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TOWARDS A BETTER UNDERSTANDING OF CORNEAL STEM CELLS BIOLOGY

BY
LEI LIU

DISSERTATION SUBMITTED 2018

AALBORG UNIVERSITY
DENMARK

TOWARDS A BETTER UNDERSTANDING OF CORNEAL STEM CELLS BIOLOGY

PHD DISSERTATION

by

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PREFACE

The purpose of this Ph.D. project was to have a better understanding of human corneal stem cell biology. The thesis is based on three individual experimental studies, which will be referred to as I-III. A list of manuscripts derived thereof is listed below.

Manuscript I:

Liu L, Nielsen FM, Riis SE, Emmersen J, Fink T, Hjortdal JØ, Bath C, Zachar V. Maintaining RNA Integrity for Transcriptomic Profiling of Ex Vivo Cultured Limbal Epithelial Stem Cells after Fluorescence-Activated Cell Sorting (FACS). ***Biological procedures online***. 2017 Dec;19(1):15.

Manuscript II:

Liu L, Emmersen J, Nielsen FM, Riis SE, Fink T, Pennesi CP, Bath C, Hjortdal JØ, Zachar V. Pigmentation is associated with stemness hierarchy of progenitor cells within cultured limbal epithelial cells. ***Stem cells***. 2018 May 20.

Manuscript III:

Liu L, Yu Y, Riis SE, Nielsen FM, Fink T, Jørgensen A, Grove A, Bath C, Hjortdal JØ, Christiansen OB, Zachar V. Human limbus harbors higher proportion of $CD146^{+}CD34^{-}$ pericyte-like stromal cells than adjacent cornea and sclera. ***Current eye research.*** Submitted.

ABBREVIATIONS

ABCB5: ATP-binding cassette sub-family B member 5

CLET: Cultivated limbal epithelial transplantation

CK3: Cytokeratin 3

FACS: Fluorescence-activated cell sorting

FMO: Fluorescence minus one;

GO: Gene ontology

KSFM: Keratinocyte serum-free medium

LESCs: Limbal epithelial stem cells

LSCD: Limbal stem cell deficiency

mRNA: Messenger RNA

P63: Transformation-related protein 63

QC: Quality control

RNA: Ribonucleic acid

RNA-seq: RNA sequencing

RIN: RNA integrity number

RNase: Ribonuclease

SCs: Stem cells

TACs: Transit-amplifying cells

ABSTRACT

It is estimated that 39.4 million people globally are affected by corneal disease, of which limbal stem cell deficiency (LSCD) is perhaps the most severe and difficult to treat. Currently, the mainstay procedure to treat the LSCD is based on transplantation of *ex vivo* cultured limbal epithelial stem cells (LESCs). However, to establish the optimal therapy has been slow and difficult due to the lack of knowledge about the biology of LESCs and their local microenvironment *in vitro*. If the identification, isolation and *ex vivo* maintenance of LESCs could be improved, more promising patient outcomes could be achieved.

The aim of this Ph.D. study was to improve the understanding of the biology of LESCs and their niche cells, with the aid of advanced biomedical technologies including, but not limited to, multicolor flow cytometry, fluorescence-activated cell sorting (FACS), and next generation RNA sequencing (RNA-seq).

In study I, we have successfully developed a FACS-based pipeline, which support accurate isolation of limbal epithelial progenitor subpopulations along with ensuring the RNA yield and quality to be sufficient to enable deep transcriptomic profiling.

In study II, a comparative transcriptome analysis of FACS-sorted human limbal epithelial cellular subpopulations was preformed, using sequencing materials obtained from study I. The results identified molecular networks regulating LESCs at an unprecedented level of detail, which, among others, include the association of pigmentation and LESCs differentiation hierarchy.

In study III, we revealed for the first time, that a subpopulation of pericyte-like cells are more enriched in limbal niche than adjacent corneal and scleral stroma by performing a comparative phenotypic analysis of stromal cells isolated from human limbus, cornea and sclera using multi-color flow cytometry.

In conclusion, these findings provide a better understanding of corneal stem cell biology, but also shed light on novel molecular tools that would be beneficial to improve the current procedure targeting LSCD.

DANSK RESUME

Hornhinden er vores vindue til verden, og den er afgørende for en normal synsfunktion og beskyttelse af øjets øvrige strukturer. Det yderste cellelag er konstant udsat for påvirkninger fra det ydre miljø og slitage f.eks. når vi blinker, og er derfor afhængig af kontinuerlig fornyelse fra hornhindestamceller. Dysfunktion eller mangel på disse stamceller kan medføre en lang række sygdomme i hornhinden, hvor hornhinden bliver uklar, tør og irriteret, hvilket i værste fald kan forårsage blindhed.

Laboratoriet for Stamcelleforskning har tidligere analyseret hornhindestamcellers gradvise udvikling til modne hornhindeceller og derudover er der udviklet et netværk over deres genekspression, som afslører reguleringen af disse stamceller.

Formålet med dette Ph.d.-projekt var at optimere laboratorieprotokollerne for dyrkning af hornhindestamceller. Dette skulle gøres gennem en bedre forståelse af den bagvedlæggende cellebiologi ved hjælp af de nyeste teknikker såsom fluorescensaktiveret cellesortering og næste generations RNA-sekventering. Alt dette for i fremtiden at kunne sikre en optimal stemcellebehandling mod uklar hornhinde og den deraf følgende blindhed.

Resultaterne fra dette Ph.d. studie viste at høj grad af intracellulær pigmentering er associeret med et meget primitivt stamcelle stadie i cellekultur; og at der findes en gruppe af pericytter i stamcellenichen, der kunne bidrage positivt til hornhindestamcellerne i den primitive fase.

På internationalt niveau vil dette projekt bidrage med vigtig ny viden inden for stamcellebiologi.

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CHAPTER 1. INTRODUCTION

As the window to the outer world, a transparent cornea has never been more important in individual's life - roughly 80% of nowadays' information are obtained through a functional visual system. Therefore, impaired version is not only universally feared by individuals, but also brings a considerable and increasing economic burden worldwide (1).

Globally, the number of blind people of all ages is estimated to be 39.4 million, of whom 1.6 million are corneal blindness (2). The epidemiology of corneal blindness is highly diverse worldwide, but mainly results from corneal scarring following infection or trauma (3). The only curative treatment available is a cornea graft or transplant, however, these treatments are hampered due to lack of essential facilities and qualified experts, but also scarcity of donor materials, due to the fact that 90% of the global cases of ocular trauma and corneal ulceration leading to corneal blindness occur in developing countries (3).

Consequently, initiatives have been launched to prevent sight loss, such as Universal Eye Health: A global action plan 2014 – 2019 (VISION 2020: The Right to Sight), which focus the use of resources towards the most cost-effective interventions to reduce avoidable visual impairment as a global public health problem (4). Furthermore, fast-growing knowledge in the field of corneal regenerative medicine and bioengineering have brought new hope to restore vision in an effective but less-costive way, where stem cell-based therapy seems to hold great promise.

As other parts of human body, corneal epithelium relies on tissue specific stem cells to renew and to keep transparent throughout an individual's lifetime (5). Despite still lacking a unique cell marker, stem cells (SCs) of human cornea are believed to reside in a specific location between transparent cornea and opaque conjunctiva, termed as limbus, which is also believed to be the niche of limbal epithelial stem cells (LESCs). Any disturbance to this microenvironment would eventually result in a disease modality known as limbal stem cell deficiency (LSCD) (6,7). This condition is often difficult to treat, the most promising treatment option remains cultivated limbal epithelial transplantation (CLET) based on *ex vivo* expanded LESCs (8). However, this procedure has a reported long-term success rate only of about 75% (9).

Despite remarkable progress during the last decade in corneal regeneration, there is still considerable room left to improve the outcome of current therapy, which requires a deeper understanding of LESCs and its niche.

