Optimizing differentiation of human pluripotent stem cells towards corneal epithelium

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TIIVISTELMÄ

Taustatieto ja tavoitteet Limbaalisten kantasolujen puutos on maailmanlaajuisesti esiintyvä sokeuttava sarveiskalvosairaus. Se voi aiheutua esimerkiksi kemiallisesta palamisesta, tulehduksesta tai perinnöllisistä syistä. Sairaudelle on tyypillistä limbaalisten kantasolujen toimintahäiriöt ja limbuksen suojavaikutuksen pettäminen. Tällöin ympäröivän sidekalvon solut tunkeutuvat sarveiskalvoon sumentaen sen. Tässä sairaudessa sarveiskalvon pinta/epiteeli ei siis voi uusiutua, joten hoitokeinoissa silmään täytyy tuoda uusia kantasoluja kudoksen uusiutumisen takaamiseksi. Limbaalisten kantasolujen puutokseen on ollut useita erilaisia lähestymistapoja, joissa hoitona on käytetty kudossiirteitä tai soluja. Tässä tutkimuksessa esitellään menetelmä, joka on kehitetty BioMediTechin silmäryhmässä Tampereen yliopistolla, joka käyttää ihmisen erittäin monikykyisiä kantasoluja sarveiskalvon epiteelisolujen kaltaisten solujen erilaistamiseksi. Tämän tutkimuksen tavoite oli optimoida kyseistä metodia ja kokeilla entsymaattista irrotusta kantasoluille.

Menetelmät Tässä tutkimuksessa solujen erilaistus toistettiin kuusi kertaa. Toistoissa solut irrotettiin alustaltaan joko mekaanisesti tai entsymaattisesti. Soluja viljeltiin pienmolekyylejä sisältävässä induktiomediumissa suspensioviljelmässä, jonka tarkoituksena oli saada solut muodostamaan soluaggregaatteja. Seitsemänpäiväisen induktion jälkeen solut siirrettiin kasvamaan kollageeni IV:llä tai kasvupinta x:llä pinnoitetuille kuoppalevyille. Solujen kiinnityttyä alustalle, niitä kasvatettiin 21 päivän ajan. Kokeiden puolivälissä ja päätepisteessä soluille tehtiin immunofluoresenssivärjäys. Käytetyillä markkereilla tunnistettiin joko limbaalisia kantasoluja tai sarveiskalvon epiteelisoluja.

Tulokset Mekaaninen ja entsymaattinen irrotusmenetelmä toimivat irrotusmenetelminä ja entsymaattisella menetelmällä saatiin paikoittain jopa suurempia solumääriä jatkoviljelyyn. Soluaggregaatit selviytyivät paremmin suspensioviljelmässä, kun kasvupinta-ala oli pienempi. Kollageeni IV ja kasvupinta x toimivat kasvupintoina, mutta solumäärät olivat suurempia kasvupinnalla x. Adherenttissa soluviljelmässä kasvupinta-alan oli tärkeää olla optimaalinen, etteivät solut kärsisi tilanpuutteesta. Kokeiden päätepisteessä viljelmissä oli sekä sarveiskalvon epiteelin kantasolumarkkereille, että erilaistuneiden solujen markkereille positiivisia soluja.

Johtopäätökset Entsymaattinen irrotus toimi solujen irrotusmenetelmänä mekaanisen irrotuksen rinnalla. Solut kasvoivat paremmin entsymaattisesti irrotetuista aggregaateista kuin mekaanisesti irrotetuista. Kasvupinta x paransi solujen kiinnittymistä ja tehosti niiden erilaistusta sarveiskalvon epiteeliksi.

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ABSTRACT

Background and aims Limbal stem cell deficiency (LSCD) is a potentially blinding corneal disease caused by trauma, such as chemical burn, inflammation, or hereditary factors. LSCD is characterized by dysfunction of limbal epithelial stem cells (LESC) and failure of barrier function of limbus. Surrounding conjunctival tissue invades transparent and avascular corneal epithelium, which leads to vascularization and haziness. In LSCD, the surface of the eye cannot recover itself due to lack or dysfunction of LESCs so the treatment needs to bring new stem cells to continue tissue regeneration. Various approaches have been proposed to treat LSCD, some using limbal tissue transplantations from healthy eye and some have been cell-based therapies. This study presents a method developed in the Eye group at the University of Tampere, which uses human pluripotent stem cells (hPSCs) to differentiate corneal epithelial-like cells. This study's aim was to further optimize this method and test enzymatic dissociation method for stem cells.

Methods In this study, cell differentiation was repeated six times. In every experiment, hPSCs were dissociated from feeder cells either mechanically or enzymatically. The cells were suspension cultured in small molecule and growth factor supplemented induction medium as embryoid bodies (EBs). After seven-day induction, the cells were plated to well plates, which were coated with collagen IV or surface x. The adherent cell culture lasted 21 days. Cells were analyzed with immunofluorescence at midpoint and end point of the experiments, to identify either LESC-like cells or corneal epithelial cells.

Results Mechanical and enzymatic dissociation methods both worked as detaching methods. Enzymatic dissociation gave partially better cell numbers than mechanical dissociation. EBs survived better in suspension culture when the growth area was smaller. Collagen IV and surface x both worked as growth surface but cell numbers were better with surface x. In adherent cell culture, the growth area proved to be important, because cells seemed to suffer from overcrowding. At the end point of the experiments, there were cells positive for both LESC and cornea epithelium markers.

Conclusions Enzymatic dissociation method worked as a dissociation method alongside the mechanical dissociation. Enzymatically dissociated EBs survived and outgrowths were better than mechanically dissociated EBs. Surface x improved cell attachment and enhanced their differentiation towards corneal epithelium.

