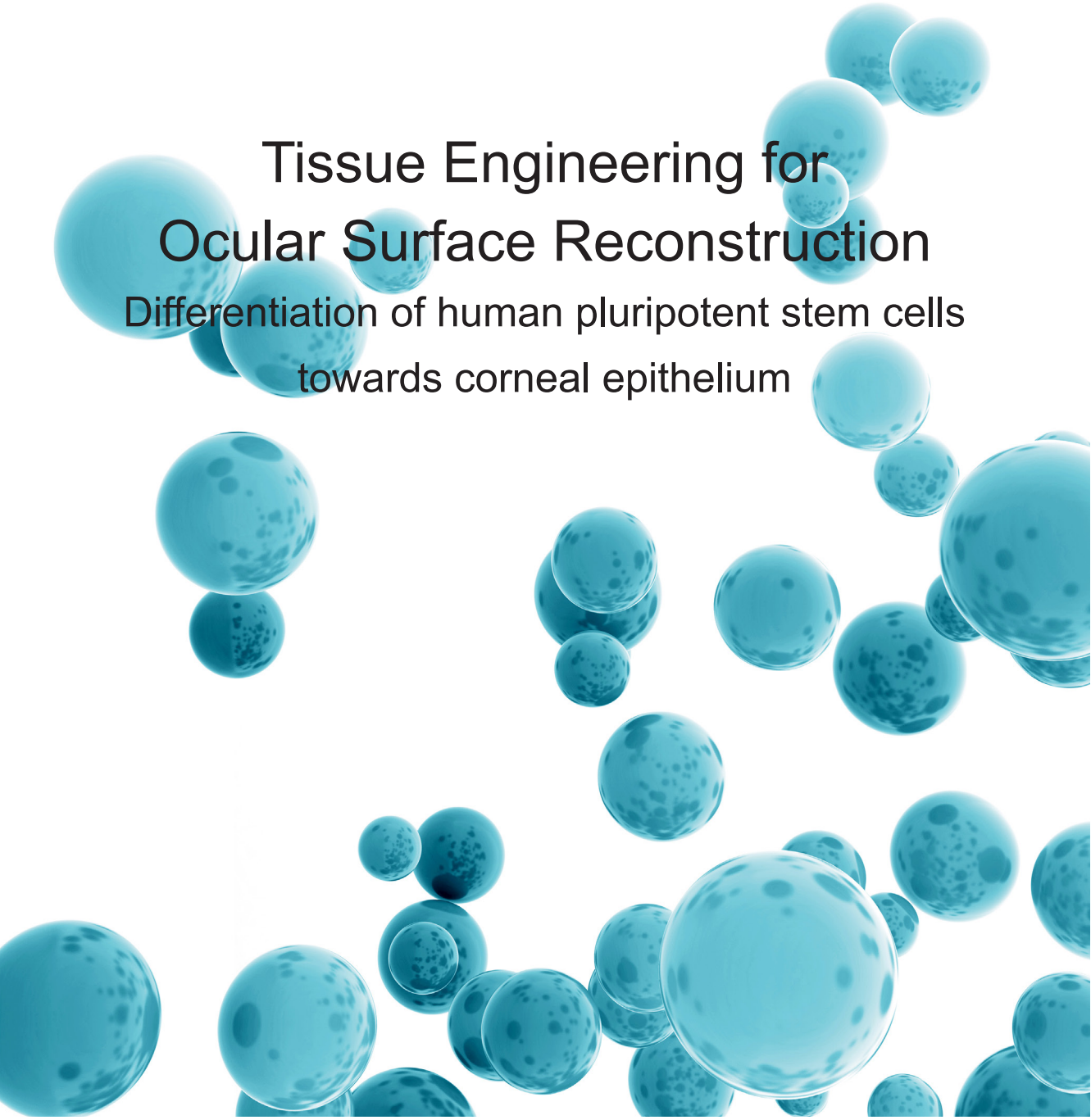
Acta Universitatis Tamperensis 2130

ALEXANDRA MIKHAILOVA

Tissue Engineering for Ocular Surface Reconstruction

Differentiation of human pluripotent stem cells towards corneal epithelium



ALEXANDRA MIKHAILOVA

Tissue Engineering for Ocular Surface Reconstruction

Differentiation of human pluripotent stem cells towards corneal epithelium

ACADEMIC DISSERTATION
To be presented, with the permission of
the Board of the BioMediTech of the University of Tampere,
for public discussion in the auditorium Pinni B 1096,
Kanslerinrinne 1, Tampere, on 5 February 2016, at 12 o'clock.

UNIVERSITY OF TAMPERE

ALEXANDRA MIKHAILOVA

Tissue Engineering for Ocular Surface Reconstruction

Differentiation of human pluripotent stem cells towards corneal epithelium

Acta Universitatis Tamperensis 2130
Tampere University Press
Tampere 2016

UNIVERSITY OF TAMPERE

ACADEMIC DISSERTATION
University of Tampere, BioMediTech
Finland

Supervised by
Associate Professor Heli Skottman
University of Tampere
Finland
PhD Tanja Ilmarinen
University of Tampere
Finland

Reviewed by
Professor Juha Holopainen
University of Helsinki
Finland
Docent Frederic Michon
University of Helsinki
Finland

The originality of this thesis has been checked using the Turnitin OriginalityCheck service in accordance with the quality management system of the University of Tampere.

Cover design by
Mikko Reinikka

Distributor:
verkkokauppa@juvenesprint.fi
https://verkkokauppa.juvenes.fi

Acta Universitatis Tamperensis 2130
ISBN 978-952-03-0010-4 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1627
ISBN 978-952-03-0011-1 (pdf)
ISSN 1456-954X
http://tampub.uta.fi

Suomen Yliopistopaino Oy – Juvenes Print
Tampere 2016

Table of Contents

Abstract

Corneal epithelium, the outermost layer of the transparent and avascular cornea, is renewed by tissue-specific stem cells, termed limbal epithelial stem cells (LESC). These stem cells are located in specialized niches of the limbus, a narrow transition zone between the cornea and sclera, which also serves as a physical barrier between the clear avascular cornea and the vascularized conjunctiva. Extensive trauma to the limbus, or LESC dysfunction as a result of certain chronic inflammatory diseases may lead to limbal stem cell deficiency (LSCD), characterized by the spread of the vascularized conjunctival tissue over the damaged ocular surface. This vision-threatening condition varies in its severity, but commonly causes severe symptoms and is difficult to treat. Conventional corneal transplantation is not a feasible treatment option, as it only replaces the central corneal epithelium, and not the damaged limbus. Therefore, various strategies aimed at replacing damaged LESCs have been explored. Limbal transplantation, with or without *ex vivo* expansion of LESCs, has shown great promise. However, patients suffering from LSCD in both eyes are common and donor corneal tissue is scarce. Alternative approaches for ocular surface reconstruction are therefore needed.

Human pluripotent stem cells (hPSC) are capable of virtually unlimited self-renewal and can differentiate into any cell type, including LESCs and corneal epithelial cells. To date, only a few studies have demonstrated successful differentiation of corneal epithelial cells from hPSCs, and these methods rely on the use of chemically undefined and xenogeneic culture components. Such factors are subject to biologic variation and pathogen transmission, and thereby require a thorough quality control to ensure the safety of transplantable cell populations. Nevertheless, hPSC-derived LESCs could provide an alternative cell source for cell-based therapy of severe ocular surface disorders. In addition, hPSC-derived LESCs could be applied to studying corneal development and modelling corneal tissue as a testing platform for *in vitro* drug development.

This dissertation aimed to investigate differentiation of LESCs and corneal epithelial cells from hPSCs under conditions that would allow for a smooth transition to clinical applications. A directed and efficient two-stage differentiation method was developed, generating LESC-like cells capable of self-renewal and

terminal differentiation towards mature corneal epithelial cells. Differentiation was carried out in close to chemically-defined and xeno-free conditions, and several modifications could be explored further. Although there was a certain degree of variation in differentiation efficiency between the studied cell lines, the method was consistent and reproducible overall.

Thorough characterization of hPSC-derived cells is crucial to confirm their authenticity. In this dissertation, several key characteristics of LESCs were examined. It was demonstrated that hPSC-derived LESCs possess the appropriate cell morphology, as well as gene and protein expression profiles. They were also able to self-renew and proliferate in culture, and terminally differentiate towards mature corneal epithelial cells. Moreover, hPSC-derived LESCs were similar to native ocular surface epithelial cells, as verified using relative mass spectrometry-based proteomics.

Cell transplantation to the ocular surface would require a transparent, mechanically durable, yet elastic carrier biomaterial. Currently, human amniotic membrane is the gold-standard material commonly used to transplant donor LESCs, despite its limitations. The final aim of this dissertation was to evaluate the suitability of a bioengineered collagen matrix for the culture and transplantation of hPSC-derived LESCs. The fabrication of this biomaterial is standardized, it is suitable for clinical use, and has shown promise in earlier biocompatibility studies using an animal model. In this study, the bioengineered collagen matrix supported the attachment and growth of hPSC-derived LESCs *in vitro*, and could therefore be applied to clinical use in the future.

In conclusion, this dissertation as a whole describes a novel tissue engineering approach to ocular surface reconstruction. These results have contributed to increasing the knowledge of hPSC differentiation towards LESCs, their characteristics, and potential for use in cell-based therapy.

Tiivistelmä

Sarveiskalvon epiteeli on läpinäkyvän ja verisuonettoman sarveiskalvon uloin kerros, jonka uusiutumisesta huolehtivat kudosspesifiset limbaaliset kantasolut. Nämä kantasolut sijaitsevat limbuksessa, sarveiskalvon ja sidekalvon rajapinnassa. Limbus toimii myös fyysisenä esteenä verisuonettoman sarveiskalvon ja verisuonitetun sidekalvon välillä. Laajat vauriot limbaalisella alueella tai limbaalisten kantasolujen vajaatoiminta kroonisen taudin seurauksena saattavat johtaa kantasolupuutokseen, missä sidekalvo verisuonineen leviää ja korvaa sarveiskalvon epiteelin. Tämän sairauden oireet ja laajuus vaihtelevat hyvinkin paljon, mutta siihen yleensä liittyy voimakkaita oireita ja sen hoito on hankala toteuttaa. Perinteinen sarveiskalvon siirto ei ole käyttökelpoinen hoitomenetelmä näissä sairauksissa, sillä siirre korvaa vain sarveiskalvon keskiosaa, eikä vaurioitunutta limbusta. Näin ollen, viime vuosikymmenien aikana on tutkittu useita erilaisia lähestymistapoja tuhoutuneiden limbaalisten kantasolujen korvaamiseen. Limbussiirto on osoittautunut lupaavaksi hoitomenetelmäksi: vauriopaikalle siirretään joko tervettä limbuskudosta tai laboratorio-olosuhteissa viljeltyjä limbaalisia kantasoluja. Limbaalisten kantasolujen vajaatoiminta esiintyy usein potilaan molemmassa silmässä, ja näiden potilaiden hoitoa rajoittaa luovuttajakudosten riittämättömyys. Täten uusia hoitomenetelmiä tarvitaan sarveiskalvovaurioiden hoitoon.

Ihmisen erittäin monikykyisillä kantasoluilla on lähes rajaton uusiutumiskyky, ja ne kykenevät erilaistumaan kehon kaikiksi solutyypeiksi, mukaan lukien limbaaliset kantasolut ja sarveiskalvon epiteelisolut. Tähän mennessä, vain muutamissa tutkimuksissa on onnistuttu erilaistamaan sarveiskalvon epiteelisoluja ihmisen erittäin monikykyisistä kantasoluista, ja nämä menetelmät hyödyntävät eläinperäisiä ja määrittelemättömiä komponentteja erilaistuksen aikaansaamiseksi. Tämäntyyppisten tekijöiden käyttö altistaa erilaistusta biologiseen vaihteluun ja taudinaiheuttajien siirtoon, joten tarkka laadunvalvonta on hyvin tärkeä turvallisuuden takaamiseksi. Menetelmiä on tärkeä edelleen kehittää, sillä kantasoluista erilaistetut limbaaliset kantasolut voivat tulevaisuudessa tarjota vaihtoehtoisia hoitomenetelmiä vakaville sarveiskalvovaurioille. Tämän lisäksi näitä

soluja voidaan hyödyntää ihmisen sarveiskalvon kehityksen tutkimukseen ja tautimallintamiseen, sekä lääkekehitykseen laboratorio-olosuhteissa.

Tämän väitöskirjatutkimuksen tavoitteena oli kehittää sarveiskalvon epiteelisolujen erilaistamista olosuhteissa, jotka helpottaisivat menetelmän tuomista kliinisiin sovelluksiin. Tutkimuksen aikana kehitettiin suunnattu ja tehokas kaksivaiheinen erilaistusmenetelmä. Tällä menetelmällä erilaistetut limbaaliset kantasolut osoittautuivat uusiutumiskykyisiksi, ja kykenivät kypsymään sarveiskalvon epiteelisoluiksi. Menetelmä on lähes täysin vapaa eläinperäisistä tai määrittelemättömistä aineista, ja sitä on mahdollista edelleen kehittää. Vaikka eri solulinjojen erilaistustehokkuuksissa esiintyi vaihtelua, menetelmä kokonaisuudessaan osoittautui erittäin toistettavaksi.

Erilaistettujen solujen kattava karakterisointi on erittäin tärkeä niiden laadun takaamiseen. Tässä väitöskirjatutkimuksessa tarkasteltiin useita solujen ominaisuuksia. Erilaistetuilla soluilla oli oikeanlainen morfologia, ja ne ilmensivät limbaalisille kantasoluille tyypillisiä geenejä ja proteiineja. Niillä oli kyky uusiutua ja jakaantua viljelyssä, sekä kypsyä sarveiskalvon epiteelisoluiksi. Lisäksi, massaspektrometriaan perustuva proteomiikkatutkimus osoitti, että erilaistetut limbaaliset kantasolut ovat samanlaisia kuin ihmisen silmän pinnan solut.

Jotta solut olisivat siirrettävissä silmän pinnalle, tarvitaan läpinäkyvä, mekaanisesti kestävä, mutta elastinen tukimateriaali. Tällä hetkellä käytetyin biomateriaali luovutettujen limbaalisten kantasolujen siirtoon on ihmisen amnionkalvo, sen monista puutteista huolimatta. Tämän väitöskirjatutkimuksen viimeisenä tavoitteena oli tutkia kollageenista valmistetun tukimateriaalin soveltuvuutta erittäin monikykyisistä kantasoluista erilaistettujen limbaalisten kantasolujen kasvatukseen ja transplantaatioon. Kyseisen biomateriaalin valmistus on standardoitu, se soveltuu kliiniseen käyttöön, ja sen on osoitettu olevan kudosyhteensopiva. Tässä työssä biomateriaali näytti tukevan erittäin monikykyisistä kantasoluista erilaistettujen limbaalisten kantasolujen kiinnittymistä sekä kasvua, ja näin ollen voisi soveltua siirtomateriaaliksi soluterapiaa varten.

Kaiken kaikkiaan tässä tutkimuksessa on kehitetty uudenlainen kudosteknologiaan perustuva menetelmä sarveiskalvovaurioiden hoitoon. Nämä tulokset ovat edistäneet tietoa erittäin monikykyisten kantasolujen erilaistuksesta sarveiskalvon epiteelisoluiksi, näiden solujen ominaisuuksista, sekä soveltuvuutta soluterapiaksi.

List of original publications

This dissertation is based on the following original publications, referred to in the text by their Roman numerals (**I-III**):

I **Mikhailova A**, Ilmarinen T, Uusitalo H, Skottman H. Small-molecule induction promotes corneal epithelial cell differentiation from human induced pluripotent stem cells. *Stem Cell Reports, 2014, 2:219-231.*

II **Mikhailova A**, Jylhä A, Rieck J, Nättinen J, Ilmarinen T, Veréb Z, Aapola U, Beuerman R, Petrovski G, Uusitalo H, Skottman H. Comparative proteomics reveals human pluripotent stem cell-derived limbal epithelial stem cells are similar to native ocular surface epithelial cells. *Scientific Reports, 2015, 5:14684.*

III **Mikhailova A**, Ilmarinen T, Ratnayake A, Petrovski G, Uusitalo H, Skottman H, Rafat M. Human pluripotent stem cell-derived limbal epithelial stem cells on bioengineered matrices for corneal reconstruction. *Experimental Eye Research, 2016, 146:26-34.*

This dissertation contains unpublished data, indicated separately in the text.

List of abbreviations

ABCB5	Adenosine triphosphate binding cassette sub-family B member 5
ABCG2	Adenosine triphosphate binding cassette sub-family G member 2
aCGH	Array comparative genomic hybridization
AMD	Age-related macular degeneration
APC	Allophycocyanin
APCM	Acellular porcine corneal matrix
ATMP	Advanced therapy medicinal product
BMI-1	Polycomb complex protein BMI-1
BMP	Bone morphogenetic protein
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
c-MYC	Myc proto-oncogene protein
C/EBPδ	CCAAT/Enhancer binding protein delta
CD	Cluster of differentiation
CEC	Corneal epithelial cells
CFE	Colony forming efficiency
CK	Cytokeratin
CLET	Cultivated limbal epithelial transplantation
COMET	Cultivated oral mucosal epithelial transplantation
DCC	Dicyclohexyl-carbodiimide
DG-3	Desmoglein-3
EBiSC	European Bank for Induced Pluripotent Stem Cells
ECM	Extracellular matrix
EDCM	1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide
EGF	Epidermal growth factor
EMA	European Medicines Agency
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FITC	Fluorescein isothiocyanate
FOX1	Forkhead box 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GMP	Good manufacturing practice
hAM	Human amniotic membrane
hDF	Human dermal fibroblasts
hESC	Human embryonic stem cells
hFF	Human foreskin fibroblasts
hiPSC	Human induced pluripotent stem cells
HLA	Human leukocyte antigen
hLF	Human limbal fibroblasts
hPSC	Human pluripotent stem cells
ICAM-1	Intercellular adhesion molecule 1
ICM	Inner cell mass
iHCE	Immortalized human corneal epithelial cell line
iTRAQ	Isobaric tag for relative and absolute quantitation
IVF	*In vitro* fertilization
KLF4	Krüppel-like factor 4
KO-DMEM	KnockOut Dulbecco's Modified Eagle Medium
KO-SR	KnockOut Serum Replacement
KRT	Keratin
LCA	Leukocyte common antigen
LEC	Limbal epithelial cells
LESC	Limbal epithelial stem cells
LSCD	Limbal stem cell deficiency
MCAM	Melanoma cell adhesion molecule
MEF	Mouse embryonic fibroblasts
miRNA	Micro ribonucleic acid
MSC	Mesenchymal stem cells
MUC18	Mucin 18
NEAA	Non-essential amino acids
NT5E	Ecto-5'-nucleotidase
OCT3/4	Octamer-binding transcription factor 3/4
PAX6	Paired box 6
PBS	Phosphate buffered saline
PCL	Poly-ε-caprolactone

PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule 1
PFA	Paraformaldehyde
PITX2	Paired-like homeodomain 2
PLGA	Poly-lactide-co-glycolide
qPCR	Quantitative polymerase chain reaction
RAFT	Real architecture for 3D tissue
REX1	Reduced expression protein 1
RHC	Recombinant human collagen
RPE	Retinal pigment epithelium
SEM	Scanning electron microscopy
SLET	Simple limbal epithelial transplantation
SOD2	Superoxide dismutase 2
SOX2	(Sex-determining region Y)-box 2
SSEA	Stage-specific embryonic antigen
TAC	Transient amplifying cells
TCF4	Transcription factor 4
TDC	Terminally-differentiated cells
TGF-β	Transforming growth factor β
TRA	Tumor-related antigen
UKSCB	United Kingdom Stem Cell Bank
UniProtKB	Universal protein knowledge base
UV	Ultraviolet
VE-cadherin	Vascular endothelial cadherin

1 Introduction

Corneal epithelium is the outermost layer of the transparent and avascular cornea. It is constantly renewed by limbal epithelial stem cells (LESC), tissue-specific stem cells located in specialized niches at the corneo-scleral junction, which also serve to maintain a physical barrier between the clear cornea and the vascularized conjunctiva (Dua & Azuara-Blanco, 2000; Ordonez & Di Girolamo, 2012). Significant loss or dysfunction of LESCs due to acute trauma or various chronic disorders can result in limbal stem cell deficiency (LSCD) – a painful and vision-threatening condition, which is difficult to treat using conventional methods (Ahmad, 2012). Various tissue engineering approaches aiming at restoring the ocular surface with the help of tissue-specific stem cells have been investigated within the past two decades. Most importantly, cultivated limbal epithelial transplantation (CLET) offers an advantage over a more traditional sector limbal transplantation – autologous or allogeneic LESCs are obtained from a small biopsy and expanded *ex vivo* prior transplantation (Baylis et al., 2011; Pellegrini et al., 1997). Several other adult stem cell types have also been studied, but the main disadvantage of such techniques is their limited capacity for self-renewal.

Human pluripotent stem cells (hPSC) can be used to generate LESCs or mature corneal epithelial cells, providing a virtually unlimited supply of transplantable cell populations (Ahmad et al., 2007; Hayashi et al., 2012). With the advance of human leukocyte antigen (HLA) haplotype-matched hPSC banking, it may soon be possible to generate readily-available hPSC lines with a minimal risk of immune response for each individual patient (Zimmermann et al., 2012). However, differentiation of LESCs often requires the use of biologically variable components such as human amniotic membrane (hAM) or cell culture medium conditioned by limbal or corneal fibroblasts. Ideally, the differentiation method needs to be robust, reproducible, and free from xenogeneic and chemically undefined components. Furthermore, differentiated hPSC-derived LESCs need to be extensively characterized, preferably using high-throughput methods, in order to ensure they possess the appropriate characteristics and behave in a way similar to that of their native counterparts. In the future, hPSC-LESCs could be applied to cell-based

therapy of severe ocular surface disorders such as LSCD, as well as studying and modelling the human cornea, or *in vitro* drug development.

In order to transplant hPSC-LESCs to the ocular surface, a carrier biomaterial is needed to provide support for the cells. Optimally, the biomaterial needs to be transparent, biocompatible and mechanically durable, yet sufficiently elastic so as to follow the natural curvature of the cornea (Menzel-Severing et al., 2013). Collagen-based bioengineered matrices, typically reinforced using cross-linking or compression, are currently widely researched for corneal reconstruction, mainly due to the fact that collagen is the main component of the corneal stroma. Several preclinical studies have demonstrated that collagen-based carriers are biocompatible *in vivo*, despite being fabricated using collagen of animal origin (Chae et al., 2015; Koulikovska et al., 2015; Petsch et al., 2014). The main benefit of using fabricated bioengineered carriers, rather than readily-available scaffolds such as hAM, is the possibility of standardized and reproducible production.

The main objectives of this dissertation were to develop an efficient and reproducible method for differentiation of hPSCs towards LESCs and mature corneal epithelial cells, to study the characteristics and functionality of hPSC-derived LESCs and compare them with native ocular surface epithelial cells, and to find a potential carrier for clinical applications of these cells.

