Effect of Substrate on Proliferation and Differentiation of Stem Cells

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Abstract

BACKGROUND Limbal epithelial stem cells (LESCs) have been localized to the palisades of Vogt and serve as a source for continuous renewal of the corneal epithelium. LESCs can be destroyed as a result of tumours or autoimmune, traumatic, congenital, infectious or iatrogenic causes, which all lead to limbal stem cell deficiency (LSCD). The results are neovascularisation, chronic inflammation as well as opacification of the cornea with symptoms of discomfort, pain, photophobia, and severely reduced vision. The most widely accepted treatment of LSCD with corneal opacification is corneal transplantation in conjunction with cultured limbal epithelium transplantation (CLET). Novel stromal substitutes together with optimized CLET procedures hold great promise for the treatment of this clinically challenging disease modality. Transplantation of LESCs is, however, hampered by the fragility of the epithelium and a cell carrier is therefore required. Several different carrier solutions have been investigated, including amniotic membrane, which is currently the golden standard, collagen scaffolds, and siloxane hydrogel contact lenses, all with varying results.

AIM The aim of this project was to investigate and compare cell adhesion, proliferation and maintenance of stem cell potential of stem cells on natural and synthetic substrates that would support differentiation towards corneal phenotype in a serum- and feeder-free system.

METHODS In this project, growth and morphology of stem cells on intact amniotic membrane, epithelially denuded amniotic membrane, acellular amniotic membrane, plastic compressed collagen membrane, CCC and PIPAA culture dishes was evaluated on adipose-derived stem cells (ASCs) as a model for LESCs. In addition, limbal epithelial cells (LECs) were isolated from corneoscleral rims with either Dispase or collagenase and grown in a xeno- and feeder-free system.

RESULTS ASCs were capable of attaching to all of the tested substrates within 24 hours of seeding. ASCs on dAM and aAM displayed the largest proliferation rate ($p < 0,05$), while there was no significant difference between the other substrates and controls after three days of culturing. Furthermore, greater numbers of LECs could be isolated from corneoscleral rings with collagenase than with Dispase, however viability of donor tissue posed some problems.

CONCLUSION Results from ASCs as a model for stem cell growth on substrates suggests its importance in regulation specific cell features, such as morphology and proliferation. Viability of donor tissue, however, posed a problem and LECs could not be grown on the substrates. It is therefore important to conduct more research on this subject.

Preface

This document is an original work by the author Jaco Botha as a final dissertation from the Master of Science programme in Medicine with Industrial Specialization.

During the past two and a half years, I have worked with various types of stem cells and culturing techniques, providing me with a good understanding of their behaviour and how to influence it. As a part of a research team, I have among other things worked on tissue engineering, gaining a better understanding about how certain cells can influence the differentiation of others, and how to apply basic knowledge to solving problems of a complex nature.

Due to an increased global alertness and requirement for supplementation of donor tissue, corneal regeneration is an emerging field in tissue regeneration. Thus, the ultimate goal of this project was to contribute to knowledge on culturing corneal epithelium and thereby facilitate the transition of theoretical treatments to a clinical reality.

This project would not have been possible if it were not for the contributions of several persons. Firstly, for providing corneoscleral rings and amniotic membranes, I would like to thank Jesper Hjortdal and the staff of the Danish Cornea Bank, Aarhus University Hospital, Aarhus, Denmark.

For excellent supervision and help on troubleshooting and problem solving, as well as being an inspiration and a good role model, I would like to thank my supervisor Vladimir Zachar for all the time and effort he has put into assisting me. For expert technical assistance in the laboratory and help with all manners of small things, I would especially like to thank Ole Jensen and Helle Skøjdt Møller. It is also my pleasure to thank the staff of the Laboratory for Stem Cell Research and my fellow students for their contributions and help throughout the duration of this project.

Finally, I would like to extend special thanks to Christine Høgsbro for putting up with me when I wasn't easy to put up with, caring more for my well-being than I did, and for comforting me when things got rough.

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1 Introduction

In 1992, in his closing paragraphs on future technologies that will affect hospitals in a paper about hospital administration, Leland R. Kaiser possibly became the first to use the term "Regenerative medicine" and described it as a new branch of medicine that will develop to change the course of chronic disease in an ageing population by regenerating failing organs[1,2]. Currently, as proposed by Mason and Dunnill, regenerative medicine aims to "replace or regenerate human cells, tissue or organs to restore or establish normal function"[3]. At present, almost no tissue or organ is exempt from research in the field of regenerative medicine and major areas include the skin, musculoskeletal system, cardiovascular system, hematopoietic system, liver, pancreas and the eye.

Worldwide, it is estimated by the World Health Organization (WHO) that between 40 and 45 million individuals are blind[4] (visual acuity of less than 3/60[5]). Furthermore, another 135 to 140 million individuals suffer from severely impaired vision[4]. Corneal blindness is the second largest cause of blindness, only surpassed by cataract[5], and its causes are numerous[6]. Even though more than five million individuals suffer from corneal blindness[5], it is estimated that only 100.000 corneal transplants are performed annually due to a limited supply of tissue from cornea banks[7]. Thus, due to the demand for corneas exceeding the supply, it is not surprising that the area of cornea regeneration is on the rise in the field of regenerative medicine.

Limbal stem cell deficiency (LSCD) is characterized by destruction of limbal epithelial stem cells (LESCs) followed by abnormalities of the corneal surface including conjunctivalization, opacification and eventual corneal blindness[8]. Corneal transplantation is in most cases inadequate in treatment of LSCD and is therefore combined with either limbal allograft[9–11], limbal autograft from a living relative or cadaveric eye[12–14], or more recently, cultured limbal epithelial transplant (CLET)[15].
In the 16 years after CLET was first performed, several techniques have been developed and tested clinically. Due to intra and inter-study variability, it is difficult to review and compare the results of different techniques[16,17]. Thus, to determine the most effective treatment for limbal stem cell deficiency,

a standardized method for culturing limbal epithelial cells on substrates as well as treatment procedure is required.

1.1 Anatomy of the Cornea

In the healthy eye, the ocular surface consists of the fully transparent and avascular cornea and its surrounding supportive structure the conjunctiva[18,19]. The transition zone between the cornea and the conjunctiva is termed the limbus, which is comprised of both corneal and conjunctival tissue[18–20].

From an anterior view, the cornea is slightly oval with an average horizontal diameter of 11,7mm[21] and a vertical diameter of 10,5mm in the adult[19]. This is due to extension of the limbus onto the cornea at its superior and inferior borders. Posteriorly, however, the cornea is circular[19]. At its centre, the cornea is about 500µm thick and its thickness gradually increases towards the limbus to 700µm in the adult, causing its shape to become prolate, which provides an aspheric optical system[18,19]. The cornea with its overlying tear film can be considered the most important refractive component of the eye providing two thirds of the total refractivity of the eye. From its posterior surface to its anterior surface, the cornea can be divided into five distinct layers: Endothelium; Descemet's membrane; stroma; Bowman's membrane; and epithelium[18,19].

The endothelium of the cornea contributes to corneal clarity by keeping the corneal stroma in a relatively deturgesced state and preventing corneal oedema. The entire posterior surface of the cornea is covered by a thin monolayer of endothelial cells that fuses with the trabecular meshwork in the periphery[22]. Corneal endothelial cells do not poses the ability to undergo mitosis *in vivo*, and during an individual's life, the amount of endothelial cells gradually decreases and the pumping function of the endothelium thereby decreases with age[22,23]. Humans are, however, born with a significant reserve and the remaining endothelial cells stretch to take over the space and function of the lost endothelial cells[23].

Located anteriorly to the endothelium and posteriorly to the corneal stroma, Descemet's membrane functions as a basement membrane for the endothelium[18,19]. From the eighth week of gestation, the endothelium constantly secretes collagen that later becomes Descemet's membrane[18], and during an individual's life, it may reach a thickness of 10µm and becomes more organized[18,19].

The stroma provides the bulk structural framework of the cornea and accounts for roughly 80 % to 85 % of its total thickness. The stroma differs from other collagenous structures in its transparency, which

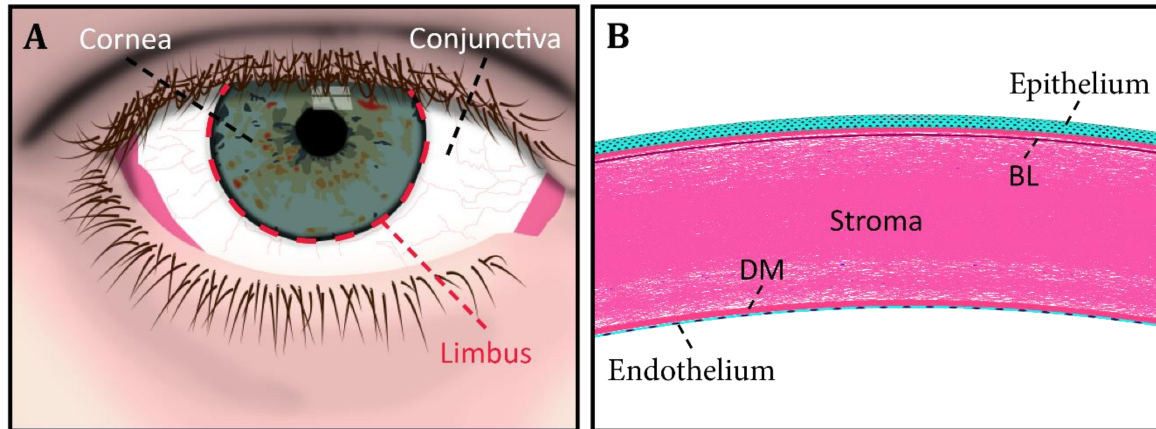


Figure 1 - Anatomy of the cornea, limbus and the conjunctiva. **(A)** Anatomy of the anterior surface of the eye depicting the locations of the cornea, limbus, and conjunctiva. **(B)** Cross-section view of the cornea. DM: Decemet's membrane; BL: Bowman's layer.

can be accounted for by the precise arrangement of its collagen fibres[24,25]. Type I and Type V collagen fibres in a thin heterodimeric complex are arranged into tightly packed parallel bundles called fibrils that in turn are arranged into lamellae in a parallel fashion. The lamellae are stacked in a fashion where each lamella lies perpendicular to the adjacent lamellae[19,25]. Surrounding the fibrils, glucosaminoglycans (GAGs) regulate the hydration of the stroma[18] and provide structural bridges between fibrils and lamellae, which contributes to the precise organization of the stroma[19]. While the highly organized character of the extracellular matrix (ECM) of the stroma reduces forward scatter of light and, thereby, contributes to transparency, it also provides structural stability and mechanical strength to the cornea[18]. Keratocytes are the major type of cells in the stroma and are located especially in the anterior stroma in the interlamellar spaces. Keratocytes are involved in maintaining and modifying the ECM in the stroma by synthesizing collagen and GAGs and by secreting matrix metalloproteases (MMPs) that digest the extracellular matrix. Keratocytes contain corneal crystallins, which might reduce the forward scatter of light caused by keratocytes and, thus, accounts for the cellular basis of corneal transparency[26]. The anterior portion of the stroma consists of an acellular layer of condensed ECM known as Bowman's membrane (or Bowman's layer)[19]. The main function of this layer is to maintain the shape of the cornea[18].

The anterior surface of the cornea is covered with stratified, non-keratinizing squamous epithelium. The epithelial layer is the first barrier to the outside environment and is an integral component of the cornea-tear film interface, which is critical to the refractive power of the eye[18]. Corneal epithelium is characterized by extreme uniformity from limbus to limbus and consists of five to six layers of cells[19]. The most superficial cells form two to three layers of flat, polygonal cells, where the cells at the surface have extensive microvilli and microplicae covered in a layer of the charged glycoprotein glycocalyx. These characteristics enable a seamless integration and spreading of tear film smoothing out any microirregularities and decreasing scatter of light[27]. The tear film also supplies the corneal epithelium with immunoglobulins and growth factors, which are essential for epithelial growth, migration and repair[18]. Superficial cells maintain tight junction complexes between adjacent cells and are impermeable to toxins[18] and even small particles such as ions[19]. Below the superficial cells, two to three layers of cuboidal to squamous cells termed wing cells with similar tight junction complexes between neighbouring cells are located[18]. The most profound layer of epithelium consists of columnar cells termed basal cells. These cells serve as a source for wing cells and superficial cells[28]. Apart from epithelial stem cells and transient amplifying cells, these cells are the only cells that are capable of mitosis, albeit very limited[29]. Unlike superficial cells and wing cells, basal cells are connected to adjacent cells by gap junctions and zonulae adherens[19] and are attached to the basement membrane with hemidesmosomes, which, due to their strong attachment, prevents the epithelium from being dissected from the underlying layers of the cornea. The basement membrane of the epithelium consists mainly of type IV collagen and laminin secreted by basal cells[18].

To replace damaged epithelium and maintain epithelial homeostasis, epithelial stem cells from the limbus proliferate, migrate centrally, differentiate to transient amplifying cells and later to post mitotic basal cells, which in turn are pushed apically in an ordered fashion, further differentiating into wing cells and transiently differentiated superficial cells[20,28]. The epithelial cells of the cornea routinely undergo involution, apoptosis and desquamation in an apically directed fashion and have an average lifespan of seven to ten days. This results in a complete turnover of corneal epithelium every week[18].

1.2 Limbal Epithelial Stem Cells and the Limbal Niche

Epithelial stem cells that give rise to corneal epithelium have been localized to the basement membrane of the palisades of Vogt and limbal crypts in the limbus[20,28]. This is supported by numerous observations on cell growth showing centripetal migration of epithelial cells from the limbus[30–32], damage to the limbus impairs regeneration of corneal epithelium[33], cells close to the limbal basement membrane display stem cell characteristics[20], and intraepithelial neoplasias often originate in the limbus[34,35] . It has also been demonstrated that transplantation of autologous cultivated limbal epithelium can restore fully functional epithelium in cases where limbal and corneal epithelium is destroyed[15].