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ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2
BMP4	Bone morphogenetic factor 4
BSA	Bovine serum albumin
CIV	Collagen IV
CK	Cytokeratin
CLET	Cultured limbal epithelium transplantation
COMET	Cultured oral mucosal epithelial transplantation
DAPI	4’,6-diamidino-2-phenylindole
DPBS	Dulbecco’s Phosphate-Buffered Saline
EB	Embryoid body
ECM	Extracellular matrix
Exp	Experiment
FGF	Fibroblast growth factor
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
hPSC	Human pluripotent stem cell
IF	Immunofluorescence
IgG	Immunoglobulin G
iPSC	Induced pluripotent stem cell
KO-DMEM	Knock-Out Dulbecco’s Modified Eagle Medium
KO-SR	Knock-Out Serum Replacement
LEC	Limbal epithelial crypt
LESC	Limbal epithelial stem cell
LSCD	Limbal stem cell deficiency
Mech	Mechanical dissociation method
PAX6	Paired box-gene 6
PFA	Paraformaldehyde
qPCR	Quantitative real-time polymerase chain reaction
RT	Room temperature
SCED	Single cell enzymatic dissociation
SLET	Simple limbal epithelial transplantation
TGF-β	Transforming growth factor β
UM	Unsupplemented medium
UM+B	Unsupplemented medium supplemented with blebbistatin
X	Surface x

1. INTRODUCTION

The cornea is transparent and avascular structure in the surface of the eye, protecting inner structures from damage. Limbal stem cell deficiency (LSCD) is a blinding corneal disease that is usually caused by trauma, such as chemical or thermal injury, or genetic condition (Notara et al., 2010). LSCD is characterized by failure of the limbus or limbal epithelial stem cells (LESCs) and their functions to regenerate corneal epithelium (Osei-Bempong et al., 2013). Limbus is a structure surrounding cornea, acting as a barrier which prevents surrounding tissues from invading cornea and as LESC reservoir (Ahmad 2012). Because corneal regeneration is dysfunctional in LSCD, its treatment is challenging. Several treatment options have been proposed, including direct limbal tissue transplant and cell-based approaches (Baylis et al., 2011, Casaroli-Marano et al., 2015). Limbal transplant-based techniques are complicated because they need sufficient amount of healthy tissue for transplantation and the tissue harvesting can harm the healthy donor eye (Baylis et al., 2011). Cell-based techniques have been promising, for example cultivated limbal epithelial transplantation (CLET), but the questions lie in long-term results, complexity and the origins of the cells (Nakamura et al., 2015). Therefore, more development and novel methods are needed to overcome existing problems.

One promising stem cell-based approach is to use human pluripotent stem cells (hPSCs) which have wide differentiation potential (Fortier, 2005). Human PSCs can be obtained either from inner cell mass of human embryo blastocyst (Ahmad et al., 2007) or by reprogramming from differentiated cells, for example fibroblasts (Takahashi et al., 2007). These reprogrammed cells, known as human induced pluripotent stem cells (hiPSCs) offer many opportunities in personalized medicine and disease modelling. There have been several studies successfully differentiating corneal epithelial cells from hPSCs (Ahmad et al., 2007, Hayashi et al., 2012, Shalom-Feuerstein et al., 2012). BioMediTech's Eye group at the University of Tampere has developed a promising directed differentiation method for hPSCs, which aims for chemically defined and xeno-free culture conditions (Mikhailova et al., 2014). They have generated relatively pure populations of corneal epithelial-like progenitor cells, which are capable of terminal differentiation toward mature corneal epithelial-like cells.

This study was carried out to further optimize the differentiation protocol of hPSCs developed by Mikhailova et al., (2014). In this study, also another cell dissociation method, single cell enzymatic dissociation (SCED) enhanced with blebbistatin, was tested beside standard mechanical dissociation method. The SCED-method is easier to standardize and faster to use than mechanical dissociation so it may give results that are more consistent. In this study, the cells were differentiated using either extracellular matrix (ECM) coating material, collagen IV, or surface x in adherent cell culture to see which is better for cell attachment and differentiation.

2. REVIEW OF THE LITERATURE

2.1 CORNEAL EPITHELIUM

Eyesight is a very important part of human social communication and it defines how we see the world. One of the most important structures of the eye is cornea, which transmits light to the retina and enables visual perception (Figure 1). Cornea is the outer layer at the front of the eye and it consists of three cellular layers: epithelium, stroma and endothelium, and two interface layers: Bowman membrane and Descemet membrane (DelMonte and Kim, 2011, Ghezzi et al., 2015). Each layer has specific features and functions. Stroma is the thickest layer, and gives the cornea mechanical support and acts as structural framework (DelMonte and Kim, 2011). Endothelial layer helps maintain corneal clarity by controlling fluid transportation (DelMonte and Kim, 2011).

Cornea is a transparent, multilayered and protective structure. To be transparent, cornea must be avascular and clear. Because cornea is the outermost layer in eye, it needs to protect the eye, and corneal epithelial cells have to regenerate continuously to maintain their function. Cornea