1.1. CORNEAL STRUCTURE AND TRANSPARENCY

As the outermost central region of the ocular surface, the transparent avascular cornea acts as the primary infectious and structural barrier. Together with the associated tear film, cornea provides two-thirds of the eye's refractive power (10,11). The human cornea consists of five distinct layers: three cellular layers (epithelium, stroma, and endothelium) and two interface layers (Bowman membrane and Descemet membrane) (Figure 1-1).

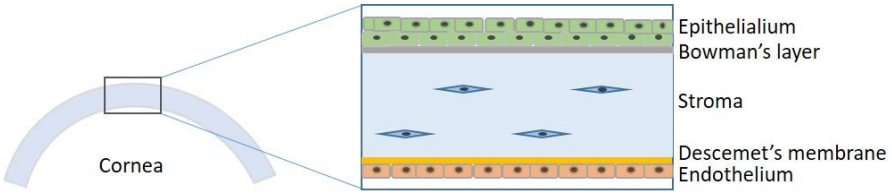

Figure 1-1: Structure of the cornea.

Corneal epithelium is composed of 4 to 6 stratified, non-keratinized, squamous cell layers (40 µm to 50 µm thick) (11). The first 2-3 layers of superficial cells, together with the underlying 2-3 layers of wing cells act as the main protective barrier. The deepest single cellular layer of corneal epithelium is the basal layer. The average lifespan of corneal epithelial cells is 7 to 10 days (12), and the whole epithelium is renewed in 9 to 12 months (13). Except for SCs and their daughter transient amplifying cells (TACs), basal cells are the only corneal epithelial cells that are able to undergo mitosis. Therefore, the basal layer plays an important role in renewing wing and superficial cells by mitotic cell divisions (14).

The limbus epithelium is a narrow ring of tissue at the transitional zone between the corneal epithelium and conjunctiva, which contains 10 to 12 cell layers and measures about 80 µm in thickness (15). As an important source of new corneal epithelium, LESCs are believed to reside in deep limbal basal epithelium, and migrate to the central cornea, while differentiating into TACs and basal cells (16). The detail process and relating theory will be discussed later in this chapter.

The acellular Bowman membrane separates corneal epithelium and corneal stroma. As the bulk of the structural framework of the cornea, the dense keratocyte-containing and collagen-rich corneal stroma comprises roughly 80% to 85% of total corneal thickness. In the corneal stroma, type I and type IV collagen fibers are arranged in bundles referred to as lamellae, which contribute to the transparency and mechanical strength of cornea (17). Furthermore, the main function of the sparsely located keratocytes are producing crystalline proteins, which is another key element to

maintain corneal transparency (18). The keratocytes closely interact with LESCs, which strongly indicates that this local environment may act as LESCs niche (16). Furthermore, it has been reported that a small population of cells in the corneal stroma displays properties of mesenchymal stem cells (MSCs) (19), which hold potential in corneal SC based therapy, and will be further discussed later in this chapter.

The posterior single cellular layer under Descemet's membrane is the corneal endothelium. The main function of corneal endothelial cells is to govern fluid and solute transport across the posterior surface of the cornea thus maintains the cornea in the slightly dehydrated state that is required for clarity. Dysfunction of corneal endothelium, due to irreversible density drop, will result in stromal edema, corneal clouding and eventual blindness (20). However, the corneal endothelium are usually quiescent and have so far not been considered to regenerate (21). Intensive research is still needed to fully understand the biology of corneal endothelial cells, and to find a proper way to transplant these cells to patients (22) .

1.2. STEM CELLS OF THE HUMAN CORNEA

1.2.1. LIMBAL EPITHELIAL STEM CELLS

Human tissues rely on SCs to replenish themselves throughout life, and corneal epithelium is no exception to this as corneal cells are lost continuously each time we blink. Epithelial stem cells of the cornea are widely known as LESCs, since they are believed to be located in the transition zone between corneal and conjunctival epithelium, also referred as limbus (16,23).

The earliest publications indicating differences between cells from central cornea and limbus can be traced back to the 1940s. In these studies a higher degree of mitoses was observed in the basal layer of peripheral cornea (24), moreover, centripetal migration of limbal pigment in response to corneal epithelial wounds provided even stronger evidence, suggesting the limbus as a source of new cells (25).

To replenish corneal epithelial cell loss, LESCs in the limbal basal layer undergo asymmetric division, which results in a stem-like daughter cell, which remains within the limbus, and a TAC detached from the basal membrane and which migrates centripetally towards the central cornea. TACs undergo multiple rounds of replication and gradually loses their "stemness", and progress to post-mitotic cells called wing cells. These cells continue to migrate anteriorly until they reach the ocular surface, and terminally differentiate into superficial squamous cells, or terminal differentiated cells. Both the post-mitotic cells and terminal differentiated cells are incapable of undergoing cell division. The above described process is also known as the X,Y,Z hypothesis (26), which is the predominant theory for corneal epithelium maintenance.

According to this theory, the cell loss from the ocular surface (Z) is equal to the sum of proliferating basal cells (X) and the centripetal migration of peripheral cells (Y). This theory has afterwards been further supported by mathematical models (27), as well as clinical data showing replacement of donor epithelium by recipient cells after keratoplasty (28). This theory was further strengthened by recent lineage tracing study of corneal epithelial progenitors, which showed that both corneal epithelial homeostasis and wound healing are mainly maintained by the activated SCs originating form limbus, but not from the corneal basal epithelial layer (29).

LESC phenotypical properties

LESCs share common features with other adult somatic SCs, including a small size, a high nuclear-cytoplasmic ration (NC ratio), a slow cell cycle, and a high proliferative potential (30). However, to date, a unique biomarker is still absent to detect LESCs either *in*- or *ex-situ*.

Nevertheless, many putative positive LESC markers have been reported in recent years (31–34), including certain structural proteins (vimentin, cytokeratin 14, 15 and 19), cell adhension molecules (integrin α6, β1, β4, P-cadherin and N-cadherin), certain enzymes (α-enolase, aldehyde dehydrogenase and cytochrome oxidase), growth factor receptors (KGF-R and NGF-R), and transcription factors (notch-1,Bmi-1, C/EBPδ,WNT7A and PAX6) (32,47–51). On the other hand, LESCs could also be phenotypically excluded by differentiated corneal epithelium markers cytokeratin 3 (CK3) and cytokeratin 12 (CK12), which have been reported negative in limbal crypts *in vivo* (34,35). Similar LESCs-excluding markers include also α9 integrin, CD45, CD31, RHAMM/HMMR, connexins 43 and 50 (46).

Beside the above-mentioned biomarkers, ATP-binding-cassette subfamily G member 2 (ABCG2) (36) and transcription factor p63 (p63) (32) have been widely used to detect LESCs. Tumor protein p63 is a member of the p53 family of transcription factors and is involved in epithelial development and proliferation (37). Furthermore, Yang et al. have shown that p63 knockout mice have major defects in their epithelial development (38). Additionally, Rama et al. have pointed out, that the percentage of ΔNp63α (a predominant p63 protein isoform) expression in limbal graft could be used as a quality control measure for CLET: cultures containing more than 3% ΔNp63α-positive cells have a success rate of nearly 80% in subsequent transplantation (8). However, recent evidence has indicated, that ΔNp63α is also expressed in the central corneal basal layer, thus it is not a unique marker for LESCs (39–41). Moreover, like other intracellular markers, its usage to purify LESCs has been limited due to the fact that cells need to be fixed and permeabilized to expose nuclear p63 antigen.