In the quest for a specific expression pattern to identify limbal epithelial stem cells (LESCs) *in vivo* and *in vitro*, several different putative markers of differentiation and stem cell potency have been suggested. Keratins have previously been identified as potential markers for determining activation of different pathways and differentiation[36,37]. In the cornea, expression of cytokeratin 3 (CK3) and cytokeratin 12 (CK12) have been associated with corneal epithelium and to some degree suprabasal limbal epithelium, while these proteins are absent in the basal cells of the limbus and in conjunctival epithelium[38,39]. p63 is a nuclear transcription factor and a member of the p53 gene family. It is especially expressed in the nuclei of keratinocytes with proliferative capacity[40]. Expression of p63 has been associated with the dividing cells of the basal epithelium and the suprabasal epithelium in the limbus as well as some basal cells of the cornea[41], and particularly of interest is the ΔNp63α isoform[42]. ATP-binding cassette subfamily G member 2 (ABCG2) is a transporter protein located on the cell membrane and in the cytoplasm of several types of stem cells[43], including the basal epithelium of the limbus displaying stem cell-like characteristics[44]. A putative model for identifying LESCs *in vivo* and *in vitro* could be defined as a lack of the differentiation markers CK3 and CK12 and maintenance of the stem cell markers ΔNp63α and ABCG2. Coupled with the centripetal migration of corneal epithelium, expression patterns of differentiation and stem cell markers suggest that the basal cells of the limbus are the least differentiated cells of the corneal epithelium, further suggesting that these cells function as stem cells for the maintenance and regeneration of corneal epithelium[38,39,42,44,45].

During their migration from the limbus to the surface of the cornea, it is thought that epithelial cells undergo several stages of differentiation, since the expression patterns of stem cell and differentiation

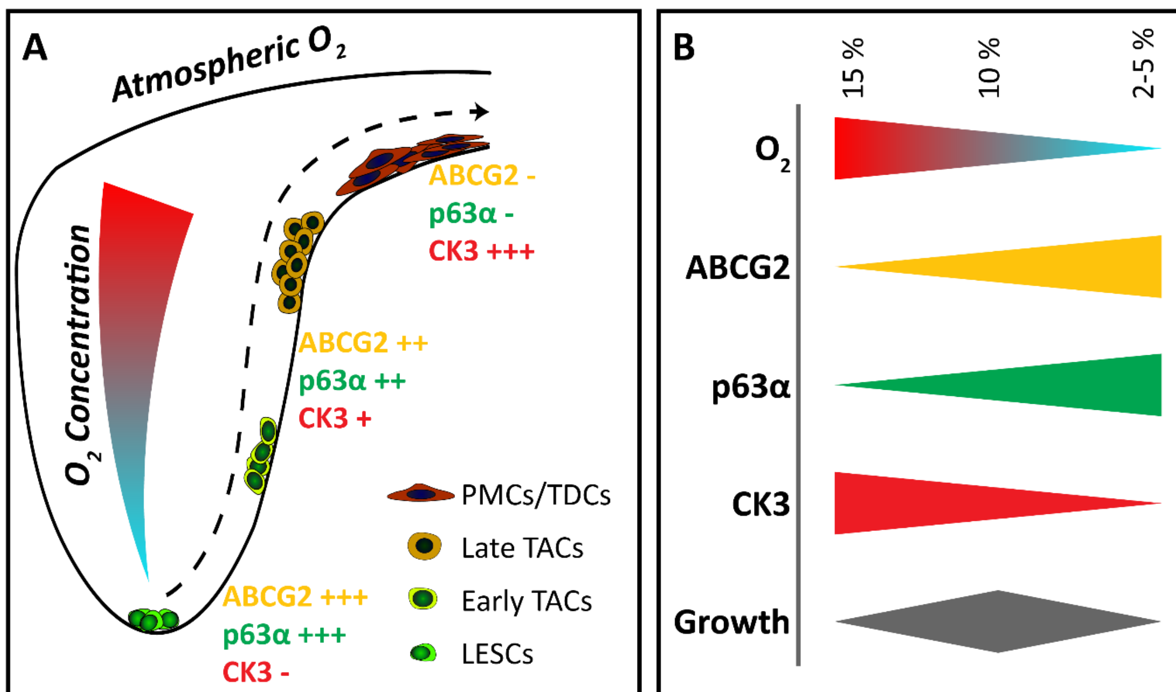


Figure 2 - Expression of stem cell markers is correlated with low oxygen concentration, whereas differentiation markers are correlated with higher oxygen concentrations. **(A)** The *in vivo* differentiation of LESCs as they migrate centripetally (dashed arrow) to maintain corneal epithelium. **(B)** *In vitro* growth and expression of ABCG2, ΔNp63α and CK3 in relation to oxygen concentration. PMC/TDC: Post mitotic cell; TAC: Transient amplifying cell; LESC: Limbal epithelial stem cell. Image adapted from Bath et al., 2012[46].

markers undergo a gradual change. After asymmetric division in the limbus, one daughter cell replenishes the stem cell population, while the other differentiates into an early transient amplifying cell (*e*TAC). As *e*TACs migrate centripetally, they proliferate and gradually differentiate into late TACs (*l*TACs) on the corneal basal membrane before maturing into post mitotic cells (PMCs) and finally terminally differentiated cells (TDCs) at the apical surface of the corneal epithelium[28,45,46].

An important structure in regulating and maintaining the stem cell potency of adult stem cells is the stem cell niche[47,48]. Keeping this hypothesis in mind, it is understandable that the limbal environment differs from that of the cornea. Anatomically, the palisades of Vogt are characterized by numerous papillae caused by stromal projections extending in an apically oriented direction[49]. In certain areas, the basement membrane of the limbus penetrates into the limbal stroma and runs circumferentially along the limbus. These structures are termed the limbal crypts[50,51]. These anatomical hallmarks suggest that there is a close interaction between LESCs and the mesenchymal cells of the stroma in the limbus. This has further been demonstrated *in vitro* by a novel technique for isolating cells from the limbus, where

limbal epithelial cells maintained close association to small mesenchymal cells that expressed stem cell markers such as Nanog, Oct4, Sox2 and Rex1. These limbal epithelial cells were capable of forming colonies in serum-free medium without the usage of 3T3-feeder cells further indicating the importance of cell-mediated interactions in the limbal niche[52–54]. Several other cells have also been suggested as candidates for acting as niche cells by protecting LESCs against factors that might promote differentiation and apoptosis, including melanocytes that provide protection against UV-radiation[55]. Of equal importance to cell-mediated interactions in the niche is cell-ECM interactions[47,48]. This fact is emphasised in the niche by the selective expression of α9 integrin[56] and N-Cadherin[57] and the complete lack of connexin 43 and 50 in LESCs as opposed to suprabasal epithelium of the limbus and corneal epithelium[58]. This is further supported by histological analyses that indicate several differences in expression of ECM-components between the basement membrane in the limbus and that of the cornea. Apart from expressing laminin (ln)-1, ln-5, fibronectin and type VII collagen, all expressed homogenously across the entire corneoscleral epithelial basement membrane, the limbal basement membrane expresses ln α2β2 chains, which the corneal basement membrane does not. Furthermore, the expression of collagen in the limbus differs from that of the cornea, where limbal chains include α1, α2 and α5 chains and central corneal chains include α3 and α5 chains[59,60]. Thus, it is likely that the composition of the limbal basement membrane contributes to the maintenance of a stem cell population in the niche. Like other stem cell niches, the components of the limbal basement membrane might also aid LESCs in maintaining their potency and keep them in a quiescent state by sequestering and modulating growth factor concentrations[28]. Finally, when taking the location of the limbal niche into consideration, it is not surprising to note that the oxygen concentration in the niche is much lower than at the surface of the cornea[61], and that corneal epithelial cells experience a steep gradient when differentiating and migrating to the surface of the cornea[46]. Both indirect evidence from other stem cell phenotypes and direct evidence from LESCs suggests that oxygen concentration plays a key role in the maintenance of stem cell potential and differentiation of stem cells[46,62–66]

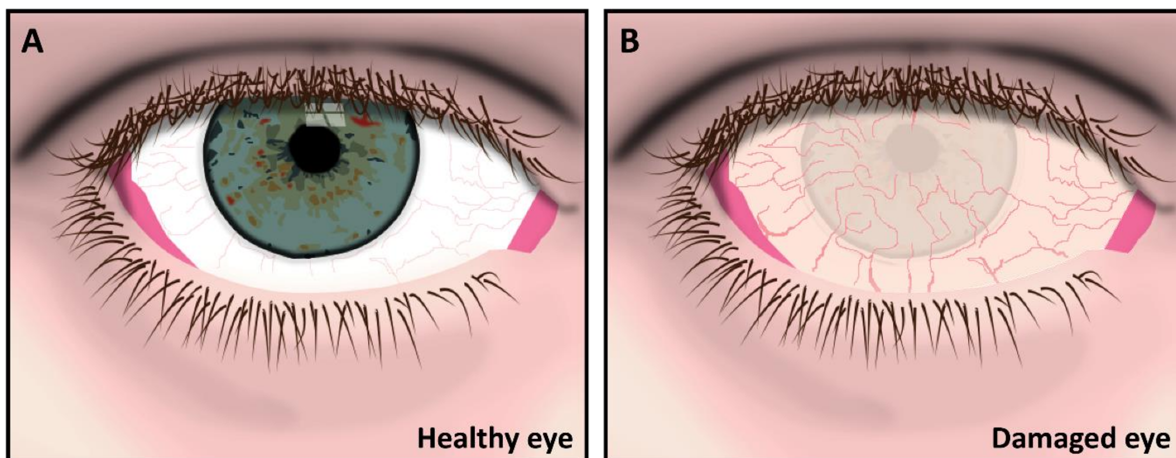


Figure 3 - Limbal stem cell deficiency. **(A)** In the healthy eye, the anterior surface is comprised of the conjunctiva, limbus and the cornea, where the latter is characterized by its complete transparency and avascular state. **(B)** When damage occurs to the cornea, the LESCs might be destroyed resulting in conjunctival invasion and neovascularization and ultimately corneal blindness.

1.3 Limbal Stem Cell Deficiency

In the healthy cornea, a delicate balance between angiogenic and anti-angiogenic factors maintains the avascular state of the cornea, in which corneal and limbal epithelial cells are thought to play an important role by producing an array of anti-angiogenic factors[67]. Also of importance is the ability of LESCs and TACs to maintain the corneal epithelium by an adequate rate of proliferation and differentiation to replace epithelial cells undergoing apoptosis or desquamation[68]. When damage occurs to the limbus and results in either partial or complete depletion of the limbal stem cell population, the angiogenic/anti-angiogenic balance might be tipped in favour of angiogenesis[67], and LESCs might not be an adequate source for maintenance of corneal epithelium[67–69]. This results in limbal stem cell deficiency (LSCD), where the apical surface of the cornea is characterized by conjunctival epithelium invasion (conjunctivalization), neovascularization, chronic inflammation, ulceration and erosion of epithelium due to poor basement membrane integrity and scarring[69].

In cases of LSCD, keratoplasty on its own is not an adequate treatment due to the requirement of LESCs[68]. Since the proposal of the XYZ hypothesis[70], several methods for transplanting limbal tissue have been developed. In cases of unilateral LSCD, limbal autografts from the healthy eye have been used to replace the depleted LESC population in the damaged eye[9–11,71]. In cases of bilateral LSCD, on the other hand, allografts from living relatives or cadaveric eyes have been used[12–14]. These treatments, however, are not uncomplicated, as autologous limbal transplantation and transplantation of limbal

tissue from a living relative bear the risk of causing iatrogenic LSCD in the donor eye, whereas all allogenic transplants stand the risk of being rejected due to histoincompatibility and, therefore, require long-term immunosuppression[72]. To decrease the risk of iatrogenic LSCD in donor eyes as well as prevent graft rejection, LESCs obtained from small biopsies have been expanded *in vitro* for transplantation, a procedure termed cultured limbal epithelial transplantation (CLET)[15].

1.4 Cultured Limbal Epithelium

With the advent of CLET, *in vitro* cultivation of LESCs has been optimized with one goal in mind: to improve the therapeutic outcome of CLET. Since the pioneering work of Pellegrini et al.[15], the preferred method of cultivating LESCs have been based on the work of Rheinwald and Green[73] on keratinocytes, where epithelial cells are cultured on lethally irradiated NIH 3T3 murine fibroblasts functioning as a feeder layer. Another factor that has been consistent in several expansion protocols is the use of amniotic membrane (AM) as a substrate for growth of LESCs[74]. In general, the differences between protocols for isolating and expanding LESCs can grossly be divided into the following three categories: 1) Isolation of cells from donor tissue; 2) use of lethally irradiated murine 3T3 fibroblasts as a feeder layer for limbal epithelial cultures; and 3) type of substrate used as a carrier for transplantation of LESCs.

Taking into mind the possibility that LESCs might be regulated by their niche *in vivo*, it can be discussed whether limbal epithelium should be separated from its underlying stroma and rendered into a single cell suspension by enzymatic digestion. Several protocols for isolating cells from donor tissue exist[15,53,75–80]. Early protocols for isolating limbal cells utilized trypsin/EDTA to digest basement membrane, cell adhesion and intracellular proteins and chelate Ca^{2+} to prevent formation of Cadherins[15,80]. Subsequent protocols replaced trypsin/EDTA with Dispase[75,76,79], which has been demonstrated to isolate intact limbal epithelial sheets[78]. Some protocols also had an additional step of trypsin/EDTA digestion to render the cell sheet into a single-cell suspension[76,79]. It is, however, questionable whether rendering the limbal epithelium into a single cell suspension is a viable technique for isolating LESCs, since cultured intact cell sheets have displayed a better maintenance of proliferative capacity, clonogenicity and p63-expression *in vitro* than single-cell cultures[52,53,81]. While cells harvested by Dispase have been capable of maintaining an expression of p63[81] and ABCG2[46], it is uncertain whether Dispase also isolates niche cells[74], and Dispase does not isolate the entire population of LESCs in the limbus[53]. Several protocols have also relied on outgrowth of LESCs from small pieces of limbal tissue to maintain association of

LESCs with their niche cells[77,82]. It is of some concern, however, whether LESCs actually migrate out from the tissue[74]. It has been reported that LESCs expanded as explant cultures are capable of migrating into the stroma and, thereby, undergo epithelial-mesenchymal transition, which results in a gradual loss of clonogenicity and proliferative capacity of the epithelial cells[83,84]. Recently, to address the aforementioned issues, a novel protocol for isolating the entire LESC population while maintaining close association with its associate niche cells was suggested using collagenase instead of Dispase without rendering the resulting cell clusters into a single-cell suspension. LESCs isolated in this manner displayed a greater proliferative capacity and clonogenicity than LESCs isolated by Dispase or single-cell cultures, irrespective of isolation method[52–54].