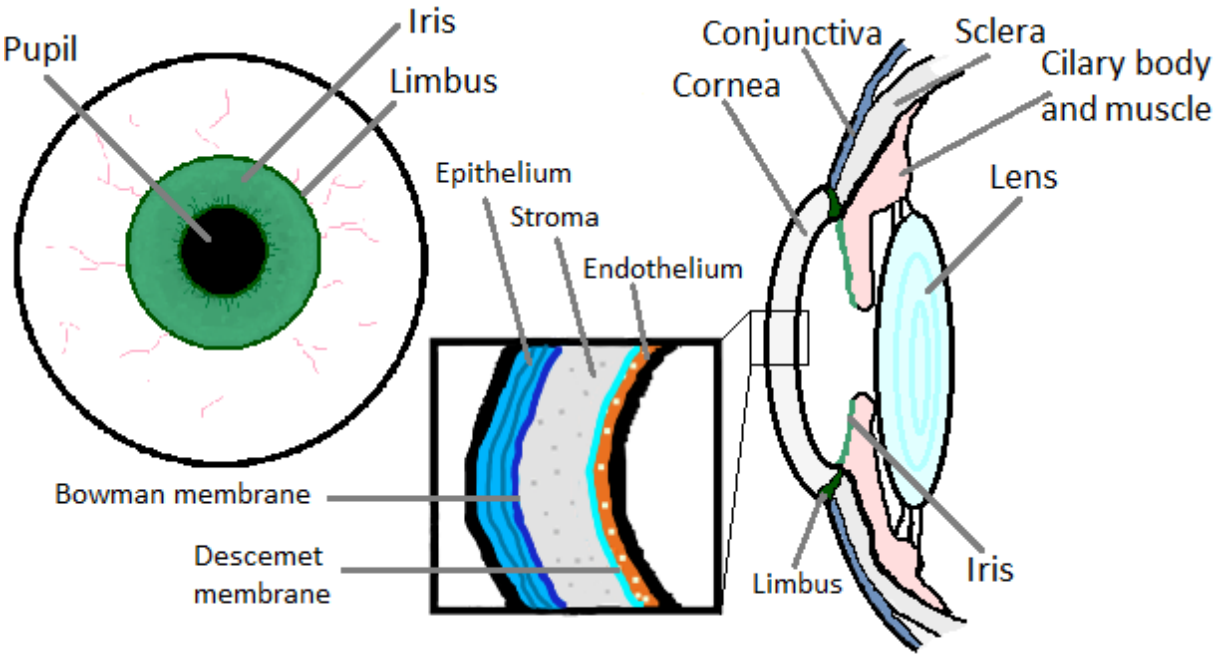

Figure 1. *Anatomy of the human eye and cornea.*
Cornea is the outermost layer of the eye and it covers pupil and iris. Cornea is surrounded by limbus that acts as barrier separating cornea from conjunctiva. Corneal epithelium is the outermost layer from the five corneal layers.

is surrounded by limbus, which acts as stem cell reservoir and separates it from the conjunctiva and sclera (Osei-Bempong et al., 2013). It is important that cornea stays separated from surrounding conjunctiva because the two of them have different structures. If limbus fails and surrounding conjunctiva starts to replace cornea it can lead to vascularization and inflammation (Frank and Frank, 2015). Because corneal epithelium needs to regenerate constantly, new corneal epithelial cells are needed. The basal limbal layer contains corneal progenitor cells, known as LESCs, which can migrate towards central cornea and differentiate into corneal epithelial cells to replace cells lost from the ocular surface (Osei-Bempong et al., 2013, He and Yiu, 2014).

2.1.1 Development of the cornea

During human development, cornea originates from one of the three embryonic layers, called ectoderm (Dhouailly et al., 2014, Ramos et al., 2015). The development of cornea is known to be guided by activation or inhibition of many signaling pathways (Fuhrmann, 2008, Dhouailly et al., 2014). The mechanisms of human corneal epithelium development are still unclear: one theory suggests that corneal epithelium's induction is the final step of inductive events in eye development and corneal inductive signals come from lens vesicle, while another theory is that corneal epithelium develops before the lens (Collomb et al., 2013, Dhouailly et al., 2014).

In the development process of the human cornea, many important factors and signaling pathways guide the development towards corneal epithelium and thus prevent other paths (Dhouailly et al., 2014). For example, Wnt signaling pathway is involved in tooth, hair or feather morphogenesis, and its inhibition in the cornea leads to non-skin development typical to corneal integument (Fuhrmann, 2008, Dhouailly et al., 2014). The activation of fibroblast growth factor (FGF) pathway narrows the developmental choices to only nasal and ocular precursors by suppressing signals in ectoderm (Dhouailly et al., 2014). There is also evidence that FGF signaling is needed for LESC proliferation (Zhang et al., 2015). Transforming growth factor β (TGF-β) regulates many responses in cell growth and if its signaling is inhibited it would enhance re-epithelization of cutaneous wounds and reduce scarring fibrosis (DaCosta Byfield et al., 2004).

2.2 STEM CELLS

Stem cells are undifferentiated cells that are able to self-renew and differentiate into several cell lineages. There are several types of stem cells and they are grouped by their differentiating abilities. Totipotent stem cells can be harvested from fertilized oocyte after the first cleavage divisions and they have most of their so-called potency left and can develop into an entire organism (Fortier, 2005). In inner cell mass of blastocyst lie pluripotent stem cells, which have the ability to form tissues from all embryonic germ layers: endoderm, mesoderm and ectoderm. However, pluripotent stem cells cannot differentiate towards extraembryonic tissues like placenta. Multipotent stem cells can differentiate into more closely related cell lineages. For example, mesenchymal and hematopoietic stem cells are multipotent. Oligopotent and unipotent stem cells are able to differentiate into only some closely related cell types, few cell types or one cell type.

Because of their unique properties, stem cells are responsible for cell replacement and tissue regeneration. Stem cells can undergo symmetric or asymmetric cell divisions. In asymmetric cell division, one of the daughter cells remains an undifferentiated stem cell and the other differentiates into less primitive cell called transient amplifying cell (Daniels et al., 2001). Transient amplifying cells have less potential and possibilities and they can further differentiate into post-mitotic cells and to their final form, terminally differentiated cells, which are unable to divide anymore, and eventually undergo apoptosis (Dua et al., 2000). A small fraction of adult individual's nucleated cells is adult stem cells that exist only in certain tissues and until recently, it was strictly believed that as such they could differentiate only into certain tissues even *in vitro* (Fortier, 2005). Nowadays there are techniques, which can overcome that boundary *in vitro* (Fortier, 2005). To yield cells that can grow indefinitely as pluripotent stem cells researches have been using human embryonic stem cells (hESCs).

The first hESC line was established in 1998 and techniques using them have been invented and nowadays used hESCs donated for reseach are derived from embryos which would otherwise be discarded (Skottman 2010).The isolation of hESCs is considered ethically questionable in some countries and their usage is regulated strictly (Davies and DiGirolamo, 2010). Due to these constraints, alternatives to hESCs have been developed. Human iPSCs are a relatively recent innovation where somatic cells are reprogrammed to embryonic-like pluripotent stem

cells (Takahashi et al., 2007). This method has revolutionized stem cell studies and provided an alternative to hESCs.