As a member of the ATP-binding cassette (ABC) family, ABCG2 is another widely accepted putative LESC surface marker (36). Watanabe et al. found ABCG2 was almost exclusively expressed in basal limbal epithelium (36). Later, ABCG2 was

found high correlation with the long-standing LESC marker ΔNp63α (31). More recently, a novel ABC transporter, ATP-binding cassette member B5 (ABCB5) was proposed as a new LESC marker (33). Besides ABCB5's high co-expression with ΔNp63α in limbal basal progenitor cells, both murine and human studies showed, that ABCB5 expressing cells were capable of fully restoring the corneal epithelium *in vitro* (42). However, as universal SC markers, both ABCG2 and ABCB5 are widely expressed in other adult SCs as well as in embryonic SCs (43). Therefore, positive selection based on these cell surface markers may not be sufficient for LESCs isolation and purification. To date, combining a panel of SC markers (e.g. p63, ABCG2) remains the most widely accepted strategy to phenotypically identify LESCs both *in* and *ex vivo*.

1.2.2. LIMBAL EPITHELIAL STEM CELL NICHE

A SC niche is a specific anatomic location that provides a microenvironment, in which SCs are present in an undifferentiated and self-renewable state. Within the niche, SCs interact with their surroundings to maintain stemness or promote differentiation. The concept of SC niche was first proposed by Schofield R in studies of the hematopoietic SCs (44); however, the most compelling evidence of the niche existence came from studies of Drosophila (fruit flies) (45). Studies of SC niche in less complex animals have led to pivotal insights into understanding the more complex mammalian SC niche architecture. Despite distinct variations among species, it appears that the fundamental anatomical components and molecular pathways of the niche environment are highly conserved, which include stromal support cells, extracellular matrix (ECM), blood vessels and neural inputs (46).

Like other human SC niches commonly occur at tissue intersections or transition zones (e.g., esophago-gastric, endo-ectocervical), it is widely believed that LESC niche locates in the transaction zone between cornea and conjunctiva, also referred as limbus. Several hypothetical niches have been proposed for the peripheral cornea over the past decade, including palisades of Vogt (5), limbal epithelial crypts (47), limbal crypts and focal stromal projections (48). The limbal palisades of Vogt is visible at the slit lamp as radial lines in the limbal regions with different degrees of pigmentation due to the presence of melanocytes (5,49). Like their function in human skin bulge area, melanocytes in the limbal palisades of Vogt may produce melanin pigments to protect LESCs from ultraviolet irradiation damage (50). Anatomically, the limbal palisades of Vogt is surrounded by a vascular network, which allows the infiltration of suppressor T-lymphocytes and antigen-presenting Langerhan's cells (51). Moreover, this highly vascularized structure also provides the residing SCs with necessary nutrient and oxygen.

In order to better identify the LESC niche, many efforts have been made in recent years to characterize the ultrastructure of the limbal palisades of Vogt. In 2005, Dua et al. first described limbal epithelial crypts, which located at the interpalisade

epithelial rete ridges of the limbal palisades of Vogt (47). Using three-dimensional image technology, they found limbal epithelial crypts extend deep into the limbal stroma (47). This feature suggested that LESCs might closely interact with underlying limbal stroma cells in the limbal palisades of Vogt, where Bowman's layer is absent. In 2007, Shortt et al. revealed further detailed ultrastructure of LESC niche using *in vivo* confocal microscopy, and proposed two additional LESC niches: limbal crypts and focal stromal projections (48). Limbal crypts are projections of limbal epithelium from the peripheral cornea into the limbal stroma; focal stromal projections are finger-like projections of limbal stroma with central blood vessels extending upward into the epithelium (48). These two structures are thrown into folds, which maximize nutrient absorption, but minimize the effect of physical damage. Moreover, some of the putative LESC markers, including p63 and ABCG2 are highly expressed in the limbal basal cells lining these papillary structures (52). More recently, using optical coherence tomography, Lathrop et al. revealed a combination of the structures described in the literature including the variety of palisade and interpalisade patterns with their intra- and inter-individual variability, along with structures that may correspond to limbal epithelial crypts, limbal crypts and focal stromal projections. Deep understanding of three-dimensional structure of LESC niche *in vivo* has greatly fostered recreating the bioengineered LESC niche. Consequently, in 2016, Hannah et al. reported to successfully having recreated the limbal crypts *in vitro* (53), which would bring great benefits to improve the outcome of limbal culture.

Interactions with their niche environment are essential for maintaining and activating LESCs *in vitro* (54–56). However, until today, little is known about the crosstalk between LESCs and their niche components. Several studies have proposed different signaling pathways, which might play important roles in the niche control, such as SDF-1/CXCR4 signaling (66), TGFβ/BMP signaling (67) and Wnt/β-catenin signaling (68). Recent studies have further pointed out, that the above signaling pathways may integrate to function, like BMP/Wnt signaling (68). Some key components involved in these pathways have also been identified, such as Frizzeled receptor 7 (69), PAX6 (70), and microRNA-103/107 (71). It has been shown that LESCs co-cultured with limbal stromal niche cells yield colonies with an average diameter five times as large as the colonies obtained when using murine 3T3 feeder layers, indicating enhanced LESCs proliferation in the presence of their niche cells (54). More recently, a study showed that LESCs and niche cells interact with each other in cell culture, migrate in spiraling patterns and self-organize to form niche-like compartments that resemble the limbal crypts (57). These new insights will help better mimicing LESC niche *in vitro* in order to support the maintenance of LESC stemness and to improve their therapeutic use in the future.

1.2.3. LIMBAL STROMAL CELLS

Although the majority of the efforts to characterize SCs in cornea have been focused on LESCs, recent investigations have sought to characterize corneal stroma, as it has

been proposed that this fiber-rich intermediate layer may also contain an adult SC population (58). These cells can be selected by FACS based on SC marker ABCG2 (58). During expansion *in vitro*, these cells express several typical markers of MSCs, such as CD166, CD90, CD73, CD105 as well as CD34 (59). Moreover, they have shown a capacity for fat, bone and cartilage differentiation (60,61), as well as corneal epithelial cell differentiation. Recently, limbal stromal SCs has also been proven to have the differentiation ability into corneal-like cells (62,63). These results indicate that SCs from limbal stroma may play an important role in both corneal bioengineering and cell-based therapeutic applications in future.

Eventhough it has been proved that limbal stromal niche cells can better support the expansion of LESCs *in vitro* than murine 3T3 feeder layer (54), the crosstalk between LESCs and their niche cells is still being explored. Higa et al. described that LESCs and limbal niche cells may directly interact *in vivo* by penetrating the epithelial basal membrane (64). Such interaction has also been observed by Dziasko et al. in a three dimensional reconstructed LESC niche (65). Interestingly, their ability to support LESCs was enhanced further by expansion of the limbal niche cells in culture conditions that maintained their SC-like phenotype (66). In the interaction of LESCs and limbal stromal SCs, the SDF-1 /CXCR4 signaling pathway was found to play an important role (55). In addition, it is interesting to note, that *in vitro*, stromal cells from limbus supported the expansion of LESCs to a higher degree than those from sclera (67), which indicates that stromal cells from different part of ocular surface may possess different phenotypical or functional characters.

1.3. LIMBAL STEM CELL DIFICIENCY (LSCD)

Once the microenvironment of the LESC niche gets disturbed, either intrinsically or extrinsically, it can result in a corneal epithelial stem-cell disorder known as limbal stem-cell deficiency (LSCD) (68). LSCD can be caused by hereditary (e.g. aniridia), acquired (e.g. thermal and chemical injuries, Stevens - Johnson syndrome, and contact lens wear), and iatrogenic factors (e.g. surgery) (69). Absence of LESCs and their barrier function will eventually lead to partial or full corneal blindness, which is characterized by conjunctivalization, neovascularization and inflammation (6). Pathology and cytology show a corneal surface covered by conjunctival epithelium containing goblet cells (70). Clinically, patients with LSCD present with pain, decreased vision, and photophobia (71). On slit-lamp examination, there is absence of the palisades of Vogt or other proposed LESC niche structure (47,72,73).

