Co-culture of corneal epithelial cells and adipose stem cells – towards the use of hydrogels in ocular surface reconstruction

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Tiivistelmä

Tutkimuksen tavoitteet: Kudosteknologiassa erilaisia yhteisviljely -tekniikoita on tutkittu laajasti lähivuosina ja tarkoituksena on ollut kehittää fysiologisesti relevantteja solurakenteita erilaisten kudosten rekonstruktiota varten. Tämän kaltaiset menetelmät ovat lupaavia käytettäväksi myös sarveiskalvon vaurioista johtuvan sokeutumisen hoitoon, johon vielä nykyäänkin käytetyin hoitomenetelmä on perinteinen kudossiirre. Kudossiirrettä ei kuitenkaan voida käyttää limbaalisten kantasolujen puutoksen hoitoon, joka on yksi suurimmista syistä sarveiskalvoperäiseen sokeutumiseen, johtuen siirteistä puuttuvista limbaalisista kantasoluista. Vaihtoehtoisia ratkaisuja siis tarvitaan. Tämän tutkimuksen tavoitteena oli löytää sopiva kasvatusmediumi ihmisen rasvan kantasoluille niistä mediumeista, jotka oli aikaisemmissa tutkimuksissa todettu sopivaksi ihmisen sarveiskalvon epiteelisoluille, ja käyttää sitä hydrogeeliin sulautettujen ihmisen rasvan kantasolujen ja immortalisoitujen ihmisen sarveiskalvon epiteelisolujen yhteisviljelyssä. Saadut tulokset voivat auttaa arvioidessa tämän kaltaisen 3D-rakenteen toiminnallisuutta sarveiskalvovaurioiden ja limbaalisten kantasolujen puutoksen hoitoon.

Materiaalit ja menetelmät: Sopivan kasvatusmediumin löytämiseksi ihmisen rasvan kantasoluja viljeltiin kolmessa eri mediumissa. Sopivan mediumin löydyttyä ihmisen rasvan kantasoluja yhteisviljeltiin immortalisoitujen ihmisen sarveiskalvon epiteelisolujen kanssa kaksiulotteisesti epäsuorassa- ja suorassa kontaktissa. Lopuksi ihmisen rasvan kantasoluja viljeltiin hyaluronihappo –hydrogeelissä. Solujen käyttäytymistä tutkimuksen eri vaiheissa arvioitiin faasikontrastimikroskoopin, proliferaatio- ja elävyys/kuolleisuus analyysien sekä immunofluoresenssivärjäysten avulla.

Tulokset ja johtopäätökset: Ihmisen rasvan kantasolut kasvoivat hyvin testatussa yhteisviljelymediumissa ja niillä näytti olevan yhteisviljelyissä parantava vaikutus immortalisoitujen ihmisen sarveiskalvon epiteelisolujen kasvuun ja erilaistumiseen. Hyaluronihappo -hydrogeeleissä ihmisen rasvan kantasolut selviytyivät ja kykenivät proliferoitumaan käytettäessä tarpeeksi suurta solutiheyttä. Saadut tulokset viittaavat siihen, että sarveiskalvon epiteelisolujen ja hydrogeeliin sulautettujen rasvan kantasolujen kolmiuloitteinen yhteisviljely saattaa omata potentiaalia tulla käytettäväksi tulevaisuudessa sarveiskalvon rekonstruktiossa.

Avainsanat: sarveiskalvo, sarveiskalvon rekonstruktio, kudosteknologia, yhteisviljely, hydrogeeli, rasvan kantasolu, sarveiskalvon epiteelisolu

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Abstract

Objectives: In a tissue engineering field, co-culture techniques of different kind have been widely researched in recent years to form physiologically relevant cell structures for different tissue reconstructions. The treatment methods of that kind are promising opportunity to be used also in the treatment of corneal blindness, which is still mainly treated with traditional tissue graft from a donor. Tissue grafts cannot still be used to treat one of the most common cause behind the corneal blindness –limbal stem cell deficiency (LSCD), due the lack of host stem cells. Thus, alternative opportunities are needed. Objectives of this research were to find a suitable medium for human adipose stem cells (hASCs), from those that had already been shown to be suitable for human corneal epithelial cells (hCECs), and use it to co-culture hydrogel- embedded hASCs with hCECs. Results will possibly help us to evaluate the functionality of this kind of 3D structure better as a treatment method of the corneal damages and LSCD.

Materials and methods: HASCs were first cultured in three different mediums to find a suitable one to use in co-culturing. Following 2D co-culturing occurred within indirect- and direct contact between the immortalized hCECs and hASCs. Research was accomplished by culturing the hASCs inside the hyaluronic acid- hydrogel. Cells' behaviour during these experiments was evaluated with phase-contrast microscope, proliferation- and live/dead assays and immunofluorescent stainings.

Results and conclusions: HASCs grew well in tested co-culture medium and appeared to have enhancing effect on the growth and differentiation of the co-cultured immortalized hCECs. When cultured in 3D hydrogel structure, hASCs were able to survive and proliferate within right cell densities. These results indicate that 3D co-cultures with hCECs and hydrogel- embedded hASCs might have potential for future applications in ocular surface reconstruction.

Keywords: cornea, corneal reconstruction, tissue engineering, co-culture, hydrogel, adipose stem cell, corneal epithelial cell

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Abbrevations

2D	Two- dimensional
3D	Three- dimensional
α-SMA	Smooth muscle α-actin
AC	Adipose cell
ADC	Anchorage- dependent cell
AdMSC	Adipose mesenchymal stem cell
AL	Airlifting
ASC	Adipose stem cell
BM	Basement membrane
BSA	Bovin serum albumin
CD44	Cell surface recepor cluster 44
CEC	Corneal epithelial cell
CI	Collagen type I
CK	Cytokeratin
ECM	Extracellular matrix
ESC	Embryonic stem cell
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HS	Human serum
hAM	Human amniotic membrane
hASC	Human adipose stem cell
hCEC	Human corneal epithelial cell
hiPSC	Human induced pluripotent stem cell
ICAM1	Intercellular adhesion molecule 1
IF	Immunofluorescent
iPSC	Induced pluripotent stem cell
LEC	Limbal epithelial cell
LSCD	Limbal stem cell deficiency
LSC	Limbal stem cell
MMP	Matrix metalloprotease
MSC	Mesenchymal stem cell
PBS	Phosphate- buffered saline
PCLA	Polylactide-co-glycolide
PFA	Paraformaldehyde
PET	Polyethylene terephthalate
PLA	Processed lipoaspirate
PLC	Poly-ε-lactone
P63α	Keratinocyte progenitor cell marker 63α
PMC	Post- mitotic cell
PU	Polyurethane
RGD	Arginine-glycine-aspartic acid
RHAMM	Hyaluronan mediated motility
SC	Stem cell
SF	Serum-free
SVF	Stromal vascular fraction

TAC	Transient amplifying cell
TDC	Terminally differentiated cell

1. Introduction

A cornea is an outermost, multilayered and avascular structure of an eye, which the main responsibilities are to separate the eye from the environment with a tear- film and act as a refractor of the light. Due the cornea is in direct contact with the surrounding environment, its epithelium layer need to regenerate constantly vie limbal stem cells. These cells are localized with low numbers to the stem cell niches in the basal region of the limbus, which is the outer edge of the corneal epithelium region that function as a physical barrier between the corneal- and conjunctival epithelium layers. From the niches, the stem cells migrate toward the corneal surface and eventually replace the lost corneal epithelial cells.

In limbal stem cell deficiency (LSCD) this corneal regeneration process is damaged by the failures of the functions of the limbus or loss or the dysfunction of the LSCs, which causes the loss of vision and even blindness. Unilateral or bilateral LSCD is one of the most common cause of the corneal blindness worldwide and it cannot be treated with traditional tissue grafts from a donor due the lack of the host stem cells. Tissue grafts are the main treatment method for different corneal damages even though there is chronic lack of them all the time. Thus, alternative opportunities to treat LSCD and other diseases causing corneal blindness are needed.

In tissue engineering, two- and three- dimensional (2D and 3D) co-culture techniques of a different kind have been widely researched in recent years. Especially when used with different stem cells, co-cultures have been researched to mimic native tissues and to form physiologically relevant cell structures to be used in different tissue reconstructions. The stem cells have been used widely in different co-cultures with terminally differentiated cells and the main purposes have been to differentiate the stem cells towards specific lineage or promote a wanted behavior of the terminally differentiated cell population including proliferation, cell adhesion and matrix production. These stem cell co-cultures have already showed to overcome some critical limitations in tissue engineering, such as vascularization.

The major advantage of 3D cell culture methods comparing with the traditional 2D methods is that they mimic the cells' natural microenvironment better. One of the most promising platforms to different 3D cell cultures have been hydrogels of a different kind due their high

water content, similarity with different tissue components and unique mean to cell encapsulation. Hydrogel- based ophthalmology applications are also already in commercial use that makes them a promising tool for corneal reconstructions. In this research, the objectives were to find a suitable culture medium from hASCs, from those that had already been shown to be suitable for hCECs, and use it to co-culture hydrogel- embedded hASCs with immortalized hCECs. Results will possibly help to evaluate the functionality of that kind of 3D co-culture structure as a treatment method towards corneal blindness and LSCD.

2. Literature Review

2.1. Cornea

2.1.1. Structure of the cornea

The cornea is an outermost, avascular and transparent structure of the eye those two main functions are to act as a primary barrier between environment and the eye and as a refractor of the light with an overlying tear- film. The cornea's refractive properties are caused by many factors, but especially due its curved shape (approximately 0,5mm thick), structural anatomy and cellular components' physiology. The cornea is composed of five different layers: epithelium, Bowman's layer, stroma, Descemet's membrane and endothelium (Figure 1) (Meek, Knupp , DelMonte, Kim 2011, Chien Y et al. 2012, Sehic A et al. 2015).

Approximately 50µm thick squamous epithelial layer that lies on the surface on the cornea constitutes approximately 8% of the thickness of it. This epithelium layer can be further divided into three different layers: a superficial-, superbasal- and basal layer (Figure 1). From these, the superficial epithelial layer creates the barrier between the eye and environment with the tear- film towards microbial invasions, chemical factors and foreign- body damages. The superficial layer consists of 2-3 squamous cellular layers and is maintained by tight junction complexes between different layers. The tear- film is produced by goblet cells from conjuctiva and supplies growth and immunological factors that are critical for example to epithelial cells' proliferation. Comparing with the superficial layer, the superbasal layer has otherwise similar structure, but it consists of the so- called wing cells instead of the squamous cells. The deepest basal layer consists of the single columnar cell layer that is attached by the hemidesmosomal system to the underlying 0,05µm thick basement membrane (BM), which consists of collagen (type IV and VII) and laminin secreted by these basal layer cells. Basal layer cells are also only epithelial cells –other than stem cells (SCs) or transient amplifying cells (TACs)– that are capable of mitosis (DelMonte, Kim 2011, Sehic A et al. 2015, Massoudi, Malecaze & Galiacy 2015, Ahmad et al. 2010, Kobayashi et al. 2015).

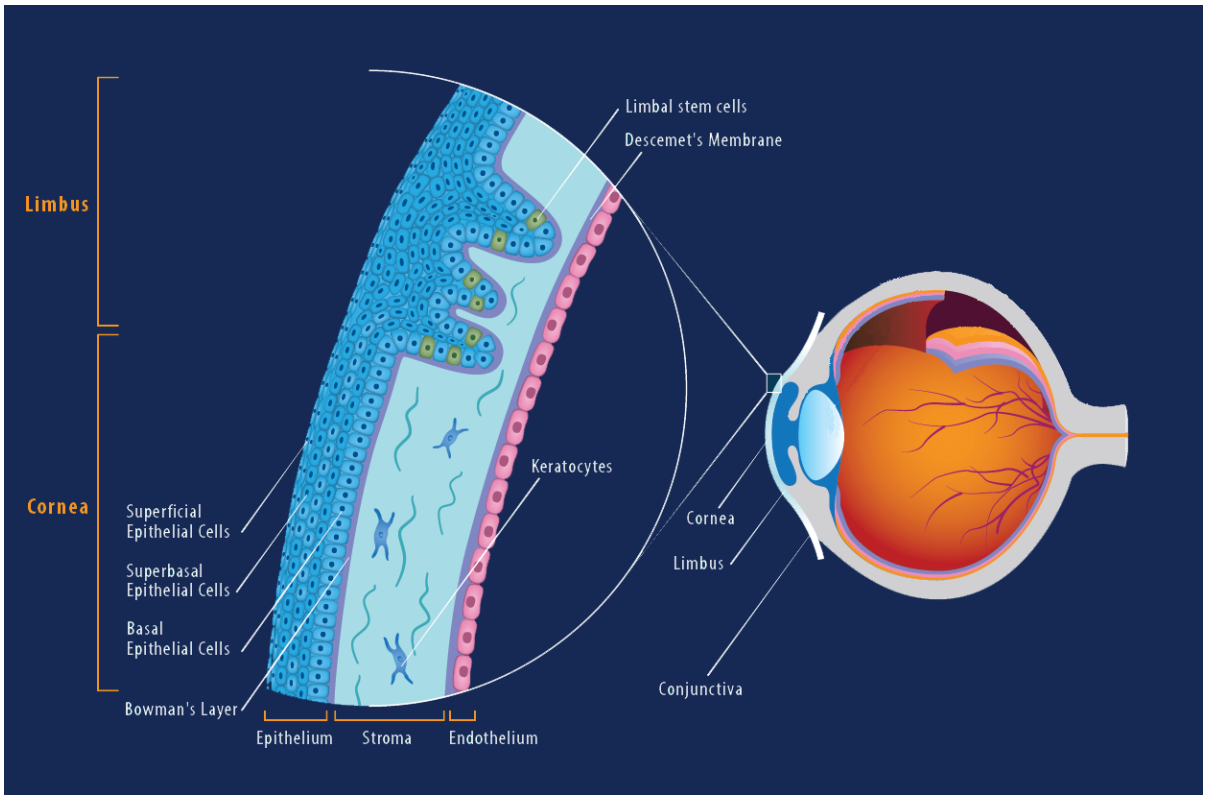

Figure 1 Structure of the human cornea (Modified from http://discovery.lifemapsc.com/library/images/the-anatomy-and-structure-of-the-adult-human-cornea; 5.9.2016)

Bowman's layer is approximately 15µm thick acellular structure in the cornea, which is composed of randomly orientated collagen fibrils (type I and V) and keratan sulfate proteoglycans within the extracellular matrix (ECM). The main function of the Bowman's layer is still unknown, but it has been suggested help maintaining the shape of the cornea, play as a barrier between epithelial- and stromal interactions or protect the stroma from injuries (DelMonte, Kim 2011, Lagali, Germundsson & Fagerholm 2009, Massoudi, Malecaze & Galiacy 2015).

