TISSUE ENGINEERED CONJUNCTIVAL EPITHELIAL TRANSPLANTS FOR THE TREATMENT OF OCULAR SURFACE DISORDERS

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Tissue Engineered Conjunctival Epithelial Transplants For The Treatment Of Ocular Surface Disorders

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2. ABBREVIATIONS

3T3	3-day transfer, inoculum 3 x 10^5 cells
ABC	Avidin-biotinperoxidase complex
ABCG2	ATP-binding cassette sub-family G member 2
ACF	Animal compound free
ADDE	Aqueous deficient dry eye
AFSC	Amniotic fluid stem cells
AM	Amniotic membrane
AMT	Amniotic membrane transplantation
ANOVA	One-way analysis of variance
ARVO	The Association for Research in Vision and Ophthalmology
α-SMA	α-smooth muscle actin
BM	Basement membrane
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CAM	Calcein-acetoxymethyl ester
CBS	Chord blood serum
CD29	β1-integrin subunit
CD40	Tumor necrosis factor receptor family-5
CD44	Hyaluronate receptor
CD62E	CD62 antigen-like family member E
CFE	Colony-forming efficiency
CK1	Cytokeratin 1
CK3	Cytokeratin 3

CK4	Cytokeratin 4
CK5	Cytokeratin 5
CK7	Cytokeratin 7
CK8	Cytokeratin 8
CK10	Cytokeratin 10
CK12	Cytokeratin 12
CK13	Cytokeratin 13
CK15	Cytokeratin 15
CLAU	Conjunctival limbal autograft
Cy2	Cyanine dye 2
Cy3	Cyanine dye 3
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide-1
DAPI	4',6-diamidino-2-phenylindole
DED	Dry eye disease
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPI	Death-to-preservation interval
DUSPs	Dual specificity phosphatases
EB	Epidermolysis bullosa
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH-1	Ethidium homodimer-1
EKC	Epidemic keratoconjunctivitis
ESC	Embryonic stem cells

FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF10	Fibroblast growth factor 10
FITC	Fluorescein isothiocyanate
GDNF	Glial cell-derived neurotrophic factor
GFRα-1	GDNF family receptor α-1
GVHD	Graft-versus-host disease
HCjE	Human conjunctival epithelial
H&E	Haematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES1	Hairy and enchancer of split-1
hESC	Human embryonic stem cells
HLEC	Human limbal epithelial cells
HPCLK	Homologous penetrating central limbo-keratoplasty
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IHC	Immunohistochemistry
iPSC	Induced pluripotent stem cells
IVCM	*In vivo* confocal microscopy
KCl	Potassium chloride
KCS	Keratoconjunctivitis sicca
KLAL	Keratolimbal allograft
KLF4	Krüppel-like factor 4
LEC	Limbal epithelial cells
LOGIC	Laryngo-onychocutaneous
lr-CLAL	Living-related conjunctival limbal allograft

LSC	Limbal stem cells
LSCD	Limbal stem cell deficiency
M	Molar
MAPK	Mitogen-activated protein kinase
MEEI	Massachusetts Eye and Ear Infirmary
MEM	Minimum Essential Medium
MEN	Multiple endocrine neoplasia
MGD	Meibomian gland dysfunction
mL	Milliliter
MMC	Mitomycin C
mM	milliMolar
MSC	Mesenchymal stem cells
MSX	Msh homeobox
MUC1	Mucin-1
MUC4	Mucin-4
MUC5	Mucin-5
MUC5AC	Mucin-5AC
MUC5B	Mucin-5B
MUC7	Mucin-7
MUC16	Mucin-16
Na_2HPO_4	Sodium phosphate dibasic
NaH_2PO_4	Monosodium phosphate
NaCl	Sodium chloride
NANOG	Tír inna n-Óc
NF-kβ	Nuclear factor-kβ
NGF	Nerve growth factor

NOTCH1	Notch homolog 1
NSAIDs	Nonsteroidal anti-inflammatory drugs
NTR	Neurotrophin low-affinity receptor
OCP	Ocular cicatricial pemphigoid
OCT	Optimal cutting temperature compound
OCT4	Octamer-binding transcription factor 4
OUH	Oslo University Hospital
p63	Tumor protein p63
PAX6	Paired box protein 6
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PED	Persistent epithelial defects
REC	The Regional Committee for Medical Research Ethics
RPE	Retinal pigment epithelial
RPMI	Roswell Park Memorial Institute
r_s	Spearman's rank correlation coefficient/rho
SCNT	Somatic cell nuclear transfer
SD	Standard deviation
SEM	Standard error of the mean
SERI	Schepens Eye Research Institute
SIX	Sina oculis
SJS	Stevens-Johnson syndrome
SLET	Simple limbal epithelial transplantation
SOX2	sex determining region Y (SRY)-box 2

SPSS	Statistical Package for the Social Sciences
SRY	Sex determining region Y
SSCE	Sequential sector conjunctival epitheliectomy
TAC	Transiently amplifying cells
TBUT	Tear break-up time
TEM	Transmission electron microscopy
TEN	Toxic epidermal necrolysis
TFLL	Tear film lipid layer
TNFR5	Tumor necrosis factor receptor family-5
Tris	Tris(hydroxymethyl)aminomethane
TrkA	Tyrosine kinase receptor A
TSP	Trisodium phosphate
UEA-1	Ulex europaeus agglutinin-1
WB	Western blot

3. LIST OF PAPERS

I. **J.R. Eidet, I.G. Fostad, M.A. Shatos, T.P. Utheim, O.A. Utheim, S. Raeder, D.A. Dartt.**
Effect of Biopsy Location and Size on Proliferative Capacity of Ex Vivo Expanded Conjunctival Tissue.
Invest Ophthalmol Vis Sci. 2012 May 14;53(6):2897-903. Print 2012 Jun.

II. **I.G. Fostad, J.R. Eidet, M.A. Shatos, T.P. Utheim, O.A. Utheim, S. Raeder, D.A. Dartt.**
Biopsy harvesting site and culture size affect the number of goblet cells.
Exp Eye Res. 2012 Nov;104:15-25. doi: 10.1016/j.exer.2012.09.007. Epub 2012 Sep 26.

III. **J.R. Eidet, O.A. Utheim, S. Raeder, D.A. Dartt, T. Lyberg, E. Carreras, T.T. Huynh, E.B. Messelt, W.E. Louch, B. Roald, T.P. Utheim.**
Effects of serum-free storage on morphology, phenotype, and viability of *ex vivo* cultured human conjunctival epithelium.
Exp Eye Res. 2012 Jan;94(1):109-16. Epub 2011 Dec 3.

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5. GENERAL INTRODUCTION

5.1 Ocular Surface

The term 'ocular surface', initially introduced by Thoft (1977), encompass the conjunctival and corneal epithelia, each of which has a distinct cellular phenotype. They interact and are both crucial for maintaining a healthy ocular surface and preventing blindness. The corneal and conjunctival epithelial cells form the ocular surface, which is covered by the tear film (Fig. 1). Conjunctival and corneal epithelia undergo continuous replacement by dividing and differentiating cells. The epithelial cells originate from stem cell populations in each tissue.

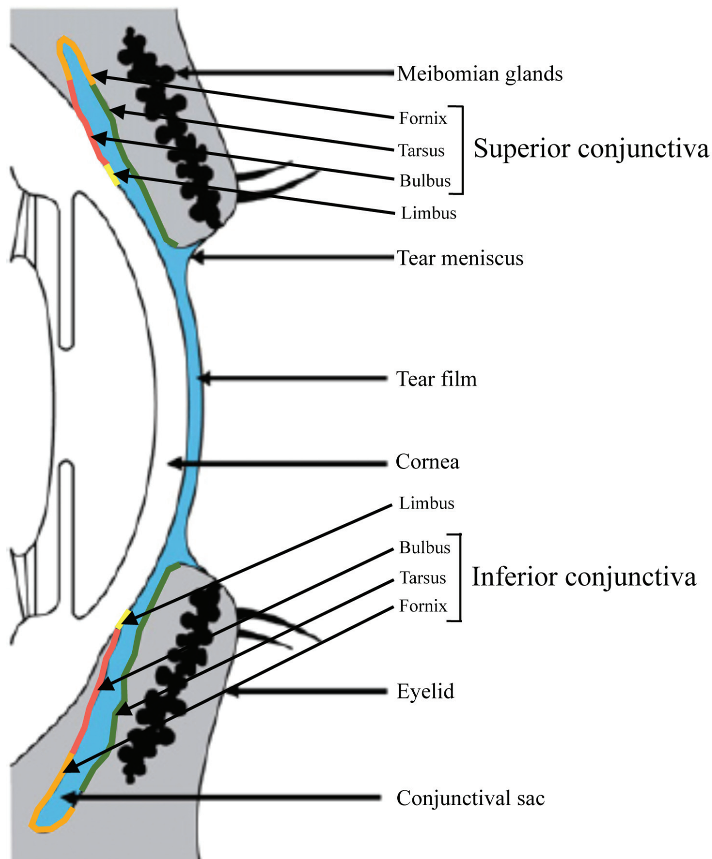

Figure 1. The ocular surface is covered by conjunctival and corneal epithelial cells. Both epithelia are covered by the tear film. The superior and inferior conjunctival epithelium can be divided into a forniceal, bulbar and tarsal part. Adapted from Gaffney et al. 2010.

5.2 The conjunctiva

5.2.1 The Conjunctival Epithelium

5.2.1.1 Structure

Conjunctival epithelium is non-keratinized and is at least two cell layers thick (Wagoner, 1997). It consists of two phenotypically distinct cell types –stratified squamous non-goblet cells (90 – 95%) and goblet cells (5 – 10%) (Fig. 2), in addition to occasional lymphocytes (Dua et al., 1994a; Steven and Gebert, 2009) and melanocytes (Latkovic and Nilsson, 1979). The conjunctival epithelium can anatomically be divided into three parts: 1) the tarsal (or palpebral) conjunctiva, which covers the backside of the eyelids; 2) the bulbar conjunctiva, which covers the anterior surface of the globe, except the cornea; and 3) the forniceal conjunctiva, which connects the bulbar and tarsal conjunctiva (Fig. 1).

5.2.1.2 Function

The conjunctival epithelium plays an important role in ensuring the optical clarity of the cornea by providing lubrication to maintain a smooth, refractive surface and by producing mucins critical for tear film stability (Wagoner, 1997; Schrader et al., 2009b). The conjunctiva also protects the eye against mechanical stress and infectious agents. It furthermore contributes water and electrolytes to the tear fluid (Yu et al., 2012). The squamous cells produce cell membrane spanning mucins, while the goblet cells secrete the gel forming mucins, both of which helps to maintain a protective tear film.

5.2.2 The Stratified Squamous Non-goblet Cells

5.2.2.1 Morphology and Identification

The squamous cells are flattened cells (Fig. 2) that cover the larger part of the conjunctival surface. In contrast to the goblet cells, the stratified squamous cells express cytokeratin 4 (CK4) (Kasper, 1991). The cells are connected together apically by zonulae occludens (tight junctions) and desmosomes (Weyrauch, 1983a). The superficial surface of the squamous cells are covered by the membrane-tethered mucins mucin-1 (MUC1), mucin-4 (MUC4) and mucin-16 (MUC16) (Gendler and Spicer, 1995; Govindarajan and Gipson, 2010).

5.2.2.2 Function

The membrane-tethered mucins MUC1, MUC4 and MUC16 are essential for tear stability and make up the innermost layer the tear film (Fig. 7) (Gendler and Spicer, 1995; Gipson et al., 2004; Govindarajan and Gipson, 2010). The squamous cells also contribute to the hydration of the ocular surface through ion transport across the apical cell membrane with accompanying osmotic water transfer (Yu et al., 2012). The forniceal region may be especially important as it has reportedly higher ion transport activity than the bulbar and palpebral region (Yu et al., 2012).

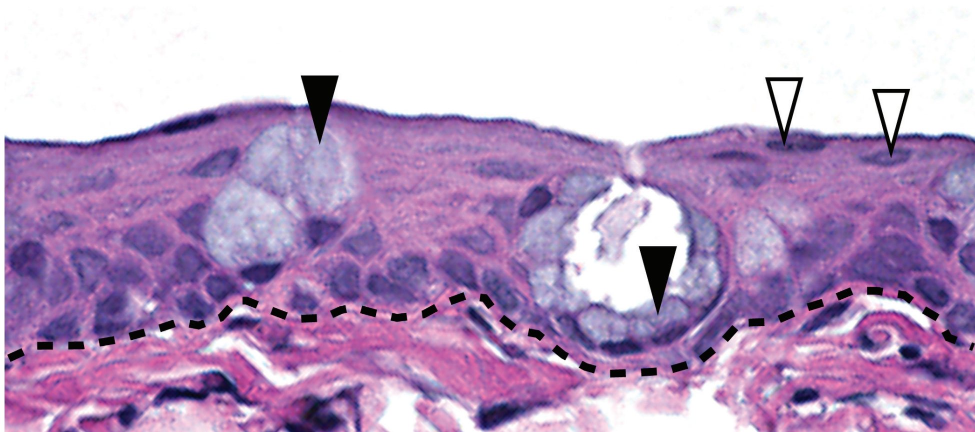

Figure 2. Photomicrograph of cross-sectioned haematoxylin and eosin (H&E)-stained rat conjunctiva showing goblet cells (black arrowheads) and stratified squamous non-goblet cells (white arrowheads). The conjunctival epithelium rests on the conjunctival basement membrane (BM) (dotted line). The loose, vascularized conjunctival stroma is situated beneath the BM. Original magnification: ×630.

5.2.3 The Goblet Cells

5.2.3.1 Morphology and Identification

Goblet cells contain mucin-granules (Fig. 2-4) and have traditionally been identified through their secretory product using markers including the Ulex europaeus agglutinin-1 (UEA-1) lectin, anti-mucin-5AC (MUC5AC) antibody and Periodic acid-Schiff (PAS) reagent that

target the goblet cell gel-forming mucins (Fig. 3) (Kawano et al., 1984; Argueso and Gipson, 2001).

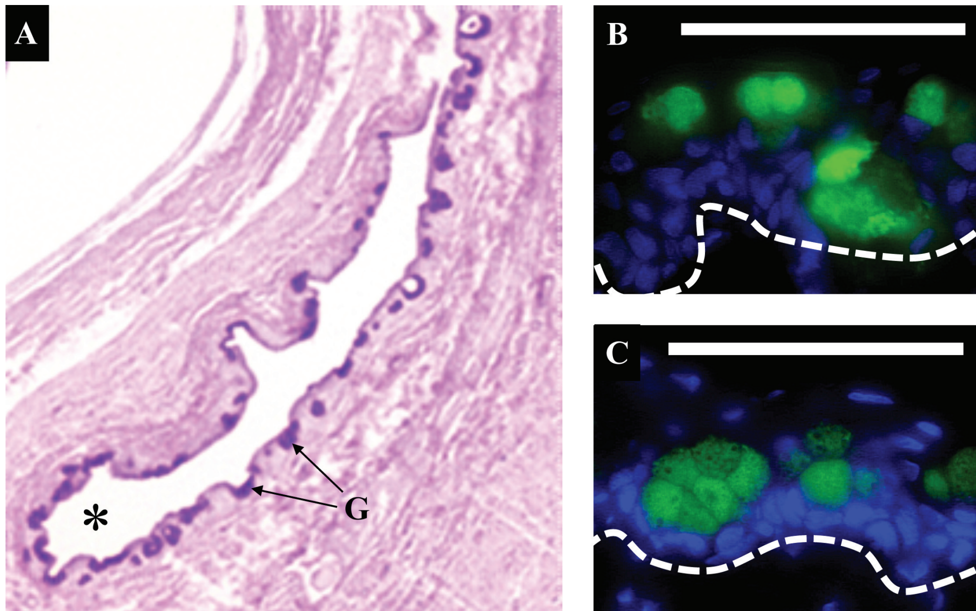

Figure 3. Conjunctival goblet cells identified by Periodic acid-Schiff (PAS) reagent, Ulex europaeus agglutinin-1 (UEA-1) and mucin-5AC (MUC5AC) staining. (A) Cross-section of PAS-stained whole rat eye showing the inferior forniceal sac (*) with numerous PAS+ (purple) goblet cells (G) in the fornix. Original magnification: ×40. (B) Immunofluorescence image displaying rat conjunctiva with UEA-1-stained goblet cell mucin (green). (C) Immunofluorescence image showing MUC5AC+ (green) goblet cells. (B and C) Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue). The white dotted line indicates the approximate position of the basal membrane. Magnification: ×630. Scale bars: 100 µm.

In haematoxylin and eosin (H&E)-stained sections the goblet cells can be easily identified through their mucin content (Fig. 2). With transmission electron microscopy (TEM), each single mucin vesicle can be discerned, often containing a central electron dense spot (Fig. 4).

As opposed to the squamous cells the goblet cells can be identified by specific anti-cytokeratin 7 (CK7) antibody clones (Kasper, 1991). In the clinic, the identification of goblet cells with impression cytology (Fig. 14B) (Tseng, 1985) or *in vivo* confocal microscopy (IVCM) (Dua et al., 2009) is one of the key diagnostic features of limbal stem cell deficiency (LSCD) (Fig. 14A).

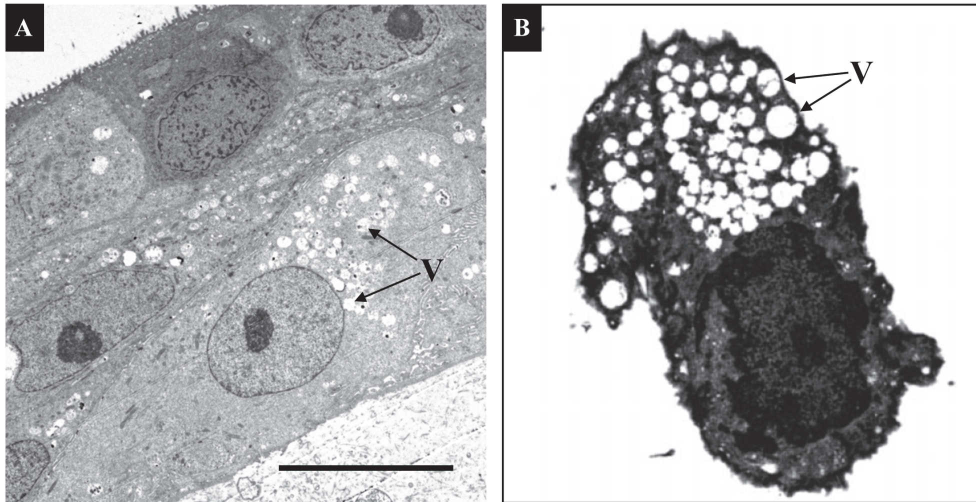

Figure 4. Transmission electron photomicrographs showing conjunctival goblet cells with characteristic perinuclear mucin vesicles. (A) Cultured human conjunctival epithelial cells. Original magnification: ×3400. Scale bar: 10 μm. (B) Cultured rat conjunctival epithelial cells. Original magnification: ×6000. Adapted from Shatos et al. 2001. (V) Goblet cell mucin vesicles.

5.2.3.2 Regulation

The MUC5AC, or gel-forming mucin, is secreted by goblet cells into the tear film by exocytosis upon stimulation of the goblet cells by contact with exogenous neurotransmitters or through activation of a neural reflex arc involving sensory afferent fibers from the cornea and conjunctiva and parasympathetic efferent fibers that surround the goblet cells (Fig. 5) (Dartt et al., 1995; Kessler et al., 1995; Dartt et al., 1996; Rios et al., 1999; Kanno et al., 2003; Dartt et al., 2011; Hayashi et al., 2012). The goblet cell secretion can also be stimulated

by the gastro-protective drug OPC-12759 (Rios et al., 2008). The production of the goblet cell specific mucin MUC5AC is induced by fibroblast growth factor 10 (FBF10) (Ma et al., 2011) and regulated by vitamin A (Driot and Bonne, 1992; Tei et al., 2000). The packing of mucin within goblet cells prior to exocytosis is dependent on a physiological level of calcium (Paz et al., 2003).

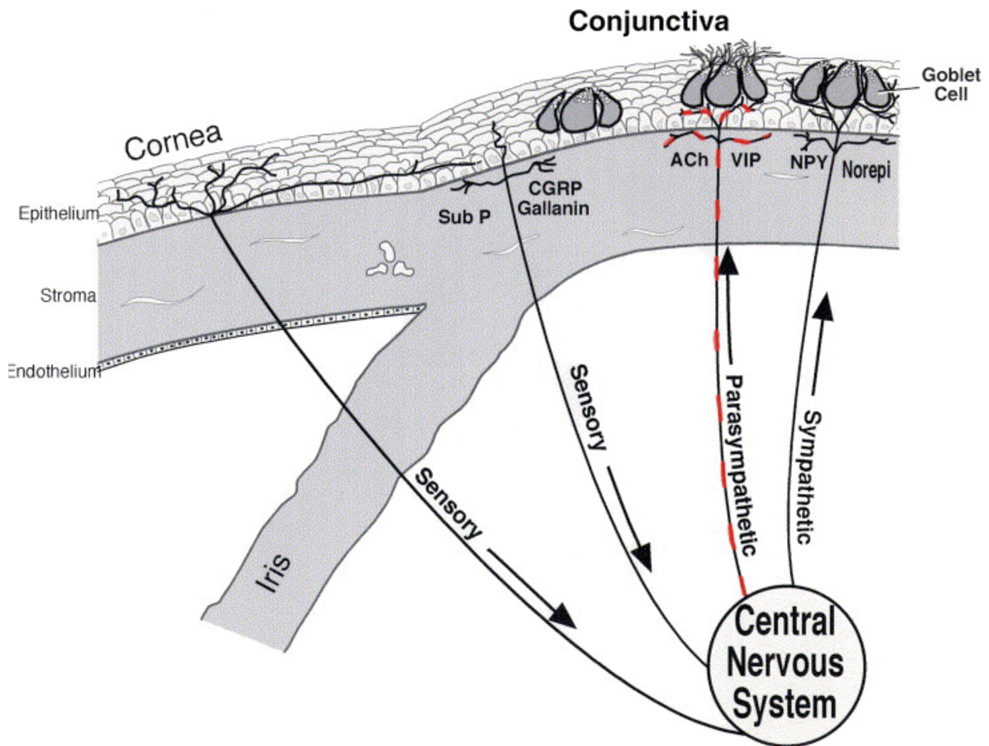

Figure 5. The neural regulation of goblet cells. Activation of a complex neural reflex arc involving corneal and conjunctival sensory afferent fibers and parasympathetic efferent fibers stimulates the goblet cells to secrete the gel-forming mucin-5AC (MUC5AC) into the tear film by exocytosis. From Dartt et al. 2004.

5.2.3.3 Function

The goblet cells secrete MUC5AC into the aqueous part of the tear film (Spurr-Michaud et al., 2007). This mucin type prevents the formation of an irregular tear film that would scatter the

light upon entering the eye (Spurr-Michaud et al., 2007), thereby maintaining visual acuity. The secreted MUC5AC also entraps and removes particles from the ocular surface (Adams, 1979) and aids in the spreading of tears on the ocular surface, thereby inhibiting the development of dry spots (Lemp et al., 1970; Holly and Lemp, 1971; Lemp and Hamill, 1973). Secretion of MUC5AC by the goblet cells has also been reported to regulate ocular surface inflammation and inhibit symptoms of dry eye disease (DED) (Contreras-Ruiz et al., 2012).

5.2.3.4 Location

In the human fetus goblet cells first appear in the fornix after nine weeks of gestational age (Miyashita et al., 1992) and later extend on to the palpebral and bulbar conjunctiva. Several studies have demonstrated that fornix contains the highest number of goblet cells (Moore et al., 1987; Huang et al., 1988; Goller and Weyrauch, 1993; Lavker et al., 1998; Fostad et al., 2012). There is a tendency for a higher goblet cell concentration in the inferior conjunctiva compared to the superior (Moore et al., 1987; Fostad et al., 2012).

Recently, Panjoohesh-Ganji *et al.* reported the identification of *corneal* goblet cells in a mouse model (Pajoohesh-Ganji et al., 2012). These goblet cells were located at the limbal-corneal junction and were, as opposed to conjunctival goblet cells, cytokeratin 12 (CK12)+, which the authors claim indicates corneal origin.

5.2.3.5 Differentiation

The differentiation of conjunctival goblet cells depends on numerous factors, including transcription factors (Ueta et al., 2005; Swamynathan et al., 2007; Swamynathan, 2010; Gupta et al., 2011; Kenchegowda et al., 2011), interleukin-13 (De Paiva et al., 2011), vitamin A (Tseng and Farazdaghi, 1988; Driot and Bonne, 1992), the substrate (Tsai and Tseng, 1988), number of cell doublings (Pellegrini et al., 1999), cell environment (Meller et al., 2002), conjunctival fibroblasts (Tsai et al., 1994) and growth factors (Li et al., 2010). Proliferation of goblet cells can be increased by the gastroprotective drug OPC-12759 (Rios et al., 2006). The goblet cell density show positive correlation with tear osmolarity in healthy subjects, but not in cases with hyperosmolar tear fluid (Julio et al., 2012).

5.2.4 The Conjunctival Basement Membrane

The conjunctival basement membrane (BM) is a thin connective tissue membrane, which is composed of collagen type IV (collagen α1 and α2 chains), laminin (α5, β2 and g1 chains), nidogen-1 and -2 and thrombospondin-4 (Schlotzer-Schrehardt et al., 2007).

5.2.5 The Conjunctival Stroma

The conjunctival stroma is made up of a densely vascularized loose connective tissue (Fig. 2) (Schrader et al., 2009b). It contains lymphoid tissue (conjunctiva-associated lymphoid tissue; CALT) (Knop and Knop, 2000; Steven and Gebert, 2009), which contributes to the defense against intrusion of ocular surface pathogens (Knop and Knop, 2003). Conjunctiva-associated lymphoid tissue develops during childhood and can be identified in healthy adult individuals (Wotherspoon et al., 1994).

