medium, both retinoic acid and DMSO were topped up daily by substituting 10% of the culture medium from each well with freshly-prepared, serum-free medium containing 10x concentration of RA/DMSO respectively. The cells were incubated at 37°C in a humidified incubator 5% CO₂ atmosphere throughout the experiment. The MTT assay was read on day 1, 3, 5, 7, 9, 11 and 14th of experiment. Each well was added with 10 μ l MTT solution, mixed and incubated at 37°C in a humidified incubator 5% CO₂ atmosphere for another 4 hours. Following incubation, 200 μ l Dimethylsulfoxide (DMSO) was added to each well to dissolve the purple formazan and the absorbance was measured at 570 nm. The total viable cell number is directly proportional to the level of absorbance produced by the purple formazan precipitate. Cell viability was determined by interpolation using a standard curve for absorbance values of 1, 5, 10, 20, 50, and 100 $\times 10^4$ cells.

5.2.4 Cell imaging

The KF cells were plated similarly as described in section 5.2.2, but for this purpose these cells were exposed to different concentrations of RA (1, 5, 10, 15, 25 μ M) as well as the DMSO control. The cells were cultured for seven days and the culture

media was changed similar to the methods described in MTT assay. Effect of retinoic acid on KF morphology was evaluated from images taken on day three and seven of experiment. These images were obtained using an inverted microscope coupled with a digital camera (Leica Microsystems, Wetzlar, Germany) using Leica Application Suite X software.

5.2.5 *qPCR on selected markers*

The evaluation on gene expression was performed in two parts: 1) day three of experiment, and 2) day seven of experiment. For the first part, only KF were used and the cells were seeded into 6-well cell culture plate with seeding density of 1×10^4 cell cm^{-2} . Similar to MTT assay, the cells underwent serum-starvation for three days and later divided into similar groups. For this part, another group of keratoconic fibroblasts cultured in serum-containing medium (FBS) was also included to represent KF in active keratoconic stage. Cells were harvested on day three of culture to isolate the RNA using RNeasy kit following manufacturer's protocol. The remaining of the protocol is as described in Chapter 4.

In the second part, both KF and HCSF were seeded into 25 cm^2 flasks with seeding density of 3.2×10^4 cell cm^{-2} . Similarly, the cells underwent the three-day serum starvation period before being introduced with RA supplementation. This time, only 1 and 5 μM RA were used, together with DMSO vehicle control. These concentrations were used in both KF and HCSF cultures. In addition to the standard culture medium change (every two days) to replenish the nutrient in the medium, both retinoic acid and DMSO were topped up daily by substituting 10% of the culture medium from each well with freshly-prepared, serum-free medium containing 10x concentration of RA/DMSO respectively. Cells were harvested on day seven of culture to isolate the RNA using RNeasy kit following manufacturer's protocol. The remaining of the protocol is as described in Chapter 4. Table 4 summarized the gene primers used in this experiment.

5.2.6 *Western Blotting*

Equal amount of proteins (20 μg) as determined by the Bradford Protein Assay (Bio-Rad), were loaded into a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were mixed with 2x Laemmli loading

| Gene | Primers 5'→3' |
|-------------------------------------|----------------------------------|
| <i>POLR2A</i> | F: cat cat cga aga caa tgg tg |
| (housekeeping gene) | R: aac aat gtc ccc atc aca ca |
| <i>Keratocan</i> | F: tat tcc tgg aag gca agg tg |
| | R: acc tgc ctc aca ctt cta gac c |
| <i>Lumican</i> | F: cct ggt tga gct gga tct gt |
| | R: tag gat ggc ccc agg a |
| <i>Decorin</i> | F: aat tga aaa tgg ggc ttt cc |
| | R: ctg ctg att ttg ttg cca tc |
| <i>Retinoic acid receptor α</i> | F: agt cct cag gct acc act at |
| (RARA) | R: cct cct tct tct tct tgt tt |
| <i>Matrix metalloproteases-1</i> | F: agg tct ctg agg gtc aag ca |
| (MMP-1) | R: ctg gtt gaa aag cat gag ca |
| <i>Matrix metalloproteases-2</i> | F: gcc aag cgt cta gca ata cc |
| (MMP-2) | R: tct ggg gca gtc caa aga ac |
| <i>Tissue inhibitor of matrix</i> | F: att gct gga aaa ctg cag gat g |
| <i>metalloproteinase-1 (TIMP-1)</i> | R: tcc aca agc aat gag tgc ca |
| <i>Lysyl oxidase</i> | F: gat acg gca ctg gct act tcc |
| (LOX) | R: ctg gcc aga cag ttt tcc tcc |
| <i>Lysyl oxidase-like 1</i> | F: agc atc cac tta tgt gca gag a |
| (LOXL1) | R: gag gaa gtc tgc tgt gcc ct |
| <i>Lysyl oxidase-like 2</i> | F: cac tgc gga tcc ctg aaa cc |
| (LOXL2) | R:ctg tct tcg ggc tga tga tcc |
| <i>Lysyl oxidase-like 3</i> | F: cag gct gcc cac atc ctc tg |
| (LOXL3) | R: atc ctc atc gtg cgt aca gtc |

Table 4. Description of primers used in qPCR for gene expression analysis.

buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue and 0.125M Tris-HCl), boiled and then separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking and washing, membranes were incubated overnight at 4°C with the primary antibodies listed in table 5. This was followed by secondary incubation using IRDye 800CW Goat-anti-rabbit IgG (Li-Cor, 925-32211), IRDye 680LT Goat-anti-mouse IgG (Li-Cor, 925-68020) and IRDye 800CW Donkey-anti-goat IgG (Li-Cor, 925-32214) at 1:10,000 dilution at room temperature for one hour. The membranes with protein bands were visualized by Li-Cor Odyssey. Alpha-tubulin was used for protein loading normalization. Quantification was performed by densitometry analysis of imaged bands using Image J v1.46 (National Institute of Health, Bethesda, MD).

| Antigen | Host | Antibody | Dilution | Source | Catalogue number |
|----------------------------------------|-------------|--------------------|-----------------|------------------------------------------------------------------|-------------------------|
| Alpha-tubulin | Rabbit | Anti-alpha tubulin | 1:1000 | abcam | ab4074 |
| Lumican | Mouse | Anti-lumican | 1:1000 | Kindly given by Dr Bruce Caterson, Cardiff School of Biosciences | - |
| Decorin | Goat | Anti-decorin | 1:1000 | Calbiochem | PC673 |
| Retinoic acid receptor α (RARA) | Rabbit | Anti-RARA | 1:1000 | St. John Labs | STJ26160 |
| MMP2 | Rabbit | Anti-MMP2 | 1:1000 | St. John Labs | STJ28003 |

Table 5. Details of primary antibodies used for Western blotting.

5.3 Results

5.3.1 *Effects of RA on keratoconic fibroblasts viability and proliferation*

Following ten days exposure to 10 μ M retinoic acid, which is the same concentration previously used in normal keratocytes (chapter 4), more viable cells were seen from day 2 to day 4 of the experiment as compared to the number of viable cells on day 1 (figure 5.1). However, these differences were not significant. There is possibility of cell mitosis after 24-hour supplementation with RA as the number of viable cells showed an increase by 15% on day two of supplementation. Furthermore, this increase was not seen in KF cultured in serum-free medium with DMSO alone over the same period of time. However, a reduction in cell viability was observed from day 5 of RA supplementation onwards. By the 7th day of supplementation, only half the cells remained viable as compared to the number of viable cells on the initial RA exposure. On the other hand, the KF cultured in serum-free medium with DMSO demonstrated the highest number of viable cells at the beginning of the experiment, but it was followed by a steady decrease in the number of viable cells from day 2 onwards. As the experiment reached the 10th day of supplementation, the total number of viable cells between both groups (with or without RA supplementation) were almost similar (figure 5.1).

Further to that, a range of RA concentrations was tested on KF cells to see if other concentrations could give more benefits to KF cells in terms of inducing mitogenesis. MTT assay was performed as it is one of the most frequently used method to quantify cell proliferation. For this experiment, the RA concentrations were divided into two groups: 1) low concentrations (1 and 5 μ M), and 2) high concentrations (10, 15 and 25 μ M). In the low concentrations group, daily RA supplementation did not seem to support the cells to proliferate higher than the control, and there has been a steady decrease of cells throughout the experiment. However, these differences were not significant when compared to the control (figure 5.2b). On the other hand, daily supplementation with 10 and 15 μ M of RA in the high concentration group were able to increase KF proliferation for the first 3 days similar to the control. However, they also showed a steady decrease in cell number from day 3 onwards (figure 5.2c). The cell number from both concentrations were significantly lower than the control by almost 50% following 1 week of RA supplementation. Unfortunately, the 25 μ M RA

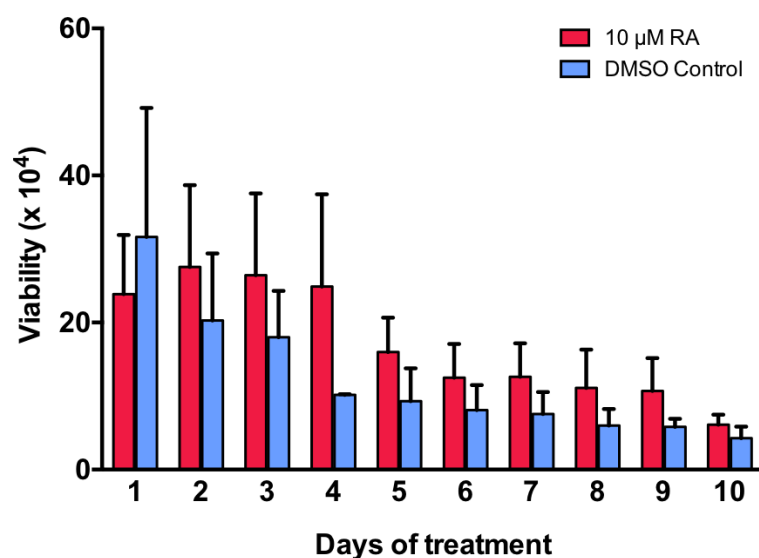


Figure 5.1 - Effects of RA on keratoconic fibroblast (KF) viability. More viable cells were seen following 24-hour exposure to RA supplementation in serum-free medium. However, as the supplementation continues less number of viable KF were recorded. The addition of DMSO into serum-free medium did not seem to support KF growth as the number of viable cells continued to decrease over time ($n = 2$).