The cornea's bulk structure, stroma, composes 80-90% of cornea's thickness and its main components are collagens (type I and V), different proteoglycans and mesenchymal origin keratocytes. The stroma's transparency and good mechanical properties are due its organized collagen structure. In the stroma, collagen fibers are arranged to fibrils and further to parallel bundles that are packed in 200 to 250 parallel-arranged lamellaes. By placing the lamellaes at the right angle within each other, they support the refractive properties of the cornea. These collagen structures are surrounded by different proteoglycans to maintain structural properties and hydration of the stroma. Keratocytes' function are to keep up stromal homeostasis and

ECM environment by producing stromal collagen fibres, glycosaminoglycans (GAGs) and matrix metalloproteases (MMPs) (DelMonte, Kim 2011, Meek, Knupp , Daniels JT et al. 2001, Griffith M et al. 2002).

The cornea's Descemet's membrane acts as a BM of the endothelium and its structure is similar comparing to the BM of the epithelium. Descement's membrane's thickness increase with age and for adults it is approximately 8-10μm thick (Massoudi, Malecaze & Galiacy 2015, Daniels JT et al. 2001).

Corneal endothelium is composed of a cell monolayer, which main function is to keep up the hydric homeostasis of the cornea by pumping fluids from hypo- osmotic stroma egresses towards hypertonic aqueous humor using the osmotic gradient. By this process, endothelium is able to maintain almost 80% water content in the stroma and the transparency of it. Endothelium is also responsible for nutrients uptake and waste release in the cornea (DelMonte, Kim 2011, Massoudi, Malecaze & Galiacy 2015, Daniels JT et al. 2001).

2.1.2. Regeneration of the corneal epithelium

Because the cornea function as a first barrier between the environment and the eye, it has to regenerate constantly due limbal stem cells (LSCs). These tissue specific SCs are localized in low numbers to the stem cell niches in the basal region of the limbus, which is the highly pigmented outer edge of the corneal epithelium region that function also as a physical barrier between the corneal and conjunctival epithelium layers (Figure 2) (Ahmad et al. 2010, Ahmad 2012, Burman S, Sangwan V 2008). In the niche regions, the SCs are well protected and due close location between epithelium and blood vessels, derived from the palisades of Vogt, cells have access also to high levels of nutrients, oxygen, growth factors and blood- borne cytokines (Mikhailova A et al. 2015, Daniels JT et al. 2001, Ahmad 2012, Dua HS, Azuara-Blanco A 2000, Davies SB et al. 2009).

Regeneration of the corneal epithelium via LSCs has been shortly described by hypothesis XYZ. That hypothesis includes a suggestion, that cell movement from the basal layer (X) and the cell movement from the periphery of the cornea (Y) are replacing the natural cell loss on the corneal epithelium surface (Z). When LSCs undergo asymmetric mitosis, only one daughter cell will

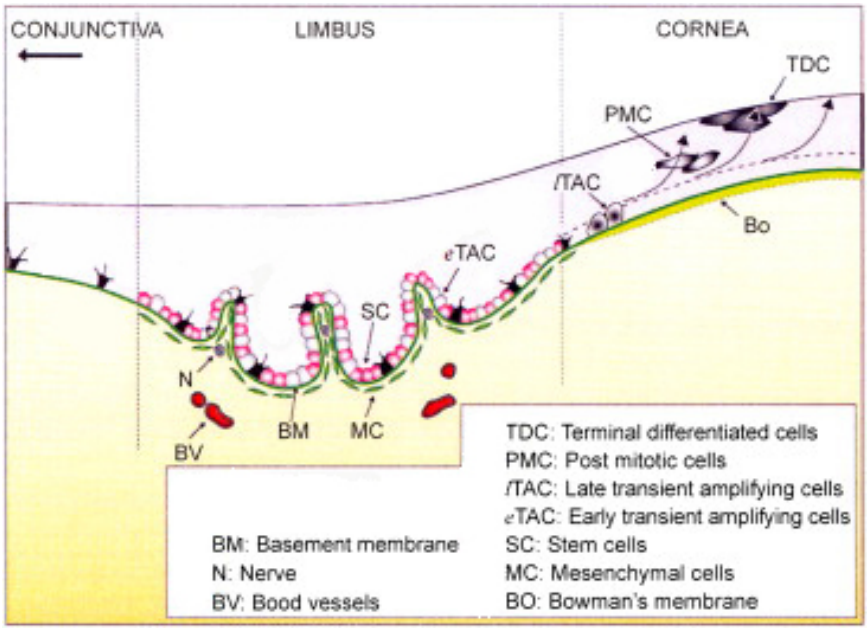

Figure 2 Hypothesis of limbal cell differentiation and migration from the stem cell niche to the corneal surface (Modified from (DelMonte, Kim 2011)

leave the niche and start to migrate towards the central cornea. Right after leaving the niche, LSC will differentiate to the TAC that has higher potential to proliferation and differentiation. TACs migrate towards the centre of the cornea and differentiate after limited numbers of divisions first to the post- mitotic cells (PMCs), and further to the terminally differentiated cells (TDCs) that replace the lost epithelial cells from the corneal surface (Figure 2). The other daughter cell will remain in the niche region and maintain its SC population. Human cornea regenerates typically in 9 to12 months (Ahmad et al. 2010, Mikhailova A et al. 2015, Daniels JT et al. 2001, Yoon JJ, Ismail S & Sherwin T 2014).

2.1.3. Corneal blindness and limbal stem cell deficiency

Corneal blindness is a worldwide problem that affects more than 10 million individuals (Yuan S, Fan G 2015, Griffith M et al. 2002). One of the biggest reason behind the corneal blindness is either unilateral or bilateral limbal stem cell deficiency (LSCD). The LSCD is caused in most cases by a failure of the functions of the limbus or loss or the dysfunction of the LSCs. From these, the dysfunction of the limbus causes the main symptom of the LSCD – conjunctivalization. In conjunctivalization, the surrounding conjunctiva migrates over the cornea and limbus. That will cause the vascularization of the cornea and thus, the loss of

transparency of the cornea, vision loss and even blindness. The loss or dysfunction of the LSCs will have an affect to the healing- and the regeneration processes of the cornea that will also cause vision loss and blindness (Daniels JT et al. 2001, Ahmad 2012). Other common symptoms of the LSCD are severe pain, redness, photophobia and chronic defects in the corneal surface.

There are many known chronic and acute factors that can cause LSCD, for example thermal and chemical burns, genetic disorders like aniridia and Stevens- Johnson's syndrome, different inflammatory diseases, radiation, drugs and even the use of contact lenses (Ahmad et al. 2010, Yoon JJ, Ismail S & Sherwin T 2014). Other big reasons behind the corneal blindness including LSCD are dysfunctions of the corneal stroma, because approximately 90% of corneal diseases and traumas have an affect to it (Arnalich-Montiel F et al. 2008).

2.3. Corneal reconstructions

Even though tissue engineering has taken huge steps ahead in these last few years, corneal blindness is still mainly treated with corneal transplant from a donor. As well as chronic lack of donor tissues, it is important to notice that that kind of transplant cannot be used in the treatment of LSCD, because of the lack of host LSCs (Baylis O et al. 2011). Due these reasons, alternative solutions to treat corneal blindness and defects have been under major interest and there have been wide ranges of different strategies for approaching this problem. Generally, different corneal reconstruction methods can be divided into two main classes: artificial scaffolds and limbal crafts. From these, artificial scaffolds are mainly used for the treatment of stromal defects and limbal crafts for the treatment of limbal defects. Still, only limbal grafts have been achieved long- term success that makes them more promising method of treating corneal defects (Meller D et al. 2011).

2.3.1. Limbal crafts

For almost 20 years, researches have been able to culture the LSCs in vitro. Thus, they have been able to introduce wide new opportunities to treat LSCD (Baylis O et al. 2011, Tominac Trcin M et al. 2015). The main requirements that the limbal grafts have, are that they have to have capability to be attached to the corneal surface either by sutures or for example fibrin

glue and anti-inflammatority, which makes sure that the transplant does not cause any scarring or haze of the stroma (Levis et al. 2015). For that purpose, the wide range of different materials –either natural or synthetic origin– have been researched. Recently, the most used materials in natural origin are human amniotic membrane, fibrin and collagen- based scaffolds. The most used materials in synthetic origin have been for example poly- ε- lactone (PCL), polyurethane (PU) and polylactide- co-glycolide (PCLA) (Tominac Trcin M et al. 2015, Massie et al. 2015). So far, the success rate of LSC transplantation studies has been high (approximately 70%), but the variety between different culture methods, surgery techniques, material sources, number of the patients, different diagnosis of the patients and short follow- up times makes the results very difficult to evaluate accurately (Baylis O et al. 2011, Rahman I et al. 2009, Meller D et al. 2011).

The human amniotic membrane (hAM) is nowadays the most used clinical method for corneal reconstructions and it has been research also as a carrier material to the LSCs. HAM is extremely suitable for this purpose, because its biological properties have showed to enhance the epithelialisation of the corneal surface and support the proliferation, differentiation and migration of the corneal epithelial cells (CECs) (Malhotra C, Jain AK 2014, Tsai et al. 2015, Baylis O et al. 2011). Due its allogenic origin, it still has remarkable disadvantages to be used in corneal reconstructions. These disadvantages include limited transparency, remarkably variety between different membranes and the possibility for viral genome material to transfer from the membrane to the patient (Massie et al. 2015). To overcome these problems, different biomaterials have been researched to use in corneal reconstructions instead of hAM. Hydrogels of different kinds have for example showed promising results already (Tsai et al. 2015).

2.4. Hydrogels

New cell- based therapeutic methods have been under great interest and expectation in recent years in tissue engineering. These methods are based on different mammalian cells delivered to the degenerated places were they can act as therapeutic agents. For that purpose, wide ranges of different biomaterials have been researched to act as three- dimensional (3D) culture platforms to the delivered cells. The major advantage of these 3D cell culture structures comparing with the traditional two- dimensional (2D) cell culturing methods is that

they mimic the cells' natural microenvironment better. Researches have for example showed that the cell-to-cell interactions and chemical- as well as mechanical signals, that coordinate the cell survival, function and differentiation in different cell cultures, are better achieved in 3D culturing methods. One of the most promising 3D platforms has been hydrogels of different kinds. Due their high water content, unique mean to cell encapsulation and similarity with different tissue components, especially with ECM, hydrogels have been widely used as matrices in different tissue reconstruction applications (Wang, Varshney & Wang 2010, Lindborg et al. 2015).

Hydrogels are hydrophilic, water- insoluble 3D structures that consist of both the natural or synthetic polymer network and water. They are capable of absorb large amounts of water or other biological fluids and still maintain their cohesion (Geckil et al. 2010). The total diffusion rate can be controlled by different pore sizes inside the hydrogel structure (Singhal, Gupta 2016). Hydrogels are typically biocompatible, biodegradable, inert, easy to modify chemically –for example by crosslinking, injectable and easy to fabricate to different shapes. Hydrogels' limitations for tissue engineering applications are their poor mechanical and physiological properties, difficulties in the sterilization process and in some cases the delayed response time in the human body. Especially the hydrogels made of natural polymers suffer from poor mechanical properties and immunogenicity more than hydrogels made from synthetic polymers. Still, due natural polymers' better capability to form interactions between cells and proteins they are widely used in tissue engineering. Ones of the most used natural polymers in ophthalmology field are collagen, hyaluronic acid (HA), gelatin and alginate (Kirchhof, Goepferich & Brandl 2015, Tsai et al. 2015, Lai 2016, Singhal, Gupta 2016, Wang, Varshney & Wang 2010).

Main requirement that different hydrogels have to enable cell culturing are that they have to contain enough anchorage- sites to the cells. Especially to the so- called anchorage- dependent cells (ADCs) the cell adhesion and spreading are essential. To enable the cell adhesion and spreading the ADCs bind to the specific motifs like arginine-glycine-aspartic acid (RGD) or other specific domains of the ECM by their integrin receptors. These different cell adhesion motifs are located to the chains of collagen, fibronectin, laminin and vitronectin. Bindings to the motifs initiate the series of intracellular events like focal adhesion in the cells that promote cell proliferation and migration. Because a bulk of the hydrogels contains typically nano- or micro- scaled pores and is bioinert as well as hydrophilic, it does not initiate these integrin

interactions without strong biochemical signals between the cells. The lack of anchorage- site interactions causes that the cells cannot spread inside the hydrogel, they will maintain their spheroidal shape caused by the encapsulating process and die typically in a couple of weeks. According to different researches, the ADCs that have survived in hydrogels have had controllable proliferation, migration and differentiations rates also as well as controllable interactions between cells and matrix –even though the cellular mechanisms behind these effects are not completely understood (Wang, Varshney & Wang 2010). Researches have also showed that if the cells do not have enough space to spread, even the cell- matrix interactions could not always deter the apoptosis (Re et al. 1994, Chen et al. 1997). Hydrogel's structure has to be also permeable to nutrients, oxygen and signalling molecules from surrounding tissue, outgoing metabolites and signalling responses to support the cell survival. The biodegradability is a preferred property to hydrogels, because due the degradation process the cells are able to produce their own cell matrix to replace the artificial one they have been encapsulated. Hydrogel materials should also cause only minimal host response when transplanted to the human body (Gasperini, Mano & Reis 2014).

Hydrogels have already approved to be use in different ophthalmology applications. From these, soft contact lenses are the most successful example. Hydrogels have also researched and used in drug delivery and with cultured cells to treat severe ocular traumas or diseases like LSCD (Kirchhof, Goepferich & Brandl 2015). Thus, they might in the future replace or optimize the other treatment methods and materials like hAM (Wright, Mi & Connon 2013). Hydrogels have also researched to regenerate the corneal stroma, but these attempts are not yet clinically feasible (Kirchhof, Goepferich & Brandl 2015). Advantages of hydrogels to be used in ophthalmology applications comparing with the hAM are that hydrogels have uniform, transparent structure that need only mild preparation conditions and cause only minimum harm when delivered to the ocular surface. Instead of the hAM, hydrogels do not need extensive screening before clinical use (Kirchhof, Goepferich & Brandl 2015, Wright, Mi & Connon 2013).

2.4.1. Hyaluronic acid

Hyaluronic acid (HA) is a linear anionic polysaccharide, which contains of repeating D-glucuronic acid and N- acetyl- D- glucosamine units. The HA is ubiquitous in the human body and found for example in the ECM and in the vitreous humour of the eye where it plays

an important role in different cellular and tissue functions. Due the HA's negative charge it is highly hydrophilic, but need chemical modifications to form a hydrogel structure (Burdick, Prestwich 2011, Lai 2016, Gasperini, Mano & Reis 2014, Lam, Truong & Segura 2014, Lindborg et al. 2015).