5.3 The Cornea

5.3.1 Structure and Composition

The cornea consists of five layers including, from outermost to innermost: epithelium, Bowman’s membrane, stroma, Descemet’s membrane and endothelium (Fig. 6). The corneal epithelium consists of five to six layers of stratified squamous, non-keratinized (Tseng et al., 1984) epithelial cells with a turnover period of four to six days (Hanna and O'Brien, 1960; Hanna et al., 1961). In the limbal region, the epithelium is thicker and comprises about ten cell layers (Wagoner, 1997). The stroma represents a dense collagenous tissue, which makes up 90% of the corneal thickness.

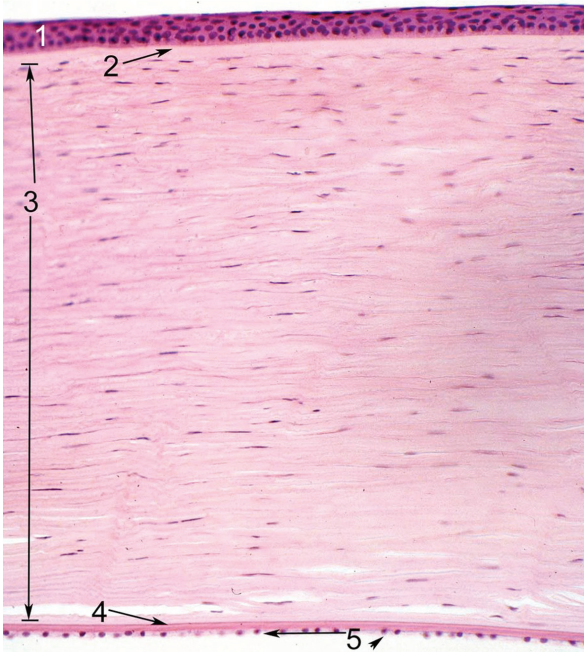

Figure 6. Corneal histology. (1) Epithelium, (2) Bowman's membrane, (3) stroma, (4) Descemet's membrane and (5) endothelium. From Mission for vision (http://www.images.missionforvisionusa.org/anatomy/2005/10/cornea-histology.html).

5.3.2 Function

The corneal epithelium protects against infection, oxidative stress (Kovaceva et al., 2007), stromal scarring and perforation (Jeng, 2006). The stroma interacts with the corneal epithelium (Mishima and Hedbys, 1967; Johnson-Muller and Gross, 1978; Johnson-Wint, 1980; Johnson-Wint and Gross, 1984; Johnson-Wint and Bauer, 1985; Strissel et al., 1995) and its highly regular structure makes the cornea transparent (Hassell et al., 1983). The endothelial cell layer pumps fluid out of the stroma, which is necessary for the maintenance of a clear cornea (Trenberth and Mishima, 1968).

5.4 The Tear Film

5.4.1 Structure and Composition

The tear film covers the ocular surface and fills the conjunctival forniceal sacs (Fig. 1). The mean total tear fluid is estimated to approximately 6-10 μl (Mishima et al., 1966; Tiffany, 2008). The tear menisci, which are visible upon fluorescein staining (Fig. 13), contain approximately 2.9 μl fluid (Bron et al., 2002). The meniscus curvature radius correlates with the total tear volume (Yokoi et al., 2004). The normal preocular tear film is 3-10 μm thick (Tiffany, 2008) and can be divided into several layers: 1) the glycocalyx; 2) inner mucous; 3) middle aqueous; and 4) outer lipid (Fig. 7). Multiple models have been suggested for the structure of the tear film, most based on the three layers proposed by Wolff (1946). Originally, these three layers consisted of an inner gelatinous mucus layer (subsequently modified to include the glycocalyx), middle aqueous layer and an outer Meibomian lipid layer. More recently, a two-layer model devoid of a free aqueous layer was presented for rats and mice. The two-layer model consists of an outer lipid layer and an inner aqueous layer that is mixed with mucous so that there is no clear separation between the mucous and aqueous compartments (Chen et al., 1997; Tran et al., 2003). So far, no consensus has been reached regarding which model best describes the human tear film.

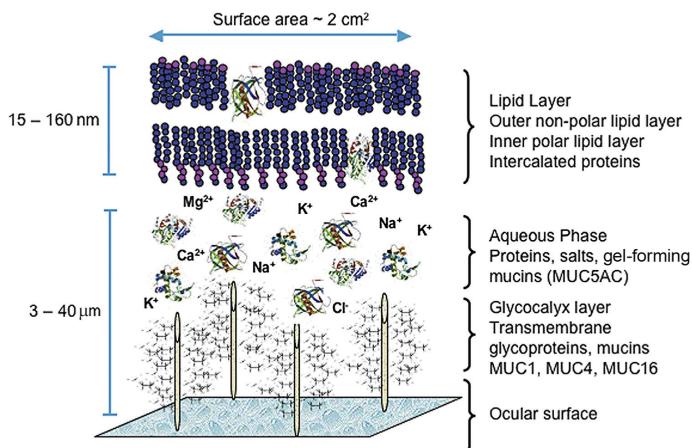

Figure 7. The layers of the tear film. The tear film covers the ocular surface and can be divided into the glycocalyx, inner mucous, middle aqueous and outer lipid layer. From Nichols et al. 2011.

5.4.2 Function

The tear film lubricates the ocular surface, ensures an optically smooth cornea by removing minute irregularities, provides nutrition (Fatt and Bieber, 1968; Tiffany, 2008), protects against oxidants (Crouch et al., 1991), promote epithelial healing (Haynes et al., 1999), acts as a pH buffer (Yamada et al., 1998) and defends the ocular surface against pathogens (Brauninger et al., 1972; Adams, 1979; Haynes et al., 1999; Fluckinger et al., 2004; Paulsen, 2008; Tiffany, 2008; Garreis et al., 2011).

5.4.3 The Glycocalyx and Inner Mucous Layer

The tear film stability depends on the ocular surface mucins, microvilli and glycocalyx. Mucins produced by conjunctival epithelial cells are either secreted directly into the tear film (MUC5AC), or membrane tethered on the tips of the microvilli, thereby participating in the formation of the glycocalyx (Fig. 8) and promoting tear film stability (Dartt, 2004; Gipson, 2004, 2007). External domains of the membrane tethered mucins can be released into the tear film by enzymatic action at the cleavage site (Lillehoj et al., 2003; Thathiah et al., 2003; Govindarajan et al., 2012). The mucins are glycosylated glycoproteins that cover the apical surface of the superficial cells (Gendler and Spicer, 1995). The main membrane-tethered mucins of the ocular surface consist of MUC1, MUC4 and MUC16 (Govindarajan and Gipson, 2010), of which the maintenance of MUC16 is dependent on Notch signaling (Xiong et al., 2011).

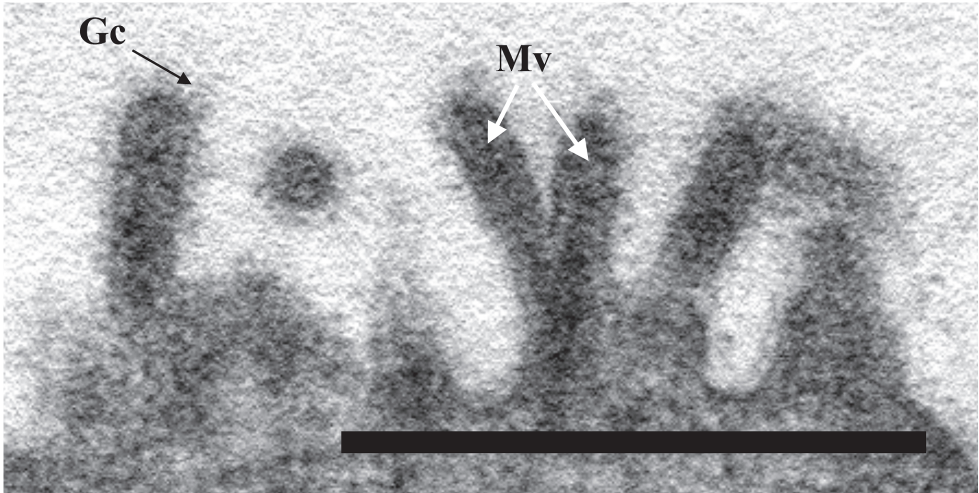

Figure 8. Transmission electron photomicrograph showing the apical surface of a cultured human conjunctival epithelial cell with numerous microvilli (Mv) covered with a faint gray "coating", the glycocalyx (Gc). Original magnification: ×9700. Scale bar: 2 µm.

5.4.4 The Middle Aqueous Layer

The aqueous part of the tear film represents the major component of tears and contains electrolytes, water, peptides (e.g., defensins (Haynes et al., 1999)), glycopeptides and proteins such as lysozyme, lactoferrin, lipocalin, secretory IgA, growth factors (van Setten et al., 1994; Tiffany, 2008) and mucins. The mucins in the aqueous layer comprise mucin-7 (MUC7) (Jumblatt et al., 2003; Paulsen, 2006; Dartt, 2009), MUC5AC and membrane-tethered mucins originating from shed ocular surface cells or released by ectodomain shedding (Spurr-Michaud et al., 2007; Govindarajan et al., 2012).

While the lacrimal glands are responsible for the major portion of the aqueous tear film, the corneal (Tiffany, 2008) and conjunctival epithelium participates directly to the hydration of the ocular surface by ion transport across the apical cell membrane with accompanying osmotic water transfer (Yu et al., 2012). The tear volume at any given time is dependent on the rate of tear influx (from the lacrimal gland (Fig. 9) and ocular surface

epithelium) and outflux (via the lacrimal puncta (Fig. 9) and tear evaporation) (Tiffany, 2008). The secretion by the lacrimal gland show diurnal variation, with a lower secretion rate during sleep (Tiffany, 2008).

5.4.5 The Superficial Lipid-layer

The superficial portion of the tear film consists of a lipid bilayer (Fig. 7) (McCulley and Shine, 1997), secreted from the Meibomian glands in the eyelids (Fig. 1 and 10). This layer stabilizes the tear film by decreasing tear evaporation (Tiffany, 1985; Nichols et al., 2011) and regulating the surface tension (Lozato et al., 2001). The thin inner polar layer consists of phospholipids (phosphatidylcholine and phosphatidylethanolamine), free fatty acids and cholesterol esters (Tiffany, 2008). The thick outer non-polar layer is dependent on the inner polar layer and comprises wax esters (fatty acids and long-chain fatty alcohol) and cholesterol esters (McCulley and Shine, 1997). In a tear film lipid layer (TFLL) model, the wax esters were found to be the most important evaporation-retardants (Rantamaki et al., 2012). The lipid layer is susceptible to temperature changes and at sub-physiological temperatures the lipids stiffens and shrinks, in which case the middle aqueous layer becomes exposed through gaps in the lipid layer (Butovich et al., 2010).

5.5 The Lacrimal Apparatus

5.5.1 Structure

The lacrimal apparatus comprises the lacrimal glands (the main and accessory lacrimal glands (Allansmith et al., 1976; Gillette et al., 1980; Tiffany, 2008)), lacrimal puncta/canaliculi, lacrimal sac and the nasolacrimal duct (Fig. 9). The lacrimal gland is a tubuloacinar, mixed seromucous gland (Prince, 1977), including acinar, ductal and myoepithelial cells (Dartt, 2009). This gland is located in the lacrimal fossa of the orbital region.

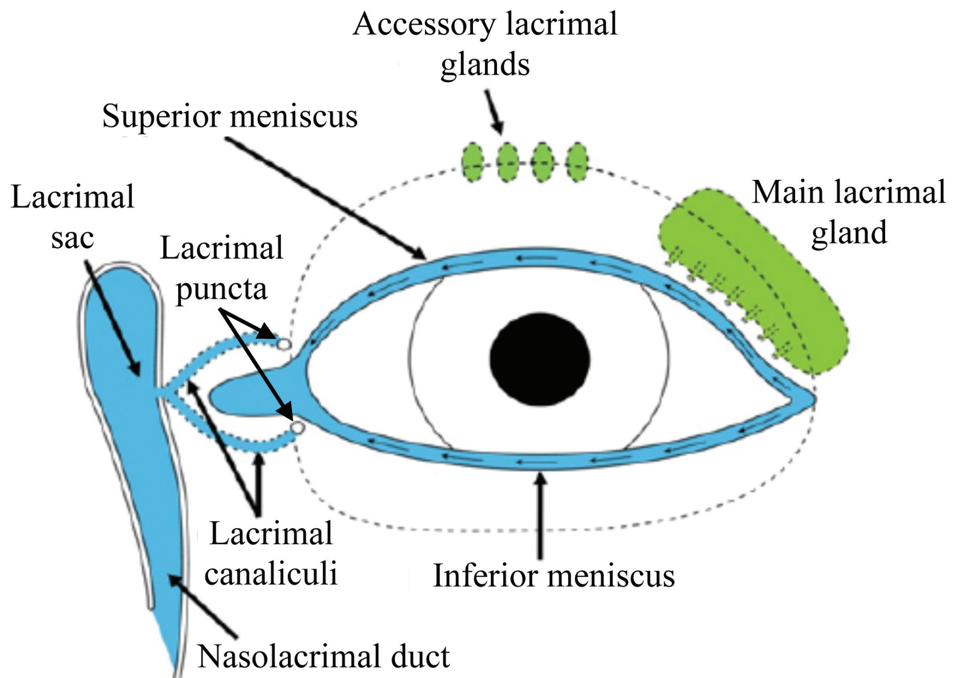

Figure 9. Tear fluid is mainly produced and drained by the lacrimal gland apparatus, which is composed of the main lacrimal gland, the accessory lacrimal glands, the lacrimal puncta, the lacrimal canaliculi, the lacrimal sac and the nasolacrimal duct. Adapted from Gaffney et al. 2010.

5.5.2 Functions

The main function of the lacrimal gland apparatus is to produce and drain tear fluid (Fig. 9). The lacrimal gland secretes the major part of the aqueous layer of the tear film, in addition to proteins (Dartt and Hodges, 2011), mucins (the secretory mucin MUC7 (Jumblatt et al., 2003; Paulsen, 2006; Dartt, 2009)) and electrolytes (Dartt, 2009). The secretory mucin-5B (MUC5B) has also been detected in the lacrimal gland (Jumblatt et al., 2003).

5.5.3 Regulation

The lacrimal gland is under strict neuronal control. Afferent sensory nerve fibers from the cornea and conjunctiva are coupled with efferent sympathetic and parasympathetic nerves that stimulate the lacrimal gland (Dartt, 2009). The acinar cells are the main secretory cells, however, the ductal cells secrete about 30% of the fluid (Mircheff, 1983) and can modify the protein and electrolyte content of the tear fluid (Ubels et al., 1994; Hodges and Dartt, 2003). The myo-epithelial cells, which can be identified by their expression of α-smooth muscle actin (α-SMA) (Ohtomo et al., 2011), surround the acinar and ductal cells (Dartt, 2009).

5.5.4 Lacrimal Gland Disease

The lacrimal gland contains progenitor cells that are capable of differentiating into several separate cell lineages (Shatos et al., 2012). This may partly explain why the lacrimal gland to a certain extent can regenerate upon damage (Zoukhri et al., 2007; Zoukhri et al., 2008). The lacrimal gland can be damaged by a number of causes, including autoimmune diseases, viral infections and trauma (Zoukhri, 2006), which subsequently lead to aqueous deficient dry eye (ADDE) (DEWS, 2007).

5.6 The Meibomian Glands

5.6.1 Structure

The Meibomian glands, first described in 1666 (Meibom), are sebaceous glands situated in the tarsal plate of the eye lids (Fig. 10A) (Nichols et al., 2011). Each gland consists of meibocytes with several acini, central and lateral ductules and a terminal excretory duct ending that deliver the secretion (meibum) to the lid margin (Fig. 10B) (Nichols et al., 2011). The upper eyelid contains 30-40 glands and the lower lid has 20-30 glands (Lawton, 1998). The Meibomian secretions contain polar and non-polar lipids and comprise of a complex mixture of different lipids (Pes, 1897; Tiffany, 1987), including wax esters, (O-Acyl)-omega-hydroxy fatty acids and cholesterol esters (Butovich, 2011).

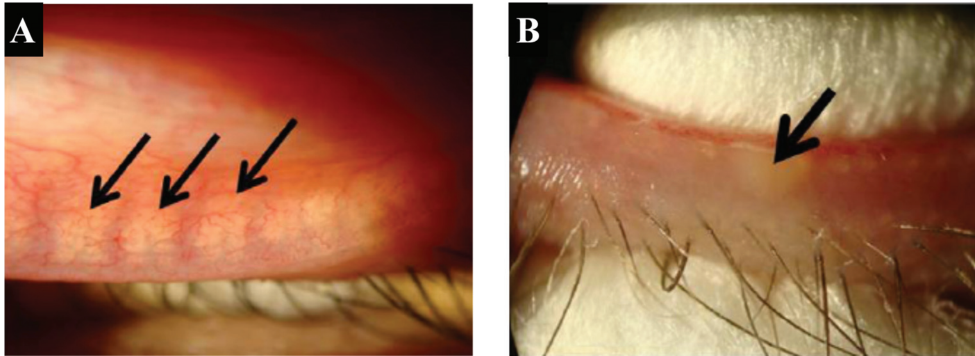

Figure 10. The Meibomian glands can be seen macroscopically on the inside of the eyelid as white stripes (A, arrows). Meibomian secretions (B, arrow) on the posterior edge of the eyelid. Adapted from Butovich et al. 2011.

5.6.2 Functions

The Meibomian glands secrete lipids and proteins onto the eyelid margins (Fig. 10B), forming the superficial layer of the tear film and thereby inhibiting tear evaporation (Tiffany, 1985; Nichols et al., 2011). The Meibomian gland secretions also aid in preserving a clear optical surface and protect against ocular surface pathogens (Garreis et al., 2011).

5.6.3 Regulation

Although the lipids are formed continuously (Butovich, 2011), the output from the glands may be affected by systemic hormones, age (Chew et al., 1993a), sex (Chew et al., 1993a) and by mechanical stimulation such as eye blinking (Tiffany, 2008). The contraction and relaxation of the orbicularis oris muscle in the eyelids during eye blinking not only promotes secretion of meibum stored in the large excretory ducts, but also removes the old secretions at the same time (Khanal and Millar, 2010).

5.6.4 Meibomian Gland Dysfunction

Meibomian gland dysfunction (MGD) (Fig. 12A) is the most common cause of DED (Nichols et al., 2011). It is a chronic, diffuse disorder of the Meibomian glands that leads to terminal duct obstruction with or without changes in the glandular secretions (Fig. 12B) (Nichols et al., 2011). In some patients the number of Meibomian glands can be visibly decreased (McCann et al., 2009).

5.7 Regenerative Medicine and Tissue Engineering

5.7.1 Definitions

A short definition of 'regenerative medicine' was suggested by Mason and Dunnill (2008):

> *"Regenerative medicine replaces or regenerates human cells, tissues or organs, to restore or establish normal function."*

'Tissue engineering', on the other hand, a more narrow term first coined by Fung in 1987 (Viola et al., 2003), does not encompass the regeneration of cells and tissues *in vivo*, but rather the process of developing functional substitutes *ex vivo* for damaged tissues (Langer and Vacanti, 1993). Transplanted cells, tissues or organs used for regenerative purposes can be 'autologous' (originating from the patient), thereby avoiding immuno-rejection, or 'allogeneic' (from another human), which necessitates immunosuppressive therapy.

5.7.2 Historical Overview

Currently estimated to be beneficial to one third of the population (Harris et al., 2007), regenerative medicine has developed tremendously since its early period in the beginning of the 20th century. Corneal transplantation, in 1924 described by Sir John Parsons in his ophthalmology textbook as being "practically never successful" (Parsons, 1924), is now the most frequently performed transplantation, with almost 50.000 operations annually in the U.S. alone (EBAA, 2010). Although the first successful corneal transplantation was performed as early as in 1905 (Zirm, 1906), considerable progress within the field of organ transplantation was first made in the 1950s and 60s, with the first kidney (Murray and Holden, 1954), bone marrow (Thomas et al., 1957), lung (Hardy et al., 1963), liver (Starzl et al., 1968) and heart (Cooper, 2001) transplantations. However, it was the discovery of the immunosuppressive drug cyclosporin A in a soil sample from Hardangervidda in Norway in 1969 (Svarstad et al.,

2000) that transformed transplantation surgery from experimental science into life-saving procedures (Borel et al., 1976). Cyclosporin A has later proven crucial for the first successful combined heart-lung transplantations in 1981 (Jamieson et al., 1983). The development of improved immunosuppressive regimens and the success of modern transplantation surgery have later dramatically increased the demand for donor organs. This, in addition to the continued challenge of allograft rejection, in spite of immunosuppressive medication, has lead to immense interest in fields such as tissue engineering.

Tissue engineering enables researchers to expand the harvested cells *ex vivo* prior to transplantation, thereby limiting donor site damage and potentially avoiding the need for organ transplantation. Using epidermis, which share many similarities with the conjunctival epithelium, Billingham and Reynolds demonstrated in 1952 the principle of expanding cells *ex vivo* before applying them to a graft bed in an animal model to restore the epidermis (Billingham and Reynolds, 1952). During the 60s and 70s the *ex vivo* culture technique was improved by the advent of culture medium growth factors (Cohen, 1986) and the co-culture technique (Rheinwald and Green, 1975), which enabled the expansion of a sufficient number of cells for transplantation. The first human therapy with cultured cells was performed in 1980 when two third degree burn patients were treated with autologous epidermal cultures (O'Connor et al., 1981; Green, 2008). In 1993, Lindberg showed that cultured human limbal epithelial cells (HLEC) could be transplanted to nude mice (Lindberg et al., 1993) and four years later Pellegrini *et al.* reported the first successful transplantation of cultured HLEC for corneal surface restoration in LSCD (Pellegrini et al., 1997). Finally, in 2000, the first transplantation of cultured human conjunctival epithelial (HCjE) cells to restore a diseased conjunctiva was reported (Scuderi et al.).

5.8 Stem Cells - Definition and Basic Principles

Edmund Beecher Wilson first introduced the English term “stem cell” in 1896, possibly inspired by Valentin Haecker’s use of “Stammzelle” (cells that give rise to the germline). Today, a stem cell is defined by its capacity to 1) self-renew and 2) produce cells that differentiate (Morrison and Kimble, 2006).

Stem sells can either divide symmetrically or asymmetrically (Morrison and Kimble, 2006). In asymmetric cell division, each stem cell divides to generate one daughter cell that is

programmed to become a stem cell (self-renewal) and one daughter cell that will differentiate (Fig. 11). Symmetric cell division is defined as the generation of daughter cells that are destined for the same fate.

Stem cells can be categorized in terms of their potency and their origin. Totipotent stem cells are able to form entire organisms, while others can only form a single cell lineage ("unipotent" stem cells). Stem cells may originate from embryos (human embryonic stem cells; hESC (Brivanlou et al., 2003)); fetal or adult tissue (Atala, 2012); amniotic fluid (amniotic fluid stem cells (AFSC) (De Coppi et al., 2007)); generated through somatic cell nuclear transfer (SCNT) (Hochedlinger et al., 2004); or induced from adult somatic cells (induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006; Teoh and Cheong, 2012)). The difference between a stem cell and a progenitor cell is not easily demonstrated in the laboratory as both cells may give rise to several different lineages (Grompe, 2012). Still, true stem cells should be capable of self-renewal for the life-time of the individual, while progenitor cells eventually cease to replicate (Grompe, 2012).

Typically, putative stem cells have been attempted identified through their proposed ability to multiply for an indefinite time, as well as their slow cycling nature *in vivo*. While the capability of cell division can be assessed by *in vitro* colony-forming assays (Barrandon and Green, 1987), the identification of slow cycling ('label-retaining') cells can be performed by administration of radioactive thymidine or 5-bromo-2'-deoxyuridine (BrdU), which incorporates into the deoxyribonucleic acid (DNA), followed by a label "washout" period to remove thymidine or BrdU from cycling cells (Potten and Hendry, 1975). Putative stem cells are considered to be able to divide for an extended time and generate large 'holoclones' *in vitro* (Barrandon and Green, 1987). *In vivo*, the stem cells are considered to divide infrequently in order to conserve their DNA, thus they are thought to be label-retaining even after several months of label washout.

More recent studies using cell lineage tracing (Grompe, 2012; Kretzschmar and Watt, 2012), a technique where the progeny of a specific stem, progenitor or differentiated cell can be traced *in vivo*, have yielded surprising results indicating that some tissues harbor more than one stem cell compartment (Li and Clevers, 2010). Co-existing stem cell compartments have

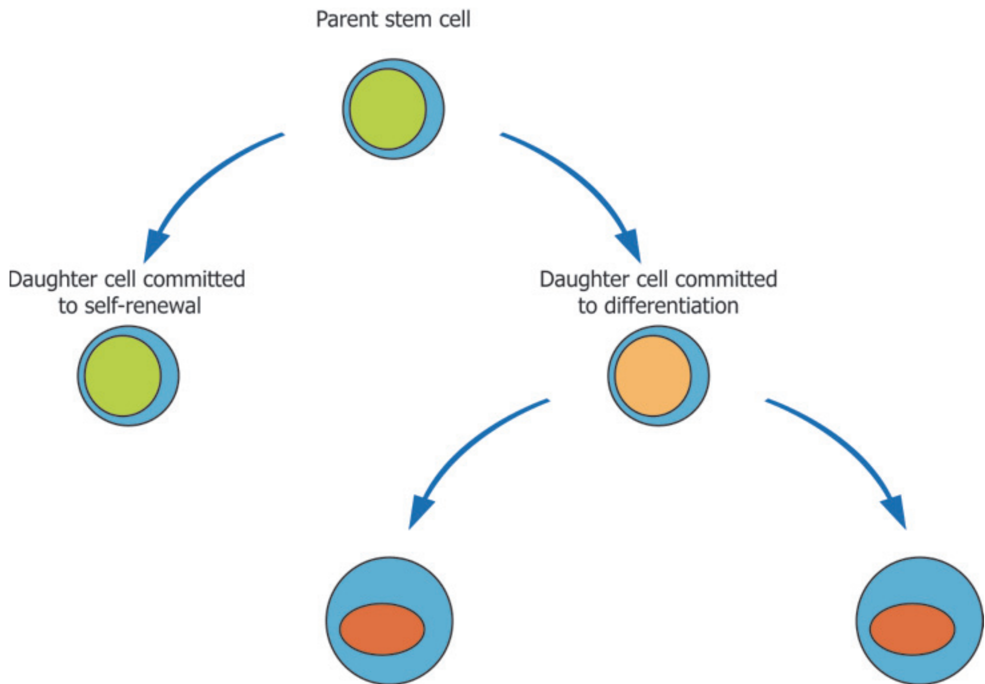

Figure 11. Stem cells can divide asymmetrically or symmetrically. In the first case they give rise to one stem cell and one cell that is destined to differentiate. When dividing symmetrically the stem cell produces two new stem cells. Courtesy of Håkon Raanes, The Oslo School of Architecture and Design, Norway.

now been detected in several tissues, including intestinal epithelium (Barker et al., 2007; Snippert et al., 2010b), epidermis (Cotsarelis et al., 1990; Jaks et al., 2010; Snippert et al., 2010a), liver (Duncan et al., 2009; Dorrell et al., 2011) and muscle (Tedesco et al., 2010). Other tissues, on the other hand, may not contain stem cells at all. In the endocrine pancreas cell lineage tracing studies have showed that the insulin+ β-cells in adult mice do not develop from insulin- stem cells, rather they are generated by the pre-existing insulin+ cells (Dor et al., 2004).