2.2.1 Human induced pluripotent stem cells

The development of hiPSC-based methods has brought many new techniques to cell technology because pluripotent stem cells could be yielded noninvasively from somatic tissues such as skin. hiPSCs have been demonstrated to be very similar to hESCs in morphology, feeder dependency and potency to differentiation (Takahashi et al., 2007). It has been shown that hiPSCs do express most of the pluripotency markers, for example hESC-specific surface antigens (Takahashi et al., 2007). Moreover, the differentiation potential of hiPSCs was studied using floating cultivation to form embryoid bodies (EBs), and the results showed that they could differentiate into three germ layers *in vitro* and in teratomas (Takahashi et al., 2007). Nevertheless, hiPSCs have the advantage of being more diverse in modeling diseases when hESCs have been limited (Vitale et al., 2012). Because hiPSCs are obtained from somatic cells, they have opened a way to generate patient- and disease-specific cell lines, which could be used for disease modelling and personalized medicine (Takahashi et al., 2007). They have still some safety issues because they may increase risk of tumorigenesis which might be caused by traditionally used retrovirus vectors but when the risks are overcome they can be used more widely (Takahashi et al., 2007). Reprogramming can also be done without virus components, when the risk to changes inducing tumor formation is said to be lower (Sareen et al., 2014).

Human iPSCs have shown potential in corneal studies and few research groups have been able to differentiate for example fibroblast-derived hiPSCs to corneal epithelial cells (Casaroli-Marano et al., 2015). So far, there have not been many corneal differentiation published studies using hiPSCs. However, the few existing studies have been successful in differentiation (Hayashi et al., 2012, Shalom-Feuerstein et al., 2012, Shalom-Feuerstein et al., 2013, Mikhailova et al., 2014, Sareen et al., 2014). Hayashi et al. (2012) have successfully induced corneal epithelial cells from hiPSCs by stromal cell-derived inducing activity differentiation method. Mikhailova et al. (2014) have developed direct, two-stage differentiation method for hiPSCs toward corneal epithelial-like cells, which are in mostly xeno-free conditions capable of terminal differentiation toward mature corneal epithelial-like cells. Shalom-Feuerstein et al. (2012) have been able to differentiate epithelial-like cells with the presence of bone morphogenetic factor 4 (BMP4) in culture medium conditioned by corneal fibroblasts, on

collagen IV-coated dishes. Later, the same group has also shown that hiPSCs from ectodermal dysplasia-related patients can undergo ectodermal development but not differentiate further towards corneal epithelium and thus they do not express certain corneal epithelial markers (Shalom-Feuerstein et al., 2013). Sareen et al. (2014) concluded that limbal epithelium-derived iPSCs could become a new functional LESC source for transplantations.

2.2.2 Limbal epithelial stem cells

Limbus is surrounding corneal epithelium and it can be located by thickening structure of the epithelium (Takacs et al., 2009). For a long time limbus and its stem cell niches, have been identified by striped structures called palisades of Vogt (Takacs et al., 2009). LESCs lie in specific niche regions in limbus called limbal crypts or limbal epithelial crypts (LEC) which are believed to contain the necessary characteristics for stem cells to stay undifferentiated (Castro-Munozledo, 2013). However, the origin of LESCs is also under debate and one theory says that LESCs are localized in basal layer of the limbal epithelium and other that they are scattered over the basal layer of the entire cornea (Majo et al., 2008, DelMonte and Kim, 2011, Dhouailly et al., 2014).

The microenvironment of limbus is said to be one of the most important factors for LESCs to maintain their stem cells state (Dua et al., 2000, Daniels et al., 2001, Osei-Bepong et al., 2013). It is suggested that limbus has different and specific ECM composition compared to corneal ECM, which may be the key to stem cell regulation in limbal crypt (Castro-Munozledo, 2013). In the limbal microenvironment, there are stem cell nutrients and growth factors, which support and regulate stem cell growth and division (He and Yiu, 2014, Wan et al., 2015). Limbus contains for example collagen type IV, different types of laminin and nidogen and some other molecules which are specific only to limbus but not to central cornea (Mei et al., 2012, Wan et al., 2015).

When stem cells migrate out of limbus, their properties change because the regulative influence of their niche does not affect them anymore (Figure 2) (Yoon et al., 2014). The decision to leave LECs is believed to be made by asymmetric cell divisions when one of the daughter cells would stay in crypt and one move towards the central cornea losing its contact with the limbal microenvironment (Castro-Munozledo et al., 2013).

When LESCs begin their migration toward peripheral cornea they divide to be transient amplifying cells and their differentiation process is then initiated (Castro-Munozledo et al., 2013, He and Yui, 2014). The migrating cells are affected by prevalent microenvironment, which regulates their differentiation (Castro-Munozledo et al., 2013). During migration, transient amplifying cells progress to post-mitotic cells, and finally, when they reach the central cornea, they become terminally differentiated cells (Yoon et al., 2014).

2.2.3 Limbal epithelial stem cell markers

The discovery of LECs and LESCs have inspired studies aiming to determine the composition and regulation mechanisms of LEC and identification methods for LESCs (Figueira et al., 2007, Schlötzer-Schrehardt et al., 2007, Li et al., 2014). Finding specific markers for LESCs has turned out to be tricky, and there has been conflicting evidence regarding marker specificity and it is believed that there is no one specific reliable LESC marker (Daniels et al., 2001, Ahmad et al., 2010). Nevertheless, some markers like cytokeratin 3 (CK3), have been shown to be specific only to mature corneal epithelial cells but not basal epithelial cells in limbus (Daniels et al., 2001). One popular LESC marker has been p63 which expression has been found in basal layer of limbus (Li et al., 2014, Menzel-Severing et al., 2013).