The biological function of the HA depends highly on its molecular weight. Naturally the HA exist as a high molecular weight polymer (10^5 to 10^6 Da) (Burdick, Prestwich 2011), but can be cleaved to smaller molecules, even under $3{,}5x10^4$ Da, by hyaluronidase enzyme. Researches have showed that the different molecular weights of the HA have an opposite effect on the cell behaviour. The HA with high molecular weight inhibits the cell proliferation and is anti- angiogenic, but also anti- inflammatory and immunosuppressive. Instead, the HA molecules with lower molecular weight have showed to promote the cell migration and angiogenesis, but also to be pro- inflammatory. On molecular level, the HA interact primary with a cell- surface receptor cluster 44 (CD44), which exists in many cell types including CECs and MSCs having an important role in tissue organization by cell-to-cell interactions, cell-matrix interactions and ECM remodelling. The HA interacts also with receptor for hyaluronan mediated motility (RHAMM) and intercellular adhesion molecule 1 (ICAM1) (Lam, Truong & Segura 2014, Gomes et al. 2004, Lindborg et al. 2015, Snyder et al. 2014, Lei et al. 2011).

Even thought the HA has ability to regulate the cell behavior it does not favor cell adhesion like many other polysaccharides without any further modifications (Gasperini, Mano & Reis 2014, Wang, Varshney & Wang 2010). Different modification methods that have showed to promote the cell adhesion in the HA- hydrogels have been for example carbodiimide crosslinking (Lai 2016) and the introduction of the adhesion motifs like arginine- glycine- aspartic acid (RGD) to the structure. Due the HA's natural origin, the HA- hydrogels have very weak mechanical properties that need also chemical modifications to fulfill the requirements of the tissue engineering material. The concentration of the used HA in the hydrogels has effect also on the different properties of the hydrogel and the cellular behaviour inside of it (Lei et al. 2011).

For tissue engineering applications in ophthalmology, the HA- hydrogels have been used to culture especially mesenchymal stem cells (MSCs) due their CD44 expression with promising results (Burdick, Prestwich 2011, Lindborg et al. 2015). Lei et al. have for example studied

the effect of different concentration of the RGDs and the HA inside the hydrogels with the MSCs from mouse embedded in the HA- hydrogels. Results showed that the higher concentration of the RGD motifs indicates faster cell migration and spreading, but as well slower cell proliferation. The higher concentration of the HA showed also to indicate the slower cell proliferation and migration as well as less cell spreading and stiffer hydrogel structure (Lei et al. 2011). In other study, hASCs cultured inside the HA- derived scaffolds showed sings to the expression of the cornea- specific proteins when transplanted to the rabbit's corneal stroma (Espandar L et al. 2012). HA- based hydrogels have been also researched and used for cell sheet delivery carriers in corneal endothelial reconstructions and for example Gomes et al. have showed that HA is able to promote the migration of the corneal epithelial cells and their interactions with cell surface CD44 receptors. These interactions contribute more likely also the wound healing process in cornea (Gomes et al. 2004, Lai 2016).

2.4.2. Collagen

Collagen is the most widely expressed protein in the human body and the main component of the ECM. Collagen consists of unique triple helix structure (Gasperini, Mano & Reis 2014, Wright, Mi & Connon 2013) and different collagen types have been found multiple. From these, the collagen type I (CI) is the most abundant in the human body and widely used in tissue engineering due its ease extraction process and adaptability (Antoine, Vlachos & Rylander 2014).

Collagen- hydrogels are suitable for tissue engineering applications due their biocompatibility and biodegradability (Tsai et al. 2015), but as disadvantages, the origin, concentration and pH of the used collagen affect the cell behaviour and survival inside the hydrogels as well as the mechanical properties of the hydrogels. Due collagen is a natural polymer the mechanical properties of the collagen- based hydrogels are poor, but can be improved for example by crosslinking. Collagen contains some adhesion motifs like RGDs naturally, which makes it a very potential material to cell- based hydrogel applications (Gasperini, Mano & Reis 2014). Comparing with the other natural polymer- origin hydrogels, the collagen hydrogels have been mainly used to culture LECs, because they have showed to maintain the cell adhesion, proliferation and differentiation of the LECs (Wright, Mi & Connon 2013).

2.5. Stem cells

SCs are undifferentiated cells that are capable of dividing indefinitely and preserve their undifferentiated status even after multiple cell- division cycles. This is enabled by asymmetric cell- division, which means that one of the produced daughter cell maintains the pool of the stem cell and the other will undergo differentiation becoming under specific signalling and conditions. These properties of SCs are also called self-renewal and potency. SCs can be divided into four different categories according to their differentiation potency: totipotent-, pluripotent-, multipotent- and unipotent stem cells. Between these categories, the potency of the SCs decreases in every step and for example totipotent SCs can differentiate to all three germ layers (ectoderm, mesoderm and endoderm) including placental cells whereas unipotent SCs can differentiate only to single cell type (Girlovanu et al. 2015, Romito, Cobellis 2016).

2.5.1. Adipose stem cells

MSCs are multipotent progenitor cells originated first from the bone marrow stroma. They have been also the traditional clinical source of SCs. Still, due the extremely low number of SCs getting from the obtain operation (approximately 1MSC per 105 adherent stromal cells), the better source of autologous SCs have been widely researched. That how the further ex vivo cell culturing to achieve the clinically relevant number of cells, which expose the cells to contamination and loss would not be necessary. Since then, MSCs have been found with low numbered for example from heart, fallopian tube, dental pulp, corneal stroma and with higher number from adipose tissue (Yao, Bai 2013, Sempere et al. 2014). From adipose tissue, MSCs where first characterized by Zuk (Zuk et al. 2001).

Adipose tissue was a long time considered being only storage for the high- energy substrates like triglycerides, lipid soluble vitamins and cholesterol. Nowadays, it is known that it provides an abundant source of mesenchymal origin SCs (Figure 3) and due adipose stem cells' (ASCs') easy isolation process, multilineage differentiation potential, genetical stability in long cultures and low immunogenicity they have been researched widely in the tissue engineering field. Zuk and her co-workers have been pioneers to research different differentiation protocols to ASCs (Lindroos, Suuronen & Miettinen 2011, Zuk et al. 2001, Zuk et al. 2002).

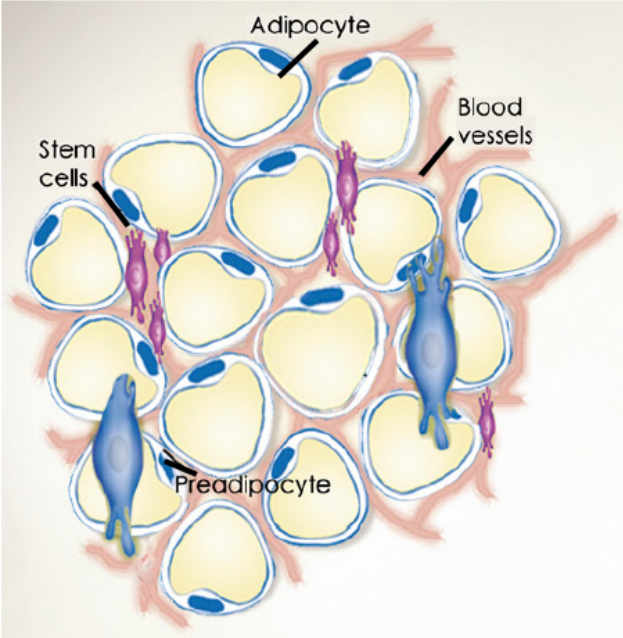

Figure 3 Schematic view from adipose tissue (Modified from (Lindroos, Suuronen & Miettinen 2011)

ASCs are one type of adult stem cells that are capable of differentiate to the wide range of mesodermal origin cells in vitro, for example to bone, adipose, cartilage and muscle cells (Patrikoski et al. 2014, Yao, Bai 2013). Recent studies have also showed that ASCs are capable of differentiate to keratocytes. MSCs' differentiation to corneal epithelial cells has been also researched, but the results have varied and that hypothesis requires further studies (Espandar L et al. 2012, Yao, Bai 2013). Isolation of the ASCs occurs mainly by liposuction aspirate or subcutaneous adipose tissue fragments. The first population of cells from these methods is called a stromal vascular fraction (SVF) and consists of adipocytes, stromal cells and ASCs. From SVF, the ASCs are further isolated by a collagenase digestion method that allows the ASCs to adhere to tissue culture flasks. These adhered cells are called ASCs as well as for example processed lipoaspirate (PLA) cells and adipose mesenchymal stem cells (AdMSCs) (Lindroos, Suuronen & Miettinen 2011, Zuk et al. 2001).

One important property of the undifferentiated ASCs' is that they cannot be indentified with only one cellular marker. It is also common that the expressions of different surface markers differ while the cells' passage is increasing. The ASCs' expression rate for for example CD29, CD44, CD73 and CD90 markers increase with increasing passage number while for example the expression rate of the hematopoietic markers like CD11, CD14 and CD45 decreases and lost with increasing passage number. Similar results are got when cultured the ASCs in

serum-free (SF) medium or medium containing human serum (HS) or fetal bovine serum (FBS). It is still important to notice that research done with HS or SF medium have been published only a few (Lindroos, Suuronen & Miettinen 2011).

Because of the ASCs' wide differentiation potency to different cell types, low immunogenicity and low immunosuppressive properties they have been widely researched in vitro and in vivo for different tissue reconstructions and treatment methods for autoimmune diseases and immunological disorders such like Crohn's disease and rheumatoid arthritis. ASCs have been researched also to treat the LSCD, because of their MSC origin. In animal models, the systemically injected MSCs have already showed to promote the wound healing and reduce the inflammation and neovascularisation of the damaged cornea as well as promote the survival, growth and proliferation of the different cell types in co-culture systems –including limbal epithelial cells (LECs) (Hu et al. 2012, Oh et al. 2009, Wen et al. 2014, Zhang et al. 2006). Ma et al. have also successfully transplanted the human MSCs cultured on amniotic membrane to alkali burned corneas of the rat (Ma et al. 2006). Clinical researches done with ASCs have been reported a few, but problems in cell preparations, homing and survival rates would need further investigations and understanding about mechanisms behind these effect as well from different delivery methods of enabling the clinical use of ASCs (Feisst, Meidinger & Locke 2015, Patrikoski et al. 2014).

2.5.2. Pluripotent stem cells

Pluripotent stem cells are divided into embryonic stem cells (ESCs), derived from the inner cell mass of preimplantation embryos, and to induced pluripotent stem cells (iPSCs), first developed in 2007 by Shinya Yamanaka. IPSCs are stem cells that are reprogrammed from differentiated somatic cells to pluripotent like stem cells by using the over expression of different genes. Traditionally, these genes have been Oct3/4, Sox2, Klf4 and cMyc (Takahashi et al. 2007, Romito, Cobellis 2016), but also other genes can be used. Comparing with the ESCs, iPSCs have less ethical issues, which have made them an extremely promising candidate to the tissue engineering researchers. IPSCs have researched to be used already in ophthalmology field for example as a source of retinal and LSCs (Liu et al. 2013, Casaroli-Marano et al. 2015) and have also great potential to be used as a source of cells to repair corneal surface (Liu et al. 2013).

2.6. Co-culturing

Co-cultures have introduced a new way to research different cell-to-cell interactions after they were developed in the early 1980s (Lawrence, Beers & Gilula 1978). In co-cultures, different distinct cell types are cultured within the same environment either directly or indirectly and either in 2D or 3D platforms (Paschos et al. 2015).

In direct co-cultures two or more different cells types are mixed and cultured together. In that kind of co-cultures the cell-to-cell interactions occur via cell-to-cell adhesion, cell-to-ECM adhesion and paracrine signaling with different soluble factors. In indirect co-cultures different cell types are separated from each other for example by porous membrane and the cell-to-cell interactions occur only via soluble factors (Paschos et al. 2015).

In different co-cultures, the used cells are also typically divided for target- and assisting cells. From these, the assisting cells produce a suitable environment for the target cells and induce the wanted behaviour including the proliferation, adhesion, differentiation and matrix production of the target cells. Target cells are the cells in the co-culture systems that will compose the engineered tissue and maintain its function. Still, also the target cells can promote the cell behaviour of the assisting cells (Paschos et al. 2015, Acharya et al. 2012).

2.6.1. Co-cultures in tissue engineering

In recent years, 3D co-culturing has become a widely used method for mimicking the native tissue structures and form physiologically relevant cell structures by controlling the target cells' behaviour with assisting cells. Different SCs have an extremely important role in these researches and when used as a target cells, the main purposes of these co-cultures have been to differentiate the SCs into specific cell types when co-cultured them with TDCs. When used as assisting cells, SCs are mainly used to support and regulate the target cell population while maintaining their potency (Paschos et al. 2015).

The differentiation of the SCs in the co-culture systems has showed to happen in direct co-cultures via cell junctions between SCs and assisting TDCs. Through these cell junctions, the TDCs exchange signals associated to SCs' behaviour and differentiation (Paschos et al. 2015, Beeres et al. 2005, Guillotin et al. 2004). In indirect co-cultures the soluble factors secreted by TDC have also showed to induce the differentiation of the SCs and for example MSCs have

differentiated towards fibroblasts lineage, when co-cultured them indirectly with ligament fibroblasts (Fan et al. 2008). As assisting cells, the MSCs have showed to regulate the proliferation, migration and gene expression for example of the indirectly co-cultured dermal fibroblasts (Smith et al. 2010). The ASCs have showed also to promote the dermal fibroblasts' wound healing potential (Kim et al. 2007).

In different tissue reconstruction researches, the SC co-cultures have showed to overcome some critical limitations, like vascularization for example in bone reconstruction (Paschos et al. 2015), when interactions that naturally do not exist are possible to create. The co-culture structures mimicking the natural tissue structures have showed promising results already and they offer a promising tool for tissue engineering scientist for different tissue reconstructions and treatment methods.

3. Objectives

Objectives of this research were to find a suitable culture medium for human ASCs (hASCs), from those that had already been shown to be suitable for human CECs (hCECs), and use it to co-culture hydrogel embedded hASCs with hCECs (Figure 4). Results will possibly help us to evaluate better the functionality of this kind of 3D structure for the treatment of corneal damages and LSCD. The hypothesis behind the research is that the hASCs would give support to the hCECs and control the inflammation reaction in the eye. That is important, because the re-epithelialisation cannot exist if the inflammation reaction is not controlled. Over time, hASCs would possibly either be replaced by- or differentiated to keratocytes. The strategy for accomplishing these objectives with hASCs and hCECs is illustrated in Figure 5.