MTT assay

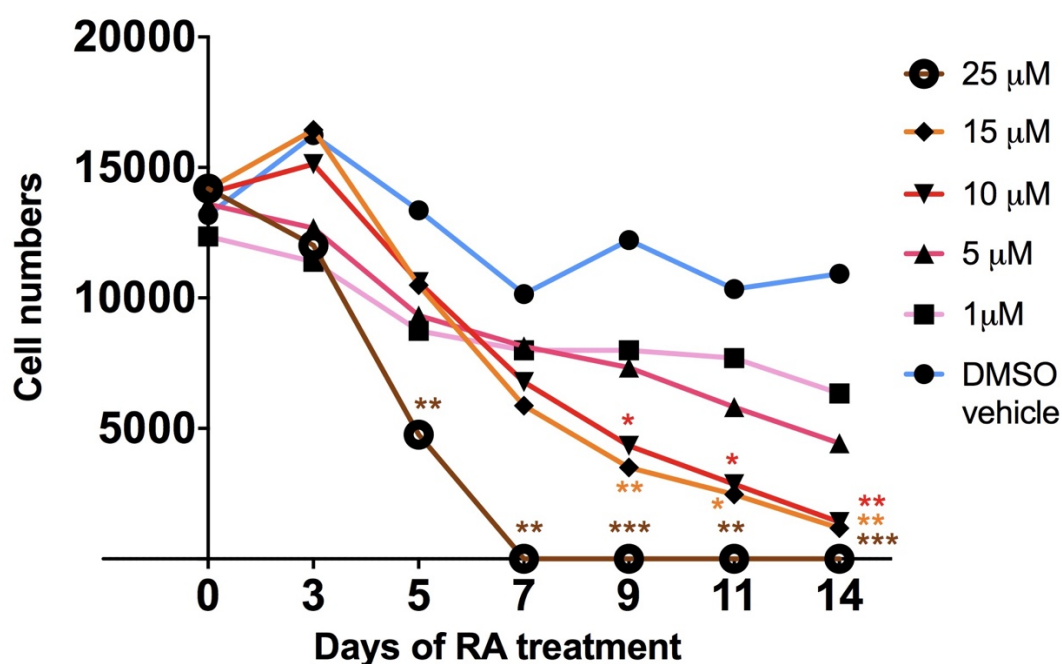


Figure 5.2 - MTT assay result on keratoconic fibroblasts supplemented with a range of RA concentrations or DMSO as control. Data (mean \pm SD) were obtained from five independent experiments ($n = 5$) and compared using t-test (* corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$).

supplementation seemed to be toxic to keratoconic fibroblasts, as no viable cells were found by 7th day of culture.

5.3.2 Effects of RA on keratoconic fibroblasts morphology

Supplementation of RA on keratoconic fibroblasts influenced some morphological changes on few of the keratoconic fibroblasts as early as day 3 of supplementation. Some of the keratoconic fibroblasts acquired a flattened, dendritic-shape morphology with triangular cell body following RA supplementation (figure 5.3c, d, e, f; figure 5.4) which was not seen in KF cultured with DMSO alone (figure 5.3a,b). Interestingly, some of these cells had some pronounced transformation from spindle-shaped fibroblasts into cells with smaller, rounded cell bodies and prominent, long dendrites, seen particularly in the 5 μ M group, and these changes became more prominent as the supplementation reached the 7th day (figure 5.3f). Some of these transformed cells also have short, branched dendrites which connect them to the neighbouring cells (figure 5.4). At the same time, more dead cells were seen in the higher concentration groups after 7 days of supplementation, which appeared as rounded, floating cells and more empty spaces were seen in between the live cells (figure 5.5a, b, c, d). As shown by the MTT assay results, no viable cells were noted in the 25 μ M group after 7 days of supplementation (figure 5.5f), supporting the possibility that this concentration is toxic to KF.

5.3.3 RA modulates the expression of selected genes: evaluation on day 3 of supplementation on keratoconic fibroblasts

Several important keratocytes-characteristic markers were analysed at the gene transcript level and compared with the control. Results showed that RA supplementation significantly increased transcription of genes coding for key corneal proteoglycans (*keratocan*, *lumican* and *decorin*) especially in the lower concentration groups (1 and 5 μ M) (figure 5.6a). In the 1 μ M group, the supplementation increased the expression of *keratocan*, *lumican* and *decorin* by 4.412 ± 0.187 , 2.381 ± 0.008 , and 1.419 ± 0.327 -fold of the control, respectively. At the same time, the 5 μ M group

Day 3

Day 7

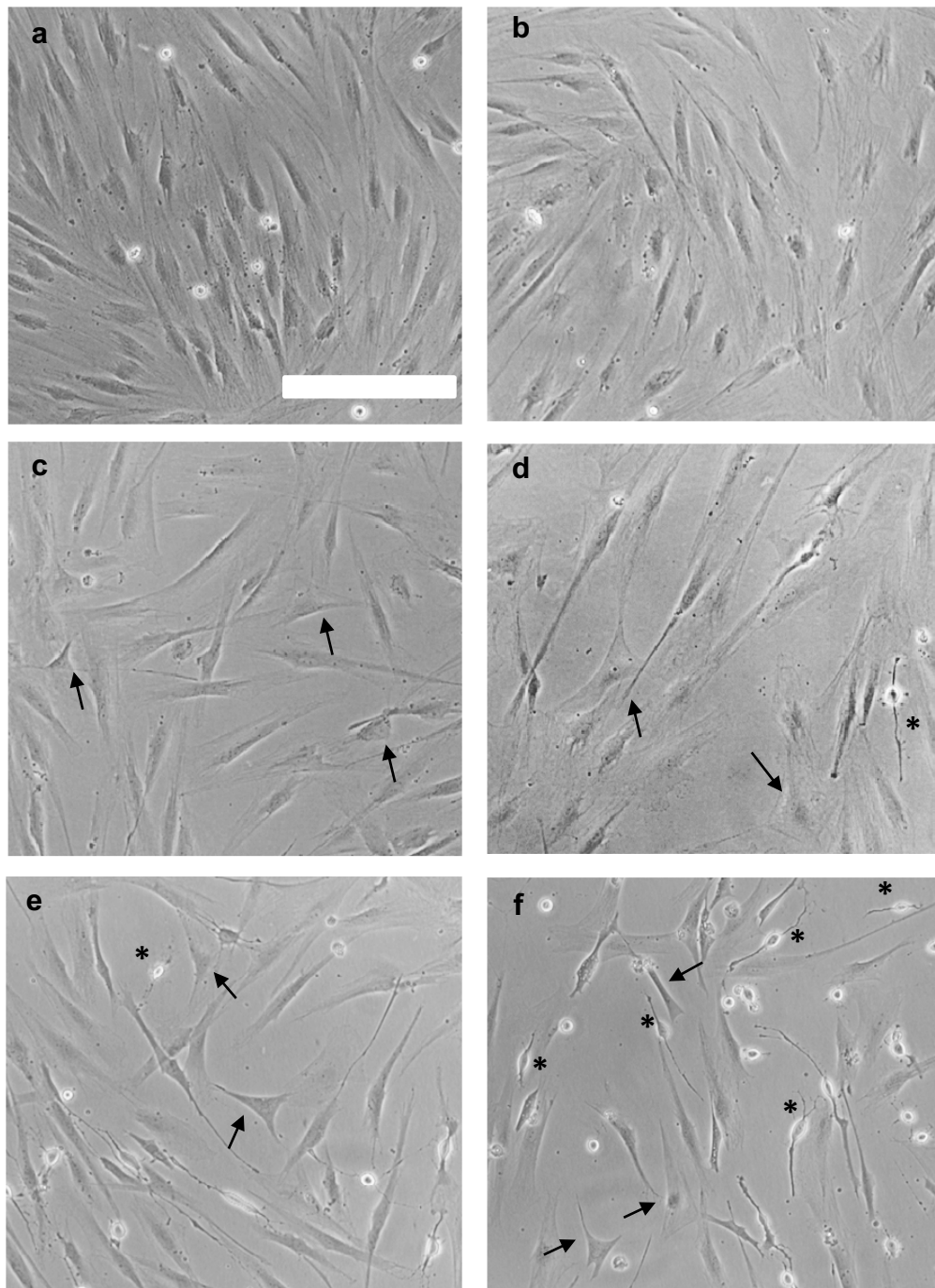


Figure 5.3 - Morphological features of keratoconic fibroblasts on day 3 of culture from DMSO control group (a, b) and groups supplemented with 1 μ M RA (c, d) and 5 μ M RA (e, f). The black arrows highlight the presence of flattened, dendritic-shape cells resembling quiescent keratocytes. The asterisks highlight KF that have transformed into cells with small, rounded cell bodies and long dendrites. All images were taken at x100 magnification. Scale bar = 50 μ m.