To prevent LESCs from differentiating after isolation and being rendered into a single-cell suspension, a common approach has been to use lethally irradiated or mitomycin C-inactivated 3T3 murine fibroblasts as a feeder layer and surrogate niche[74]. By seeding isolated cells onto the feeder layer directly after isolation and, thereby, establishing direct cell contact between the isolated epithelial cells and the 3T3 fibroblasts, clonogenicity and proliferative capacity are restored in the corneal epithelial cells[15,74,80]. Even though direct epithelial-mesenchymal interactions seems to be implicated in maintaining clonogenicity and proliferative potential of epithelial cells *in vitro*, indirect interaction by soluble factors seems to play an equally important role. Co-culture experiments with limbal epithelium and 3T3 fibroblasts have been capable of delaying differentiation of epithelial cells[85], while a 3T3 feeder layer culturing system has been used to promote expression the stem cell factor CK15 in corneal epithelial cells cultured on AM[86]. Using 3T3 feeder cells as surrogate niche cells does, however, pose a risk of xenotoxicity and transferral of murine diseases to humans if used in constructing a graft for CLET, and there are strict guidelines issued by the FDA for use of 3T3 fibroblasts in surgical grafts[74]. Recently, circumvent regulatory concerns with 3T3 feeder systems, a novel serum- and feeder-free culturing system has been developed to expand corneal epithelium on AM while maintaining the LESC population[87]. This system has also been tested without AM and was able to maintain expression of stem cell markers and proliferation capacity of isolated corneal epithelium[46].

1.5 Substrates for CLET

Due to the fragility of a single sheet of corneal epithelium, a carrier for transplantation is necessary[6]. Since CLET was in its infancy, AM has been the golden standard for culturing corneal epithelium for transplantation[74]. Apart from maintaining the clonogenicity and proliferative capacity of LESCs, AM also possesses other properties such as inhibition of inflammation, scarring and angiogenesis[88,89], while also being able of restoring a normal corneal surface in cases of partial LSCD without containing cultured corneal epithelium[90]. Furthermore, cells of the AM do not express HLA-A, B, C, making it an ideal substrate for CLET[91]. Both intact AM (iAM)[75] and epithelially denuded AM (dAM)[76,77,79,80,82] have been used for CLET. The rationale for using either iAM or dAM in CLET is not explained in early literature, however iAM maintains the LESC phenotype and slow-cycling properties to a larger extent than dAM[92], which ultimately requires a 3T3 feeder layer when used as a substrate for CLET[74]. Furthermore, inactivated epithelial cells on iAM have been demonstrated to be superior to 3T3 feeder layers in maintaining clonogeneity, proliferation and differentiation potential of LESCs, which explains why expanding limbal epithelium on iAM does not require a 3T3 feeder layer[93]. Finally, iAM can maintain differentiation potential and proliferation of cultured corneal epithelium for several passages in serial expansion, further supporting the hypothesis that iAM provides an environment similar to that of the limbal niche[94]. There are, however, certain concerns with AM, including inter-donor variability, expensive screening procedures for pathogens without a guarantee for complete success and, finally, AM is not fully transparent, further limiting its capabilities as a substrate for CLET[95].

To circumvent these concerns, several synthetic substrates have been proposed as replacements for AM. Not surprisingly, several collagen-based substrates have been proposed, as collagen displays several features favourable to transplantation including biocompatibility, low immunogenicity, and allowing cells to be fully incorporated into its matrix where they can remodel its structure. Furthermore, collagen substrates are inexpensive to produce and can be mass-produced without great difficulty[95]. Production of most collagen substrates requires a step of fibrillogenesis where collagen solutions become hydrated gels[96–100]. Hydrated collagen gels are, however, relatively unstable and quite vulnerable to changes in pH, high temperatures and enzymatic degradation[101]. Therefore, to increase their stability, several approaches have been investigated. Vitrification of collagen gels is a process where the water content in a collagen gel is evaporated over a long period of time at cold temperatures, resulting in a strong, glass-

like, transparent substrate capable of supporting growth of corneal epithelium and maintenance of differentiation potential of LESCs[100]. However, due to the extensive evaporation process, it is not possible to seed keratocytes into vitrified membranes and, thereby, mimic the native limbal niche[95]. *In vivo*, collagen in connective tissue is stabilized by cross-linking of fibrils by aldehyde residues formed by the enzyme lysyl oxidase[102–104]. Thus, cross-linking of collagen gels with several cross-linking agents has been attempted for corneal tissue engineering, including glutaraldehyde[96] and carbondiimides[97], both capable of supporting corneal epithelial cell growth with cells expressing stem cell and differentiation markers, as well as supporting growth of keratocytes and endothelium[96,97]. Cross-linking collagen scaffolds is not a perfect solution as cross-linking agents are often toxic when released after tranasplantation[105]. This is, however, circumvented by carbondiimides, as they are "zero length" cross-linking agents that are water soluble, thus they are not incorporated into the scaffold and can be washed away with water[97,106]. Regardless of cross-linking agent, however, cross-linking collagen with artificial cross linkers reduces its biomimetic properties and, thereby, the ability of cells to modify the substrate[95]. Another possibility for increasing the mechanical strength of a collagen substrate is by plastic compression of the hydrogel, resulting in a thin, mechanically robust membrane of reasonable transparency capable of supporting growth of limbal epithelium and fibroblasts. Furthermore, during the preparation of the hydrogel, it was possible to add keratocytes to the mixture and thus incorporating them into the substrate and mimicking the limbal niche to a greater degree[107].

Several alternative substrates for growth of LESCs have also been suggested, including contact lenses[108,109], silk fibroin[110] and petroleum gauze[15]. In an attempt to maintain cell-cell and cell-ECM contact, a novel system for harvesting single cells sheets without the use of enzymatic cleavage of cell adhesion proteins has been developed by Nishida *et al.*[111–113]. The system consists of culture ware coated with poly-(N-isopropylacrylamide) (PIPAA), a temperature responsive polymer that is hydrophobic at 37°C enabling cells to attach, while being hydrophilic below 32°C causing cells and ECM to detach in a single sheet. Limbal epithelium cultured on PIPAA substrates maintained their clonogenicity and differentiation potential, and expressed both stem cell and differentiation markers. To prevent damage or destruction of the graft, it can be transferred to a transfer membrane and placed directly onto the recipient eye [111–113].

2 Aim

Currently, there are still several issues to address concerning the treatment of LSCD with CLET. Due to intra and inter-study variability, it is difficult to compare the efficiency of different treatments[16,17]. Even though amniotic membrane is the golden standard in CLET, several issues persist including handling and preparation, donor variability and expensive and questionable donor screening procedures[94]. To circumvent these issues, several other substrates have been produced including cross-linked collagen substrates[96,97,114] and plastic compressed collagen substrates[107], the former potentially being toxic[105] and exhibiting inadequate biocompatibility[95], while the latter might be unstable and vulnerable to pH changes and enzymatic digestion[101]. Several alternative substrates have been suggested including PIPAA substrates from which cell-sheets can be harvested[111–113]. Furthermore, many methods still make use of a 3T3 feeder layer, which poses a risk of xenotoxicity and cross-species spread of diseases and, thus, there are very strict regulations for the usage of 3T3 feeder layers[74]. Finally, low oxygen concentrations have been demonstrated to maintain stem cell potential in several stem cell phenotypes[62–64], including LESCs[46,65,66].

Thus, the aim of this project is to investigate and compare cell adhesion, proliferation and maintenance of stem cell potential of stem cells on natural and synthetic substrates that would support differentiation towards corneal phenotype in a serum- and feeder-free system.

3 Methods

3.1 Cell Cultures and Medium

3.1.1 NIH/3T3 Murine Fibroblast Cell Cultures

NIH/3T3 fibroblasts were in stock (Laboratory for Stem Cell Research, Aalborg University, Aalborg, Denmark) and kept at -140°C in 3T3 medium supplemented with 5 % (v/v) DMSO (#D8418; Sigma Aldrich, Broendby, Denmark). 3T3 medium consisted of Dulbecco's Modified Eagles Medium (DMEM; #D5030; Sigma Aldrich) with 10 % Foetal Bovine Serum (FCS; #12133C; Sigma Aldrich), 100 units/mL penicillin, 100mg/mL streptomycin and 50mg/mL gentamycin (Life Technologies, Naerum, Denmark). Medium was sterile filtered through a 0,22μm filter membrane (#FP204250; FilterMax FU, Frisenette, Knebel, Denmark) and kept at 4°C to 8°C. NIH/3T3 fibroblasts were expanded and serially passaged in T175 polystyrene culture flasks (#660975; Greier Bio-One GMBH, Frickenhausen, Germany). Before seeding a feeder layer, cells were split by incubation of cells with 0,25 % trypsin (#27250018; Life Technologies) and 0,02 % EDTA (#444125D; Bie and Berntsen, Herlev, Denmark) at 37°C for 5 minutes. After incubation, the trypsin was inactivated by dilution with 3T3 medium (1:2) and cell suspension was centrifuged at 250 x G for 5 minutes at 25°C and the supernatant discarded. The cell pellet was re-suspended in medium and counted with a haemocytometer. The cell suspension was then diluted to a concentration of 10^5 cells/mL in a 50mL centrifuge tube (#227283; Greiner Bio-One GMBH) for inactivation by γ-irradiation.

3.1.2 γ-Irradiation of NIH/3T3 Fibroblasts

Centrifuge tubes containing 3T3 fibroblasts were sealed with Parafilm® M (Thermo Fischer Scientific, Waltham, MA). 3T3 fibroblasts were inactivated by γ-irradiation (Department for Clinical Immunology, Aalborg University Hospital, Aalborg, Denmark) at 40 Gy. After inactivation, cells were seeded at a density of 4×10^4 cells/cm^2 for feeder layer and 3×10^3 cells/cm for control of inactivation on 12-well CellBIND® culture dishes (#CLS3336; Corning, NY) and left overnight for cells to attach. Before LECs were seeded, the cells were washed three times with sterile phosphate buffered saline (sPBS; #14200-067; Gibco, Taastrup, Denmark).

3.1.3 Green Fluorescent Protein (GFP) Expressing Human Adipose-derived Stem Cells (ASCs)

GFP expressing ASCs were available in stock (Laboratory for Stem Cell Research, Aalborg University, Aalborg Denmark) and kept at -140°C in ASC growth medium supplemented with 10 % (v/v) DMSO. ASC growth medium consisted of Alpha Modified Essentials Medium (α-MEM) with Glutamax® (#32571093; Life Technologies) supplemented with 10 % FCS, 100 units/mL penicillin and 100mg/mL streptomycin. Prior to use, medium was sterile filtered through a 0,22μm filter membrane and kept at 4°C to 8°C. ASCs were expanded and serially passaged in T175 polystyrene culture flasks. Cells were passaged as described above for NIH/3T3 fibroblasts. Optimal seeding density was between 1500 and 2000 cells/cm^2.

3.1.3 Limbal Epithelial Cell Medium for 3T3 Feeder System

Limbal epithelial cell medium for 3T3 feeder system consisted of DMEM and Hams F12 (#51651C; Sigma Aldrich) (2:1) supplemented with 10 % FCS, glutamine (4mM; #G3126; Sigma Aldrich), hydrocortisone (0,4μg/mL; #H0888; Sigma Aldrich) , insulin (5μg/mL; #I3536; Sigma Aldrich), transferrin (5μg/mL; #T8158; Sigma Aldrich), triiodothyronine (2nM; #IRMM469; Sigma Aldrich), adenine (0,18mM; A8626; Sigma Aldrich), epidermal growth factor (EGF; 10ng/mL; #E9644; Sigma Aldrich), choleratoxin (0,1nM; #C8052; Sigma Aldrich), penicillin (50 units/mL; #P3032; Sigma Aldrich) and streptomycin (50 mg/mL; #S6501; Sigma Aldrich).

3.1.4 Serum- and Feeder-Free Limbal Epithelial Cell Medium

Limbal epithelial cells (LECs) were cultured in Epilife® Basal Medium (#M-EPICF-500; Life Technologies, Naerum, Denmark) supplemented with Human Corneal Growth Supplement (HCGS; #S0095; Life Technologies).

3.1.5 Isolation of Human Limbal Epithelial Cells from Corneoscleral Rings

Human LECs were isolated from corneal scleral rings kindly donated by the Danish Cornea Bank (Aarhus University Hospital, Aarhus, Denmark). The posterior surface of the rings were initially cleared of iris pigmentation and endothelium by gentle scraping with a sterile scalpel and subsequently washed four times in s-PBS to remove any medium residues. After washing, rings were placed in either pre-warmed Dispase II (2,4 U/mL; #D4693; Sigma Aldrich) in sPBS at 37°C for 2 hours or collagenase type IV (400 U/mL; #17104019; Life Technologies) in either sPBS or Epilife® Basal Medium at 37°C for 20 to 24 hours. Following incubation, the anterior surfaces of rings were scraped with a sterile scalpel to completely remove the epithelium and subsequently washed three times in s-PBS. The enzyme solution

and the sPBS used for washing the rings were collected in a 50mL centrifuge tube and centrifuged at 250 x G for 10 minutes at 25°C. The supernatant was discarded and the resulting pellet was re-suspended in pre-warmed TrypLE™ Express without phenol red (#12604021; Life Technologies) and incubated at 37°C for eight minutes. TrypLE® was inactivated by dilution with sPBS (1:3) and the suspension was filtered through a 70μm cell strainer (#352350; BD Biosciences, CA) to obtain a single cell suspension. The cell suspension was centrifuged at 250 x G for 10 minutes at 25°C and the supernatant discarded. The cell pellet was re-suspended in medium, counted with a haemocytometer and diluted to system specific seeding densities. Optimal seeding densities are 1500 to 2000 cells/cm^2 in Epilife® with HCGS and 300-500 cells/cm^2 on 3T3 feeder cells.