5.9 Conjunctival Stem Cells

Conjunctival stem cells continuously regenerate the conjunctiva by giving rise to both stratified squamous non-goblet and goblet cells (Wei et al., 1997; Pellegrini et al., 1999), thereby maintaining a healthy tear film (Mason et al., 2011). Disorders that damage these stem cells cause varying extents of keratinization, which disrupts the protective tear film and ultimately lead to LSCD and visual impairment or blindness.

5.9.1 Location

The location of the conjunctival stem cells has been a matter of controversy. Conjunctival epithelial stem cells have been suggested to reside in the limbus (rat (Pe'er et al., 1996)), bulbar conjunctiva (mouse (Nagasaki and Zhao, 2005) and human (Pellegrini et al., 1999; Qi et al., 2010)), forniceal conjunctiva (rabbit (Wei et al., 1993), mouse (Wei et al., 1995; Lavker et al., 1998) and human (Pellegrini et al., 1999)), palpebral conjunctiva (rat (Chen et al., 2003)) and mucocutaneous junction (rat (Pe'er et al., 1996) and rabbit (Wirtschafter et al., 1999)).

5.9.2 Characterization

Putative conjunctival stem cells may be identified by immunomarkers, colony-forming efficiency (CFE) (Pellegrini et al., 1999; Schrader et al., 2010; Su et al., 2011), BrdU label-retaining cells (Su et al., 2011), cell movement (Su et al., 2011), microarray gene analysis (Akinci et al., 2009) and based on the success of conjunctival transplantations (Tsubota et al., 2002).

5.9.2.1 Putative Immunomarkers for Conjunctival Epithelial Progenitor Cells

Possible markers of conjunctival progenitor cells include ATP-binding cassette sub-family G member 2 (ABCG2) (Budak et al., 2005; Schrader et al., 2010; Pauklin et al., 2011), tumor protein p63 (p63) (Vascotto and Griffith, 2006; Rosellini et al., 2007; Pauklin et al., 2011), p63α (Schrader et al., 2010), cytokeratin 5 (CK5) (Liu et al., 2007), cytokeratin 8 (CK8) (Liu et al., 2007), cytokeratin 10 (CK10) (Rosellini et al., 2007), cytokeratin 13 (CK13) (Rosellini et al., 2007), cytokeratin 15 (CK15) (Schrader et al., 2010; Pauklin et al., 2011), β1-integrin subunit (CD29) (Vascotto and Griffith, 2006), tumor necrosis factor receptor family-5

(TNFR5/CD40) (Vascotto and Griffith, 2006), hyalunorate receptor (CD44) (Vascotto and Griffith, 2006), muscarinic reseptor subtype m3 (Liu et al., 2007), nestin (Rosellini et al., 2007), tyrosine kinase receptor A (TrkA) (Qi et al., 2010), nerve growth factor (NGF) (Qi et al., 2010), glial cell-derived neurotrophic factor (GDNF) (Qi et al., 2010), GDNF family receptor α-1 (GFRα-1) (Qi et al., 2010), neurotrophin low-affinity receptor (NTR) p75 (Qi et al., 2010), epidermal growth factor receptor (EGFR) (Vascotto and Griffith, 2006) and integrin β-1, α-enolase (Qi et al., 2010). In addition to the presence of stem cell markers, conjunctival epithelial stem cells are devoid of differentiation-associated markers, such as cytokeratin 4 (CK4) (Kasper, 1991), MUC5AC (Argueso and Gipson, 2001) and UEA-1 (Table 1) (Shatos et al., 2001).

Table 1 Immunocharacterization of Conjunctival Epithelial Cells

Epithelial location	Marker	Cell type	Reference
Basal	ATP-binding cassette sub-family G member 2 (ABCG2)	Progenitor cells	(Budak et al., 2005)
	Tumor protein p63 (p63)	Progenitor cells	(Rosellini et al., 2007)
	p63α	Progenitor cells	(Schrader et al., 2010)
	Cytokeratin 5 (CK5)	Progenitor cells	(Liu et al., 2007)
	Cytokeratin 8 (CK8)	Progenitor cells	(Liu et al., 2007; Merjava et al., 2011a)
	Cytokeratin 10 (CK10)	Progenitor cells	(Rosellini et al., 2007)
	Cytokeratin 15 (CK15)	Progenitor cells	(Schrader et al., 2010)
	β1-integrin subunit (CD29)	Progenitor cells	(Vascotto and Griffith, 2006)
	Tumor necrosis factor receptor family-5 (TNFR5/CD40)	Progenitor cells	(Vascotto and Griffith, 2006)

Epithelial location	Marker	Cell type	Reference
	Hyalunorate receptor (CD44)	Progenitor cells	(Vascotto and Griffith, 2006)
	Muscarinic reseptor subtype m3	Progenitor cells	(Liu et al., 2007)
	Nestin	Progenitor cells	(Rosellini et al., 2007)
	Tyrosine kinase receptor A (TrkA)	Progenitor cells	(Qi et al., 2010)
	Nerve growth factor (NGF)	Progenitor cells	(Qi et al., 2010)
	Glial cell-derived neurotrophic factor (GDNF)	Progenitor cells	(Qi et al., 2010)
	GDNF family receptor α-1 (GFRα-1)	Progenitor cells	(Qi et al., 2010)
	Neurotrophin low-affinity receptor (NTR) p75	Progenitor cells	(Qi et al., 2010)
	Epidermal growth factor receptor (EGFR)	Progenitor cells	(Vascotto and Griffith, 2006)
	Integrin β-1, α-enolase	Progenitor cells	(Qi et al., 2010)
	Cytokeratin 7 (CK7)	Goblet cells	(Kasper, 1991)
	Mucin-5AC (MUC5AC)	Goblet cells	(Argueso and Gipson, 2001)
	Ulex europaeus agglutinin-1 (UEA-1)	Goblet cells	(Shatos et al., 2001)
	Proliferating cell nuclear antigen (PCNA)	Proliferating cells	(Eidet et al., 2012a)
	Ki67	Proliferating cells	(Nishida et al., 1999)
Suprabasal	p63	Progenitor cells	(Qi et al., 2010)
	CK7	Goblet cells	(Kasper, 1991)

Epithelial location	Marker	Cell type	Reference
	MUC5AC	Goblet cells	(Argueso and Gipson, 2001)
	UEA-1	Goblet cells	(Shatos et al., 2001)
	Cytokeratin 16 (CK16)		(Merjava et al., 2011b)
Superficial	CK4	Stratified squamous non-goblet cells	(Kasper, 1991)
	CK7	Goblet cells/ stratified squamous non-goblet cells	(Kasper, 1991; Jirsova et al., 2011; Merjava et al., 2011b)
	MUC5AC	Goblet cells	(Argueso and Gipson, 2001)
	UEA-1	Goblet cells	(Shatos et al., 2001)

5.9.2.2 Genes Associated with Conjunctival Epithelial Progenitor Cells

Microarray analysis has demonstrated upregulation of Msh homeobox (MSX), MEIS, ID, hairy and enchancer of split-1 (HES1), sina oculis (SIX), cytochrome P450, family 1, subfamily A, polypeptide-1 (CYP1A1), dual specificity phosphatases (DUSPs), paired box protein 6 (PAX6) and CD62 antigen-like family member E (CD62E) in conjunctival side population cells, which may represent conjunctival stem cells (Akinci et al., 2009). Polymerase chain reaction (PCR) analysis detected similar expression of the stem cell-related genes for CK15, p63, Tír inna n-Óc (NANOG), octamer-binding transcription factor 4 (OCT4), sex determining region Y (SRY)-box 2 (SOX2), KIT, Krüppel-like factor 4 (KLF4), Notch homolog 1 (NOTCH1) and nestin in human bulbar and forniceal conjunctiva (Pauklin et al., 2011). In the latter study, only ABCG2 of the assayed stem cell related genes was more expressed in fornix compared to bulbus.

5.9.2.3 Colony-forming Efficiency

The CFE assay is used to estimate the percentage of seeded cells that are able to form colonies in culture, an estimate which is indicative of the number of putative stem cells in the given cell sample. Su and co-workers compared the CFE of the palpebral, forniceal and bulbar conjunctiva and found that the palpebra yielded the highest CFE (Su et al., 2011). Others have found a uniform distribution of colony-forming cells throughout the superior and inferior fornix and bulbus, as well as the nasal and temporal bulbus of a single patient (Pellegrini et al., 1999). According to size, the colonies can be identified as holoclones, meroclones and paraclones; the holoclones are considered to result from putative stem cells (Barrandon and Green, 1987; Majo et al., 2008). The latter colonies are rapidly growing and the largest. Meroclones, which are intermediate in size, consist of both growing and terminal colonies. The paraclones are the smallest, which include only terminal colonies with highly limited growth potential.

5.9.2.4 5-bromo-2'-deoxyuridine Labelling

As described in *section 5.8*, label-retaining cells are considered to be quiescent stem cells. They have been identified in the palpebral conjunctiva of rabbits (Budak et al., 2005; Su et al., 2011) and Wistar rats (Chen et al., 2003), the fornix of rabbits (Budak et al., 2005) and at the mucocutaneous junction of rabbits (Wirtschafter et al., 1997; Wirtschafter et al., 1999). Nagasaki *et al.*, though only investigating the bulbar conjunctiva, found that label-retaining cells were uniformly distributed throughout the bulbar region of mice (2005). The number of label retaining cells, or putative conjunctival stem cells, has been reported to be decreased in DED (Chen et al., 2007).

5.9.2.5 Cell Movement

Analogous to the centripetal movement of the corneal epithelial cells from the limbus to the center of the cornea (Hanna, 1966; Buck, 1979; Auran et al., 1995; Collinson et al., 2002; Nagasaki and Zhao, 2003), the migration of cells within the conjunctival epithelium has also been investigated to determine the location of the conjunctival stem cells. Nagasaki *et al.* found no cell movement in the bulbar region and suggested the stem cells were uniformly distributed in the bulbus (Nagasaki and Zhao, 2005). In a controversial article, Majo and co-

workers, on the other hand, suggested that bulbar conjunctival epithelial cells move towards the limbus (2008). This was later strongly rejected by Sun and associates who claimed that conjunctival cells do not migrate, at least not towards the limbus (2010). In a short-term BrdU label-retaining study in rabbits, label-retaining cells were identified progressively further from the mucocutaneous junction and closer to the fornix after one, three and five days. The authors, therefore, suggested that transiently amplifying cells (TAC) move from the stem cell rich mucocutaneous junction to the fornix (Wirtschafter et al., 1997). Recently, a nearly identical experiment, also in rabbits, has confirmed the study by Wirtschafter *et al.* (Su et al., 2011).

5.9.2.6 Clinical Observations

Tsubota and co-workers reported successful conjunctival restoration after transplantation of a limbal-conjunctival (bulbar and forniceal conjunctiva) allograft in a nine-year old patient with total keratinization of the ocular surface due to Stevens-Johnson syndrome (SJS) (Tsubota et al., 2002). This could indicate that conjunctival epithelial stem cells reside in either bulbus or fornix.

5.10 Corneal Stem Cells

The corneal epithelium is replenished by the limbal stem cells (LSC) that give rise to highly proliferative daughter cells that migrate centripetally and differentiate before being shed from the surface of the cornea (Schermer et al., 1986; Thoft et al., 1989; Kruse et al., 1990; Lindberg et al., 1993).

5.10.1 The Limbal Dogma

The corneal stem cells have long been considered to be located in the limbal region, more specifically at the palisades of Vogt (Davanger and Evensen, 1971). In this location the LSC are close to blood vessels (Van Buskirk, 1989) and are protected against shearing forces (Gipson, 1989). More recently, the limbal crypts, extending from the palisades of Vogt, have been suggested as a niche for LSC (Dua et al., 2005; Shanmuganathan et al., 2007) due to expression of putative stem cell markers. In 2008, Majo and associates questioned the limbal dogma by reporting that corneal epithelial cells from several different species were capable of

generating holoclones, which are considered a stem cell characteristic (Majo et al., 2008). This finding, however, has been controversial and while having some support (Dua et al., 2009), others argue that the finding of putative stem cells in the central cornea was caused by the use of corneas from young children and non-human species, but not adult human corneas (Sun et al., 2010).

5.10.2 Characterization

Several different methods, including immunostaining, CFE assays and label-retaining assays, have been used to try to identify putative limbal stem cells. A great number of potential stem cell markers have been proposed, however, no specific marker of LSC has been detected so far. Currently, LSC can be indicated by the presence of the putative stem cell markers ABCG2 and ΔNp63α, combined with the absence of differentiation markers, including cytokeratin 3/12 (CK3/12) (de Paiva et al., 2005; Di Iorio et al., 2005; Schlotzer-Schrehardt and Kruse, 2005).

5.11 Ocular Surface Stem Cell Niche

Stem cells are surrounded, and affected, by a three-dimensional microenvironment known as a niche (Schofield, 1983; McNairn and Guasch, 2011; Ordonez and Di Girolamo, 2012). The niche comprises of numerous components, including stromal cells, soluble factors, extracellular matrix (ECM), mechanical/spatial cues and signaling molecules that dictates stem cell function (Watt and Hogan, 2000; Scadden, 2006; Jones and Wagers, 2008).

Stem cell niches have been localized for several different types of stem cells, including haematopoietic stem cells (HSC) (Calvi et al., 2003; Zhang et al., 2003; Kiel et al., 2005), neural stem cells (NSC) (Doetsch et al., 1999; Palmer et al., 2000), epidermal stem cells (Watt, 2002; Tumbar et al., 2004), intestinal stem cells (ISC) (Barker et al., 2007), spermatogonial stem cells (SSC) (Chiarini-Garcia et al., 2003; Yoshida et al., 2007), skeletal muscle stem cells (Collins et al., 2005; Kuang et al., 2007) and LSC (Dua et al., 2005; Shanmuganathan et al., 2007). As mentioned in *section 5.9.1*, the location of conjunctival stem cells and thereby their niche, are still a matter of controversy, however. The limbal stem cell niche has been reported to contain specific ECM proteins (Schlotzer-Schrehardt et al., 2007). Moreover, the specific composition of the ECM reportedly show topographical

variations throughout the ocular surface (Schlotzer-Schrehardt et al., 2007). Thus, there may also exist differences in the composition of the ECM across the conjunctiva, which affect the location of the conjunctival stem cells. Secretion of hormones from stromal cells, such as fibroblast growth factor 10 (FGF10) has been shown to induce the production of the mucins MUC1, MUC4 and mucin-5 (MUC5), as well as stimulate growth, of conjunctival epithelial cells (Ma et al., 2011). *In vitro* simulation of the conjunctival epithelial niche using co-cultures with human bulbar subconjunctival fibroblasts did also support more conjunctival epithelial progenitor cells than co-cultures with conventional fibroblasts (3-day transfer, inoculum 3 x 10^5 cells; 3T3) (Schrader et al., 2010). Recently, Schrader and co-workers reported that conjunctival fibroblasts might enhance the maintenance of conjunctival epithelial progenitor cells by regulating the activation of the Wnt pathway (Schrader et al., 2012a). In another study, co-cultures with conjunctival fibroblasts reportedly induced the development of more goblet cells than co-cultures with 3T3 fibroblasts (Tsai et al., 1994). Hence, the conjunctival stem cell niche may be influenced by the topographical differences in the ECM and the secretion of fibroblast growth factor (FGF) from subconjunctival fibroblasts.

5.12 Ocular Surface Disease

A wide variety of diseases and injuries can affect the ocular surface and cause different degrees of inflammation, scarring and/or tissue loss. The main ocular surface disorders that are of particular interest to regenerative ophthalmology are described below.

5.12.1 Dry Eye Disease

Dry eye disease is a multifactorial disorder involving a dysfunctional tear film and abnormalities in the ocular surface (DEWS, 2007).

5.12.1.1 Symptoms

The occurrence of symptoms, which indicates activation of sensory nerves at the ocular surface (Belmonte et al., 2004), include feeling of ocular dryness, foreign body sensation and irritation, light sensitivity and visual disturbance (Rieger, 1992; Liu and Pflugfelder, 1999; Goto et al., 2002; Begley et al., 2003; Adatia et al., 2004; Vitale et al., 2004).

5.12.1.2 Signs

Signs of DED depends on the underlying cause and may include decreased tear break-up time (TBUT), decreased tear meniscus height (Fig. 13), decreased Schirmer's test score, Meibomian gland dysfunction (MGD) with impaired meibum expression and/or meibum quality (Fig. 12), conjunctival injection (Fig. 12), conjunctival and/or corneal staining, keratitis, corneal ulceration, mucous clumping, increased tear film debris (Holly, 1973; Bron, 2001; Goto et al., 2003), trichiasis, sympblepharon and ocular surface keratinization (DEWS, 2007).

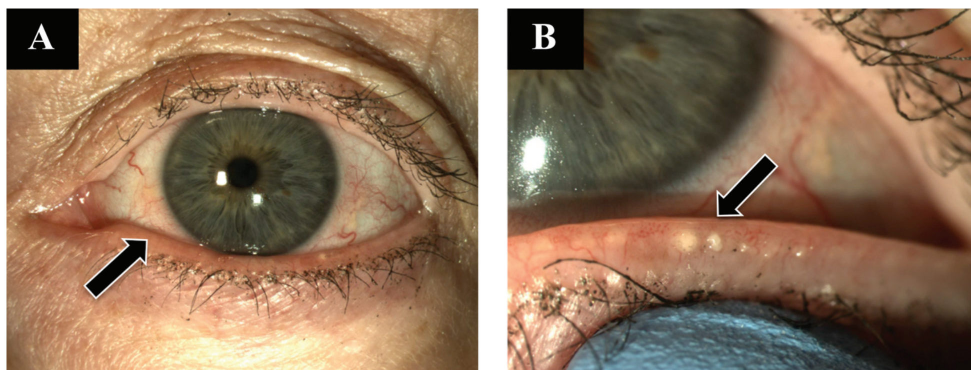

Figure 12. Photomicrographs showing a patient with dry eye disease due to aquous deficiency and Meibomian gland dysfunction. (A) The eyelid margins are slightly inflamed (arrow). Characteristic dry eye-related hyperemia in the form of a horizontal band over the conjunctiva limited to the interpalpebral fissure can also be seen. (B) When assuming gentle pressure on the eyelid the Meibomian glands extrude pathological toothpaste-like secretions. Courtesy of Dr. Sten Raeder, MD, PhD, Tørreøyneklinikken, Oslo, Norway (http://torreoyneklinikken.no).

5.12.1.3 Prevalence

The global prevalence of DED is 5-35% (DEWS, 2007) and the prevalence increases with age. Hence it is expected that this disease will affect an even bigger group in the years to come as the population above 50 years old increases (Schaumberg et al., 2001).

5.12.1.4 Etiology

Dry eye disease can be subdivided into an aqueous deficient type and evaporative type (DEWS, 2007). The former can be further separated into dry eye caused by Sjögren's syndrome and non-Sjögren's syndrome (Feltsan et al., 2012). Besides lacrimal deficiency (Scherz and Dohlman, 1975), the non-Sjögren's group includes primary (formerly denoted "Keratoconjunctivitis sicca" (KCS)) (Mathers et al., 1996) and secondary (Scherz and Dohlman, 1975) lacrimal gland deficiency, as well as cases with lacrimal gland obstruction caused by scarring ocular surface diseases, such as trachoma (Guzey et al., 2000), cicatrizing conjunctival disease (Dart, 2005; Eschle-Meniconi et al., 2005; Hingorani and Lightman, 2006), erythema multiforme/SJS/toxic epidermal necrolysis (TEN) (Power et al., 1995) and chemical/thermal burns (Lemp, 1992). Also included in the non-Sjögren group are cases with hyposecretion due to for example adverse effects of systemic drugs (Moss et al., 2004).

The evaporative type includes those caused by intrinsic and extrinsic factors. The intrinsic causes are represented (most often) by MGD (Foulks and Bron, 2003; Bron and Tiffany, 2004; Bron et al., 2004; Nichols et al., 2011), in addition to defective lid/globe interaction leading to an exposed evaporative surface (Lemp, 1973) and low blink rate (Abelson et al., 2002). There are numerous extrinsic causes that mainly lead to dry eye through damage to the ocular surface corneal and conjunctival goblet/non-goblet cells, which again lead to imperfect surface wetting and destabilization of the tear film. Such extrinsic disorders include vitamin A deficiency (Tei et al., 2000; Hori et al., 2004), chronic ocular surface diseases (DEWS, 2007) and allergic conjunctivitis (Abelson et al., 2003). In addition, environmental factors, such as low relative humidity, can also cause tear film parameters similar to those of DED patients (Abusharha and Pearce, 2012). For any of the above mentioned causes of dry eye, there may be a concomitant loss of goblet cell numbers, which further aggravate symptoms (Ralph, 1975a).

5.12.1.5 Pathogenesis

Although still largely unknown, the pathogenesis of DED includes an inflammatory component (Pflugfelder et al., 1999; Tsubota et al., 1999), involving the mitogen-activated protein kinase (MAPK) (Luo et al., 2005) and nuclear factor-kβ (NF-kβ) pathway (Lan et al., 2012), that is activated upon tear film instability and tear hyperosmolarity (Gilbard, 1994; Murube, 2006; Tomlinson et al., 2006; Stevenson et al., 2012). Secretion of pro-inflammatory

cytokines (Cejkova et al., 2007b) cause oxidative stress (Cejkova et al., 2007a) and lead to squamous metaplasia and a decrease in the differentiation of goblet cells (De Paiva et al., 2007; Chen et al., 2010). During the course of the disease the epithelial cell density and thickness increases (Fabiani et al., 2009). Goblet and non-goblet conjunctival cells are also lost due to induction of apoptosis (Yeh et al., 2003), where the loss of goblet cells can be seen as a result of chronic conjunctival inflammation (Kunert et al., 2002). Expression of the goblet cell secreted MUC5AC is reduced in dry eye (Zhao et al., 2001; Argueso et al., 2002) and glycosylation of MUC16 is changed in non-Sjögrens dry eye (Gipson et al., 2004). Thus, a diseased conjunctival epithelium, including alterations in both the secreted and membrane bound mucins, is an important contributing factor to tear film instability in DED.

5.12.1.6 Diagnostic Tests

The tear film can be analyzed be means of its stability (Lemp and Hamill, 1973), volume (Schirmer, 1903; Jones, 1966; Kurihashi, 1976; Yokoi et al., 1999) (Fig. 13), osmolarity (Pensyl and Benjamin, 1999), completeness (Feenstra and Tseng, 1992), quality (Norn, 1994), evaporation rate (Mathers et al., 1993) and physical properties (Tiffany et al., 1989; Tiffany, 1991). The Meibomian gland output can be objectively measured by meibometry (Chew et al., 1993a; Chew et al., 1993b). Dry eye associated cell morphology characteristics can be visualized by impression cytology and IVCM (Kojima et al., 2010).

Based on the severity of symptoms and signs DED patients can be grouped into four levels (DEWS, 2007). Treatment will then be tailored according to the severity level.

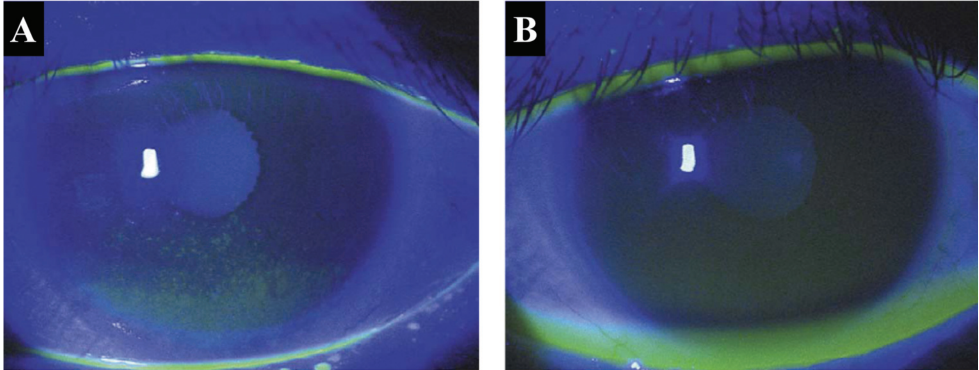

Figure 13. Fluorescein staining of the tear fluid can be used to assess the tear film and tear volume. In cases of decreased tear production (A) the tear meniscus height (green) is <100 μm and less than in healthy eyes (B). Adapted from Koh et al. 2006.