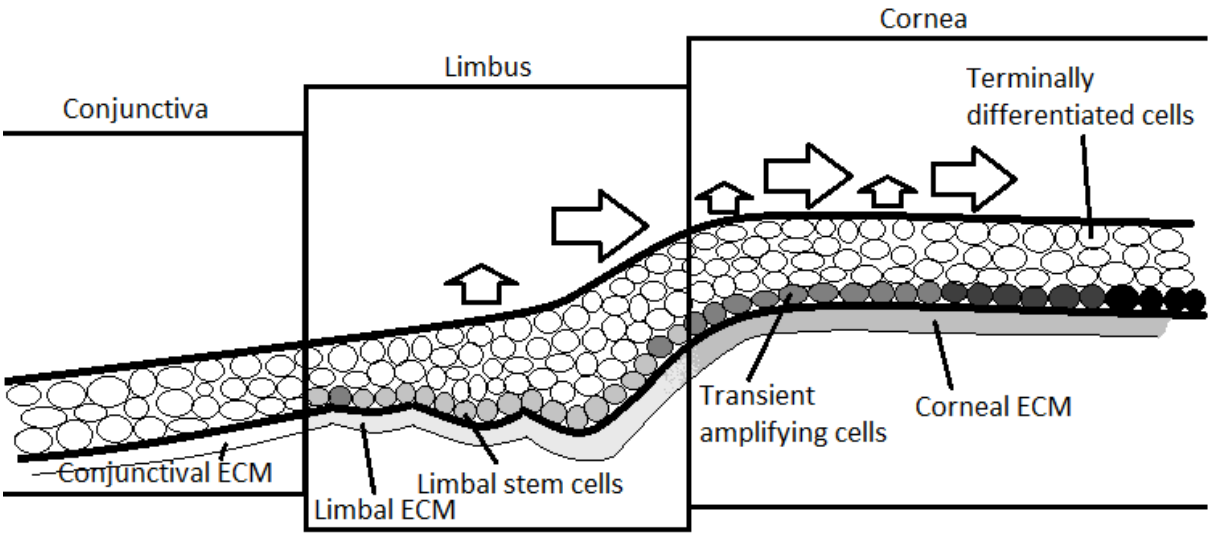

***Figure 2**. Limbal epithelial crypt.*
Arrows show LESC migration towards central cornea and how they differentiate along the way first to transient amplifying cells and finally to terminally differentiated cell. The differentiation starts when cells lose their contact with limbal ECM (Modified from Menzel-Severing et al., 2013).

Currently there are several markers that are used for identification of limbal or corneal epithelial cells (Figueira et al., 2007). For example, PAX6, and cytokeratins 12 (CK12) and 3 are differentiation markers from which CK12 is said to be cornea-specific (Daniels et al., 2001, Castro-Munozledo, 2013, Nakamura et al. 2015). Integrin α9, N-cadherin, cytokeratin 15 CK15), ABC-binding cassette sub-family G member 2 (ABCG2) and p63 are considered markers for LESCs (Schlötzer-Schrehardt et al., 2007, Notara et al., 2010, Nakamura and Kinoshita, 2011, Wan et al., 2015). Ki67 is proliferating cell marker and it is used to identify actively cycling cells (Schlötzer-Schrehardt and Kruse, 2005). In the lack of certainly specific markers, researchers use multiple markers simultaneously and identify LESCs by analyzing their niches and regulatory functions (Ahmad, 2012, Ramos et al., 2015, Wan et al., 2015).

2.3 CORNEAL DISEASES AND THERAPIES

Cornea is one of the most important functional structures in the human eye but it is also exposed to damage (Takacs et al., 2009). Corneal regeneration is mediated by LESCs and it is essential that they replace lost corneal epithelial cells so that cornea stays functional and is able to protect interior structures of the eye (DelMonte and Kim, 2011). Corneal epithelium cooperates with tear film, which is the primary protector of the corneal surface from microbes, chemicals, toxic and foreign-body damage (DelMonte and Kim, 2011). However, it is possible that corneal protection fails and consequences can be severe.

There are many corneal diseases and injuries that can harm eyesight. Corneal injuries are usually mechanical, but chemical and thermal injuries are also possible (DelMonte and Kim, 2011). Limbal stem cell diseases, caused by some kind of dysfunction such as destruction or innate lack of LESCs, can be really painful and difficult to treat (Ahmad, 2012). LSCD is a major cause of blindness worldwide (Frank and Frank, 2015).

2.3.1 Limbal stem cell deficiency

LSCD is characterized by dysfunction or lack of LESCs (He and Yiu, 2014). In LSCD, cornea is unable to regenerate itself and the barrier effect of limbus fails (Ahmad, 2012). Conjunctival epithelium starts to invade cornea, which is followed by neovascularization and inflammation causing scarring of corneal stroma (Casaroli-Marano et al., 2015, Nakamura et al., 2015). The following events will decrease vision, cause pain, and the chronic inflammation causes more

LESC loss, which complicates the survival of epithelial cells leading to deterioration of the situation (Casaroli-Marano et al., 2015).

There are many different, partly uncertain, causes of LSCD such as anatomical abnormalities, physical or chemical damages, inflammatory diseases or contact lens-associated causes (Ahmad, 2012). LSCD can also be caused by hereditary disease, such as iris coloboma (iris defect) or aniridia where the eye develops without an iris (Notara et al., 2010, Casaroli-Marano et al., 2015). The most frequent cause of damages leading to LSCD are chemical burns (Casaroli-Marano et al., 2015). LSCD can be diagnosed with detecting conjunctival epithelial specific cells from corneal epithelium, which can be done by for example PCR-strip-based diagnostic system, impression cytology or an application of the confocal microscope (He and Yiu, 2014).

2.3.2 Therapies for limbal stem cell deficiency

Many different treatment strategies for LSCD have been described, some of which have been successful and lead to clinical use. The difficulty in finding treatment methods is due to sustainability problems, because cornea's natural regeneration ability is decreased or disabled due to lack of healthy LESCs (Baylis et al., 2011). Treatment approaches for LSCD are divided into three main categories: 1) transplants and bio-replacement of tissue, 2) cell-based therapy by *ex vivo* cell culture expansion and 3) symptomatic and alternative treatment: keratoprosthesis implantation, provisional debridement of conjunctival corneal tissue, therapeutic contact lenses and drug therapy (Casaroli-Marano et al., 2015).