3.2 Substrates

3.2.1 Preparation of Amniotic Membrane

Cryopreserved human amniotic membrane (AM) was kindly donated by the Danish Cornea Bank. AM was stored at -80°C until used. Before use, AM was thawed at room temperature for one hour. After thawing, the amniotic membranes were transferred to 6-well plates, where they were washed three times with sPBS. AM was either decellularized, epithelially denuded or kept intact. Intact amniotic membranes (iAMs) were completely unmodified and kept at 4°C in sPBS saturated with chloroform (#1024441000; Merck, Darmstadt, Germany) until further use.

3.2.2 Production of Acellular Amniotic Membrane (aAM)

Before decellularization, AMs were washed for 9 hours at room temperature with hypotonic Tris-buffered saline (TBS) (10mM Tris (#T1503; Sigma Aldrich), 50mM sodium chloride (#S7653; Sigma Aldrich) and 0,01 % (w/v) EDTA; pH = 8,0) and wash was discarded. Subsequently, AMs were incubated in decellularization buffer consisting of 0,3 % (w/v) sodium dodecyl sulphate (SDS; L3771; Sigma Aldrich) in isotonic TBS (50mM Tris, 150mM sodium chloride and 0,01 % (w/v) EDTA; pH = 7,6) for 48 hours at room temperature with agitation. Subsequently, decellularization buffer was discarded and AMs were washed three times in large volumes of isotonic TBS (1:5) for 24 hours each time to remove SDS residues. Finally, aAMs were washed by three changes of sPBS and kept at 4°C in sPBS saturated with chloroform until further use. To ensure viability of decellularization, a 1cm x 1cm piece of each aAM was removed and stained with Hoechst 33342 as specified below.

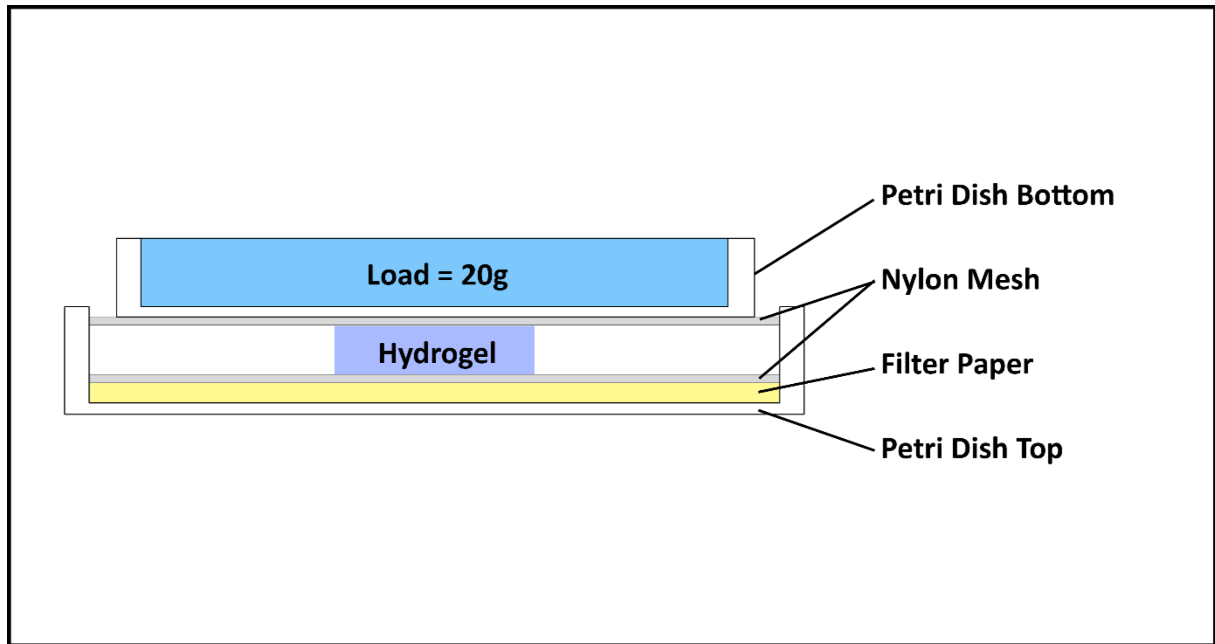


Figure 4 - Schematic of plastic compression of collagen hydrogels to produce mechanically stable collagen membranes.

3.2.3 Production of Epithelially Denuded Amniotic Membrane (dAM)

AMs were incubated in 0,25 % trypsin and 0,02 % EDTA at 37°C for 30 to 45 minutes. After incubation, trypsin was inactivated by dilution with s-PBS (1:3) and washed by three changes of s-PBS. The epithelial layer was removed by gentle scraping with blunt forceps. Finally, dAMs were washed three times with sPBS and kept at 4°C in s-PBS saturated with chloroform until further use. To ensure viability of denudation, a 1cm x 1cm piece of each dAM was removed and stained with Hoechst 33342 as specified below.

3.2.4 Production of Plastic Compressed Collagen Membranes

Collagen solutions were prepared on ice by neutralization of 5,33mL sterile rat-tail type I collagen (#A1048301; Gibco), 0,7mL 10x sPBS (#14200-067; Gibco) and 0,83mL sterile filtered de-ionized water (dH_2O) with 1N sodium hydroxide. 1,75mL collagen solution was added to each of four ice-cold 12mm diameter custom circular moulds and incubated at 4°C for 60 minutes for small bubbles to disperse. After cold incubation, gels were incubated at 37°C for 60 minutes to undergo fibrillogenesis. After fibrillogenesis, gels were gently removed from the moulds, sandwiched between two layers of nylon membrane and placed on filter paper in the lid of a 100mm petri dish (#CLS430591; Corning). Gels were then subjected to unconfined plastic compression for five minutes by placing the bottom of the petri dish on top of the gel and filling it with 15mL of water. Finally, the bottom of the petri dish was removed and the resulting membrane was rehydrated with sPBS and gently peeled from the nylon mesh with

blunt forceps and a scalpel. Plastic compressed collagen membranes were washed by three changes of sPBS and kept at 4°C in sPBS saturated with chloroform until further use.

3.2.5 Preparation of Substrates for Cell Cultures

Before cells were seeded, substrates were washed by three changes of sPBS. iAM, aAM, dAM and plastic compressed collagen membranes were cut into 1cm x 1cm pieces with a sterile scalpel. iAM, aAM, dAM, plastic compressed collagen and Collagen Cell Carrier™ were placed onto 24-well Cell-Bind® plates (#CLS3337; Corning) containing 200µL sPBS in each well, and sPBS was subsequently removed and discarded. Membranes were left to partially dry for 30 minutes at room temperature to remove any air trapped underneath the substrates. Finally, before cells were seeded, the substrates were incubated in either ASC growth medium or Epilife® with HCGS for 30 minutes at 37°C. Cells were seeded at densities specified above and medium was changed three times a week.

3.2.6 Cell Growth Assay

To test viability of substrates to support cell growth, GFP expressing ASCs were seeded at a density of 1500 to 2000 cells/cm2 onto the substrates and incubated overnight at 37°C at 5 % O2. Cell growth was monitored with a AxioObserver.Z1 fluorescence microscope (Carl Zeiss International, Göttingen, Germany) for five days (ASCs).

3.2.7 Poly-(N-isopropylacrylamide) (PIPAA)

PIPAA is immobilized as a thin film in normal cell culturing conditions at 37°C facilitating normal cell adhesion to the culture ware, whereas PIPAA swells and becomes hydrated at temperatures below 30°C resulting in detachment of cells without the use of enzymatic cleavage[111–113]. Prior to seeding of cells, PIPAA culture dishes (#174904; Thermo Fischer Scientific) were incubated with either ASC growth medium or LEC medium at 37°C for 1 hour. Cells were seeded at densities specified above and medium was changed three times a week. When cells were confluent, the wells were removed and all but 50-100µL medium was removed from PIPAA culture dishes. The dishes were incubated at room temperature for 30 minutes for cells to detach, and cell-sheets were removed with a poly(vinylidene difluoride) membrane (supplied with PIPAA culture dishes ;Thermo Scientific) and transferred to a CellBIND® dish (#CLS3295; Corning).

3.3 Fixation and Staining of Cells

Before fixation, samples were washed by three changes of sPBS. Samples were fixated by incubation with 4 % formaldehyde for 30 minutes at 37°C. After fixation, samples were washed by two changes of sPBS

and nuclei were stained with a 1:1000 Hoechst 33342 solution followed by an additional washing step of three sPBS changes. Samples were kept at 4°C in sPBS.

3.4 Microscopy

Phase contrast pictomicrographs were obtained with a PixeLink PL-A782 camera (Olympus Denmark A/S, Ballerup, Denmark) attached to a CKX41 inverted phase contrast microscope (Olympus Denmark A/S) and sampled with PixeLink v.1.4.5 software (Olympus Denmark A/S) at 4x, 10x and 20x magnifications. Fluorescent images were acquired with either an AxioCam MRm (Carl Zeiss International) or an *Orca* Flash 4.0 camera (Carl Zeiss International) attached to an AxioObserver.Z1 microscope (Carl Zeiss International) at 5x and 10x magnifications.

3.5 Data Analysis

In the cell growth assay, ASCs were counted at days 1, 3 and 5 after seeding and LECs were counted 14 days after seeding using the Cell Counter plugin for ImageJ (National Institutes of Health, MD). Variances were analysed by Levene's test for equality of variances. Depending on distribution, statistical significance was analysed by either a one-way analysis of variance (ANOVA) or Kruskal-Wallis analysis of variance by ranks and Mann-Whitney U test for determining inter-group variability.

4 Results

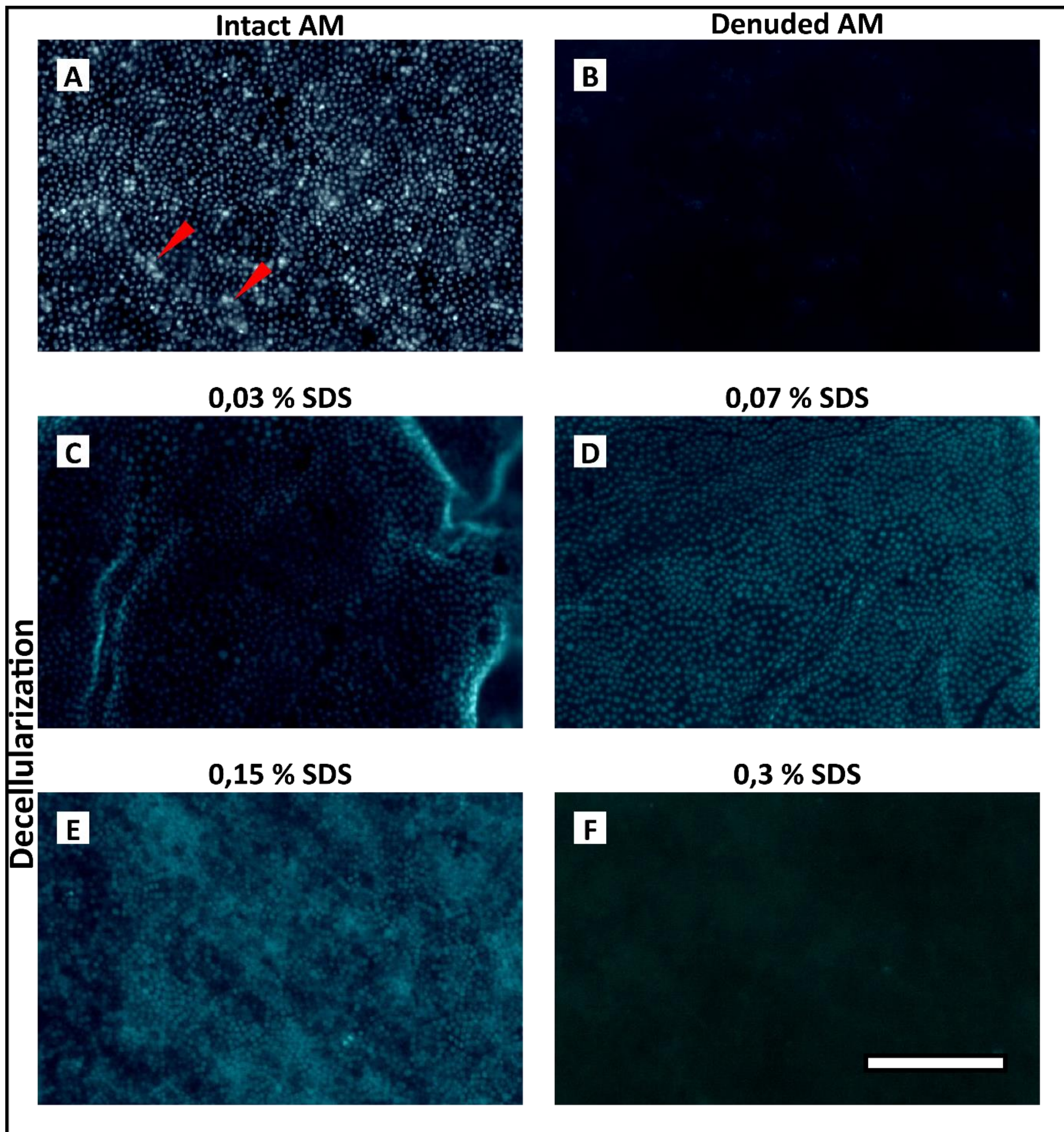


Figure 5 - Decellularization without pre-treatment with hypotonic TBS and denudation of human amniotic membrane. **(A)** The apical surface of intact AM is covered by a thin monolayer of epithelium with mesenchymal cells residing in the underlying stroma (red arrowheads). **(B)** Treatment of AM with trypsin/EDTA results in complete detachment of the epithelial basement membrane, which can subsequently be removed by scraping. **(C-F)** Treatment of AM with different concentrations of SDS without prior treatment with hypotonic TBS was not sufficient at removing epithelium at low concentrations **(C-E)**, but no cells could be observed at a concentration of 0,3 % **(F)**. Nuclei are stained with Hoechst 33342 (blue). Scalebar depicts 200 µm. AM: Amniotic membrane; SDS: Sodium dodecyl sulphate; iAM; Intact AM; dAM; Epithelially denuded AM.