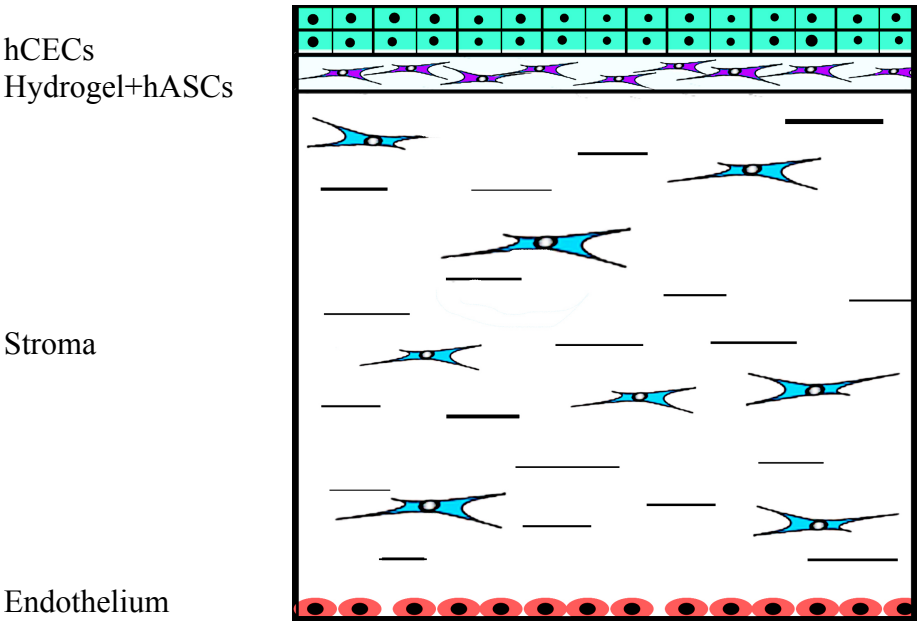

Figure 4 Shematic picture from co-culture of the ASCs embedded in hydrogel and hCECs (Modified from http://www.stembook.org/node/588.html; 28.3.2016)

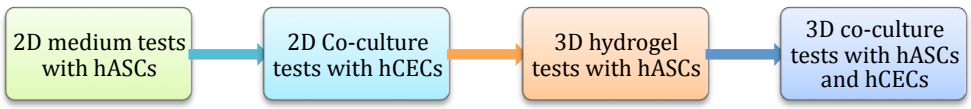

Figure 5 Strategy to accomplish the objectives of this research

4. Materials and methods

All tests in this research were done using hASC line 11/15 from Susanna Miettinen's Adult stem cell group (BioMediTech). In this group, the hASCs are isolated from subcutaneous adipose tissue by the protocol from Zuk (Zuk et al. 2001). In co-culture tests, immortalized human corneal epithelial cells (hCECs) (Araki-Sasaki et al. 1995) from Hannu Uusitalo's research group (the University of Tampere) were used. Cells were always handled in sterile conditions and cultured in humidified incubators at 37°C and 5% CO_2. Both cell lines were cultured in T75-culture flasks and fresh medium were changed to the cells three times a week.

4.1. Medium tests

4.1.1. Cell culture conditions

The hASCs were cultured on 24- well plates (Sigma-Aldrich: Corning Cellbind surface, St. Louis, USA) with and without human CI (Sigma-Aldrich, 1mg/ml) 6µg/cm^2 coating. Coatings were prepared by treating the well plates with CI (diluted in phosphate- buffered saline (PBS), Lonza group Ltd, Basel, Switzerland) over night at +4°C. After removing the CI- solution the well plates were left over night to the laminar hood to dry out before plating the cells. Cells were cultured in three different test mediums: CnT-Prime, CnT-30 and CnT-Prime-CC (all from CellnTech, Bern, Switzerland). From these, the CnT-Prime is a corneal proliferation medium, CnT-30 corneal differentiation medium, and CnT-Prime-CC co-culture medium. They have been all found to be suitable for hCECs in previous studies (data not shown). Into all mediums, 1% penicillin/streptomycin (P/S) was added. 5% HS medium (DMEM F/12, 1% Glutamax, 1% P/S, 5% HS Biowest) was used as control medium. Cells were cultured for 13 days and fresh medium (1ml) were changed three times a week.

4.1.2. Cell viability

On the 2nd day of culturing, the proliferation rates of the cells were analyzed by treating the cells with 400µl PrestoBlue Cell Viability Reagent (Life Technologies Thermo Fisher Scientific, Waltham, USA) solution (diluted 1:10 in control medium) for 30 minutes in the incubator (37°C and 5% CO_2). After incubation, solution was pipetted to 96-well plate

(100µl/well). Blank control samples were used to eliminate non- specific absorbance. Absorbances were measured at 544 nm with Victor 1420 Multilabel Counter (Wallack, Turku, Finland).

4.1.3. Immunocytochemistry

On the 13th day cells were immunofluorescent (IF)- stained. Staining was started rinsing the cells twice with PBS and fixing with 4% paraformaldehyde (PFA) for 15 minutes. After three washes with PBS cell membranes were permeabilized with 0.1% Triton-X-100 (diluted in PBS) for 15 minutes. After three 5- minute washes with PBS, unspecific binding sites were blocked using 3% Bovine Serum Albumin (BSA, diluted in PBS). All the samples were then treated with one or two primary antibodies (Table 1) over night at +4°C. All antibodies were diluted in 0.5% BSA in PBS and they were manufactured by Millipore Corporate, Billerica, USA (Ki67 and vimentin), R&D Systems, Minneapolis, USA (α-SMA) and Santa Cruz Biotech, Dallas, USA (keratocan). From these, the Ki67 is a proliferating marker, vimentin a mesenchymal marker, keratocan a keratocyte marker and α-SMA a α-smooth muscle actin marker.

Table 1 Primary and secondary antibodies

Antibody	Host species	Dilution
Ki67	Rabbit	1:500
α-SMA	Mouse	1:400
Keratocan	Rabbit	1:200
Vimentin	Goat	1:200
Anti- rabbit	Donkey	1:800
Anti- mouse	Donkey	1:800
Anti- goat	Donkey	1:800

Next day, samples were washed again with three 5- minute periods with PBS and treated with secondary antibodies from Invitrogen (Table 1) diluted in 0.5% BSA in PBS and protected from light before counter- staining of the cell nuclei with VectaShield mounting medium containing DAPI (Vector Laboratories Inc.). After mounting 13mm diameter cover glasses were put on the samples. All other reagents except PBS (Lonza) and primary antibodies were from Invitrogen Thermo Fisher Scientific. Samples were visualized with fluorescent microscope (Olympus 1X51) and images were edited using ImageJ and Adobe Photoshop Elements.

4.2. 2D co-culture tests

4.2.1. Cell culture conditions

Co-culture tests with hCECs and hASCs were accomplished by co-culturing the immortalized hCECs directly and indirectly with hASCs. Culturing occurred directly in 24- well plates (Sigma-Aldrich: Corning Cellbind surface) and in 1,0µm PET filter inserts (Millipore). HCECs were also co-cultured indirectly in 1,0µm PET filter inserts (Millipore) on top of the hASCs cultured in 24- well plates. As control samples, hCECs were cultured separately inside the PET inserts and hASCs in 24- well plates (Figure 6). Co-culture started after culturing the hASCs for 1 day and by adding the hCECs to inserts and well plates in 1:1 ration on 2nd day. Airlifting (AL) was started to half of the insert replicates and continued for five days starting on 6th co-culturing day. Fresh CnT-Prime-CC medium was changed to cells three times a week.

4.2.2. Immunocytochemistry

On the 6th and 11th co- culture day, the cells were IF- stained the same way as in the 2D medium tests. Inserts were cut to four pieces after fixation and stainings were continued in 8-well chamber sliders (Life Technologies Thermo Fisher Scientific). Used primary antibodies for the control hASCs and indirectly co- cultured hASCs were the same as in the 2D medium tests (Table 1). These same antibodies were also used for the control hCECs, indirectly co-cultured hCECs and directly co-cultured hASCs and hCECs with cytokeratin 3 (CK3) (Abcam, Cambrigde, USA), CK12 (Santa Cruz Biotech) and keratinocyte progenitor cell marker 63α (p63α) (Cells Signaling Techonogy, Danvers, USA) in 1:200 dilutions. Samples in wells were visualized using fluorescent microscope (Olympus 1X51) and insert samples using confocal microscope (Zeiss LSM780) to evaluate the localization of the proteins and possible 3D structures. Images were edited using ImageJ and Adobe Photoshop Elements.

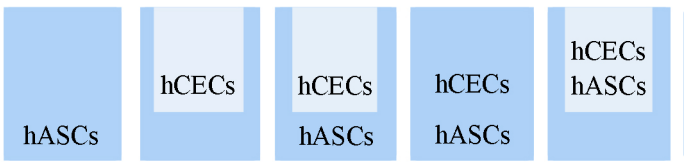

Figure 6 Shematic view from the direct and indirect co-culturing of the ASCs and hCECs

4.3. Hydrogel tests

4.3.1. Cell culture conditions

HASCs were embedded in the hydrogels with 45 000, 67 500, 90 000, 180 000, 200 000 and 500 000 cell densities. All of these cell densities are declared into perspective of 300µl hydrogels. Like in 2D cultures, the fresh CnT-Prime-CC medium was changed to the cells three times a week.

4.3.2. Hydrogel materials

Used hydrogel in this research was a HA- hydrogel, mixed from two differently modificated components (HA1 and HA2) given from the Tampere University of Technology from Professor Minna Kellomäki's Laboratory for Biomaterials and Tissue Engineering- group. Hydrogels were formed with and without added CI from human (Sigma-Aldrich) and rat origin (isolated by another research group from University of Tampere) and made to 96- well plates (Sigma-Aldrich), 8- well chamber sliders (Life Technologies Thermo Fisher Scientific) as well as in 1,0µm polyethylene terephthalate (PET) filter inserts (Millipore).

To prepare HA1+HA2- hydrogels without added CI, dry HA- components were diluted to 10% sucrose (diluted in water) solution at room temperature with over night magnetic stirring. Before use, the solutions were filtered to sterilize them. To form the hydrogel structure the centrifuged cell pellet was mixed first to the HA1- component and then to HA2- component. Solution was then pipetted quickly to used platforms and left to an incubator for 1h. The used medium was then added to lower (800µl) and upper chamber (300µl) of the insert, to 96- well plates (200µl) or to chamber sliders (300µl). Half of the inserts were coated with CI in the first experiments such as in medium tests. The coatings were prepared similar way as for the 2D cultures.

When adding the CI to the HA- hydrogels, the preparation of the HA1- and HA2- components was made similarly than for the hydrogels without CI, but the used medium for different components were changed (Table 2). When mixed the components together, the collagen was added by two different ways: to the HA1- cell suspension before adding the HA2, or by suspending the cells straight to the CI and then adding the HA1- and HA2- components.

The same HA1- and HA2- components were used to form hydrogels without added CI. Volumes of the hydrogels differed between different platforms and were 300µl for hydrogels made to inserts, 100µl to 96- well plates and 100µl as well as 200µl for chamber sliders.

Table 2 Hydrogel components with added collagen

Polymer component	Medium	Consentration (mg/ml)
HA2	Water	22,5
HA1	Water +PBS	11,25
Collagen I (rat)	Mild acetic acid	2
Collagen I (human)	Mild acetic acid	1

4.3.3. Evaluation of the adipose stem cells embedded in hydrogels

During the culturing, the cells were visualized using Zeiss (AX10) phase-contrast microscope. On the 3th day, the viability of the hASCs embedded in the hydrogels were analyzed using the Live/Dead assay- kit (Invitrogen Thermo Fisher Scientific) and treating the cells for 30 minutes with calcein-AM (1mM, 1:200); EthD (2mM, 1:8000) solution in the incubator after washing them once with PBS. Cells' viability was then analyzed with fluorescent microscope (Olympus 1X51). Images were edited using ImageJ and Adobe Photoshop Elements.

To evaluate the expression of different proteins, cells were IF- stained the same way and with the same antibodies as 2D cultured cells only with longer fixation time (30 minutes). After fixation, the hydrogels made to PET inserts were cut out from the inserts and to four pieces. Single hydrogel pieces were then placed in 8-well chamber sliders where the staining was continued. Samples were visualized with the fluorescent microscope (Olympus 1X51).

5. Results

5.1. Medium tests

The hASCs were first cultured in the CnT-Prime-CC and CnT-Prime medium with and without CI coating. Proliferation analysis on 2nd day showed that cells proliferated more efficiently in CnT-Prime-CC medium. In CnT-Prime medium, the cells' proliferation rate was very low. When the same culturing was repeated with the higher number of cells with Cnt-Prime-CC and CnT-30 medium, the results for CnT-Prime-CC medium were the same as in the first experiment. Like in CnT-Prime medium, cells' proliferation rate in CnT-30 medium was very low. In control samples, the number of cells decreased to half for the second experiment due the confluence of the cells in the first experiment. CI coatings showed to increase the proliferation rate of the cells cultured in CnT-Prime-CC and CnT-Prime medium and decrease it when cultured the cells in CnT-30 medium. The effect of the CI coatings on the control samples differed between different experiments (Figure 7).

During the culturing, cells were visualized with the phase-contrast microscope. In CnT-Prime-CC medium, cells grew and proliferated well, but in the CnT-Prime medium,

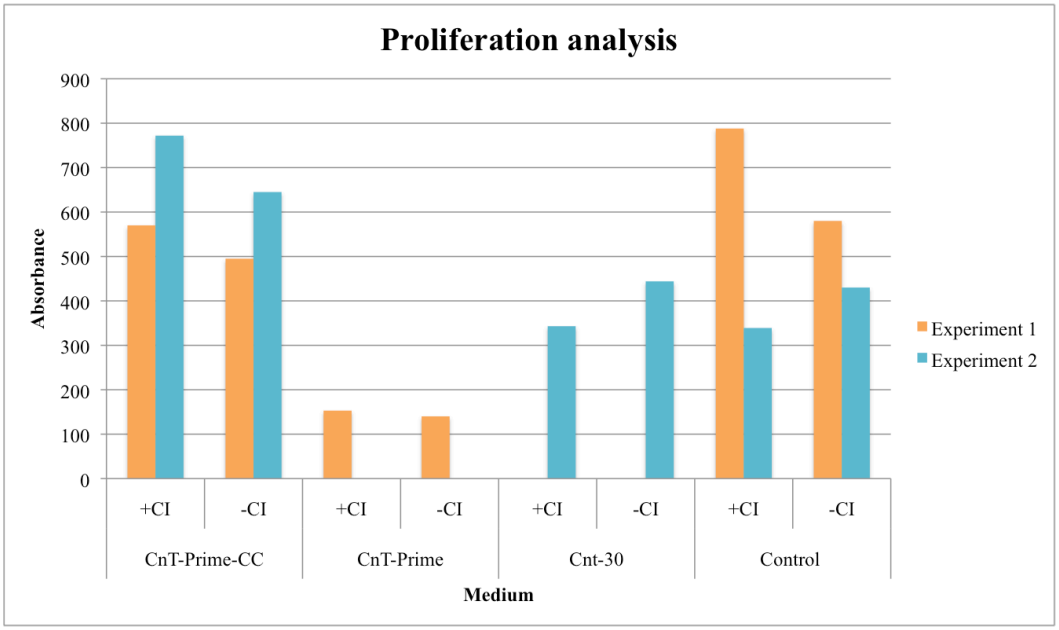

Figure 7 Proliferation analysis from the hASCs cultured in different mediums. In the experiment 2 the cell density was increased comparing with the experiment 1