5.12.1.7 Treatment Options

Treatment depends on the level of disease severity (Table 2) (DEWS, 2007). Severe DED (i.e. level 4) may benefit from transplantation of a conjunctival tissue substitute.

Table 2 - Treatment Modalities According to Severity Level of Dry Eye Disease (DED)		
Level 1	Education and dietary adjustments	(Tsubota and Nakamori, 1993; Barabino et al., 2003)
	Eliminating adverse medications	(Mader and Stulting, 1991)
	Artificial tear substitutes, gels and ointments	(Gilbard et al., 1989)
	Eyelid therapy	(DEWS, 2007)
Level 2	Topical anti-inflammatories	(Laibovitz et al., 1993; Gunduz and Ozdemir, 1994; Pflugfelder et al., 2004)
	Tetracyclines	(Jansen and Plewig, 1997)
	Punctal plugs	(Foulds, 1961)
	Tear stimulation	(Tauber et al., 2004)
	Moisture chamber spectacles	(Gresset et al., 1984)
Level 3	Serum eye drops	(Noble et al., 2004; Kojima et al., 2005; Jirsova et al., 2008)
	Contact lenses	(Bacon et al., 1994; Pullum et al., 2005)
	Permanent punctal occlusion	(Beetham, 1935)
Level 4	Systemic anti-inflammatory agents	(DEWS, 2007)
	Surgery: lid surgery; mucous membrane, salivary gland and amniotic membrane transplantation.	(Geerling et al., 1998)

5.12.2 Limbal Stem Cell Deficiency

5.12.2.1 Definition

A hallmark of LSCD is conjunctivalization of the cornea (Fig. 14A) (Tseng, 1989). The diagnosis can be made clinically (Schwartz and Holland, 2005), by impression cytology (Fig. 14B) (Egbert et al., 1977; Thiel et al., 1997) or by IVCM (Dua et al., 2009; Vera et al., 2009; Zheng et al., 2009; Nubile et al., 2012).

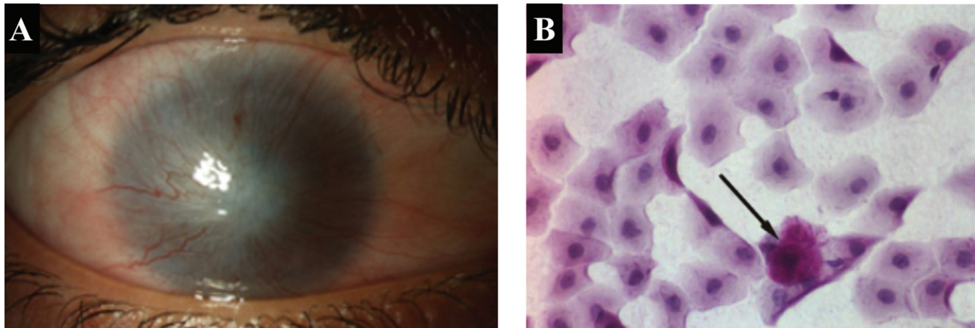

Figure 14. Limbal stem cell deficiency (LSCD). (A) Clinically, LSCD can be diagnosed by ingrowth of conjunctival cells resulting in an opaque, vascularized cornea. (B) Impression cytology of the cornea of a patient with LSCD showing a Periodic acid-Schiff (PAS)+ (arrow) goblet cell. Adapted from Sangwan et al. 2012 and Garcia et al. 2012.

5.12.2.2 Etiology

Limbal stem cell deficiency may be primary, as in aniridia, more commonly however, it is secondary to external damaging processes, of which chemical burns are most usually the cause (Table 3) (Dua et al., 2000). The stem cell deficiency may be diffuse, covering the entire cornea, or partial, affecting only a sector of the cornea (Dua et al., 2000).

Table 3 Etiology of Limbal Stem Cell Deficiency (LSCD)		
Idiopathic		(Espana et al., 2002a)
Hereditary	Aniridia	(Nishida et al., 1995)
	Congenital aniridia variant: minimally abnormal irides with severe limbal stem cell deficiency (LSCD)	(Skeens et al., 2011)
	Dominantly inherited keratitis Autosomal dominant keratitis	(Kivlin et al., 1986) (Pearce et al., 1995)
	Multiple endocrine neoplasia (MEN)	(Puangsricharern and Tseng, 1995)
	Ectrodactyly-ectodermal dysplasia-clefting syndrome	(Di Iorio et al., 2012)
	Ectodermal dysplasia syndrome	(Anderson et al., 2003)
	Keratitis-icthyosis-deafness syndrome (KID syndrome)	(Gicquel et al., 2002; Messmer et al., 2005)
	Xeroderma pigmentosa	(Fernandes et al., 2004)
	Lacrimo-auriculo-dento-digital syndrome (LADD syndrome or Levy-Hollister syndrome)	(Cortes et al., 2005; Inan et al., 2006)
	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)	(Tarkkanen and Merenmies, 2001)
	Bilateral LSCD with chromosomal translocation of 3p and 9p	(Usui et al., 2010)
	Epidermolysis bullosa (EB) dystrophica hallopeau-Siemens	(Thanos et al., 2010)
	Iris coloboma	(Espana et al., 2002c)
	Dyskeratosis congenital	(Aslan and Akata, 2010)

Table 3 Etiology of Limbal Stem Cell Deficiency (LSCD)		
	Gelatinous drop-like dystrophy	(Shimazaki et al., 2002)
Trauma	Chemical and thermal burn	(Rama et al., 2010)
	Sulfur-mustard	(Kadar et al., 2009; Kadar et al., 2011)
	Delayed-onset mustard gas keratitis	(Pleyer et al., 1999; Javadi et al., 2005; Javadi et al., 2011)
Eye disease	Pterygium	(Kwok and Coroneo, 1994; Mahdy and Bhatia, 2009)
	Pseudopterygium	(Vemuganti et al., 2009)
	Neurotrophic keratitis	(Puangsricharern and Tseng, 1995)
	Infections (including trachoma)	(Dua and Azuara-Blanco, 1999; Holland and Schwartz, 1999; Schwab et al., 2000; Kremer et al., 2009; Ledbetter et al., 2012)
	Atopy	(Tsai et al., 1990)
	Sclerocornea	(Tsai and Tseng, 1994)
	Keratoconjunctivitis sicca (KCS)	(Vemuganti et al., 2009)
	Vernal keratoconjunctivitis, keratoconjunctivitis	(Gomes et al., 2009; Sangwan et al., 2011b)
	Tumors	(Vemuganti et al., 2009)
	Corneal intraepithelial dysplasia	(Tan et al., 1996)
	Peripheral ulcerative keratitis (Mooren's ulcer)	Peripheral ulcerative keratitis (Mooren's ulcer)
	Bullous keratopathy	(Satake et al., 2009)

Table 3 Etiology of Limbal Stem Cell Deficiency (LSCD)		
	Limbal dermoid	(Hong et al., 2010)
Iatrogenic	Multiple ocular surface operations	(Holland and Schwartz, 1997; Schwartz and Holland, 1998, 2001)
	Multiple intravitreal injections	(Capella et al., 2011)
	Cryotherapy	(Puangsricharern and Tseng, 1995)
	Phototherapeutic keratectomy	(Nghiem-Buffet et al., 2003; Cannon et al., 2004)
	Radiotherapy	(Fujishima et al., 1996; Smith et al., 2000)
	Contact lens use	(Clinch et al., 1992; Jenkins et al., 1993; Bhatia et al., 2009)
	Topical treatment with cytostatic drugs and mitomycin-C	(Pires et al., 2000; Dudney and Malecha, 2004; Sauder and Jonas, 2006; Lichtinger et al., 2010)
	Systemic chemotherapy	(Ellies et al., 2001; Solomon et al., 2002; Ding et al., 2009)
	Ultraviolet radiation	(Di Girolamo et al., 2002)
	Posttreatment for recurrent ocular surface melanoma	(Di Girolamo et al., 2009)
Systemic	Graft-versus-host disease (GVHD)	(Sale et al., 1994; Meller et al., 2009)
	Vitamin A deficiency	(Vemuganti et al., 2009)

Table 3 Etiology of Limbal Stem Cell Deficiency (LSCD)		
Immune-mediated	Stevens-Johnson syndrome (SJS)	(Puangsricharern and Tseng, 1995)
	Ocular cicatricial pemphigoid (OCP)	(Dua, 1995; Elder et al., 1996; Dua et al., 2000)
	Multiple endocrine deficiency	(Mohammadpour et al., 2006)

5.12.2.3 Symptoms

Symptoms of LSCD include epiphora, irritation, blepharospasm, photophobia, pain and decreased visual acuity (Dua and Azuara-Blanco, 2000).

5.12.2.4 Signs

Depending on the severity of the LSCD, signs include loss of palisades of Vogt (Schwartz and Holland, 2005), late epithelial staining with fluorescein (Dua et al., 1994b), corneal vascularization (Holland, 1996), peripheral pannus (Dua and Azuara-Blanco, 2000; Dua et al., 2000), punctate epithelial keratopathy, epithelial defects, corneal ulceration and perforation, conjunctivalization (Kruse et al., 2003) and finally epithelial keratinization and stromal scarring.

5.12.2.5 Treatment

Limbal stem cell deficiency is mainly treated by surgical means. In addition, eye drops (Poon et al., 2001; Geerling et al., 2004; Young et al., 2004; Jeng et al., 2011), insertion of scleral lenses (Schornack, 2011) and recently, even the application of pulsed electromagnetic fields may be attempted (Rezaei Kanavi et al., 2012). An important surgical treatment of LSCD is the use of keratoprosthesis (Dohlman et al., 2005). Several other surgical procedures have been developed, including sequential sector conjunctival epitheliectomy (SSCE) (Dua, 1998; Dua et al., 2010), keratolimbal allograft (KLAL) (Holland, 1996), conjunctival limbal autograft (CLAU) (Kenyon and Tseng, 1989; Holland, 1996), living-related conjunctival

limbal allograft (lr-CLAL) (Holland, 1996), homologous penetrating central limbo-keratoplasty (HPCLK) (Reinhard et al., 1999; Reinhard et al., 2004; Spelsberg et al., 2004; Egarth et al., 2005) and amniotic membrane transplantation (AMT) (Kim and Tseng, 1995; Tseng et al., 1998; Pires et al., 2000; Anderson et al., 2001; Gomes et al., 2003; Sangwan et al., 2004).

The replacement of the limbal stem cells by transplantation of *ex vivo* cultured limbal cells has been much explored and is successful in approximately three out of four cases (Pellegrini et al., 1997; Schwab, 1999; Schwab et al., 2000; Tsai et al., 2000; Koizumi et al., 2001a, b; Rama et al., 2001; Grueterich et al., 2002; Tseng et al., 2002; Nakamura et al., 2003b; Sangwan et al., 2003a; Sangwan et al., 2003b; Harkin et al., 2004; Nakamura et al., 2004b; Daya et al., 2005; Sangwan et al., 2005; Nakamura et al., 2006b; Sangwan et al., 2006; Ang et al., 2007; Fatima et al., 2007; Kawashima et al., 2007; Shimazaki et al., 2007; Shortt et al., 2008; DeSousa et al., 2009; Di Girolamo et al., 2009; Gomes et al., 2009; Meller et al., 2009; Baradaran-Rafii et al., 2010; Colabelli Gisoldi et al., 2010; Di Iorio et al., 2010; Kolli et al., 2010; Meller et al., 2010; Nakamura et al., 2010; Pauklin et al., 2010; Rama et al., 2010; Thanos et al., 2010; Sangwan et al., 2011a; Sharma et al., 2011; Marchini et al., 2012; Shigeyasu and Shimazaki, 2012). Recently, Sangwan *et al.* reported successful treatment of unilateral LSCD using a novel technique, ‘simple limbal epithelial transplantation’ (SLET), in which a 2x2 mm limbal biopsy from the healthy contralateral eye is divided into eight to ten smaller pieces and allowed to grow *in vivo* on the damaged cornea (Sangwan et al., 2012). According to the authors, using SLET will cut laboratory expenses of treating LSCD and reduce the risk of infection.

In addition to using limbal cells, several non-limbal cell types have been attempted, including oral mucosal epithelial cells (Nakamura et al., 2003a; Nakamura and Kinoshita, 2003; Nakamura et al., 2004a; Hayashida et al., 2005; Ang et al., 2006; Inatomi et al., 2006; Nakamura et al., 2007; Satake et al., 2008; Chen et al., 2009; Ma et al., 2009; Shimazaki et al., 2009; Liu et al., 2011; Nakamura et al., 2011; Priya et al., 2011; Takeda et al., 2011; Burillon et al., 2012), conjunctival epithelial cells (Sangwan et al., 2003a; Sangwan et al., 2003b; Tanioka et al., 2006; Ono et al., 2007; Di Girolamo et al., 2009; Ang et al., 2010), embryonic stem cells (ESC) (Homma et al., 2004), bone-marrow-derived mesenchymal stem cells (MSC) (Ma et al., 2006; Omoto et al., 2009; Jiang et al., 2010; Reinshagen et al., 2011), epidermal epithelial cells (Yang et al., 2007; Yang et al., 2008), immature dental pulp stem

cells (Monteiro et al., 2009; Gomes et al., 2010), hair follicle-derived stem cells (Meyer-Blazejewska et al., 2011) and umbilical cord lining stem cells (Reza et al., 2011). Irrespective of the cell type used, however, in the absence of a healthy conjunctiva replacement of limbal stem cells are prone to failure (Mason et al., 2011).

5.12.3 Conjunctival Inflammation

5.12.3.1 Etiology

Conjunctival inflammation (conjunctivitis) is the most common cause of red eye and is usually caused by self-limiting viral and bacterial infections (Morrow and Abbott, 1998). Adenovirus is the major viral culprit (Morrow and Abbott, 1998; Oliver et al., 2009). Bacterial conjunctivitis can be hyperacute (within 24 hours of exposure), acute (typically lasting seven to ten days) or chronic (lasting more than three weeks) (Morrow and Abbott, 1998). It is mostly caused by to streptococcus pneumoniae and haemophilus influenzae (children), whereas adults are predominantly infected by staphylococcus aureus (Morrow and Abbott, 1998; Oliver et al., 2009). Non-infectious conjunctivitis includes self-limiting and mild diseases, as well as severe sight-threatening disorders (Table 4).

5.12.3.2 Symptoms

Symptoms are unspecific and include tearing, burning sensation, grittiness and stinging. Pain, photophobia and foreign body sensation usually indicate involvement of the cornea.

5.12.3.3 Signs

Signs may include conjunctival injection, chemosis, haemorrhages, pseudomembrane formation, infiltration, follicular reaction and subconjunctival scarring (Fig. 15)

Table 4 - Etiology of Conjunctivitis			
Infectious	Viral	Adenovirus	(Skevaki et al., 2011)
		Herpes simplex	(Skevaki et al., 2011)
	Bacterial	Staphylococcus aureus	(Wright et al., 2008; Oliver et al., 2009)
		Streptococcus pneumonia	(Pichichero, 2011)
		Haemophilus species	(Pichichero, 2011)
		Moraxella	(Pichichero, 2011)
		Neisseria species	(Pichichero, 2011)
		Enteric gram-negativ rods	(Pichichero, 2011)
		Chlamydia trachomatis	(Burton, 2007)
	Other	Fungi	(Schubach et al., 2005)
		Parasites	(Shen et al., 2006)
Non-infectious	Allergic	Seasonal/perennial allergic conjunctivitis	(Stahl and Barney, 2004)
		Atopic keratoconjunctivitis (AKC)	(Akova et al., 1994)
		Vernal keratoconjunctivitis (VKC)	(Kari and Saari, 2012)
		Giant papillary conjunctivitis (GPC)	(Kari and Saari, 2012)
	Immune-mediated	Ocular cicatricial pemphigoid (OCP)	(Nguyen and Foster, 1996)
		Stevens-Johnson syndrome (SJS)	(Ignat and Oprescu, 1989)
		Toxic epidermal necrolysis (TEN)	(Prendiville et al., 1989)
		Sjögren’s syndrome	(DEWS, 2007)

Table 4 - Etiology of Conjunctivitis			
	Miscellaneous	Superior limbic keratoconjunctivitis	(Cher, 2000)
		Foreign body conjunctivitis	(Meyer and Tangas, 2003)
		Toxic conjunctivitis	(Thygeson, 1957)
		Graft-versus-host disease (GVHD)	(Townley et al., 2011)
		Xeroderma pigmentosa	(Hadj-Rabia et al., 2012)
		Meibomian gland dysfunction (MGD)	(Nichols et al., 2011)

5.12.3.4 Treatment Options

Treatment depends on the disease etiology and severity, but conservative measures may include bandage contact lens (Watson et al., 2002); lid hygiene (Tetz et al., 1997); topical application of artificial tears/lubricants (Bielory, 2002a, b), decongestants (Wallace et al., 2008), mast cell stabilizers (Gokhale et al., 2012), antihistamines (McCabe and McCabe, 2012), nonsteroidal anti-inflammatory drugs (NSAIDs) (Bielory, 2007, 2008), antibiotics (Sheikh et al., 2012), steroids (Bielory and Friedlaender, 2008; Oner et al., 2012), mitomycin C (MMC) (Jain and Sukhija, 2006) or immunosuppressive/modulating drugs (Kheirkhah et al., 2011; Meyer-Rusenberg et al., 2011; Garnock-Jones, 2012; Gonzalez-Lopez et al., 2012; Turan-Vural et al., 2012); systemic administration of antibiotics (Poli et al., 2010), antihistamines (Bhargava et al., 1998; Butrus and Portela, 2005), immunosuppressive drugs (Poli et al., 2010; Gokhale et al., 2012); environmental improvement; and reduction of transmission through hygienic measures (Meyer-Rusenberg et al., 2011).

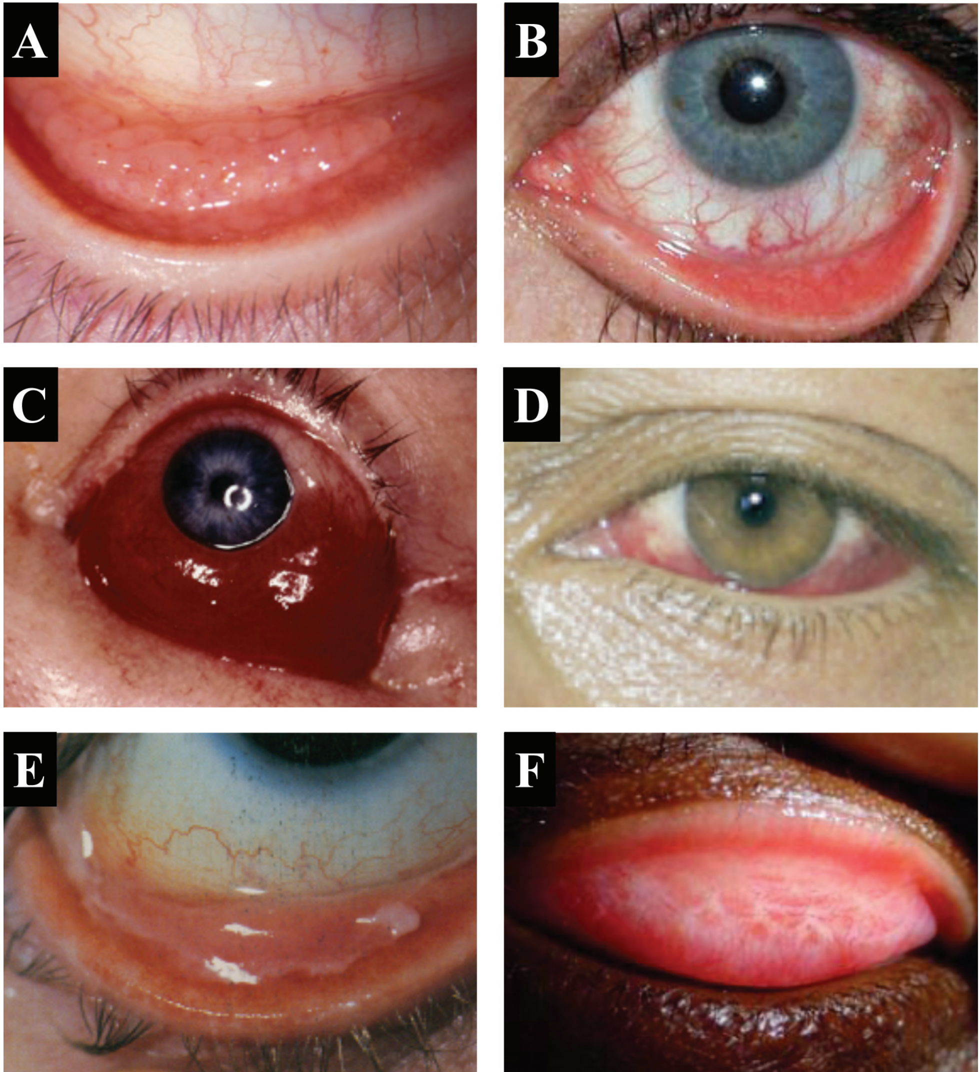

Figure 15. Photomicrographs showing signs of conjunctival inflammation, including (A) follicular reaction, (B) conjunctival injection, (C) chemosis, (D) subconjunctival hemorrhage, (E) pseudomembrane and (F) subconjunctival scarring. Adapted from Burton and Mabey 2009; Engin et al. 2009; Jabs et al. 1989; Torkildsen 2005; and Vose et al. 2006.

5.12.4 Conjunctival Scarring

5.12.4.1 Etiology

A large number of disorders can lead to scarring of the conjunctiva (Table 5). Scarring varies in severity and can be self-limited, such as in infectious diseases due to adeno- and herpes viruses, or progressive, as in ocular cicatricial pemphigoid (OCP).

Table 5 - Etiology of Severe Conjunctival Scarring		
Immune-mediated	Ocular cicatricial pemphigoid (OCP)	(Nguyen and Foster, 1996)
	Stevens-Johnson syndrome (SJS)	(Ignat and Oprescu, 1989; Prendiville et al., 1989)
	Toxic epidermal necrolysis (TEN)	(Prendiville et al., 1989; Magina et al., 2003)
	Sjögren syndrome	(McCarthy, 1988)
Trauma	Injuries	(Kaufman and Thomas, 1979)
	Chemical/thermal burns	(Zurabov, 1956)
Allergy	Atopic keratoconjunctivitis (AKC)	(Akova et al., 1994)
Iatrogenic	Eye lid surgery	(Tarr and Constable, 1980; Miller and Boynton, 1987)
Infections	Herpesvirus	(Andrew, 2001)
	Adenovirus (epidemic keratoconjunctivitis; EKC)	(Hammer et al., 1990)
	Trachoma	(Pavisic, 1953)
Systemic diseases	Epidermolysis bullosa (EB)	(Letko et al., 2006)
	Graft-versus-host disease (GVHD)	(Allan et al., 2011)
	Sarcoidosis	(Flach, 1978)

5.12.4.2 Pathogenesis

Ocular surface mucin expression may increase initially especially in cases of allergic conjunctivitis (Foster et al., 1986; Kunert et al., 2001), however, in later stages alterations in mucin biosynthesis (Argueso et al., 2003) and loss of goblet cells (Nelson and Wright, 1984) lead to decreased surface wetness with the development of dry eye as described above (*section 5.12.1*). During the course of chronic inflammation the conjunctiva becomes keratinized with expression of epidermal epithelial cytokeratin 1 (CK1) and CK10 (Nakamura et al., 2001). Symblepharon, which represents shortening or obliteration of the fornices, may develop with resulting concomitant ineffective blinking and lagophthalmos (Fig. 16) (Solomon et al., 2003). This aggravates the ocular surface damage, causing persistent epithelial defects (PED) (Fu et al., 2012) and worsening the tear film instability due to obliteration of the normal tear meniscus and distortion of the eye lid/globe interaction. Extensive symblepharon may even cause restrictive diplopia (Casas et al., 2008).

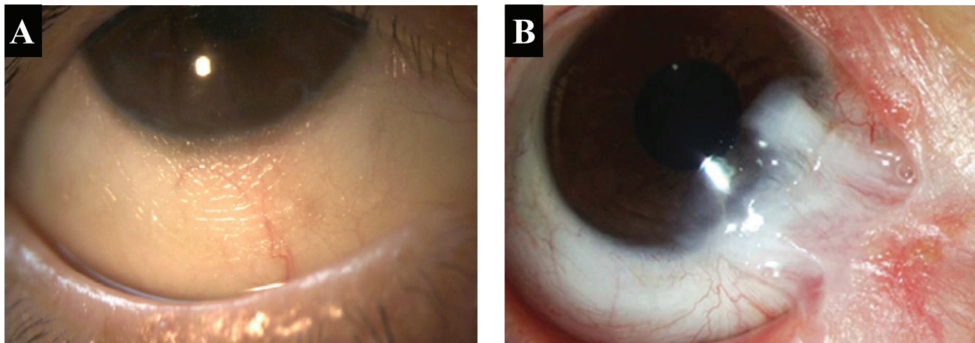

Figure 16. Chronic conjunctival inflammation can lead to a dry keratinized conjunctiva (A) and the formation of symblepharon (B), the latter in which case the eyelid becomes adherent to the sclera. Adapted from Hsu et al. 2012 and Kheirkhah et al. 2012.

5.12.4.3 Treatment

Depending on the disease etiology, conservative treatment options may include those mentioned in *section 5.12.3.4*. For controlling complications of conjunctival scarring, surgery can be considered. Surgical treatment of symblepharon includes removal of the scar tissue to

reestablish the deep fornix (Solomon et al., 2003). The surgical defect is then covered by the application of a tissue substitute. This is usually followed by different means to prevent re-obliteration. These include the application of different mechanical (Ralph, 1975b; Choy et al., 1977; Patel et al., 1998), physical (Fein, 1979) or chemical (Donnenfeld et al., 1999) means, or by grafting of conjunctival or mucous membrane (McCarthy, 1988; Solomon et al., 2003).

5.13 Surgical Restoration of the Conjunctiva

Surgical techniques for restoration of a diseased conjunctiva have utilize different conjunctival substitutes, although most studies have employed oral mucosal autografts and AM, either separately or combined (Table 6).