One early approach is limbal transplantation using autologous or allogeneic grafts straight from a healthy eye's limbal or conjunctival tissue to replace damaged tissue (Casaroli-Marano et al., 2015, Holland, 2015). During the past two decades, many *ex vivo* and cell-based approaches such as simple limbal epithelial transplantation (SLET), cultured limbal epithelial transplantation (CLET), and cultured oral mucosal epithelial transplantation (COMET) have been described (He and Yiu, 2014, Casaroli-Marano et al., 2015, Holland, 2015). Of these, CLET is the most commonly used *ex vivo* method of limbal transplantation where epithelial cells are harvested from donor, cultured *in vitro* and transplanted to damaged cornea (Holland, 2015). SLET is an advanced method of previous grafts that uses piece of limbal tissue from donor eye dissected into smaller pieces and seeded on the amniotic membrane covered cornea

(He and Yiu, 2014). COMET uses cultivated oral mucosal epithelial cells, which are transplanted to reconstruct damaged corneal epithelium (He and Yiu, 2014, Casaroli-Marano et al., 2015).

Cell-based therapies for LSCD are considered the most promising approaches due to their transformability and lesser invasiveness (Notara et al., 2010, Casaroli-Marano et al., 2015). The problem with cell-based approaches is their complexity and need for xenogeneic products in culturing (Pellegrini et al., 2014). However, the limbal tissue transplantation is simpler and it does not need xenoproducts, but it has disadvantage in standardization, causing immune rejection and durability issues, especially when the explant is allogeneic (Ahmad, 2012, Casaroli-Marano et al., 2015). Preferable method to treat LSCD would be harvesting patient's own limbal tissue from healthy eye, but if the damage is in both eyes, a different approach is necessary and harvesting tissue could harm the donor eye (Notara et al., 2010, Casaroli-Marano et al., 2015). This is where cell-based therapies can be useful, and these studies are evolving rapidly and alternative cell sources are being tested (Pellegrini et al., 2014).

There are always risks in transplantation therapies where biopsy has to be taken from the healthy eye and allogeneic transplantation poses a risk of immune rejection (Ahmad 2012, Casaroli-Marano et al., 2015). Therefore, using hiPSCs would be a promising method because the cells can be harvested noninvasively, immune rejection can be potentially avoided and cells differentiated to the target cell type (Hayashi et al., 2012, Casaroli-Marano et al., 2015). Alternatively, hESCs could be used. It seems that hiPSCs would be an ideal source for ocular clinical applications but there are still safety concerns, which require further studies (Sareen et al., 2014, Casaroli-Murano et al., 2015).

3. AIMS OF THE STUDY

The purpose of the study was to optimize the differentiation of hPSCs towards corneal epithelium. The optimization was done in order to create better, standardized conditions for stem cells to differentiate and grow. Optimization process had many different main points, which were tested. Two detaching methods, mechanical and enzymatic dissociation, were used for the cells and compared at the end. Induction, coating and plating conditions were also optimized. The hypothesis was that enzymatic dissociation could be used as a method and surface x (Appendix 1) could mimic limbal microenvironment better than collagen IV.

4. MATERIALS AND METHODS

4.1 CELL CULTURE

The experiment was repeated six times and the culturing conditions were optimized along the way (Figure 3, Table 1). All conditions are described in more detail in the following chapters. All cells used in this study were handled in sterile conditions and cultured in humidified incubator (Thermo Electron Corp., Waltham, MA, USA) at +37 °C and 5 % CO_2.

Table 1. *Outline of the different conditions in every experiment repeat.*
Abbreviations: CIV: collagen IV, X : surface x.

	Undiff. RNA-sample	***Induction***	***Plating***	***Coating concentrations***	***End point***	***End point RNA-sample***
Exp1	No	6-well plate	48-well delta surface plate	5 µg/cm^2 CIV low concentration of X	Day 22	No
Exp2	Yes	24-well plate	48-well delta surface plate	5 µg/cm^2 CIV low concentration of X	Day 28	No
Exp3	Yes	24-well plate	48-well delta surface plate	5 µg/cm^2 CIV high concentration of X	Day 28	No
Exp4	No	24-well plate	48-well delta surface plate	5 µg/cm^2 CIV high concentration of X	Day 28	No
Exp5	Yes	24-well plate	24-well cellbind plate	5 µg/cm^2 CIV high concentration of X	Day 28	Yes
Exp6	Yes	24-well plate	24-well cellbind plate	5 µg/cm^2 CIV high concentration of X	Day 28	Yes

4.1.1 Pluripotent stem cells

The undifferentiated hiPSCs used in this study were from cell line UTA.04511.WT (46XY) reprogrammed from human fibroblast using the CytoTune®-iPS Sendai Reprogramming Kit (Invitrogen, Carlsbad, CA) by Professor Katriina Aalto-Setälä's group at the University of Tampere (Ojala et al., 2015). The cell line was routinely maintained in hPSC medium consisting of KnockOut Dulbecco's Modified Eagle's Medium (KO-DMEM) supplemented with 20 % KnockOut Serum Replacement (KO-SR), 2 mM Glutamax, 0.1 mM 2-mercaptoethanol (all

from Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1 % Non-essential amino acids, 50 U/ml penicillin/streptomycin (both from Lonza Group Ltd, Basel, Switzerland), and 8 ng/ml human basic fibroblast growth factor (bFGF; PeproTech, Rocky Hill, NJ) on mitotically inactivated human foreskin fibroblast (CRL-2429; American Type Culture Collection, ATCC, Manassas, VA) feeder cells. The undifferentiated stem cell colonies were enzymatically passaged to fresh feeder cell layers every tenth day. The cell line was routinely karyotyped and characterized for self-renewal and differentiation capacities to confirm its quality.

4.1.2 Initiation of differentiation

Undifferentiated hiPSCs were detached from feeder cells using two methods: mechanical or enzymatic dissociation. Mechanical detaching was carried out by manually dissecting undifferentiated colonies of hiPSCs into smaller pieces with sterile scalpel. The pieces were detached with sterile needle and transferred to an ultra-low attachment surface 6-well plate (Costar®, Corning Inc., New York, USA). The pieces were then cultured in induction medium, where they spontaneously formed EB-like structures. Induction medium was based on unsupplemented medium (UM) which consisted of hPSC culture medium but using only 15 % KO-SR and with higher bFGF concentration. UM was supplemented with 10 µM TGF-β inhibitor SB-505124 hydrochloride hydrate (Sigma-Aldrich, St. Louis, Missouri), 10 µM Wnt inhibitor IWP-2 (Merck Millipore, Darmstadt, Germany) and 50 ng/ml bFGF (R&D Systems Inc., Minneapolis, Minnesota, USA) and used as induction medium.