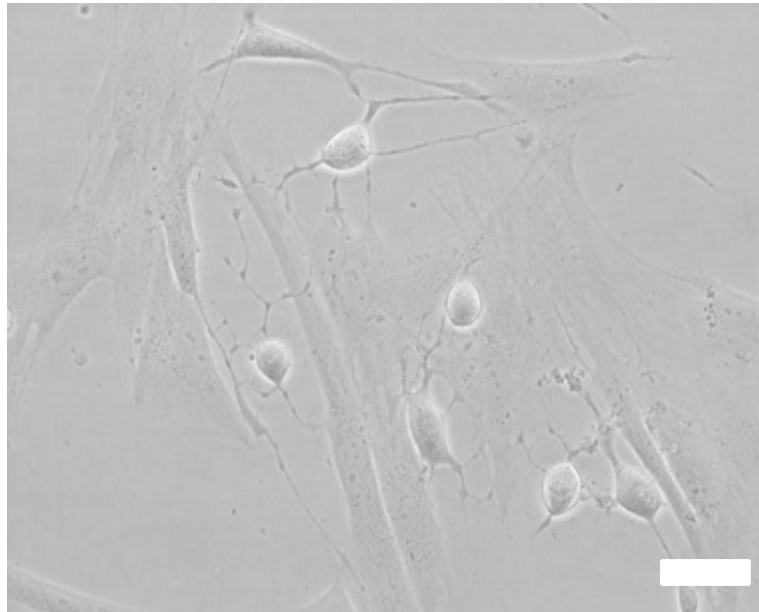


Figure 5.4 - Closer view of transformed keratoconic fibroblasts from group supplemented with 5 μ M RA on day 7 of supplementation. The image shows cells with short-branched dendrites which connect them to the neighbouring cells. Their cell bodies also appeared small and rounded as compared to the flat, triangular shape of the adjacent cells. Image was taken at x400 magnification. Scale bar = 50 μ m.

exhibited a similar increase in the expression of *keratocan*, *lumican* and *decorin* by 2.854 ± 0.256 , 3.285 ± 0.007 , and 2.123 ± 0.287 - fold of the control, respectively.

Supplementation with 10 μ M RA managed to push a slight increase the expression of *lumican* and *decorin* (1.667 ± 0.008 and 1.224 ± 0.429 - fold of the control, respectively) but not *keratocan* (0.875 ± 0.313 - fold of the control). Other concentrations of RA (15 and 25 μ M) produced a much lower expression of corneal proteoglycans, some of which were almost similar to that expressed by KF in serum-containing group (FBS).

More importantly, supplementation of KF serum-free medium with 1 and 5 μ M RA have also managed to suppress the production of *MMP-1* following 3 days of supplementation (0.191 ± 0.749 and 0.532 ± 0.643 - fold of the control, respectively) as shown in figure 5.6b, but these differences were not significant when compared to the control. Surprisingly, KF cultured in higher RA concentration groups displayed an increasing trend of *MMP-1* expression. Supplementation with 10 μ M RA showed an

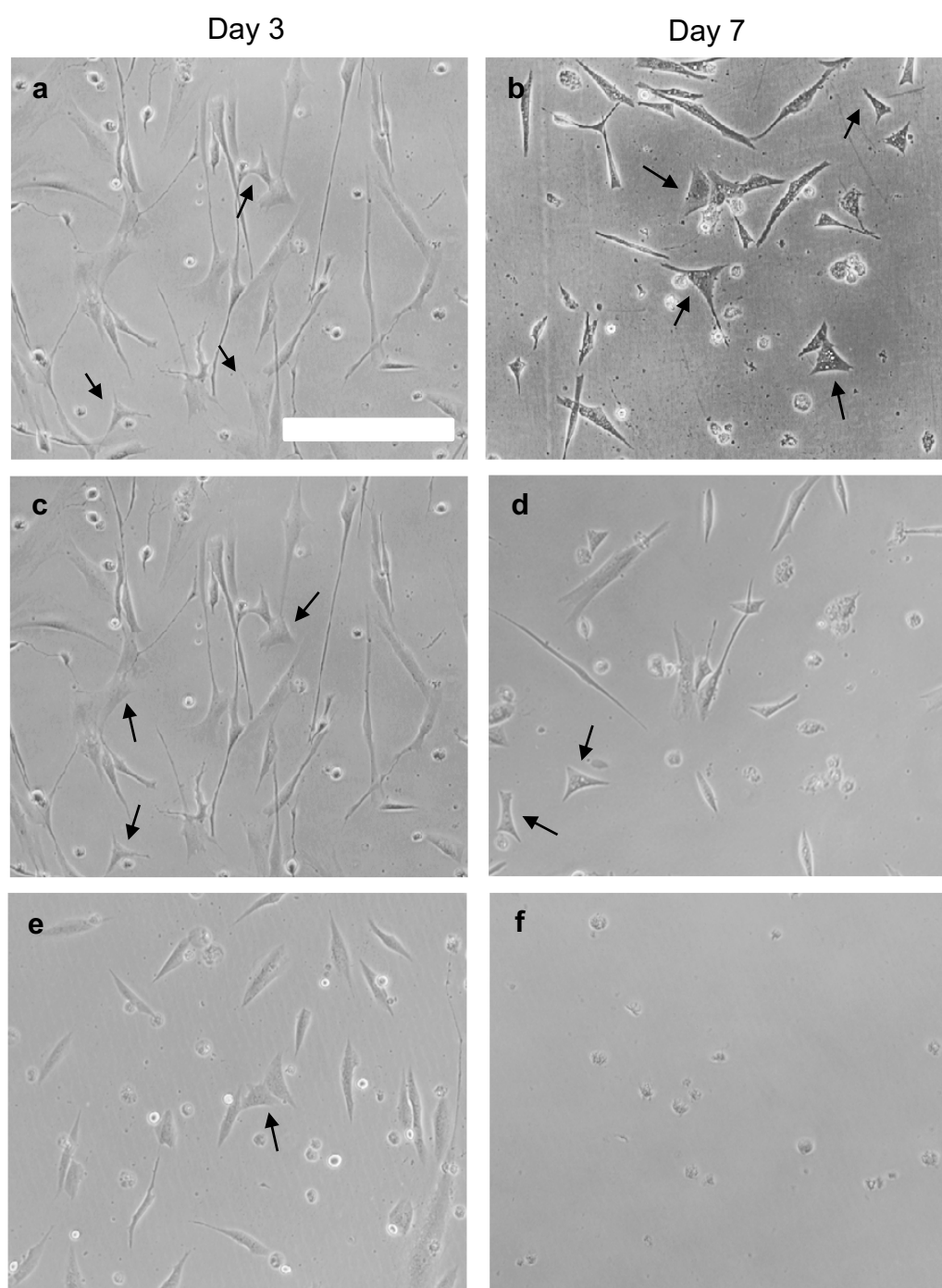


Figure 5.5 - Morphological features of keratoconic fibroblasts on day 3 and 7 of culture from groups supplemented with 10 μ M RA (a, b), 15 μ M RA (c, d) and 25 μ M RA (e, f). The black arrows highlight the presence of flattened, dendritic-shape cells resembling quiescent keratocytes. On day 7 of supplementation, fewer cells were seen in the 10 and 15 μ M groups as compared to the cells in lower concentration groups. No viable cells were observed in the 25 μ M group after one week of supplementation. All images were taken at x100 magnification. Scale bar = 50 μ m.

almost similar expression of *MMP-1* as compared to the control (1.2 ± 0.924 - fold of the control), while KF cultured in medium supplemented with 15 and 25 μM RA showed at least 10 times higher *MMP-1* expression as compared to KF cultured in control medium without the presence of RA (11.37 ± 0.887 and 46.02 ± 0.803 - fold of the control, respectively) (figure 5.6b).

As for the *MMP-2* expression, all RA concentrations showed slightly higher expressions of *MMP-2* as compared to the control, except for KF cultured in 25 μM RA. Instead, the *MMP-2* expression was found to be slightly lower in the 25 μM group (0.868 ± 0.862 - fold of the control), but this difference was not statistically significant. As expected, the KF cultured in serum-containing medium (FBS) showed the highest expression of *MMP-1* and *MMP-2* (92.52 ± 0.694 and 24.01 ± 0.747 - fold of the control, respectively), which are what usually found in corneal stroma during the active phase of keratoconus.

Based on these results, only the lower RA concentrations (1 and 5 μM) were used for further assessments on both gene and protein expressions by both KF and human corneal stromal fibroblast (HCSF) following 7 days of RA supplementation. The HCSF was included to represent normal stromal keratocytes which have been activated into fibroblasts by the action of serum in the culture medium prior to experiment.

5.3.4 Keratocytes-characteristic markers: effects of RA on KF versus HCSF

For KF, the earlier increase of keratocyte-characteristic markers observed on day 3 post supplementation were unable to be maintained by both groups (1 and 5 μM) as the supplementation reached the 7th day. Supplementation with 1 μM RA gave almost similar expressions of *keratocan* and *decorin* as compared to the control (0.869 ± 0.002 and 0.665 ± 0.445 - fold of the control, respectively), however the expression of *lumican* were significantly reduce by almost 70% than that of the control (0.326 ± 0.001 - fold of the control) (figure 5.7a, b, c). Meanwhile, the expressions of *keratocan*, *lumican* and *decorin* in 5 μM group were significantly reduced than the DMSO control (0.02 ± 0.001 , 0.019 ± 0.0004 , and 0.02 ± 0.214 - fold of the control, respectively).