Today, treatment of LSCD depends on the extent of damage of the limbus (partial vs total) and on the involvement of number of eyes (unilateral vs bilateral). For partial LSCD, mechanical removal of invading conjunctival epithelium from the corneal surface (conjunctival epitheliectomy) can be enough to restore corneal transparency

as the healthy limbus will promote stem cells to cover the defect and maintain cornea homogenies afterwards (74). On the other hand, treatment of total LSCD often requires LESC transplantation, since they will act an indispensable barrier to the afterwards irreversible conjunctival invasion (7).

The concept of cultured epithelial stem-cell therapy was first described in the 1970s (75). However, it was not until 1997, that Pellegrini et al. showed that autologous *ex vivo* cultured LESCs, which afterwards is referred to as "cultivated limbal epithelial transplantation" (CLET), could be used to restore corneal epithelium (76). In this procedure, epithelial progenitor cells are obtained by a small limbal biopsy, and expanded *ex vivo*; amniotic membrane or a fibrin-based substrate is often used as the carrier for cell culture and transplantation. Despite many efforts have been made afterwards to improve the outcome of CLET (77–81), its long-term success rate remains lower than 80% (8) (82). This may partly be due to the fact, that the cell culture processes have not yet been standardized and therefore, vary greatly between research groups.

Although CLET is widely applied in recent years, it cannot be applied to patients with bilateral LSCD, since these patients do not possess their own LESCs. However, transplantation of limbal allograft requires long-term systemic immunosuppression, and the reported outcomes are not optimal (83,84). Therefore, alternative tissues or cells have been tested as possible epithelial cell sources for treating bilateral LSCD, including MSCs, embryonic SCs, oral mucosa epithelium, hair follicles, and dental pulp (85). Among the alternative SC resources , only oral mucosal epithelium based transplantation has been evaluated clinically for safety and efficiency (86).

Currently, to enhance clinical outcome of LSCD, investigations are mainly focusing on optimizing the cell culture conditions; developing novel substrates to better support stem cell proliferation, maintenance and differentiation; and evaluating the therapeutic potential of different kinds of autologous SCs. However, lack of knowledge about the biology of LESCs and their local microenvironment remains the greatest barrier to success. If LESCs can be better identified, isolated, and maintained *ex vivo*, more promising patient outcomes can be achieved in the future.

CHAPTER 2. OVERVIEW OF PH.D. WORK

2.1. OBJECTIVES

The aim of this dissertation was to obtain a better understanding of the biology of human corneal stem cells. In order to achieve this aim, this dissertation had following major objectives:

1. To develop a protocol for high output and high quality isolation of RNA from FACS sorted LESCs to enable subsequent transcriptomic profiling by next generation RNA-seq.

2. To gain new insight into gene regulatory network on LESCs maintenance and differentiation within limbal cultures by performing a detailed transcriptomic analysis of FACS sorted LESCs.

3. To further characterize ocular surface stromal cells by phenotypic profiling using multi-color flow cytometry.

To address the above aims, three studies were conducted as described below and outlined in Figure 2-1:

I. Maintaining RNA integrity for transcriptomic profiling from intracellular sorted LESCs subpopulations.

II. Comparative transcriptome analysis of FACS-sorted LESCs subpopulations.

III. Comparative phenotypic analysis of stromal cells isolated from human limbus, cornea and sclera.

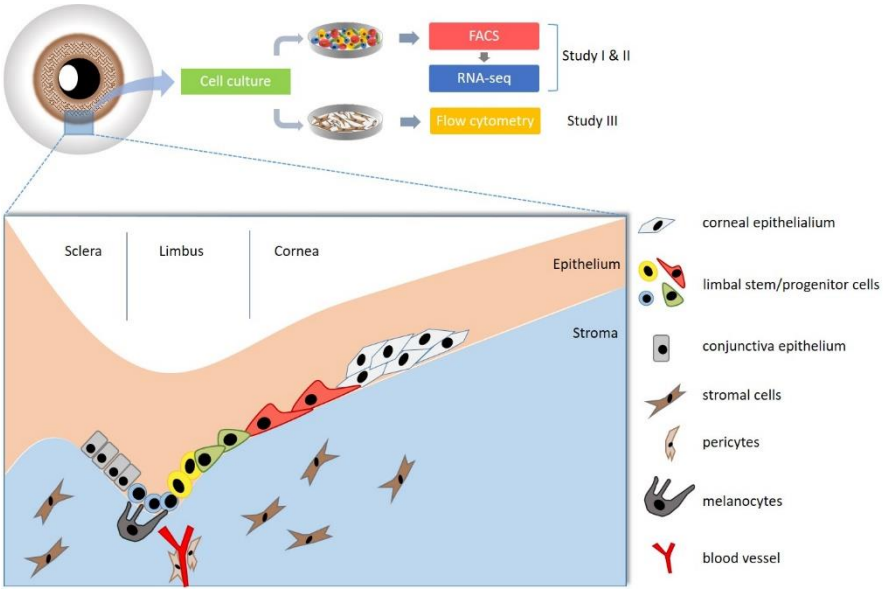

Figure 2-1: Outline of study design. Histology of the cornea, with a focus on limbal epithelium and stroma, is depicted in this simplified drawing. Different cell populations dissected from donated corneoscleral tissue for downstream experiments conducted in study I, II, and III, respectively.

2.2. EXPERIMENTAL METHODOLOGY

Key experimental methodologies applied in each study is summarized in Table 2-1. Detailed description of all materials and methods used in this dissertation is given in the individual manuscripts. In this section, choice of materials and methods will be elucidated more in depth, explained or discussed wherever found relevant.

	Study I	**Study II**	**Study III**
Primary cell culture	•	•	•
Immunofluorescence staining	•	•	•
Flow cytometry	•	•	•

Fluorescence-activated cell sorting	•	•	
Massive parallel sequencing	•	•	
Bioinformatics		•	

Table 2-1. An overview of methodologies applied in the studies I-III

2.2.1. HUMAN DONOR MATERIALS

Donor materials for primary cultures of corneal epithelial and stromal cells were obtained from the Department of Ophthalmology, Aarhus University Hospital (Aarhus, Denmark). After post-mortem eye donation, the central 16 mm of the donated eye globes were excised and stored in organ culture medium. Central cornea button was routinely removed for keratoplasty; residual human corneoscleral tissue, otherwise meant for destruction, were anonymized, stored in organ culture medium, and shipped to the Laboratory for Stem Cell Research, Aalborg University (Aalborg, Denmark) every second week and stored at 4 °C until further processing. The storage medium was based on MEM with HEPES and GlutaMAX (Gibco, Life Technologies, Naerum, Denmark) added 100 µg/ml biklin (Bristol-Meyers Squibb, Lyngby, Denmark), 2 mg/ml piperacillin (Laboratorio Farmaceutico, Sanremo, Italy), 2.5 µg/ml fungizone (Bristol-Myers Squibb) 8% fetal bovine serum (PAA Pharmacosmos, Holbaek, Denmark), and 8% Dextran 500 (Pharmacosmos, Holbaek, Denmark).

As a rich source of human material, the obtained corneoscleral tissue was handled according to Danish healthcare law and after guidance from the local ethical committee and the Danish Health and Medicines Agency in subsequent studies.

2.2.2. CHOICE OF CULTURE SYSTEMS

LESC culture

The dissociation culture system used for the isolation of LESCs in this study was initially developed by Bath et al. (87), but was further optimized in this Ph.D. study to achieve a better cell culture outcome.