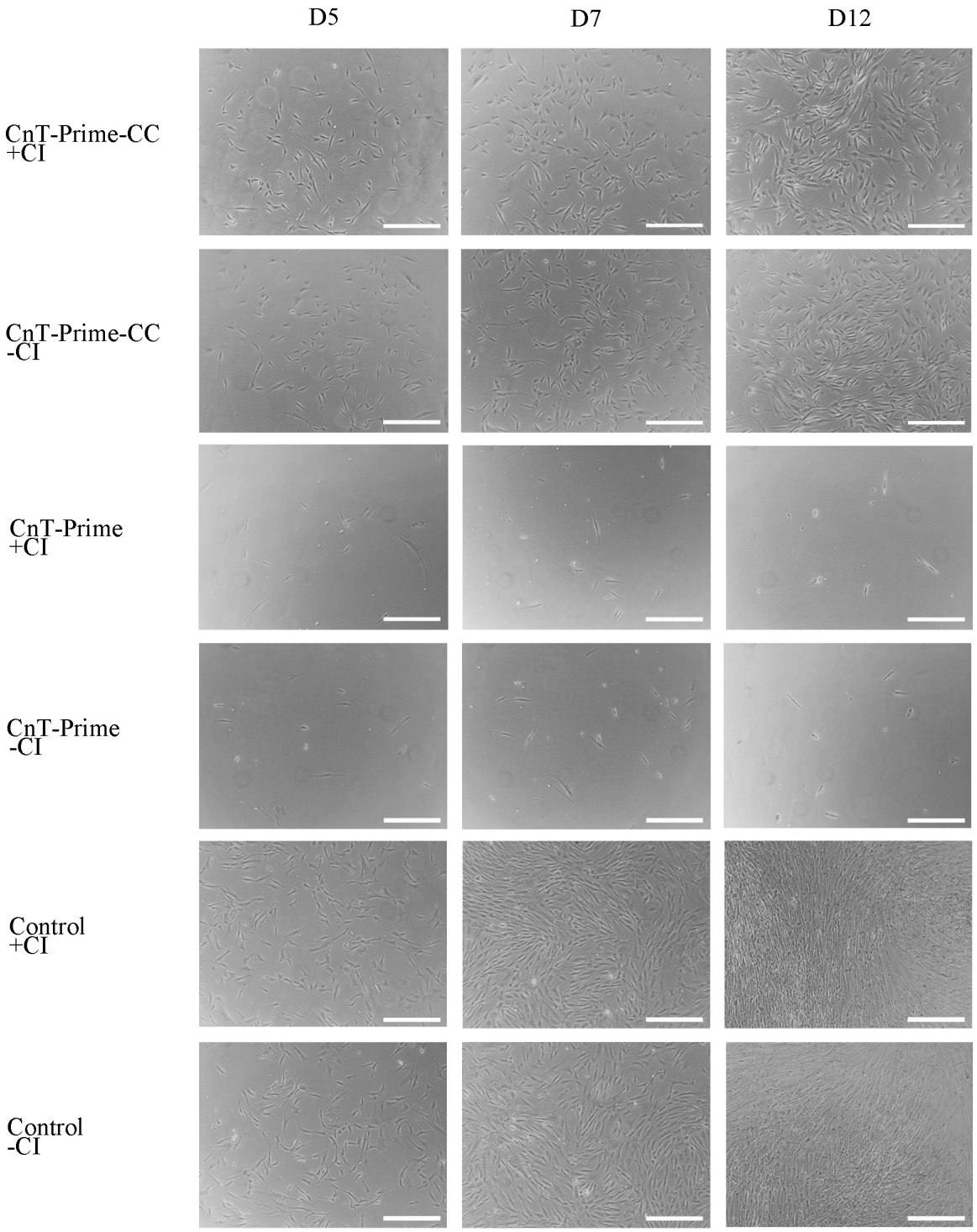

Figure 8 HASCs cultured in different mediums. Scale bars 500µm

the number of cells was extremely low from the beginning of the culturing until the 13th day (Figure 8). Similar results were got with CnT-30 medium as with CnT-Prime medium (data not shown). On 13th day, cells cultured in CnT-Prime-CC medium were IF- stained with Ki67, α-SMA, vimentin and keratocan antibodies and cells cultured in control medium with

keratocan and α-SMA antibodies. Between CI coated samples and non- coated samples, no significant differences between staining results were observed. Cells cultured in the CnT-Prime-CC medium were positive to Ki67 and vimentin (Figure 9 and 10), but not to α-SMA or keratocan. Cells cultured in control medium were not positive either to α-SMA or keratocan.

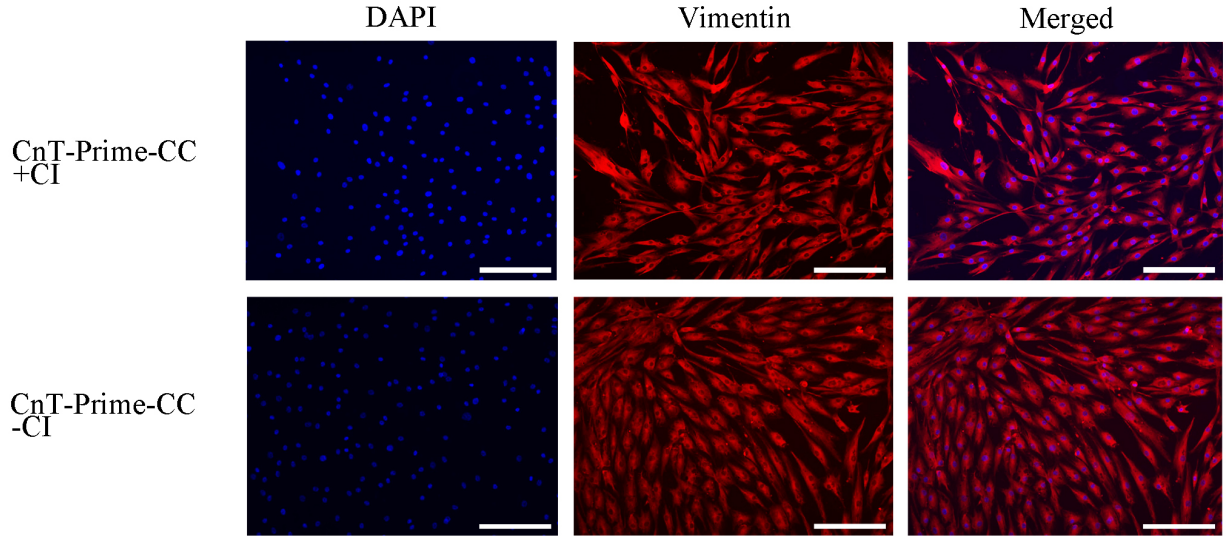

Figure 9 HASCs stained with vimentin (red) and presented as single stainings with the nuclear counter-staining DAPI (blue). Scale bars 200µm

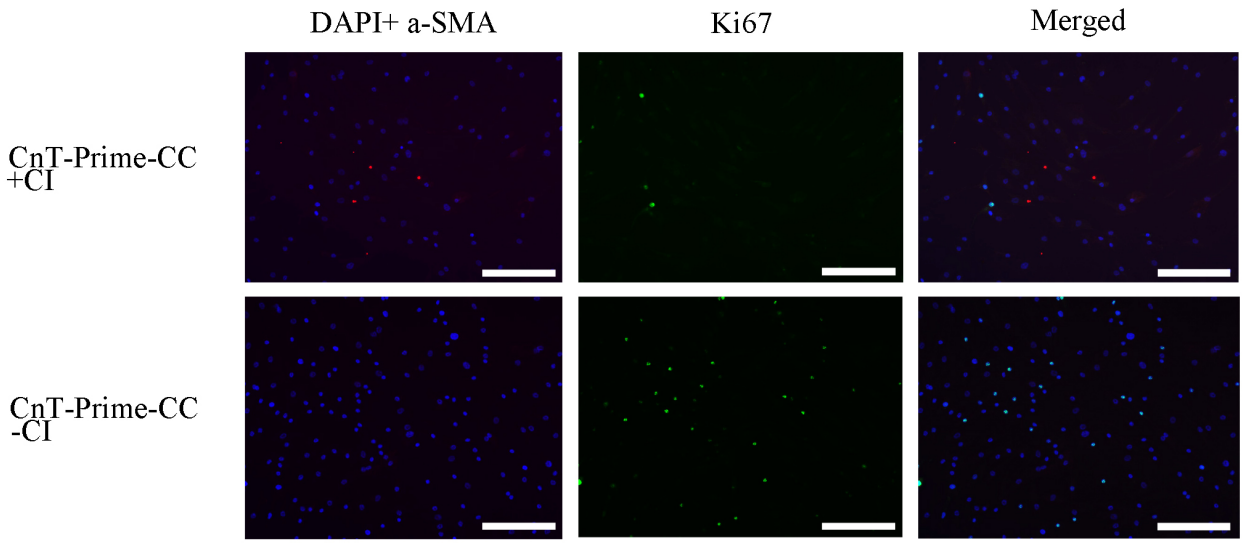

Figure 10 HASCs stained with α-SMA (red) and nuclear counter-staining DAPI (blue) presented as double stainings, and with Ki67 (green) presented as a single stainings. Scale bars 200µm.

5.2. 2D co-culture tests

On 6th day of culturing, the indirectly co-cultured hCECs and hASCs grew well with similar morphology as control cells. The hCECs also have higher cell density comparing with the control cells. Directly co-cultured hCECs in the PET inserts also grew well and with similar morphology comparing with the control cells, but with lower cell density comparing with the indirectly co-cultured hCECs. Directly co-cultured hASCs in the inserts grew in groups and with cell-to-cell connections between different groups. Directly co-cultured hCECs' cell density in the wells was extremely high and they grew in extremely tight groups. They also have layered in these groups. Otherwise, all the other hCECs grew on two layers at the most. Most of the hCECs in the wells looked dead by phase-contrast microscope, but after fixation most of the cells still remained in the wells. The hASCs in the wells were hard to observe under the hCECs, but when observed, they looked growing in groups as well as constantly with similar morphology as control cells. In both direct co- cultures, the hCECs grew mostly on top of the hASCs.

On 6th day, IF- stainings showed that in the control hASCs samples the cells were not positive to any other antibodies except very weakly to Ki67 (data not shown). Most of the control hCECs were positive to vimentin, Ki67 and CK3 (Figure 11). Few control hCECs were also weakly positive to p63α (data not shown). Indirectly co-cultured hCECs were positive to vimentin, Ki67 and CK3, CK12 and also weakly to α-SMA (Figure 11). Indirectly co-cultured hASCs were not positive to any of the antibodies like the control cells (data not shown). Directly in the wells co-cultured hASCs and/or hCECs showed strong positivity to Ki67 and vimentin. Some hCECs on the edges of the well with lower cell density were also positive to α-SMA (data not shown). Possibly a few of directly in the inserts co-cultured hASCs were positive to CK12 when hCECs were positive to vimentin, CK12 and α-SMA, some cell on the edges also to the CK3 (Figure 11). Keratocan positivity was not observed in any of the samples.

AL started on 6th and continued until the 11th day to half of the insert replicates of the control hCECs, indirectly co-cultured hCECs and direct co-cultures. AL showed, that the layer of the hCECs or hCECs and hASCs in all samples was permeable to the medium, because thin medium layers were observed on top of all the inserts.

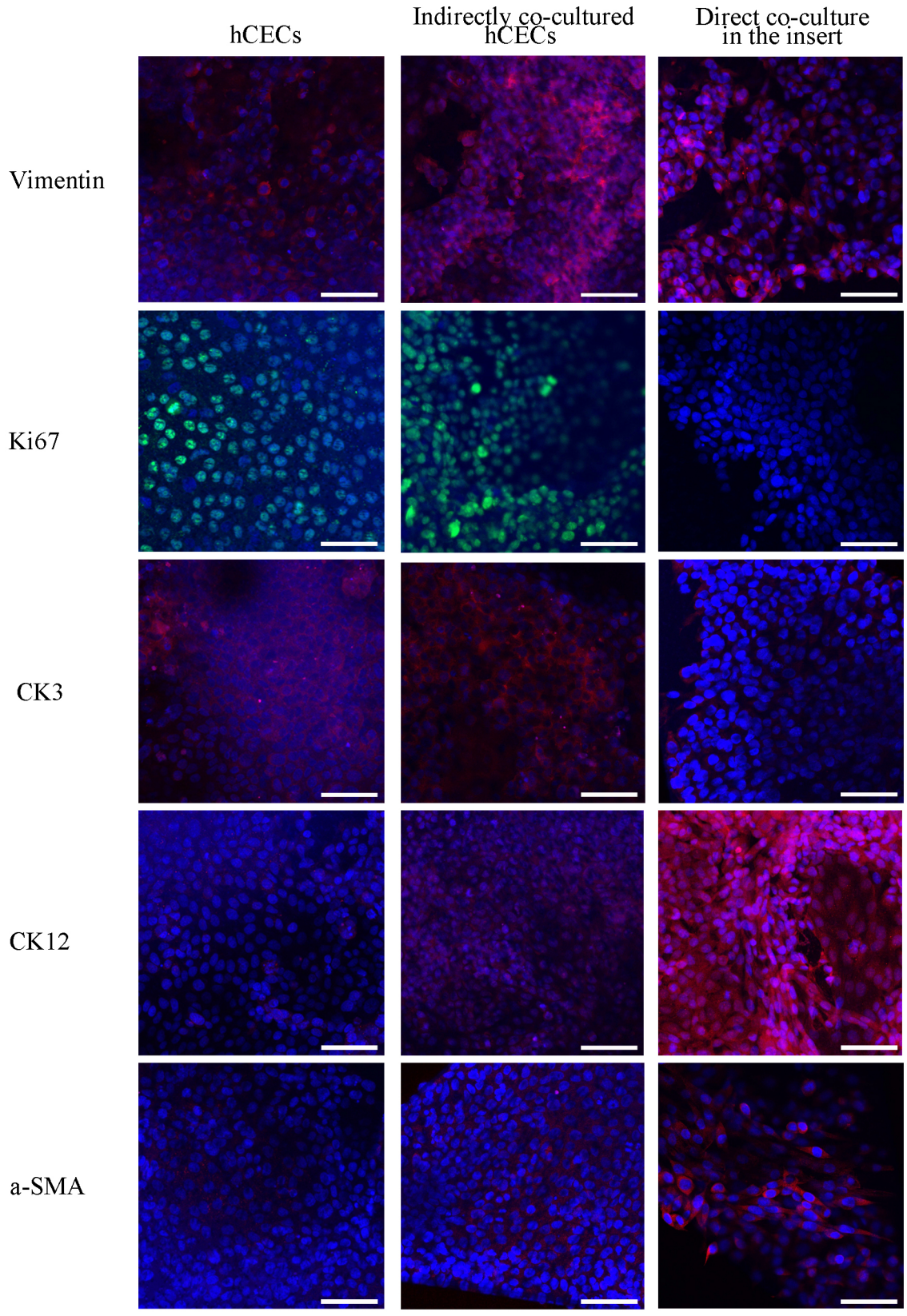

Figure 11 IF- staining results from co-cultures on 6th day. Vimentin, CK3, CK12 and α-SMA (all red) are presented as single stainings with the nuclear counter-staining DAPI (blue). Ki67 (green) is also presented as single staining with DAPI. Scale bars 100µm.