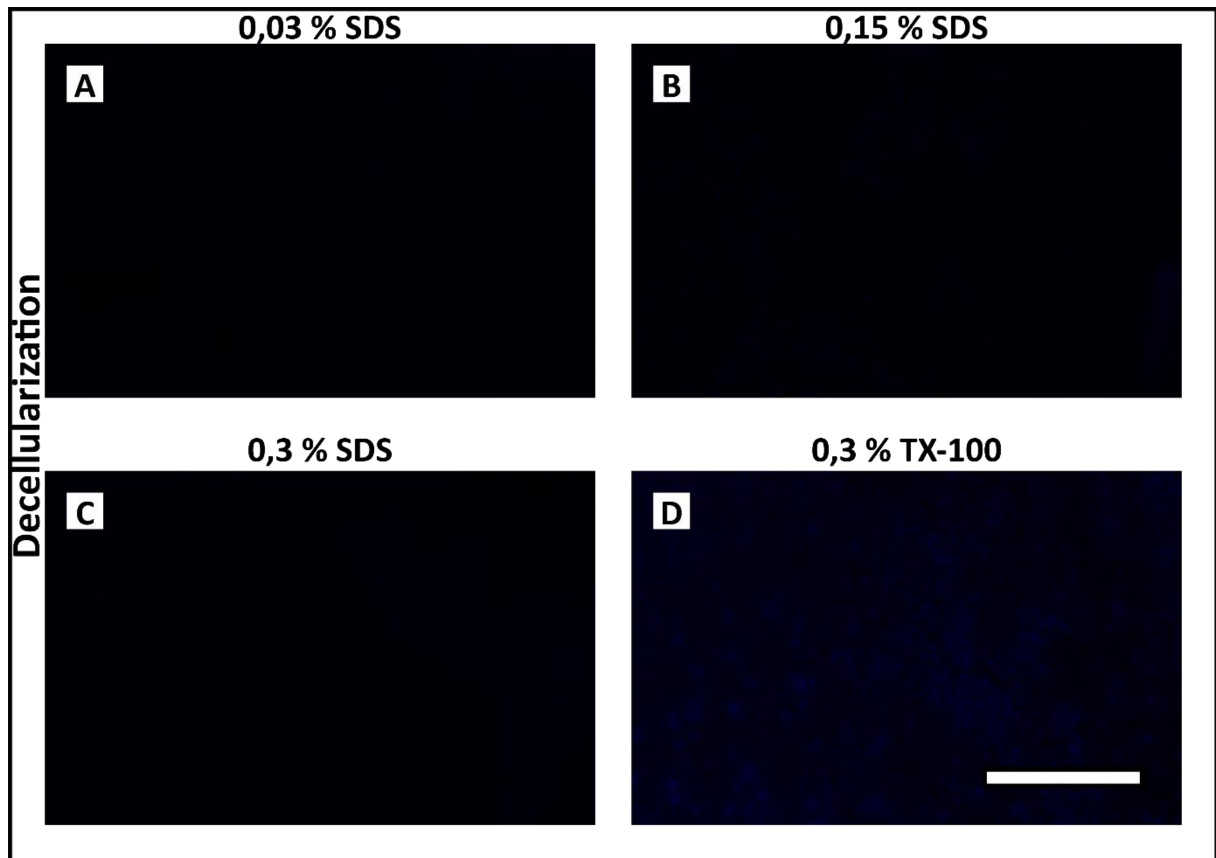


Figure 6 - Decellularization with pre-treatment with hypotonic TBS of human amniotic membrane. **(A-C)** Treatment of AM with SDS with prior hypotonic TBS treatment resulted in complete acellularity regardless of concentration. **(D)** Treatment of AM with TX-100 did not sufficiently remove epithelium. Nuclei are stained with Hoechst 33342 (blue). Scalebar depicts 200 µm. AM: Amniotic membrane; SDS: Sodium dodecyl sulphate; TX-100: Triton X-100.

4.1 Production of Acellular and Epithelially Denuded Amniotic Membrane

Cryopreserved AM was either kept intact, decellularized or epithelially denuded. iAM was placed into sPBS immediately after thawing. Intact amniotic membrane was slightly opaque and could be physically manipulated without being damaged.

For decellularization, AM was subjected to treatment with or without hypotonic TBS followed by incubation in different concentrations ranging from 0,03 % to 0,3 % of SDS to determine the correct concentration for decellularization. Hoechst 33342 stain detected a fine monolayer of epithelium on the apical surface of iAM as well as stromal cells with large nuclei below the epithelium **(figure 5A)**. The monolayer of epithelium was also detected on AM treated with 0,03 %, 0,07 % and 0,15 % SDS without

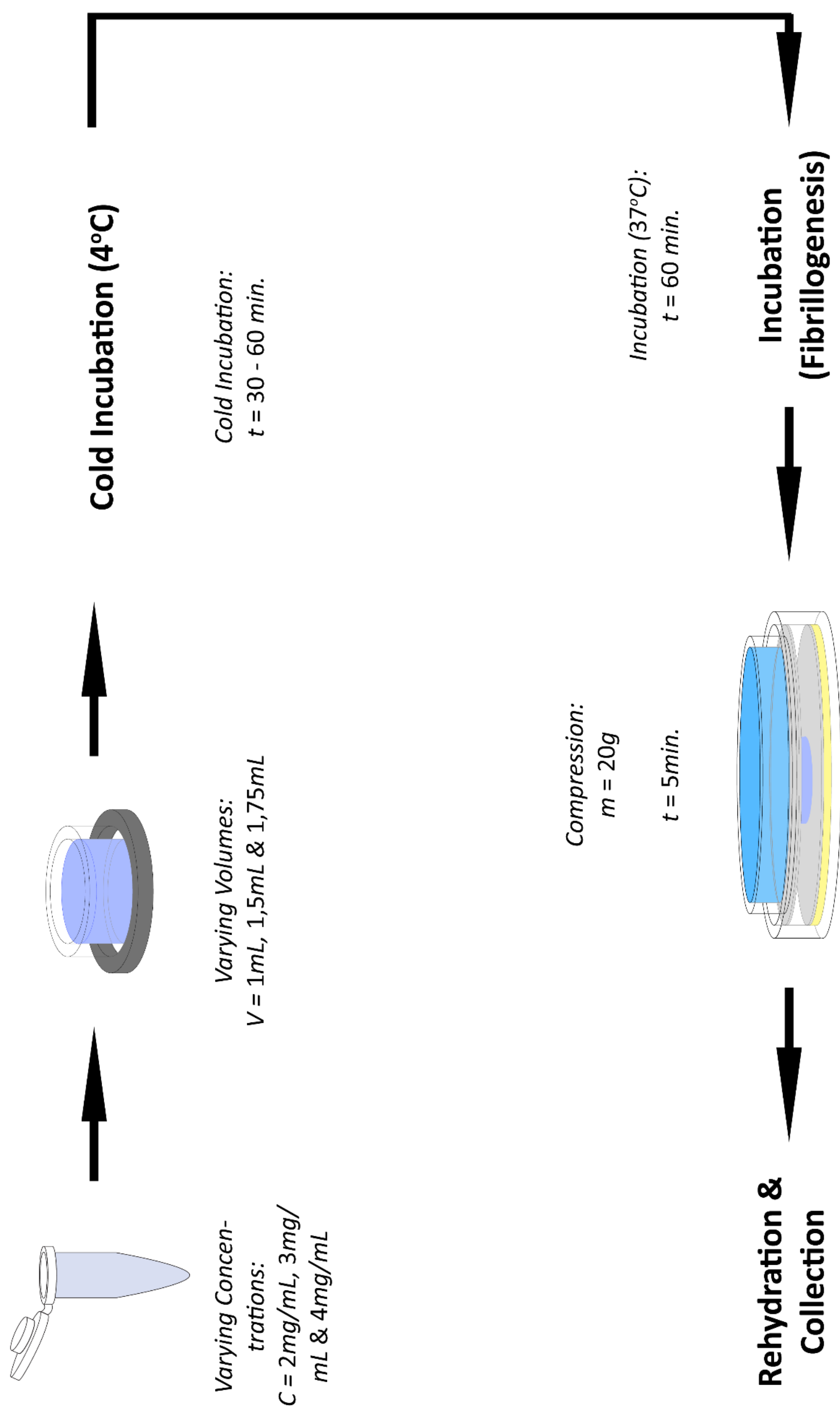
hypotonic TBS treatment, however, no nuclei from stromal cells could be seen **(figure 5C-E)**. Treatment with 0,3 % SDS without prior treatment with TBS resulted in the complete absence of nuclei on the entire AM and, thus, complete acellularity **(figure 5F)**. Treating AM with hypotonic TBS prior to treatment with SDS resulted in complete acellularity at all tested concentrations **(figure 6B-D)**. This was, however, not the case for TX-100, where nuclear remnants could be seen after 48 hours of incubation **(figure 6E)**. The resulting aAM had similar physical properties to iAM and could be physically manipulated without being damaged.

To remove the epithelial layer, AM subjected to enzymatic digestion and inhibition of Ca^{2+}-dependent Cadherins by trypsin/EDTA treatment followed by scraping with blunt forceps to remove the epithelial layer. After trypsinization, the epithelial layer was partially detached from the stroma of the AM and could subsequently be removed with forceps. Hoechst 33342 stain did not detect the epithelial monolayer, however, several large stromal nuclei could be detected **(figure 5B)**. The resulting dAM was much thinner than iAM and, therefore, more transparent. dAM was, however, more fragile than iAM and aAM and extra care had to be taken when handling dAM.

4.2 Production of Plastic Compressed Collagen Membranes

Plastic compressed collagen membranes were produced by preparing a collagen hydrogel and subjecting it to unconfined compression afterwards. Several combinations of concentrations and volumes have been tested in different combinations to produce a thin, mechanically stable collagen membrane. Initially, to determine the concentration needed to produce a stable hydrogel, the concentration of collagen was set to 2mg/mL, 3mg/mL and 4mg/mL in a 1mL solution. After incubation, hydrogels were subjected to plastic compression. Incubation of the collagen solutions resulted in stable hydrogels for all conditions. Membranes created from hydrogels with 2mg/mL and 3mg/mL collagen concentrations were very thin, mechanically unstable, and could not be removed from the nylon mesh without being damaged. Membranes produced from hydrogels with concentrations of 4mg/mL were, however,

Figure 7 - Production and optimization of plastic compressed collagen membranes. First, the concentration of collagen in the hydrogel was varied and the volume kept constant to determine the amount of collagen necessary to produce a stable hydrogel and plastic compressed membrane. Thereafter, the total amount of collagen was kept constant and the volume varied. All collagen solutions were incubated at 4°C to allow small bubbles to disperse followed by fibrillogenesis at 37°C. Hydrogels were subjected to unconfined compression and the resulting membranes rehydrated and collected with forceps.


Varying Concen-
trations:
C = 2mg/mL, 3mg/
mL & 4mg/mL
Varying Volumes:
V = 1mL, 1,5mL & 1,75mL
Cold Incubation (4°C)
Cold Incubation:
t = 30 - 60 min.
Incubation
(Fibrillogenesis)
Incubation (37°C):
t = 60 min.
Compression:
m = 20g
t = 5min.
Rehydration &
Collection