The majority of previous established LESCs isolation protocols, regardless of cell-suspension or explant culture, were based on dispase digestion, as reviewed in Table 2-2. Dispase cleaves fibronectin and collagen IV to release epithelial cells from cornea

basal membrane (88). However, digestion with collagenase was recently shown to better maintain the niche cells in LESCs cultures by dissolution of the basement membrane components (54). In the initial stage of this project, different enzymatic digestion methods were compared. Both collagenase and dispase digestion allow satisfactory cell culture outcome, but when using collagenase digestion in our laboratory we observed irreversible contamination of fibroblasts in LESCs culture (Figure 2-2a). Therefore, dispase II was chosen to digest limbal tissue to make sure that stromal fibroblasts did not influence subsequent investigations of LESCs. Afterwards the digested tissue was incubated with TrypLE (Invitrogen) to recover the epithelial cells (89,90). Cell-suspension technique was applied in study I and II, as it has been shown superior to explant culture technique in terms of SCs content (91).

Enzyme	**Work dilution (units/mL)**	**Temperature (°C)**	**Digestion time (hours)**	**Additional digestion with Trypsin/EDTA**	**Reference**
Dispase	1.2	37	1	No	(92)
	1.2	37	2	Yes	(91)
	2.4	37	1-2	Yes	(33,73)
	5	4	16	Yes	(54,93,94)
	25	4	18	No	(95)
Collagenase	200	37	18	Yes	(54,94)

Table 2-2: A review of enzyme digestion parameters applied in LESCs cell-suspension culture studies.

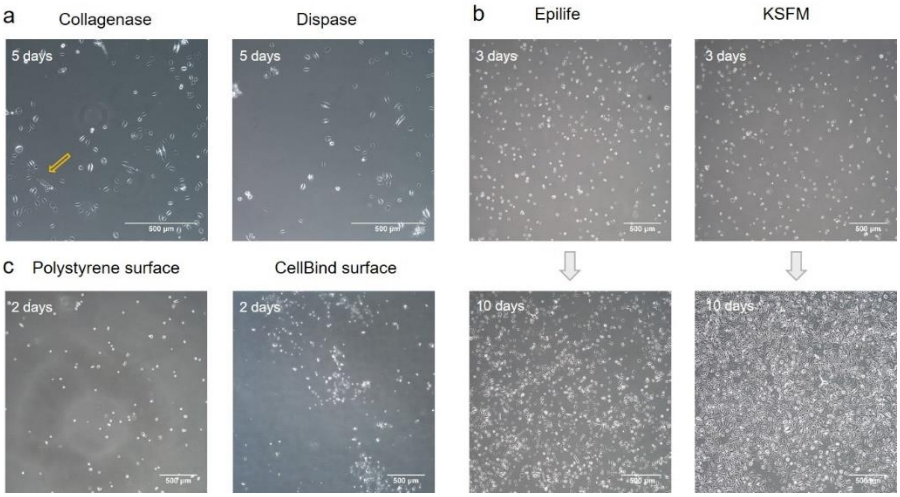

Figure 2-2: Optimization of limbal epithelial stem cell culture protocol. (a) Comparison of cell culture outcome after enzyme digestion by collagenase or dispase. Cells were cultured in complete K-SFM at 20% O_2, 37°C for 3 days. Yellow arrow points to a fibroblast-shaped cell. (b) Comparison of cell culture outcome in different culture media. Cells were cultured in complete K-SFM or Epilife at 20% O_2, 37°Cfor 3 days (upper panel) and 10 days (lower panel). (c) Comparison of cell culture outcome in different culture surface. Cells were cultured in complete K-SFM at 20% O_2, 37°C for 2 days on normal polystyrene surface (left panel) or CellBind surface (right panel). Scale bar depicts 500µm.

Even though feeder cells like NIH/3T3 are unquestionably supporting growth and morphology of LESCs cultured *in vitro* (96), a feeder-free and serum-free culture system was preferred in study I and II to avoid unwanted contamination for downstream analyses. Epilife basal medium supplied with Human Corneal Growth Supplement (both from Life Technologies, Naerum, Denmark) was previously shown to support the growth of human corneal epithelial equivalents, and also to minimize the risk of contamination during culture (39,96). However, a more recent study have indicated that cells cultured in Keratinocyte Serum-free Medium (KSFM) supplied with human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract (all from Life Technologies) included a higher percentage of limbal stem/progenitor cells, compared to the ones cultured in Epilife (97). In our study, LESCs grew generally faster in KSFM than in Epilife, while displayed no obvious difference in cell morphology (Figure 2-2b).

One disadvantage of a serum free culture system is a decreased cell attachment, since it often lacks calcium and other attachment factors found in serum (98). Corning CellBIND surface (Sigma–Aldrich, Copenhagen, Denmark) is proven to improve cell attachment by incorporating significantly more oxygen into the cell culture surface,

making it easier to maintain successful primary cell culture under difficult conditions, such as serum-free medium (99). In study I and II, after enzymatic and mechanical isolation from corneoscleral rings, more epithelial cells were attached to corning CellBIND surface than normal polystyrene cell culture flasks, resulting in higher cell yields for subsequent analysis (Figure 2-2c).

Ocular surface stromal cell culture

In study III, to compare the phenotypes of the stromal cells from limbus, cornea and sclera, it was necessary to optimize the protocol even further to distinctively isolate each population. To the best of our knowledge, no attempts have ever been made to isolate stromal cells from distinctive parts of an individual's ocular surface, therefore, several parameters were for this purpose optimized based on previous reports (61,100–103). In order to accurately separate the limbus from the adjacent cornea and sclera, tissue dissection was performed in aid of a stereo dissection microscope (Nikon SMZ-2B, Nikon, Tokyo, Japan), as shown in Figure 2-3a. During optimization, we found that a combination of dispase II and collagenase IV digestion allowed for satisfactory isolation of stromal cells from distinctive part of single-donor's ocular surface. According to the enzyme manufacturer (Life Technologies), comparing with other collagenase preparations, collagenase type IV is designed to be especially low in tryptic activity to limit the damage to membrane proteins and receptors. In order to achieve a better tissue digestion, collagenase type IV is often applied together with dispase, as described by Nayar S et al. (104). This combination thus possesses normal to above normal collagenase activity, but also the advantage of better preservation of the surface antigens, reducing the loss of cell surface markers necessary for the subsequent flow cytometric analysis (104).

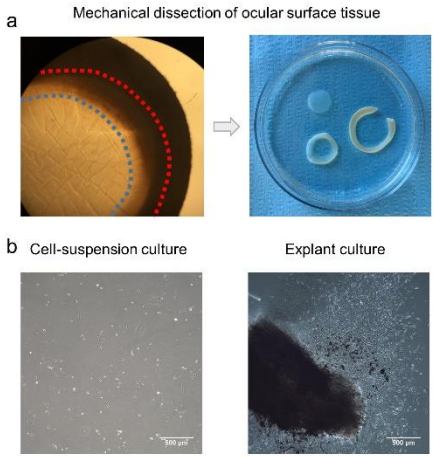

Figure 2-3: Optimization of ocular surface stromal cell culturing protocol. (a) Left panel: observation of ocular surface tissue under a stereo dissection microscope. Blue dashed line separates cornea from limbus; red dashed line separates limbus from sclera. Right panel: cornea, limbus and sclera tissue obtained for subsequent cell culture after mechanical dissection. (b) Comparison of cell culture outcome obtained from cell-suspension culture technique (left panel), or explant culture technique (right panel). Cells (or explant) were cultured in DMEM at 20% O_2, 37℃ for 5 days. Scale bar = 500µm.

After enzymatic digestion, processed ocular surface tissue was used to test the cell-suspension technique and explant technique. Our finding revealed that both culture systems gave a satisfactory cell culture outcome. However, the explant culture was shown superior in terms of cell attachment and proliferation (Figure 2-3b). One possible explanation could be both extracellular matrix and cellular components are active during the process of primary explant culture, which can exert several advantages for cell attachment and growing, as discussed by Hendijani F in his recently published review (105).