On 11th day in the airlifted control hCEC samples, the cell density was possibly a little bit higher comparing with the non- airlifted samples, but the differences were extremely small. Similar effect was seen in the indirectly co-cultured hCECs samples. In the direct co-culture inserts, the hCECs' cell density differences between airlifted and non-airlifted samples were more significant and higher cell density was observed in airlifted samples. Indirectly or directly in the inserts co-cultured hCECs were growing on two layers at the most. The hASCs in direct co-culture inserts were growing more clearly in groups in non- airlifted samples. In the airlifted samples, the hASCs were even hard to observe under the hCECs (Figure 12). Direct co-culture samples in the wells looked similar than on 6th day, only in a few replicates the cell density of the hCECs was increased a little. Indirectly co-cultured hASCs were very confluent but stayed well attached like the control cells (Figure 13).

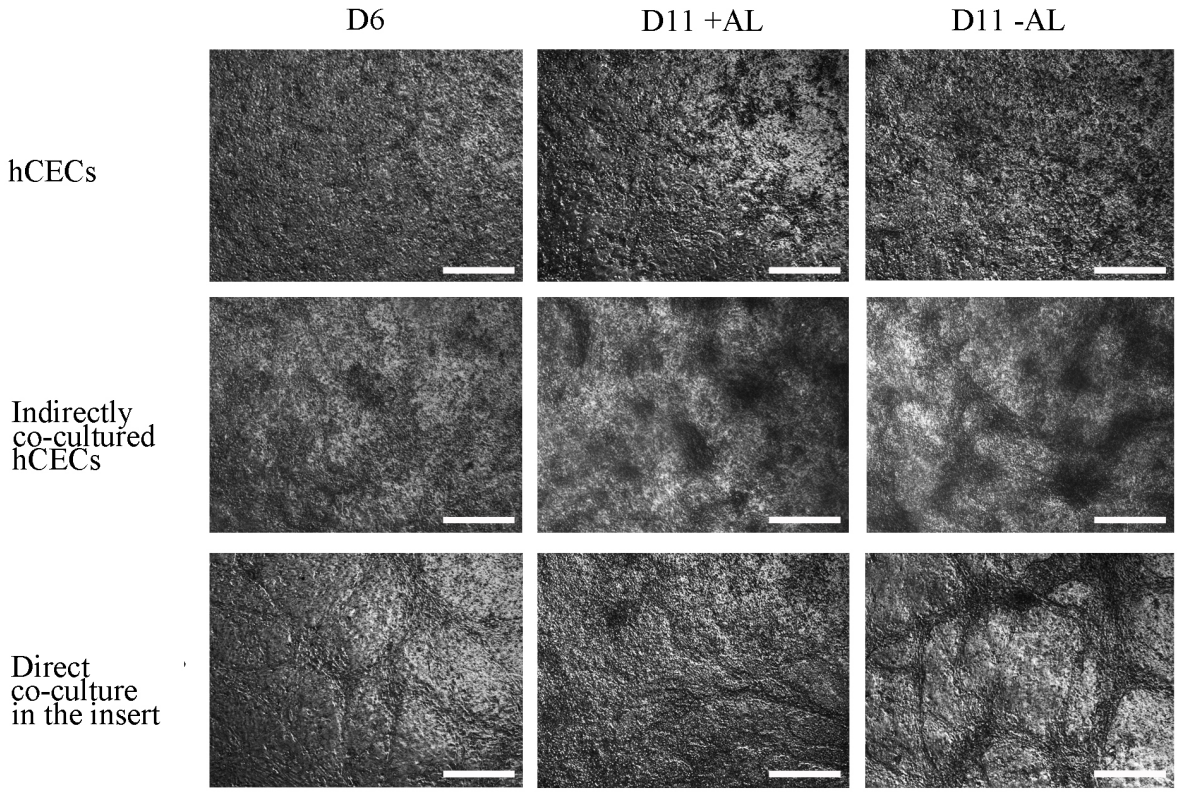

Figure 12 HCECs cultured separately, indirectly and directly with hASCs in CnT-Prime-CC medium. Scale bars 500µm.

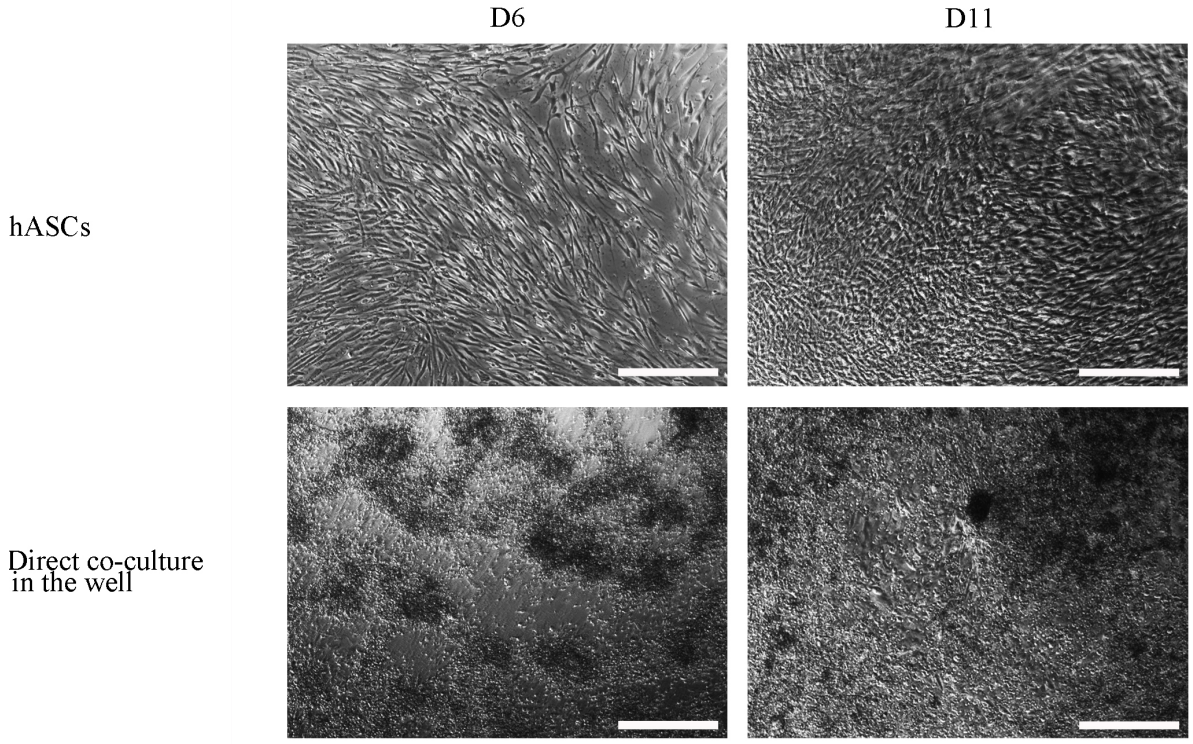

Figure 13 HASCs cultured separately and directly with hCECs in CnT-Prime-CC medium. Scale bars 500µm.

IF-staining results on 11th day showed that the control airlifted hCECs were strongly positive to vimentin, Ki67, weakly to the CK3 and few cells also weakly to CK12, α-SMA (Figure 14) and p63α (data not shown). Non-airlifted control hCECs were weakly positive to vimentin, CK3, CK12 and on the edges of the inserts to Ki67 (Figure 14). Only a few cells were extremely weakly positive to α-SMA (Figure 14) and p63α (data not shown). Airlifted hCECs co-cultured indirectly were positive to vimentin and CK3, weakly positive to Ki67 on the edges of the sample and to the CK12 and α-SMA (Figure 14). Non-airlifted indirectly co-cultured hCECs were strongly positive to Ki67, weakly to the α-SMA and very weakly to CK3 and CK12 (data not shown). Indirectly co-cultured and control hASCs were positive to vimentin and few cells on the edges to Ki67 (data not shown). Directly co-cultured hASCs in the wells showed similar results as on 6th day (data not shown). Airlifted hCECs co-cultured directly with the hASCs in the inserts were positive to vimentin, Ki67, CK3, CK12 and α-SMA. The hASCs in the same samples were positive to Ki67 (Figure 14). Non-airlifted hCECs co-cultured directly with the hASCs in the inserts were positive to Ki67 and α-SMA as well as weakly positive to CK3 and CK12. The hASCs in the same samples were positive to Ki67 (data not shown). No keratocan positivity was observed in any of the samples.

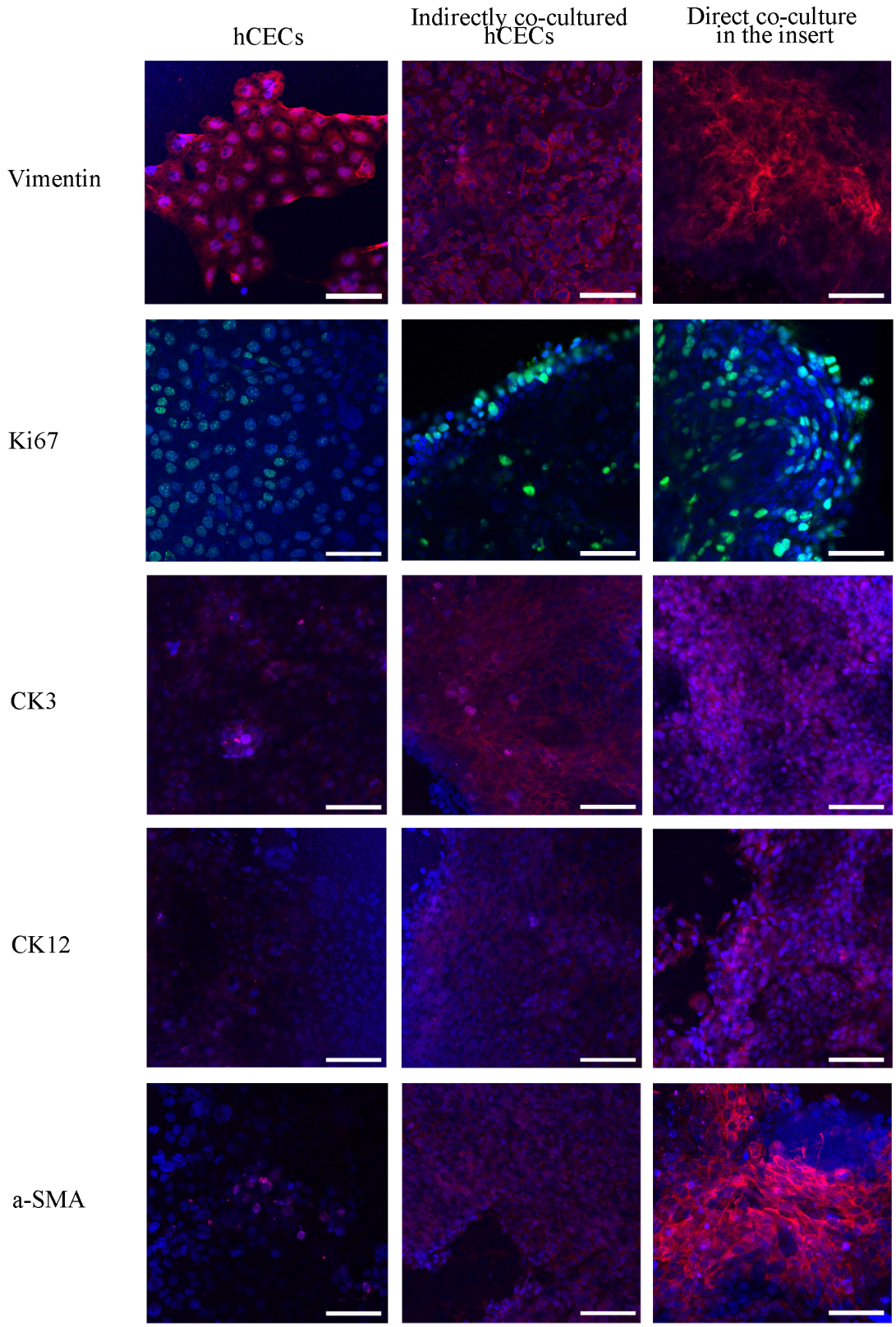

Figure 14 IF- staining results from co-cultures on 11th day after AL Vimentin, CK3, CK12 and α-SMA (all red) are presented as single sainings with the nuclear counter-staining DAPI (blue). Ki67 (green) is also presented as single staining with DAPI. Scale bars 100µm.

5.3. Hydrogel tests

Hydrogel tests started with HA1+HA2- hydrogels (components diluted to 10% sucrose), with and without CI coating on the inserts and with the cell density of 45 000 cells/ hydrogel (data not shown). The hASCs embedded in hydrogels were cultured in CnT-Prime-CC medium for 13 days and some replicates even for 21 days. The hASCs were growing on approximately 2-3 layers, mostly on top part of the hydrogels or even on the surface of the hydrogels. Between different replicates, the number of cells varied widely during the whole culturing. On 10^{th} day, a few spreaded cells were observed in CI- coated control samples with higher cell density. Spreaded cells were also observed in some non-coated control samples with higher number of cells on 12^{th} day. While the culturing continued, the number of spreaded cells increased slowly. All cells cultured in CnT-Prime-CC medium had spheroidal shape and the number of cells on top of the hydrogels decreased dramatically during the culturing. On 13^{th} day, the number of cells was extremely low and only a few cells were observed in CnT-Prime-CC samples. Stil, the IF- staining results were the same as in 2D culturing and the hASCs were positive to vimentin, Ki67 but no to α-SMA or keratocan (data not shown). On 21^{th} day, only single spheroidal cells were observed in CnT-Prime-CC samples, but in control samples the spreaded cells were confluent and only a few spheroidal shaped cells were observed.

By increasing the number of the embedded cells to 67 500 cells/hydrogel and 90 000 cells/hydrogel, the number of cells after 3^{rd} and 7^{th} days of culturing was higher, but still extremely low although the cells were localized more constantly in the hydrogels. No spreaded cells were observed.

Research was continued with the cell density of 90 000 cells/hydrogels and 180 000 cells/hydrogel, but without any CI coatings on the inserts. Instead, CI from human and rat origin were added to hydrogels. The hydrogels without added CI were made from the same components as CI added ones (diluted in to PBS and water). On 3^{rd} day, all the cells had spheroidal shape and live/dead analysis done to the CnT-Prime-CC samples showed that there were no living cells in any of the hydrogels with the cell density of 90 000 cells/hydrogel. With higher cell density, a few living cells were observed in hydrogels without added CI (data not shown).