Enzymatic dissociation was carried out using single cell enzymatic dissociation (SCED). The cells were detached using StemPro® Accutase® Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Detaching was initiated by aspirating culture media and rinsing cells twice with Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza Group AG, Basel, Switzerland). After that, 1 ml of room temperature (RT) Accutase was added to each well and incubated at +37 °C for about 2 min and the rounded cells were completely detached by Accutase trituration. After detaching, 2.5 ml pre-warmed UM was added to each well and the resulting single-cell suspensions were pooled to 15 or 50 ml Falcon tube. The single-cell suspensions were centrifuged 1500 rpm for 5 min. After that, the supernatant was aspirated and cell pellet resuspended in 1 ml of culture medium. Cells were counted from 10 µl of cell suspension using Bürker cell counting chamber. Cell suspension was diluted using UM

to 300 000 cells/ml, and 1 ml suspension transferred to an ultra-low attachment well plate (Corning Inc., Corning, New York, USA). In the first experiment, a 6-well ultra-low attachment plate was used, while in the remaining five experiments, a 24-well plate was used. To advance the formation of EBs from single cell suspensions, 5 µM blebbistatin (Sigma-Aldrich, St.Louis, Missouri, USA) was added to each well. The blebbistatin treatment to the EBs was performed overnight and on the second day after initiation, the medium was changed to appropriate volume of pre-warmed induction medium.

The induction period lasted seven days (Figure 3) and the induction medium was changed in both methods three times a week. Images were acquired with Nikon phase contrast light microscope (Nikon Corp., Tokyo, Japan) before the medium was changed. The induction medium was protected from light and stored at +4 °C for 1 week at most.

4.1.3 Cell plating

On the seventh day after initiation of induction, EBs were plated to 48-well plate (Nunclon delta surface, Thermo Fisher Scientific, Waltham, Massachusetts, USA) or to 24-well plate (Corning® CellBIND® Corning Inc., Corning, NY, USA). Before plating, the wells were coated with collagen IV from human placenta (Sigma-Aldrich), or surface x. The growth surface materials were performed with 5 µg/cm^2 collagen IV and either low or high concentration of surface x. Surface x coating preparations are presented in Appendix 1. The wells were incubated with the collagen IV coating solution at +4 °C at least overnight. Just before plating cells, coating solutions were aspirated and EBs plated in commercial CnT-30

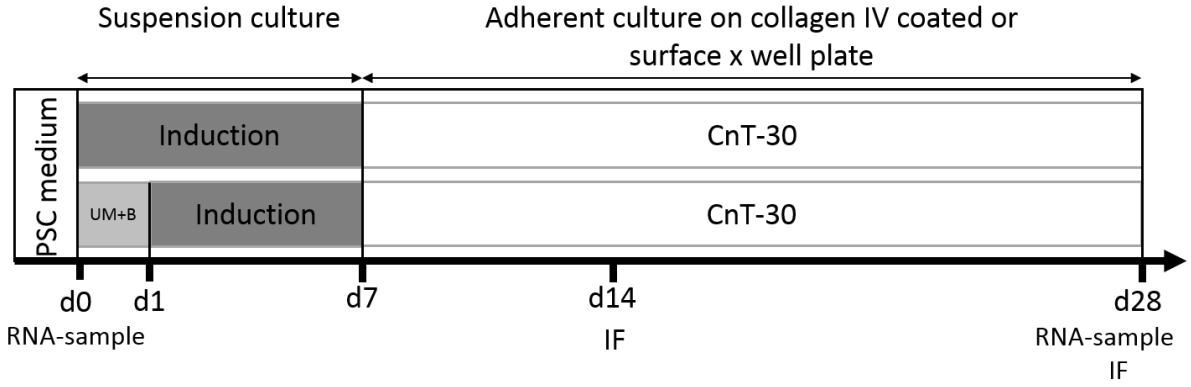

***Figure 3**. Outline of the study.*
The culture conditions, duration for each stage and main analyses are shown. Upper represents mechanical dissociated cells and lower enzymatic. Abbreviations: PSC: pluripotent stem cell, UM+B: unsupplemented medium supplemented with blebbistatin, IF: immunofluorescence.

medium fabricated for native corneal epithelial cell culture (CellnTec Advanced Cell Systems AG, Bern, Switzerland). In two last experiments, the wells were washed twice with CnT-30 medium before plating. The prepared wells were not allowed to dry at any point.

Mechanically detached EBs were dissected to smaller pieces with sterile scalpel before plating. After aspirating the collagen IV and surface x coating solutions and adding appropriate volume of pre-warmed CnT-30 medium, the EB suspension was divided equally (1:2) to these wells. Before plating SCED-EBs, they were pooled to 50 ml Falcon tube and the induction medium was aspirated. Appropriate volume of pre-warmed CnT-30 medium according to number of prepared wells was added to SCED-EBs and the cell suspension was divided to aspirated prepared wells equally. Same amount of cells were plated to 48- and 24-well plates.

4.1.4 Adherent cell differentiation

After plating, four conditions were studied: mechanically detached EBs and SCED-EBs on collagen IV coating and on surface x (Figure 4). The different conditions were handled the same way during differentiation.