2 Literature review

2.1 The human cornea

The cornea is located at the outer surface of the eye (Figure 1), surrounded by the limbus, conjunctiva, and sclera (DelMonte & Kim, 2011). Its main functions are to protect the eye from external environmental factors, including mechanical damage, pathogens and ultraviolet (UV) light, while allowing accurate focusing of light to produce a sharp image on the retina (Ghezzi et al., 2015). The human cornea is approximately 0.5 mm thick and composed of three cellular layers (Figure 1).

Corneal epithelium is the topmost layer, consisting of four to six layers of non-keratinized squamous epithelial cells. The epithelium is covered by a tear film, which is secreted by the various types of lacrimal glands (e.g. major and minor lacrimal glands and Meibomian glands) and conjunctival goblet cells (Bolanos-Jimenez et al., 2015). Tear film provides protection from external noxious stimuli and pathogens, while supplying various growth factors and cytokines important for epithelial health, proliferation and repair (DelMonte & Kim, 2011). The epithelial basement membrane is composed primarily of type IV collagen and several laminin isoforms, with regional heterogeneity from central cornea to limbus to conjunctiva (Torricelli et al., 2013). Bowman's layer is described as an acellular condensation of the anterior stroma, composed primarily of collagen types I, III, and V, and lacking an apparent critical function in corneal physiology (Wilson & Hong, 2000). Corneal stroma comprises roughly 80-90% of the entire corneal thickness. It consists primarily of densely and regularly packed collagen fibrils (predominantly type I, with smaller amounts of type V), interspersed with four proteoglycans (decorin, lumican, keratocan and mimecan), arranged in a highly organized network to maintain the optical transparency and mechanical strength of the tissue (Ghezzi et al., 2015; Hassell & Birk, 2010). The normally quiescent stromal keratocytes reside in the anterior stroma and maintain homeostasis by synthesizing collagen molecules and glycosaminoglycans, as well as matrix metalloproteinases (DelMonte & Kim, 2011; Meek & Knupp, 2015). The Descemet's membrane is a thin acellular layer composed of collagen fibrils, separating the stroma from the endothelium (Bolanos-Jimenez et al., 2015). Corneal endothelium is a monolayer of hexagonal

cells, the main function of which is to provide the upper layers with nutrients from the anterior chamber, and to keep the water content of the stroma at around 78%, thereby maintaining its transparency (Bolanos-Jimenez et al., 2015; DelMonte & Kim, 2011). The *in vivo* wound healing capacity of the corneal endothelium is limited, although several studies have demonstrated that slow-cycling progenitor cells exist at the endothelial periphery (Espana et al., 2015; He et al., 2012; Whikehart et al., 2005).

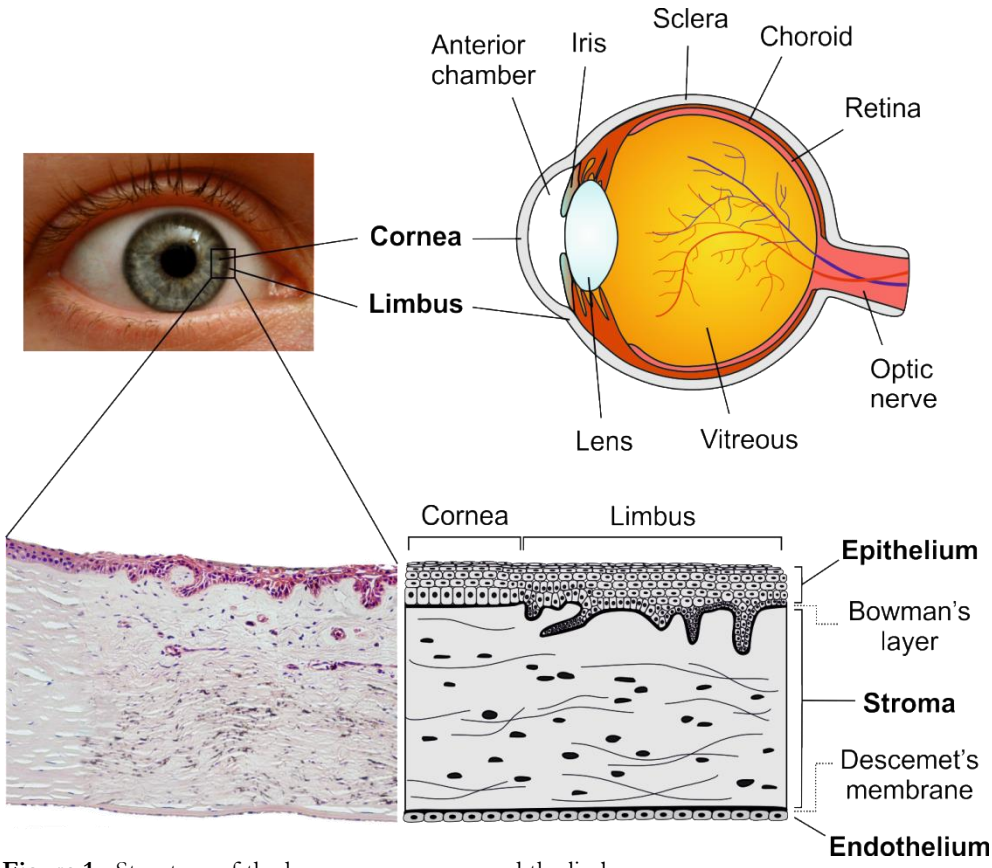


Figure 1. Structure of the human eye, cornea and the limbus.

The limbus is a specialized narrow transitional zone, located circumferentially along the periphery of the cornea, at its junction with the sclera and the conjunctiva (Figure 1). It is generally accepted that the stem cells of the corneal epithelium, known as limbal epithelial stem cells (LESC), and their immediate progeny reside within limbal crypts in the palisades of Vogt (Dua et al., 2005; Grieve et al., 2015; Shanmuganathan et al., 2007). Moreover, the limbus acts as a physical barrier to the

conjunctiva and its blood vessels (Osei-Bempong et al., 2013). LESCs will be described in greater detail in later chapters.

Transparency, lack of vascularization and dense innervation are the main structural features of the cornea. In the healthy eye, blood and lymphatic vessels do not enter the corneal stroma, but rather surround it underneath the peripheral limbal epithelium (Dhouailly et al., 2014). This unique property of the cornea is referred to as angiogenic privilege.

2.1.1 Corneal development

In humans, the primitive bi-layered corneal epithelium first becomes apparent between five and six weeks of gestation (DelMonte & Kim, 2011; Zieske, 2004). It derives from the ocular surface ectoderm, a multipotent region of head ectoderm, shortly after lens vesicle detachment (Collomb et al., 2013; Zhang et al., 2015). The corneal stroma and endothelium, on the other hand, derive from migrating neural crest cells (Dhouailly et al., 2014). By the seventh week of gestation, two waves of neural crest migration into the space between the primitive epithelium and the lens take place: the primitive endothelium forms during the first wave and the corneal stroma forms during the second wave (Collomb et al., 2013; DelMonte & Kim, 2011). Upon opening of the eyelids, around 24 weeks of gestation, the primitive epithelium starts to stratify and undergoes morphological changes, finally forming the mature corneal epithelium with four to six distinct cell layers (Zieske, 2004).

Corneal development involves inductive interactions between the ocular surface ectoderm and the underlying mesenchyme. In addition to corneal epithelium, ocular surface ectoderm gives rise to lens and conjunctival epithelia, as well as the epidermis of the eyelids (Gage et al., 2014; Zhang et al., 2015). The specification of corneal epithelium development is guided by the inhibition of bone morphogenetic protein (BMP) signaling, while lens epithelium formation is dependent on active BMP signaling (Collomb et al., 2013). Shortly after lens vesicle detachment, fibroblast growth factor (FGF) signaling is required for cell proliferation in the ocular surface ectoderm, which then differentiates into corneal epithelium (Zhang et al., 2015). Furthermore, inhibition of the canonical Wnt signaling pathway is crucial for corneal epithelial commitment, and establishing its angiogenic privilege (Dhouailly et al., 2014; Gage et al., 2014). Meanwhile, primitive corneal stroma appears to be involved in stabilizing paired box 6 (PAX6) expression in the corneal epithelium (Collomb et al., 2013). Overall, corneal epithelium is finally committed

during stroma formation in the seventh week of gestation, as determined by the persistence of intrinsic PAX6 signaling (Collomb et al., 2013; Dhouailly et al., 2014). Loss of PAX6, on the other hand, leads to skin-like differentiation, indicating that PAX6 expression is a central event in corneal cell fate control (Li et al., 2015). Although the main features of corneal development are relatively well established, most studies are done using animal models, and the precise signaling mechanisms behind human corneal morphogenesis remain largely unknown.

2.1.2 Corneal epithelial renewal

In the fully-developed human eye, corneal epithelial cells have an average lifespan of seven to ten days (DelMonte & Kim, 2011). This continuous renewal is explained by the XYZ hypothesis of corneal epithelial homeostasis: the proliferation and movement of cells from the basal layers (X) and centripetal movement from the periphery of the cornea (Y) replace cells lost from the central corneal surface (Z), giving the equation X+Y=Z (Ahmad, 2012; Osei-Bempong et al., 2013). The process of corneal epithelial renewal is schematically presented in Figure 2. As mentioned earlier, the unipotent LESCs are primarily responsible for corneal epithelial renewal, under both normal and wound healing conditions. They reside in the specialized niches in the limbal region of the eye, providing a protective environment for the LESCs, and helping maintain their undifferentiated state (Ordonez & Di Girolamo, 2012). LESCs are normally slow-cycling, but have a high proliferative potential (Dua & Azuara-Blanco, 2000). Only about 5% of all cells at the human limbus are considered to be true LESCs, while the rest are transient amplifying cells (TAC) at varying levels of maturity (Pellegrini et al., 2013). Corneal TACs have a more limited proliferative potential, and are considered to be committed on the pathway to replace the terminally-differentiated cells (TDC) constantly shed from the corneal surface (Dua & Azuara-Blanco, 2000; Pellegrini et al., 1999).

Although it is widely accepted that stem cells responsible for corneal epithelial cell renewal reside exclusively in the limbus, several studies have recently challenged this view. Serial transplantation of the mouse corneal epithelium was shown to be possible, implying on existence of progenitor cells in the central cornea as well as the limbus (Majo et al., 2008). Additionally, an observational study of five LSCD patients revealed clear central corneal islands despite the lack of functional LESCs (Dua et al., 2009). Nevertheless, these findings remain

outnumbered by the extensive evidence in support of the limbus as the principal source of corneal epithelial stem cells in humans. More specifically, centripetal migration of corneal epithelial cells has been documented through lineage tracing in mice (Amitai-Lange et al., 2015; Di Girolamo et al., 2015), LESCs have been shown to possess slow-cycling characteristics and superior *in vitro* proliferative capacity (Figueira et al., 2007; Pellegrini et al., 1999), and most importantly, there are numerous clinical reports of LESCs restoring the corneal epithelium once transplanted to the damaged ocular surface (Baylis et al., 2011; Zhao & Ma, 2015). Overall, although it is likely that some early-stage progenitor cells are in fact distributed throughout the basal layers of the corneal epithelium, the limbus appears to be crucial for long-term corneal epithelial renewal.

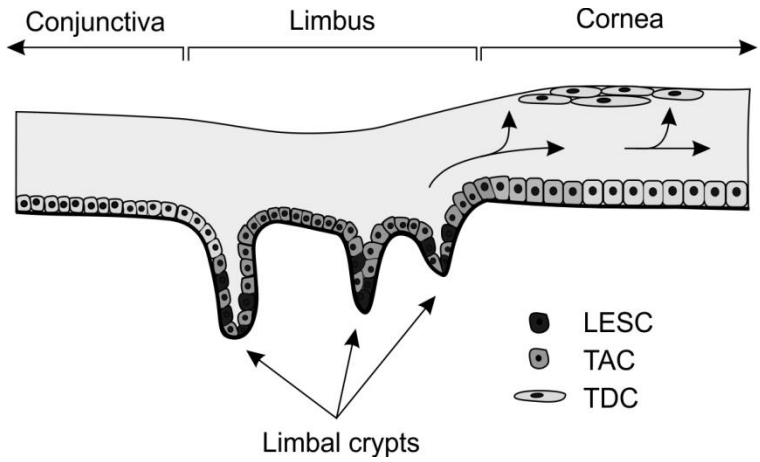


Figure 2. Corneal epithelial renewal. LESCs and their immediate progeny reside within limbal crypts, where they self-renew and give rise to TACs, which move towards the central cornea and differentiate to replace TDCs lost from the ocular surface.

Despite the ongoing efforts to find a molecular marker specific for LESCs, distinguishing between true LESCs and their immediate progeny remains a challenge. Therefore, it is currently common practice to assess a wider molecular signature of cell populations, selected from a growing set of positive and negative makers (Table 1). One of the most widely used LESC markers is the nuclear transcription factor p63, which appears to regulate corneal epithelial renewal through control of cell proliferation, and was demonstrated to be functionally significant and important for graft survival in a clinical trial (Pellegrini et al., 2001;

Rama et al., 2010). More than six isoforms of p63 have been described, and ΔNp63α is predominantly expressed within the ocular surface epithelia (Kawasaki et al., 2006; Robertson et al., 2008). Unlike nuclear transcription factors, cell surface proteins such as adenosine triphosphate (ATP)-binding cassette sub-family B member 5 (ABCB5) or ATP-binding cassette sub-family G member 2 (ABCG2) enable antibody-based sorting and enrichment of heterogeneous cell populations for clinical use (Ksander et al., 2014).

Table 1. Expression and localization of widely used corneal and limbal epithelial markers. The following references were used: Bian et al., 2010; Figueira et al., 2007; Joe & Yeung, 2014; Ksander et al., 2014; Lu et al., 2012; Lyngholm et al., 2008; Nieto-Miguel et al., 2011; Notara et al., 2010; Qu et al., 2015; Schlotzer-Schrehardt & Kruse, 2005.

Marker	Corneal cell type	Localization
ABCB5	Limbal epithelium	Cell membrane
ABCG2	Limbal epithelium	Cell membrane
α-enolase	Limbal epithelium, basal corneal epithelium	Cytoplasm
BMI-1	Limbal epithelium	Nucleus
C/EBPδ	Limbal epithelium	Nucleus
Connexin 43	Basal corneal epithelium	Gap junctions
Cytokeratin 3	Mature corneal epithelium	Cytoskeleton
Cytokeratin 12	Mature corneal epithelium	Cytoskeleton
Cytokeratin 14	Limbal epithelium	Cytoskeleton
Cytokeratin 15	Limbal epithelium	Cytoskeleton
Cytokeratin 19	Limbal epithelium	Cytoskeleton
Desmoglein 3	Limbal epithelium	Cytoskeleton
ΔNp63α	Limbal epithelium	Nucleus
Integrin α9	Limbal epithelium	Cell surface
Integrin β1	Limbal epithelium	Cell surface
Involucrin	Mature corneal epithelium	Cytoplasm
Ki67	Limbal epithelium	Nucleus
Nestin	Mature corneal epithelium	Cytoplasm
Notch1	Limbal epithelium	Nucleus
PAX6	Limbal and corneal epithelia	Nucleus
P-cadherin	Limbal epithelium	Cell membrane
Periostin	Limbal epithelium	Cytoplasm
SOD2	Limbal epithelium	Mitochondrion
TCF4	Limbal epithelium	Cytoplasm
Vimentin	Limbal epithelium	Cytoskeleton
Wnt-4	Limbal epithelium	Cell membrane, secreted

Abbreviations: ABCB5, ATP-binding cassette sub-family B member 5; ABCG2, ATP-binding cassette sub-family G member 2; BMI-1, polycomb complex protein BMI-1; C/EBPδ, CCAAT/Enhancer binding protein delta; PAX6, paired box 6; SOD2, superoxide dismutase 2; TCF4, transcription factor 4

Recently, several microRNAs (miRNA), a type of non-coding regulatory RNA involved in modulating post-transcriptional gene expression, have been identified in the corneal and limbal epithelia. Specifically, miR-184 was detected during early eye development in the mouse, and in a pluripotent stem cell model, demonstrating its importance in corneal lineage specification (Shalom-Feuerstein et al., 2012). In addition, miR-103 and miR-107 were found to be preferentially expressed in the basal limbal epithelium of mice, regulating the stem cell characteristics of LESCs and contributing to their slow-cycling phenotype (Peng et al., 2015). Finally, miR-143 and miR-145 were found to be expressed predominantly in the limbal epithelium of the human donor corneas, and miR-145 was shown to be involved in regulating corneal epithelial formation and maintenance (Lee et al., 2011). Nevertheless, more research is needed to fully understand the complex signaling cascades involved in LESC maintenance and self-renewal.

2.2 Limbal stem cell deficiency

Corneal diseases are major causes of blindness especially in developing countries, where they are second only to cataract (Whitcher et al., 2001). Ocular surface diseases where there is either a significant loss or dysfunction of LESCs are collectively referred to as limbal stem cell deficiency (LSCD). These disorders are characterized by disruption of corneal epithelial renewal and loss of barrier function of the limbus, leading to the invasion of conjunctival epithelium onto the cornea (Osei-Bempong et al., 2013). Consequently, patients with LSCD suffer from recurrent epithelial defects, persistent pain and inflammation, loss of corneal clarity, decreased visual acuity, and in severe cases blindness (Ahmad, 2012). Each year, corneal vascularization and opacity have been estimated to cause blindness in eight million people worldwide – roughly 10% of total cases (Whitcher et al., 2001). The severity of LSCD varies depending on the extent of the injury – it can be partial or total, and either unilateral or bilateral (Dua & Azuara-Blanco, 2000). In addition to the damaged epithelium, the stroma is often involved in LSCD cases. Complete wound healing of a stromal injury can take months or even years, and thus affects corneal clarity long after primary wound healing has occurred (DelMonte & Kim, 2011).

There are many known causes of LSCD: hereditary diseases such as aniridia, inflammatory disorders including ocular cicatricial pemphigoid and Stevens-Johnson syndrome, prolonged contact lens wear, extensive cryotherapy or surgery,

chemical burns and other acute trauma of the ocular surface (Ahmad, 2012; Osei-Bempong et al., 2013). There are also idiopathic cases of LSCD, meaning that there is no known cause for the disorder. For each patient, the etiology of LSCD and its degree of severity need to be taken into consideration when deciding on a treatment plan.

2.2.1 Strategies for ocular surface reconstruction

For partial LSCD not affecting the patient's vision, topical lubricant drops and anti-inflammatory drugs could help sufficiently reduce ocular discomfort and nurture the remaining viable LESCs, and no surgical intervention may be necessary (Dua et al., 2010). However, in more severe cases, surgery is required to first clear the ocular surface from the vascularized conjunctival epithelium, and then transplant healthy tissue. Patients with LSCD are generally poor candidates for conventional corneal transplantation, as it does not permanently reconstitute the function of the limbus (Dua & Azuara-Blanco, 2000; Pellegrini et al., 2013). Sector limbal transplantation of autologous or allogeneic tissue has been reported, where a limbal biopsy is taken from a healthy donor eye (patient's own, living related donor, or cadaveric) and transplanted to the injured eye (Dua & Azuara-Blanco, 2000; Dua et al., 2010; Kenyon & Tseng, 1989). Autologous grafts generally give the best results, but are only available in cases of unilateral LSCD. Moreover, the technique is limited by the need for fairly large amounts of donor tissue, putting the healthy donor eye at risk (Osei-Bempong et al., 2013). Therefore, other strategies have been developed and are beginning to gain popularity. The most commonly implemented and widely studied techniques for treatment of partial and total LSCD are summarized in a flow diagram at the end of this chapter (Figure 3).

2.2.2 Cultivated limbal epithelial transplantation

About 18 years ago, the first successful clinical use of cultivated limbal epithelial transplantation (CLET) was reported, using autologous limbal tissue obtained from a 1-2 mm^2 biopsy expanded *ex vivo* to treat two patients (Pellegrini et al., 1997). Subsequently, the technique has become fairly widely used worldwide, and the overall success rate of the procedure is around 76%, based on the data gathered over a period of 13 years from a total of 583 patients (Baylis et al., 2011). LSCD is a highly heterogeneous disease with respect to cause and severity, and the methods

for the isolation and cultivation of LESCs vary greatly among laboratories, making it difficult to compare the efficacy of each technique (Baylis et al., 2011; Zhao & Ma, 2015). Typically, limbal biopsies are subjected to either mechanical disruption (explant culture) or enzymatic digestion (single-cell suspension), followed by 2-3 weeks of culture on mitotically-inactivated mouse fibroblast feeder cells or a transplantable carrier, such as human amniotic membrane (hAM), fibrin, or contact lens (Joe & Yeung, 2014). Most of the available culture protocols rely on the use of xenogeneic and undefined culture components, although efforts are being made to eliminate such potential contaminants and standardize the techniques (Kolli et al., 2010; Shortt et al., 2008; Zakaria et al., 2014). On the other hand, some laboratories continue using fetal calf serum and mouse feeder cells, provided they are clinical-grade and good manufacturing practice (GMP) certified (Pellegrini et al., 2013). In early 2015, the first advanced-therapy medicinal product (ATMP) containing stem cells was granted conditional approval by the European Medicines Agency (EMA). This product, Holoclar (Holostem Terapie Avanzate, Modena, Italy), consists of autologous LESCs expanded *ex vivo* and transplanted to treat severe LSCD caused by chemical or thermal burn (Dolgin, 2015; http://www.ema.europa.eu). Notably, expansion of LESCs is carried out on mouse feeder cells in the presence of animal serum, thereby requiring thorough quality assessment to guarantee the safety of the product prior transplantation. Most importantly, Holoclar is not applicable to treating bilateral LSCD, as it requires a biopsy of healthy autologous limbus.

Although CLET alone may stabilize the ocular surface, the patient's vision often remains poor due to corneal neovascularization and stromal scarring, making it necessary to perform corneal transplantation later on (Joe & Yeung, 2014; O'Callaghan & Daniels, 2011; Zakaria et al., 2014). Additionally, localized conjunctival invasion of the cornea is sometimes observed within the first year after transplantation (Kolli et al., 2010). Surprisingly, there appears to be no significant difference in success rates between autologous and allogeneic CLET, while the cause of LSCD does have an effect on graft survival (Baylis et al., 2011). There are several factors that may contribute to CLET outcome, regardless of whether autologous or allogeneic LESCs are used. Firstly, LESC cultures used for CLET are transplanted as heterogeneous cell populations, and therefore likely contain a mixture of LESCs, TACs, as well as mature corneal and conjunctival epithelial cells. This may contribute to graft failure, as it is believed that only true LESCs are capable of long-term tissue homeostasis (Joe & Yeung, 2014). Secondly, clinical evidence shows a correlation between p63 expression and CLET success – grafts containing less than 3% of p63-positive cells were shown to have a

significantly higher risk for failure (Rama et al., 2010). Finally, the mechanisms through which CLET functions remain unclear – the transplanted cell populations either replace the patient's damaged or lost LESCs, or stimulate them to reactivate (O'Callaghan & Daniels, 2011). Contradictory evidence exists, with a clinical study showing that donor LESCs are observed for up to 3.5 years after allogeneic limbal tissue transplantation (Djalilian et al., 2005), yet a different study demonstrating an absence of donor cells nine months after allogeneic CLET (Daya et al., 2005). Overall, it may be that the mode of action depends on LSCD etiology, the extent of damage and whether the LESC niche environment is compromised.