By increasing the cell density to 200 000 cells/hydrogel and 500 000 cells/hydrogel and making the hydrogels to chamber sliders, spreaded cells were observed already on 1st day in some control samples with cell density of 500 000 cells/hydrogel. On 3rd day, live/dead analysis showed that in human CI added hydrogels there were no living cells in any of the samples, but in rat CI added hydrogels, few living cells were observed in control samples with both cell densities (Figure 15). Only a few dead cells were observed in all hydrogels without added CI (Figure 15). In control medium samples, some cells obtained also spreaded shape especially with higher cell density (Figure 16).

Experiment was repeated with hydrogels without added CI and with the cell density of 500 000 cells/hydrogel. Hydrogels were made on 96- wells. On 1st day, spreaded cells were observed in some replicates in both CnT-Prime-CC and control mediums and number of them increased during the culturing. On 3th day, the live/dead analysis showed only a few dead cells in all hydrogels (Figure 16). In control samples, the cells were almost confluent. In every hydrogel, the cells were growing at approximately 2-3 levels in the beginning. On 3th day, the cells were mostly at one level close to, or on the well plate surface. On 5th day, solid hydrogels were not observed anymore, and especially in CnT-Prime-CC medium the cells grew in tight groups. In control samples, the situation was not changed as much and the

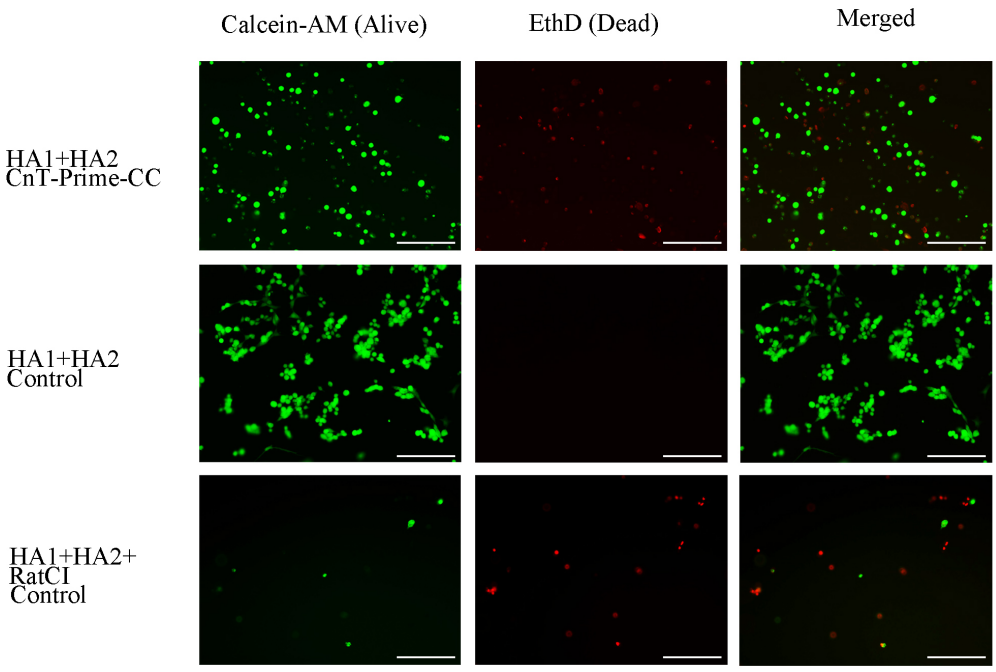

Figure 15 Live/dead analysis on 3th day for hASCs embedded in hydrogels with cell density of 200 000 cells/hydrogels. Scale bars 200µ.

number of cells was even increased. Culturing was still continued until the 7th day when new live/dead analysis was accomplished. Results showed that the cells were confluent in both mediums and alive (Figure 17). Only a few dead cells were observed in the CnT-Prime-CC medium samples. On 7th day, the cells cultured in CnT-Prime-CC medium were positive only to Ki67. In control samples, more Ki67 positive cells were observed (data not shown).

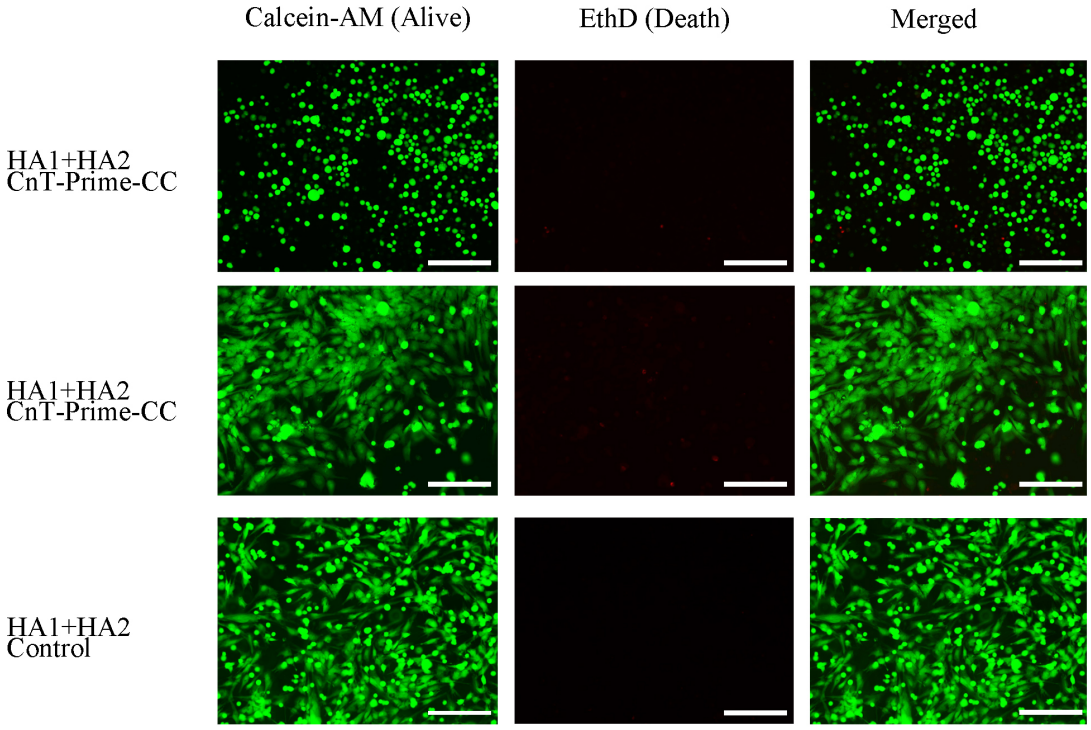

Figure 16 Live/dead analysis on 3th day for hASCs embedded in hydrogels with cell density of 500 000 cells/hydrogels. Scale bars 200µm.

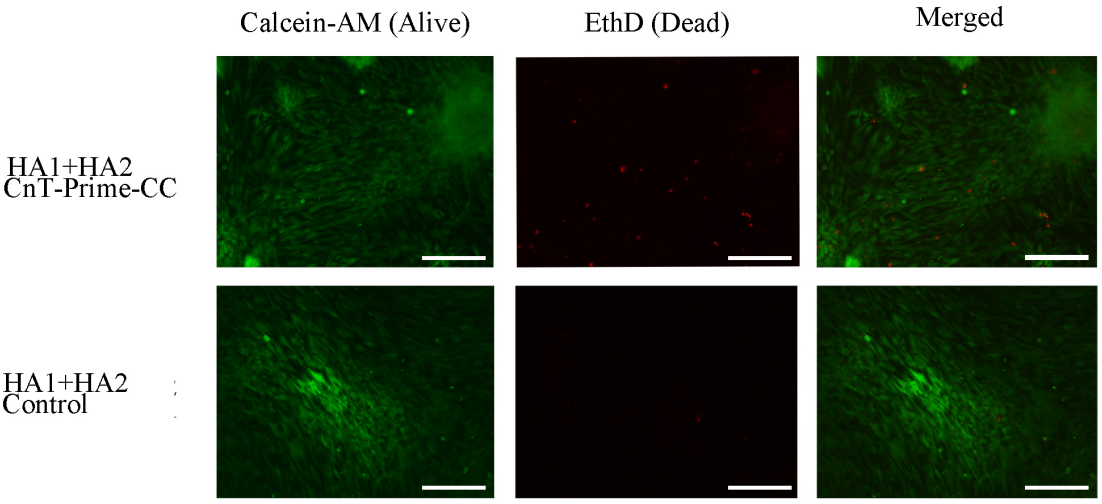

Figure 17 Live/dead analysis on 7th day for hASCs embedded in hydrogels with cell density of 500 000 cells/hydrogels. Scale bars 200µm.

6. Discussion

Different 3D co-culture methods in vitro have been under major interest and research in recent years. Especially with SC co-cultures, researchers have tried to form physiologically relevant tissue structures. The advantages that 3D cell cultures have comparing with the traditional 2D cell culturing methods are that they mimic the cells' natural microenvironment better. One of the most used 3D structures in the tissue engineering field have been hydrogels of a different kind due their ability to mimic the cells' ECM especially well (Wang, Varshney & Wang 2010, Lindborg et al. 2015, Paschos et al. 2015). The objectives of this study were to find in vitro co- culture conditions to hASCs and hCECs for potential future applications in ocular surface reconstruction. The study was performed by culturing the hASCs in mediums known suitable to hCECs and co-culturing the hCECs with hASCs directly and indirectly in 2D. HASC culture was also performed in 3D hydrogel structure.

6.1. Medium tests

To these tests, the cell density of the hASCs was based on the knowledge from preliminary studies done with immortalized keratocytes and corneal epithelial cells (data not shown). Because the first culturing showed that the cell density was very low to the hASCs it was increased to the second culturing. This is showed well in the proliferation analysis results from the cells cultured in the CnT-Prime-CC medium. Even thought the results got from Cnt-Prime and CnT-30 mediums are also for that reason not directly comparable, the differences between these mediums and CnT-Prime-CC medium were clear. It is still possible that if the used cell density had been higher, the CnT-30 medium would have showed better results, because the differences between the non-coated samples of CnT-Prime-CC- and CnT-30 medium were not so big.

Because collagen naturally promotes the cell adhesion, it was expected that it would increase the proliferation rates in the cultures when used as a coating material. Surprisingly, this effect was observed only slightly and not significantly. Althought the CI coating showed to increase the proliferation rate of the hASCs cultured in CnT-Prime-CC medium, the significance of

this result is very hard to evaluate because the differences between different samples are small. Only small differences in proliferation rates were also observed between CnT-Prime samples. Between CnT-30 medium samples, the differences were a little bit bigger. Still, because the tests with CnT-Prime and CnT-30 were done only once, the significance of the effect of the CI is hard to evaluate. The opposite results got from hASCs cultured in the control medium were interesting, but the most likely due the problems with pipetting when running the proliferation analysis the second time. If the tests had been done more than once or twice, the significance of the CI coating would have been possibly easier to evaluate.

IF- results got on 13th day were expected, because hASCs are mesenchymal origin cells and thus positive to vimentin. Positivity to Ki67 was expected, because the number of cells increased during the culturing. Positivity to α- SMA and keratocan would have been surprising if observed in hASCs without any additional keratocyte differentiation signals such as pellet culture, because keratocan is a marker for keratocan protein in the corneal stroma and α-SMA is a protein that is expressed by keratocytes in corneal wound healing as well as vimentin (Ishizaki Masamichi et al. 1993, Myrna, Pot & Murphy 2009). Both of these are also cytoskeletal markers and for that reason, a few red spots observed in Figure 10 are only non-specific fluorescence.

6.2. 2D co-culture tests

In this study, co-culture tests were used to examine the interactions between hASCs and hCECs in different culture conditions. The aim was to see, if the hASCs act as assisting cells for hCECs and support their attachment, growth or differentiation. HASCs and hCECs were co-cultured indirectly and directly in PET inserts as well as directly in 24-wells and the results were evaluated with the phase–contrast microscope and IF- stainings.

During the co-culturing, the number of indirectly co-cultured hCECs was all the time a little higher comparing to the control hCECs evaluating by the phase-contrast microscope. Also the IF- results showed that the number of proliferating cells was a little higher in indirect co-culture samples. In direct co-culture insert samples, the same effect was not observed as clearly and there were no proliferating cells observed at all on 6th day. Also in the direct co-

culture wells, most of the proliferating cells appeared to be hASCs and only proliferating hCECs were observed on the edges of the wells with lower cell density. On 11th day, the situation changed in direct co-culture inserts and a lot of proliferating cells were observed in both –airlifted and non- airlifted– samples. Also few proliferating hASCs were observed on 11th day in indirect co-cultures although on 6th day no proliferating cells were observed. These results indicate that the hASCs have some kind of promoting effect on the hCECs proliferation rates, but also to the other way around. Because different cell types were extremely hard to identify in direct co-cultures and in the co-culture wells most of the proliferating cells showed to be hASCs instead of hCECs, the promoting effect was hard to point out only to one cell type. Mutual benefittings of that kind are typical of different SC co-cultures (Paschos et al. 2015). Because of the hASCs' MSC origin, it was very presumable that at least hASCs would have promoting effect to the hCECs (Hu et al. 2012, Zhang et al. 2006). A possible explanation for that proliferation of hCECs' was not observed in direct co-culture inserts until the 11th day, could be that the cell-to-cell interactions slowed down the secretion of the important soluble factors of the MSCs, which induces the proliferation of the hCECs. It is also possible that the hASCs did not like the PET coating in the inserts. The reason why a few proliferating hCECs were observed in direct co-culture wells already on 6th day could be because the wells are a traditional 2D platform to the hASCs and they were able to secrete more soluble factors or support otherwise better the hCECs within these conditions. The cell death is more likely the reason why the number of proliferating hCECs did not increase between the 6th and 11th day, which could be caused for example by the lack of PET coating.