5.13.1 Pre-surgery Considerations

Chronic inflammation may adversely affect the outcome of conjunctival surgery (Schrader et al., 2009b). Thus, prior to surgery measures must be taken to control inflammation, e.g. by postponing surgery until inflammation has subsided after chemical burns (Kheirkhah et al., 2012) or by administering systemic immunosuppressive medication in cases of cicatrizing disease (Kheirkhah et al., 2012). In addition, severe dry eye decreases the chance of successful surgery and should be alleviated by e.g. punctal occlusion (Kheirkhah et al., 2012).

5.13.2 Post-surgery Considerations

Post-operative medications for patients receiving autologous tissue may include topical lubricants, antibiotics, autologous serum and steroids (Pauklin et al., 2010; Kheirkhah et al., 2012). Patients treated with allogeneic tissue in addition receive systemic immunosuppressive medication, such as cyclosporin, tacrolimus (Pauklin et al., 2010; Holland et al., 2012) and prednisone (Holland et al., 2012). Outcome evaluation encompasses regular post-operative check-ups with e.g. slitlamp examinations, determination of visual acuity and fluorescein staining of the ocular surface to assess integrity.

Table 6 - Transplanted Tissue Substitutes in Conjunctival Disorders		
Tissue substitute	**Disorder**	
Conjunctival graft	Pterygium	(Vastine et al., 1982; Dayanir et al., 2009; Kawano et al., 2011)
Hard palate mucosal graft	Malignant tumors of the eyelids	(Ito et al., 2001; Ito et al., 2007)
Oral mucosal graft	Ocular cicatricial pemphigoid (OCP)	(Shore et al., 1992)
	Trachoma	(Hosni, 1974)
	Trauma	(Karesh and Putterman, 1988)
Nasal mucosal graft	Chemical and thermal burns	(Kuckelkorn et al., 1996; Wenkel et al., 2000)
	Neurodermatitis	(Wenkel et al., 2000)
	Ocular cicatricial pemphigoid (OCP)	(Wenkel et al., 2000)
	Stevens-Johnson syndrome (SJS)	(Wenkel et al., 2000)
	Systemic cicatricial mucosal disease	(Wenkel et al., 2000)
	Toxic epidermal necrolysis (TEN)	(Wenkel et al., 2000)
Tenon sheets	Chemical and thermal burns	(Kuckelkorn et al., 1995)
Venous wall graft	Malignant tumors of the eyelids	(Barbera et al., 2008)
Amniotic membrane (AM) and conjunctival graft	Mooren's ulcer	(Chen et al., 2004)

Table 6 - Transplanted Tissue Substitutes in Conjunctival Disorders		
Tissue substitute	**Disorder**	
AM and oral mucosal graft	Chemical and thermal burns	(Kheirkhah et al., 2012)
	Graft-versus-host disease (GVHD)	(Kheirkhah et al., 2012)
	Mucous membrane pemphigoid (MMP)	(Kheirkhah et al., 2012)
	Stevens-Johnson syndrome (SJS)	(Kheirkhah et al., 2012)
	Xeroderma pigmentosa	(Kheirkhah et al., 2012)
AM and scleral graft	Marfan's syndrome	(Rodriguez-Ares et al., 1999)
AM with/without oral mucosal/conjunctival graft	Chemical and thermal burns	(Kheirkhah et al., 2008)
	Idiopathic chronic cicatricial conjunctivitis	(Kheirkhah et al., 2008)
	Malignant tumors of the eyelids	(Kheirkhah et al., 2008)
	Mucous membrane pemphigoid (MMP)	(Kheirkhah et al., 2008)
	Multiple previous surgeries	(Kheirkhah et al., 2008)
	Pseudopemphigoid	(Kheirkhah et al., 2008)
	Pterygium	(Kheirkhah et al., 2008)
	Stevens-Johnson syndrome (SJS)	(Kheirkhah et al., 2008)
AM	Allergic conjunctivitis/hay fever	(Solomon et al., 2003)

Table 6 - Transplanted Tissue Substitutes in Conjunctival Disorders		
Tissue substitute	**Disorder**	
	Chemical and thermal burns	(Solomon et al., 2003; Tseng et al., 2005)
	Dystrophic epidermolysis bullosa (EB)	(Goyal et al., 2006)
	Junctional EB	(Goyal et al., 2006)
	Laryngo-onychocutaneous (LOGIC) syndrome	(Goyal et al., 2006)
	Obstetric trauma	(Solomon et al., 2003)
	Ocular cicatricial pemphigoid (OCP)	(Solomon et al., 2003; Tseng et al., 2005)
	Pseudopterygium	(Tseng et al., 2005)
	Pterygium	(Solomon et al., 2003; Ang et al., 2005; Tseng et al., 2005)
	Stevens-Johnson syndrome (SJS)	(Honavar et al., 2000; Solomon et al., 2003; Tseng et al., 2005)
	Strabismus surgery	(Solomon et al., 2003)
	Superior limbic keratoconjunctivitis	(Gris et al., 2010)
	Toxic epidermal necrolysis (TEN)	(Solomon et al., 2003)
	Vernal keratoconjunctivitis (VKC)	(Guo et al., 2012)
Autologous conjunctival and limbal epithelial cells cultured on AM	Limbal stem cell deficiency (LSCD) and symblepharon due to chemical burn	(Sangwan et al., 2003a)

Table 6 - Transplanted Tissue Substitutes in Conjunctival Disorders		
Tissue substitute	**Disorder**	
Autologous conjunctival epithelial cells cultured on AM	Extensive conjunctival nevus	(Tan et al., 2004)
	Persistent leaking trabeculectomy blebs	(Tan et al., 2004)
	Pterygium	(Tan et al., 2004; Ang et al., 2005)
	Recurrent viral papillomata	(Ang and Tan, 2005)
	Superior limbic keratoconjunctivitis	(Tan et al., 2004)
Autologous conjunctival epithelial cells cultured without AM	Acanthamoeba corneal infection with conjunctival disepithelialization	(Scuderi et al., 2002)
	Congenital divided naevus	(Scuderi et al., 2002)
	Iatrogenic symblepharon	(Scuderi et al., 2002)
	Xeroderma pigmentosa	(Scuderi et al., 2002)

5.13.3 Tissue Grafts

5.13.3.1 Conjunctival Autografts

Conjunctival autografts has been used to cover small conjunctival defects after removal of pterygium (Thoft, 1977; Vastine et al., 1982). Obvious limiting factors when using autografts are the size of the defect to be covered as the amount of healthy conjunctiva is limited. In addition, in cases where the native conjunctiva is diseased, such as in SJS and OCP, harvesting of autografts is not recommended as it may reactivate the disease (Schrader et al., 2009b). These limitations have led scientists to look for alternative tissues grafts, such as oral mucus membrane and nasal mucosa.

5.13.3.2 Oral Mucous Membrane Grafts

Oral mucosal grafts have been used for reconstruction of the conjunctival fornices (Hosni, 1974; Shore et al., 1992). Although available in large amounts, the large possibility of donor site morbidity, the esthetic discrepancy in thickness and color compared to the conjunctiva and the lack of goblet cells in patients with SJS and OCP, are some of the drawbacks.

5.13.3.3 Nasal Mucous Grafts

Nasal mucous grafts have been successfully employed for fornix reconstruction in cases of chemical and thermal burns (Kuckelkorn et al., 1996) as well as in patients with OCP (Wenkel et al., 2000). One benefit of using nasal mucosa is that it contains a large number of epithelial goblet cells. Nasal autografts have reportedly been capable of sustaining goblet cells for ten years post-operative (Wenkel et al., 2000). Moreover, the transplanted goblet cells also increased ocular surface mucin (Wenkel et al., 2000). Still, the complicated surgical harvesting of nasal mucosa, with possibly grave donor site morbidity, prevents nasal mucosa autografts from being widely used.

5.13.4 Tissue Engineered Conjunctival Substitutes

A tissue engineered conjunctival epithelial transplant needs to be easily manipulated surgically, must not cause an inflammatory reaction and has to be biocompatible. So far, conjunctival epithelial cells cultured on AM have been considered the most promising (Ang et al., 2005; Schrader et al., 2009b).

5.13.4.1 Ex Vivo Expansion of Conjunctival Epithelial Cells

Although conjunctival epithelial cells have been cultured in the laboratory for at least more than 50 years (Cieciura et al., 1956), the culture of conjunctival epithelial cells is still a challenge due to the specialized goblet cells. In paper II we show that differentiated mucin-producing goblet cells generally do not migrate as far from the explant as less differentiated goblet cells without mucin production. This loss of mucin-production during cell migration from the explant may represent cell dedifferentiation, which is typical of differentiated cells initially during primary culture (MacDonald, 1994). Retinal pigment epithelial (RPE) cells, another highly specialized ocular epithelium, are also known to dedifferentiate in culture,

hence posing some difficulty when attempting to transplant a fully functioning RPE cell sheet (Valtink and Engelmann, 2009). For transplantation of LSC, the focus has been on transplanting the most undifferentiated cells, as this has been shown to enhance clinical outcome (Rama et al., 2010). No clinical studies have explored the effect of the degree of conjunctival transplant differentiation on the clinical outcome.

5.13.4.2 Culture Substrates

The conjunctival phenotype is partly dependent on the substrate. Several studies have demonstrated presence of goblet cells when using glass or plastic as substrate (Shatos et al., 2001; Shatos et al., 2002; Horikawa et al., 2003; Kanno et al., 2003; Shatos et al., 2003; Hodges et al., 2007; Gu et al., 2008; Rios et al., 2008; Shatos et al., 2008; Shatos et al., 2009a; Shatos et al., 2009b; Dartt et al., 2011). Conjunctival epithelial cells cultured on amniotic membrane (AM), however, usually primarily present a non-goblet phenotype (Cho et al., 1999; Meller and Tseng, 1999; Meller et al., 2002). Our group, on the other hand, detected numerous MUC5AC+ superficial cells when using AM (Eidet et al., 2012b). Goblet cells have been detected in 3D organotypic cultures on gelatin sponges (Rosellini et al., 2007), when culturing conjunctival cells inside type I collagen gels (Niiya et al., 1997) and when culturing on top of collagen gels (Tsai and Tseng, 1988). Goblet cells were also seen in culture when using Matrigel as substrate, in which case the conjunctival epithelium also stratified, as opposed to when using collagen gel substrates (Tsai and Tseng, 1988). Regarding the stratified squamous non-goblet conjunctival epithelial cells (which are CK4+), lack of laminin β2 in the ECM substrate has been shown to lead to loss of CK4 expression (Kurpakus and Lin, 1999).

5.13.4.3 Explant Culture

Explant culture is a well-established method for studying the conjunctival epithelial cell physiology (Diebold et al., 1997). As the explants already contain ECM with growth stimulating, subepithelial fibroblasts, cultures are usually not co-cultured with xenobiotic feeder cells. This is an advantage when it comes to clinical use, in which case the explant method so far has been the only method for culturing conjunctival epithelial transplants (Tan et al., 2004; Ang et al., 2005; Di Girolamo et al., 2009). The effects of soluble and insoluble ECM factors on the outgrowing cells should, however, be recognized. Our group showed that

the size of conjunctival explants affects how effectively the cells grow from the explant, which could be related to ECM factors (Eidet et al., 2012a). Human limbal epithelial cells reportedly loose stem cell characteristics with increasing distance to the explant (Kolli et al., 2008). Topographical variations in the composition of the ECM throughout the ocular surface (Schlotzer-Schrehardt et al., 2007) may affect the cells when employing explant cultures.

5.13.4.4 Cell Culture

For cell culture, cells are initially harvested from their native tissue by the use of different enzymes. Chen *et al.* (2011) demonstrated that complete removal of the ECM from the cells, by the use of e.g. dispase, resulted in poorer outgrowth and clonal growth compared the use of collagenase, in which case some of the ECM remains. If no specific substrates (*see 5.13.4.2*) or culture techniques (*see 5.13.4.6*) are used, cell cultures generally do not stratify, as opposed to explant cultures. Cell cultures co-cultured with 3T3 fibroblasts may show higher mechanical strength than explant cultures due to more desmosomal junctions between cells (Koizumi et al., 2002).

5.13.4.5 Culture Media

Fetal bovine serum (FBS) has traditionally been used as growth supplement in culture media. FBS contains vitamin A that has been shown to promote the expression of conjunctival epithelial cytokeratins (Fuchs and Green, 1981). There is, however, considerable interest in developing animal compound free (ACF) media mostly due to the risk of transmitting diseases when using FBS. Culture medium supplemented with umbilical chord blood serum (CBS) has been shown to be comparable to media supplemented with FBS in terms of supporting proliferative capacity, CFE and number of cell generations (Ang et al., 2011). When growing HCjE cells for transplantation, Tan and co-workers employed a serum-free culture media (2004). That culture medium, however, was not completely free of animal compounds as it contained bovine pituitary extract (BPE).

5.13.4.6 Air-lifting

Air-lifting has been shown to induce the development of MUC5AC+ goblet cells and stratification of HCjE cultures (Meller and Tseng, 1999; Chung et al., 2007; Lee et al., 2011).

Others have not found MUC5AC+ goblet cells despite the use of air-lifting (Tanioka et al., 2006). Cell polarity with microvilli, tight junctions and hemidesmosomes have been shown to be more prominent in air-lifted conjunctival cultures (Meller and Tseng, 1999). The molecular mechanisms involved in this technique include the p38 mitogen-activated protein kinase and Wnt signaling pathways, which were shown to be activated in conjunctival explants cultured with an airlift technique (Tan et al., 2011).

5.13.4.7 Feeder Layers

Human conjunctival epithelial cells may be cultured in the presence (Ang et al., 2004; Schrader et al., 2009a; Schrader et al., 2012b) or absence (Ang et al., 2004; Nizam et al., 2008) of a feeder layer. Although a feeder layer is generally considered to increase the proliferative capacity of epithelial cells in culture (Rheinwald and Green, 1975), conjunctival epithelial cells propagated in serum-free media reportedly demonstrated a similar *in vivo* proliferative capability, as compared to serum-containing media with 3T3 feeder cells (Ang et al., 2004). Moreover, co-cultures with human bulbar subconjunctival fibroblasts supported more conjunctival epithelial progenitor cells than co-cultures with conventional 3T3 fibroblasts (Schrader et al., 2010). In the search of a completely ACF culture system, HCjE cells cultured with human lung fibroblasts (MRC-5) and 5% human serum displayed similar proliferative capacity and number of progenitor cells as conventionally cultured HCjE cells (3T3 feeder cells and FBS) (Schrader et al., 2012b).

5.13.4.8 Transplantation of Amniotic Membrane Alone

Seventy years after its first use in ophthalmology, the AM, which constitutes the innermost layer of the fetal membranes, has a prominent role in ocular surface reconstruction (Tseng, 2001). The AM is especially suited for clinical use due to its effects on promoting epithelialization (Touhami et al., 2002), reducing scaring (Lee et al., 2000), immune-suppression (Ueta et al., 2002), pain reduction and decreasing inflammation (Solomon et al., 2001; Tseng, 2001). Prior to AM transplantation (AMT), the AM is cryopreserved, which kills all the AM cells (Kruse et al., 2000). Hence, AM grafts function primarily as a matrix and not by virtue of transplanted functional cells. The membranes have most commonly been cryopreserved in a basal cell medium at -80°C (Lee and Tseng, 1997), but a technique for freeze-drying the AM has also been developed (Nakamura et al., 2004c). Freeze-dried AM

can be sterilized by gamma-irradiation (Nakamura et al., 2004c), however, AM treated this way may release less amount of growth factors than conventionally cryopreserved membranes (Russo et al., 2012). In addition, the AM can be sterilized with peracetic acid/ethanol and air-dried (von Versen-Hoeynck et al., 2008). The latter technique is, however, reported to yield inferior results compared to cryopreserved AM with respect to cell outgrowth rate, release of wound-healing factors and preservation of the AM BM (Thomasen et al., 2009). Amniotic membrane transplantation can be performed using the AM either as a permanent BM substitute with epithelium side facing outwards (e.g. in cases with PED (Seitz, 2007)), as a temporary "patch" with the epithelial side facing inwards (e.g. in cases of acute burns (Meller et al., 2000)) or by using a combination of the two methods; the sandwich technique (e.g. in deep corneal ulcerations (Seitz, 2007)). AM has been used for conjunctival reconstruction after complete tumor removal where the AM functions as a BM substitute, promoting wound healing by the native conjunctival epithelial cells (Paridaens et al., 2001; Espana et al., 2002b). It has been suggested as a tissue substitute after removal of conjunctival scars and symblepharon, especially when the surrounding conjunctival tissue is close to normal (Tseng et al., 1997). In cases with chronic inflammation, however, there is a tendency for recurrent shrinkage and symblepharon (Henderson and Collin, 2008). The success of transplanting AM is therefore dependent on the underlying disease (Schrader et al., 2009b).

5.13.4.9 Amniotic Membrane-based Cell Constructs

It has generally been considered that goblet cells do not develop when culturing conjunctival cells on AM (Meller and Tseng, 1999; Ang et al., 2010). Our group, however, demonstrated large numbers of CK7+/MUC5AC+ goblet cells in the superficial cell layers when using AM as culture substrates (Eidet et al., 2012b). Meller and colleagues (2002) showed that although mucin-filled (MUC5AC+) goblet cells were undetected in rabbit conjunctival epithelial cells cultured on AM, strong PAS+ materials presumably representing mucin-filled goblet cells were found eleven days subsequent to transplantation into Balb/c athymic mice.

5.13.4.10 Conjunctival Restoration with Cultured Conjunctival Epithelial Cells

Cultured conjunctival epithelial cells have been used to repair conjunctival defects following pterygium surgery (Fig. 17) (Tan et al., 2004; Ang et al., 2005), removal of conjunctival nevus (Fig. 18) (Tan et al., 2004) and recurrent viral papillomata (Fig. 19) (Ang and Tan, 2005),

leaking trabeculectomy blebs (Tan et al., 2004) and bilateral superior limbic keratoconjunctivitis (Tan et al., 2004). Sangwan and co-workers reported a technique combining the culture of limbal and conjunctival epithelial cells for the treatment of LSCD and symblepharon due to chemical burns (Sangwan et al., 2003a).

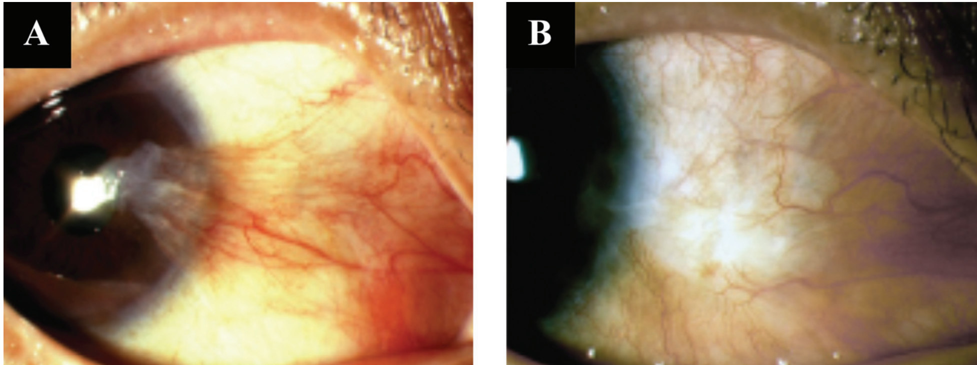

Figure 17. Transplantation of cultured human conjunctival epithelial (HCjE) cells for the treatment of pterygium. Photographs showing pre- (A) and post- (B) transplantation of cultured HCjE cells. Adapted from Tan et al. 2004.

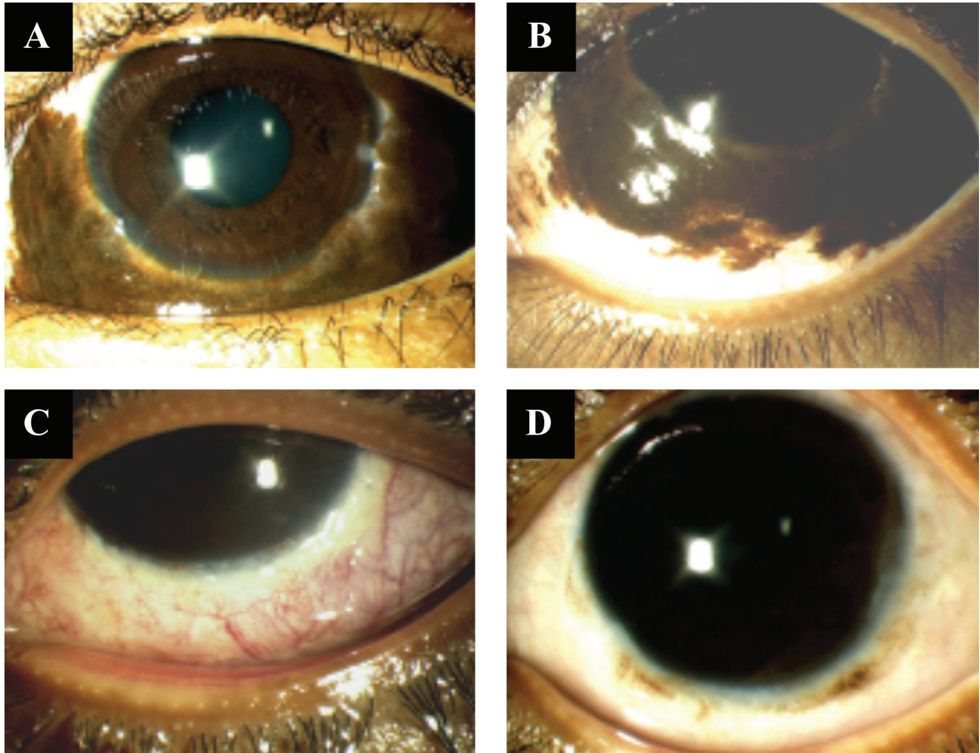

Figure 18. Transplantation of cultured human conjunctival epithelial (HCjE) cells for the treatment of an extensive nevus. Photographs showing pre- (A and B) and post- (C and D) transplantation of cultured HCjE cells. Adapted from Tan et al. 2004.

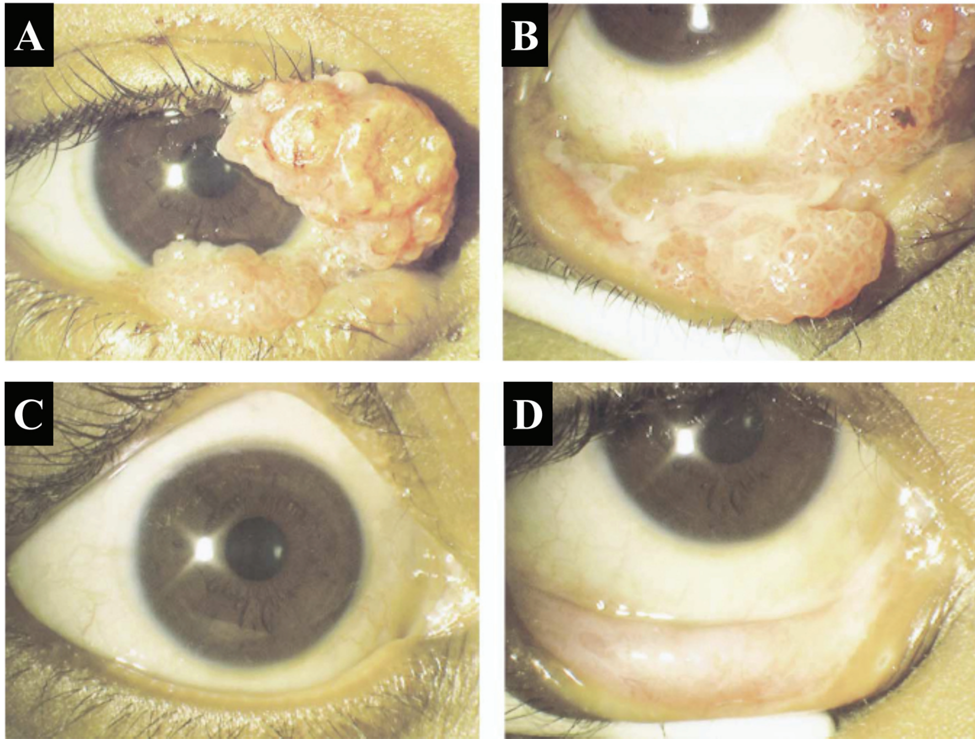

Figure 19. Transplantation of cultured human conjunctival epithelial cells for the treatment of a recurrent viral papilloma. Photographs showing pre- (A and B) and post- (C and D) transplantation of cultured human conjunctival epithelial cells. Adapted from Ang and Tan 2005.

5.13.5 Tissue Engineered Conjunctival Substitutes with Non-conjunctival Epithelial Cells

Epidermal epithelial cells cultured on AM have been transplanted to Rhesus monkeys to replace damaged conjunctiva (Lu et al., 2011). A multilayered, viable transplant was still present two weeks after the transplantation. The presence of conjunctival markers in the transplant, including MUC4 and CK4, was confirmed with immunohistochemistry (IHC). Goblet cells, however, were not detected.

5.13.6 Corneal Restoration with Cultured Conjunctival Epithelial Cells

Transplantation of cultured conjunctival epithelial cells has been used to treat contact lens-related corneal abnormalities in humans (Clinch et al., 1992) and LSCD in rabbits (Tanioka et al., 2006; Ono et al., 2007; Ang et al., 2010) and humans (Fig. 20) (Di Girolamo et al., 2009; Ricardo et al., 2012).