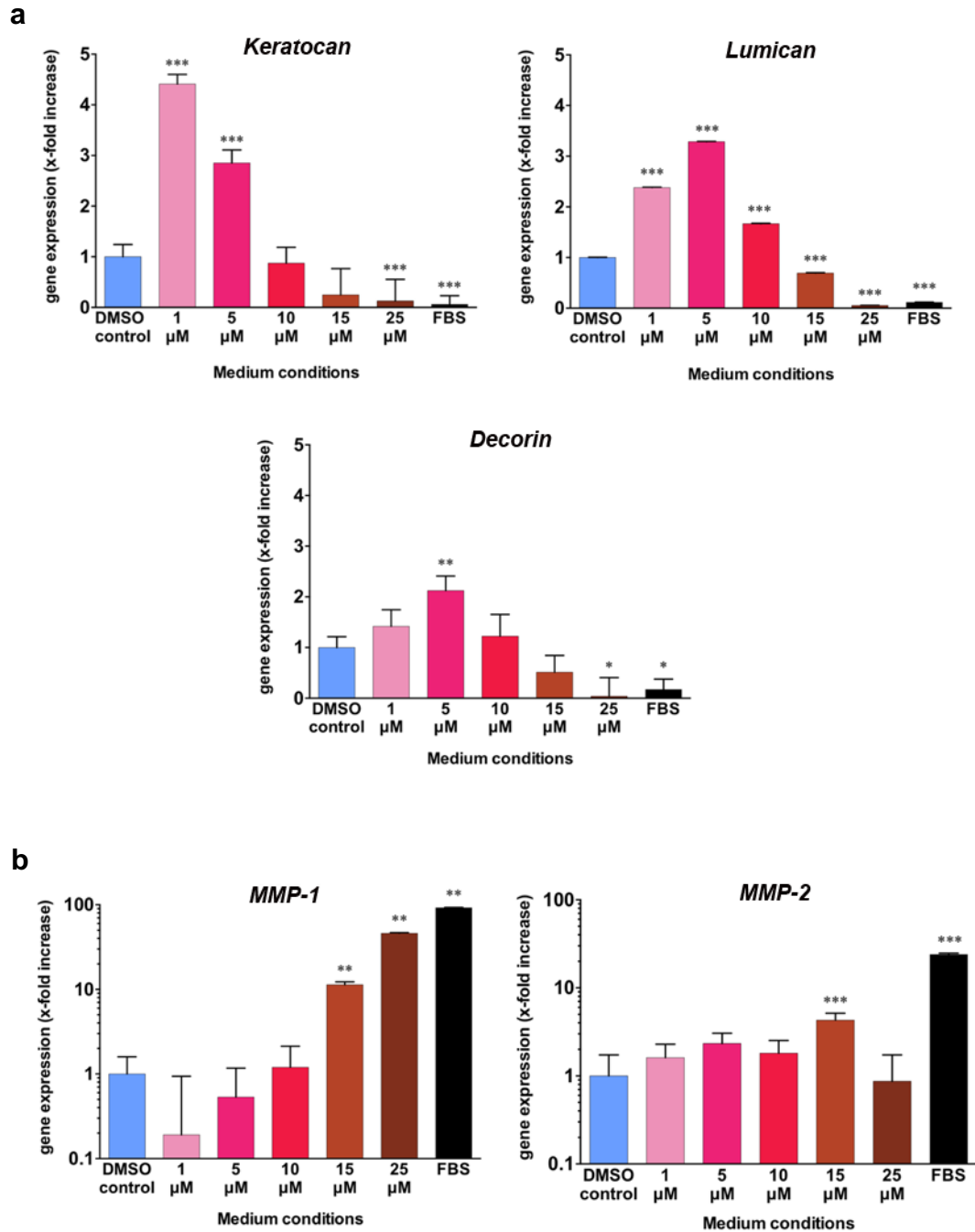


Figure 5.6 - Expression of (a) keratocyte-characteristic markers and (b) MMPs by keratoconic fibroblasts cultured with or without RA supplementation after 3 days of supplementation. KF cultured in serum-containing medium (FBS) was also included to represent cells in active keratoconic stage. Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared using ANOVA with Bonferroni's post hoc correction (* corresponds to $p < 0.05$, ** corresponds to $p < 0.01$, and *** corresponds to $p < 0.001$).

For HCSF, similar observations were noted when these cells were supplemented with 1 and 5 μ M RA (figure 5.7a, b, c). Again, both supplementations were unable to influence the cells to produce more markers than that of the control at transcript level. However, between the 2 groups, HCSF supplemented with 1 μ M RA gave a consistently higher expression of *keratocan* (0.238 ± 0.1), *lumican* (0.153 ± 0.193) and *decorin* (0.184 ± 0.198) than that in 5 μ M RA group (0.042 ± 0.226 , 0.108 ± 0.28 , and 0.055 ± 0.143 - fold of the control, respectively).

Comparing the gene expressions between both cells, KF expressed significantly higher *keratocan* as compared to HCSF when both cells were supplemented with 1 μ M RA. Although the *keratocan* expression in 5 μ M RA groups also displayed obvious difference between the two cells, this difference was not statistically significant. Nevertheless, the expressions of *lumican* and *decorin* were similar between KF and HCSF upon supplementation with either 1 or 5 μ M RA.

5.3.5 *RARA* expression: effects of RA on KF versus HCSF

As seen in figure 5.7d, the expression of retinoic acid receptor alpha (*RARA*) in KF were lower in both 1 and 5 μ M RA groups as compared to the control (0.645 ± 0.32 and 0.601 ± 0.574 - fold of control, respectively). For HCSF, supplementation with 1 μ M RA showed a very similar *RARA* expression as compared to the control (0.95 ± 0.147 - fold of control), whereas the 5 μ M RA supplementation on HCSF managed to significantly induce higher *RARA* expression by 2 folds than those cultured in DMSO control (2.3 ± 0.242 - fold of control). Overall, the difference in *RARA* expression between KF and HCSF was only significant when both cells were supplemented with 5 μ M RA (figure 5.7d).

5.3.6 *Modulation of MMPs and TIMP-1 expressions by RA on KF versus HCSF*

Interestingly, keratoconic fibroblasts cultured in serum-free medium supplemented with lower concentrations of RA gave a better impact on the modulation of *MMP-2* expression as compared to *MMP-1* after 7 days of supplementation (figure 5.8b). Keratoconic fibroblasts, in response to 1 μ M RA supplementation, expressed only 0.328- fold of *MMP-2* compared to the control. Such low expression value was not

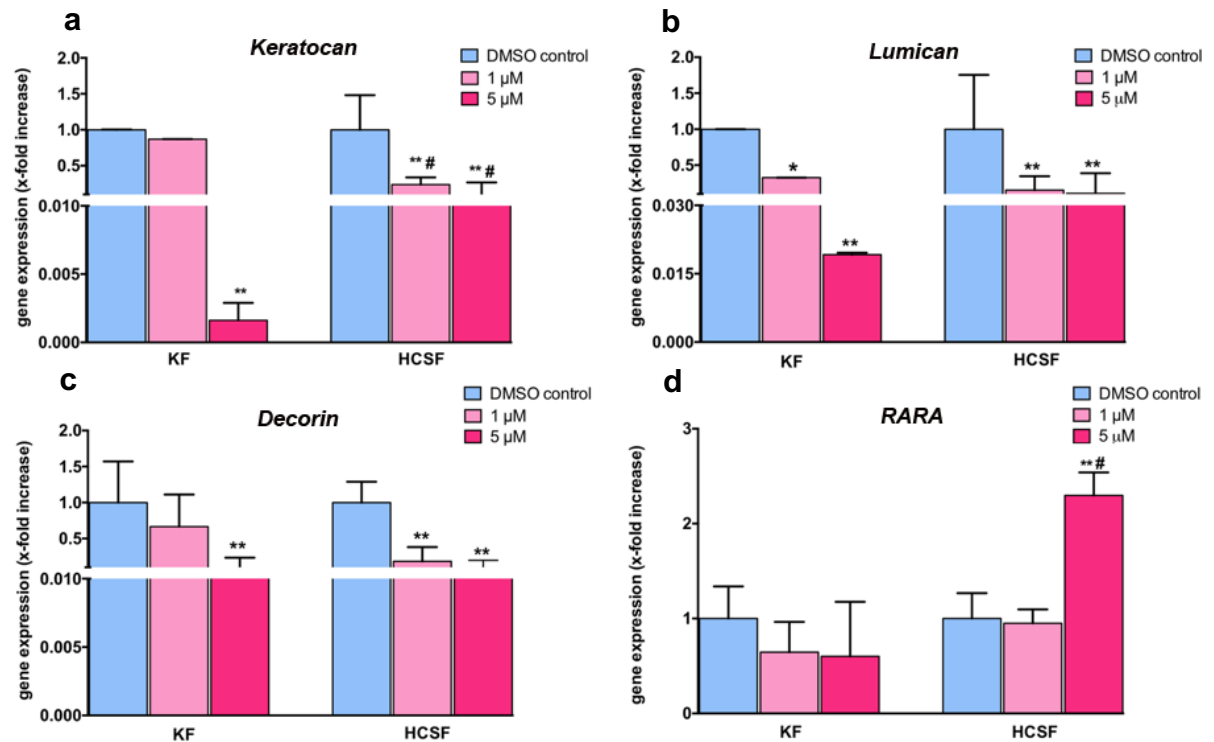


Figure 5.7 - Expression of keratocyte-characteristic markers (a, b, c) and RARA (d) by keratoconic fibroblasts (KF) and human corneal stromal fibroblasts (HCSF) cultured with or without RA supplementation after 7 days of supplementation. Data (mean \pm SD) were obtained from five independent experiments ($n = 5$) and compared using ANOVA with Bonferroni's post hoc correction (** corresponds to $p < 0.01$). * denotes significant difference between RA groups of the same cell compared to the control. # denotes significant difference between two cell types.

only statistically significant, but was also lower than the values expressed earlier at day 3 (figure 5.6b). Meanwhile, KF cultured with 5 μ M RA too expressed lower *MMP-2* on day 7 (1.213 ± 0.478 - fold of the control) as compared to day 3 (2.334 ± 0.713 - fold of control). For *MMP-1*, only keratoconic fibroblasts cultured with 1 μ M RA expressed at least 50% less *MMP-1* than the control (0.571 ± 4.479 - fold of control) (figure 5.8a). However, this difference was not statistically significant. At the same time, supplementation of KF with 5 μ M RA resulted in slight increase of *MMP-1* expression on day 7 (2.279 ± 8.334 - fold of the control) as compared to day 3 (0.532 ± 0.643 -fold of control).