2.2.3 Simple limbal epithelial transplantation

In 2012, a novel surgical technique for the treatment of unilateral LSCD was described. Similarly to CLET, a small limbal biopsy (2 mm^2) is first obtained from the healthy eye. However, rather than expanding LESCs *ex vivo*, the tissue is divided into 8-10 small pieces, which are then distributed evenly over a sheet of hAM placed on the corneal surface, secured with fibrin glue (Sangwan et al., 2012). The technique, termed simple limbal epithelial transplantation (SLET), is therefore a relatively affordable single-stage procedure, not requiring a specialized GMP certified facility for cell culture. So far, only a small number of patients have been treated with SLET, although the clinical outcomes show promise (Amescua et al., 2014; Bhalekar et al., 2013; Sangwan et al., 2012; Vazirani et al., 2013). The long-term efficacy of the technique is yet to be demonstrated.

2.2.4 Alternative cell sources

LSCD more commonly presents bilaterally, rather than unilaterally (Utheim, 2015). In cases of bilateral and total LSCD, there is not enough autologous limbal tissue for CLET, and alternative autologous cell sources may be considered. In 2004, cultivated oral mucosal epithelial transplantation (COMET) was first introduced in two separate studies, each demonstrating successful treatment of four LSCD patients (Nakamura et al., 2004; Nishida et al., 2004). Subsequently, many other clinical studies have been reported (Hirayama et al., 2012; Kolli et al., 2014; Utheim, 2015). Although the definition of clinical success varies among different studies, the overall success rate of COMET is around 72%, similar to that of CLET (Utheim, 2015). Complications include peripheral corneal

neovascularization, corneal epithelial defects, increased ocular pressure and infections (O'Callaghan & Daniels, 2011; Utheim, 2015).

In addition to oral mucosal epithelium, several other autologous sources of epithelial stem cells have been explored in hopes of finding an alternative to CLET. Conjunctival epithelium is the only other tissue besides oral mucosal epithelium which has been tested in human patients (Ricardo et al., 2013). Finally, many pre-clinical studies in search of a novel cell therapy for LSCD are in progress using mesenchymal stem cells (MSC) of various origin (Holan et al., 2015; Katikireddy et al., 2014; Nieto-Miguel et al., 2013; Tsai et al., 2015), corneal stromal stem cells (Hashmani et al., 2013), hair follicle stem cells (Blazejewska et al., 2009), umbilical cord lining stem cells (Reza et al., 2011) and nasal mucosal epithelium (Kobayashi et al., 2015a). The main disadvantage of using somatic cells or adult stem cells is their limited capacity for self-renewal, resulting in limited yields of transplantable cells. Therefore, more extensive studies are needed to determine whether these cell types are capable of self-sustaining corneal reconstruction. It could also be beneficial to investigate alternative cell sources with a higher potential for proliferation and self-renewal, such as human pluripotent stem cells (hPSC).

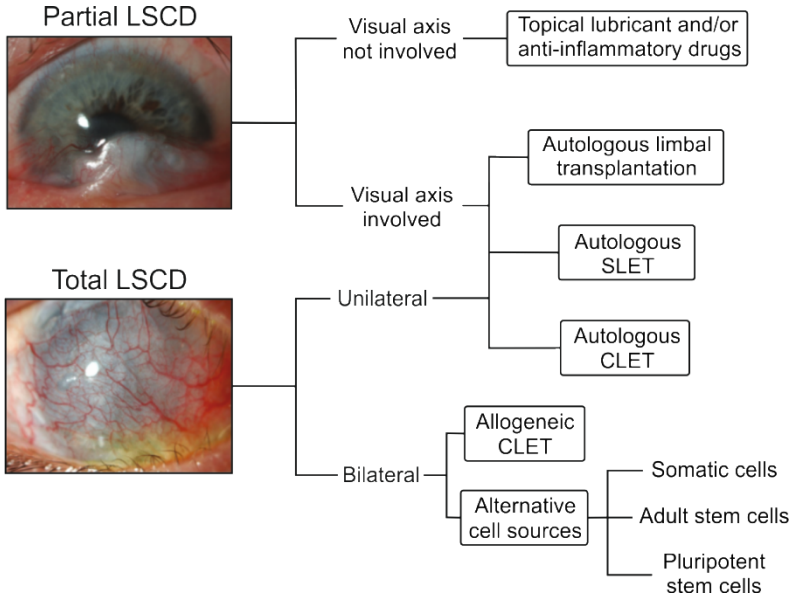


Figure 3. Strategies for ocular surface reconstruction, depending on the severity of the disorder. Representative images of partial and total LSCD modified from Zakaria et al., 2014.

2.3 Human pluripotent stem cells

Stem cells are identified by two characteristics: they have the capacity to self-renew, and differentiate into specific cell lineages (Fortier, 2005). Human stem cells are typically classified by their differentiation potential. Unipotent and multipotent stem cells are found in adult tissues: unipotent stem cells are only capable of differentiating into one other cell type, while multipotent stem cells can differentiate into several cell types. LESCs are an example of unipotent stem cells, as described earlier in Chapter 2.1.2. Hematopoietic stem cells, on the other hand, are an example of multipotent stem cells, giving rise to all types of blood cells (Fortier, 2005). Pluripotent and totipotent cells are only found in the fertilized embryo, and have a much wider differentiation potential. Totipotent cells have the ability to give rise to an entire individual, and exist until the eight-cell stage of the morula. Later, once the morula develops into a blastocyst, the cells within this structure become segregated into two distinct populations. The inner cell mass (ICM) cells have the capacity to form the embryo, while the outer trophoblast cells form extraembryonic tissues such as the placenta. Although the pluripotent cells of the ICM are capable of forming all tissues and cell types of the human body, they cannot give rise to an entire individual due to their inability to form extraembryonic tissues. Human PSCs offer insights into human development, drug discovery, toxicology and personalized medicine, as well as cell-based therapy (Pera et al., 2000). There are two sources of hPSCs – human embryonic stem cells (hESC) are derived from early-stage embryos, while human induced pluripotent stem cells (hiPSC) are obtained by reprogramming somatic cells to a pluripotent state.

2.3.1 Human embryonic stem cells

The first hESC lines were established in 1998, by culturing embryos to the blastocyst stage, isolating ICM cells, and plating them onto mouse embryonic fibroblast (MEF) feeder cell layers to give rise to hESC colonies (Thomson et al., 1998). The preimplantation embryos used for hESC derivation are produced by *in vitro* fertilization (IVF), donated for research by couples undergoing IVF treatment (Hasegawa et al., 2010). In Finland, both partners are required to sign an informed consent form after receiving both an oral and written description of the research, and no financial compensation is provided to the donors (Skottman, 2010). During

the last two decades, rapid progress in the field of hESC research has been made worldwide, and currently 1304 hESC lines are registered at the International Stem Cell Registry (http://www.iscr-admin.com) and 683 hESC lines at the European Human Pluripotent Stem Cell Registry (http://hpscreg.eu/). In addition to the conventional hESC derivation on feeder cell layers, feeder-independent derivation of hESC lines on surfaces coated with recombinant laminin-521 and E-cadherin has recently been described (Rodin et al., 2014). Elimination of feeder cells, regardless of whether or not they are of animal origin, improves the reproducibility of hESC culture. Currently, Phase I/II clinical trials using hESC-derived retinal pigment epithelial (RPE) cells to treat advanced age-related macular degeneration (AMD) and Stargardt's macular dystrophy are ongoing, showing promising results regarding the safety of treatments (Schwartz et al., 2015; Song et al., 2015). Furthermore, clinical trials aiming to treat spinal cord injury, post-infarction heart failure and type 1 diabetes mellitus using hESC-derived progenitor cells are currently recruiting participants (https://clinicaltrials.gov/).

2.3.2 Human induced pluripotent stem cells

In 2007, the first hiPSC line was generated from adult human dermal fibroblasts (hDF) by retroviral transduction of four transcription factors (octamer-binding transcription factor 3/4 (*OCT3/4*), (sex-determining region Y)-box 2 (*SOX2*), Krüppel-like factor 4 (*KLF4*) and myc proto-oncogene protein (*c-MYC*)), and shown to behave similarly to hESCs (Takahashi et al., 2007). After successful reprogramming, these transcription factors become silenced, indicating that hiPSCs do not depend on continuous expression of the transgenes for self-renewal (Takahashi et al., 2007). Although the technique was introduced less than a decade ago, it has been widely used and modified. Various somatic tissues besides fibroblasts and different combinations of reprogramming factors have been used to successfully generate hiPSC lines (Hu, 2014; Trokovic et al., 2014; Zhou et al., 2012). As recognition for his ground-breaking work in reprogramming adult cells to hPSCs, Professor Yamanaka received the Millennium Technology Prize and the Nobel Prize for Physiology or Medicine in 2012.

The discovery of hiPSCs has opened new opportunities in the field of regenerative medicine. For instance, hiPSC technology enables the generation of disease-specific cell lines, useful for understanding disease mechanisms, drug screening, and toxicology (Park et al., 2008; Takahashi et al., 2007). Regenerative

medicine is the most widely studied application of both types of hPSCs. A unique property of hiPSCs is that it is possible to generate patient-specific cell lines to be used for autologous cell therapy. In fact, the first clinical trial using cells differentiated from autologous hiPSCs was initiated in late 2014 in Japan, aiming to treat AMD with hiPSC-derived RPE cells (Kamao et al., 2014). Unfortunately, the trial was suspended in March 2015, after several mutations were detected in hiPSCs of a prospective second patient, likely caused by the reprogramming procedure (Garber, 2015). In light of the setbacks concerning hiPSC-derived cells, there are still some issues that need to be addressed before wider clinical implementation. In attempts to minimize the risk of harmful mutations and transgene reactivation, several non-integrating methods for reprogramming have been developed, utilizing the non-integrating Sendai virus, recombinant proteins, or synthetic modified mRNA (Fusaki et al., 2009; Warren et al., 2010; Zhou et al., 2009). Moreover, reprogramming methods in feeder-independent and chemically-defined conditions have been described (Beers et al., 2015; Chen et al., 2011). It has been noted that genomic stability of hiPSCs is mainly affected by the reprogramming methods and culture conditions, highlighting the importance of protocol optimization (Bai et al., 2013). Interestingly, an extensive karyotype analysis on >1700 hiPSC and hESC cultures collected from 97 investigators revealed no notable difference in the incidence of chromosomal aberrations between the two hPSC types (Taapken et al., 2011). On the other hand, it appears that hiPSCs partially retain the DNA methylation patterns of parental somatic cells, suggesting that transcription-factor based reprogramming is associated with incomplete epigenetic reprogramming (Lister et al., 2011; Ma et al., 2014). However, the biological consequence of these aberrations remains unclear and requires further studies (Lund et al., 2012a). Ultimately, there is substantial molecular and functional evidence showing similarity between hiPSCs and hESCs, and the choice of cell type for differentiation studies is largely dictated by the end application. In addition, guidelines defining acceptable levels of genomic and epigenetic stability need to be established for both hPSC types.

2.3.3 Culture and characterization of hPSCs

Human PSCs are conventionally cultured as colonies on feeder cell layers (MEF or human foreskin fibroblasts (hFF)), or on Matrigel, a complex mixture of matrix proteins derived from Engelbreth-Holm-Swarm mouse sarcomas, in a medium

containing xenogeneic serum or serum albumin (Hasegawa et al., 2010; Hoffman & Carpenter, 2005; Skottman & Hovatta, 2006). Ideally, culture conditions will include a defined matrix and a defined medium supplemented with recombinant proteins to allow the establishment of more reproducible hPSC cultures (Hoffman & Carpenter, 2005; Villa-Diaz et al., 2013). Recently, various methods of culturing hPSCs in chemically-defined and xeno-free conditions, on surfaces coated with recombinant extracellular matrix (ECM) proteins, or synthetic coatings, have been described (Chen et al., 2011; Rodin et al., 2014; Villa-Diaz et al., 2013). Passaging of hPSCs is done either mechanically, by manually selecting and transferring undifferentiated colonies onto fresh substrates, or enzymatically, where hPSC colonies are passaged as single cell suspensions or clusters (Hasegawa et al., 2010; Hoffman & Carpenter, 2005). Mechanical passaging is generally regarded as the gentler technique, yet it is fairly laborious and subject to variation. Enzymatic passaging, on the other hand, allows for more uniform plating of hPSCs, but has been shown to increase the risk of genetic and epigenetic instability (Bai et al., 2015; Garitaonandia et al., 2015).

The quality of hPSCs in culture needs to be routinely verified. Human PSCs have a high nucleus to cytoplasm ratio, prominent nucleoli, and grow in flat colonies with distinct borders. Undifferentiated hPSCs are commonly characterized by their gene and protein expression of cell surface markers and transcription factors associated with an undifferentiated state, such as stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor-related antigen (TRA)-1-60, TRA-1-81, OCT3/4, reduced expression protein 1 (REX1), SOX2 and NANOG (Hovatta et al., 2014; Pera et al., 2000; Takahashi et al., 2007). Telomerase and alkaline phosphatase activities are also linked with pluripotency, and often assessed in hPSCs. Additionally, hPSCs are required to maintain the potential to form derivatives of all three embryonic germ layers (mesoderm, endoderm and ectoderm). This may be evaluated *in vitro*, using spontaneous differentiation in embryoid body culture, or *in vivo*, by injecting undifferentiated cells into nude mice and following teratoma formation (Hasegawa et al., 2010; Hoffman & Carpenter, 2005; Itskovitz-Eldor et al., 2000). Furthermore, a normal euploid karyotype despite continuous passaging is essential in hPSCs. Karyotyping can be done by chromosomal G-band analysis or higher resolution techniques such as array comparative genomic hybridization (aCGH), which enable the detection of unbalanced genomic changes at the kilobase level (Hovatta et al., 2014; Lund et al., 2012a). In addition to evaluation of genomic stability, it is becoming increasingly evident that epigenetic stability of hPSCs also needs to be studied. For instance, X-

chromosome inactivation and variation in DNA methylation of a subset of imprinted and developmental genes is fairly common in both hESC and hiPSC lines (Lund et al., 2012a).

2.3.4 Human PSC-derived corneal epithelium

There are several reasons why the ocular surface is a good target for therapy using hPSCs. Firstly, the eye is easily accessible, making the surgery and follow-up procedures less invasive than for internal organs. Secondly, it is possible to treat only one eye and use the fellow eye as the control, whereby assessing efficacy is more reliable. Finally, the eye is an immune-privileged organ, and the cornea lacks blood vessels, meaning that there is a lower risk of immune rejection than in other tissues. This, however, may be altered in the case of severe LSCD, making regular follow-up very important in order to recognize possible signs of rejection or graft dysfunction early on. Efficient production of hPSC-derived LESCs or stratified corneal epithelium (Figure 4) would potentially solve the issues related to CLET, most importantly donor tissue shortage. In addition, hPSC-LESCs could be applied to studying and modelling the human cornea, as well as drug development *in vitro* (Figure 4).

Although corneal transplantation and CLET are often successfully performed without matching for human leukocyte antigen (HLA), it may be beneficial especially for high risk cases with extensive vascularization (Van Essen et al., 2015). This is an important aspect to take into consideration also when dealing with hPSC-LESCs, which are likely to be allogeneic. In order to make cell-based therapy more readily available and minimize the risk of immune rejection posed by allogeneic hPSCs, HLA haplotype-based banking of hPSC lines could be considered (Wilmut et al., 2015; Zimmermann et al., 2012). A haplotype is a set of alleles encoded by a group of closely linked genes, and is usually inherited as a unit, making HLA haplotype-matching feasible. Selection of donors with homozygous HLA haplotypes for hiPSC production and banking would provide cell lines matching large groups of patients (Wilmut et al., 2015; Zimmermann et al., 2012). For instance, it was estimated that as few as 30 HLA-homozygous hiPSC lines would provide a match for 82% of the Japanese population (Nakatsuji et al., 2008). Similarly to Japan, there is a limited amount of HLA allele diversity and fewer haplotypes in Finland than in countries with a more mixed population (Haimila et al., 2013). The more diverse the HLA haplotypes in a certain population, the more

homozygous hiPSC lines would be needed to provide a possible match for a patient. There are currently two hPSC banks in Europe – The European Bank for Induced Pluripotent Stem Cells (EBiSC), and The UK Stem Cell Bank (UKSCB). Nevertheless, the idea of collecting homozygous hiPSC lines has not yet been implemented.

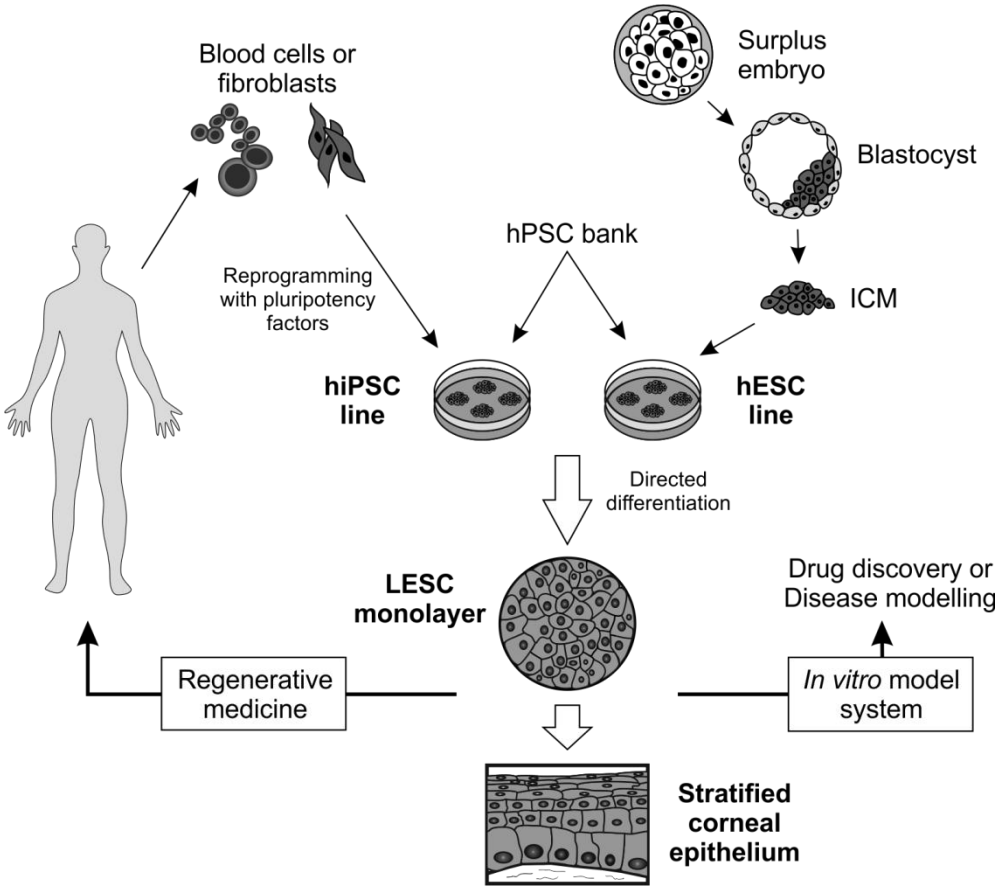


Figure 4. Establishment of hPSC lines and generation of hPSC-LESCs and corneal epithelium. Human ESC lines are derived from the ICM of the early-stage surplus embryos, while hiPSC lines are obtained by reprogramming somatic cells with a combination of transcription factors. Collecting hPSC lines in specialized banks can allow generation of HLA haplotype-matched cell populations. Differentiation of hPSCs can be directed towards corneal epithelium, first producing monolayers of LESC-like cells, which stratify upon further maturation. Finally, hPSC-derived LESC-like cells or corneal epithelium can be used to treat ocular surface disorders, or as an *in vitro* model system.

The first study describing differentiation of corneal epithelial cells from hPSCs was published in 2007, where corneal epithelial-like cells were obtained from hESCs by *in vitro* replication of the corneal epithelial stem cell niche with the help of a differentiation medium conditioned by human limbal fibroblasts (hLF) and collagen IV-coated substrate (Ahmad et al., 2007). Since then, hESC and hiPSC differentiation towards mature or progenitor corneal epithelial cells has been reported by several groups (Table 2).

Table 2. Published corneal epithelial differentiation methods using hPSCs.

hPSC	Substrate	Key medium components	Duration	Reference
hESC	Collagen IV	Limbal fibroblast CM, contains FCS, hydrocortisone, insulin, adenine, tri-iodothyronine, cholera toxin, EGF	up to 21 days	Ahmad et al., 2007
hESC	Fixed MEF	FCII, adenine, HEPES, hydrocortisone, cholera toxin, insulin, EGF	21 days	Hewitt et al., 2009
hESC	Bowman's membrane	FBS	~30 days	Hanson et al., 2013
hiPSC	PA6 feeder cells	KO-SR, NaPyr, NEAA, 2-mE	12-16 weeks	Hayashi et al., 2012
hiPSC	Collagen IV	Corneal fibroblast CM, contains FCII, adenine, hydrocortisone, cholera toxin, insulin, EGF, BMP-4	12 days	Shalom-Feuerstein et al., 2012 and 2013
hESC	Collagen IV and APCM	LESC CM, contains FBS, EGF, hydrocortisone, insulin, transferrin, BPE	23 days	Zhu et al., 2013
hESC	Matrigel	Limbal fibroblast CM	21 days	Brzeszczynska et al., 2014
hiPSC	FCL, hAM, or human cornea	mTeSR1, gradually changed to EpiLife®, B27, N2, HKGS, EGF	15 days	Sareen et al., 2014

Abbreviations: 2-mE, 2-mercaptoethanol; APCM, acellular porcine corneal matrix; B27, growth supplement (contains BSA); CM, conditioned medium; Epilife®, chemically-defined medium for keratinocyte culture (Invitrogen); FCII, FetalClone II™ serum (HyClone™); FCL, mixture of fibronectin, collagen type IV and laminin; FCS, fetal calf serum; HKGS, human keratinocyte growth supplement (contains BPE, human insulin-like growth factor 1, hydrocortisone, bovine transferrin, and EGF); N2, chemically-defined and xeno-free growth supplement; NaPyr, sodium pyruvate; PA6, mouse stromal cells. Other abbreviations are listed on pages 12-14.

Generally, differentiation is driven by a combination of two factors – the culture substrate and an appropriate cell culture medium, commonly relying on undefined or xenogeneic components. A favorable culture substrate is usually obtained with the help of ECM coatings, feeder cells or biological scaffolds, while cell culture medium is often conditioned by limbal or corneal fibroblasts, so as to offer the necessary differentiation cues. Characterization of hPSC-derived cells is largely

based on verification of LESC and corneal epithelial marker expression using RT-PCR, immunocytochemistry, or flow cytometry. The duration required for adequate differentiation varies from 12 days to 16 weeks, and efficiency is rarely quantified. Moreover, two of the studies have shown that limbal epithelial-derived hiPSCs differentiate into corneal epithelium more efficiently than dermal fibroblast-derived hiPSCs, likely due to their epigenetic memory (Hayashi et al., 2012; Sareen et al., 2014).