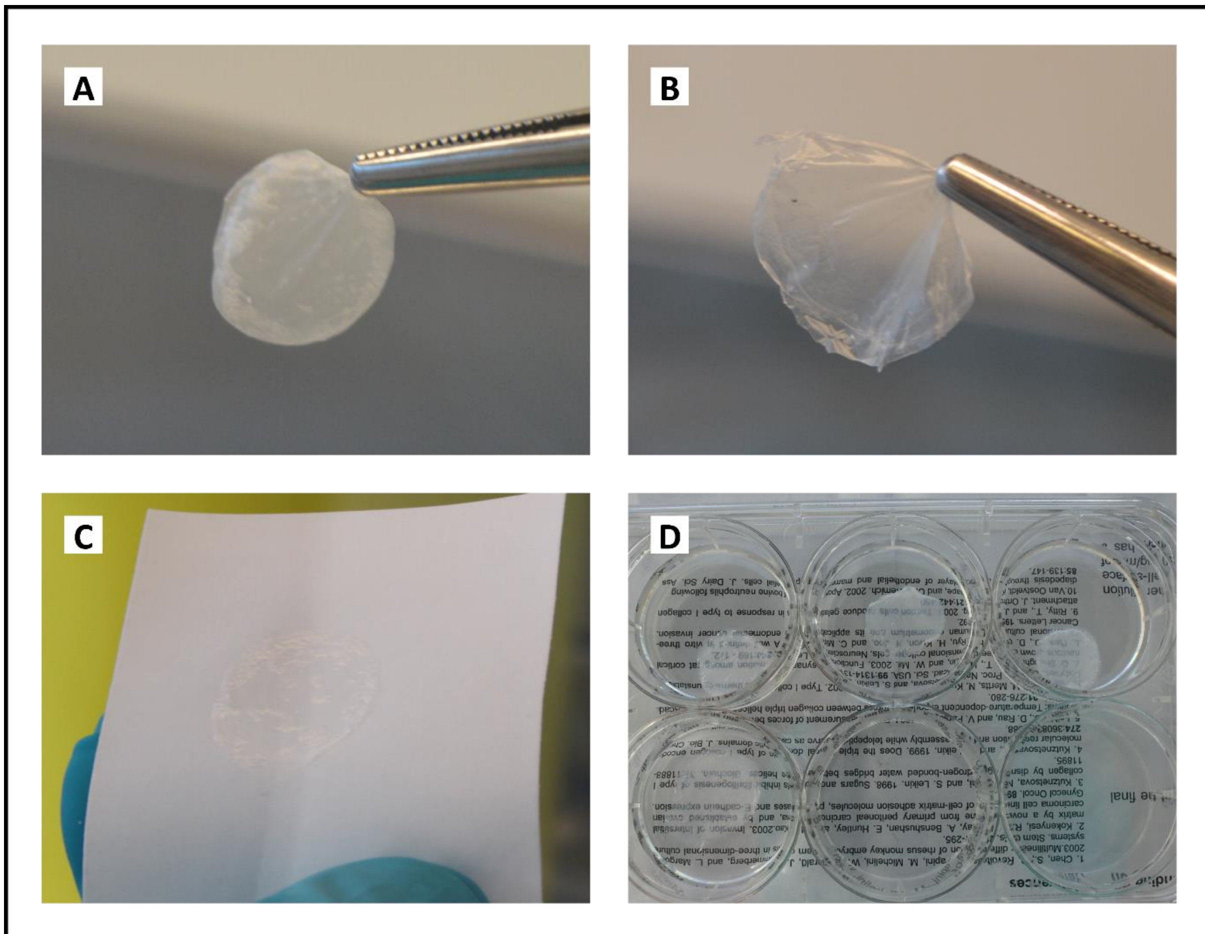


Figure 8 - Plastic compressed collagen membranes. **(A)** Plastic compressed collagen membrane produced from a 4mg/mL, 1mL suspension. **(B - D)** Plastic compressed collagen membranes produced from a 2,28mg/mL, 1,75mL suspension. **(C)** After compression, collagen membranes were stuck to the nylon mesh and had to be rehydrated before removal with forceps. **(A & B)** Even though the total amount of collagen was equal in both conditions, membranes produced from a 2,28mg/mL, 1,75mL hydrogel tended to be slightly larger, thinner and more transparent. **(D)** Membranes were, however, not completely transparent.

mechanically stable and could be removed from the nylon mesh with forceps. To facilitate the use of collagen at different stock concentrations of collagen, a test of stability of membranes with the same total collagen content but different concentrations was devised, where the volume of the solutions was set to 1mL, 1,5mL and 1,75mL. Incubation of all solutions resulted in stable hydrogels. Unconfined compression of all hydrogels resulted in stable membranes that could be removed with forceps. Membranes produced from 1,75mL hydrogels, however, tended to be slightly larger and thinner than membranes produced from 1mL and 1,5mL hydrogels **(figures 7 and 8)**.

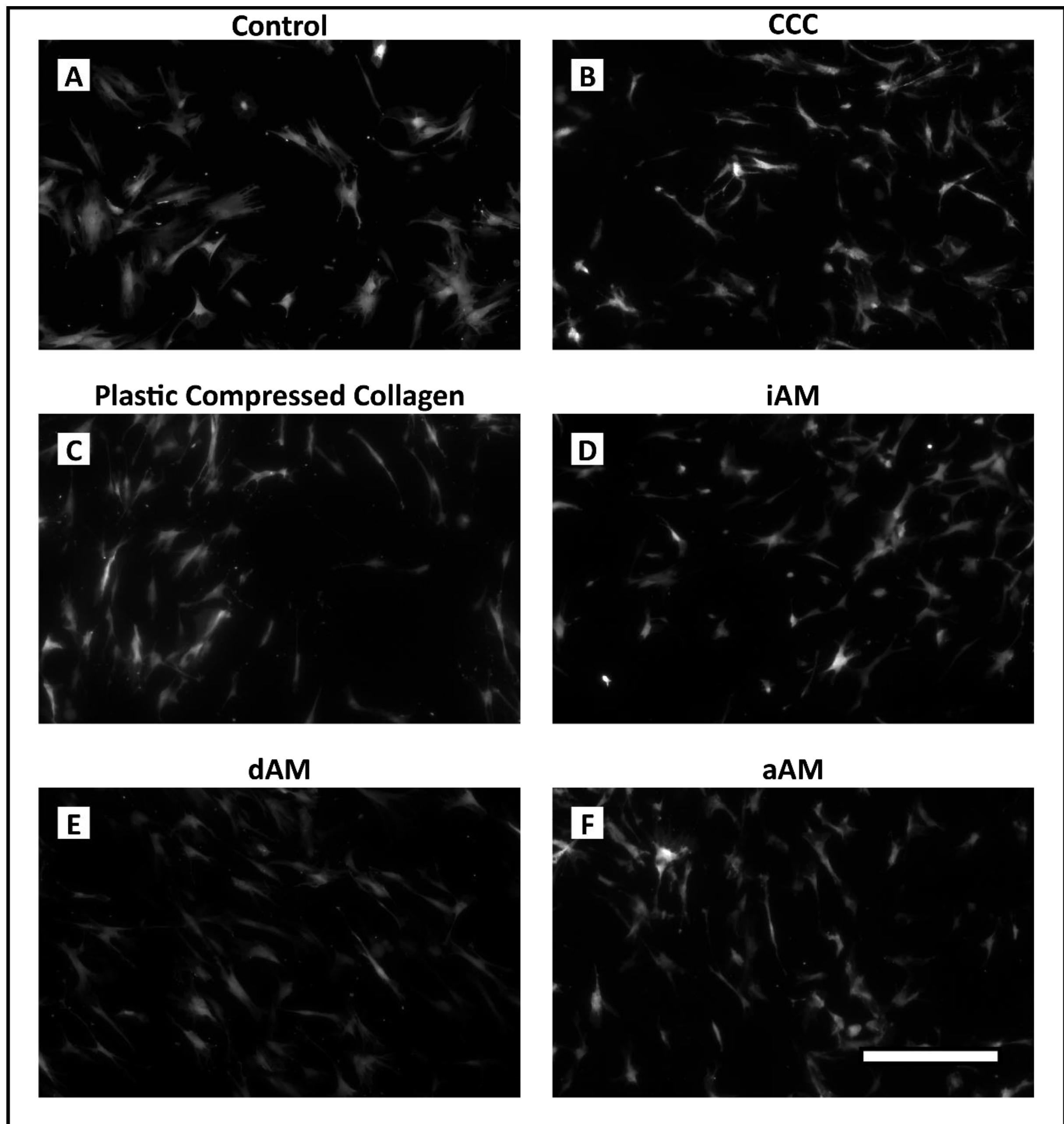


Figure 9 - Attachment of GFP expressing ASCs onto substrates. **(A)** Polystyrene tissue culture dish. **(B)** Collagen Cell Carrier™. **(C)** Plastic compressed collagen membranes. **(D)** Intact AM. **(E)** Epithelially denuded AM. **(F)** Acellular AM. Original magnification 5x. Scalebar depicts 200 µm. AM: Amniotic membrane; CCC: Collagen Cell Carrier™; iAM; Intact AM; dAM; Epithelially denuded AM; aAM; Acellular AM.

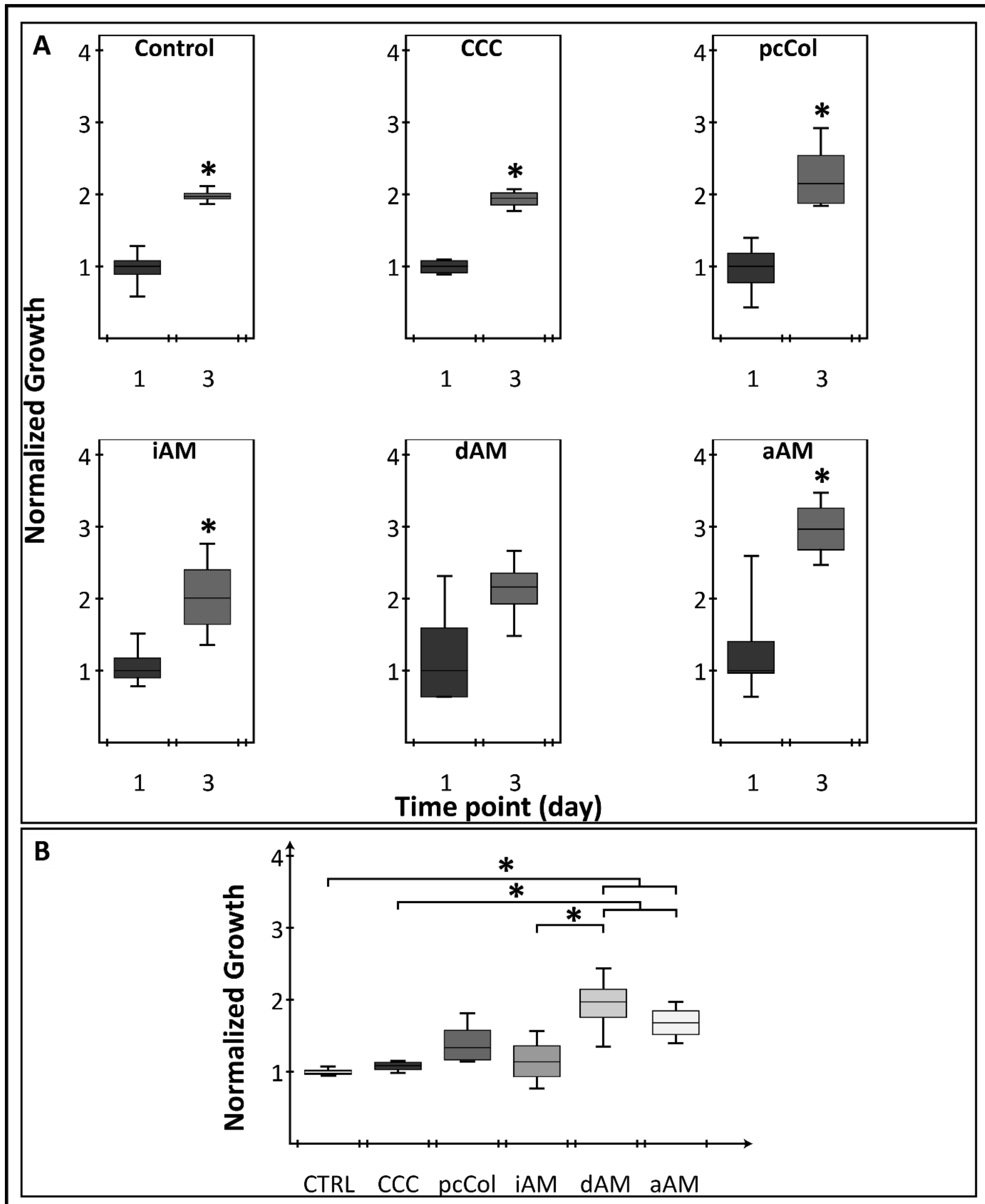


Figure 10 - Normalized growth of GFP expressing ASCs on substrates. The attachment and growth of ASCs was assessed in two independent experiments with duplicates of each sample on days 1 and 3. **(A)** Growth of cells on individual substrates from day 1 to day 3. Numbers were normalized for each substrate with respect to its own day 1. **(B)** Relative growth of cells on substrates at day 3. Numbers were normalized for each substrate with respect to control wells. Number of samples per condition: $n = 4$. AM: Amniotic membrane; CCC: Collagen Cell Carrier™; pcCol: Plastic compressed collagen membranes; iAM: Intact AM; dAM; Epithelially denuded AM; aAM: Acellular AM. Asterisk (*) represents statistically significant differences ($p < 0,05$).

4.3 Cell Growth Assay

To test the viability of the substrates as potential carriers, GFP expressing ASCs were seeded duplicates of each substrate in two independent experiments and left overnight to allow attachment of cells. At day 1, day 3 and day 5, fluorescent micrographs were taken of ASCs, cells were counted and cell density calculated, and cell morphology was analysed.

ASCs attached to all substrates within 24 hours of seeding **(figure 9)**. At day 1, no significant differences was observed in cell concentrations on the substrates ($p > 0,05$). Regardless of substrate, cells grew at a steady rate and cell density was approximately doubled at day three in control wells ($p = 0,0033$), on CCC ($p = 0,0001$), plastic compressed collagen membranes ($p = 0,0076$) and iAM ($p = 0,0442$). On dAM, however, this was not statistically significant ($p > 0,05$). On aAM, cell density was tripled at day three ($p = 0,0207$) **(figure 10)**. No significant differences were observed between controls, CCC and iAM at day 3. However, a greater cell density could be observed on dAM ($p = 0,0250$) and aAM ($p = 0,0111$) compared to controls **(figure 10)**. No differences in cell morphology were noted between substrates and controls at any of the time points.

4.4 Growth and Detachment of Cells from PIPAA

ASCs and LECs were seeded onto PIPAA culture dishes in two separate experiments to determine growth on PIPAA culture dishes and feasibility of harvesting and transplanting cell sheets. Both ASCs and LECs displayed growth on PIPAA culture dishes similar to that on polystyrene culture dishes **(figure 11A and B)**. Lowering the temperature of the culture dishes to 20°C resulted in the complete detachment of ASCs, which allowed for transplantation onto another culture dish **(figure 11C)**. Transplanted ASCs, however, did not thrive after transplantation and stopped dividing while still sub-confluent **(figure 11E)**. LECs did not detach when temperature was lowered to 20°C and could not be transplanted **(figure 11D)**.