In contrast to the above pattern of MMPs expression in KF, HCSF cultures gave better modulation of *MMP-1* following supplementation with lower concentrations of RA for 1 week compared to *MMP-2*. From figure 5.8a, HCSF cultured with 1 μ M RA

demonstrated lower expression of *MMP-1* at 0.458 ± 0.058 - fold of the control, while cells in the 5 μ M RA group were able to maintain the *MMP-1* expression comparable to that of the control (1.195 ± 0.441 - fold of the control). In the case of *MMP-2*, both RA concentrations were unable to influence HCSF to reduce the gene transcription as compared to HCSF cultured in DMSO alone (figure 5.8b).

On the other hand, the expression of tissue inhibitor of matrix metalloproteinase 1 (*TIMP-1*), which functions to counter the action of MMPs, was also investigated. Interestingly, supplementation with low RA concentrations on KF cultures, particularly with 5 μ M RA, were able to significantly induce higher *TIMP-1* expressions as compared to their effects on HCSF cultures (figure 5.8c). This result, coupled with their low expressions on *MMP-2*, provides interesting supporting evidence that RA supplementation may improve the balance between the enzymes and inhibitors that play a role in keratoconus development. However, RA supplementation did not seem to affect the *TIMP-1* production in normal corneal fibroblasts (HCSF), as the expression was not positively-induced by the presence of RA in the culture medium. This low *TIMP-1* expression in HCSF also coincides with the higher expression of *MMP-2* by the same cells even with the supplementation of RA in the culture medium.

5.3.7 LOXs activities were down-regulated in both KF and HCSF following RA supplementation

In this study, the expression of both LOX and LOX-like genes (*LOXL1*, *LOXL2* and *LOXL3*) were investigated on both KF and HCSF supplemented with low concentrations of RA. The results showed that both RA concentrations caused significant reductions in the expression of LOX and LOX-like genes by both cells as compared to the control following 7 days of supplementation (figure 5.9). However, the pattern of reduction in these gene expressions were different between KF and HCSF. Supplementation with 1 μ M RA in KF reduced the expression of *LOX* and *LOXL1* by half as compared to that in HCSF (figure 5.9a, b), whereas for *LOXL2* and *LOXL3* the genes were similarly expressed by both cells (figure 5.9c, d). On the other hand, supplementation with 5 μ M RA in KF only managed to induce one-third of that in HCSF (figure 5.9c), while the the remaining LOX and LOX-like genes (*LOXL1* and *LOXL3*) showed similar transcript expression in both cells after 7 days supplementation (figure 5.9a, b, d).

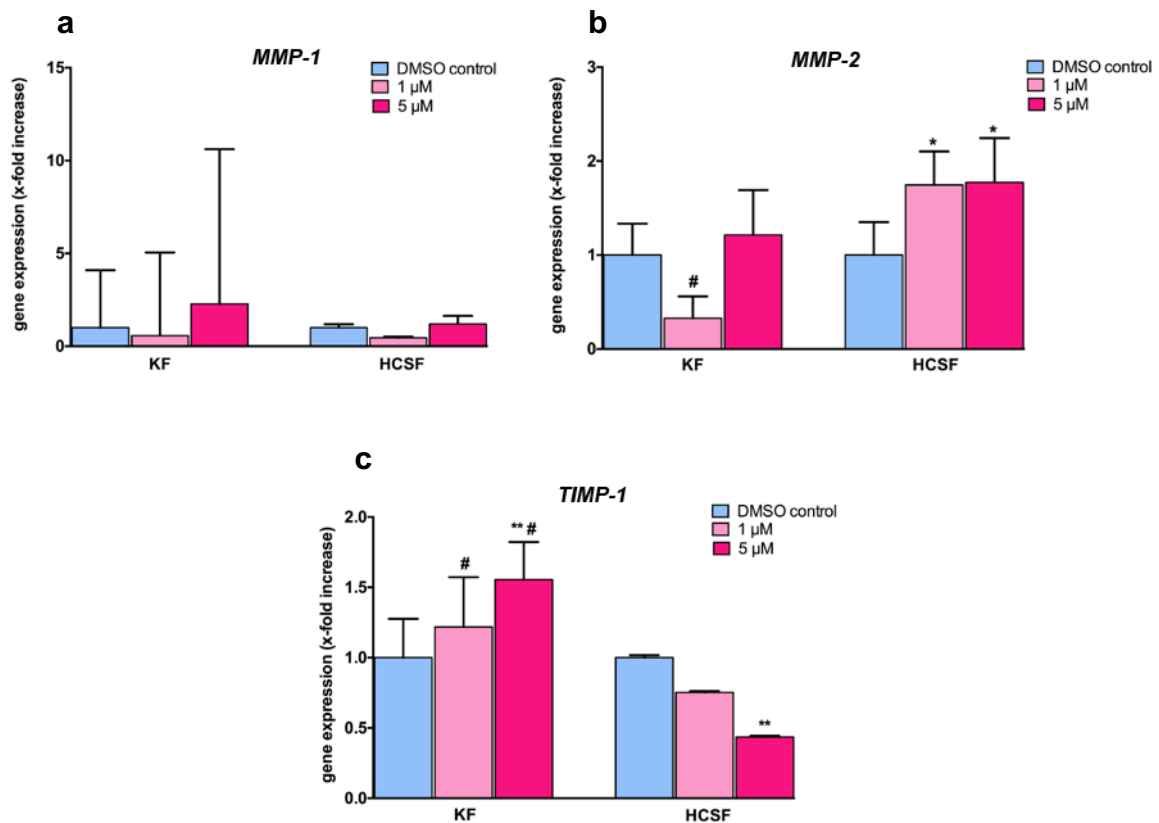


Figure 5.8 - Expression of MMP-1 (a), MMP-2 (b) and TIMP-1 (c) by keratoconic fibroblasts (KF) and human corneal stromal fibroblasts (HCSF) cultured with or without RA supplementation after 7 days of supplementation. Data (mean \pm SD) were obtained from five independent experiments ($n = 5$) and compared using ANOVA with Bonferroni's post hoc correction (* corresponds to $p < 0.05$ and ** corresponds to $p < 0.01$). * denotes significant difference between RA groups of the same cell compared to the control. # denotes significant difference between two cell types.

In the case of LOX and LOX-like gene transcription, keratoconic fibroblasts appeared to be more sensitive to RA supplementations since their transcriptions were greatly affected as compared to HCSF.

5.3.8 Protein expressions by KF following exposure to RA

To further investigate the effects of retinoic acid on *in vitro* keratoconic fibroblasts cultures, relative protein expression of some markers of keratoconic fibroblasts cultured with or without retinoic acid using western blotting method was carried out and quantified. Keratoconic fibroblasts cultured in serum-containing medium (FBS) was also included to represent cells in active fibroblastic state. Here, both lumican