2.2.3. IMMUNOFLUORESCENCE STAINING

Combined surface and intracellular staining to detect LESCs

To phenotype *ex vivo* expanded LESCs, antibody-based epitope recognition of ABCB5, p63 and CK3 was applied. In study I and II, direct fluorochrome-conjugated antibodies were regarded as first choice whenever available, since they can greatly simplify the staining procedure, thus avoid unnecessary extrinsic ribonuclease (RNase) contamination or prolonged time resulting from secondary antibody staining. After validation and titration, chosen antibodies were further tested for cross-reactivity and fluorescence spillover. Antibody staining was tested using two types of negative controls to ensure specificity: firstly, appropriate negative controls using isotype-matched conjugated antibodies were performed; secondly, negative controls omitting two intracellular antibodies, also referred as Fluorescence Minus One (FMO) control were performed for cell surface epitopes.

In immunofluorescence, the choice of fixative and the following permeablization reagent have great influence on the outcome, since they can affect epitope detection (106), but also affect subsequent analysis of extracted biomolecules, like mRNA (107,108). Cross-linking fixatives including formaldehyde were reported to hamper RNA-based analyses (109), therefore, a more RNA friendly alcohol-based fixative 70% ethanol was used (110–112) in study I and II to preserve high quality RNA for downstream RNA-seq.

The major source of failure in any attempt to produce high quality RNA is RNase contamination. RNases are very stable and effective enzymes, erroneous introduction of RNase either from instrumentation, immunostaining, FACS, end user, or endogenously from the sample itself will lead to unavoidable RNA degradation. Therefore, precautions was needed to avoid contamination during intracellular antibody staining, as well as subsequent FACS (discussed below). RNase inhibitor has been proven to effectively prevent the enzymatic degradation of mRNA and total RNA for molecular biological studies (113). In study I and II, RNasin® Plus RNase Inhibitor (Promega, Roskilde, Denmark), which is widely used in transcriptomic studies for purpose of maintaining high quality RNA (114,115), was applied to each step after cells were permeablized, as well as during FACS sorting.

Multi-surface antigen staining to phenotype ocular surface stromal cells

The multi-surface antigen staining protocol used in study III was obtained from our previous report (114). All antibodies were titrated, and then tested for cross-reactivity and fluorescence spillover, as previously described, before application in this scenario.

2.2.4. FLOW CYTOMETRY

Flow cytometric analysis

Flow cytometric analysis took place immediately after immunofluorescence staining. After raw data was obtained on a MoFlo® Astrios™ sorter (Beckman Coulter, Brea, CA), subsequent analysis and sorting were performed in Summit Software v4.3 (Beckman Coulter). A typical workflow of flow cytometric analysis is illustrated in Figure 2-4. To exclude debris and dead cells after processing, primary gates based on forward and side scatter were set to select the overall population of interest. Gates for detecting positive staining were set against isotype controls for intracellular antigens, and FMO for surface antigen. Sorting gates were initially set so that approximately 2.5% of the events in the negative control were above the threshold. For multi-color flow cytometric analysis, compensation values were calculated for applied fluorochromes using the BD CompBeads Set (BD Biosciences, Brøndby, Denmark) according to the manufacturer's protocol, and using unstained and singly stained cells as compensation controls.

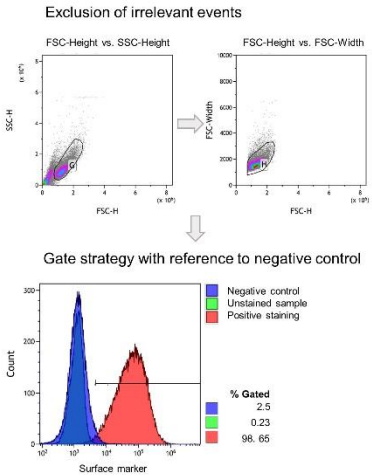

Figure 2-4: A workflow of flow cytometric analysis in Kaluza 1.3 software. FSC-Height vs. SSC-Height (upper left panel) and subsequent FSC-Height vs. FSC-width (upper right panel) were used to exclude debris and dead cells from the analysis. Gate strategy (lower panel) was set with reference to isotype or FMO controls, and

discrimination limit for positive events was set at a fluorescence intensity above which 2.5% of controls stained positive.

Fluorescence-activated cell sorting (FACS)

One of the biggest obstacles in study I was to maintain a RNA-friendly environment during FACS. To maintain RNA integrity, following precautions were taken during sorting.

Before launching, the flow cytometer was thoroughly decontaminated from outer sorting chamber to inner streaming system including sample line, sheath line, as well as waste tank following manufacture's instruction. Once decontamination was complete, a test sort using RNase-free water, followed by RNase-detecting reagent RNaseAlert (Life Technologies) was performed to ensure the instrument and sheath fluid was completely RNase-free following manufacture's instruction.

To perform an optimal sorting, appropriate concentration, single-cell suspension and proper pH maintenance are key parameters during sample preparation. An obvious advantage of a high-speed sorter, like MoFlo Astrios (Beckman Coulter, Brea, CA) used in this study, is the opportunity for a sample concentration up to $2x10^7$ /mL, and a sorting speed up to 20,000 events per second, which would dramatically shorten the sorting time, thus avoiding potential time-lead RNA degradation during sorting. However, a dense cell concentration often results in formation of aggregates, which will eventually lead to nozzle blockage. In study I, single cell suspension was maintained by the proteolytic and collagenolytic enzyme-based Accumax solution (Sigma-Aldrich, Brøndby, Denmark) to dissociate clumped cells. An additional filtration through the 70 µm Pre-Separation Filters (Miltenyi Biotec, Bergisch Gladbach, Germany) was used to remove cell aggregates or large particles prior to FACS.

RNA degradation can be directly caused by high pressure within the instrument during sorting (115). To minimize harmful shear force, large nozzle size (100 µm) and low running pressure (20 Psi) was used when sorting. On the other hand, high pressure also compromise phosphate and carbonate solution's buffer capacity (116), and eventually lead to RNA degradation due to pH variation (117). In study I, the buffering capacity was boosted by adding a non-phosphate system HEPES (Life Technologies) at a final concentration of 25mM (118) during staining, sorting as well as sample collection to maintain a neutral pH environment optimal to maintain RNA integrity.

After enough events was collected for FACS gating, sort mode was set as "purify" to ensure accurately separation of LESCs subpopulations. Polypropylene round-bottom FACS tubes was chosen to collect the sorted samples to minimize cell loss from binding to the tube (119). It is advantageous to sort directly into extraction reagent such as Trizol LS (Invitrogen) or RLT buffer (Qiagen) in order to minimize

downstream handling and inadvertent gene expression changes (120). However, these buffers' capacity to maintain RNA integrity is highly concentration-dependent (121), and often compromised by unavoidable dilution by sheath fluid during long-time sorting (121). Therefore, in study I, cells were directly sorted into a PBS based collection buffer, and pelleted for flash-freezing after sorting.

2.2.5. RNA SEQUENCING AND BIOINFORMATICS

For study I and II, RNA extraction and subsequent RNA-seq was performed by AROS Applied Biotechnology (Arhus, Denmark). A workflow is shown in Figure 2-6, methods in detail is presented in Manuscript I and II, respectively. Obtained raw sequencing data were submitted to Gene Expression Omnibus (GEO, http://www. ncbi. nlm. nih. gov/geo/) under accession number PRJNA387095.