Ideally, the differentiation method should be robust and reproducible, carried out in chemically-defined conditions free from xenogeneic components. Most importantly, hPSC-derived corneal epithelial cells should be similar to native human corneal epithelium with regard to cell morphology, gene and protein expression and functionality.

2.4 Biomaterials for ocular surface reconstruction

Transplantation of LESC-like cells to the ocular surface requires a carrier that is biocompatible, mechanically stable, transparent, and capable of supporting cell attachment and proliferation both in culture and after transplantation (Menzel-Severing et al., 2013). Currently, the gold standard for culture and transplantation of LESCs is hAM, the inner of the two fetal membranes obtained following elective Cesarean section, with a long-standing history in corneal applications (Gomes et al., 2005; de Rotth, 1940; Sorsby & Symons, 1946). The main advantages of hAM are its anti-inflammatory, anti-angiogenic and anti-microbial properties, combined with the ability to promote epithelialization and inhibit fibrosis (Gomes et al., 2005; Zhao & Ma, 2015). The disadvantages of hAM include lack of standardization, biological variability in morphological, chemical, and optical properties, poor mechanical strength and difficulty of handling, as well as limited availability (Connon et al., 2010; Ghezzi et al., 2015; Menzel-Severing et al., 2013).

Various biomaterials are being investigated in the hopes of finding an alternative superior to hAM. While some approaches aim at replacing the corneal epithelium alone, others attempt using a thicker construct to simultaneously reconstruct part of the stroma. Some of the most recent studies investigating transplantable carriers for human LESC-like cells are summarized in Table 3. Biomaterials of natural origin have been more widely studied than synthetic scaffolds. Some of the most widely studied biomaterials include various hydrogels

(such as alginate, collagen and fibrin), silk fibroin or keratin films, and electrospun membranes (Table 3). Hydrogels are a particularly promising type of transplantable carrier, owing to their highly hydrated network structure and the possibility of co-culturing two types of cells – supporting stromal cells incorporated within the hydrogel, and epithelial cells cultured on the surface (Wright et al., 2013a). This type of construct could potentially replace part of the damaged corneal stroma along with the epithelium.

Table 3. Some of the most recently developed transplantable carriers for LSCD treatment.

Biomaterial	Modification	Cell type	Clinical status	Reference
Alginate hydrogel	Oxidation, ColIV incorporation	iHCE	*In vitro*	Wright et al., 2014
Chitosan-gelatin membrane	Cross-linking	iHCE or LESC	*In vitro*	de la Mata et al., 2013
Collagen I (rat tail)	Concentration (up to 90 mg/ml)	LESC	*In vitro*	Tidu et al., 2015
Collagen I (bovine)	Plastic compression (RAFT™)	LESC	*In vitro*	Levis et al., 2013
Collagen I (bovine)	Vitrification	LESC	*In vivo* (rabbit)	Chae et al., 2015
Collagen I (porcine)	Cross-linking	iHCE	*In vivo* (rabbit)	Koulikovska et al., 2015
Collagen I (equine)	Cross- linking	hOME	*In vivo* (rabbit)	Petsch et al., 2014
RHCI or III	Cross-linking	iHCE	*In vivo* (pig)	Liu et al., 2008
Collagen-chitosan	Cross-linking	iHCE	*In vivo* (pig)	Rafat et al., 2008
Fibrin	None	LESC	Clinical trial	Rama et al., 2010
Human corneal stroma	Sections, 200 μm thickness	LESC	*In vitro*	Lin et al., 2012
Human lens capsule	None	LESC	*In vitro*	Albert et al., 2012; Galal et al., 2007
Keratin film	None	iHCE	*In vitro*	Feng et al., 2014; Reichl et al., 2011
PCL	Electrospinning, plasma treament	iHCE and LESC	*In vitro*	Sharma et al., 2014
PLGA	Electrospinning	LESC (rabbit or human)	*In vitro*	Deshpande et al., 2013
Silk fibroin film	None	iHCE	*In vitro*	Liu et al., 2012
Silk fibroin dual layer	Film and fibrous mat	LESC and L-MSC	*In vitro*	Bray et al., 2012
Silicone contact lens	None	LESC or CjE	Clinical trial	Bobba et al., 2015

Abbreviations: ColIV, collagen type IV; CjE, conjunctival epithelium; hOME, human oral mucosal epithelium; iHCE, immortalized human corneal epithelial cell line; L-MSC, limbal mesenchymal stromal cells; PCL, poly-ε-caprolactone; PLGA, poly-lactide-co-glycolide; RAFT, real architecture for 3D tissue; RHCI or III, recombinant human collagen type I or III.

Collagen hydrogels have gained exceptional popularity as a potential LESC carrier, mainly due to the fact that collagen is the main structural component of the corneal stroma (Hassell & Birk, 2010). Collagen forms highly hydrated and inherently weak hydrogels, and is therefore often modified either by plastic compression (Levis et al., 2010; Mi et al., 2010) or chemical cross-linking (Koulikovska et al., 2015; Liu et al., 2008; Petsch et al., 2014). Recently, commercial kits designed to simplify and standardize plastic compression of collagen hydrogels such as the RAFT™ 3D Cell Culture System (Lonza Group Ltd., Basel, Switzerland) have become available. Another interesting approach to corneal reconstruction was proposed using a thermoresponsive polymer Mebiol Gel: autologous rabbit LESCs were cultured on the surface of a solid hydrogel, but transplanted as a liquid gel, the transition being triggered by a temperature change (Sitalakshmi et al., 2009). Additionally, several biomaterials are being studied for their compatibility with corneal stromal cells (Mi et al., 2010; Wu et al., 2014), or as cell-free scaffolds aimed at corneal stromal reconstruction (Fagerholm et al., 2014; Van Essen et al., 2013).

With regard to transplantable carriers for hPSC-LESCs, so far only two *in vitro* studies have been published – one using hAM (Sareen et al., 2014) and the other using acellular porcine corneal matrix (Zhu et al., 2013). Neither of these scaffolds is optimal, mainly due to their biological variability and potential for pathogen transmission. More research is needed before a suitable carrier for hPSC-LESCs is thoroughly characterized *in vitro*, the construct's safety verified in an animal model, and eventually tested in human patients.

3 Aims of the study

The aim of this dissertation was to study the differentiation of hPSC lines towards functional LESC-like cells capable of terminal differentiation towards mature corneal epithelial cells. Moreover, it was important to minimize the use of undefined and animal-derived components, aiming for clinical applications in the long run. The specific aims of the dissertation were:

1. To develop a directed and efficient differentiation method for hPSC-LESCs in serum-free and feeder-free conditions (**Study I**).

2. To characterize the molecular properties and functionality of hPSC-LESCs (**Studies I** and **II**).

3. To compare hPSC-LESCs with their *in vivo* counterparts (**Study II**).

4. To evaluate *in vitro* the suitability of a bioengineered collagen matrix for clinical applications using hPSC-LESCs (**Study III**).

4 Materials and methods

4.1 Ethical considerations

The use of human embryos for research purposes at BioMediTech has been approved by the National Authority for Medicolegal Affairs in Finland (Dnro 1426/32/300/05). The institute also has supportive statements of the Ethical Committee of the Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines (Skottman/R05116), and use hiPSC lines derived in other laboratories for ophthalmic research (Skottman/R14023). The research groups of Prof. Timo Otonkoski at the University of Helsinki and Prof. Katriina Aalto-Setälä at the University of Tampere have the appropriate permissions of the Ethics Committee for generation of hiPSC lines. No new cell lines were established for the studies conducted as part of this dissertation.

Use of human corneal donor tissue unsuitable for corneal transplantation for research purposes was approved by the local ethics research committee (Valvira, Dnro 7797/05.01.00.06/2011).

Collection of human corneal tissue was carried out in Debrecen, Hungary, with approval by the National Medical Ethics Committee of Hungary (14415/2013/EKU-183/2013 and DEOEC RKEB/IKEB 3094/2010), and in compliance with the guidelines of the Helsinki Declaration. Hungary follows the EU Member States' Directive 2004/23/EC on presumed consent practice for tissue collection.

4.2 Human tissue collection

Donor corneas unsuitable for transplantation were obtained from Regea Cell and Tissue Center, to be used as positive control in **Study I**. The epithelium was collected from the surface of the corneas by mechanical scraping, washed with phosphate buffered saline (PBS) and pelleted by centrifugation. Cell pellets were lysed and stored at -80 °C until total RNA extraction. Additionally, corneal tissue

was fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO), embedded in paraffin and sectioned for immunofluorescence antibody verification.

Limbal epithelial cell (LEC) and corneal epithelial cell (CEC) samples were obtained within 12 hours post-mortem by gently scraping the surface of the limbus or the central cornea, respectively. All samples were collected in sterile PBS, pelleted by centrifugation and stored as dry pellets at -80 °C until protein extraction for comparative proteomics (**Study II**) or Western blotting (**Study III**).

4.3 Culture of hPSC lines

Three hESC lines and three hiPSC lines were used in the original publications. The hESC lines Regea08/017 (**Studies I, II** and **III**), Regea08/023 and Regea11/013 (**Study I**) were previously derived at the University of Tampere (Skottman, 2010). The hiPSC lines FiPS5-7, A116 and HEL24.3 (**Study I**) were generated from neonatal hFFs (FiPS5-7 and HEL24.3) or adult hDFs (A116) by Professor Timo Otonkoski's research group at the University of Helsinki (Hussein et al., 2011; Toivonen et al., 2013; Trokovic et al., 2015). The hiPSC line UTA.04511.WT (**Studies II** and **III**) was generated from adult hDFs by Professor Katriina Aalto-Setälä's research group at the University of Tampere (Ojala et al., 2015).

All hPSC lines were routinely cultured on mitotically inactivated hFF feeder cells (CRL-2429, ATCC, Manassas, VA) in a basic hPSC culture medium consisting of KnockOut Dulbecco's Modified Eagle Medium (KO-DMEM) supplemented with 20% KnockOut Serum Replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all from Invitrogen, Carlsbad, CA), 1% Non-Essential Amino Acids (NEAA), 50 U/ml penicillin/streptomycin (both from Lonza Group Ltd.), and 8 ng/ml human basic FGF (bFGF; PeproTech, Rocky Hill, NJ). Undifferentiated colonies were enzymatically passaged onto fresh feeder layers at ten-day intervals.

All hPSC lines were regularly characterized for their pluripotency and ability to generate derivatives of all three embryonic germ layers as previously described (Skottman, 2010). Karyotype analysis was performed either using conventional G-banding at United Medix Laboratories Ltd in Helsinki (**Study I**), or using a high-throughput bead-based KaryoLite™ BoBs™ assay (Lund et al., 2012b) at the Finnish Microarray and Sequencing Center in Turku (**Studies II** and **III**).

4.4 LESC differentiation and culture

Differentiation was initiated by manually dissecting the hPSC colonies, transferring them to suspension culture, and directing differentiation towards surface ectoderm using an induction medium supplemented with 10 μM of transforming growth factor β (TGF-β) inhibitor SB-505124, 10 μM of Wnt inhibitor IWP-2 (both from Sigma-Aldrich) and 50 ng/ml bFGF (PeproTech). During this induction stage, three-dimensional cell aggregates would form, and they were maintained for 4-7 days, changing the induction medium daily. The cell aggregates were then plated onto well-plates (Corning CellBIND; Corning, NY) coated with human placental collagen IV (Sigma-Aldrich), and differentiation was continued as adherent culture in the commercial serum-free and defined corneal epithelium medium CnT-30 (CELLnTEC Advanced Cell Systems, Bern, Switzerland). Differentiated cells were characterized at several time-points, as described in the following chapters, and considered to have reached the LESC-like state after a total of 30 ±5 days of differentiation. Further maturation until day 44 resulted in corneal epithelial cells.

In **Study I**, xeno-free RegES medium (Rajala et al., 2010) modified by omitting retinol and activin A was used as the base for surface ectoderm induction medium. The effect of the small molecules was assessed by comparing differentiation efficiency in supplemented and unsupplemented RegES media. In addition, differentiation in only RegES medium (i.e. spontaneous differentiation) or only CnT-30 medium throughout the duration of the study was evaluated. In **Studies II** and **III**, instead of RegES medium, hPSC medium modified by lowering KO-SR concentration to 15% was used as the base for the induction medium.

In **Study III**, bioengineered matrices fabricated using porcine atelo-collagen I as transparent membranes of uniform 100 μm thickness (LinkoCare Life Sciences AB, Linköping, Sweden), were tested as carriers for hPSC-LESCs. For culture on the bioengineered collagen matrices LESC-like cells (28-33 days in differentiation culture) were enzymatically detached from their substrate, and plated onto the bioengineered matrices or well-plates coated with human placental collagen IV at a density of 20 000 cells/cm^2. After re-plating, hPSC-LESCs were maintained in the progenitor cell targeted serum-free and defined medium CnT-20 (CELLnTEC Advanced Cell Systems).

4.5 Cell characterization methods

During hPSC-LESC differentiation, cell growth and morphology were regularly monitored using Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments, The Netherlands). In **Study I**, three days after plating cell aggregates onto collagen IV-coated substrate, the adhesion ratios were quantified and compared between the three studied induction media. In **Study III**, cell attachment and proliferation on bioengineered collagen matrices were regularly monitored using Zeiss Axio Vert A1 inverted brightfield microscope (Carl Zeiss, Jena, Germany).

4.5.1 Quantitative PCR

Quantitative polymerase chain reaction (qPCR) was used to evaluate differentiation efficiency in different culture conditions (**Study I**). Total RNA extraction, cDNA synthesis and qPCR protocols are described in the original publication. The qPCR reactions were run in triplicates using the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA). The studied genes and respective TaqMan primers (Applied Biosystems) are presented in Table 4.

Table 4. Gene expression studied using qPCR.

Gene symbol	TaqMan assay	Cell type	Time-point
OCT3/4	Hs00999632_g1	Pluripotent stem cells	d0, d4, d44
NANOG	Hs02387400_g1	Pluripotent stem cells	d0, d4, d44
SOX2	Hs01053049_s1	Pluripotent stem cells	d0, d4, d44
c-MYC	Hs00153408_m1	Pluripotent stem cells	d0, d4, d44
PITX2	Hs04234069_mH	Surface ectoderm	d0, d4, d44
BMP4	Hs00370078_m1	Surface ectoderm	d0, d4, d44
FOX1	Hs01125659_m1	Surface ectoderm	d0, d4, d44
PAX6	Hs01088112_m1	Eye precursors, cornea	d0, d4, d44
TP63	Hs00978339_m1	LESCs	d0, d44
KRT15	Hs00267032_m1	LESCs	d0, d44
KRT3	Hs00365074_m1	Corneal epithelium	d0, d44
KRT12	Hs00165015_m1	Corneal epithelium	d0, d44

Abbreviations: OCT3/4, octamer-binding transcription factor 3/4; SOX2, (sex determining region Y)-box 2; PITX2, paired-like homeodomain 2; BMP4, bone morphogenetic protein 4; FOX1, forkhead box 1; PAX6, paired box 6; KRT, keratin.

Relative expression analyses were performed using the 2ΔΔCt method (Livak & Schmittgen, 2001), with Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1) as the endogenous control gene, and undifferentiated hPSCs (day 0) as the calibrator. Corneal epithelium obtained from human donor corneas was used as a positive control of LESC and corneal epithelial gene expression.

4.5.2 Immunofluorescence

Protein expression of the putative LESC markers and proteins specific to the mature corneal epithelium, as well as their subcellular localization, were evaluated using immunofluorescence. The primary antibodies are presented in Table 5. Their detection was carried out with the following secondary antibodies, diluted 1:800: donkey anti-goat Alexa Fluor 568, donkey anti-mouse Alexa Fluor 488 or 568, donkey anti-rabbit Alexa Fluor 488 or 568 (all from Molecular Probes®, Thermo Fisher Scientific, Waltham, MA). The staining protocol is described in detail in the original publications (**Studies I, II** and **III**).

Table 5. Protein expression studied using immunofluorescence

Antibody	Host	Manufacturer	Dilution	Analysis	Study
ABCG2	Mouse	Millipore	1:200	Qual	**I**, **II**, **III**
CK3	Mouse	Abcam	1:300	Qual, quant	**I**, **III**
CK10/13	Mouse	Santa Cruz Biotech.	1:400	Qual	**III**
CK12	Goat	Santa Cruz Biotech.	1:100	Qual, quant	**I**, **III**
CK15	Mouse	Thermo Fisher Scientific	1:200	Qual, quant	**I**, **III**
DG-3	Mouse	US Biological	1:100	Qual	**I**
Ki67	Rabbit	Millipore	1:500	Qual, quant	**I**, **III**
p40	Mouse	Biocare Medical	1:200	Qual	**III**
p63	Goat	Santa Cruz Biotech.	1:100	Qual, quant	**I**, **II**, **III**
p63α	Rabbit	Cell Signaling Tech.	1:200	Qual	**III**
OCT3/4	Goat	R&D Systems	1:400	Qual	**I**
PAX6	Mouse	DSHB	1:500	Qual	**I**
PAX6	Rabbit	Sigma-Aldrich	1:300	Qual	**III**
TCF4	Mouse	Santa Cruz Biotech.	1:400	Qual	**II**, **III**

Abbreviations: ABCG2, ATP-binding cassette subfamily G member 2; CK, cytokeratin; DG-3, desmoglein 3; OCT3/4, octamer-binding protein 3/4; PAX6, paired box 6; TCF4, transcription factor 4; qual, qualitative; quant, quantitative.

Qualitative evaluation was performed by staining LESC-like cells directly on their culture substrate (**Studies I**, **II** and **III**). Quantitative immunofluorescence was carried out by preparing and staining cytospin samples, and counting the positively stained cells in relation to the total nuclei (**Studies I** and **III**). In addition, p63 expression was quantified at ten-day intervals in **Study I**, from cells cultured on collagen IV-coated hanging cell culture inserts. Images of stained cells were captured using Olympus IX51 fluorescence microscope (Olympus, Hamburg, Germany), or Zeiss LSM700 confocal microscope (Carl Zeiss).

4.5.3 Western blotting

Protein expression of CK3 and CK12 in hiPSC-LESCs cultured on bioengineered collagen matrices and human native LECs was analyzed using Western blotting (**Study III**). The following primary antibodies were used: mouse anti-CK3 (diluted 1:1000, Abcam), goat anti-CK12 (diluted 1:500, Santa Cruz Biotechnology), and mouse anti-β-actin (diluted 1:2000, Santa Cruz Biotechnology) as loading control. Their detection was carried out using horseradish peroxidase-conjugated goat anti-mouse and rabbit anti-goat secondary antibodies (both diluted 1:3000, Santa Cruz Biotechnology). The detailed protocol is described in the original publication.

4.5.4 Flow cytometry

Protein expression of the LESC marker BMI-1 in hPSC-LESCs was analyzed using flow cytometry (**Study II**). The detailed protocol is described in the original publication. Briefly, single-cell suspensions were treated either with the fluorescein isothiocyanate (FITC)-conjugated mouse anti-human BMI-1 antibody or the FITC-conjugated mouse anti-human IgG2A isotype control (both from R&D Systems, Minneapolis, MN), and analyzed using the BD Accuri C6 Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

In addition, hiPSC-LESCs (HEL24.3 and UTA.04511.WT cell lines), and their undifferentiated counterparts were analyzed for protein expression of the cell surface markers presented in Table 6. Single-cell suspensions were incubated with the appropriate fluorochrome-conjugated antibody for 30 min, protected from light. Before and after the staining, samples were washed with a buffer containing 0.5% bovine serum albumin (BSA) and 0.01% NaN_3, and collected by

centrifugation. Matched isotype controls were used to account for unspecific binding. Samples were analyzed using the BD Accuri C6 Flow Cytometer.

Table 6. Additional cell surface markers analyzed by flow cytometry

Antibody	Host	Conjugate	Clone	Manufacturer
CD18 / Integrin β2	Mouse IgG1	FITC	TS1/18	BioLegend
CD29 / Integrin β1	Mouse IgG1	APC	MAR4	BD Pharmingen
CD31 / PECAM-1	Mouse IgG1	FITC	HEC/75	Immunotools
CD34	Mouse IgG1	APC	4H11[APG]	Immunotools
CD45 / LCA	Mouse IgG1	APC	HI30	BD Pharmingen
CD49a / Integrin α1	Mouse IgG1	PE	TS2/7	BioLegend
CD49d / Integrin α4	Mouse IgG1	PE	9F10	BD Pharmingen
CD51 / Integrin αV	Mouse IgG2A	FITC	NKI-M9	BioLegend
CD54 / ICAM-1	Mouse IgG1	FITC	BBIG-I1	R&D Systems
CD73 / NT5E	Mouse IgG1	PE	AD2	BD Pharmingen
CD117 / c-kit	Mouse IgG1	PE	Ab81	Miltenyi Biotec
CD144 / VE-cadherin	Mouse IgG2B	PE	123413	R&D Systems
CD146 / MCAM / MUC18	Mouse IgG1	PE	P1H12	BD Pharmingen
Tra-1-81	Mouse IgM	FITC	Tra-1-81	BD Pharmingen

Abbreviations: APC, allophycocyanin; CD, cluster of differentiation; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule 1; LCA, leukocyte common antigen; MCAM, melanoma cell adhesion molecule; MUC18, mucin 18; NT5E, ecto-5'-nucleotidase; PE, phycoerythrin; PECAM-1, platelet endothelial cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin

4.5.5 Cell passaging and colony forming efficiency assay

Like other types of tissue-specific stem cells, LESCs are known to be capable of self-replication. To test this function in hPSC-LESCs, differentiated cells were enzymatically detached from their substrate, collected by centrifugation, and re-plated onto human placental collagen IV-coated well-plates. Plating densities of 20 000 cells/cm^2 and 5 000 cells/cm^2 were used for cell passaging and colony forming efficiency (CFE) assays, respectively. Re-plated hPSC-LESCs were maintained in the progenitor cell targeted CnT-20 medium. Cells were serially passaged upon reaching sub-confluency, roughly at two-week intervals, until cultures no longer proliferated.

In order to assess their CFE, hPSC-LESCs were cultured in CnT-20 medium for 14 days, after which they were fixed with 4% PFA for 15 min and stained with

0.1% Rhodamine B for 15 min, with PBS washes in between each step. The visibly-stained colonies were counted and CFE calculated as follows:

$$CFE = \frac{\#\ of\ colonies}{\#\ plated\ cells} \times 100\%.$$

4.5.6 Cell proliferation assay

In **Study III**, cell proliferation on bioengineered collagen matrices and human placental collagen IV-coated well-plates was evaluated using the WST-1 Cell Proliferation Assay (Takara Bio Inc., Shiga, Japan). This method is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases in living cells. The formazan produced by viable cells is quantified after 4 h incubation at +37 °C by measuring the absorbance at 450 nm. The protocol is described in more detail in the original publication.