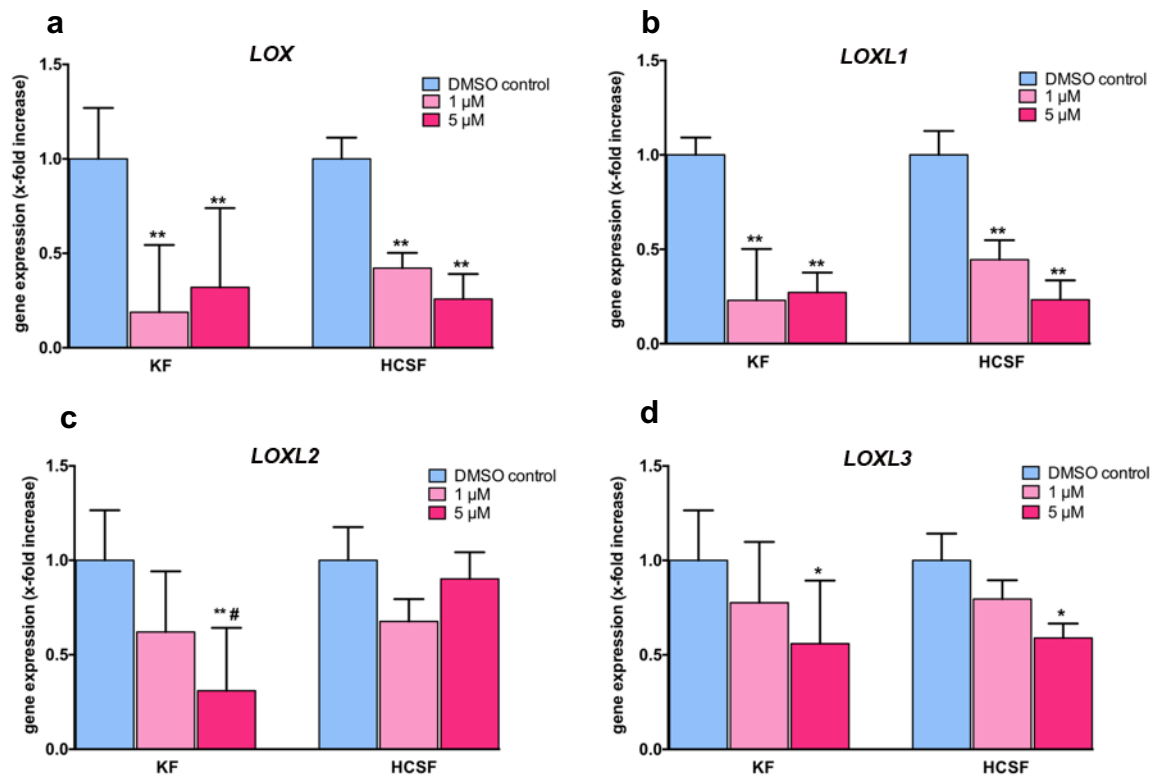


Figure 5.9 - Expression of lysyl oxidase (LOX) and lysyl-oxidase-like (LOXL1, LOXL2 and LOXL3) markers by keratoconic fibroblasts (KF) and human corneal stromal fibroblasts (HCSF) cultured with or without RA supplementation after 7 days of supplementation. Data (mean \pm SD) were obtained from five independent experiments ($n = 5$) and compared using ANOVA with Bonferroni's post hoc correction (* corresponds to $p < 0.05$ and ** corresponds to $p < 0.01$). * denotes significant difference between RA groups of the same cell compared to the control. # denotes significant difference between two cell types.

and decorin (keratocyte-characteristic markers) protein bands as well as protein bands for retinoic acid-receptor α (RARA) and MMP-2 were measured. The expression of lumican in both groups cultured with DMSO control and 1 μ M RA were higher as compared to the cells cultured in FBS (5.231 ± 8.548 and 9.521 ± 6.010 , respectively), but the observed differences were not statistically significant (figure 5.10b). This did not occur in KF cultures supplemented with 5 μ M RA as the cells expressed similar amount of lumican protein as compared to the cells cultured in FBS (1.046 ± 0.081).

On the other hand, both decorin and RARA proteins were expressed similarly in all groups with or without RA supplementation, including KF cultured in FBS (figure 5.10c, d). The most obvious and significant difference between KF supplemented with RA and those cultured in FBS was seen in MMP-2 (figure 5.10e). The protein

expression in cultures supplemented with RA (1 and 5 μ M) were massively reduced relative to those cultured in FBS (0.107 ± 0.216 and 0.204 ± 0.033 , respectively). The MMP-2 expression of KF cultured with DMSO was also reduced to less than half of the amount produced by cells cultured in FBS (0.474 ± 0.216). This result suggests that supplementation of keratoconic fibroblasts with low concentrations of RA in serum-free medium is able to modulate the expression of MMP-2 at both protein and gene levels.

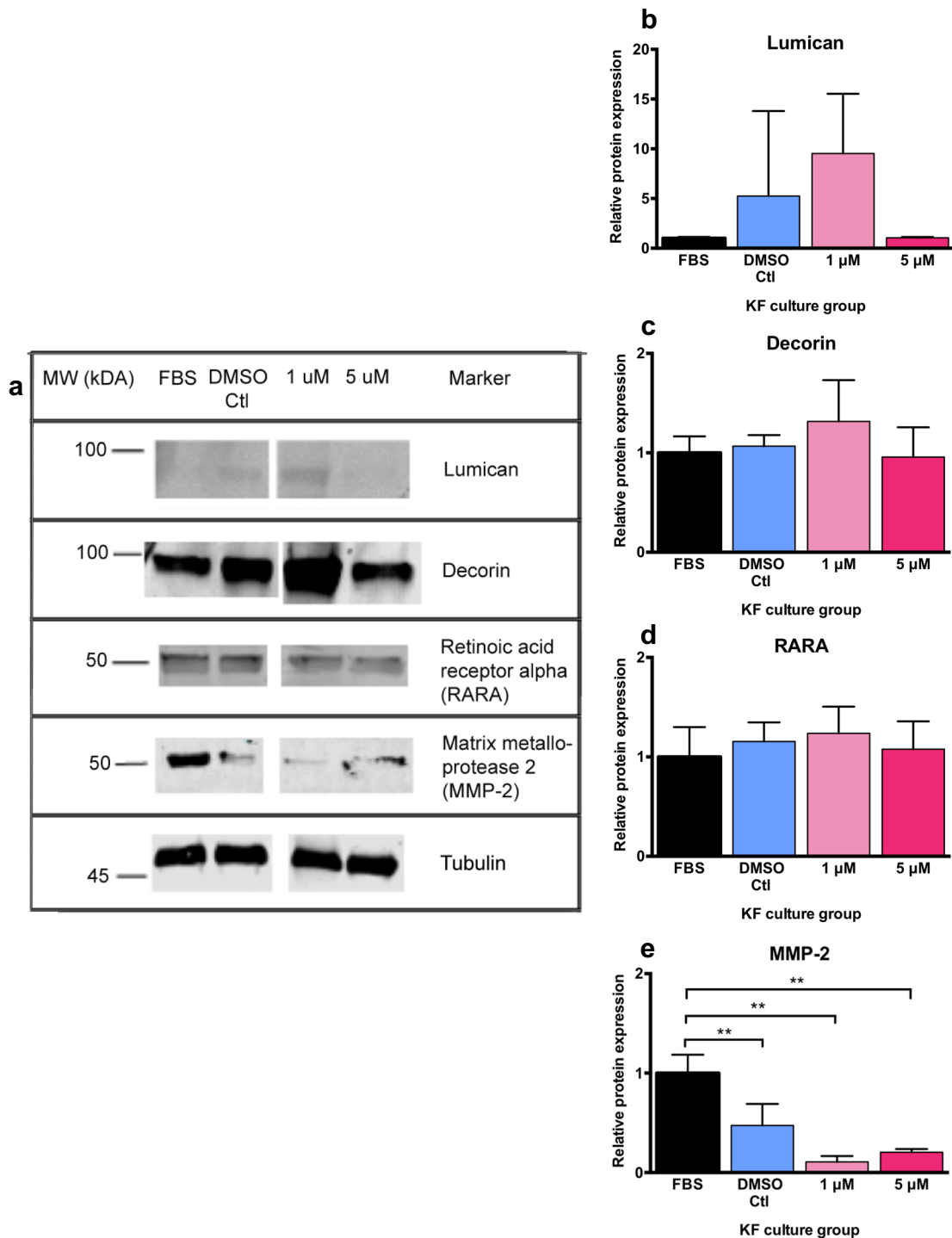


Figure 5.10 - Western Blotting of selected protein markers. (a) Lysates from keratoconic fibroblasts cultured in FBS (FBS), or keratoconic fibroblasts supplemented with RA (1 and 5 μ M) or DMSO control (DMSO Ctl) in serum-free medium were extracted and analyzed by reducing SDS-PAGE followed by immunoblotting. (b-e) Quantification of protein expression was performed by immunoblot densitometry for all markers. Protein expression was normalized relatively to that of group cultured in FBS. Data (mean \pm SD) were obtained from three independent experiments ($n = 3$) and compared ANOVA with Bonferroni's post hoc correction (* correspond to $p < 0.05$).

5.4 Discussion

Keratoconus is an ocular condition in which the cornea assumes a conical shape due to focal thinning and protrusion. The thinning effect comes from overwhelming activities of protease enzymes that cause continuous extracellular matrix degradation. This disruption of collagen organization as well as on-going keratocyte apoptosis in the stroma both contribute to corneal biomechanical instability that eventually results in corneal protrusion. Retinoic acid (RA) has been shown to be a beneficial supplement to corneal keratocytes as it is able to modulate the expression of matrix metalloproteases as well as promoting cellular proliferation when used in serum-free cultures (Gouveia and Connon, 2013; Abidin *et al.*, 2015). Therefore, it is hypothesized that RA is able to modulate the expression of MMP-1 and MMP-2 in cultured keratoconic fibroblasts which are associated with keratoconus disease progression. At the same time, we are also interested to look into the effects of retinoic acid supplementation on keratoconic fibroblasts' viability and their ability to revert back into quiescent keratocytes by assessing their morphology as well as their ability to express keratocyte-characteristic markers. The expression of lysyl oxidase enzymes by keratoconic fibroblasts following RA supplementation is also investigated. To our knowledge, this is the first study that explore the effect of RA on keratoconic fibroblasts in serum-free condition.