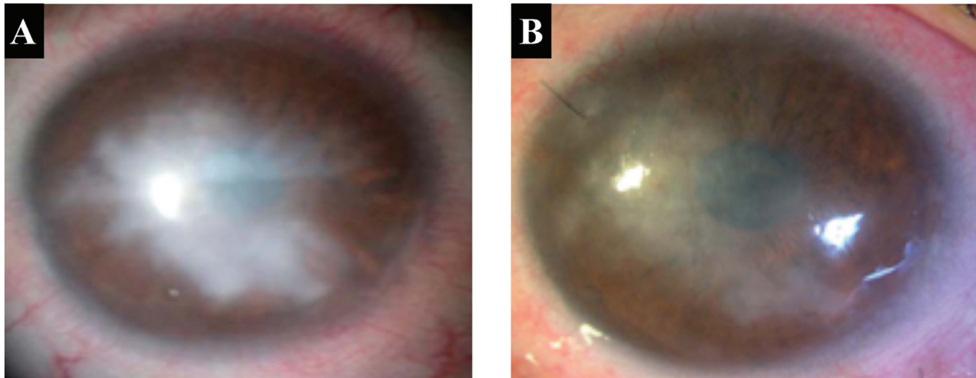

Figure 20. Transplantation of cultured conjunctival epithelial cells for the treatment of limbal stem cell deficiency (LSCD). (A) Prior to surgery. (B) Twelve months post-surgery. Adapted from Ricardo et al. 2012.

5.13.7 Eyelid Reconstruction

The eyelid can be divided into an anterior and posterior lamella, where the former consists of the skin and orbicularis muscle and the latter the tarsus and conjunctiva (Schrader et al., 2009b). In cases with a full thickness eyelid defect exceeding 25% of the width of the lid the superior and inferior lids cannot be closed directly. In such cases the patient needs a full thickness graft (Spinelli and Jelks, 1993).

5.14 Storage

One of the main benefits of storing cells is to create a logistical window in which one can plan further use of the cells. The longer the cells can be stored while maintaining quality and the less preparation needed after storage before the cells can be used, the longer this time window

becomes. Cell suspensions can be cryopreserved for long periods, but the cells usually must be expanded *ex vivo* before transplantation as well as cultured to form the organized tissue it is replacing (exceptions include injections of cells into the patient's eye as a cell suspension, e.g. RPE cells (Durlu and Tamai, 1997)). Storage of organized cells or tissues, on the other hand, has the benefit of being instantly transplantable upon end of storage. Hence, the benefit of storing "ready-to-use" tissues it that it can be shipped on very short notice from culture laboratories to transplantation clinics, which do not need cell culture facilities or expertise. Moreover, it does not necessitate the use of nitrogen freezer.

5.14.1 Whole Cornea Storage

Corneas have been and are still being, stored differently throughout the world. For same-day corneal transplantations enucleated eyes can be kept in moist chambers (Ehlers et al., 2009). For longer duration storage there are three current approaches: cold storage, cryopreservation and organ culture. Corneal cold storage at 4°C, first introduced in 1974 (McCarey and Kaufman) employs the Optisol-GS medium which consists of a traditional culture medium (TC-199) supplemented with antibiotics, dextran (anti-swelling) and antioxidants. Using cold-storage, corneas can be preserved for maximally ten days (Ehlers et al., 2009). Different cryopreservation techniques have been explored, with (Capella et al., 1965) or without crystallization (vitrification) (Rich and Armitage, 1992); or the inclusion of an additional processing step with organ culture (Ehlers et al., 1982; Erdmann and Ehlers, 1993). The poor ability to preserve the corneal endothelium and the complex technique of cryopreserving corneas have prevented this method from being widely used, however (Armitage, 2009). Using the organ culture technique (Summerlin et al., 1973; Bourne et al., 1977) the corneas are maintained in a closed bottle at 31°C in a basal medium supplemented with FBS for two to four or seven weeks, if the medium is changed (Ehlers et al., 1999; Ehlers et al., 2009). Among the benefits with organ culture preservation are the possibility for longer preservation and the feasibility of sterility control prior to surgery (Ehlers et al., 2009), which decreases the chance of primary graft failure (Ehlers et al., 2009). The technique of organ culture storage has also been investigated for conjunctival tissue (Berry and Radburn-Smith, 2005).

In addition to the storage method, the age of the donor and the death-to-preservation interval (DPI) may affect the quality of the corneas (Kryczka et al., 2011). It is generally

suggested that the corneas should be harvested as soon as possible after death (Slettedal et al., 2008). Cold-storage, however, can potentially minimize the adverse metabolic effects on the cornea due to a long DPI (Kryczka et al., 2011).

5.14.2 Above Zero Degree Storage of Cultured Ocular Surface Cells

The rational for storing cells and tissues above freezing point is to avoid cryopreservation-associated cell damage. Nevertheless, some degree of temperature reduction is usually involved during storage to protect the cells against degradation (Rauen and de Groot, 2002). Storing cells at temperatures below 37°C reduces their metabolism by slowing down the rate of enzymatic reactions (Rauen and de Groot, 2002). Although this may preserve the stored tissue, by decreasing the rate of injurious cell processes, temperatures that are too cold (e.g. 0°C to 10°C) can conversely have a detrimental effect on cell viability owing to electrolyte disturbances and oxidative stress (Rauen and de Groot, 2002).

5.14.2.1 Adherent Cell Cultures

To our knowledge, only one study has demonstrated successful above zero degree storage of cultured HCjE cells (Eidet et al., 2012b). Scuderi and co-workers, however, described a few hours storage of cultured HCjE cells prior to transplantation, though they did not report the tissue viability prior to transplantation (Scuderi et al., 2002). Our group have showed the feasibility of storing cultured HLEC on AM at 23°C in minimum essential medium (MEM), which was buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with FBS, for one week (Utheim et al., 2007). Storage at 23°C yielded higher viability and better morphology than storage in Optisol-GS at 5°C and in organ culture at 31°C degrees, while maintaining phenotype of the stored cells (Raeder et al., 2007b). Using a structurally modified calcium alginate hydrogel as substrate instead of AM, Wright *et al.* also demonstrated the possibility of storing cultured limbal epithelial cells (LEC) at ambient temperature (2012). Epidermal cells are considered a potential cell source for treating ocular surface disorders, such as LSCD. Storage of cultured epidermal epithelial cells for up to 20 days showed superior results at 22-24°C compared to storage at 4°C and 37°C (Bolivar-Flores et al., 1990), which supports our groups previous studies on cultured HLEC.

5.14.3 Below Zero Degree Storage - Cryopreservation

5.14.3.1 Cell Suspensions

Cell suspensions have traditionally been cryopreserved, which is the most widely used cell storage method. There are two principal mechanisms of cell injury during freezing cell suspensions: 1) intracellular ice-formation and 2) solution effects (Mazur, 1970, 1984; Armitage, 2009). The extent to which cells are damaged by any of these processes depends on the cooling rate (Mazur, 1970, 1984). The formation of extracellular ice crystals removes pure water but leaves the solutes, thereby exposing the cells to a hyperosmolar extracellular fluid. This, however, causes osmotic efflux of intracellular water, thereby shrinking the cells preventing intracellular ice formation. Higher cooling rates, on the other hand, lead to intracellular ice formation as the intracellular water freeze before it leaves the cells. In addition, the rate of warming the cells will affect the cell viability (Miller and Mazur, 1976). Cryopreserved conjunctival epithelial cell suspensions demonstrated comparable CFE and number of cell doublings as cells that were not cryopreserved (Schrader et al., 2009a).

5.14.3.2 Tissues

Tissues, including cell sheets, are usually more susceptible to cryopreservation damage than cell suspension (Armitage, 2009). This is due to factors including the presence of a higher cell concentration and intercellular junctions, which may promote spreading of intracellular ice to neighboring cells (Taylor and Pegg, 1983; Berger and Uhrik, 1996; Acker et al., 2001). As cryopreserved cell sheets are especially vulnerable to intracellular ice-formation it is essential to avoid too rapid cooling-rates (Routledge and Armitage, 2003). Some studies, however, have reported high viability after cryopreservation of monolayer cultures of conjunctival epithelial cells (Zheng et al., 2000; Oh et al., 2007). Oh and co-workers cultured rabbit conjunctival epithelial cells and demonstrated 95% viability after one-week cryopreservation of the cell sheets (2007). The conjunctival cells were stored in a medium containing 50% FBS and the cultures were co-cultured with 3T3 fibroblasts. Zheng and co-workers did not describe the storage medium used. They reported resuscitation of 90% of the cultured HCjE cells after 30 days of cryopreservation. Only one study has mentioned successful cryopreservation of stratified HCjE cultures (Scuderi et al., 2002), however, they did not report the percentage of viable cells upon thawing nor what storage medium they used or how

long they preserved the cells. Cryopreservation of stratified cultures with cultured LEC has demonstrated far lower viability (Kito et al., 2005; Yeh et al., 2008).

5.14.3.3 Vitrification

Vitrification is essentially freezing without ice-formation, a technique through which the cryopreservation-associated cell injuries mentioned above potentially could be avoided (Armitage, 2009). This technique necessitates an extremely high solution viscosity to inhibit the formation of ice crystals, in addition to a more rapid cooling rate (Armitage, 2009). Disadvantages of vitrification include the need for very high concentrations of cryoprotectants and that the method is exceedingly complex and time consuming (Armitage, 2009).

5.14.4 Hibernation

In contrast to simple lowering of the ambient temperature to extend cell survival, hibernation represents an innate mechanism to ensure long-term survival in a hypometabolic state (Storey, 2010). Through hibernation the cell induces global metabolic depression (Storey and Storey, 2004, 2007) and suppresses transcription/translation (Frerichs et al., 1998; Osborne et al., 2004), while at the same time critical genes are upregulated and access to DNA transcription are moderated by histones (Morin and Storey, 2006). Other key preserving factors during hibernation are antioxidant defense (Buzadzic et al., 1990; Drew et al., 2002; Carey et al., 2003; Tan et al., 2005; Ohta et al., 2006; Okamoto et al., 2006; Morin and Storey, 2007; Morin et al., 2008), chaperones, the unfolded protein response (Schroder, 2008) and the inhibition of inflammation by serpins (Gettins, 2002). If hibernation could be induced in cells *before* storage several of the harmful effects of exposing the tissue to cold-temperature during storage could possibly be avoided (Storey, 2010). Indeed, reversible hibernation has been pharmacologically induced in HeLa cells (Vecchio et al., 2006). Hence, understanding and implementing techniques for cell hibernation during storage of tissue-engineered substitutes may have great potential.

5.15 Unresolved Issues with Current Methods of *Ex Vivo* Expansion of Conjunctival Epithelial Cells

5.15.1 Site of Biopsy Harvesting

In order for a conjunctival substitute to be successful, it must contain goblet cells (Schrader et al., 2009b; Mason et al., 2011). Despite the importance of goblet cells in conjunctival regeneration, the impact on the cultured transplant of harvesting conjunctival tissue from different regions has never been investigated. In the human fetus goblet cells first appear in the fornix and then move towards the tarsal and bulbar regions at nine weeks (Miyashita et al., 1992). Goblet cells are located at the highest density in the forniceal conjunctiva in mice (Lavker et al., 1998; Nagasaki and Zhao, 2005), rats (Setzer et al., 1987; Yamabayashi and Tsukahara, 1987; Huang et al., 1988), canines (Moore et al., 1987; Goller and Weyrauch, 1993), domestic ruminants (Weyrauch, 1983b) and humans (Vujkovic et al., 2002). Like intestinal and respiratory epithelial stem cells, conjunctival stem cells are capable of committing to both non-goblet and goblet cell lineage (Bjerknes and Cheng, 1981; McDowell et al., 1987; Pellegrini et al., 1999). The location of the conjunctival stem cells has been a matter of some controversy. Conjunctival stem cells have been reported to reside in the limbus (rat (Pe'er et al., 1996)), forniceal conjunctiva (rabbit (Wei et al., 1993)), mouse (Lavker et al., 1998)), palpebral conjunctiva (rat (Chen et al., 2003)) and mucocutaneous junction (rat (Pe'er et al., 1996)) and rabbit (Wirtschafter et al., 1999)). Nagasaki *et al.* (2005) reported that mice bulbar conjunctival cells were capable of self-renewal *in situ* and concluded that conjunctival stem cells are evenly distributed in bulbar conjunctiva. Others have suggested that pockets of conjunctival stem cells reside throughout the bulbar conjunctiva (Qi et al., 2010). These studies support the findings of Pellegrini *et al.* (1999), who proposed that the conjunctival stem cells are evenly distributed in human bulbar and forniceal conjunctiva. Few studies have used tissue harvested from different conjunctival regions when culturing goblet cells (Nizam et al., 2008) and no studies have determined the optimal harvesting site for culturing goblet cells.

5.15.2 Conjunctival Epithelial Cell Storage

Storage of cultured HCjE cells for some days, preferably a week, have several advantages: Firstly, storage in a closed container with a septum renders microbiological testing and quality

control possible, both of which will be increasingly more important due to increasingly stricter EU directives. Secondly, storage enables transportation, making the products available worldwide. Thirdly, the possibility of preserving the cells makes large-scale production in centralized and highly specialized centers attractive. Finally, the method alleviates the logistical challenges by giving both the patient and surgeon an open interval for the operation to take place (Raeder et al., 2007b; Utheim et al., 2007). We have previously shown that cultured HLEC can be successfully stored at 23°C for one week (Raeder et al., 2007b; Utheim et al., 2007). If cultured conjunctival epithelial cells also may be stored under similar conditions this will increase the accessibility to treatment for not only corneal surface diseases, but to diseases affecting the entire ocular surface and eye lids.

6. AIMS OF THE PRESENT STUDY

6.1 Overall Aims of the Study

The primary overall objectives of the study were to (1) improve the method for *ex vivo* expansion of conjunctival epithelial cells and (2) improve the storage method for conjunctival explant cultures.

6.2 Aims of the Individual Studies

Study I To evaluate the effect of location and size of biopsy on phenotype and proliferative capacity of cultured rat conjunctival epithelial cells.

Study II To investigate if the number of goblet cells *ex vivo* is affected by the biopsy harvesting site and distance from the explant.

Study III To investigate the feasibility of storing cultured HCjE cells in HEPES-MEM and Optisol-GS at 23°C for four and seven days.

7. MATERIALS AND METHODS

7.1 Conjunctival Epithelial Cultures

7.1.1 Paper I and II - Conjunctival Explant Cultures on Glass Coverslips and Tissue Culture Plates

Male Sprague-Dawley rats were used as previously described (Shatos et al., 2001). Conjunctival tissue was excised at the 12 and 6 o'clock positions and immediately placed into phosphate-buffered saline (PBS; 145 milliMolar (mM) sodium chloride (NaCl), 7.3 mM sodium phosphate dibasic (Na_2HPO_4), 2.7 mM monosodium phosphate (NaH_2PO_4) [pH 7.2]). Tissue from each eye was divided into six pieces, superior and inferior bulbus, fornix and tarsus. The fornix was identified as the band running along the most posterior part of the fold at the junction of the bulbar and tarsal conjunctiva. The superior fornix was grasped and lifted and then excised from the conjunctiva. Thereafter, the superior tarsal and bulbar tissue were collected in similar fashion. Finally, the inferior tissues were excised accordingly after first identifying the inferior fornix. A total of six pieces, each measuring about 1-2 mm × 4 mm, were collected from each rat. The tissue was further divided into 0.5-1 mm^2 pieces that were anchored onto glass coverslips placed within six-well culture dishes. With a yield of six explants from each conjunctival region, a total of 36 explants were obtained from each animal. One explant was anchored in each tissue culture well. The culture dishes contained just enough medium to cover the bottom of the dish so that the tissue would receive nutrients through surface tension. The cell medium used to feed explants consisted exclusively of Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 100 μg/ml penicillin/streptomycin. This medium was changed every two days and the cultures grown for eight days under routine culture conditions of 95% air - 5% CO_2 at 37°C.

7.1.2 Paper III - Conjunctival Explant Cultures on Amniotic Membranes

Human conjunctival tissues, which were harvested from the superior and inferior forniceal regions, were rinsed three times with Dulbecco's Modified Eagle Medium (DMEM) containing 50 μg/ml gentamycin and 1.25 μg/ml amphotericin B. The donor samples were then subdivided into 2 × 2 mm explants under a dissecting microscope. Human AM was preserved as previously reported (Lee and Tseng, 1997), with some modifications.

Specifically, following caesarean section, the fetal membranes with placenta were transferred into 500 milliliter (mL) of transport medium (pH 7.4) consisting of sterile washing buffer (0.14 Molar (M) NaCl, 0.004 M potassium chloride (KCl), 0.001 M trisodium phosphate (TSP) and 0.01 M glucose), 50 U/mL penicillin (Sigma-Aldrich), 50 μg/mL streptomycin (Sigma-Aldrich) and 2.5 μg/mL amphotericin B (Sigma-Aldrich). The fetal membranes were separated from the placenta using scissors, washed in sterile washing buffer with added antibiotics and cleansed of blood clots. The AM with chorion was stretched out on nitrocellulose paper (0.45 μm pore size, OneMed, Vantaa, Finland) and the chorion was removed using forceps, leaving only the AM attached to the nitrocellulose paper. AM was stored in sterile cryo-tubes (Nunc, Roskilde, Denmark) at –70°C in a 1:1 (vol/vol) mixture of DMEM and glycerol (Sigma-Aldrich). Prior to use, the AM was thawed and washed three times with sterile PBS (Sigma-Aldrich). AM with the BM facing up was fastened to the polyester membrane of Costar Netwells (24 mm diameter, 74 µm pore size, Costar Corning, Corning, NY) using Ethicon Ethilon 6-0 monofilament sutures. Conjunctival explant cultures were prepared by placing the explants on the centre of each AM insert in a supplemented hormonal epithelial medium made of DMEM/Ham's F12 (1:1) containing HEPES and sodium bicarbonate (Sigma-Aldrich). The medium was supplemented with 5% FBS (Sigma-Aldrich), 0.5% dimethyl sulfoxide (Sigma-Aldrich), 2 ng/mL human epidermal growth factor (EGF) (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), 5 μg/mL transferrin (Sigma-Aldrich), 5 ng/mL selenium (Sigma-Aldrich), 3 ng/mL hydrocortisone (Sigma-Aldrich), 30 ng/mL cholera toxin (Biomol, Exeter, UK), 50 μg/mL gentamycin and 1.25 μg/mL amphotericin B. Cultures were incubated for two weeks in a Heraeus HERAcell 240 l CO_2 copper incubator (former Kendro Laboratory Products, Newtown, CT) at 37ºC in an atmosphere of humidified 5% CO_2 and 95% air and the medium was changed every two to three days.

7.2 Storage of Cultured Conjunctival Epithelial Cells

7.2.1 Paper III

Cultured HCjE cells were prepared for storage as previously reported with some modifications (Utheim et al., 2007). The polyester mesh membrane with the cultured epithelium attached was released using a steel blade. Unlike in previous experiments from our group where the grafts were transferred to an air- and watertight container, the cultured HCjE

cells were transferred to a secondary six-well culture plate for storage at 23°C. The HCjE cells were stored for four and seven days in 5 mL serum-free medium containing either MEM with 12.5 mM HEPES, 7.5% sodium bicarbonate, 50 μg/ml gentamycin, 100 μg/ml vancomycin (Abbott Laboratories, Abbott Park, IL) and 2.5 μg/ml amphotericin B; or Optisol-GS (Bausch & Lomb, Irvine, CA).

7.3 Transmission Electron Microscopy

7.3.1 Paper III

Human conjunctival epithelial cells cultured for two weeks without subsequent storage (control; n = 3), with subsequent four days of storage (HEPES-MEM; n = 4 or Optisol-GS; n = 4) or seven days of storage (HEPES-MEM; n = 5 or Optisol-GS; n = 4) were processed for TEM analysis as previously described (Raeder et al., 2007a). In brief, ultrathin sections (60-70 nm thick) were cut on a Leica Ultracut Ultramicrotome (Leica, Wetzlar, Germany) and examined using a CM120 transmission electron microscope (Philips, Amsterdam, the Netherlands). The average number of microvilli/mm of epithelial length (Koufakis et al., 2006) was measured using ImageJ software (Ver1.44 m). The number of cell layers was counted by two independent investigators.

7.4 Histology and Immunostaining

7.4.1 Paper I and II

7.4.1.1 Histochemistry and Immunohistochemistry

Sections were deparaffinized, rehydrated and subjected to antigen retrieval before histochemistry and IHC. The antigen retrieval step was added as formaldehyde fixation is known to mask antigens due to cross-linking of proteins (Abcam). Antigen retrieval was performed by exposing the sections to 95-100°C for 20 minutes submersed in a buffer solution consisting of 10 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% Tween-20.

Sections were incubated in blocking buffer, to decrease unspecific staining, for one hour at room temperature. For lectin histochemistry, this was followed by incubation for one hour at room temperature with UEA-1 lectin (Vector Laboratories, Burlingame, CA), a marker for goblet cell selective high molecular weight glycoconjugates, conjugated directly to

fluorescein isothiocyanate (FITC) diluted 1:3000 in PBS. Cells were incubated overnight at 4°C with antibodies for markers of goblet cells and non-goblet ocular surface epithelial cells: CK7 (RCK105) (Santa Cruz Biotechnology), which recognizes a goblet cell–specific keratin (Kasper, 1991) (1:200); MUC5AC (45M1) (Thermo Fischer Scientific, Fremont, CA), specific for mucin produced by goblet cells (Argueso and Gipson, 2001) (1:100); CK4 (6B10) (Abcam Inc., Cambridge, MA), specific for stratified, squamous, non-goblet epithelial cells (Kasper, 1991) (1:200); ABCG2 (M-70) (Santa Cruz Biotechnology, Santa Cruz, CA), a putative stem cell marker (Budak et al., 2005) (1:200); and Proliferating cell nuclear antigen (PCNA) (FL-261) (Santa Cruz Biotechnology), a marker for proliferative capacity (Hall et al., 1990) (1:200). The anti-mouse or anti-rabbit immunoglobulin G (IgG) secondary antibodies (Jackson Immuno Research Laboratories, Inc., West Grove, PA), conjugated to either cyanine dye 2 (Cy2) or cyanine dye 3 (Cy3), were diluted 1:100 and 1:300, respectively, in PBS and incubated for one hour at room temperature. Slides were washed three times in PBS, after which coverslips were mounted on the slides with mounting media containing 100 mM Tris (pH 8.5), 25% glycerol, 10% polyvinyl alcohol, 2.5% 1,4-diazobicyclo-[2.2.2]-octane and 4', 6-diamidino-2-phenylindole (DAPI).

Sections adherent to microscope slides were visualized with an epifluorescence microscope (Eclipse E 800; Nikon, Tokyo, Japan) or laser confocal microscopy (Leica TCS-SP5; Leica Microsystems, Wetzlar, Germany). Negative controls consisted of substituting PBS for the primary antibody. Positive controls included fixed sections of whole rat eyes with eyelids containing structures with known positive staining for each of the antibodies used. Semi-quantitative immunohistochemical analysis of the sections was carried out at a magnification of ×630 and scored as previously described (Eidet et al., 2012b). Positive staining in the sections was graded as 0 (undetectable), + (detectable in <¼ of the cells), ++ (detectable in ¼-½ of the cells), +++ (detectable in ½-¾ of the cells) and ++++ (detectable in >¾ of the cells).

7.4.1.2 Cytochemistry and Immunocytochemistry

Explant cultures on coverslips were methanol-fixed for 15 minutes, rinsed in PBS and then incubated for one hour at room temperature in blocking buffer that consisted of 1% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS. Cells were then incubated overnight at 4°C with the following dilutions of primary antibodies in PBS: Antibody to p63 (1:100), a

marker for undifferentiated and highly proliferative cells (Hsueh et al., 2004); anti-ABCG2 (1:200) (M-70; Santa Cruz Biotechnology, Santa Cruz, CA); anti-PCNA (1:200) (FL-261; Santa Cruz Biotechnology); antibody against CK7 (1:200; RCK105; Santa Cruz Biotechnology); MUC5AC (1:100) (45M1; Thermo Fischer Scientific, Fremont, CA); UEA-1 conjugated directly to FITC was diluted 1:1000 in PBS; and CK4 (1:200) (6B10; Abcam Inc., Cambridge, MA). The secondary antibodies, conjugated to either Cy2-or 3, were diluted 1:100 or 1:300, respectively, in PBS and incubated for one hour at room temperature. Coverslips were washed three times in PBS, after which coverslips were mounted on microscope slides with mounting media containing 100 mM Tris (pH 8.5), 25% glycerol, 10% polyvinyl alcohol and 2.5% 1,4-diazobicyclo-[2.2.2]-octane. Cell cultures adherent to glass coverslips were visualized with an epifluorescence microscope (Eclipse E 800; Nikon). Negative controls consisted of substituting PBS for the primary antibody. Positive controls included fixed sections of whole rat eyes with eyelids containing structures with known positive staining for each of the antibodies used. Expression of the markers was assessed at a magnification of ×630 and 100 cells in six fields were counted in each culture by two independent investigators. The number of positive cells/total number of cells × 100% was calculated.

7.4.2 Paper III

7.4.2.1 Histochemistry and Immunohistochemistry

The cultures were fixed in neutral buffered 4% formaldehyde and embedded in paraffin. Haematoxylin and eosin staining and IHC with a panel of proposed conjunctival stem cell, progenitor, differentiation, proliferation and apoptosis markers was performed on serial sections of 4.5 μm using Benchmark XT Antibody diluent (251-018) and Detection Kit Ventana ultraView Universal DAB (760-500), an automated immunostaining system based on the avidin-biotin-peroxidase complex (ABC) method, with negative and positive controls (Ventana Medical Systems, Inc., Tucson, AZ, USA). The immunoreactivity was graded as 0 (undetectable), + (detectable positivity in <¼ of the cells), ++ (detectable positivity in ¼-½ of the cells), +++ (detectable positivity in ½-¾ of the cells) and ++++ (detectable positivity in >¾ of the cells). All scores were assigned at a magnification of ×630 by two independent, experienced investigators blinded to the origin of the samples.