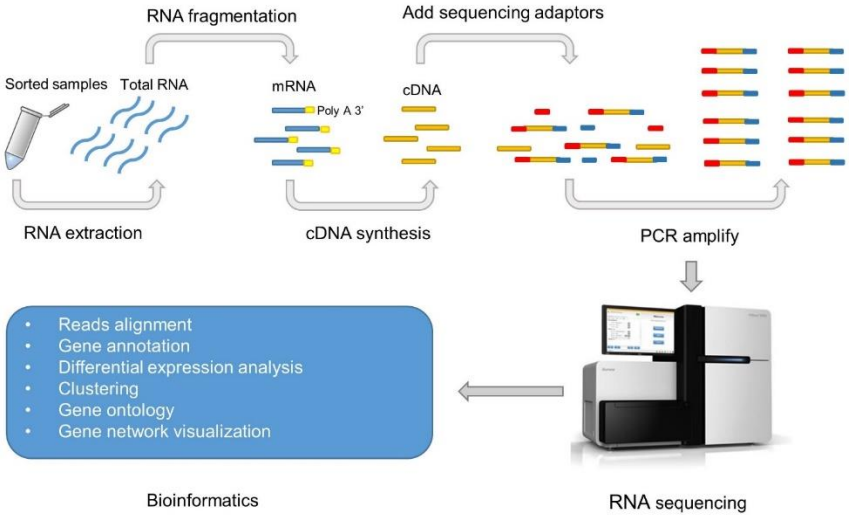

Figure 2-5: Workflow of total RNA sequencing and bioinformatics

In comparison with traditional transcriptomic analysis based on microarrays, high-throughput RNA-seq requires a lower amount of input matrtials, but provides significantly higher sensitivity to detect novel genes and differential expression (122). It is widely accepted that at least 30 million paired-end reads are needed to evaluate similarity between transcriptional profiles (123). Therefore, in study I and II, a depth of 100 million paired-end reads was applied for sequencing to ensure accurately detect differential gene expression among sorted LESCs subpopulations.

A systematic quality control (QC) of RNA quality, raw sequencing data, as well as reads alignment was performed to ensure a meaningful bioinformatic analysis afterwards, as shown in Figure 2-7.

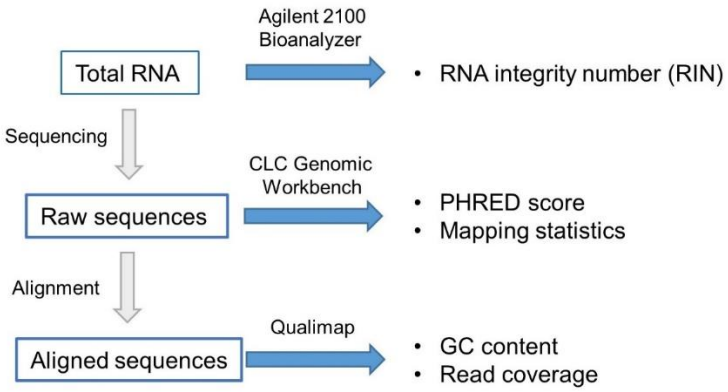

Figure 2-7: Quality control of RNA sequences. GC-content: guanine-cytosine content; A Phred (quality) score is a measure of the quality of the identification of the nucleobases generated by sequencing (124,125).

Data analysis, or bioinformatics, remains one of the biggest challenges in a successful RNA-seq experiment (122). In study II, bioinformatics was focus on differential gene expression among sorted LESCs subpopulations, as well as subsequent gene ontology (GO) network analysis to reveal to what extent selective markers (p63, ABCB5 and CK3) were associated with gene activation characteristic of limbal stem or precursor cells.

CHAPTER 3. SUMMARY OF RESULTS

Detailed results from the study I-III are presented in manuscript I-III, respectively (see appendices). An overview of the main findings is presented below.

Study I:

Objective:

To develop a reliable FACS-based procedure supporting accurate isolation of LESCs subpopulations along with a RNA yield and a RNA quality sufficient to enable deep transcriptomic profiling.

Results & Conclusions:

1. Ethanol fixation together with optimized downstream procedures provided for a pipeline that preserved good discrimination between the individual LESC immunophenotypes, but also yielded high quality total RNA in amounts to readily support the RNA-seq procedure: the average RNA integrity number was 7.7 ± 0.4, and the average yield was 4.6 ± 1.7 pg of RNA per cell.

2. Optimized pipeline would allow transcriptomic analysis of FACS-sorted/purified cells in the absence of a specific surface molecular marker.

Study II:

Objective:

To perform a comparative transcriptome analysis of four phenotypic subpopulations of cultured limbal epithelial cells, based on co-expression of reputed stem/progenitor markers ABCB5 and p63, along with corneal differentiation marker CK3, with the aid of FACS and RNA-seq.

Results & Conclusions:

1. Transcriptomic analysis showed pigmentation was among the leading biological processes in less-differentiated LESCs.

2. P63 identified a highly pigmented, stem/progenitor-like population, whereas ABCB5 was associated with a non-pigmented, late-stage differentiation cell population in LESCs culture.

3. Pigment epithelium-derived factor (PEDF), of which transcription is directly regulated by p63, might be a key factor regulating LESCs self-renewal.

Study III

Objective:

To perform a comparative phenotypic analysis of stromal cells isolated from human limbus, cornea and sclera.

Results & Conclusions:

1. Heterogeneity in MSCs populations was observed in human ocular surface stroma.

2. A significant higher proportion of pericyte-like cells featured by CD146+CD34- expression was observed in cultured stromal cells from limbus than those from cornea or sclera.

CHAPTER 4. DISSCUSSION

The advancement of biological technologies in recent years has greatly facilitated SC research, among which, bioinformatics in combination with transcriptomic analysis or RNA-seq has been proven to be a powerful and effective tool to better understand SC biology (126). However, its usefulness in corneal SC research is greatly limited, partly due to the absence of an unequivocal surface marker to allow for FACS enrichment. By successfully overcoming the difficulty of maintaining RNA integrity during intracellular sorting (127), we have provided a novel insight into the diversity of corneal SC development hierarchy (128). Meanwhile, a comprehensive multicolor flow-cytometric analysis revealed a heterogeneity of MSCs populations in human ocular surface stroma, as described in study III, which will foster a better understanding of their role in cornea maintenance as well as in future SC-based therapies.

Diversity of the stem/progenitor cell populations in human corneal epithelial cell culture

Percentage of SCs (characterized by holoclonal activity and high p63 expression) in an *ex vivo* cultured limbal graft has been recognized as a key prognosis indicator for CLET (8). However, the SCs percentage alone is still insufficient to guarantee 100% successful outcomes in patients with LSCD (129).

A possibly overlooked factor in graft failure is the distribution of epigenetic states in limbal culture. The diversity of stem/progenitor cell population, featuring a variety of different epigenetic states with different propensities for proliferation and differentiation, is recently recognized to be the key to maximize tissue performance (130,131).

Many tissues possess two different populations of SCs: a quiescent population and a primed population (132). Recent lineage tracing assays involving epithelial tissues revealed that adult SCs are not homogeneous, even under normal physiological conditions (133–135). A lineage tracing study revealed that such diversity also exists in the corneal SC pool (136). Under normal homeostasis, quiescent SCs are slow proliferating and responsible for self-renewal maintenance; under stress, a fraction of quiescent SCs is stimulated to become a primed population and gives rise to TACs, which proliferate actively and migrate centripetally to restore and regenerate the corneal epithelium (137).

Moreover, the role of TACs as an important component for tissue development and regeneration as well as their feedback regulation on SCs in the niche has received increased attention. In human hair follicle, TACs generated by primed SCs can express Sonic Hedgehog (SHH), which promotes quiescent-SC to start proliferation

(138). The knowledge about TAC-SC-crosstalk in limbal niche as well as in limbal graft could be essential to improve the unsatisfactory outcome of CLET, due to the majority of SCs in a limbal graft tend to remain almost quiescent, and take too long to proliferate (129,137).

However, it has been challenging to identify markers that distinguish TACs from SCs. Expression of C/EBPδ and Bmi1, which are responsible for mitotic quiescence (139) are believed to be associated with quiescent SCs; whereas expression of p63, which features proliferative potential (32), is not restricted to SCs, but also shared by TACs at early differentiation stage (140). Therefore, high p63 expression alone is not sufficient to distinguish high proliferative SCs from TACs.