Other IF- results were harder to evaluate due to the extremely weak and unclear cytochemical labels, which could have been due the used hCECs' immortalization reprogramming. Immortalized cells are mutated cells that are capable of cell division although it would not be natural to them otherwise. Immortalized hCECs were used in this research, because they offer a good source of corneal epithelial cells to study and they have showed similar properties as normal hCECs (Araki-Sasaki et al. 1995), but because of the reprogramming processes, immortalized cells also express typically abnormal characteristics (Maqsood et al. 2013). Still, some more significant differences occur when comparing the staining for example between results got from indirect and direct co-cultures and from control samples. Indirectly and directly in the inserts co-cultured hCECs were stronger positive to α-SMA without AL and stronger positive to vimentin, CK3, CK12 and α-SMA with AL comparing with the

control hCEC samples. That would indicate that the hASCs have some enhancing effect to the hCECs. Comparing the airlifted and non- airlifted samples, the CK3 and CK12 labels were stronger in airlifted samples. Stronger positivity to the CEC surface markers (CK3, and CK12) (Dhamodaran et al. 2016) in airlifted insert replicates was expected, because AL typically enhances hCEC differentiation and stratification. Vimentin positivity of the hCECs was the strongest in the indirectly co-cultured samples but the effect of the AL could not be evaluated, because the non- airlifted sample disappeared during the IF- staining process. Still, since the hCECs were positive to vimentin on 6^{th} day as well as on 11^{th} day with AL, it can be assumed that also the non- airlifted cells would have been positive to it. Vimentin is expressed naturally in the corneal stroma in wound healing process instead of the epithelial layer (Ishizaki Masamichi et al. 1993), but some cultured epithelial cells have been shown to express it (SundarRaj et al. 1992). That would indicate that the hCECs expressed abnormal characteristics either due the reprogramming or the culturing. Because α-SMA is also expressed naturally in corneal stroma in wound healing, the expression of it in this research is more likely due the reprogramming of the hCECs. This problem was also observed when p63α label was observed in cytoskeleton of the cells in every other samples instead of control hCECs. Naturally this protein is expressed in the nucleus of the cells (Pellegrini et al. 2001).

When evaluating the IF- results of the hASCs, it was surprising to see that hASCs cultured separately for six days were not positive to vimentin at all although they were positive to it in CnT-Prime-CC medium after 11 days and after 13 days in the first medium tests. That is most likely explained by the high passage number of hASCs, because the expression rates of the different surface markers can increase or decrease while passage increases (Lindroos, Suuronen & Miettinen 2011). Because also the control hASCs were positive to vimentin after 11 days like the indirectly co-cultured hASCs, the effect could not be caused only by the hCECs. In this experiment, the passage of the hASCs was 15 and in medium tests 3 to 8. To be able to compare these IF- results significantly, the same passage has to be used.

Co-culturing of the MSCs and hASCs with direct cell-to-cell contact with TDCs have been shown in many studies to induce TDC differentiation (Ball, Shuttleworth & Kielty 2004, Liu et al. 2009, Strassburg et al. 2010, Qing, Wei-ding & Wei-min 2011). The hypothesis also behind this research was that the used hASCs could either be differentiated to keratocytes or the hASCs' immunomodulatory properties can be utilized to support the healing of the corneal surface after epithelial stem cell transplantation. Differentiation of the hASC- induced

by the hCECs to keratocyte-like cells was not observed in this research. However, the ASCs appeared to enhance the differentiation of the hCECs.

6.3. Hydrogel tests

After evaluating the different interactions between hASCs and hCECs, the hydrogel tests were started with embedding the hASCs in the hydrogels and evaluating their viability and adhesion to the hydrogel structure during the culturing.

6.3.1. Hydrogel structure

Many researches have shown that mechanical and physiological properties of the hydrogel structure are extremely important to the behaviour of the cells encapsulated inside of it (Antoine, Vlachos & Rylander 2014, Gasperini, Mano & Reis 2014) and they can regulate also the cell behaviour of SCs in co-culture systems (Paschos et al. 2015). These different properties are still far from easy to control. During the preparation of the hydrogels, there are many steps that can affect dramatically different properties of hydrogels with only slight modifications. That was seen for example by Lindborg et al. (Lindborg et al. 2015) when they were researching chitosan- HA- based hydrogel- colloidal structure with hMSCs and by other research group in BioMediTech when they were researching new hydrogel materials for 3D cell culturing (data not shown). The preparation process of the hydrogels used in this study would also need further studies and analysis due to differences in hydrogel properties between different experiments although the used preparation methods were the same. It would be good to see especially how different blending methods (by pipette or in the well) would affect hydrogels' structure. The main problem with the blending especially in the first experiments was that when adding the HA1- cell suspension- component to the HA2- component, material solidified extremely fast and cells were not blended in the hydrogel evenly and remained even on the surface of the hydrogels. These cells were then most likely removed by medium change, which would explain the loss of cells during the culturing. Also in some experiments, the cells did not spread during the three- day culturing whereas in some experiments, spreaded cells were observed already on 1st day that has more likely something to do with changed hydrogel structure.

The observed loss of hydrogel material after five days had most likely happened also in the earlier experiments when the cells were cultured for 13, and even for 21 days, because when cutting the inserts to accomplish the IF- stainings, the hydrogels were not clearly observed. The lack of experience was the reason why this loss was not observed certainly and earlier. Similar results were observed also in other experiments without embedded cells already on 3rd day (Figure 18). The reason for this loss of the hydrogel material is more likely the swelling and eventual liquidification of the hydrogels. When using the inserts as a platform for the hydrogels, the swelling could be caused by the medium change under the hydrogel as well as on top of it. Still, it is important to notice that this problem occurred also when hydrogels were made into wells. That indicates that the pores inside the hydrogel are too big and promote too high diffusion and the swelling rates comparing with the mechanical strength of the hydrogel structure (Singhal, Gupta 2016). Another cause for the loss of hydrogel could be too fast degradation process. Even thought the degradation was not observed during the hydrogel tests done without medium contact (data not shown), the solid environment can lead to the dissolution of the polymer chains or the chemical bonds (Nicodemus, Bryant 2008).

6.3.2. Hydrogel- embedded adipose stem cells

Most of the current 3D cell culture structures, including hydrogels, have showed lack of cell anchorage- sites, which causes that the cell spreading cannot occur and cells will not survive (Wang, Varshney & Wang 2010). That was also seen in this research. Because hASCs embedded to hydrogels maintain their spheroidal shape during the culturing it can be assumed that there were not enough anchorage- sites in the hydrogel structures to them to adhere. Due the MSC origin of the ASCs, the cell adhesion is necessary to hASCs' survival in the hydrogels (Wang, Varshney & Wang 2010, Salzig et al. 2016). The assumption about the lack

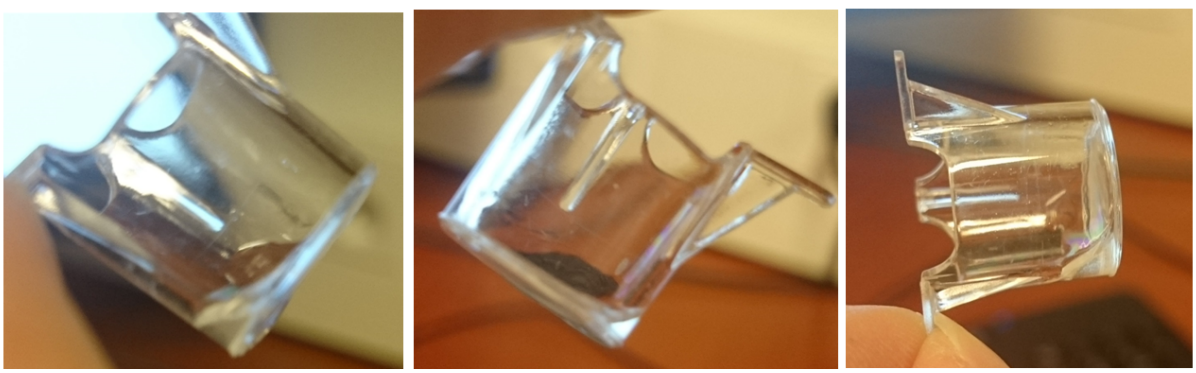

Figure 18 Hydrogels on 3th day. Picture by Laura Koivusalo.

of anchorage- sites in the hydrogels is strengthened by the fact that the cells spread immediately after migrating or sinking to the bottom of the used platform. Maintaining the spheroidal shape of the cells could be also caused by too tight hydrogel structure, but because the cells could move inside the hydrogel easily that is unlikely.

The different anchorage- sites can be added to the hydrogel structures for example by different chemical modifications like crosslinking, but very often these methods change the hydrogels' mechanical and physiological properties and even reduce the cell binding ability. One major disadvantage of the crosslinking modification is that the crosslinking decreases even more the number of cell adhesion motifs inside the hydrogels, same time as it approves the mechanical properties of the hydrogel. Different mechanical methods can be also toxic to the cells (Lindborg et al. 2015). Other possibilities to increase the cell adhesion inside the hydrogels are for example adding cell- adhesion motifs like RGDs straight to the hydrogel structure (Lei et al. 2011) or by modification of the degradation rate of the hydrogels since spheroidal shaped MSCs have been able to migrate inside the hydrogel to form cell-to-cell interactions better with the right degradation rate of the hydrogel (Nicodemus, Bryant 2008). In that case, the hydrogel structure has to bee a lot thicker so there would be a transplantable material left after the culturing and degradation process.

As expected, the cell density was an extremely important factor in our 3D culturing, due to the facts that cells grow naturally close to each other and require different biochemical signals from other cells to survive. That requirement was clearly observed in this research, because the cells did not survive even three days inside the hydrogel with too low cell densities. When cell density was increased step by step, more and more living cells were observed in the culturing and with the highest used cell densities the live/dead analyses also showed the spheroidal shaped cells to be alive in the hydrogels. The number of spreaded cells increased as cell density increased. That was assumed, but the reason behind it is not obvious. Alternative explanations could be stronger biochemical signals between separately growing cells with higher cell density (Wang, Varshney & Wang 2010) or the higher cell density just enables more cells to migrate or sink to the bottom of the used inserts or wells where they are able to spread normally. With our experience, the sinking is a more likely option, because of the loss of hydrogel material. The migration also requires the adhesion sites to the cells.

Because the live/dead analysis showed a lower number of dead hASCs in the control medium samples comparing with the CnT-Prime-CC medium samples, it is assumable that the HS used in the control medium has positive effect on the hASCs' survival, because the CnT-Prime-CC medium is a SF medium. This was expected, because the FBS have for example showed to promote the MSCs' growth. ASCs still have been shown to be able to proliferate and survive in the SF conditions (Wan Safwani et al. 2016). That was observed also in this research and because the use of SF conditions will ease the possible future clinical researches, there is no need to use any serum in the mediums in the further studies.

It was surprising to see that the hASCs survived so much worse inside the HA- hydrogels with added CI from human and rat origin than without any added CI. Because the collagen consists of the required RGD motifs naturally (Gasperini, Mano & Reis 2014) and collagen-based hydrogels have been shown to be suitable for 3D cell culturing, the better cell survival was expected. The poor cell survival in CI- added hydrogels might be explained by the fact that multiple factors of the used collagen have an affect to the cell behavior and survival in the hydrogels. Antoine et al. have for example showed that for example the origin, pH and concentration of the used collagen have an affect to the cellular behavior as well as mechanical properties of the hydrogel (Antoine, Vlachos & Rylander 2014). The pH was measured from the used rat origin CI only by pH-paper that showed it to be close to 7. This was the wanted value, but an extremely inaccurate method. If the true pH-value was not close enough to 7, that might have caused the poor cell survival in the culture. The origin of the rat CI was also one strong candidate to cause that effect, because it was got and isolated by another research group in the University of Tampere and was not a commercial product, which would offer more stable constitute. The assumption, that the origin of the rat CI would be a causing effect on poor cell survival, is supported by the results obtained from other research group from BioMediTech. In that group, used rat CI was a commercial product and used SCs survived well in their natural polymer hydrogel (data not shown). In the case of human CI, the reason behind the cell death is harder to evaluate, because the CI was the same used in 2D medim tests coatings. Because the passage of the cultured hASCs was also extremely high during the hydrogel test (7 to 14), it might have an affect also to the cell survival results.

Within righ cell densities the hASCs' positivity to Ki67 after seven days was not a surprise, because the phase-contrast microscopy had showed that the number of cells increased during

the culturing. What was interesting to observe, was that the hASCs were no longer positive to vimentin. Reason for that is most likely the extremely high passage of the hASCs'. In Susanna Miettinen's research group, the hASCs are used until passage 10 at the highest and for example Gonzales et al. have showed that the phenotype of the bone marrow MSCs is maintained only until the passage eight (González et al. 2016). It has also been shown that the different protein expression rates increases or decreases while the passage of the hASCs increases (Lindroos, Suuronen & Miettinen 2011). The positivity to Ki67 still showed that the cells grew actively.

6.4. Future perspectives

Because the suitable medium for the hASCs culturing was found, hASCs appeared to have enhancing effect on the hCECs' differentiation and growth and hASCs survived in the HA-hydrogels, this research project can be continued by trying to co-culture the hASCs and hCECs within HA- hydrogel structures. In this study, there was not enough time to do that.

Still, before that, new modifications of the HA- hydrogel materials need to be developed to support better the hASCs' cell adhesion inside the hydrogel and decrease the swelling rate of the hydrogel. After better modifications have been developed, hASCs' survival rate in this new hydrogel material can be studied. If the new material supports the hASCs survival, the co-cultures are continued with hASCs embedded in the hydrogel and hCECs on the surface of it. Used hCECs are most likely the iPSC-derived hCECs, instead of immortalized hCECs due to the difficulties in the evaluation process in this research. Used iPSCs will be differentiated by Heli Skottman's group's own serum-free protocol. After the co-culture state is managed, the transplantation issues and preclinical ex- and in vivo studies of this 3D structure can be considered.

If better modifications to the hydrogels cannot be developed, one option is also research commercial hydrogel materials. Lonza have for example developed a rat CI- based RAFT^{TM} 3D cell culturing system (http://www.lonza.com/products-services/bio-research/primary-cells/raft-3d-cell-culture-system/raft-3d-kits; 16.3.2016) that could be suitable for groups' purposes.

7. Conclusions

Objectives of this research were to find a suitable medium for hASCs, from those that had already been shown to be suitable for hCECs, and use it to co-culture hCECs with hydrogel-embedded hASCs. The study was performed by finding the suitable culture medium for hASCs, use that medium to co-culture hCECs with hASCs in 2D within direct- and indirect contact and study the suitability of the HA- hydrogel structure as a 3D cell culturing platform for hASCs. There was not enough time in this study for 3D co-culturing.

The medium tests showed that hASCs were capable of growing in the tested SF co-culture medium (CnT-Prime-CC) separately as well as with hCECs. Co-culture tests showed that hASCs have some kind of enhancing effect on the hCECs' growth and differentiation with indirect- and direct co-cultures in the inserts especially with AL. In direct co-cultures in the well, the hCECs did not survive. Hydrogel tests showed that the hASCs were able to grow and survive in the HA- hydrogel structure with right cell densities although they did not spread until they sank or migrate to the bottom of the used platform. These results indicate that this research project can be continued with 3D co-cultures with hCECs and hydrogel-embedded hASCs, and that kind of 3D structure might have potential for future applications in ocular surface reconstruction.

One of the most important factors to take into account in this study was the high passage number of the hASCs and the immortalizing reprogramming of the hCECs. Both of these factors had more likely effect on the IF- staining results got in different tests. The high passage of the hASCs more likely also effected the cell survival rate in the hydrogels. To be able to evaluate the got results more significantly, a lower passage number of the hASCs need to be used and research what kind of effect the immortalizing reprogramming has to the hCECs if they are used in future studies. Also the different preparing methods of the hydrogels and their effect on the hydrogels' structure need to be evaluated more carefully.

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