7.5 Cell Viability Assays

7.5.1 Paper III

Viability staining was performed using a calcein-acetoxymethyl ester (CAM)/ethidium homodimer 1 (EH-1) (Invitrogen, Carlsbad, California) assay as described previously by the group (Fig. 21) (Utheim et al., 2009b). In brief, HCjE cells cultured for two weeks but not stored (n = 7), stored for four days in HEPES-MEM (n = 7) and Optisol-GS (n = 8) and stored for seven days in HEPES-MEM (n = 4) and Optisol-GS (n = 5) were incubated in PBS containing 2 mM CAM and 2 mM EH-1 for 45 min at 23°C, then rinsed with PBS. Epithelial discs were trephined using a 6 mm Kai biopsy punch (Kai Industries, Gifu, Japan) and mounted on coverslip glass slides. Fluorescence images of the basal layer were recorded using an Axiovert 100 LSM 510 laser scanning confocal microscope (Carl Zeiss Microscopy, Oberkochen, Germany). The number of live and dead cells (green and red fluorescence, respectively) was counted in five fields at a magnification of ×250 and the percentage of live cells calculated. Human conjunctival epithelial cells cultured for two weeks and then exposed to methanol for 1 hour (n = 2) were used as positive controls for dead cells.

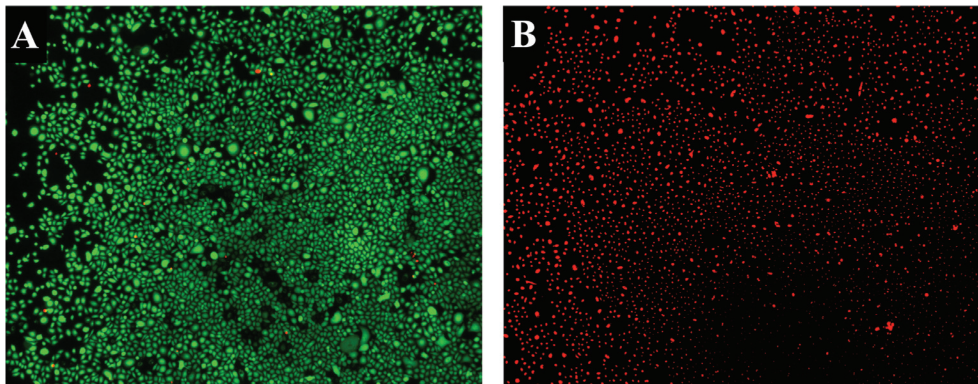

Figure 21. Epifluorescence microscopy photomicrographs showing that live conjunctival epithelial cells (A) were generally calcein-acetoxymethyl ester (CAM) positive (green) and ethidium homodimer-1 (EH-1) negative (red), while methanol-fixed dead cells (B) were EH-1 positive and CAM negative. Original magnification: ×40.

7.6 Colony-forming Efficiency Assay

7.6.1 Paper I

The clonal growth capacity of epithelial cells from the superior and inferior bulbar, forniceal and tarsal membrane of the conjunctiva was determined by a CFE assay (n = 9). Conjunctival tissues from six regions were left in Dispase II overnight at 4°C, thereafter trypsinized for ten minutes at 37°C to achieve single cells. Mitomycin C treated 3T3 feeder cells were seeded onto six- and 12-well plates at a density of $1 \times 10^5/cm^2$ 24 hours before plating conjunctival cells at a clonal density of 50 cells/cm^2. A colony was defined as a group of eight or more contiguous cells (Fig. 22) (Nakamura et al., 2006a). Colonies were fixed on day eight, stained with Rhodamine B Fluka (ready to use solution, Sigma) and counted independently by two investigators; data were then averaged. Colony-forming efficiency was defined as follows: CFE (%) = total number of colonies formed at the end of growth period/total number of cells seeded × 100%.

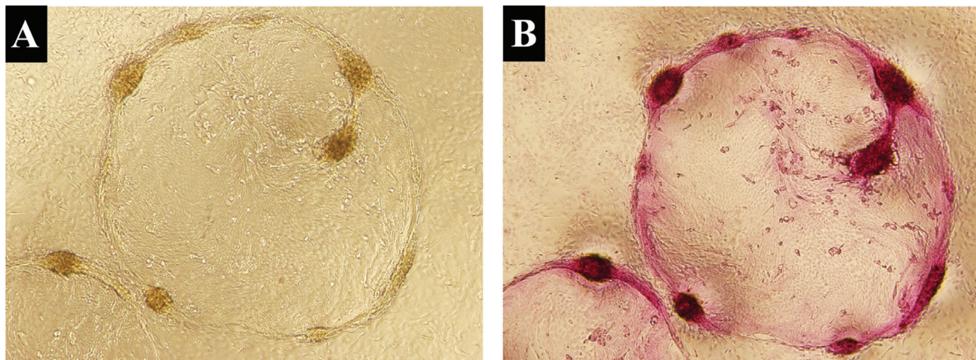

Figure 22. Colony-forming efficiency (CFE) assay. Phase contrast photomicrographs showing two circular conjunctival epithelial cell cultures before (A) and after (B) staining with Rhodamine B Fluka (red). The CFE assay in paper I was performed by counting the number of colonies using an inverted phase contrast microscope. Eight or more contiguous cells were defined as a colony. Original magnification: ×40.

7.7 Outgrowth Measurements

7.7.1 Paper I and II

The degree to which the cells grow out from the explant can be used as a measure of proliferative and migratory capacity (Fig. 23). In paper I and II we measured the outgrowth in primary culture to assess the proliferative capacity of different harvesting sites in the conjunctiva and to investigate the effect of distance to the explant on phenotype.

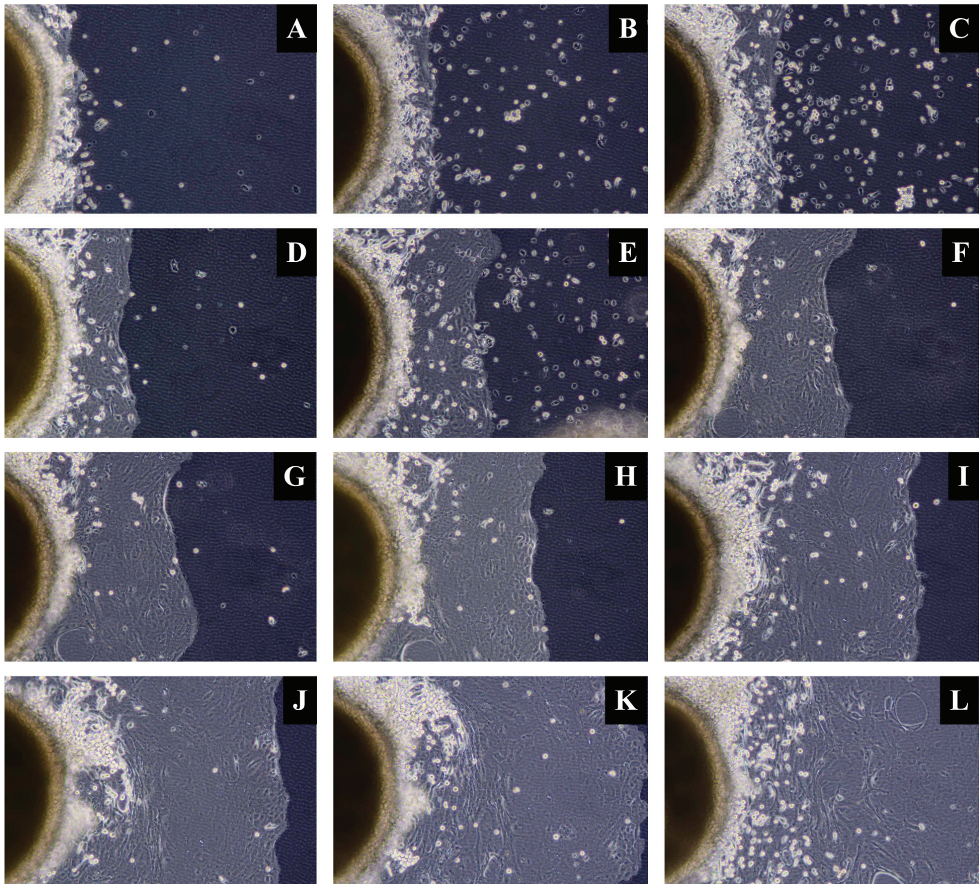

Figure 23. Photomicrographs of the same conjunctival explant culture captured at consecutive time points showing how the conjunctival epithelial cells grow out from the explant. Original magnification: ×100.

Nuclei of epithelial cells in primary culture grown from explants from the superior and inferior bulbar, forniceal and tarsal areas of the conjunctiva were stained with DAPI and outgrowth was visualized with a fluorescence microscope (Eclipse E800; Nikon) at a magnification of ×40 (n = 6). Occasional areas with fibroblasts were recognized by morphology and excluded (Fig. 24). After eight days in culture outgrowth size and explant size were quantified using ImageJ software (National Institute of Health, Bethesda, MD). Fold growth in each primary culture was quantified as follows: outgrowth size/explant size.

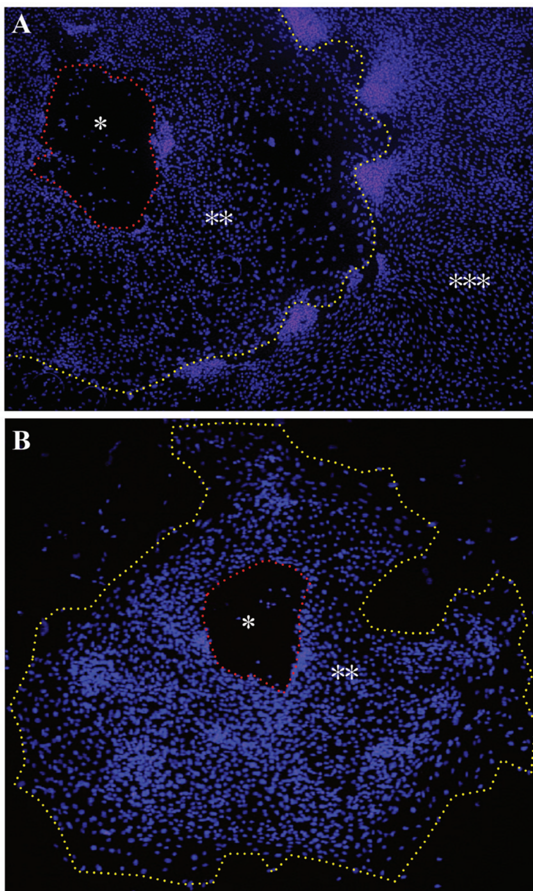

*Figure 24. Photomicrograph displaying a rat conjunctival epithelial explant culture extensively contaminated with conjunctival fibroblasts (A). An explant culture without fibroblast contamination is shown in comparison (B). * Explant area. ** Epithelial outgrowth. *** Fibroblast contamination identified by their elongated nuclei and wavy growth pattern. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Red dotted line: Explant demarkation line. Yellow dotted line: Border between epithelial cells and fibroblasts. Original magnification: ×40.*

7.8 Statistical Analysis

7.8.1 Paper I

The Mann–Whitney test was used to compare measurements of fold growth between all six conjunctival regions. In contrast to these measurements, all the CFE measurements were paired, hence the Wilcoxon test was used for comparison of CFE between the six conjunctival regions. To analyze the relationship between fold growth, outgrowth size and explant size, as well as between outgrowth size, explant size and phenotypic data, the Spearman's rank order correlation test was used. Data were expressed as mean ± standard error of the mean (SEM). A significance level of $P < 0.05$ was used throughout the study (Statistical Package for the Social Sciences (SPSS) ver. 18.0).

7.8.2 Paper II

The Mann–Whitney test was used to compare phenotypic data between all six conjunctival regions as well as between cells near the explant and close to the leading edge. To analyze the relationship between outgrowth size and phenotypic data, the Spearman's rank-order correlation test was used. Spearman's rank-order correlation test and a paired sample T-test were employed to assess observer agreement. Data were expressed as mean ± SEM. A significance level of $P < 0.05$ was used throughout the study (SPSS ver. 18.0).

7.8.3 Paper III

Data were presented as mean ± standard deviation (SD). To assess the difference between the groups, a one-way analysis of variance (ANOVA), followed by Tukey's test to control for multiple comparisons were performed. A significance level of $P < 0.05$ was used throughout the study (SPSS ver.18.0).

8. SUMMARY OF RESULTS

8.1 Paper I

Paper I evaluated the effect of location and size of biopsy on phenotype and proliferative capacity of cultured rat conjunctival epithelial cells. Epithelial cells from six regions of rat conjunctiva were grown in RPMI 1640 with 10% FBS for eight days. Analysis included immunocytochemistry (ICC), outgrowth measurements and a CFE assay. Superior (23.9 ± 2.9 fold growth) and inferior (22.4 ± 1.2 fold growth) forniceal tissues yielded significantly more outgrowth with respect to explant size than superior bulbar (13.4 ± 1.9 fold growth; $P < 0.05$ and $P < 0.01$, respectively), inferior bulbar (13.6 ± 1.6 fold growth; $P = 0.01$ and $P < 0.01$, respectively) and inferior tarsal tissues (14.0 ± 1.3 fold growth; $P = 0.01$). Outgrowth size correlated positively with explant size (Spearman's rank correlation coefficient/rho (r_s) = 0.54; $P < 0.001$), whereas explant size correlated negatively with fold growth ($r_s = 0.36$; $P < 0.001$). Superior forniceal cells displayed higher CFE (3.6% ± 0.9%) than superior bulbar (1.1% ± 0.3%; $P < 0.05$) and inferior bulbar cells (1.6% ± 0.8%; $P < 0.05$). Percentage of p63+ and PCNA+ cells correlated positively with explant and outgrowth size. Small forniceal conjunctival explants grow the most effectively. However, for transplantation purposes the loss of p63+ and PCNA+ cells with small explants must be considered.

8.2 Paper II

In paper II we investigated if the number of goblet cells *ex vivo* is affected by the biopsy harvesting site and distance from the explant. Sections of whole rat conjunctiva were processed for IHC. Explants from six regions of rat conjunctiva were grown in RPMI 1640 with 10% FBS on coverslips for eight days and the cultures were processed for ICC and outgrowth measurements with ImageJ. In the conjunctival sections all three goblet cell markers showed fewest goblet cells in the superior bulbar region, followed by the inferior bulbus, while the highest concentrations were found in the forniceal and tarsal regions. Distribution of the CK4+ stratified, squamous non-goblet epithelial cells was mainly opposite that of the goblet cells, with higher concentrations in the bulbar regions than in the forniceal regions. Cultures from superior and inferior fornix contained the most goblet cells, with 60.8% ± 9.2% and 64.7% ± 6.7% being CK7+, respectively, compared to the superior tarsal (26.6% ± 8.4%; $P < 0.05$), superior bulbar (31.0% ± 4.0%; $P < 0.05$), inferior bulbar (38.5% ±

9.3%; $P < 0.05$) and inferior tarsal regions (27.7% ± 8.3%; $P < 0.05$). While 28.4% ± 6.3% of CK7+ goblet cells co-labeled with PCNA, only 7.4% ± 1.6% of UEA-1+ goblet cells did ($P < 0.01$). The number of goblet- and non-goblet cells *ex vivo* was affected by distance from the explant and culture size. We conclude that *ex vivo* expansion of forniceal conjunctiva generates the highest number of goblet cells. CK7+/UEA-1- cells seem to have the highest proliferative potential for goblet cell transplantation.

8.3 Paper III

Paper III investigated the feasibility of storing HCjE cells in the serum-free media HEPES-MEM and Optisol-GS at 23°C for four and seven days, respectively. The five experimental groups were analyzed by light microscopy, IHC, TEM and a viability assay. The ultrastructural integrity of cultured HCjE cells was well preserved following four days of storage, however, seven days of storage resulted in some loss of cell-cell contacts and epithelial detachment from the AM. The number of microvilli in cultured HCjE cells not subjected to storage was 2.03 ± 0.38 microvilli/μm. In comparison, after four and seven days of HEPES-MEM storage this number was 1.69 ± 0.54 microvilli/μm; $P = 0.98$ and 0.89 ± 1.0 microvilli/µm; $P = 0.28$, respectively. After Optisol-GS storage for four and seven days, the mean number of microvilli was 1.07 ± 1.0 microvilli/μm; $P = 0.47$ and 0.07 ± 0.07 microvilli/μm; $P = 0.03$, respectively. The number of cell layers in cultured HCjE cells not subjected to storage was 4.4 ± 0.3 cell layers, as opposed to 4.0 ± 0.9 cell layers; $P = 0.89$ after four days of HEPES-MEM storage and 2.8 ± 0.6 cell layers; $P = 0.01$ after seven days of storage in HEPES-MEM. The number of cell layers after four and seven days of storage in Optisol-GS was 3.7 ± 0.2 cell layers; $P = 0.46$ and 3.4 ± 0.4 cell layers; $P = 0.18$, respectively. The expression of markers for undifferentiated cells (ΔNp63α, ABCG2 and p63), proliferating cells (Ki67 and PCNA), goblet cells (CK7 and MUC5AC), stratified squamous epithelial cells (CK4) and apoptotic cells (caspase-3) in cultured HCjE cells appeared to be unchanged after four and seven days of HEPES-MEM and Optisol-GS storage. The percentage of viable cells in cultured HCjE cells not subjected to storage (91.4% ± 3.2%) was sustained after four and seven days of storage in HEPES-MEM (94.1% ± 4.5%; $P = 0.99$ and 85.1% ± 13.7%; $P = 0.87$, respectively) as well as after four and seven days of storage in Optisol-GS (87.7% ± 15.2%; $P = 0.97$ and 79.8% ± 15.7%; $P = 0.48$, respectively). We conclude that cultured HCjE

cells may be stored for at least four days in serum-free conditions at 23°C while maintaining the phenotype and viability. HEPES-MEM appears to be comparable to Optisol-GS for serum-free storage with preservation of the ultrastructure for at least four days.

9. GENERAL DISCUSSION

9.1 Methodological Considerations

9.1.1 Choice of Culture Method

The three papers employed two slightly different culture protocols; explants were cultured on either AM for two weeks or on glass coverslips for eight days. The culture media used included either DMEM/F12 or RPMI-1640, both supplemented with FBS.

In paper I and II we built on the experience of the Dartt laboratory with their rat conjunctival primary culture protocol for generating goblet cells *in vitro*. Dartt and colleagues have demonstrated successful culturing of both rat (Shatos et al., 2001) and human (Shatos et al., 2003) goblet cells when culturing explants *in vitro* in RPMI 1640 supplemented with FBS. In paper III we sought to develop a method for storing cultured HCjE cells for subsequent clinical use. We cultured the HCjE cells on AM since all previous studies demonstrating transplantation of cultured HCjE cells to restore the human conjunctiva (Tan et al., 2004; Ang and Tan, 2005; Ang et al., 2005) and cornea in LSCD (Ricardo et al., 2012), have used AM as culture substrate. As we aimed at developing the storage and not the culture method of HCjE cells, we did not culture the cells in an ACF medium. Although not a completely xenobiotic-free culture medium, in Tan and Ang's studies they used a serumfree medium, with the only animal compound being bovine pituitary extract (Tan et al., 2004; Ang and Tan, 2005; Ang et al., 2005). Ricardo *et al*. employed a culture medium much similar to the one utilized for paper III, although with a 10% instead of 5% concentration of FBS (Ricardo et al., 2012).

We consistently applied an explant culture protocol instead of cell suspension culture. There are several reasons for this. First, the use of explants in our studies is encouraged by previous successful clinical studies (Tan et al., 2004; Ang et al., 2005; Di Girolamo et al., 2009; Ricardo et al., 2012). Second, there are qualitative differences between cell suspension culture and explant culture. Chen *et al.* (2011) demonstrated that complete removal of the ECM (e.g following dispase treatment), resulted in poorer outgrowth and clonal growth compared to the use of collagenase, in which case some ECM remains. Third, the use of cell suspension culture *in vitro* usually warrants a co-culture technique including feeder cells (Wei et al., 1993), thereby introducing another undefined factor (xenobiotic cells) in the culture protocol that may decrease the reliability of the results, transfer infectious agents, as well as

possibly causing subsequent immunorejection if the cells were transplanted (Martin et al., 2005).

9.1.2 Storage Method

9.1.2.1 Storage Medium

The development of an ACF storage media is currently an area of immense interest not only within ophthalmology, but also within all fields of organ transplantations and regenerative medicine. At the present time, there is an unmet demand for ACF media and it is of high priority to avoid the use of animal compounds, such as FBS, because of multiple accompanying disadvantages when applied in a clinical setting. Such disadvantages include the risk of bovine spongiform encephalopathy and infection with animal viruses and bacteriophages (Geier et al., 1975; Kniazeff et al., 1975; Frueh and Bohnke, 2000); the risk of inducing subsequent immunorejection due to the introduction of animal proteins in the transplant (Martin et al., 2005); the risk of possible alterations in the components of FBS used (Engelmann et al., 1998); and finally, the possibility for high variability between the FBS batches renders the standardization of storage media and hence their validation by the health authorities, very difficult (Thuret et al., 2005). Due to these reasons, the HCjE cells in paper III were stored in a defined basal cell culture medium, MEM, without the addition of any animal compounds. The choice of this storage medium was partly based on our research group’s previous studies (unpublished) on HLEC showing superior cell preservation when using MEM without FBS compared to when including FBS.

9.1.2.2 Storage Temperature

We only employed above-zero degree temperatures for storage, thus we did not include cryopreservation. There are various reasons for the omission of cryopreservation studies. The most important one being our aim at improving short-term and not long-term, storage. The possibility of storing cultured cells for at least a few days (i.e. short-term storage) would be enough to ease logistical difficulties (O'Callaghan and Daniels, 2011) regarding transplantations and potentially improve outcomes by providing time for quality control of the transplant (Utheim et al., 2009b). Furthermore, short-term storage is sufficient to enable transportation of tissue (Ahmad et al., 2010), which will become increasingly important in

regenerative medicine as various technologies leave the experimental stage to be used in mainstream medicine. Whereas cryopreservation is the standard method for storing cells in suspension, cryopreservation of adherent stratified ocular epithelia has been less successful (Kito et al., 2005; Yeh et al., 2008). In addition, when only short-term storage is required, above-zero degree storage methods are more convenient than cryopreservation, which necessitates expensive cryopreservation facilities and expertise.

9.1.2.3 Storage Conditions

For the storage experiments in paper III, culture plates were stored in a commercial wine cooler set at 23°C. To be able to store the cells in regular air the storage medium contained HEPES, as the HCO_3^- buffer does not maintain physiological pH without CO_2 incubation (Mackenzie et al., 1961; Itagaki and Kimura, 1974). The HEPES buffer is also ideal for hypothermic cell storage as it is effective over the entire 0°C to 40°C temperature range (Baicu and Taylor, 2002). As HEPES is known to be photo-toxic the stored cells were protected from light (Zigler et al., 1985; Lepe-Zuniga et al., 1987).

9.1.3 Analyses

In the three studies the cell morphology was assessed by bright-field light microscopy of H&E sections and TEM; protein expression was investigated by ICC/IHC; cell proliferation by cell outgrowth measurements and CFE assay; and viability by a CAM/EH-1 assay.

9.1.3.1 Light and Electron Microscopy

Morphology was subjectively assessed throughout the studies, mostly to assess cell health. Visual signs of cell damage depend on the magnifications used and properties of the tissue sample. On H&E-stained sections signs of cell damage range from cytosolic granulation, loss of cell attachment, cell fragmentation, nuclear halo/condensation/fragmentation and cellular edema.

Electron microscopy, enabling the visualization of the ultrastructure of the cells, can reveal more detailed images of cell destruction not otherwise seen on lower magnification images. Signs of cell membrane damage, such as membrane discontinuities and membrane blebbing can easily be detected. Transmission electron microscopy gives detailed images of

both intra/extracellular changes, including loss of cell organelles, chromatin rearrangement and loss of hemidesmosomes and desmosomes. Based on morphology cell death can be characterized as apoptotic or necrotic or as a combination. In brief, apoptotic cell death conserves the organelles and cell membrane for some time whereas the nucleus undergoes early degeneration. In necrotic cell death, however, the nucleus stays relatively intact while the cell membrane and organelles show early degeneration (Vitale et al., 1993). Apoptotic cells typically first show signs of cap-shaped chromatin margination (Falcieri et al., 1994; Stuppia et al., 1996). Cytosol condensation, pyknosis and cell membrane blebbing may also be seen (Fig. 25). The nucleus then develops several electron dense micronuclei, which are often released to the extracellular space. Finally, the cells split into numerous apoptotic bodies. *In vitro*, however, the apoptotic cells undergo a late phase of necrosis. A key event in necrosis is early cell membrane damage (Buja et al., 1993; Ziegler and Groscurth, 2004). Pyknotic and fragmented cell nuclei are not common in necrosis, instead, the cells either show 1) autophagic cell death characterized by numerous cytosol vacuoles filled with cellular remnants; or 2) a non-lysosomal cell death with swell and dilation of organelles (Clarke, 1990; Ziegler and Groscurth, 2004).

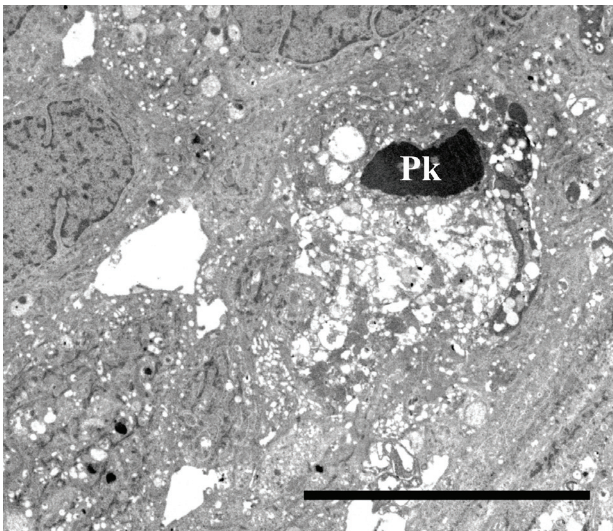

Figure 25. Transmission electron photomicrograph of a human conjunctival epithelial cell with a condensed and pyknotic nucelus (Pk), thus showing signs of apoptosis. Original magnification: ×3400. Scale bar: 10µm.