4.5.7 Comparative proteomics

In **Study II**, hESC-LESCs and hiPSC-LESCs were compared with native human CECs and LECs using isobaric tag for relative and absolute quantitation (iTRAQ) proteomics. The detailed protocols for all the following steps are available in the original publication. Briefly, human corneal and limbal epithelial cells were collected from three cadaveric donors as described in Chapter 4.2. Both hPSC lines were differentiated towards LESC-like cells as described in Chapter 4.4, and enzymatically detached from their substrate after 30-35 days in differentiation culture. Protein was extracted from all samples, and equal amounts (25 μg/sample) were digested with Trypsin (AB Sciex, Concord, Canada). Digested peptides were labeled with iTRAQ reagents and analyzed in duplicate by Nano-RPLC-TripleTOF instrumentation using Eksigent 425 NanoLC coupled to high speed TripleTOF™ mass spectrometer (AB Sciex). Raw data processing was carried out in ProteinPilot software (AB Sciex), and all identified proteins were converted to the Universal Protein Knowledgebase (UniProtKB) accession numbers. Data normalization was performed using log transformation and central tendency normalization. Finally, only proteins that were detected in at least two of the biological replicates were used for further biological interpretation.

4.6 Fabrication and characterization of bioengineered matrices

In **Study III**, bioengineered collagen matrices were evaluated as potential carriers for hPSC-LESCs. These matrices were fabricated from medical-grade, high-purity porcine atelo-collagen type I. Briefly, the 18% collagen solution was cross-linked with 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDCM; Sigma-Aldrich) and dicyclohexyl-carbodiimide (DCC; Thermo Fisher Scientific), and molded between glass plates with a 100 μm thick spacer. The resulting matrices were cured at room temperature in 100% humidity chambers for 25 h, and de-molded by immersion in PBS for 1 h. Prior to cell culture experiments, the sheet of bioengineered collagen matrix was cut into round pieces (1 cm in diameter) using a sterile trephine, and the pieces were sterilized by soaking in antibiotic solution (150 U/ml penicillin/streptomycin in PBS), with thorough PBS washes after each step. Finally, each piece of the matrix was placed in a separate well of a 48-well plate, and incubated in CnT-20 cell culture medium overnight at +37 °C, 5% CO_2.

Water content and swelling capacity of bioengineered matrices and research grade human donor corneas (obtained from the Eye Bank of Canada) were calculated by comparing dry and hydrated masses of five replicate samples as follows: $Water\ content = \left(\frac{m_{hydrated} - m_{dry}}{m_{hydrated}}\right) \times 100\%$ and $Water\ uptake = \left(\frac{m_{hydrated} - m_{dry}}{m_{dry}}\right) \times 100\%$. To evaluate the transparency of the matrices, light transmission and scatter measurements were performed for white light and for narrow spectral regions. Finally, the microstructure of the matrix surface and cross-section was visualized using scanning electron microscopy (SEM). The detailed protocols describing the fabrication and characterization methods are available in the original publication (**Study III**).

4.7 Statistical analyses

Mann-Whitney U test was used to assess the statistical significance of differences between culture conditions in gene expression and p63 protein expression data in **Study I**, as well as differences in cell proliferation and Ki67 and p63 protein expression in **Study III**. Analyses were carried out in IBM SPSS Statistics Software, and results were considered significant if $p < 0.05$, and highly significant if $p < 0.01$.

5 Summary of the results

5.1 Differentiation of hPSCs towards LESC-like cells

The aim of **Study I** was to develop an efficient differentiation method for production of corneal epithelial cells and their progenitors from hPSCs. Four different conditions were tested: 1) spontaneous differentiation, 2) commercial medium for corneal epithelial cell culture (CnT-30), 3) small-molecule induction, and 4) induction in unsupplemented medium, both followed by maturation in CnT-30 medium. All four conditions were free from serum and feeder cells. During the first four days (i.e. induction stage), differentiation was carried out in suspension culture as three-dimensional cell aggregates, followed by adherent culture on human placental collagen IV-coated substrate (schematic outline in **Study I**/Figure 1).

Spontaneous differentiation proved to be very inefficient and did not yield detectable amounts of LESCs or CECs. In contrast, pigmented cells were often observed in spontaneous differentiation cultures (**Study I**/Figure 3). Differentiation in the commercial CnT-30 medium was adequate, yet subject to a high degree of variation. Supplementing the induction medium with two small molecule inhibitors (SB-505124 and IWP-2) and bFGF was seen to promote corneal epithelial differentiation. Of all the studied differentiation conditions, small-molecule induction followed by maturation in CnT-30 was deemed superior. This method was therefore utilized in **Studies II** and **III**, modified by replacing RegES medium with 15% KO-SR hPSC medium as the induction medium base.

5.1.1 Cell morphology during differentiation

During the induction stage in suspension culture, three-dimensional cell aggregates would form, and they would then be plated onto cell culture substrate coated with human placental collagen IV. Degree of adhesion to collagen IV was substantially higher after small-molecule induction, than in the other studied conditions (**Study I**/Figure 2). Under these differentiation conditions, cell outgrowths from

the attached aggregates possessed predominantly fibroblast-like cell morphology at first, gradually changing to polygonal morphology typical to epithelial cells, and spontaneously stratifying after prolonged culture (**Study I**/Figure 3). LESC-like cells were typically obtained after approximately 30 days of differentiation. They possessed polygonal morphology and were small in size (**Study III**/Figure 3). Representative phase contrast microscopy images of an undifferentiated hPSC colony, three-dimensional cell aggregates, and differentiated LESC-like cells are presented in Figure 5.

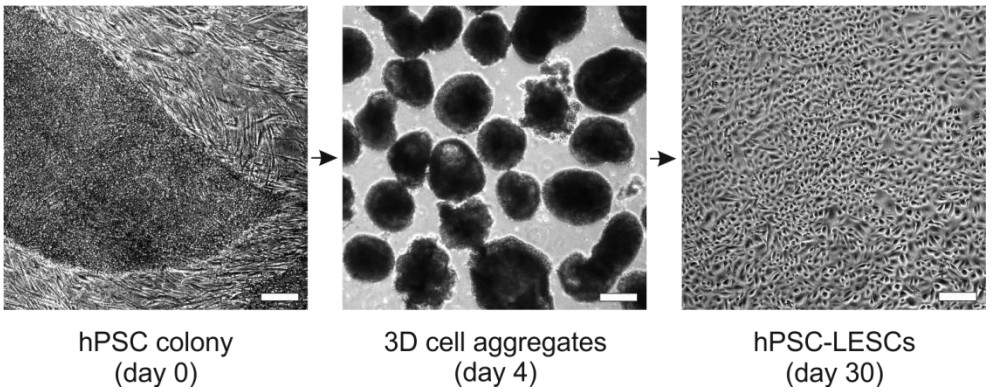


Figure 5. Differentiation of hPSCs towards LESC-like cells. Scale bars 200 μm.

5.1.2 Gene expression during differentiation

Differentiation efficiency was assessed using qPCR, relative to undifferentiated hPSCs (**Study I/**Figures 2 and 6). Undifferentiated hiPSCs (HEL24.3 cell line) expressed endogenous pluripotency markers *OCT3/4*, *NANOG*, *SOX2* and *c-MYC*. Expression of these genes decreased after the four-day induction towards surface ectoderm, and even more so by the end-point of the study at day 44. Gene expression of the eye progenitor and corneal marker *PAX6* increased to about 100-fold after the four-day induction stage, and remained upregulated until the end-point of the study. Gene expression of *OCT3/4* and *PAX6* was analyzed for four additional hPSC lines (A116, Regea08/017, Regea08/023 and Regea11/013), showing similar trends after the four-day induction stage (**Study I**/Figure S1). Transcription factors involved in early eye development (*PITX2*, *BMP4* and *FOX1*) were slightly upregulated after the four-day induction stage, but their expression decreased by the end-point of the study. Finally, LESC-markers *TP63* and *KRT15*,

and corneal epithelial markers *KRT3* and *KRT12* were highly upregulated after 44 days of differentiation. At this time-point, gene expression of *TP63* and *KRT15* was at levels comparable to native human cornea, while *KRT3* and *KRT12* were expressed at substantially lower levels (**Study I**/Figure 6). Small-molecule induction promoted corneal epithelial differentiation, with the most pronounced differences in gene expression compared to undifferentiated hPSCs.

5.1.3 Protein expression during differentiation

Differentiating hPSC-LESCs were analyzed for their protein expression using immunofluorescence (**Studies I**, **II** and **III**) and flow cytometry (**Study II** and unpublished results). Several of the key antibodies were verified by staining paraffin sections of native human corneal tissue. Putative LESC markers p63, CK15 and ABCG2 were localized to the limbus, while CK3 and CK12 were expressed exclusively in central corneal epithelium (Figure 6).

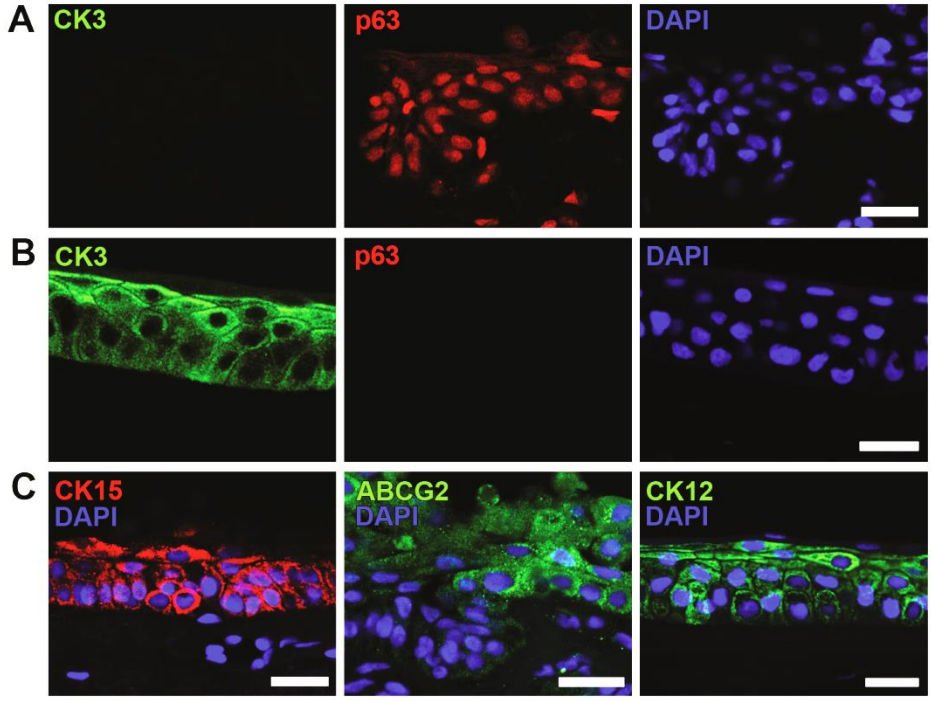


Figure 6. Localization of LESC and mature corneal epithelial markers in native human cornea. CK3 was not detected at the limbus (A), but was strongly expressed in the central corneal epithelium (B), while the opposite was true for p63. CK15 and ABCG2 were expressed at the limbus and CK12 in central corneal epithelium (C). Scale bars 20 μm.

Already after 20 days of differentiation, hiPSC-LESCs (HEL24.3 cell line) were shown to express several putative LESC markers, namely ABCG2, CK15, DG-3, Ki67, p63, and PAX6 (**Study I**/Figure 4). Moreover, the pluripotency marker OCT3/4 was no longer expressed, and CK3 and 12 were faintly visible already at this time-point (**Study I**/Figure 4). On the other hand, LESC-like cells differentiated from UTA.04511.WT and Regea08/017 cell lines did not express CK3 or 12 after 28 days of differentiation, but did express several putative LESC markers (**Study III**/Figure 3). In addition, after 28-35 days of differentiation, co-localization of p63 and TCF4 and a uniform expression of ABCG2 (**Study II**/Figure 6) were observed in hPSC-LESCs (UTA.04511.WT and Regea08/017 cell lines). Protein expression of the ΔNp63α isoform was indirectly verified via co-localization of p40 and p63α proteins (**Study III**/Figure 3). Importantly, protein expression of CK10/13, a marker of epidermal differentiation, was not detected in hPSC-LESCs (**Study III**/Figure 3).

Efficiency of LESC differentiation was assessed by counting cells positively stained for the clinically-relevant p63 protein at ten-day intervals (**Study I**/Figure 5). Small-molecule induction promoted differentiation towards p63-positive LESC-like cells. For HEL24.3 cell line, an average of 50% of cells expressed this protein at day 10, and by day 30 up to 95% of cells were p63-positive. The studied cell lines showed variation in overall differentiation efficiency. For instance, A116 and UTA.04511.WT hiPSC lines yielded an average of 64% p63-positive cells by day 30 (**Study I**/Figure S1 and **Study III**/Figure 3), while only 50% of analyzed cells of Regea08/017 hESC line were clearly positive at this time-point (**Study III**/Figure 4). Additionally, the proliferation marker Ki67 was expressed in approximately 50% of hPSC-LESCs (UTA.04511.WT and Regea08/017 cell lines) after 28-35 days of differentiation (**Study III**/Figure 4).

Additional quantitative measurements of protein expression were obtained using flow cytometry. The putative LESC marker BMI-1 showed an average of 80% and 84% positivity for Regea08/017 and UTA.04511.WT cell lines, respectively (**Study II**/Figure 6). Additionally, protein expression of several cell surface markers was analyzed in hiPSC-LESCs (UTA.04511.WT and HEL24.3 cell lines) and their undifferentiated counterparts (Unpublished results, Figure 7). Most importantly, the pluripotency marker TRA-1-81 was highly expressed in undifferentiated hiPSCs, but virtually undetected in hiPSC-LESCs.

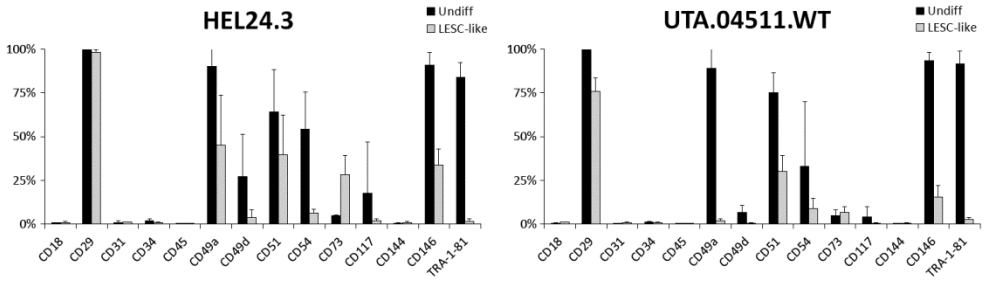


Figure 7. Cell surface marker expression in undifferentiated hiPSCs (undiff) and hiPSC-LESCs

5.1.4 Self-renewal properties of hPSC-LESCs

In order to evaluate whether hPSC-LESCs are capable of self-renewal, the differentiated cells (UTA.04511.WT and Regea08/017 cell lines) were serially passaged in progenitor cell targeted CnT-20 medium. Passaging hPSC-LESCs three times at two-week intervals did not seem to affect their overall cell morphology (Figure 8), and p63 protein expression was maintained in more than 50% of cells.

Additionally, CFE assay was carried out for two hiPSC lines (UTA.04511.WT and HEL24.3). Differentiated hiPSC-LESCs were seeded onto human placental collagen IV-coated well-plates at clonal density (5000 cells/cm^2) in CnT-20 medium. During the two week culture period, colonies of LESC-like cells would appear (Figure 8). The colonies were visualized and counted following Rhodamine B staining. The average CFE of hiPSC-LESCs was 0.029% (±0.02%) and 0.03% (±0.02%) for UTA.04511.WT and HEL24.3 cell lines, respectively.

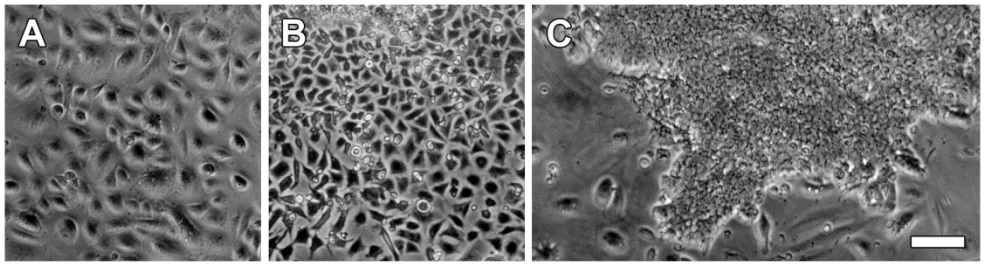


Figure 8. Self-renewal properties of hPSC-LESCs. Representative light microscopy images of hiPSC-LESC morphology before (A) and after (B) passaging. When seeded at clonal density, colony formation was observed (C). Scale bar 100 μm.

5.2 Comparison of hPSC-LESCs with their native counterparts

In **Study II**, high-throughput mass spectrometry-based proteomics were used to compare hPSC-LESCs (UTA.04511.WT and Regea08/017 cell lines) with CECs and LECs obtained from human cadaveric donors (schematic outline in **Study II**/Figure 1). Using this approach, a total of 860 unique proteins expressed in all four samples were identified (**Study II**/Figure 2). Approximately 57% of these proteins were present in at least two of the three biological replicates, and were selected for further analyses. Roughly two thirds of these proteins were similarly expressed in hPSC-derived LESCs and their native counterparts (**Study II**/Figure 3). Identified proteins were grouped according to their function: proteins involved in maintaining stem cell or TAC behavior (i.e. cell cycling, proliferation, differentiation and apoptosis), as well as various niche components of the ocular surface, and corneal and limbal markers (**Study II**/Figures 4-6). Relatively few proteins involved in cell adhesion, immune response or angiogenesis were identified, likely due to the fact that hPSC-LESCs are maintained in far poorer conditions than what native cells are exposed to at the ocular surface. Most importantly, protein expression of CK3 and 12 in hPSC-LESCs was higher than in LECs, but lower than in CECs. The opposite was true for the putative LESC markers CK19, S100A8 and S100A9. Together, these observations indicate that hPSC-LESCs are perhaps more mature than LECs, but less mature than CECs.

The overall protein expression profiles of LESC-like cells obtained from the two hPSC lines were very similar, demonstrating that the differentiation method is highly reproducible and yields homogeneous cell populations. Surprisingly, protein expression profiles of CECs and LECs were also very similar to each other, likely attributable to the innate heterogeneity of limbal cell populations, biological variation between human donors and tissue collection method used in this study. Nevertheless, hPSC-LESCs were clearly similar to the native ocular surface epithelial cells, and possessed LESC-like characteristics.

5.3 Bioengineered matrices as carriers for hPSC-LESCs

In **Study III**, bioengineered matrices fabricated using medical-grade porcine collagen type I were evaluated as carriers for hPSC-LESCs (UTA.04511.WT and Regea08/017 cell lines). These 100 μm thick bioengineered matrices had a parallel

lamellar microstructure and were fully transparent, transmitting over 92% of light at visible wavelengths, and scattering less than 4% (**Study III**/Figures 1 and 2). They also exhibited water content of 91% (±0.2%), and were capable of absorbing water 9.6 times of their dry weight – both measurements slightly higher than those of the native human cornea (**Study III**/Figure 1).

Growth of hPSC-LESCs was supported by the bioengineered matrices in serum-free conditions. Proliferative activity of hPSC-LESCs cultured on the matrices was approximately four times higher than on well-plates coated with human placental collagen IV (**Study III**/Figure 5). Moreover, protein expression of LESC markers p63 and CK15, along with the proliferation marker Ki67 was maintained for at least 30 days in culture on bioengineered matrices (**Study III**/Figure 5). Finally, upon stimulation by the corneal epithelium medium CnT-30, hPSC-LESCs were induced to differentiate, as demonstrated by co-localization of proteins CK3 and 12, yet the construct remained fully transparent (**Study III**/Figure 6). Protein expression of CK3 and 12 was also analyzed using Western blotting, and these proteins were found to be expressed at lower levels in hiPSC-LESCs than in native human LECs (**Study III**/Figure 6).

6 Discussion

6.1 Directed differentiation of hPSCs towards LESCs

The first aim of this dissertation was to optimize a directed and efficient differentiation method, minimizing the use of undefined and xenogeneic components. Differentiation efficiency of several hPSC lines towards corneal epithelial cell lineage was evaluated by following gene and protein expression of several putative LESC markers, as well as CK3 and 12 – both specific to mature corneal epithelium.

Spontaneous differentiation in a cell culture medium lacking inductive molecules did not yield detectable amounts of CECs or their progenitors, but rather resulted in heterogeneous cell populations. It is known that the default pathway of hPSC differentiation is towards neuroectoderm (Vallier et al., 2004), and even that is subject to a high degree of variation between cell lines (Osafune et al., 2008; Toivonen et al., 2013). Being a derivative of the surface ectoderm, a directed differentiation method is likely needed in order to obtain LESC-like cells. To date, most of the available corneal epithelial differentiation methods rely on the use of niche components, such as conditioned medium (Ahmad et al., 2007; Brzeszczynska et al., 2014; Shalom-Feuerstein et al., 2012; Zhu et al., 2013), hAM (Sareen et al., 2014), or Bowman's membrane (Hanson et al., 2013). The reasoning behind using these culture components is to mimic the ocular surface environment, thereby providing appropriate signals to drive hPSC differentiation towards corneal epithelial cell fate. Although these methods are relatively successful in producing CECs, their reproducibility and scalability suffer due to the biological variability of such undefined components. Primary LESCs and hLFs secrete proteins that affect cell growth and proliferation (Shimmura et al., 2006; Wright et al., 2013b), which may vary depending on the culture conditions. This makes the differentiation methods using conditioned medium subject to batch-to-batch variation. Similarly, there is variation among hAM obtained from different donors in respect to growth factor secretion (Hopkinson et al., 2006). Furthermore, the handling and processing of hAM prior cell seeding affects cell viability and proliferation (Shortt et al., 2009). Finally, culture components of

animal origin, such as fetal bovine serum (FBS), pose a risk of infection by nonhuman pathogens, and incorporation of immunogenic nonhuman sialic acids (Hoffman & Carpenter, 2005; Martin et al., 2005). Overall, standardized conditions for LESC differentiation and culture are needed in order to obtain cell populations applicable to the clinical setting.

In this dissertation, hPSC differentiation was induced towards surface ectoderm by mimicking the early eye development. As described in Chapter 2.1.1, corneal epithelial development involves activation of FGF signaling, as well as inhibition of the canonical Wnt signaling pathway (Dhouailly et al., 2014; Zhang et al., 2015). These mechanisms were replicated *in vitro* using two small molecule inhibitors and the recombinant growth factor bFGF. This induction was shown to promote early-stage differentiation by down-regulating pluripotency markers and up-regulating *PAX6* and several surface ectodermal transcription factors. Moreover, expression of LESC markers at later stages of differentiation was also enhanced by the small molecule induction. Plating the three-dimensional cell aggregates onto human placental collagen IV coupled with the transition to corneal epithelium medium CnT-30 was aimed at further directing differentiation towards LESC-like cells. Collagen type IV was chosen for two reasons: it is one of the main components of the corneal epithelial basement membrane (Torricelli et al., 2013), and there is evidence of LESCs preferentially adhering to type IV collagen (Bian et al., 2010; Li et al., 2005). The commercial cell culture medium was used in order to provide a corneal epithelial environment to the differentiating cells without the use of conditioned medium. Successful differentiation of hPSC-LESCs was verified through gene and protein expression of several markers, as well as appropriate cell morphology and spontaneous stratification upon prolonged culture.