Our study demonstrates for the first time that ABCB5 could be used to identify TACs with more limited proliferative potential, which is in stark contrast to previously published studies concluding that ABCB5 identifies SCs in both *in* and *ex vivo* (33,42). Despite the clinical success in these studies, the contribution of implanted ABCB5+ cells in mice with induced LSCD remains unclear (141), moreover, corneal abnormalities arising from ABCB5 deficiency could be ascribed to the anti-apoptotic role of ABCB5 protein (142), or absence of TACs, which has been demonstrated to be the key to develop a stratified epithelium (143).

Pigmentation identifies stem/progenitor cells with high proliferative potential

Interestingly, the bioinformatic analysis in study II revealed that apart from p63+ABCB5- phenotype to identify SCs with highest proliferative potential, these cells are also characterized by highest degree of pigmentation. This conclusion is in accordance with previous observation by transmission electron microscopy, showing that pigmentation distinguish early progenitor cells from TACs at late differentiation stage (30). In the above-mentioned study, Schlötzer-Schrehardt U and Kruse FE described two different cell types in the basal layer of limbal epithelium: primitive appearing putative SCs or early TACs containing melanin granules; and another group of similar cells without pigment granules, but with two centrioles associated with the process of mitosis, which are believed to be late TACs (30).

It is widely believed that SCs in the limbal niche receive melanin from surrounding melanocytes to protect them from UV irradiation. However, recent studies showed that melanocytes interact closely with SCs, and might be involved in their self-renewal regulation within limbal niche (49,144–146). Indeed, the concept of limbal stem cells was firstly inspired by the observation of limbal melanocytes migrate into the corneal epithelium during wound healing (147). The movement of pigmentation during corneal wound healing is possibly due to melanocytes interacting with a constant number of surrounding SCs to form the “melanin unit” (49). The presence of the “melanin unit” in the limbal niche was recently confirmed by serial block-face

scanning electron microscopy (144). These findings partly explained why pigmentation is associated with stemness, as the gene ontology analysis revealed in study II. However, following questions remain unanswered: 1) what is the key molecular involved in pigmentation-SC regulation? 2) why could pigmented progenitors be exclusively detected by p63, but not ABCB5 expression?

To answer the above questions, we propose that pigment epithelium-derived factor (PEDF) might play a key role in the pigmentation-p63-stemness association. Abundant evidence have demonstrated PEDF's role in SCs self-renewal regulation from different human tissues (148–151). However, its role in LESCs regulation has not been recognized until 2013 (152). In their study, Ho et al. showed that synthetic PEDF could enhance the proliferation potential of LESCs in vitro, by evidence of ΔNp63α, Bmi-1, and ABCG2 expression; in addition, they also proposed that this effect might be mediated by phosphorylations of p38 MAPK and STAT3 (152). However, there is still no evidence showing the presence of intrinsic PEDF in LESC culture. Nevertheless, PEDF was previously reported to be a direct target gene of p63 (153), and could be detected in early stage melanosome (154). Thus, there is a possibility that high p63-expressing LESCs may be able to produce endogenous PEDF, which might located in their intracellular pigment granules.

Pericytes as an indispensable component in limbal stroma

Recent years have witnessed an increasing interest in the study of stromal cells in human cornea. This is partly due to their exhibition of stem cell phenotype and potential for epithelial transdifferentiation, which might be a resource for cell-based therapeutics (19,58,59,61,101,155–157). Unlike the self-renewing epithelium, homeostasis of the corneal stroma dose not rely on the presence of an active population of SCs, thus the role of these SC-like stromal cells as well as their cross talk with LESCs in the limbal niche remains elusive. Therefore, it is worthwhile to explore how stromal cells from limbus are different from their counterparts from adjacent cornea and sclera.

Results from study III revealed that a significant higher proportion of pericytes are present in limbal stroma, by evidence of their CD146+ CD34-marker profile (158). Pericytes are an integral cellular component of vascular structures, which play a critical role in vascular development (159). Recently, many newly discovered functions of pericytes, which could functionally distinguish them from other stromal cells, have been reported, including neutrophil recruitment (160), maintenance of the integrity of the blood-brain-barrier (161), multiple-lineage differentiation (162), and functioning as therapeutic agents (163). In 2013, Corselli M et al. reported for the first time that FACS purified pericytes are capable of supporting the self-renewal and proliferation of transplantable human cord blood hematopoietic SCs *in vitro*, through cell-to-cell contact and at least partly via paracrine effects (164). Afterwards, the interest in pericytes as a niche component for adult SCs has been significantly

growing (165–167). However, to our best knowledge, the role of pericytes in limbal niche has still not been identified, which is undoubtedly needed be further investigated in future to have a better understanding of corneal SC biology.

Limitations

Ideally, gene expression experiment in study II should have been performed on freshly isolated limbal epithelial cells, since it is well known that LESCs change phenotype after culture. However, the number of cells available represents an insurmountable technical problem. Even after processing approx. 15 corneal rings, which represents on average 3 months´ supply from the Danish Cornea Bank, and seeding into T25, the cell yield is barely appreciable. This is due to the fact that the limbal crypt cell numbers are in the order of few thousands (168). Our optimized pipeline (127), in order to produce sufficient quality RNA for the least represented phenotype (p63+ABCB5+), relies on the use of the total of 75 x 10^6 cells (this includes controls for setting the gates and sorting parameters, and the sorted cells). The minimal number of cells for reliable RNA isolation was stipulated by a company specialized in deep RNA-seq. Thus, until a quantum leap occurs in the fields of cell sorting and deep RNA-seq, the *in vitro* culture remains the approach of choice.

Another concern raised is the choice of antibodies. Conjugated polyclonal antibodies are chosen in study I and II, since an extra step to label the unconjugated monoclonal antibody would severely prolong the experiment and lead to unnecessary RNA degradation. Instead of commonly used ΔNp63α antibody, which is believed to better identify LESCs (169), we have chosen an antibody (#167531-FITC, US Biological, Salem, MA) that recognizes variant ΔN-p63 isoforms proteins, which is indeed in accordance with Pellegrini et al. in their milestone paper that demonstrated p63 identifies keratinocyte stem cells (32).

At last, when performing bioinformatic analysis, the complex gene ontology network in large scale was manually trimmed to highlight the association between “pigmentation” and “stemness”. Therefore, the raw data, which has been deposited in The Gene Expression Omnibus (GEO, http://www. ncbi. nlm. nih. gov/geo/) under the accession number PRJNA387095(ID 387095), is still worth further investigation to get a better insight of LESC cell biology regulation.

CHAPTER 5. CONCLUSIONS

In conclusion, the studies described in this dissertation have provided new insight into the corneal SC biology. The results presented throughout study I and II have opened up a new area of LESCs research: it is time to revise the role of pigmentation in LESCs self-renewal regulation, especially its association with p63-expression, which could be mediated by PEDF. Moreover, comparing with other proposed molecular markers, identification of early limbal progenitors by pigmentation is undoubtfully more convenient and straightforward. In addition, these results also implied ABCB5 as a marker to identify TACs, which would allow a better understanding of their role in corneal SCs differentiation dynamics as well as in SC-based therapies for sight-threatening corneal diseases, such as LSCD or corneal scaring. In Study III, a diversity of MSCs population is observed in human ocular surface stroma. A deeper understanding of MSCs diversity in corneal stroma might bring benefits to better mimicing LESC niche *in vitro* in order to support the maintenance of LESC stemness and to improve their therapeutic use in the future.

Together, these findings may serve as an inspiration for the research of corneal SCs of the future. If the diversity of SCs in corneal epithelium as well as in corneal stroma can be better identified, more promising culture outcomes can be achieved. The way forward is thus to develop strategies to identify the role of pigmentation as well as pericytes in LESCs *ex vivo* maintenance.

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