9.1.3.2 Immuno- and Histochemical Stainings

Immunostaining methods are based on the specific binding of an antibody to the epitopes of the antigen (Janeway et al., 2001). The location of the antibody can then be detected by linking it, directly or indirectly, to fluorophores or enzymes that cleave a colorless chromogen into colored products. The protein expression in the three papers was visualized by IHC using immunofluorescence (paper I-II) and the ABC method (paper III) and the protein expression was quantified subjectively by blinded independent investigators. Prior to immunostaining the tissue samples were fixed with formalin/formaldehyde or methanol. For IHC, the fixed tissue samples were then embedded in paraffin and sectioned. Formalin-fixed paraffin-embedded samples enable superior morphology compared to frozen sections. Using the latter method the non-fixed samples are embedded in optimal cutting temperature compound (OCT), then frozen and sectioned. However, formalin is known to mask antigens due to cross-linking of proteins (Abcam). For this reason, an antigen-retrieval step typically has to be included when using formalin-fixed paraffin-embedded tissue. Antigen retrieval can be performed by exposing the samples to heat or enzymes (Abcam).

9.1.3.3 Outgrowth Measurements and Colony-forming Efficiency Assay

Cell proliferative capacity was assessed by outgrowth measurements and a CFE assay. The outgrowth area in each culture is not merely a measure of the proliferative capacity of the growing cells, but also of the degree of cell migration and culture stratification. The outgrowth measurements in paper I should therefore not be taken as direct quantifications of proliferation. By looking at outgrowth instead of using more conventional proliferation assays, such as the MTT cell proliferation assay which is based on the reduction of the yellow tetrazolium salts by metabolically active cells (ATCC), we were able to exclude areas in the cultures covered with fibroblasts and concentrate on epithelial cell growth. In addition, we were able to compare directly the mm^2 of primary culture obtained when using biopsies of different sizes and from different conjunctival regions. Such information is practically useful for surgeons attempting to cover a conjunctival defect of a given size.

The CFE assay protocol used in paper I was based on that of Nakamura and co-workers (2006a), who cultured the cells for no more than eight days. In addition to describing the CFE percentage, other groups have, based on colony size, differentiated between holo-, mero- and paraclone colonies (Barrandon and Green, 1987), where the former are considered

to rise from stem cells. Those groups, however, cultured the cells for almost two weeks, thus in paper I we were not able to detect any differences regarding the types of colonies grown from the different conjunctival regions. A longer culture period may have revealed differences between the groups.

9.1.3.4 Calcein-acetoxymethyl Ester/Ethidium Homodimer-1 Assay

Viability, demonstrated by a CAM/EH-1 assay (Invitrogen), was scored subjectively by blinded independent investigators. The CAM/EH-1 assay has the benefit of giving low background fluorescence as the CAM and EH-1 have to interact with the cell to become visibly fluorescent (Invitrogen). Calcein-acetoxymethyl is cell membrane permeable and enters the cytosol where it is enzymatically cleaved to the fluorescent calcein by esterases that are ubiquitously present in living cells (Poole et al., 1993). Calcein is retained by living cells, which become intensely green fluorescent. While EH-1 does not traverse the healthy cell membrane, cell membranes of dead cells, however, are widely permeable. Upon entering the cell, EH-1 reacts with nucleic acids to become 40-fold more fluorescent, after which the dead cell nucleus becomes intensely red fluorescent.

9.2 Discussion of the Results

9.2.1 Effect of Explant and Culture Size on the Proliferative Conjunctival Cells

In paper I, the higher number of p63+ and PCNA+ (proliferative) cells in cultures from large explants may have been related to the finding that small explants yielded a relatively larger outgrowth area after eight days in culture. In split-skin transplantation, mincing of the split-skin into smaller fragments has been shown to increase the fold growth without necessitating a longer growth period (Nanchahal, 1989), thus suggesting that smaller explants lead to faster outgrowth. As a result of a higher relative growth yield, cultured cells from small explants may have gone through more cell divisions at eight days in culture, compared with the large explants. Kolli *et al.* (2008) reported a loss of p63+ cells with increasing distance to the explants; in addition, the number of p63+ cells in the explant falls as the culture reaches confluence (Joseph et al., 2004). By day eight, cultured cells from the small explants might have gone through more cell divisions than cells from large explants, thereby reaching

senescence earlier and becoming p63– PCNA–. In paper I, we also reported higher p63 and PCNA expression in larger cultures. This could be explained by the findings: 1) of a positive correlation between explant size and culture size and 2) that large explants yield less fold growth after eight days *ex vivo*.

9.2.2 Proliferative Capacity of Conjunctival Epithelial Cells

Our results in paper I showing the highest outgrowth relative to explant size in forniceal cultures correspond to previous studies (Wei et al., 1993; Nizam et al., 2008). We also showed greater clonal growth capacity of the superior fornix. Few studies have investigated the difference between the superior and inferior conjunctiva with respect to proliferative or clonal growth capacity (Pellegrini et al., 1999). In contrast to our results, Pellegrini *et al.* (1999) concluded that similar CFE occurred in conjunctiva from the superior and inferior fornix and the four bulbar quadrants. The difference in findings might be explained by the fact that only one experiment with biopsies from a single human organ donor was employed and the CFE was higher in the superior fornix (18%) than in the inferior fornix (10%).

9.2.3 Significance of Explant Size on Outgrowth

The finding that smaller explants yielded relatively more growth *ex vivo* may be of importance in improving culture protocols for conjunctival transplantation, as well as decreasing the donor site defects by necessitating less donor tissue. Ang *et al.* (2004; 2005) described an explant culture technique where they minced the conjunctival biopsies into small pieces (about 0.5 x 0.5 mm) before culturing. The technique of growing a single ocular surface transplant from numerous small explants has also been utilized successfully by others (Sangwan et al., 2003a; Sangwan et al., 2003b; Sangwan et al., 2005; Sangwan et al., 2006; Sangwan et al., 2011a; Basu et al., 2012). However, these authors did not investigate the significance of explant size on the cultured cells. The principle of dividing biopsy tissue to cover a larger wound area has long been used in split-skin transplantation (Tanner et al., 1964). Using the mesh split-skin technique, between 1.5:1.0- to 6.0:1.0-fold expansion has been possible. This has been increased to a 20:1 expansion by mincing the epidermal biopsy into numerous smaller fragments (40 x 40 μm) (Nanchahal, 1989). Recently, Sangwan *et al*. reportedly necessitated less limbal donor tissue than with conventional limbal autografting

when adopting a novel technique involving transplantation of numerous small limbal epithelial explants (Sangwan et al., 2012).

The reason why small biopsies give relatively larger outgrowth may be hypothesized to result partly from the shorter average distance from the cut edge of the biopsy to the cells. Cytokine discharge from damaged epithelial cells can induce growth factor release from stromal fibroblasts (Li and Tseng, 1995). In skin biopsies, keratinocytes close to the cut edge show a shift toward the hyperproliferative phenotype (Komine et al., 2001).

9.2.4 Number of Goblet Cells with Regard to Conjunctival Region

9.2.4.1 In Vivo

Consistent with previous studies (Moore et al., 1987; Huang et al., 1988; Goller and Weyrauch, 1993; Lavker et al., 1998), the sections of whole rat eyes in paper II showed higher concentration of goblet cells, as indicated by CK7, UEA-1 and MUC5AC, in the forniceal regions than in the bulbar regions. In addition, the superior bulbar region had fewer goblet cells than the inferior bulbar region. A tendency for higher goblet cell concentration in the inferior conjunctiva compared to the superior (Moore et al., 1987) was reported for the first time in humans in 1910 (Virchow). The presence of a higher concentration of goblet cells in the inferior conjunctiva has been explained by the level of surface hydration (Kessing, 1968), which is increased in the lower lid sac due to the effect of gravity on the aqueous tear fluid (Moore et al., 1987).

9.2.4.2 Ex Vivo

In accordance with Nizam *et al.* (2008), in paper II we detected goblet cells in explant cultures originating from all conjunctival regions. In addition, we found that the densities of goblet cells in *ex vivo* cultures from specific locations in the conjunctiva corresponded with the location in sections from conjunctiva removed *in vivo*, with significantly more goblet cells in the forniceal cultures and least in superior bulbar cultures. Conjunctival transient amplifying cells are capable of differentiating into stratified epithelium and goblet cells (Pellegrini et al., 1999), which may suggest that forniceal explants contain the most progenitor cells committed to the goblet cell lineage. Moreover, it is well documented that goblet cells are able to proliferate in *ex vivo* culture (Shatos et al., 2001; Shatos et al., 2003;

Shatos et al., 2008). Thus, the higher percentage of goblet cells in forniceal cultures may also result from *ex vivo* proliferation of the larger quantity of goblet cells already present in the forniceal explants.

9.2.5 Phenotype with Regard to Location in Culture

9.2.5.1 Stratified Squamous, Non-goblet Epithelial Cells

Kolli *et al.* (2008) reported a progressively more differentiated limbal epithelium with increasing distance from the explant. In contrast, limbal cell cultures grown in the presence of feeder layer cells show undifferentiated cells mostly at the leading edge, while differentiated cells are found at the stratified center of the colonies (Meyer-Blazejewska et al., 2010). The latter study is in agreement with paper II showing stratified squamous epithelial cells (CK4+) present close to the explants, but absent close to the leading edge.

9.2.5.2 Goblet Cells

Cytokeratin 7 is a secondary cytokeratin of simple epithelia, which can be upregulated in reactive conditions, including various types of injury such as inflammation or atrophy (Moll et al., 2008). Upregulation of CK7 is also seen in the stratified oral mucosal epithelium *ex vivo*, even though this epithelium is devoid of CK7 *in vivo* (Garzon et al., 2009). Thus, in paper II, the CK7+/UEA-1- cells at the leading edge in the primary cultures could represent cells with reactive upregulation of CK7 rather than true goblet cells. The density of CK7+ cells at the leading edge, on the other hand, showed significant positive correlation with the percentage of UEA-1+ goblet cells close to the explant in the same culture (data not shown). This suggests that the CK7+ cells at the leading edge were in fact related to the mucin-filled goblet cells.

In cases of LSCD with few goblet cells, detection of CK7+ cells can reportedly confirm conjunctival overgrowth of the cornea (Jirsova et al., 2011). That study, though, used a different anti-CK7 antibody clone (OV-TL 12/30) than we used in paper II, which stained all conjunctival cells and not specifically the goblet cells. In paper III, however, we employed the OV-TL 12/30 clone for CK7 detection. Similarly to Jirsova *et al.*, this antibody clone demonstrated CK7 expression in all the cultured HCjE cells.

Although the migration pattern of stratified epithelia is not completely understood, corneal leading edge cells have been shown to become displaced to the apical epithelial layer as migration moves forward (Danjo and Gipson, 2002). If this migration pattern also applies for conjunctival epithelium, CK7+ cells at the leading edge are expected to be displaced to the apical layer during migration, thereafter possibly differentiating into mucin-filled goblet cells. Our finding that CK7+/UEA-1- cells, but not the CK7+/UEA-1+ cells, occasionally co-express the putative stem cell marker ABCG2 could support this speculation.

9.2.6 Goblet Cell Differentiation and Proliferative Capacity

The percentage of CK7+ cells *ex vivo* in paper II was similar in both large and small cultures, while the concentration of UEA-1+ cells was lowest in the large cultures. This could mean that the CK7+ goblet cells are more proliferative than the mucin-filled (UEA-1+) goblet cells or that mucin-production ceases during goblet cell migration from the explant. One could argue that the CK7+/UEA-1- cells growing from the explant have simply secreted their mucin granules and therefore are not less developed than the CK7+/UEA-1+ goblet cells. Still, the former cells would then not be expected to be more proliferative than the latter, as reported in our study. The maintenance of a differentiated state is linked to cell cycle arrest and when stimulating post-mitotic ocular cells to proliferate, e.g. by exposing them to culture conditions with a medium containing FBS, the cells may lose some of their differentiated characteristics (Valtink and Engelmann, 2009). Transitory cell dedifferentiation initially during primary culture is typical of differentiated cells (MacDonald, 1994).

In paper II we used the same culture protocol as in previous reports that demonstrated mucin-producing goblet cells in both primary (Shatos et al., 2001; Shatos et al., 2003; Hayashi et al., 2012) and passaged cell culture (Shatos et al., 2001; Hayashi et al., 2012). Knowing that mucin-production can re-establish when passaging the cells using our culture protocol, we hypothesize that the loss of mucin-production during migration may represent temporary goblet cell dedifferentiation. Tracheal epithelial cells have been shown to briefly lose their mucin granules during the first phase of culture, apparently signalizing a transitory phase of dedifferentiation during cell proliferation (Wasano et al., 1988). The loss of mucin-filled goblet cells during conjunctival cell migration has similarly been reported in studies on conjunctival wound healing (Friedenwald, 1951; Shapiro et al., 1981; Geggel et al., 1984). Collectively, these reports indicate that goblet cell mucin-production is temporarily halted

during the phase of migration/proliferation. The CK7+/UEA-1- cells might therefore represent immature goblet cells, i.e. goblet cells that can further develop into fully differentiated mucin-filled goblet cells. This finding may explain why proliferating goblet cells identified by their secretory product are difficult to detect *in vivo*.

9.2.7 Conjunctival Niche

9.2.7.1 Phenotype of Cultured Cells

The higher percentage of mucin-filled goblet cells (UEA-1+ and MUC5AC+) close to the explant compared to near the leading edge may signify effects of different gradients of soluble or insoluble ECM proteins. The study by Meller and colleagues (2002) showing mucin-filled goblet cells after transplantation into Balb/c athymic mice may be an example of a niche effect. Others have showed that the composition of the BM and the ECM varies throughout the ocular surface (Schlotzer-Schrehardt et al., 2007). As we used explant culture in paper II, dissimilarities in the BM and ECM in each conjunctival harvesting site could have influenced the *ex vivo* phenotype. There were, however, no significant differences in the size of the explants obtained from the six conjunctival regions. Still, the relative distribution of subconjunctival tissue and epithelial cells in the explants was nevertheless unknown and variation in this distribution could also have affected the phenotype of the cultured conjunctival cells. Previous co-culture studies have shown that the specificities of the fibroblasts, as well as the FGF secreted by these cells, affect the amount of conjunctival progenitor and goblet cells (Tsai et al., 1994; Schrader et al., 2010; Ma et al., 2011). Hence, in paper II, the differentiation of goblet cells *ex vivo* could be partly related to the amount of subconjunctival tissue and the specific composition of the BM/ECM, in the explants.

9.2.7.2 Explant and Outgrowth Size

As the outgrowth measurements in paper I were performed on explant cultures, those measurements may have been affected by differences in the composition of the ECM in each conjunctival origin. Similarly, the outgrowth obtained from explants of different sizes may also have been related to the amount of growth-stimulating ECM components in each explant. A cell suspension approach could have normalized for such effects; however, we used the explant method for reasons mentioned in *section 5.13.4.3*.

9.2.8 Morphology of Cultured Human Conjunctival Epithelial Cells after Storage

In paper III, the number of microvilli had largely disappeared after seven days of storage in Optisol-GS. As described in *section 5.4.3*, the microvilli/glycocalyx has an important role in promoting tear film stability and thereby a healthy ocular surface. Due to this, a decrease in the number of microvilli is undesired. A low number of microvilli is found in Sjögren's syndrome (Koufakis et al., 2006) and Cennamo *et al.* (2008) demonstrated a direct correlation between the number of microvilli and the degree of tear film abnormalities and subjective sensation of dry eye. In our study, only storage for seven days in Optisol-GS showed a significant decrease in the number of microvilli compared to the unstored control. However, the mean number of microvilli had declined in all groups after storage. For instance, this number was approximately reduced by 50% in the four-day Optisol-GS storage group as well as in the seven-day HEPES-MEM storage group, compared to the HCjE cultures that were not stored. This suggests that the number of samples (n = 3-5), or the statistical power, were too low to obtain significance. Loss of microvilli is recognized as one of the early common events in various types of apoptosis (Kondo et al., 1997). In addition, a deficiency of nutrients has been speculated to cause alterations of the microvilli (Cennamo et al., 2008).

With respect to the number of cell layers, a significant decrease in this number was only observed in the seven-day HEPES-MEM storage group. However, it is worth noting that the number of cell layers had declined in all storage groups. The lack of significant results for these groups could be attributed to the low number of samples (n = 3-4). A decrease in the number of cell layers with time in culture has been reported with HLEC and oral mucosal cells, where the number was more than halved from day 15 to day 28 (Hayashi et al., 2010). Furthermore, reduction of epithelial thickness has been registered after organ culture storage of corneas at 37°C (Van Horn et al., 1975).

Storage for seven days demonstrated detachment of epithelial cells and detachment of the epithelia from the AM. Moreover, dilated intercellular spaces and an increase in the number of intracellular vacuoles were observed. We speculate that detachment of epithelial cells might be avoided or minimized by increasing cell stratification and enhancing the formation of cell-cell and cell-substrate adhesion by various techniques, such as denuding the AM, air-lifting and increasing the calcium concentration. However, any technique that

enhances stratification might simultaneously decrease the proliferative potential of the cells, thus reducing the long-term regenerative capability for cellular renewal. Consequently, a delicate balance is necessary. The use of a different substrate could also be a technique to enhance cell attachment (Tian et al., 2008).

9.2.9 Phenotype of Cultured Human Conjunctival Epithelial Cells after Storage

Numerous localizations, including the fornix, have been suggested for the conjunctival stem cells (Wei et al., 1993; Pellegrini et al., 1999; Wirtschafter et al., 1999), but no consensus has been reached. In paper III we cultured conjunctival tissue originating solely from the fornical region. After storage for four and seven days in both HEPES-MEM and Optisol-GS, positive immunostaining with several proposed stem/progenitor cell markers (ΔNp63α, ABCG2 and p63) was observed. No studies have reported immunostaining of cultured and stored HCjE cells, hence no data is available for direct comparison with our immunohistochemical findings. However, the results are in agreement with previous studies of short-and long-term eye bank storage of HLEC in serum-based media (Raeder et al., 2007b; Utheim et al., 2007; Utheim et al., 2009b) and serum-free media (Utheim et al., 2009a).

Raeder and colleagues (2007b) demonstrated that despite great variations in morphology of cultured HLEC due to different storage conditions for one week, the phenotype was still preserved. The expression of the proliferation markers Ki67 and PCNA was sustained during storage in both media. Preservation of Ki67 staining during storage of HLEC cultured on AM has been demonstrated previously by Utheim *et al.* (2007). While the number of PCNA positive epithelial cells has been reported to decrease with time in corneal organ culture (Gan et al., 1998), the retained PCNA and Ki-67 staining during storage in our study might have resulted from the use of AM as substrate, which contains mitogenic components, including growth factors. Freeze-dried AM, that might contain 50% lesser amount of EGF than non-freeze-dried AM, still induces significantly more PCNA positive corneal epithelial cells during healing of corneal ulcers, compared to application of contact lenses (Kim et al., 2009).

Interestingly, our culture and storage protocol resulted in immunoreactivity against the goblet cell markers CK7 and MUC5AC, but not against the stratified squamous marker CK4.

Investigators in other studies have demonstrated variable results, with some reports showing absence (Meller and Tseng, 1999; Ang et al., 2010) or presence (Wei et al., 1997) of goblet cells in cultured conjunctival epithelium. The variability in results may reflect the difference in goblet cell proliferation under various cell culture conditions. In addition, the use of the CK7 antibody clone OV-TL 12/30 in paper III, an antibody clone which in previous studies has stained all conjunctival cells (Jirsova et al., 2011), may not have been specific for goblet cells.

9.2.10 Viability of Cultured Human Conjunctival Epithelial Cells after Storage

The viability in paper III was maintained at a high level throughout storage as indicated by few caspase-3 positive cells and a low percentage of EH-1 stained dead cells. These findings are consistent with a previous study on storage of cultured HLEC (Utheim et al., 2011). Hayashi *et al.* (2010) reported a drop in the viability of cultured HLEC and oral mucosal cells from 93.2% to 64.1% from day 15 to day 28. Our studies and their study are not directly comparable due to the differences in the culture protocol and cell type. However, their finding suggests that a reduction in viability could be expected with increasing time in storage.

9.3 Strengths and Limitations of the Studies

9.3.1 Study Designs

All studies were designed as randomized controlled trials, thereby enabling inferences regarding causality. However, the studies are generally based on small samples, thus caution should be exercised when generalizing the data to a wider population. Small sample studies could be especially problematic when describing equality between groups, such as between the control cells and stored tissue in paper III. However, although possible significant results are missed due to an underpowered study, the lack of large effects is still demonstrated.

9.3.2 Analyses

In all studies, the comparisons of expressed proteins were subjectively assessed. To lessen the chance of observer bias two independent investigators blinded to the origin of the material performed the analyses. Although supplemental methods, such as Western blot (WB) would

have strengthened the quantification of expressed proteins, the use of ICC/IHC had some advantages. In paper II, the rat primary cultures were generally small, yielding little amount of tissue. In this case ICC, which in contrast to WB necessitates only a small number of cells, was chosen to assess differences in expressed proteins between the groups. Detection of co-expressed proteins in single cells in paper I also required the use of ICC compared to WB and PCR analyses. Assessment of the agreement between the two independent investigators confirmed high reliability of the phenotypic data. Thus, although a subjective method for quantifying proteins, ICC was indispensable for that paper and produced reliable results.

9.3.3 Generalizability of the Results

The studies have been performed using two different sources of conjunctival epithelial cells. The first two studies utilized rat conjunctival tissue, thus the results are not directly transferable to humans. However, rat conjunctival epithelial cells are an excellent alternative to human conjunctiva due, amongst other reasons, to lack of available human tissue and the high biological similarity between rat and human conjunctival epithelium (Dartt et al., 2011). Biological variations are also likely to be smaller between rats of the same age that are bred under defined conditions than between human donors. The third study took use of human donor tissue instead of rat tissue and, therefore, possibly leading to larger biological variation between the samples. The tissues were obtained from Centro de Oftalmologia de Barraquer in Barcelona where a local surgeon was responsible for the tissue harvesting. There may therefore have been some variation in biopsy harvesting site to which we were unaware. Judging from the results of study I and II, such variations could have had effects on the phenotype and proliferative ability of the cultured cells in paper III. Using human donor tissue however, has the obvious strength of being more clinically relevant.

9.4 Ethics

9.4.1 Use of Animal Tissues

The Schepens Eye Research Institute (SERI) Animal Care and Use Committee approved the two studies employing rat conjunctival tissue. In addition, all removal of tissue and subsequent manipulations of animals conformed to the guidelines established by the

Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

9.4.2 Use of Human Donor Tissues

Study three was approved by The South-Eastern Norway Regional Committee for Medical Research Ethics (REC) and was reported to the Biobank Registry. Human conjunctival tissue was obtained with research consent from the Institut Universitari Barraquer/Universitat Autonoma de Barcelona (Barcelona, Spain) and human AM was obtained with research consent from the Department of Obstetrics and Gynecology, Oslo University Hospital (OUH), Ulleval. All human tissues were used in accordance with the Declaration of Helsinki. Tissue and the information derived from all samples are stored in a specific biobank at OUH (biobank 2010/1022). Wenche Reed is responsible for the biobank, but in daily practice, project manager Torstein Lyberg can be contacted regarding practical questions. The biobank is planned to last until 2018. After this, material and information will be destroyed / deleted according to internal guidelines.

10. FUTURE PERSPECTIVES

10.1 Culture of Conjunctival Epithelial Cells

More work needs to be done to establish the optimal harvesting site for conjunctival epithelial progenitor cells. Although several reports conclude that fornix is the best location for collecting conjunctival cells, this should be proven in clinical studies with human donors. Moreover, the optimal harvesting site for transplanting goblet cells should also be explored using human cells as this so far has not been investigated.

10.2 Storage of Conjunctival Epithelial Cells

Our study showing that HCjE cells can be stored for at least four days on AM warrants further animal/clinical studies to demonstrate that the HCjE cells show continued survival in the recipient's eye. The effect of storage temperature needs also be further elaborated by culturing cells stored at different temperatures to look for changes in proliferative capacity, degree of differentiation and cell damage.

11. CONCLUSIONS

11.1 General Conclusion

Small forniceal conjunctival explants grow the most effectively; however, for transplantation purposes, the loss of p63+ and PCNA+ cells with small explants must be considered. Forniceal explants yield the highest number of goblet cells *ex vivo* and thereby seem to be optimal for goblet cell transplantation. In addition, we suggest that CK7+/UEA-1- cells represent highly proliferative immature goblet cells that might have the capacity to develop into mucin-filled goblet cells. The CK7+/UEA-1- cells may be of importance during conjunctival migration as they are mostly located close to the leading edge and their density does not decrease with increasing outgrowth size. Cultured HCjE cells may be subjected to storage for at least four days in serum-free HEPES-MEM at 23°C. Knowledge regarding conjunctival epithelial cell response during serum-free storage conditions may aid in the process of developing methods for ocular surface reconstruction.

11.2 Conclusions of the Individual Papers

Paper I We concluded that forniceal explants appeared the most proliferative and thereby seem to be optimal for conjunctival epithelial cell transplantation. The use of small, rather than large, explants is most effective for *ex vivo* expansion, potentially resulting in smaller donor site defects. The loss of p63+ and PCNA + cells when using small explants must be taken into consideration, however, favoring the use of larger explants.

Paper II We conclude that forniceal explants yield the highest number of goblet cells *ex vivo* and thereby seem to be optimal for goblet cell transplantation. We also suggest that CK7+/UEA-1- cells represent highly proliferative immature goblet cells. These cells could be important during conjunctival migration as they are mostly located close to the leading edge and their density does not decrease with increasing outgrowth size.

Paper III We concluded that the phenotype and viability of HCjE cells cultured for two weeks were preserved during subsequent storage for seven days in serum-free HEPES-MEM and Optisol-GS. The number of epithelial microvilli and cell layers showed a decreasing tendency with time during storage. Thus, cultured HCjE cells appear to be preserved in serum-free storage media at 23°C for at least four days.

12. ERRATA

Paper III, section 2.2.: The concentrations of insulin and transferrin were 5 μg/mL.

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14. PAPERS

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