Ideally, LESC-like cells would be differentiated from hPSCs in entirely xeno-free and chemically-defined conditions. In this work, although differentiation is carried out in the absence of a biological substrate or serum, there are several issues which could still be addressed. First of all, hPSCs used in this study were maintained on hFF feeder cells, in undefined culture conditions. It remains to be seen whether or not hPSCs maintained in feeder-independent and chemically-defined conditions behave differently and require a modified differentiation method. Secondly, the recombinant bFGF added to the induction medium is a growth factor, which could be replaced with a small molecular compound. The use of chemically-defined small molecules rather than growth factors is generally more affordable and reliable as they tend to be more specific in their mode of action. Thirdly, human placental collagen IV is used as a coating for adherent culture, and

it is subject to batch-to-batch variation. Various synthetic coatings incorporating specific binding sequences are currently on the market, and they may provide a more optimal culture substrate than human placental collagen (Villa-Diaz et al., 2013). Finally, although the corneal epithelium medium CnT-30 is chemically-defined, it does contain unspecified animal-derived components. The novel simplified and xeno-free media CnT-Prime and CnT-Prime-2D, manufactured by the same company, could be tested to see if they could replace CnT-30. Overall, further studies are needed to refine the differentiation method in fully-defined and xeno-free conditions.

6.2 Characteristics of hPSC-derived LESCs

After adhering to the human placental collagen IV coating, cell migration and outgrowth from three-dimensional cell aggregates was primarily fibroblast-like. Upon reaching confluence, cells would obtain the compact epithelial morphology similar to that of primary LESCs. In this dissertation, LESC-like cells were obtained from hPSCs within approximately 30 days of differentiation. At this time-point, protein expression of the clinically significant marker p63 was at its highest, decreasing slightly by day 44 (**Study I**). Furthermore, the putative LESC marker BMI-1 was expressed in over 80% of cells at this time-point (**Study II**), and positive expression of ABCG2, CK15, DG-3, p40, p63α, PAX6, and TCF4 was assessed qualitatively (**Studies I, II** and **III**). Meanwhile, protein expression of an epidermal differentiation marker CK10/13 was not detected. Gene and protein expression of CK3 and 12, markers specific to mature corneal epithelium, was observed by day 44 in differentiation, and faint protein expression was detected already at day 20 (**Study I**). Confirming positive expression of several putative LESC markers is essential, because there is currently no known marker capable of distinguishing between LESCs and early-stage TACs. Importantly, co-localization of p40 (i.e. ΔNp63) and p63α proteins indirectly verifies expression of the ΔNp63α isoform. Judging by their gene and protein expression profiles, hPSC-derived cells obtained in this work do in fact possess LESC-like characteristics, although it cannot be said with certainty whether they are closer to true LESCs or early-stage progenitor cells.

To compare, the first study demonstrating successful differentiation of CECs from hESCs reported p63 expression to peak after six days of differentiation, yielding 15-25% positive cells (Ahmad et al., 2007). However, close to 60% of cells

were also expressing CK3/12 at the same time-point, and gene and protein expression of CK10 was also detected in the same cultures, suggesting that skin-like epithelial cells are also obtained using this differentiation method. Similarly, other differentiation studies carried out in the presence of conditioned medium also show that hPSC-derived cells bypass the LESC-like state fairly early on and express primarily CK3 and 12 after roughly two weeks of differentiation (Brzeszczynska et al., 2014; Shalom-Feuerstein et al., 2012). In addition, two separate studies found that hiPSCs derived from limbal epithelium have a much higher propensity for corneal epithelial differentiation than hiPSCs derived from hDFs (Hayashi et al., 2012; Sareen et al., 2014). In particular, using mouse feeder cells to differentiate hiPSCs derived from hDFs for 12-16 weeks yielded RPE and lens epithelial cells in addition to low amounts of CK12 and 14 positive cell colonies (Hayashi et al., 2012). Using a mixture of fibronectin, type IV collagen and laminin as an ECM coating was also fairly inefficient at inducing corneal differentiation of hiPSCs derived from hDFs: after two weeks of culture only 20-30% of cells expressed CK14 and 15, and close to 10% expressed ΔNp63 (Sareen et al., 2014). For comparison, hiPSCs derived from limbal epithelium generated about 60% CK14 and 15 positive cells, and close to 20% ΔNp63 positive cells under identical culture conditions. Lastly, differentiation on hAM or denuded human cornea was more effective, supporting the premise that a LESC-like niche microenvironment plays an important role in guiding hPSC differentiation (Sareen et al., 2014). Nevertheless, validation of LESCs is challenging because their identity has not been clearly defined. This is a hindrance especially when considering LESC-like cells derived from another cell type, such as somatic cells, adult stem cells or hPSCs. Thus, there is a need for a consensus regarding the basic characteristics and qualities that are sufficient for identification of LESC-like cells.

6.2.1 Cell surface marker expression

Protein expression of several cell surface markers was analyzed using flow cytometry in LESC-like cells differentiated from two hiPSC lines, and compared to their undifferentiated counterparts (unpublished results). Most importantly, the pluripotency marker TRA-1-81 was expressed in 73–97% (seven biological replicates) of undifferentiated hiPSCs, and in 0.6–3.6% (six biological replicates) of hiPSC-LESCs after 30-35 days of differentiation (Figure 7). In order to ensure safety of cell-based therapy, it is important that hPSC-LESCs do not contain

potentially tumorigenic pluripotent cells. Recently, a novel strategy to eliminate pluripotent cells from potentially heterogeneous cell populations has been introduced, where a small molecular compound selectively eliminates undifferentiated hPSCs by inhibiting oleic acid biosynthesis (Ben-David et al., 2013). Alternatively, enrichment of LESC-like cell populations can be implemented via cell sorting, utilizing a LESC-specific cell surface marker such as ABCB5 or ABCG2. Tumorigenic potential of hPSC-derived cells, whether or not they have been enriched or purified, could be assessed *in vivo* using immune-deficient rodent models (Kanemura et al., 2014).

Integrins are cell adhesion molecules essential for cell attachment to various ECM proteins. Different types of cells express different integrins, and primary LESCs have previously been shown to express integrins α1, α2, α6, β1 and β4 (Albert et al., 2012; Vereb et al., 2013). To compare, hiPSC-LESCs expressed high levels of integrin β1, and moderate levels of integrin αV, while protein expression of integrins α1 and α4 was low and variable between replicates (Figure 7). Moreover, the MSC markers CD31, CD34 and CD45, as well as the leukocyte cell adhesion molecule integrin β2 and VE-cadherin were not expressed in hiPSC-LESCs. Finally, protein expression of ICAM-1, CD73, c-kit and MCAM was quite low, and varied between replicates. MCAM and c-kit were previously detected in primary LESCs, but not in mature CECs, while the opposite was true for ICAM-1 expression (Vereb et al., 2013). The expression differences between hPSC-LESCs derived and analyzed in this dissertation and primary LESCs reported by other laboratories may be explained by the poor serum-free culture conditions that hPSC-LESCs are differentiated and maintained in, coupled with the lack of interactions with other cell types and ocular niche factors. Further research using animal models or *ex vivo* organotypic culture is needed to see whether or not this changes if hPSC-LESCs are transplanted onto the ocular surface.

6.2.2 Self-renewal and proliferation

LESCs are tissue-specific stem cells, and therefore have a capacity for producing cell generations, and a potential for self-renewal. To test their proliferative capacity *in vitro*, hPSC-LESCs were serially passaged at roughly two-week intervals in a serum-free progenitor cell targeted medium CnT-20. The LESC-like cells retained their morphology for at least three passages, and p63 expression was maintained in over 50% of cells. Further passaging of hPSC-LESCs was not attempted, and could

be tested in the future. Primary LESCs cultured on mouse feeder cells in a medium containing FBS have been shown to maintain their phenotype for up to 14 passages before reaching senescence (Pellegrini et al., 1999). However, frequent enzymatic dissociation subjects cells to stress and may induce chromosomal aberrations (Bai et al., 2015; Hoffman & Carpenter, 2005). Therefore, extensive cell passaging may not be desirable in practice, especially if cell therapy is the target.

The CFE assay is an *in vitro* functionality test commonly used for evaluating the self-renewal properties of a cell population. Generally, single-cell suspensions are plated onto mitotically inactivated feeder cells at clonal densities, although the assay has also been carried out in feeder-independent conditions, on ECM coatings (Albert et al., 2012). There is a high degree of variation in the average CFE values for primary LESCs among different laboratories, which is likely caused by discrepancies in isolation and culture methods (Albert et al., 2012; Kolli et al., 2010; Li et al., 2005; Pellegrini et al., 1999). Also, only a small amount of cells at the limbus are authentic LESCs, while most are considered to be TACs of varying maturity levels (Pellegrini et al., 1999). In this work, because hPSC-LESCs were differentiated and cultured in the absence of feeder cells, the CFE assay was performed using human placental collagen IV coating. The values obtained for LESCs differentiated from two hiPSC lines in serum-free conditions are low, and would require a more careful testing and validation. It remains to be seen whether purifying hPSC-LESC populations or enriching the culture conditions for the duration of the assay would enhance their CFE. For instance, using feeder cells or adding serum to the culture medium may in fact create a more favorable microenvironment and promote the clonal growth of hPSC-LESCs.

6.2.3 Comparison with native corneal and limbal epithelia

High-throughput characterization methods are generally more informative than conventional characterization techniques, allowing a broader analysis of target cell populations. Therefore, mass spectrometry-based iTRAQ proteomics was used to compare hPSC-LESCs with their native counterparts obtained directly from the ocular surface of cadaveric human donors. **Study II** was the first study to utilize a high-throughput proteomics approach for hPSC-LESC characterization. A total of 860 unique proteins present in all samples were identified, including various LESC niche components, proteins involved in cell cycling, proliferation, differentiation and apoptosis, and most importantly corneal and limbal markers. Judging by their

overall protein expression profiles, it appears that hPSC-LESCs fall in between LESCs and terminally-differentiated CECs. To compare, 2737 proteins have been previously identified in the corneal epithelium, yet only a fraction of them was quantified (Dyrlund et al., 2012). The limitation of the iTRAQ method is that it is only capable of detecting proteins present in all analyzed samples. Therefore, proteins expressed exclusively in the native ocular surface epithelial cells, or exclusively in hPSC-LESCs, were not detected. This explains why only a few proteins involved in angiogenesis or immune response were identified in this study – hPSC-LESCs lack interactions with other cell types or blood vessels, and are not exposed to pathogens in the same way as the ocular surface *in vivo*. Mechanisms such as angiogenesis and immune response are therefore not necessarily needed, and this is reflected in the protein expression profile. In the future, it would be interesting and important to characterize entire proteomes of hPSC-LESCs and native ocular surface epithelial cells, to better assess the differences between these cell populations. Nevertheless, iTRAQ proteomics did reveal clear similarities between hPSC-LESCs and their native counterparts, providing valuable information for further studies.

6.3 Bioengineered collagen matrix as hPSC-LESC carrier

Transplantation of an epithelial cell sheet to the ocular surface requires a supportive carrier. Collagen is the most abundant structural component of the corneal stroma, and therefore has been widely researched in attempts to provide a better alternative to hAM. Collagen is also biodegradable, possesses low immunogenicity and has shown promising results *in vitro* and *in vivo*, as a cell-free scaffold, or in combination with primary LESCs (Chae et al., 2015; Fagerholm et al., 2014; Levis et al., 2013). Conventional collagen hydrogels are fairly soft due to high water content, requiring plastic compression or chemical cross-linking to enhance their mechanical strength (Ahn et al., 2013; Levis et al., 2010; Mi et al., 2010).

The aim of **Study III** was to evaluate the suitability of a cross-linked collagen hydrogel fabricated as thin membranes to act as a carrier for hPSC-derived LESCs. The bioengineered matrix possessed excellent optical properties, had high water content and was mechanically stable yet elastic. The cross-linking agents used during fabrication do not become incorporated into the hydrogel, eliminating the risk of toxic degradation products being released into the tissue (Ahn et al., 2013).

The biocompatibility of the similarly-fabricated, yet thicker bioengineered matrices was previously verified *in vivo*, by implanting cell-free matrices into the corneal stroma of rabbits (Koulikovska et al., 2015). Although the collagen used for fabrication of these matrices is of porcine origin, and immunosuppressive medication was not used post-operatively, no adverse immune reaction was observed. This offers hope for possible clinical applications, as long as the matrix production is standardized and carefully monitored according to GMP standards. The *in vitro* study presented as part of this dissertation demonstrated that the bioengineered matrix supports the adhesion and proliferation of hPSC-LESCs in serum-free conditions. Further studies are needed to assess the performance of this tissue engineered construct *in vivo*, using animal models. In addition, immunogenic properties of hPSC-LESCs and bioengineered matrix could be studied using various *ex vivo* assays, such as mixed lymphocyte culture, lymphocyte transformation tests or the enzyme-linked immunospot assay, which is able to detect cytokine production on a single-cell level (Lindemann, 2014). Finding a suitable surgical technique for transplantation onto the ocular surface will also require attention. Ideally, this type of tissue engineered construct combining a sufficiently thick carrier with LESC-like cells could serve as a replacement for damaged corneal stroma, while providing a self-renewing source of LESCs and corneal epithelium.

6.4 Future perspectives

The field of regenerative medicine is relatively young – the first hESC lines were derived in 1998 (Thomson et al., 1998) and the first hiPSC lines were generated in 2007 (Takahashi et al., 2007). However, both cell types have rapidly progressed towards cell-based therapies in the recent years. Transplantation of hPSC-derived LESC-like cells could be possible in the future, yet several issues need to be addressed to ensure the high quality and safety of the approach.

Although the native corneal epithelium is composed of four to six cell layers, evidence supporting transplantation of LESC-like cell monolayers is rapidly accumulating. First of all, clinical studies show that a monolayer of primary LESCs transplanted on hAM is capable of differentiating and stratifying on the ocular surface post-transplantation (Kolli et al., 2010; Shortt et al., 2008). Secondly, a recent *in vitro* study investigating the effects of air-lifting on cell functionality revealed that primary LESCs lose their ability to re-epithelialize a wounded area

upon stratification (Massie et al., 2014). And finally, culturing human limbal explants at an air-liquid interface was shown to induce squamous metaplasia – abnormal epidermal differentiation confirmed by co-localization of the corneal CK12 and epidermal CK10 (Li et al., 2008). Taken together, these results suggest that *in vitro* stratification of LESC-like cells prior transplantation may in fact be disadvantageous. Nevertheless, the ability to differentiate and give rise to the stratified corneal epithelium is a key characteristic of LESCs, and it may therefore be a requirement to demonstrate the functionality of hPSC-derived LESCs pre-clinically. However, the eye likely provides a more optimal environment to induce cell differentiation and stratification than an *in vitro* culture system, due to the various niche components and growth factors present at the ocular surface (Bolanos-Jimenez et al., 2015; Ordonez & Di Girolamo, 2012).

The interactions between the corneal epithelium and the underlying corneal stroma may play an important role in maintaining corneal integrity. For instance, incorporating hLFs into plastically-compressed collagen hydrogels was shown to enhance the production of basement membrane components by LESCs (Levis et al., 2010). However, the ability of LESCs to re-epithelialize a wounded area was not affected by hLF incorporation (Massie et al., 2014). Interestingly, an *in vitro* study conducted in a different laboratory demonstrated that human corneal epithelial cells only stratified if human corneal fibroblasts were incorporated in the culture system (Kobayashi et al., 2015b). The conflicting results are possibly due to the differences in cell isolation and culture methods. Furthermore, the recently identified limbal niche cells, also known as limbal mesenchymal cells, were shown to support LESCs in co-culture, emphasizing the importance of a niche microenvironment for LESC function (Li et al., 2014; Nakatsu et al., 2014). Alternatively, co-culture and co-transfer of mouse LESCs with bone marrow-derived MSCs was shown to inhibit local inflammatory reactions and support the healing process in a mouse model (Zajicova et al., 2010). MSCs may be beneficial even when administered systemically. Studies using mouse models have shown that MSCs possess the ability to migrate to the inflamed ocular surface and suppress inflammation, thereby improving allograft survival (Lan et al., 2012; Oh et al., 2012; Omoto et al., 2014). It remains to be seen whether similar effects can be achieved in human patients. More research is needed to determine the optimal strategy for hPSC-LESC transplantation and whether stromal cells are needed either as part of the graft, or administered systemically.

In addition to regenerative medicine, hPSCs offer novel opportunities for tissue modeling and drug development. More specifically, hPSC-derived LESCs or fully

stratified corneal epithelial constructs could be used as an *in vitro* model to study drug absorption, permeability and transport (Vellonen et al., 2014). Traditionally, animal models, most commonly rabbits, are used to evaluate ocular drug absorption and chemical irritation. For instance, the Draize eye irritation test performed on rabbits has been widely criticized due to its lack of reproducibility, overestimation of human responses, and animal cruelty (Bartok et al., 2015). Alternative *ex vivo* models using porcine corneas have been developed to study transcorneal drug permeation and predict eye irritation of cosmetic ingredients (Pescina et al., 2015; Van den Berghe et al., 2005). Moreover, various *in vitro* cell culture models have been established, and there are currently two commercially-available tissue models for ocular toxicity and irritation studies: SkinEthic™ Reconstructed Human Corneal Epithelium (EpiSkin, Lyon, France) and EpiOcular™ (MatTek Corporation, Ashland, MA). Utilizing primary and immortalized cell lines have shown promise as a possible alternative to the Draize test (Bartok et al., 2015; Reichl, 2008), and hPSC-derived corneal epithelium could be evaluated in a similar way. Ideally, an *in vitro* model for permeation and toxicity studies should exhibit a multilayered structure with tight junctions and barrier properties similar to that of the native corneal epithelium. For this purpose, differentiation and culture does not necessarily need to be carried out in chemically-defined and xeno-free conditions, as long as a functional corneal epithelium-like structure is obtained.

To conclude, the novel tissue engineering approach described in this dissertation provides a valuable and clinically relevant treatment strategy for ocular surface reconstruction. The method could be translated to the clinic after further optimization and testing in animal models in accordance with the regulatory guidelines for ATMPs defined by EMA.

7 Conclusions

The aim of this dissertation was to examine the ability of several hPSC lines to differentiate towards LESC-like cells capable of self-renewal and terminal differentiation. LESC differentiation was carried out in the absence of feeder cells and serum, in order to minimize biological variation and improve reproducibility. The resulting hPSC-derived LESCs were characterized and compared with native ocular surface epithelial cells using high-throughput proteomics. Finally, a bioengineered collagen matrix was evaluated as a possible carrier for transplantation of hPSC-derived LESCs, cultured in serum-free conditions. Based on the results of these studies, the following conclusions can be drawn:

1. Several hPSC lines were successfully differentiated towards LESCs.

    - Spontaneous differentiation did not yield detectable amounts of LESCs.
    - Mimicking *in vivo* corneal development using two small-molecule inhibitors along with bFGF promoted corneal epithelial differentiation.
    - Small-molecule induction followed by maturation in a commercial corneal epithelium medium CnT-30 resulted in efficient and reproducible differentiation of LESC-like cells.

2. Human PSC-derived LESCs possessed appropriate cell morphology, gene and protein expression, and were capable of both self-renewal and terminal differentiation – features typical to authentic LESCs.

3. Comparative proteomics revealed a total of 860 unique proteins that hPSC-derived LESCs have in common with their native counterparts. Their overall protein expression profile demonstrated a similarity between the cell types, and strengthened the evidence for LESC-like properties of hPSC-derived LESCs.

4. Bioengineered collagen matrices supported the growth of hPSC-derived LESCs in serum-free conditions *in vitro*, showing potential for use as a transplantable carrier of these cells in clinical applications. However, further *in vivo* testing using animal models will be necessary to definitively test the functionality of hPSC-derived LESCs.

Acknowledgements

The research for this dissertation was carried out at the Institute of Biosciences and Medical Technology (BioMediTech), University of Tampere, during the years 2011-2015. I am grateful to the Dean of the institute Hannu Hanhijärvi for maintaining excellent research facilities and a collaborative working environment during my studies.

I would like to thank the Doctoral Program in Biomedicine and Biotechnology at the University of Tampere, the University of Tampere Foundation, the Finnish Funding Agency for Technology and Innovation (TEKES), the Finnish Eye and Tissue Bank Foundation, the Emil Aaltonen Foundation, the Foundation Supporting Research in Tampere, and the Finnish Concordia Fund for financially enabling my research and journeys to international conferences.

Most importantly, I am deeply grateful to my supervisors Associate Professor Heli Skottman and Tanja Ilmarinen, PhD. Both of you are an inspiration and I greatly admire your expertise, enthusiasm, and overall passion for science. Heli, you introduced me to the fascinating world of stem cells already during my undergraduate studies, and afterwards gave me the opportunity to work with the corneal differentiation project. Thank you for granting me scientific freedom, yet always finding the time to provide guidance. Tanja, thank you for all the help, reassurance and optimism during these years – they have been invaluable for this dissertation and my sanity. Also, your multitasking skills are incredible, and deserve special recognition.

The members of my thesis committee Professor Hannu Uusitalo and Docent Susanna Miettinen are warmly thanked for the encouraging annual meetings and thought-provoking discussions. I also owe my gratitude to Professor Juha Holopainen and Docent Frederic Michon for finding the time to review this dissertation and providing constructive feedback to improve its quality. I am thankful to my co-authors Ulla Aapola, Roger Beuerman, Antti Jylhä, Janika Nättinen, Goran Petrovski, Mehrdad Rafat, Anjula Ratnayake, Jochen Rieck, and Zoltán Veréb – without your contributions to the publications, this dissertation would not have been possible.

The entire staff of BioMediTech, especially the regenerative medicine research groups, is thanked for such a friendly working environment. Regea Cell and Tissue Center personnel, particularly Annika Hakamäki and Minna Sjöblom, are thanked for providing human donor corneas for research during this dissertation, and Marja-Leena Koskinen is acknowledged for skillful paraffin embedding and sectioning of these corneas.

I wish to thank the past and present members of the Eye Group – it's been a pleasure to work with all of you! Outi Heikkilä, Outi Melin and Hanna Pekkanen, thank you for technical assistance and more importantly for outstanding company in the cell culture lab. Kati Juuti-Uusitalo, thank you for scientific advice during these years. Hanna Hiidenmaa and Heidi Hongisto, thank you both for supervising me back in the days of my undergraduate studies and showing me that research (especially in the right company) can be fun. Also special thanks to Heidi for all the help and support while I was writing this thesis, and sorry for all the distractions I caused you! Anni Sorkio – the other half of the A-team – thank you for sharing the fun times, existential crises, and cheesecake. You are a wonderful person and an amazing friend.