The involvement of matrix metalloprotease (MMP) has been the subject of interest in trying to understand the pathophysiology behind keratoconus (Brown *et al.*, 1993; Brown *et al.*, 2004; Smith *et al.*, 2006). Elevation of the MMPs, which includes MMP-1 and -2, have been documented in tears of keratoconic patients, suggesting their involvement in the pathogenesis of the disease (Balasubramanian *et al.*, 2012). MMP-1 degrades collagen type 1 and 3 which form the major components of corneal extracellular matrix (Pilcher *et al.*, 1997). This contributes to the gradual thinning and ectasia of keratoconic cornea (Collier, 2001; Balasubramanian *et al.*, 2012). At the same time, the upregulation of gelatinase (or MMP-2) expression has been linked to an increase in extracellular matrix degradation which also leads to corneal thinning in keratoconus (Kenney *et al.*, 1989). An increase in MMP activity in keratoconus is usually attributed to the imbalance between the expression of MMP and its inhibitor, for example tissue inhibitor of matrix metalloprotease (TIMP). TIMP-1 is known to carry both MMP inhibitory function as well as having anti-apoptotic properties

(Matthews *et al.*, 2007). Low levels of TIMP-1 has been reported in keratoconus corneas (Kenney *et al.*, 2005). However, there is also study which found no difference in TIMP-1 expression between normal corneal fibroblasts and KF in vitro even when there is an increase in gelatinase activity within the same KF cultures (Smith *et al.*, 1995). Another possibility to explain such increase in MMP activity is surplus of proenzyme (proMMP) which later facilitates enzyme activation. Examples of proMMPs are interleukin-6 (IL-6) and membrane-bound MT1-MMP (MMP-14). IL-6 mediates the synthesis of MMP-1 induced by tumor necrosis factor- α (TNF- α) in cultured keratoconic fibroblasts (Du *et al.*, 2016). The expression of MT1-MMP in the epithelium and stroma of keratoconus cornea is significantly elevated than in normal cornea (Collier *et al.*, 2000), which in turn can activate latent MMP-2. This might represent a more dominant factor to the MMP2:TIMP1 imbalance as compared to reduction in TIMP-1 expression (Smith *et al.*, 2006).

More to that, involvement of inflammatory cytokines (interleukin-1) is also believed to play important role in modulating the expression of metalloprotease in keratoconus. Interleukin-1 (IL-1) receptor is found to be enhanced in keratocytes from keratoconus corneas, as high as 4x the expression in normal cornea (Bureau *et al.*, 1992; Zhou *et al.*, 1996). This highlights the abundance of IL-1 activity in the system produced by epithelial or endothelial cells of keratoconus corneas. Such increase in IL-1 is believed to be due to epithelial microtrauma from eye rubbing, contact lens wear and atopy which are all related to keratoconus (Wilson, 1999). This pro-inflammatory cytokine consists of two subtypes; α and β subtypes. Both subtypes share the same receptors, thereby competing with each other for binding sites. IL-1 α induces autocrine loop in keratocytes leading to keratocytes apoptosis which is a hallmark in keratoconus (Kim *et al.*, 1999). Meanwhile, IL-1 β is responsible for the induction of matrix metalloproteinases (MMPs), and these MMPs are responsible for collagen degradation in the stroma. It has been shown that the expression of metalloproteases by corneal stromal cells can be modulated in the presence of IL-1 β (Girard *et al.*, 1991). Interestingly, expression of MMP-2 by corneal fibroblasts in the presence of IL-1 β is enhanced when accompanied by an increased in mechanical strain caused by thinning of the cornea (Feng *et al.*, 2016). This is very significant to keratoconus as both factors (presence of IL-1 β and thinning of cornea) are well-documented in the disease.

Based on our findings, we found that the expressions of both MMP-1 and MMP-2 in our keratoconic fibroblasts (KF) differ when the cells were cultured in medium with or without serum, which is noticeable as early as day 3 of culture (figure 5.6b). Not only that, the modulation of MMP-2 following retinoic acid supplementation was more significant in KF cultures as compared to normal stromal cells culture (HCSF). This is not surprising as the upregulation in MMP-2 was also found to be more evident in keratoconic stromal cell cultures as compared to stromal cells cultured from normal corneas (Smith *et al.*, 2006). More importantly, the addition of RA at 1 μ M concentration was able to further down-regulate the MMPs expression until day 7 of supplementation at both gene and protein level. Bauer *et al.* in 1983 recorded a maximal inhibition of collagenase activity by skin fibroblasts upon supplementation of these cells with retinoic acid between 0.25 – 1 μ M concentration, but higher amount of retinoic acid is required for the same cells to inhibit gelatinase activity (3 – 6 μ M concentration). However, we found that in keratoconic fibroblasts higher concentrations of retinoic acid (5 μ M and above) were not able to reduce both MMP-1 and MMP-2 expressions as much as in 1 μ M group (figure 5.6b and figure 5.8) despite a slightly higher TIMP-1 gene expression recorded by the 5 μ M group (figure 5.8c). Such difference in retinoic acid activity might be due to the different types of fibroblasts (skin versus cornea) used in both studies.

Nevertheless, the modulation of MMPs in KF following 1 μ M RA supplementation is not reciprocated by significant increase in TIMP-1 expression. Previous studies have shown that keratoconic fibroblasts are capable to increase the synthesis TIMP-1 in vitro (Kenney *et al.*, 1998). On top of that, RA has been shown to significantly induce TIMP expression in chondrocytes fibroblasts in vitro, however, this effect could be heightened when used in combination with growth factors such as basic fibroblasts growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) or transforming growth factor (TGF) (Bigg and Cawston, 1995; Bigg and Cawston, 1996). There is possibility that TIMP-1 expression can be up-regulated in KF cells supplemented with RA in the presence of these growth factors. Besides TIMP, other possibilities include direct inhibition of RA on IL-1 β or by modulating the expression of MMP proenzymes. Kimura *et. al* (2016) has recently demonstrated the ability of RA to suppress the activities of both MMP-1 and MMP-2 on corneal fibroblasts cultures exposed to IL-1 β using similar concentration (1 μ M). However,

since the level of IL-1 β in our cultures were not measured (before and after RA supplementation), this ability to counter-act the effects of IL-1 β on keratoconic stromal cells remains inconclusive.

One interesting observation from this study is the morphological changes that occurred to some of the keratoconic fibroblasts following RA supplementation. Previous studies have shown that retinoic acid induces morphological changes on neuroblastoma cells as they were exposed to retinoic acid in their culture medium. These changes, termed as 'cellular differentiation', induces neurite-formation in neuroblastoma cells with long neuritic extensions which increases their adherence to neighbouring cells (Oppenheimer *et al.*, 2007; Mandili *et al.*, 2011). Similarly, exposure of keratoconic fibroblast to RA in serum-free conditions resulted in morphological changes especially in the culture groups supplemented with 5 and 10 μ M RA. These cells differentiated from being spindle-shape like cells into 'neurite-like' cells. Therefore, it is tempting to say that the morphological changes observed in keratoconic fibroblasts in this study was due to exposure to RA. However, it is uncertain if this change is only seen in pathological conditions, and not on normal, healthy keratocytes.

At the same time, the changes in KF morphology following RA supplementation is associated with reduction in cell viability, especially at higher concentrations of RA (10 μ M and above). Although we have previously shown that RA supplementation at 10 μ M is able to maintain cell viability and induce cell proliferation on normal corneal keratocytes, similar results was not achieved in KF cultures. Keratoconic fibroblasts appear to be sensitive to retinoic acid at higher concentrations, especially at 25 μ M where it appears to be toxic to the cells. Surprisingly, the use of dimethyl sulfoxide (DMSO) alone (as the control) in serum-free medium produced better results in terms of supporting KF viability in culture. We postulate that this happen as a result of successful reduction of oxidative stress level. Oxidative stress is one of the important aspects that has been discussed in the development and progression of keratoconus (Galvis *et al.*, 2015). Alteration in antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase, and antioxidant molecules have been reported in keratoconus cornea (Behndig *et al.*, 2001; Arnal *et al.*, 2011) resulting in abnormally high reactive oxygen/nitric species (ROS/RON) production, increase in catalase/caspase activities and loss of cell viability (Chwa *et al.*, 2006).

Meanwhile, DMSO is a known reactive oxygen species scavenger (Salim, 1992). DMSO may have played an excellent role in reducing the amount of harmful free radicals thereby improving the survival of KF in culture. Since RA is also prepared in DMSO, supplementation with lower concentrations of RA have been shown to be able to maintain certain level of KF viability although it was not as good as in DMSO control.

Since DMSO is able to retain more KF in culture to remain viable, and the fact that these cells were cultured in serum-free conditions, this may have induced them to become quiescent keratocytes and started producing more keratocyte-characteristic markers (keratocan, lumican, decorin) as compared to those in RA-supplemented groups. This effect might also take place secondary to effective scavenging of free radicals by DMSO. The use of antioxidant riboflavin in keratoconic stromal cell culture has been shown to give beneficial effect in upregulating both keratocan and lumican gene expression (Cheung *et al.*, 2014). However, there has been mixed results on the levels of proteoglycans present in keratoconic corneas. Some has found that the disease causes a reduction in corneal proteoglycans, including keratocan, lumican and decorin (Chaerkady *et al.*, 2013; García *et al.*, 2016). Such reduction in core protein production may be due to downregulation of transcription at gene level, or due to corneal thinning secondary to ECM degradation (Zhang *et al.*, 2013). An increased in keratocan expression from stromal layer of keratoconus cornea has also been reported, although it has been associated with altered fibrillogenesis in the stroma and possibly structural defect (Wentz-Hunter *et al.*, 2001).

Lysyl oxidase (LOX) is a copper-dependent amine oxidase which is secreted as a glycosylated pro-enzyme with a molecular weight (Mw) of 50 000 (Kagan, 1986; Trackman *et al.*, 1992). It involves directly in maintaining the structural integrity of ECM by cross-linking the side chain of collagen and elastin (Kagan and Trackman, 1991). This intermolecular cross-linking helps to increase the tensile strength of collagen fibers by making them more insoluble and resistant to enzymes, which are important for normal connective tissue function and wound healing (Vater *et al.*, 1979). To date, there are 4 isoforms of LOX, known as LOX-like proteins (LOXL) which are functional but genetically distinct from one another (Molnar *et al.*, 2003). Its deregulation has been reported in ocular disorders, such as proliferative diabetic retinopathy and keratoconus (Ollivier *et al.*, 2007; Coral *et al.*, 2008; Dudakova *et al.*,

2012). There are conflicting reports while discussing the level of LOX in keratoconus. An earlier study by Nielsen et al. in 2003 showed an increase LOX expression in the epithelium of keratoconus cornea. On the contrary, recent studies suggested the opposite, showing significant reduction in LOX transcript levels in keratoconus epithelial cells and patients tear fluids (Dudakova *et al.*, 2012). We found that supplementation of retinoic acid significantly reduced the LOX expression both on KF as well as normal stromal fibroblasts cultures, particularly LOX and LOX-like 1 gene. However, the reason behind this is still unknown. Further investigations are needed to validate the result as well as providing understanding on the negative-relationship between RA-LOX.