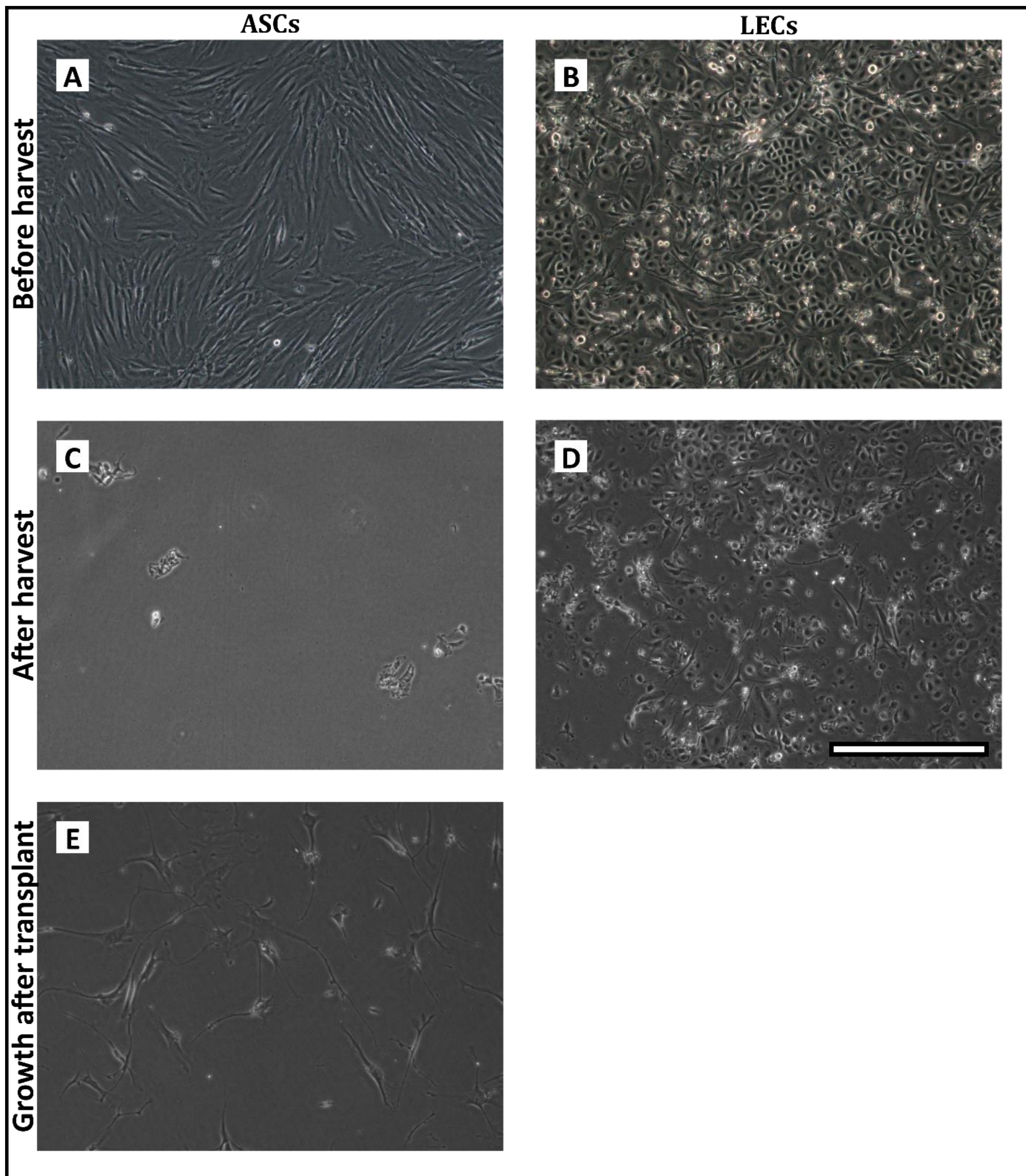


Figure 11 - Growth and detachment of GFP expressing ASCs and LECs three and 13 days after seeding respectively. **(A)** Confluent ASCs on PIPAA prior to harvest of cell sheet. **(B)** Sub-conflueent LECs on PIPAA. **(C)** Lowering the temperature to 20°C resulted in detachment of ASC cell sheet after 40 minutes. **(D)** LECs did not detach when temperature was lowered to 20°C. **(E)** Ten days after transplant of ASCs from PIPAA. ASCs did not thrive after transplantation and became mitotically inactive. Original magnification 4x. Scale-bar depicts 50 μm.

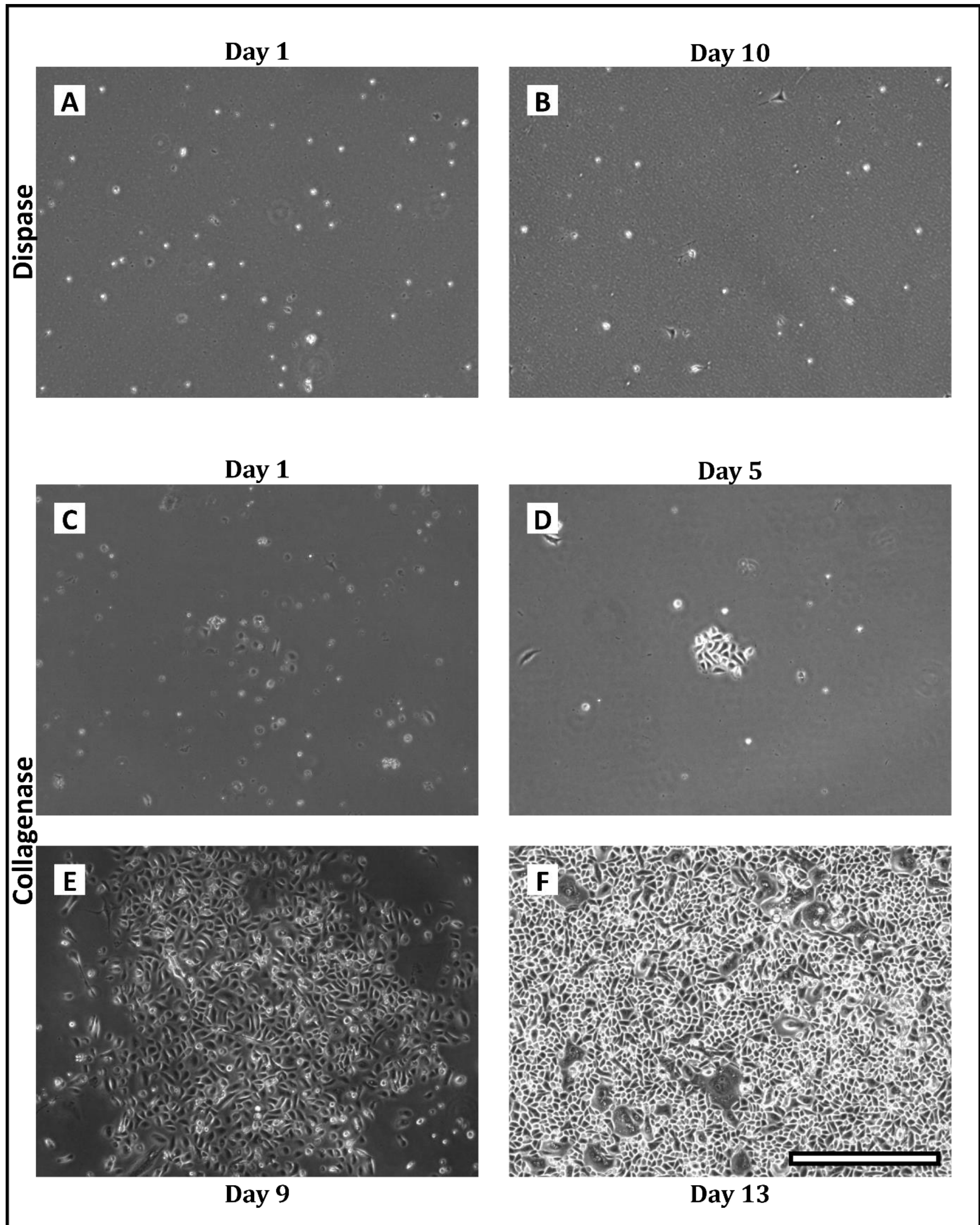


Figure 12 - LECs isolated by Dispase II or Collagenase on CellBIND® culture plate in Epilife® with HCGS. **(A&B)** Few cells are isolated by Dispase II treatment, cultures remain mitotically incative and only few cells are seen to attach to the culture ware. **(C-F)** During the first three to five days after isolation, LECs are in a quiescent state and are mitotically inactive. After the first signs of mitosis, confluence is reached within seven to fourteen days. Original magnification 4x. Scalebar depicts 50 µm.

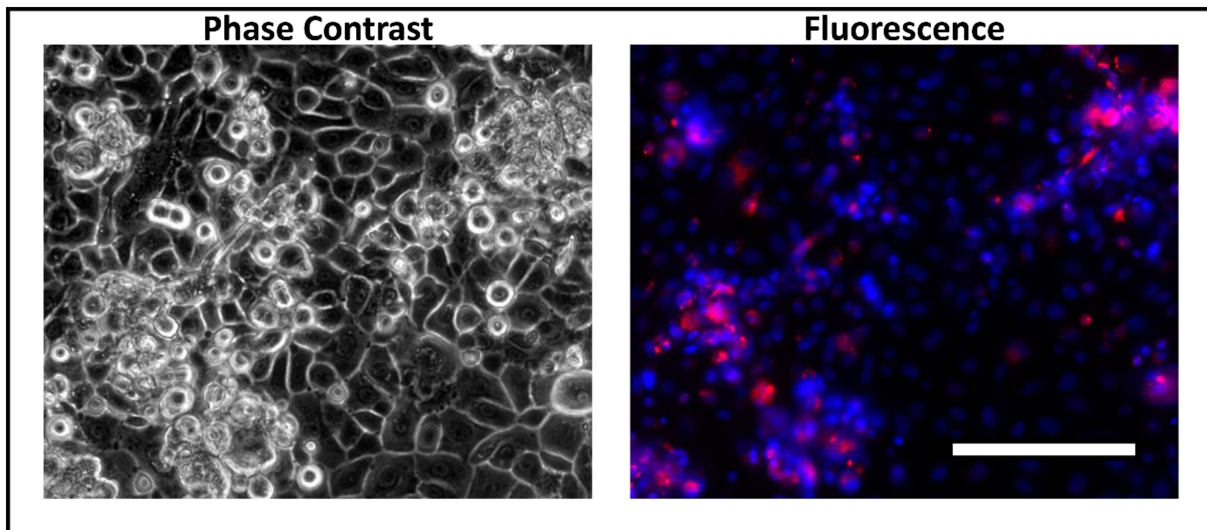


Figure 13 - Multilayered clusters of label-retaining cells in LEC cultures isolated by collagenase treatment. (A) Phase contrast. (B) Fluorescence. Cells were labelled with PKH26 (red) prior to seeding and fixed and stained with Hoechst 33342 (blue) at day 13. Original magnification 10x. Scalebar depicts 50 µm.

4.5 Isolation of Limbal Epithelial Cells from Corneoscleral Rings

LECs were isolated from corneoscleral rings by primary enzymatic digestion with either Dispase II or collagenase IV to loosen the epithelial sheet and secondary enzymatic digestion with TrypLE Express to render epithelium into a single cell suspension. Although Dispase isolation loosened the epithelial sheet after 1,5 hours of incubation, subsequent digestion with TrypLE Express did not render the epithelium into a single cell suspension and an average of 2388 (SD = 283) cells per ring were isolated from seven rings. Cells isolated by Dispase digestion attached to culture ware but remained mitotically inactive **(figure 12A and B)**. Collagenase digestion differed greatly from Dispase digestion as the epithelial sheet was almost completely dissolved in all cases after 18 to 24 hours, and subsequent incubation with TrypLE Express rendered cells into a single cell suspension. With collagenase, an average of 397566 (SD = 21754) cells per ring were isolated from 26 rings. Cells harvested by collagenase digestion tended to be quiescent for between three and five days before they became mitotically active, and the cultures became confluent within seven to fourteen days after first signs of mitosis **(figure 12C to F)**. Cultures consisted of epithelial cells seen as small polygonal cells with a “cobble stone”-like appearance with interspersed fibroblasts seen as elongated bipolar or multipolar cells **(figure 12E and F)**. In cultures labelled with PKH26 prior to seeding, multi-layered clusters of label retaining cells as opposed to a monolayer of cells without label could be observed after confluence was reached **(figure 13)**. In cases where the viability of a batch of rings was in question due to low cell counts during harvest or high cell mortality after seeding, LECs

were isolated from three randomly selected corneoscleral rings from the batch and seeded onto a feeder layer consisting of 3T3 fibroblasts inactivated by γ-irradiation in parallel with CellBIND® and Epilife®. Several batches of corneoscleral rings were excluded from further experimentation in this manner **(figure 14)**.

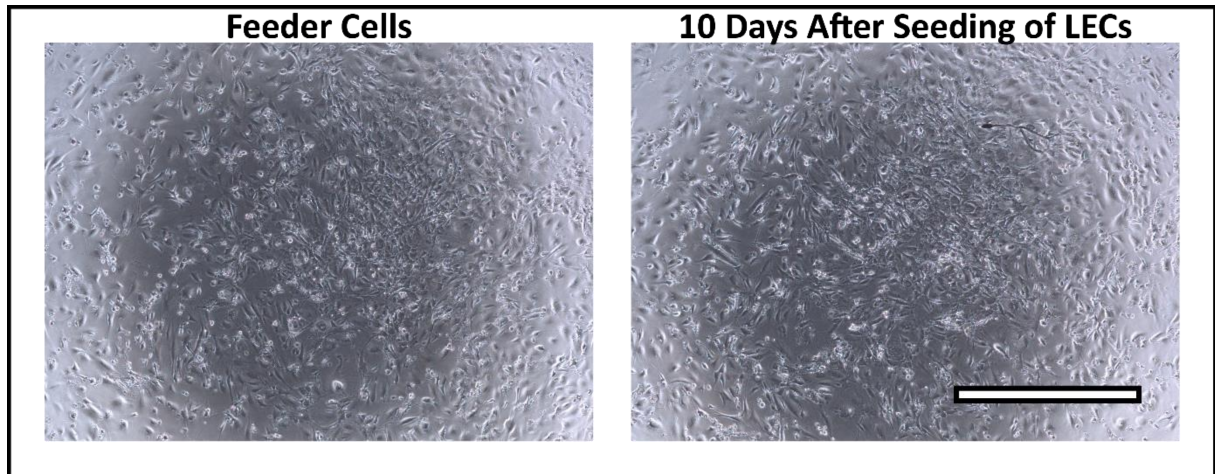


Figure 14 - 3T3 feeder system was used to test the viability of corneoscleral rings. **(A)** Feeder layer prior to seeding of LECs. **(B)** Feeder layer devoid of epithelial colonies ten days after seeding of LECs. Original magnification 4x. Scalebar depicts 100 µm.