Thanks to my friends from the university world for the annual picnics, sangria evenings and other traditions, and for sharing the ups and downs of life as a researcher. Thanks to "the gang" for your friendship and silliness, and for reminding me that there's more to life than science. And last but not least, I am eternally grateful to my family for encouraging me in all my endeavors, and to Johan for the much needed moral support and understanding during these stressful times. I am lucky to have you in my life.

Tampere, December 2015

Alexandra

References

Ahmad, S. (2012). Concise review: limbal stem cell deficiency, dysfunction, and distress. Stem Cells Transl. Med. *1,* 110-115.

Ahmad, S., Stewart, R., Yung, S., Kolli, S., Armstrong, L., Stojkovic, M., Figueiredo, F., and Lako, M. (2007). Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells *25,* 1145-1155.

Ahn, J.I., Kuffova, L., Merrett, K., Mitra, D., Forrester, J.V., Li, F., and Griffith, M. (2013). Crosslinked collagen hydrogels as corneal implants: effects of sterically bulky vs. non-bulky carbodiimides as crosslinkers. Acta Biomater. *9,* 7796-7805.

Albert, R., Vereb, Z., Csomos, K., Moe, M.C., Johnsen, E.O., Olstad, O.K., Nicolaissen, B., Rajnavolgyi, E., Fesus, L., Berta, A., and Petrovski, G. (2012). Cultivation and characterization of cornea limbal epithelial stem cells on lens capsule in animal material-free medium. PLoS One *7,* e47187.

Amescua, G., Atallah, M., Nikpoor, N., Galor, A., and Perez, V.L. (2014). Modified simple limbal epithelial transplantation using cryopreserved amniotic membrane for unilateral limbal stem cell deficiency. Am. J. Ophthalmol. *158,* 469-475.e2.

Amitai-Lange, A., Altshuler, A., Bubley, J., Dbayat, N., Tiosano, B., and Shalom-Feuerstein, R. (2015). Lineage tracing of stem and progenitor cells of the murine corneal epithelium. Stem Cells *33,* 230-239.

Bai, Q., Desprat, R., Klein, B., Lemaitre, J.M., and De Vos, J. (2013). Embryonic stem cells or induced pluripotent stem cells? A DNA integrity perspective. Curr. Gene Ther. *13*, 93-98.

Bai, Q., Ramirez, J.M., Becker, F., Pantesco, V., Lavabre-Bertrand, T., Hovatta, O., Lemaitre, J.M., Pellestor, F., and De Vos, J. (2015). Temporal analysis of genome alterations induced by single-cell passaging in human embryonic stem cells. Stem Cells Dev. *24,* 653-662.

Bartok, M., Gabel, D., Zorn-Kruppa, M., and Engelke, M. (2015). Development of an in vitro ocular test system for the prediction of all three GHS categories. Toxicol. in. Vitro. *29,* 72-80.

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

Beers, J., Linask, K.L., Chen, J.A., Siniscalchi, L.I., Lin, Y., Zheng, W., Rao, M., and Chen, G. (2015). A cost-effective and efficient reprogramming platform for large-scale production of integration-free human induced pluripotent stem cells in chemically defined culture. Sci. Rep. *5,* 11319.

Ben-David, U., Gan, Q.F., Golan-Lev, T., Arora, P., Yanuka, O., Oren, Y.S., Leikin-Frenkel, A., Graf, M., Garippa, R., Boehringer, M., Gromo, G., and Benvenisty, N. (2013). Selective elimination of human pluripotent stem cells by an oleate synthesis inhibitor discovered in a high-throughput screen. Cell. Stem Cell. *12,* 167-179.

Bhalekar, S., Basu, S., Lal, I., and Sangwan, V.S. (2013). Successful autologous simple limbal epithelial transplantation (SLET) in previously failed paediatric limbal transplantation for ocular surface burns. BMJ Case Reports bcr2013009888.

Bian, F., Liu, W., Yoon, K.C., Lu, R., Zhou, N., Ma, P., Pflugfelder, S.C., and Li, D.Q. (2010). Molecular signatures and biological pathway profiles of human corneal epithelial progenitor cells. Int. J. Biochem. Cell Biol. *42,* 1142-1153.

Blazejewska, E.A., Schlotzer-Schrehardt, U., Zenkel, M., Bachmann, B., Chankiewitz, E., Jacobi, C., and Kruse, F.E. (2009). Corneal limbal microenvironment can induce transdifferentiation of hair follicle stem cells into corneal epithelial-like cells. Stem Cells *27,* 642-652.

Bobba, S., Chow, S., Watson, S., and Di Girolamo, N. (2015). Clinical outcomes of xeno-free expansion and transplantation of autologous ocular surface epithelial stem cells via contact lens delivery: a prospective case series. Stem Cell. Res. Ther. *6,* 23.

Bolanos-Jimenez, R., Navas, A., Lopez-Lizarraga, E.P., de Ribot, F.M., Pena, A., Graue-Hernandez, E.O., and Garfias, Y. (2015). Ocular surface as barrier of innate immunity. Open Ophthalmol. J. *9,* 49-55.

Bray, L.J., George, K.A., Hutmacher, D.W., Chirila, T.V., and Harkin, D.G. (2012). A dual-layer silk fibroin scaffold for reconstructing the human corneal limbus. Biomaterials *33,* 3529-3538.

Brzeszczynska, J., Samuel, K., Greenhough, S., Ramaesh, K., Dhillon, B., Hay, D.C., and Ross, J.A. (2014). Differentiation and molecular profiling of human embryonic stem cell-derived corneal epithelial cells. Int. J. Mol. Med. *33,* 1597-1606.

Chae, J.J., Ambrose, W.M., Espinoza, F.A., Mulreany, D.G., Ng, S., Takezawa, T., Trexler, M.M., Schein, O.D., Chuck, R.S., and Elisseeff, J.H. (2015). Regeneration of corneal epithelium utilizing a collagen vitrigel membrane in rabbit models for corneal stromal wound and limbal stem cell deficiency. Acta Ophthalmol. *93,* e57-66.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., *et al.* (2011). Chemically defined conditions for human iPSC derivation and culture. Nat. Methods *8,* 424-429.

Collomb, E., Yang, Y., Foriel, S., Cadau, S., Pearton, D.J., and Dhouailly, D. (2013). The corneal epithelium and lens develop independently from a common pool of precursors. Dev. Dyn. *242,* 401-413.

Connon, C.J., Doutch, J., Chen, B., Hopkinson, A., Mehta, J.S., Nakamura, T., Kinoshita, S., and Meek, K.M. (2010). The variation in transparency of amniotic membrane used in ocular surface regeneration. Br. J. Ophthalmol. *94,* 1057-1061.

Database of clinical trials conducted around the world. Available at https://clinicaltrials.gov/. *Accessed: September 23, 2015.*

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

de la Mata, A., Nieto-Miguel, T., Lopez-Paniagua, M., Galindo, S., Aguilar, M.R., Garcia-Fernandez, L., Gonzalo, S., Vazquez, B., Roman, J.S., Corrales, R.M., and Calonge, M. (2013). Chitosan-gelatin biopolymers as carrier substrata for limbal epithelial stem cells. J. Mater. Sci. Mater. Med. *24,* 2819-2829.

de Rotth, A. (1940). Plastic repair of conjunctival defects with fetal membranes. Arch. Ophthalmol. *23*, 522-525.

DelMonte, D.W., and Kim, T. (2011). Anatomy and physiology of the cornea. J. Cataract Refract. Surg. *37,* 588-598.

Deshpande, P., Ramachandran, C., Sefat, F., Mariappan, I., Johnson, C., McKean, R., Hannah, M., Sangwan, V.S., Claeyssens, F., Ryan, A.J., and MacNeil, S. (2013). Simplifying corneal surface regeneration using a biodegradable synthetic membrane and limbal tissue explants. Biomaterials *34,* 5088-5106.

Dhouailly, D., Pearton, D.J., and Michon, F. (2014). The vertebrate corneal epithelium: from early specification to constant renewal. Dev. Dyn. *243,* 1226-1241.

Di Girolamo, N., Bobba, S., Raviraj, V., Delic, N.C., Slapetova, I., Nicovich, P.R., Halliday, G.M., Wakefield, D., Whan, R., and Lyons, J.G. (2015). Tracing the fate of limbal epithelial progenitor cells in the murine cornea. Stem Cells *33,* 157-169.

Djalilian, A.R., Mahesh, S.P., Koch, C.A., Nussenblatt, R.B., Shen, D., Zhuang, Z., Holland, E.J., and Chan, C. (2005). Survival of donor epithelial cells after limbal stem cell transplantation. Invest. Ophthalmol. Vis. Sci. *46,* 803-807.

Dolgin, E. (2015). Next-generation stem cell therapy poised to enter EU market. Nat Biotech *33,* 224-225.

Dua, H.S., and Azuara-Blanco, A. (2000). Limbal stem cells of the corneal epithelium. Surv. Ophthalmol. *44,* 415-425.

Dua, H.S., Miri, A., and Said, D.G. (2010). Contemporary limbal stem cell transplantation - a review. Clin. Experiment. Ophthalmol. *38,* 104-117.

Dua, H.S., Shanmuganathan, V.A., Powell-Richards, A.O., Tighe, P.J., and Joseph, A. (2005). Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. Br. J. Ophthalmol. *89,* 529-532.

Dua, H.S., Miri, A., Alomar, T., Yeung, A.M., and Said, D.G. (2009). The role of limbal stem cells in corneal epithelial maintenance: testing the dogma. Ophthalmology *116,* 856-863.

Dyrlund, T.F., Poulsen, E.T., Scavenius, C., Nikolajsen, C.L., Thogersen, I.B., Vorum, H., and Enghild, J.J. (2012). Human cornea proteome: identification and quantitation of the proteins of the three main layers including epithelium, stroma, and endothelium. J. Proteome Res. *11,* 4231-4239.

Espana, E.M., Sun, M., and Birk, D.E. (2015). Existence of corneal endothelial slow-cycling cells. Invest. Ophthalmol. Vis. Sci. *56,* 3827-3837.

European Medicines Agency. Available at http://www.ema.europa.eu. *Accessed: September 23, 2015.*

Fagerholm, P., Lagali, N.S., Ong, J.A., Merrett, K., Jackson, W.B., Polarek, J.W., Suuronen, E.J., Liu, Y., Brunette, I., and Griffith, M. (2014). Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold. Biomaterials *35,* 2420-2427.

Feng, Y., Borrelli, M., Meyer-Ter-Vehn, T., Reichl, S., Schrader, S., and Geerling, G. (2014). Epithelial wound healing on keratin film, amniotic membrane and polystyrene in vitro. Curr. Eye Res. *39,* 561-570.

Figueira, E.C., Di Girolamo, N., Coroneo, M.T., and Wakefield, D. (2007). The phenotype of limbal epithelial stem cells. Invest. Ophthalmol. Vis. Sci. *48,* 144-156.

Fortier, L.A. (2005). Stem cells: classifications, controversies, and clinical applications. Vet. Surg. *34,* 415-423.

Fusaki, N., Ban, H., Nishiyama, A., Saeki, K., and Hasegawa, M. (2009). Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc. Jpn. Acad. Ser. B. Phys. Biol. Sci. *85,* 348-362.

Gage, P.J., Kuang, C., and Zacharias, A.L. (2014). The homeodomain transcription factor PITX2 is required for specifying correct cell fates and establishing angiogenic privilege in the developing cornea. Dev. Dyn. *243,* 1391-1400.

Galal, A., Perez-Santonja, J.J., Rodriguez-Prats, J.L., Abad, M., and Alio, J. (2007). Human anterior lens capsule as a biologic substrate for the ex vivo expansion of limbal stem cells in ocular surface reconstruction. Cornea *26,* 473-478.

Garber, K. (2015). RIKEN suspends first clinical trial involving induced pluripotent stem cells. Nat Biotech *33,* 890-891.

Garitaonandia, I., Amir, H., Boscolo, F.S., Wambua, G.K., Schultheisz, H.L., Sabatini, K., Morey, R., Waltz, S., Wang, Y., Tran, H.*, et al.* (2015). Increased risk of genetic and epigenetic instability in human embryonic stem cells associated with specific culture conditions. PLoS ONE *10,* e0118307.

Ghezzi, C.E., Rnjak-Kovacina, J., and Kaplan, D.L. (2015). Corneal tissue engineering: recent advances and future perspectives. Tissue Eng. Part B. Rev. *21,* 278-287.

Gomes, J.A., Romano, A., Santos, M.S., and Dua, H.S. (2005). Amniotic membrane use in ophthalmology. Curr. Opin. Ophthalmol. *16,* 233-240.

Grieve, K., Ghoubay, D., Georgeon, C., Thouvenin, O., Bouheraoua, N., Paques, M., and Borderie, V.M. (2015). Three-dimensional structure of the mammalian limbal stem cell niche. Exp. Eye Res. *140,* 75-84.

Haimila, K., Peräsaari, J., Linjama, T., Koskela, S., Saarinen, T., Lauronen, J., Auvinen, M.-K., and Jaatinen, T. (2013). HLA antigen, allele and haplotype frequencies and their use in virtual panel reactive antigen calculations in the Finnish population. Tissue Antigens *81,* 35-43.

Hanson, C., Hardarson, T., Ellerstrom, C., Nordberg, M., Caisander, G., Rao, M., Hyllner, J., and Stenevi, U. (2013). Transplantation of human embryonic stem cells onto a partially wounded human cornea in vitro. Acta Ophthalmol. *91,* 127-130.

Hasegawa, K., Pomeroy, J., and Pera, M. (2010). Current technology for the derivation of pluripotent stem cell lines from human embryos. Cell. Stem Cell. *6,* 521-531.

Hashmani, K., Branch, M.J., Sidney, L.E., Dhillon, P.S., Verma, M., McIntosh, O.D., Hopkinson, A., and Dua, H.S. (2013). Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation. Stem Cell. Res. Ther. *4,* 75.

Hassell, J.R., and Birk, D.E. (2010). The molecular basis of corneal transparency. Exp. Eye Res. *91,* 326-335.

Hayashi, R., Ishikawa, Y., Ito, M., Kageyama, T., Takashiba, K., Fujioka, T., Tsujikawa, M., Miyoshi, H., Yamato, M., Nakamura, Y., and Nishida, K. (2012). Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLoS One *7,* e45435.

He, Z., Campolmi, N., Gain, P., Ha Thi, B.M., Dumollard, J., Duband, S., Peoc'h, M., Piselli, S., Garraud, O., and Thuret, G. (2012). Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. Stem Cells *30,* 2523-2534.

Hewitt, K.J., Shamis, Y., Carlson, M.W., Aberdam, E., Aberdam, D., and Garlick, J.A. (2009). Three-dimensional epithelial tissues generated from human embryonic stem cells. Tissue Eng. Part A. *15,* 3417-3426.

Hirayama, M., Satake, Y., Higa, K., Yamaguchi, T., and Shimazaki, J. (2012). Transplantation of cultivated oral mucosal epithelium prepared in fibrin-coated culture dishes. Invest. Ophthalmol. Vis. Sci. *53,* 1602-1609.

Hoffman, L.M., and Carpenter, M.K. (2005). Characterization and culture of human embryonic stem cells. Nat. Biotechnol. *23,* 699-708.

Holan, V., Trosan, P., Cejka, C., Javorkova, E., Zajicova, A., Hermankova, B., Chudickova, M., and Cejkova, J. (2015). A Comparative Study of the Therapeutic Potential of Mesenchymal Stem Cells and Limbal Epithelial Stem Cells for Ocular Surface Reconstruction. Stem Cells Transl. Med. *4*, 1052-1063.

Hopkinson, A., McIntosh, R.S., Tighe, P.J., James, D.K., and Dua, H.S. (2006). Amniotic membrane for ocular surface reconstruction: donor variations and the effect of handling on TGF-beta content. Invest. Ophthalmol. Vis. Sci. *47,* 4316-4322.

Hovatta, O., Rodin, S., Antonsson, L., and Tryggvason, K. (2014). Concise review: animal substance-free human embryonic stem cells aiming at clinical applications. Stem Cells Transl. Med. *3,* 1269-1274.

Hu, K. (2014). All roads lead to induced pluripotent stem cells: the technologies of iPSC generation. Stem Cells Dev. *23,* 1285-1300.

Human Pluripotent Stem Cell Registry. Available at http://hpscreg.eu/. *Accessed: September 23, 2015.*

Hussein, S.M., Batada, N.N., Vuoristo, S., Ching, R.W., Autio, R., Narva, E., Ng, S., Sourour, M., Hamalainen, R., Olsson, C*., et al.* (2011). Copy number variation and selection during reprogramming to pluripotency. Nature *471,* 58-62.

International Stem Cell Registry. Available at http://www.iscr-admin.com. *Accessed: September 23, 2015.*

Itskovitz-Eldor, J., Schuldiner, M., Karsenti, D., Eden, A., Yanuka, O., Amit, M., Soreq, H., and Benvenisty, N. (2000). Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers. Mol. Med. *6,* 88-95.

Joe, A.W., and Yeung, S.N. (2014). Concise review: identifying limbal stem cells: classical concepts and new challenges. Stem Cells Transl. Med. *3,* 318-322.

Kamao, H., Mandai, M., Okamoto, S., Sakai, N., Suga, A., Sugita, S., Kiryu, J., and Takahashi, M. (2014). Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application. Stem Cell Reports *2,* 205-218.

Kanemura, H., Go, M.J., Shikamura, M., Nishishita, N., Sakai, N., Kamao, H., Mandai, M., Morinaga, C., Takahashi, M., and Kawamata, S. (2014). Tumorigenicity studies of induced pluripotent stem cell (iPSC)-derived retinal pigment epithelium (RPE) for the treatment of age-related macular degeneration. PLoS One *9,* e85336.

Katikireddy, K.R., Dana, R., and Jurkunas, U.V. (2014). Differentiation potential of limbal fibroblasts and bone marrow mesenchymal stem cells to corneal epithelial cells. Stem Cells *32,* 717-729.

Kawasaki, S., Tanioka, H., Yamasaki, K., Connon, C.J., and Kinoshita, S. (2006). Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. Exp. Eye Res. *82,* 293-299.

Kenyon, K.R., and Tseng, S.C. (1989). Limbal autograft transplantation for ocular surface disorders. Ophthalmology *96*, 709-722.

Kobayashi, M., Nakamura, T., Yasuda, M., Hata, Y., Okura, S., Iwamoto, M., Nagata, M., Fullwood, N.J., Koizumi, N., Hisa, Y., and Kinoshita, S. (2015a). Ocular surface reconstruction with a tissue-engineered nasal mucosal epithelial cell sheet for the treatment of severe ocular surface diseases. Stem Cells Transl. Med. *4,* 99-109.

Kobayashi, T., Shiraishi, A., Hara, Y., Kadota, Y., Yang, L., Inoue, T., Shirakata, Y., and Ohashi, Y. (2015b). Stromal-epithelial interaction study: The effect of corneal epithelial cells on growth factor expression in stromal cells using organotypic culture model. Exp. Eye Res. *135,* 109-117.

Kolli, S., Ahmad, S., Lako, M., and Figueiredo, F. (2010). Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. Stem Cells *28,* 597-610.

Kolli, S., Ahmad, S., Mudhar, H.S., Meeny, A., Lako, M., and Figueiredo, F.C. (2014). Successful application of ex vivo expanded human autologous oral mucosal epithelium for the treatment of total bilateral limbal stem cell deficiency. Stem Cells *32,* 2135-2146.

Koulikovska, M., Rafat, M., Petrovski, G., Vereb, Z., Akhtar, S., Fagerholm, P., and Lagali, N. (2015). Enhanced regeneration of corneal tissue via a bioengineered collagen construct implanted by a non-disruptive surgical technique. Tissue Eng. Part A. *21,* 1116-1130.

Ksander, B.R., Kolovou, P.E., Wilson, B.J., Saab, K.R., Guo, Q., Ma, J., McGuire, S.P., Gregory, M.S., Vincent, W.J., Perez, V.L.*, et al.* (2014). ABCB5 is a limbal stem cell gene required for corneal development and repair. Nature *511,* 353-357.

Lan, Y., Kodati, S., Lee, H.S., Omoto, M., Jin, Y., and Chauhan, S.K. (2012). Kinetics and function of mesenchymal stem cells in corneal injury. Invest. Ophthalmol. Vis. Sci. *53,* 3638-3644.

Lee, S.K., Teng, Y., Wong, H., Ng, T., Huang, L., Lei, P., Choy, K., Liu, Y., Zhang, M., Lam, D.S., Yam, G.H., and Pang, C. (2011). MicroRNA-145 regulates human corneal epithelial differentiation. PLoS ONE *6,* e21249.

Levis, H.J., Brown, R.A., and Daniels, J.T. (2010). Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. Biomaterials *31,* 7726-7737.

Levis, H.J., Massie, I., Dziasko, M.A., Kaasi, A., and Daniels, J.T. (2013). Rapid tissue engineering of biomimetic human corneal limbal crypts with 3D niche architecture. Biomaterials *34,* 8860-8868.

Li, D.Q., Chen, Z., Song, X.J., de Paiva, C.S., Kim, H.S., and Pflugfelder, S.C. (2005). Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp. Eye Res. *80,* 581-590.

Li, W., Hayashida, Y., Chen, Y., He, H., Tseng, D.Y., Alonso, M., Chen, S., Xi, X., and Tseng, S.C.G. (2008). Air exposure-induced squamous metaplasia of human limbal epithelium. Invest. Ophthalmol. Vis. Sci. *49,* 154-162.

Li, Y., Inoue, T., Takamatsu, F., Kobayashi, T., Shiraishi, A., Maeda, N., Ohashi, Y., and Nishida, K. (2014). Differences between niche cells and limbal stromal cells in maintenance of corneal limbal stem cells. Invest. Ophthalmol. Vis. Sci. *55,* 1453-1462.

Li, G., Xu, F., Zhu, J., Krawczyk, M., Zhang, Y., Yuan, J., Patel, S., Wang, Y., Lin, Y., Zhang, M., *et al.* (2015). Transcription factor PAX6 (Paired box 6) controls limbal stem cell lineage in development and disease. J. Biol. Chem. *290*, 20448-20454.

Lin, J., Yoon, K.C., Zhang, L., Su, Z., Lu, R., Ma, P., De Paiva, C.S., Pflugfelder, S.C., and Li, D.Q. (2012). A native-like corneal construct using donor corneal stroma for tissue engineering. PLoS One *7,* e49571.

Lindemann, M. (2014). Ex vivo assessment of cellular immune function - applications in patient care and clinical studies. Tissue Antigens *84,* 439-449.

Lister, R., Pelizzola, M., Kida, Y.S., Hawkins, R.D., Nery, J.R., Hon, G., Antosiewicz-Bourget, J., O'Malley, R., Castanon, R., Klugman, S.*, et al.* (2011). Hotspots of aberrant epigenomic reprogramming in human induced pluripotent stem cells. Nature *471,* 68-73.

Liu, J., Lawrence, B.D., Liu, A., Schwab, I.R., Oliveira, L.A., and Rosenblatt, M.I. (2012). Silk fibroin as a biomaterial substrate for corneal epithelial cell sheet generation. Invest. Ophthalmol. Vis. Sci. *53,* 4130-4138.