In conclusion, the interaction between retinoic acid and keratoconic fibroblasts appears to be quite complex. Keratoconic fibroblasts appears to be sensitive to retinoic acid beyond certain concentration gradients. Our findings indicate that supplementation with 1 μ M RA may be the most appropriate concentration for keratoconic fibroblasts as it is able to significantly reduce MMPs expressions as well as maintaining cell viability under serum-free environment. This study also demonstrates the capability of DMSO to modulate the expression of MMPs and keratocyte-characteristic markers in keratoconic fibroblasts while maintaining the highest number of cell viability in vitro, possibly through direct scavenging of free radicals. Further studies are needed to look at the therapeutic potential of DMSO in the clinical management of keratoconus.

Chapter 6: General conclusion and future directions

This thesis has extensively discussed the role of mechanical and chemical cues to preserve corneal cells function. Specifically, the use of decellularised bovine cornea to study the effect of substrate stiffness (as the main biomechanical cue) in controlling corneal epithelial stem cell fate has led to the conclusion that these stem cells are more sensitive towards compliant environment as compared to stiffer environment. Since the corneal stem cells source comes from corneal limbus alone, this inclination towards compliant environment might reflect the protective mechanism the nature provides to these cells from getting easily differentiated especially when stiffness shift occurs in the limbus.

In order to arrive to the above conclusion, we began the study by carefully selecting decellularisation protocols as majority of the protocols established are meant for human corneas which are not always suitable for bovine cornea works. As mentioned previously, our aim is to determine which decellularisation methods provide the best overall outcome in terms of effectiveness in cellular removal, preservation of the ECM and its ability to support corneal cell growth post-decellularisation. Despite such effort, there were still unfavourable consequences following decellularisation such as swelling of corneal scaffolds which altered the extracellular matrix (ECM) compartment. This unexpected corneal edema has caused the scaffolds to become softer thereby affecting the overall mechanical property of the decellularised scaffold. This is a very important point that needs to be taken into great consideration for any researcher who has the interest to use decellularised cornea to study substrate stiffness in the future. Another obstacle that we faced was choosing the 'best' markers for corneal epithelial stem cell identification. Until now, majority of corneal epithelial stem cell markers proposed for stem cell studies remain as 'putative' rather than 'definitive'. Future works related to mechanosensing and substrate stiffness using decellularised cornea may need to use a different approach to get a better definitive measure rather than relying on stem cell markers alone, for example by measuring Rho GTPase activity. There has been an emerging interest in using Rho GTPase and their effectors to identify the response elicited by mechanical stimuli on cellular signalling cascade. A good example of Rho GTPase is focal adhesion kinase (FAK) in cell proliferation and migration as well as regulator in stem cell differentiation. It is interesting how mechanical signal from decellularised matrix stimulates the

newly-attached cells to proliferate, migrate and differentiate through the regulation of Rho GTPase and their effectors. The use of Rho kinase inhibitors in limbal epithelial stem cells culture may also need to be considered as it has been shown to be able to fabricate stratified epithelial cells when used on cultured keratinocytes. At the moment, the interest in Rho kinase inhibitor is steered towards studies on corneal endothelial diseases and corneal endothelial cell cultures. This inhibitor has beneficial effects which aid the recovery of diseased or injured endothelial cells, and helps improve human corneal endothelial cell cultures through inhibition of apoptosis and increasing cellular attachment and proliferation.

At the same time, this thesis has also looked into the role played by chemical cues in the control and maintenance of the corneal stromal cells (or keratocytes) phenotype in order to investigate its biology *in vitro*. Any modification made by chemical cues present in the medium may significantly give impact to the cellular maintenance environment; hence alter the long-term research results. These chemical signals come from all the elements present in the culture medium, including additional medium supplement. The use of retinoic acid (RA) as supplement to serum-free medium has opened up a new strategy in promoting serum-free cultures for corneal keratocytes particularly in 3D environment. RA supplementation maintains keratocytes viability and induces cell proliferation in serum-free environment for extended period of time. This is important to tissue engineering as engineered constructs, such as the cornea, typically require many days in culture. Our RA-supplemented 3D stromal constructs success may provide an alternative to decellularised cornea to further understand the effect of substrate stiffness on corneal epithelial stem cell fate. This is because the stiffness of our 3D stromal construct can be manipulated to mimic the actual stiffness of the limbus or central cornea by controlling the level of tissue hydration during collagen gel compression step. At the same time, our 3D stromal model may also provide the epithelial-stromal crosstalk required during stem cell proliferation and differentiation as the embedded keratocytes are kept in their quiescence state similar to their condition in native cornea.

On the other hand, the interaction between RA and fibroblasts taken from keratoconus cornea appears to be quite complex. The first few months of work using retinoic acid in keratoconic fibroblasts cultures were very exhaustive with frustrating

results. The cells appear to be sensitive to retinoic acid using the same concentration previously shown to be useful in normal healthy keratocytes. Further extension to the work reveals that only low levels of retinoic acid appear to contribute to reducing the levels of matrix metalloproteases, however they still affect keratoconic fibroblasts' ability to remain viable as well as producing other markers known to be involved in the pathogenesis of the disease. On top of that, the exact mechanism to how retinoic acid affects keratoconic fibroblasts viability as well as their capability to reduce the matrix metalloproteases levels remains unknown. Nevertheless, we found that there is possibility to improve keratoconic fibroblasts cultures in serum-free condition by using elements that can remove free radicals from the culture environment, in this case using DMSO. Such incidental finding is very intriguing as oxidative stress has been suggested as one of the most important regulator in the pathogenesis of keratoconus. This study can be extended further by looking into the potential role of RA and DMSO on keratoconic fibroblasts culture from oxidative stress point of view. These results may provide a 'hint' towards a more detailed study in the future which focus on the potential use of other anti-oxidant elements in the clinical management of keratoconus. On a different note, since RA has been shown to be able to modulate the expression of matrix metalloproteases from diseased cornea, its potential use can also be explored on other inflammatory corneal diseases associated with elevated levels of matrix metalloproteases, for example bullous keratoplasty and vascularised corneal scar.

Based on our earlier hypotheses, our studies showed that:

1. Decellularised cornea could be used as scaffold to study the effect of mechanical stiffness on limbal epithelial stem cells differentiation.
2. There was no significant difference in limbal epithelial stem cells phenotype when they were cultured on decellularised limbal or central cornea.
3. The mechanical properties of bovine cornea could be controlled by enzymatic digestion (artificial softening) which could affect human limbal epithelial stem cells fate.
4. In 3D environment, chemical cues from retinoic acid supplementation was able to modulate keratocytes proliferation, the expression of keratocyte-characteristic markers as well as improving the overall hydration and weight of the 3D construct.

5. Early exposure of keratoconic fibroblast to retinoic acid supplementation was able to modulate the MMPs expression while de-differentiating the fibroblasts into more native, quiescent keratocytes through regulation of keratocyte-characteristic markers, but these effects did not sustain beyond three days of supplementation.

In conclusion, both mechanical and chemical cues have been shown to give significant effects on corneal cells behaviour. Mechanical cue from underlying matrix stiffness is very influential in directing corneal epithelial stem cells response *in vitro*, which ultimately may affect their therapeutic potential to restore and regenerate native tissue. Biochemical cue is another important element that guides corneal cells to remain as close as possible to their quiescent phenotype. Therefore, any future corneal models will have to incorporate both signals in order to achieve the best corneal model for *in vitro* works.

Presentations and paper publication

Abidin, F.Z., Hamley, I., Connon, C.J. (2014, January). *Histological evaluation of recellularised bovine corneas prepared from different decellularisation methods*. Poster presented at Hopkin's Postgraduate Research Day, University of Reading, Reading, UK.

Abidin, F.Z., Gouveia, R.M. and Connon, C.J. (2015, March). *Retinoic acid promotes cell proliferation and ECM production on encapsulated human corneal keratocytes*. Poster presented at NICR/IGM Joint Postgraduate Research Day, Newcastle University, Newcastle UK.

Abidin, F.Z., Gouveia, R.M. and Connon, C.J. (2015, June). *Application of retinoic acid improves form and function of tissue engineered corneal construct*. Paper presented at the International Conference of Tissue Engineering, Lisbon, Portugal.

Abidin, F.Z., Gouveia, R.M. and Connon, C.J. (2015, October). *Developing a robust tissue-engineered corneal construct using retinoic acid-supplemented serum-free medium*. Paper presented at the North East Postgraduate Conference 2015, Newcastle, UK.

Abidin, F.Z., Karamichos, D., Figueiredo, F. and Connon, C.J. (2016, May). *The effects of retinoic acid on keratoconic fibroblasts in vitro under serum-free conditions*. Paper presented at the annual meeting of the Association for Research in Vision and Ophthalmology (ARVO), Seattle, Washington USA.

Abidin, F.Z., Gouveia, R.M. and Connon, C.J. (2015) 'Application of retinoic acid improves form and function of tissue engineered corneal construct', *Organogenesis*, 11(3), pp. 122-136.

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