5 Discussion

The main goal of this current project was to replicate substrates used for CLET and study their potential in facilitation of cell adhesion and maintenance of proliferation potential in cells. Furthermore, LECs were to be harvested from corneoscleral rings and cultured on the substrates in a xeno- and feeder-free system at both atmospheric and sub-atmospheric conditions to further assess the potential of substrates to function as carriers for CLET in a clinical environment based on their ability to maintain proliferation and differentiation potential of LESCs.

5.1 Human Amniotic Membrane

When treated with different concentrations of SDS, it was discovered that concentrations below 0,3 % are not sufficient to completely decellularize amniotic membranes. However, with the addition of prior incubation with hypotonic TBS, all tested concentrations of SDS were sufficient to decellularize AM, but AM treated with TX-100 still contained nuclear residues. Hypotonic buffer solutions have long been used for the purpose of lysing cells[115–118]. Furthermore, hypotonic solutions have been demonstrated to be sufficient for decellularization of tissue[119]. Hypotonic and hypertonic solutions are, however, often used in combination with detergents such as SDS or TX-100 to increase their efficiency[119–122]. This is corroborated by the results of this experiment. Apart from causing cell lysis, a possible explanation for the increased efficiency of detergents by hypotonic buffers is that transient holes develop in the cell membrane during treatment with hypotonic buffer[117], which makes the cells more susceptible to chemical[116] and mechanical damage[123]. The decreased efficiency of TX-100 compared to SDS is, however, converse to reports indicating that TX-100 has a lower LC_{50} in cell cultures[124] and has a greater detergent efficiency than SDS[125]. Thus, this result requires further experimentation to be confirmed. It has previously been reported that AM can be successfully decellularized with hypotonic TBS and 0,03 % SDS. Histological and histochemical analyses demonstrated that the aAM produced in this manner had histological, chemical and physical properties similar to iAM[126]. This is also in support of the observations in this experiment.

Incubation of AM in trypsin/EDTA resulted in the detachment of the epithelial basement membrane from the stroma. Subsequent scraping with blunt forceps removed all traces of epithelium from the basement membrane. This is supported by several other studies[76,77,79,80,90]. On the one hand, dAM is much

thinner than iAM and aAM, and it is not as mechanically stable and has a tendency to tear when manipulated. On the other hand, this issue is potentially outweighed by its relative transparency compared to iAM and aAM making it an attractive solution for CLET.

5.2 Plastic Compressed Collagen Membranes

Stable plastic compressed collagen membranes were created with a total collagen amount of 4mg in 12mm diameter circular moulds from collagen hydrogels with different volumes and concentrations. It has been reported that the mechanical strength of collagen tissues depends on the concentration of collagen fibrils in the tissue, organization of the collagen fibrils and the average diameter of collagen fibrils[127]. Furthermore, collagen hydrogels display a highly irregular arrangement of fibrils and a high water content compared to the collagen content[128]. Thus, it is plausible that higher collagen concentrations in hydrogels in this present project are related to greater hydrogel and membrane stability.

When keeping the total amount of collagen stable in the hydrogels while increasing their volumes, plastic compression produced stable membranes that were thinner and slightly larger. A partial explanation for this could be that unconfined compression and capillary forces cause fluid to leave the hydrogel in one direction only. Due to obstruction by collagen fibrils, the fluid leaving surface of the membrane functions as an ultrafiltration membrane allowing water to pass through while retaining collagen fibrils, where the latter becomes compacted and forms a "cake"[128]. Due to the directionality of fluid leaving the hydrogel and the increased height of the hydrogel, only a slight expansion in diameter of the membrane occurs during plastic compression, while a larger amount of collagen fibrils becomes compacted in the central region of the membrane. It was interesting to note that plastic compression of collagen membranes produced from hydrogels with larger volumes produced a more transparent membrane than membranes produces from hydrogels with smaller volumes. This could, however, also be explained by the relative thickness of the resulting membranes. Membranes were, however, not completely transparent regardless of hydrogel volume due to the irregular arrangement of collagen fibrils causing scatter of light. Conversely, it has been reported that plastic compression of collagen hydrogels can produce clear membranes with a similar histological structure to that of the cornea[107]. Several approaches have been suggested to create transparent collagen structures for corneal regeneration, including vitrification[100], cross linking with "zero-length" cross-linkers[99] and magnetic alignment of collagen fibrils and fibroblasts during fibrillogenesis[98], as collagen substrates are a desirable replacement

for amniotic membrane due to their low production cost, biomimetic properties, possibility for mass production and low immunogenicity.

5.3 Attachment, Proliferation and Morphology of Cells

5.3.1 Amniotic Membrane

GFP expressing ASCs seeded onto iAM, aAM and dAM were capable of attachment and proliferation. Data also suggested that cell proliferation might differ on the three substrates, as cell density on day 3 was 2 times higher on dAM and 1,5 times higher on aAM than in controls, while there was no difference between iAM and controls. It has previously been reported by Koizumi *et al.* that removal of the epithelial layer from amniotic membrane results in an initial rapid proliferation and stratification of LECs in a larger degree than on iAM[129]. This has furthermore been confirmed by Shortt *et al.*, who demonstrated that culturing ASCs on dAM caused rapid proliferation and differentiation of LECs, which resulted in early confluence with a low cell density[130]. iAM, on the other hand, maintained a monolayer of undifferentiated, slow-cycling cells, thus displaying slower proliferation in both studies[129,130] as well as the ability to maintain stem cell potential for multiple cell passages, thus functioning as niche cells[93,94]. The findings from the aforementioned studies are consistent with observations from this current project reporting that dAM enhances proliferation compared to iAM. Observations from aAM are similar to those from dAM, which further suggests that the epithelium of the AM plays a role in maintenance of differentiation and proliferation potential. Cells cultured on dAM and iAM took on a bipolar, spindle shape with small "star-shaped" cells interspersed between them. These observations correlate to observations on human foreskin fibroblasts, bovine bone marrow stromal cells and 3T3 murine fibroblasts cultured on dAM and iAM from a study by Guo *et al*[131]. Cells on aAM, however, tended to be small, polygonal cells with multiple filopodia, and only few bipolar cells were observed. It is possible that removal of the amniotic epithelium without removal of its basement membrane could cause ASCs take on this morphology, as the structure and composition of the ECM has been demonstrated to influence the morphology of cells[132].

5.3.2 Plastic Compressed Collagen and CCC™

Collagen constitutes the greatest quantity of protein in the human body, and it is by far the most abundant protein in the extracellular matrix[133]. Collagen has been used to create several tissue scaffolds[95,107,133] and tested on several cell types[134]. Thus, in this project, it is not surprising that GFP expressing ASCs were capable of attachment and proliferation on both plastic compressed collagen

membranes and CCC™ similar to that on polystyrene. The morphology of ASCs on both CCC™ and plastic compressed collagen membranes was a mixture between elongated bipolar cells and small multipolar cells similar to that on iAM and dAM.

5.3.3 PIPAA

On PIPAA culture plates, attachment, growth and cell morphology of LECs and ASCs was similar to polystyrene. ASC cell sheets could be harvested by lowering the temperature of the well to 20°C, as described by Nishida *et al*[57,112,113]. When transplanting the cell sheet, however, only few cells attached after overnight incubation of cell sheet attached to nitrocellulose paper on a normal polystyrene culture dish. Cells did not thrive after transplantation, and stopped proliferating at sub-confluence. A possible explanation for this could be that during the incubation at 20°C, cells were exposed to atmospheric concentrations of CO_2 for extensive periods and succumbed due to alkalosis.

When incubating LECs at 20°C, cells did not detach even after 60 minutes. Nishida *et al.*, however, reported that corneal epithelial cells as well as oral mucosal epithelial cell sheets could be harvested by temperature reduction[111,112]. There is, however, one main difference between the culturing techniques utilized by Nishida *et al.* and this study: This project utilizes a serum- and feeder-free system to culture LECs, while Nishida's group utilizes the classic feeder system. Corneal epithelium was cultured on Mitomicin inactivated 3T3 murine fibroblasts, while oral mucosal epithelium was cultured on a cell culture insert separating the cells from the 3T3 feeder cells. Furthermore, the epithelial cultures in Nishida's experiment underwent spontaneous stratification after full confluence was reached, while LEC cultures in this study remained in a monolayer state. Based on the results from this project and taking the differences between the methods utilized in this project and by Nishida's group into mind, it can be hypothesized that culture conditions greatly affect the efficiency of PIPAA.

5.3 Isolation of LECs from Corneoscleral Rings

5.3.1 Dispase

Isolation of LECs from corneoscleral rings with primary digestion with Dispase II and secondary digestion with TrypLE Express did not produce a sufficient quantity of viable LECs for experimentation. It has been reported that Dispase digestion causes detachment of the epithelial sheet from the stroma, and that subsequent treatment with trypsin/EDTA renders the epithelial sheet into a single cell suspension[76,79].

To identify the issue with the procedure, each step of the procedure was investigated. In this experiment, 2,4u/mL Dispase II treatment for 2 hours at 37°C resulted in complete dissociation of the epithelial sheet from the corneoscleral rings, indicating that Dispase indeed causes dissociation of the epithelial basement membrane. Subsequently, corneoscleral rings were scraped in sPBS to ensure removal of the epithelium. All basement membrane residues were then incubated in either TrypLE Express or 0,25 % trypsin/0,02 % EDTA for 15 minutes. Samples from both suspensions were transferred to a haemocytometer and viewed with a phase contrast microscope to determine whether cells were released from the basement membrane. Cells could be seen in both suspensions and no differences could be observed. The TrypLE and trypsin/EDTA suspensions were inactivated by dilution with sPBS (1:3) and poured through two cell strainers each to prevent cells from being caught in the remaining connective tissue. Finally, the cells were pelleted and re-suspended in Epilife®. Subsequent counting revealed that the amount and viability of cells harvested from rings were still insufficient for experimentation, and a new method was sought for isolating LECs from corneoscleral rings.

5.3.2 Collagenase

Recently, it has been reported that a protocol similar to Dispase II isolation utilizing collagenase instead can be used in isolation of LECs from corneoscleral rings, and that it has a greater efficiency than Dispase II at isolating limbal basal cells and cells from the limbal crypts[52,53,135]. To adapt this method to the existing Dispase II protocol, 400u/mL collagenase IV incubation for 18 to 24 hours replaced the Dispase II incubation step. Incubation of corneoscleral rings in collagenase for 18 to 24 hours resulted in almost complete digestion of the epithelial basement membrane. Furthermore, after incubation with TrypLE and filtration, a large quantity of cells was obtained.

5.3.3 LEC Cultures and Viability of Corneoscleral Rings

LECs isolated by collagenase tended to be quiescent for three to five days in Epilife® before the first signs of mitosis could be observed, and cultures were confluent within seven to fourteen days as has been reported[74]. The cultures did not consist of pure epithelial cells as a variable quantity of fibroblast-like cells were interspersed between LECs. Collagenase isolation has been demonstrated to isolate a side population of small fibroblast cells expressing several stem cell[53]. The authors noted a better maintenance of proliferative and differentiation potential of LECs with this method compared to Dispase II isolation, and thus suggested that these cells function as a niche[52,53,135]. Furthermore, in a feeder-free environment, small epithelial populations with stem cell characteristics in close association with their niche cells

formed 3-dimentional structures[53], which was supported by observations from this study demonstrating 3-dimentional structures of label-retaining cells providing more evidence for the hypothesis that these cells are stem cells. Further experimentation is, however, needed to confirm this.

Finally, low cell counts and poor cell survival in culture has resulted in several batches of corneoscleral rings being excluded from further experimentation, and more attention needs to be paid to collection and storage of donor tissue.

6 Conclusion

The aim of this project is to investigate and compare cell adhesion, proliferation and maintenance of stem cell potential of LECs on natural and synthetic substrates that would support differentiation towards corneal phenotype in a serum- and feeder-free system.

During the course of this project, six different substrates were acquired or produced. iAM was either used as a substrate, epithelially denuded with trypsin/EDTA with subsequent scraping, or decellularized with a combination of a hypotonic buffer and a mild detergent. Furthermore, a mechanically stable collagen membrane was produced by plastic compression of stable collagen hydrogels. Finally, a commercial collagen membrane, CCC™, and thermo-responsive PIPAA culture dishes were acquired. GFP expressing ASCs were used to study adhesion, proliferation and cell morphology. Due to technical difficulties with donor tissue it was, however, not possible to study how the substrates affect cell adhesion, proliferation and maintenance of stem cell potential in the limbal epithelial phenotype. Thus, more attention needs to be paid to acquisition and handling of donor tissue before the substrates can be tested on LECs.

7 Perspectives

Currently, there are still several issues with CLET including determining the best substrate and growth conditions, lack of a well-defined xeno-free culture system, and difficulty to compare reported treatments due to intra- and inter-study variability. Even though this project did not test the effect of the substrates on the limbal epithelial phenotype, it has shed some light on the importance of the substrate on the growth and morphology of stem cells and contributed to current knowledge on the subject, albeit on a different stem cell phenotype. Furthermore, results from isolation of cells from corneoscleral rings using collagenase supports current knowledge on the subject, although it contributes in its own way by being in a xeno- and feeder-free culture system. Finally, by including a large number of substrates and combining it with a novel method for isolating cells from donor tissue and xeno- and feeder-free system, this project ultimately contributes by paving the way to eliminate current issues with CLET and improve its clinical outcomes. It is therefore adamant for research on this subject to be continued.

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