Liu, W., Merrett, K., Griffith, M., Fagerholm, P., Dravida, S., Heyne, B., Scaiano, J.C., Watsky, M.A., Shinozaki, N., Lagali, N., Munger, R., and Li, F. (2008). Recombinant human collagen for tissue engineered corneal substitutes. Biomaterials *29,* 1147-1158.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25,* 402-408.

Lu, R., Qu, Y., Ge, J., Zhang, L., Su, Z., Pflugfelder, S.C., and Li, D.Q. (2012). Transcription factor TCF4 maintains the properties of human corneal epithelial stem cells. Stem Cells *30,* 753-761.

Lund, R.J., Narva, E., and Lahesmaa, R. (2012a). Genetic and epigenetic stability of human pluripotent stem cells. Nat. Rev. Genet. *13,* 732-744.

Lund, R.J., Nikula, T., Rahkonen, N., Narva, E., Baker, D., Harrison, N., Andrews, P., Otonkoski, T., and Lahesmaa, R. (2012b). High-throughput karyotyping of human pluripotent stem cells. Stem Cell. Res. *9,* 192-195.

Lyngholm, M., Vorum, H., Nielsen, K., Ostergaard, M., Honore, B., and Ehlers, N. (2008). Differences in the protein expression in limbal versus central human corneal epithelium--a search for stem cell markers. Exp. Eye Res. *87,* 96-105.

Ma, H., Morey, R., O'Neil, R.C., He, Y., Daughtry, B., Schultz, M.D., Hariharan, M., Nery, J.R., Castanon, R., Sabatini, K.*, et al.* (2014). Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature *511,* 177-183.

Majo, F., Rochat, A., Nicolas, M., Jaoude, G.A., and Barrandon, Y. (2008). Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature *456,* 250-254.

Martin, M.J., Muotri, A., Gage, F., and Varki, A. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat. Med. *11,* 228-232.

Massie, I., Levis, H.J., and Daniels, J.T. (2014). Response of human limbal epithelial cells to wounding on 3D RAFT tissue equivalents: effect of airlifting and human limbal fibroblasts. Exp. Eye Res. *127,* 196-205.

Meek, K.M., and Knupp, C. (2015). Corneal structure and transparency. Prog. Retin. Eye Res. *49,* 1-16.

Menzel-Severing, J., Kruse, F.E., and Schlotzer-Schrehardt, U. (2013). Stem cell-based therapy for corneal epithelial reconstruction: present and future. Can. J. Ophthalmol. *48,* 13-21.

Mi, S., Chen, B., Wright, B., and Connon, C.J. (2010). Plastic compression of a collagen gel forms a much improved scaffold for ocular surface tissue engineering over conventional collagen gels. J. Biomed. Mater. Res. A. *95,* 447-453.

Nakamura, T., Inatomi, T., Sotozono, C., Amemiya, T., Kanamura, N., and Kinoshita, S. (2004). Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. Br. J. Ophthalmol. *88,* 1280-1284.

Nakatsu, M.N., Gonzalez, S., Mei, H., and Deng, S.X. (2014). Human limbal mesenchymal cells support the growth of human corneal epithelial stem/progenitor cells. Invest. Ophthalmol. Vis. Sci. *55,* 6953-6959.

Nakatsuji, N., Nakajima, F., and Tokunaga, K. (2008). HLA-haplotype banking and iPS cells. Nat Biotech *26,* 739-740.

Nieto-Miguel, T., Calonge, M., de la Mata, A., Lopez-Paniagua, M., Galindo, S., de la Paz, M.F., and Corrales, R.M. (2011). A comparison of stem cell-related gene expression in the progenitor-rich limbal epithelium and the differentiating central corneal epithelium. Mol. Vis. *17,* 2102-2117.

Nieto-Miguel, T., Galindo, S., Reinoso, R., Corell, A., Martino, M., Perez-Simon, J.A., and Calonge, M. (2013). In vitro simulation of corneal epithelium microenvironment induces a corneal epithelial-like cell phenotype from human adipose tissue mesenchymal stem cells. Curr. Eye Res. *38,* 933-944.

Nishida, K., Yamato, M., Hayashida, Y., Watanabe, K., Yamamoto, K., Adachi, E., Nagai, S., Kikuchi, A., Maeda, N., Watanabe, H., Okano, T., and Tano, Y. (2004). Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. N. Engl. J. Med. *351,* 1187-1196.

Notara, M., Alatza, A., Gilfillan, J., Harris, A.R., Levis, H.J., Schrader, S., Vernon, A., and Daniels, J.T. (2010). In sickness and in health: Corneal epithelial stem cell biology, pathology and therapy. Exp. Eye Res. *90,* 188-195.

O'Callaghan, A.R., and Daniels, J.T. (2011). Concise review: limbal epithelial stem cell therapy: controversies and challenges. Stem Cells *29,* 1923-1932.

Oh, J., Lee, R., Yu, J., Ko, J., Lee, H., Ko, A., Roddy, G., and Prockop, D. (2012). Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. Mol. Ther. *20,* 2143-2152.

Ojala, M., Prajapati, C., Pölönen, R., Rajala, K., Pekkanen-Mattila, M., Rasku, J., Larsson, K., and Aalto-Setälä, K. (2015). Mutation-specific phenotypes in hiPSC-derived cardiomyocytes carrying either myosin-binding protein C or a-tropomyosin mutation for hypertrophic cardiomyopathy. Stem Cells Int. *In press.*

Omoto, M., Katikireddy, K., Rezazadeh, A., Dohlman, T., and Chauhan, S. (2014). Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation. Invest. Ophthalmol. Vis. Sci. *55,* 6631-6638.

Ordonez, P., and Di Girolamo, N. (2012). Limbal epithelial stem cells: role of the niche microenvironment. Stem Cells *30,* 100-107.

Osafune, K., Caron, L., Borowiak, M., Martinez, R., Fitz-Gerald, C., Sato, Y., Cowan, C.A., Chien, K.R., and Melton, D.A. (2008). Marked differences in differentiation propensity among human embryonic stem cell lines. Nat. Biotechnol. *26,* 313-315.

Osei-Bempong, C., Figueiredo, F.C., and Lako, M. (2013). The limbal epithelium of the eye--a review of limbal stem cell biology, disease and treatment. Bioessays *35,* 211-219.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008). Disease-specific induced pluripotent stem cells. Cell *134,* 877-886.

Pellegrini, G., Dellambra, E., Golisano, O., Martinelli, E., Fantozzi, I., Bondanza, S., Ponzin, D., McKeon, F., and De Luca, M. (2001). P63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. U. S. A. *98,* 3156-3161.

Pellegrini, G., Golisano, O., Paterna, P., Lambiase, A., Bonini, S., Rama, P., and De Luca, M. (1999). Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. J. Cell Biol. *145,* 769-782.

Pellegrini, G., Rama, P., Matuska, S., Lambiase, A., Bonini, S., Pocobelli, A., Colabelli, R.G., Spadea, L., Fasciani, R., Balestrazzi, E.*, et al.* (2013). Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. Regen. Med. *8,* 553-567.

Pellegrini, G., Traverso, C.E., Franzi, A.T., Zingirian, M., Cancedda, R., and De Luca, M. (1997). Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. The Lancet *349,* 990-993.

Peng, H., Park, J.K., Katsnelson, J., Kaplan, N., Yang, W., Getsios, S., and Lavker, R.M. (2015). MicroRNA-103/107 family regulates multiple epithelial stem cell characteristics. Stem Cells *33,* 1642-1656.

Pera, M.F., Reubinoff, B.E., and Trounson, A. (2000). Human embryonic stem cells. J. Cell. Sci. *113,* 5-10.

Pescina, S., Govoni, P., Potenza, A., Padula, C., Santi, P., and Nicoli, S. (2015). Development of a convenient ex vivo model for the study of the transcorneal permeation of drugs: histological and permeability evaluation. J. Pharm. Sci. *104,* 63-71.

Petsch, C., Schlotzer-Schrehardt, U., Meyer-Blazejewska, E., Frey, M., Kruse, F.E., and Bachmann, B.O. (2014). Novel collagen membranes for the reconstruction of the corneal surface. Tissue Eng. Part A. *20,* 2378-2389.

Qu, Y., Chi, W., Hua, X., Deng, R., Li, J., Liu, Z., Pflugfelder, S.C., and Li, D.Q. (2015). Unique expression pattern and functional role of periostin in human limbal stem cells. PLoS One *10,* e0117139.

Rafat, M., Li, F., Fagerholm, P., Lagali, N.S., Watsky, M.A., Munger, R., Matsuura, T., and Griffith, M. (2008). PEG-stabilized carbodiimide crosslinked collagen-chitosan hydrogels for corneal tissue engineering. Biomaterials *29,* 3960-3972.

Rajala, K., Lindroos, B., Hussein, S.M., Lappalainen, R.S., Pekkanen-Mattila, M., Inzunza, J., Rozell, B., Miettinen, S., Narkilahti, S., Kerkela, E*., et al.* (2010). A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. PLoS One *5,* e10246.

Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M., and Pellegrini, G. (2010). Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. *363,* 147-155.

Reichl, S. (2008). Cell culture models of the human cornea - a comparative evaluation of their usefulness to determine ocular drug absorption in-vitro. J. Pharm. Pharmacol. *60,* 299-307.

Reichl, S., Borrelli, M., and Geerling, G. (2011). Keratin films for ocular surface reconstruction. Biomaterials *32,* 3375-3386.

Reza, H.M., Ng, B.Y., Gimeno, F.L., Phan, T.T., and Ang, L.P. (2011). Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. Stem Cell. Rev. *7,* 935-947.

Ricardo, J.R., Cristovam, P.C., Filho, P.A., Farias, C.C., de Araujo, A.L., Loureiro, R.R., Covre, J.L., de Barros, J.N., Barreiro, T.P., dos Santos, M.S., and Gomes, J.A. (2013). Transplantation of conjunctival epithelial cells cultivated ex vivo in patients with total limbal stem cell deficiency. Cornea *32,* 221-228.

Robertson, D.M., Ho, S.I., and Cavanagh, H.D. (2008). Characterization of DeltaNp63 isoforms in normal cornea and telomerase-immortalized human corneal epithelial cells. Exp. Eye Res. *86,* 576-585.

Rodin, S., Antonsson, L., Niaudet, C., Simonson, O.E., Salmela, E., Hansson, E.M., Domogatskaya, A., Xiao, Z., Damdimopoulou, P., Sheikhi, M*., et al.* (2014). Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat. Commun. *5,* 3195.

Sangwan, V.S., Basu, S., MacNeil, S., and Balasubramanian, D. (2012). Simple limbal epithelial transplantation (SLET): a novel surgical technique for the treatment of unilateral limbal stem cell deficiency. Br. J. Ophthalmol. *96,* 931-934.

Sareen, D., Saghizadeh, M., Ornelas, L., Winkler, M.A., Narwani, K., Sahabian, A., Funari, V.A., Tang, J., Spurka, L., Punj, V.*, et al.* (2014). Differentiation of human limbal-derived induced pluripotent stem cells into limbal-like epithelium. Stem Cells Transl. Med. *3,* 1002-1012.

Schlotzer-Schrehardt, U., and Kruse, F.E. (2005). Identification and characterization of limbal stem cells. Exp. Eye Res. *81,* 247-264.

Schwartz, S.D., Regillo, C.D., Lam, B.L., Eliott, D., Rosenfeld, P.J., Gregori, N.Z., Hubschman, J.P., Davis, J.L., Heilwell, G., Spirn, M.*, et al.* (2015). Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet *385,* 509-516.

Shalom-Feuerstein, R., Serror, L., Aberdam, E., Muller, F.J., van Bokhoven, H., Wiman, K.G., Zhou, H., Aberdam, D., and Petit, I. (2013). Impaired epithelial differentiation of induced pluripotent stem cells from ectodermal dysplasia-related patients is rescued by the small compound APR-246/PRIMA-1MET. Proc. Natl. Acad. Sci. U.S.A. *110,* 2152-2156.

Shalom-Feuerstein, R., Serror, L., De La Forest Divonne, S., Petit, I., Aberdam, E., Camargo, L., Damour, O., Vigouroux, C., Solomon, A., Gaggioli, C.*, et al.* (2012). Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. Stem Cells *30,* 898-909.

Shanmuganathan, V.A., Foster, T., Kulkarni, B.B., Hopkinson, A., Gray, T., Powe, D.G., Lowe, J., and Dua, H.S. (2007). Morphological characteristics of the limbal epithelial crypt. Br. J. Ophthalmol. *91,* 514-519.

Sharma, S., Gupta, D., Mohanty, S., Jassal, M., Agrawal, A.K., and Tandon, R. (2014). Surface-modified electrospun poly(ε-caprolactone) scaffold with improved optical transparency and bioactivity for damaged ocular surface reconstruction. Invest. Ophthalmol. Vis. Sci. *55,* 899-907.

Shimmura, S., Miyashita, H., Higa, K., Yoshida, S., Shimazaki, J., and Tsubota, K. (2006). Proteomic analysis of soluble factors secreted by limbal fibroblasts. Mol. Vis. *12,* 478-484.

Shortt, A.J., Secker, G.A., Lomas, R.J., Wilshaw, S.P., Kearney, J.N., Tuft, S.J., and Daniels, J.T. (2009). The effect of amniotic membrane preparation method on its

ability to serve as a substrate for the ex-vivo expansion of limbal epithelial cells. Biomaterials *30,* 1056-1065.

Shortt, A.J., Secker, G.A., Rajan, M.S., Meligonis, G., Dart, J.K., Tuft, S.J., and Daniels, J.T. (2008). Ex vivo expansion and transplantation of limbal epithelial stem cells. Ophthalmology *115,* 1989-1997.

Sitalakshmi, G., Sudha, B., Madhavan, H.N., Vinay, S., Krishnakumar, S., Mori, Y., Yoshioka, H., and Abraham, S. (2009). Ex vivo cultivation of corneal limbal epithelial cells in a thermoreversible polymer (Mebiol Gel) and their transplantation in rabbits: an animal model. Tissue Eng. Part A. *15,* 407-415.

Skottman, H. (2010). Derivation and characterization of three new human embryonic stem cell lines in Finland. In Vitro Cell. Dev. Biol. Anim. *46,* 206-209.

Skottman, H., and Hovatta, O. (2006). Culture conditions for human embryonic stem cells. Reproduction *132,* 691-698.

Song, W.K., Park, K.M., Kim, H.J., Lee, J.H., Choi, J., Chong, S.Y., Shim, S.H., Del Priore, L.V., and Lanza, R. (2015). Treatment of macular degeneration using embryonic stem cell-derived retinal pigment epithelium: preliminary results in Asian patients. Stem Cell. Reports *4,* 860-872.

Sorsby, A., and Symmons, H.M. (1946). Amniotic membrane grafts in caustic burns of the eye (burns of second degree). Br. J. Ophthalmol. *30*, 337-345.

Taapken, S.M., Nisler, B.S., Newton, M.A., Sampsell-Barron, T.L., Leonhard, K.A., McIntire, E.M., and Montgomery, K.D. (2011). Karyotypic abnormalities in human induced pluripotent stem cells and embryonic stem cells. Nat. Biotechnol. *29*, 313-314.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell *131,* 861-872.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282,* 1145-1147.

Tidu, A., Ghoubay-Benallaoua, D., Lynch, B., Haye, B., Illoul, C., Allain, J.-M., Borderie, V.M., and Mosser, G. (2015). Development of human corneal epithelium on organized fibrillated transparent collagen matrices synthesized at high concentration. Acta Biomater. *22*, 50-58.

Toivonen, S., Ojala, M., Hyysalo, A., Ilmarinen, T., Rajala, K., Pekkanen-Mattila, M., Aanismaa, R., Lundin, K., Palgi, J., Weltner, J.*, et al.* (2013). Comparative analysis of targeted differentiation of human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells reveals variability associated with incomplete transgene silencing in retrovirally derived hiPSC lines. Stem Cells Transl. Med. *2,* 83-93.

Torricelli, A.A., Singh, V., Santhiago, M.R., and Wilson, S.E. (2013). The corneal epithelial basement membrane: structure, function, and disease. Invest. Ophthalmol. Vis. Sci. *54,* 6390-6400.

Trokovic, R., Weltner, J., Nishimura, K., Ohtaka, M., Nakanishi, M., Salomaa, V., Jalanko, A., Otonkoski, T., and Kyttala, A. (2014). Advanced feeder-free generation of induced pluripotent stem cells directly from blood cells. Stem Cells Transl. Med. *3,* 1402-1409.

Trokovic, R., Weltner, J., and Otonkoski, T. (2015). Generation of iPSC line HEL24.3 from human neonatal foreskin fibroblasts. Stem Cell. Res. *15,* 266-268.

Tsai, C.L., Chuang, P.C., Kuo, H.K., Chen, Y.H., Su, W.H., and Wu, P.C. (2015). Differentiation of Stem Cells From Human Exfoliated Deciduous Teeth Toward a Phenotype of Corneal Epithelium In Vitro. Cornea. *34* 1471-1477.

Utheim, T.P. (2015). Concise review: transplantation of cultured oral mucosal epithelial cells for treating limbal stem cell deficiency - Current status and future perspectives. Stem Cells *33,* 1685-1695.

Vallier, L., Reynolds, D., and Pedersen, R.A. (2004). Nodal inhibits differentiation of human embryonic stem cells along the neuroectodermal default pathway. Dev. Biol. *275,* 403-421.

Van den Berghe, C., Guillet, M.C., and Compan, D. (2005). Performance of porcine corneal opacity and permeability assay to predict eye irritation for water-soluble cosmetic ingredients. Toxicol. in. Vitro. *19,* 823-830.

Van Essen, T.H., Lin, C.C., Hussain, A.K., Maas, S., Lai, H.J., Linnartz, H., van den Berg, T.J., Salvatori, D.C., Luyten, G.P., and Jager, M.J. (2013). A fish scale-derived collagen matrix as artificial cornea in rats: properties and potential. Invest. Ophthalmol. Vis. Sci. *54,* 3224-3233.

Van Essen, T.H., Roelen, D.L., Williams, K.A., and Jager, M.J. (2015). Matching for human leukocyte antigens (HLA) in corneal transplantation - To do or not to do. Prog. Retin. Eye Res. *46*, 84-110.

Vazirani, J., Basu, S., and Sangwan, V. (2013). Successful simple limbal epithelial transplantation (SLET) in lime injury-induced limbal stem cell deficiency with ocular surface granuloma. BMJ Case Reports bcr2013009405.

Vellonen, K.S., Malinen, M., Mannermaa, E., Subrizi, A., Toropainen, E., Lou, Y.R., Kidron, H., Yliperttula, M., and Urtti, A. (2014). A critical assessment of in vitro tissue models for ADME and drug delivery. J. Control. Release *190,* 94-114.

Vereb, Z., Albert, R., Poliska, S., Olstad, O., Akhtar, S., Moe, M.C., and Petrovski, G. (2013). Comparison of upstream regulators in human ex vivo cultured cornea limbal epithelial stem cells and differentiated corneal epithelial cells. BMC Genomics *14,* 900.

Villa-Diaz, L.G., Ross, A.M., Lahann, J., and Krebsbach, P.H. (2013). Concise review: The evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings. Stem Cells *31,* 1-7.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A.*, et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7,* 618-630.

Whikehart, D.R., Parikh, C.H., Vaughn, A.V., Mishler, K., and Edelhauser, H.F. (2005). Evidence suggesting the existence of stem cells for the human corneal endothelium. Mol. Vis. *11,* 816-824.

Whitcher, J.P., Srinivasan, M., and Upadhyay, M.P. (2001). Corneal blindness: a global perspective. Bull. World Health Organ. *79,* 214-221.

Wilmut, I., Leslie, S., Martin, N.G., Peschanski, M., Rao, M., Trounson, A., Turner, D., Turner, M.L., Yamanaka, S., and Taylor, C.J. (2015). Development of a global network of induced pluripotent stem cell haplobanks. Regen. Med. *10,* 235-238.

Wilson, S.E., and Hong, J.W. (2000). Bowman's layer structure and function: critical or dispensable to corneal function? A hypothesis. Cornea *19,* 417-420.

Wright, B., De Bank, P.A., Luetchford, K.A., Acosta, F.R., and Connon, C.J. (2014). Oxidised alginate hydrogels as niche environments for corneal epithelial cells. J. Biomed. Mater. Res. A. *102,* 3393-3400.

Wright, B., Mi, S., and Connon, C.J. (2013a). Towards the use of hydrogels in the treatment of limbal stem cell deficiency. Drug Discov. Today *18,* 79-86.

Wright, B., Hopkinson, A., Leyland, M., and Connon, C.J. (2013b). The secretome of alginate-encapsulated limbal epithelial stem cells modulates corneal epithelial cell proliferation. PLoS ONE *8,* e70860.

Wu, J., Rnjak-Kovacina, J., Du, Y., Funderburgh, M.L., Kaplan, D.L., and Funderburgh, J.L. (2014). Corneal stromal bioequivalents secreted on patterned silk substrates. Biomaterials *35,* 3744-3755.

Zajicova, A., Pokorna, K., Lencova, A., Krulova, M., Svobodova, E., Kubinova, S., Sykova, E., Pradny, M., Michalek, J., Svobodova, J., Munzarova, M., and Holan, V. (2010). Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. Cell Transplant. *19,* 1281-1290.

Zakaria, N., Possemiers, T., Dhubhghaill, S.N., Leysen, I., Rozema, J., Koppen, C., Timmermans, J.P., Berneman, Z., and Tassignon, M.J. (2014). Results of a phase I/II clinical trial: standardized, non-xenogenic, cultivated limbal stem cell transplantation. J. Transl. Med. *12,* 58.

Zhang, J., Upadhya, D., Lu, L., and Reneker, L.W. (2015). Fibroblast growth factor receptor 2 (FGFR2) is required for corneal epithelial cell proliferation and differentiation during embryonic development. PLoS One *10,* e0117089.

Zhao, Y., and Ma, L. (2015). Systematic review and meta-analysis on transplantation of ex vivo cultivated limbal epithelial stem cell on amniotic membrane in limbal stem cell deficiency. Cornea *34,* 592-600.

Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y.*, et al.* (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell *4,* 381-384.

Zhou, T., Benda, C., Dunzinger, S., Huang, Y., Ho, J.C., Yang, J., Wang, Y., Zhang, Y., Zhuang, Q., Li, Y.*, et al.* (2012). Generation of human induced pluripotent stem cells from urine samples. Nat. Protoc. *7,* 2080-2089.

Zhu, J., Zhang, K., Sun, Y., Gao, X., Li, Y., Chen, Z., and Wu, X. (2013). Reconstruction of functional ocular surface by acellular porcine cornea matrix scaffold and limbal stem cells derived from human embryonic stem cells. Tissue Eng. Part A. *19,* 2412-2425.

Zieske, J.D. (2004). Corneal development associated with eyelid opening. Int. J. Dev. Biol. *48,* 903-911.

Zimmermann, A., Preynat-Seauve, O., Tiercy, J.M., Krause, K.H., and Villard, J. (2012). Haplotype-based banking of human pluripotent stem cells for transplantation: potential and limitations. Stem Cells Dev. *21,* 2364-2373.

Original publications