Adherent cells were cultured in CnT-30 medium, which is commercially available defined and serum-free corneal epithelium medium. The medium was changed three times a week. Medium aspiration was done under stereomicroscope to avoid cell detachment. The cultures were observed and images were acquired with Nikon phase contrast light microscope (Nikon Corp.,

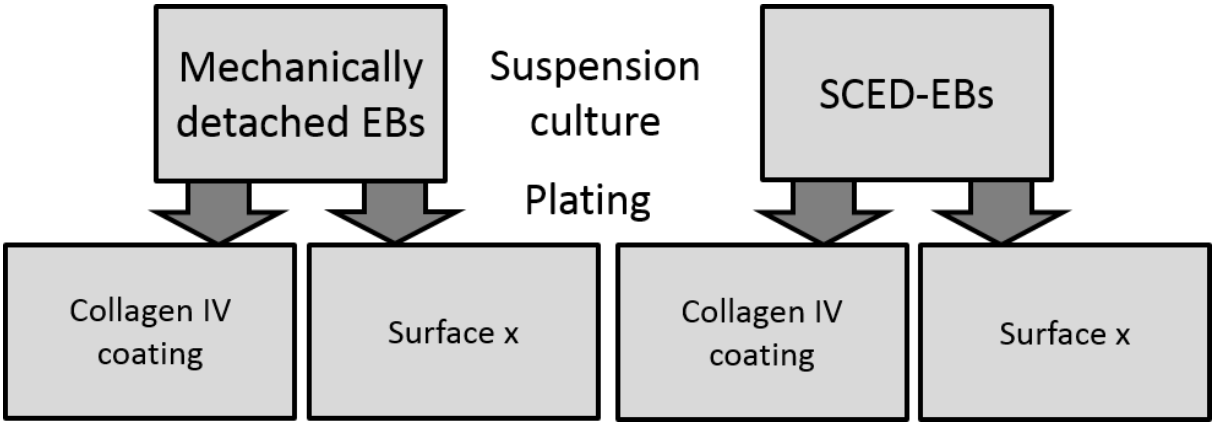

Figure 4. *Differentiation conditions.*
First, the undifferentiated hiPSCs were detached using two methods: mechanical and enzymatic dissociation. The suspension cell culture period was called induction. After induction the EBs were plated to four different conditions, two from each dissociation method. Abbreviations: EB: Embryoid body, SCED: single cell enzymatic dissociation.

Tokyo, Japan) three times a week, before changing the medium. Differentiation period was 21 days long except in one experiment it was 15 days long. Cells were characterized after 14 days of differentiation, and at the end point (d22 or d28) of the study. During the culturing, cell morphology and progression of differentiation were followed and images were taken.

4.2 RNA extraction

Total RNA was extracted from undifferentiated hiPSCs in four experiments and end point RNA-samples from differentiated cells in two experiments. The sample collection for undifferentiated hiPSCs was done by detaching colonies mechanically and transferring the detached colony pieces to 1.5 ml Eppendorf and centrifuged 1500 rpm for 4 min. The end point sample collections was done using sterile cell scraper and after detaching cells were transferred to 1.5 ml Eppendorf and centrifuged 1500 rpm for 5 min. After centrifugations, supernatants were aspirated and the cell pellets lysed with 350 µl RA1-lysis buffer (Macherey-Nagel, Düren, Germany) containing 2-mercaptoethanol (Sigma-Aldrich). Cells were homogenized with sterile needle and syringe by passing 5 times through it. After homogenization, samples were stored at -80 °C.

Total RNA extraction was carried out using Qiagen RNase mini Kit (Qiagen N.V., Venlo, Netherlands) following the manufacturer's protocol. The RNA samples were eluted with 30 µl of RNAse-free water. The RNA concentration of each sample was measured using NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

4.3 Cytospin

At halfway time point (d14), the differentiation analysis was done from cytospin samples. The samples were collected from one well of each condition in every experiment. In the first experiment, d14 cytospin sample was not taken and in the third experiment, d14 stainings were done directly to well plate. The cells were detached using TrypLE Select (Life technologies, Thermo scientific Fisher, Waltham, Massachusetts, USA): culture medium was aspirated and cells were rinsed twice with DPBS, 200 µl of TrypLE Select was added to each well and allowed to take effect at RT for about 2-4 min. When the cells had detached from the bottom of the well, 200 µl of UM was added and cells were filtered through a sterile 40 µm cell strainer to 50 ml Falcon tube. Cells were transferred to 1.5 ml Eppendorf tube and centrifuged at 1000 rpm for 5

min at RT. Subsequently, supernatant was aspirated and cells resuspended in 300 µl of UM. Cells were counted from 10 µl of suspension in Bürker cell counting chamber. Based on the cell number of cells they were divided to 2-4 batches which consisted 10 000-100 000 cells each. Cells were washed with DPBS once and then diluted to appropriate volume of 150 µl/sample. After that, each sample was added to cytofunnel, which was attached to a glass slide and slide carrier. The apparatus was inserted to Cellspin I (Tharmac GmbH, Waldsolms, Germany) and cytocentrifuged at 500 rpm for 5 min at RT. After centrifugation, cells in the glass slides were moistened with DPBS until continuing with immunocytochemistry.

4.4 Immunofluorescence

At time points d14 and d28, cell differentiation was assessed with immunocytochemistry. Cells were stained as cytospin samples at d14, and directly on the 48- or 24-well plates at d28. First, the samples were washed twice with DPBS and fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich) for 15 min at RT. After three washes with DPBS, cell membranes were permeabilized with 0.1 % Triton X-100 (Sigma-Aldrich) diluted in DPBS for 10 min at RT and washed three times with DPBS. Unspecific binding sites were blocked with 3 % Bovine Serum Albumin (BSA, Sigma-Aldrich), diluted in DPBS, for 1 h. After blocking, the samples were treated overnight at +4 °C with one or two primary antibodies listed in Table 2. All antibodies were diluted in 0.5 % BSA in DPBS.

Primary antibodies were localized by incubating cells with Alexa Fluor 488 and 568-conjugated secondary antibodies for 1 h protected from light. Secondary antibody solutions were prepared with one or two of secondary antibodies (Table 3) diluted in 0.5 % BSA in DPBS. The secondary antibody host species was corresponding to that of the primary antibody. Before and after secondary antibodies the samples were washed with DPBS three times for 5 min.

After washes, the samples were mounted using VectaShield (Vector Laboratories Inc., Burlingame, CA, USA), a mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI), to counter-stain cell nuclei, and covered with cover slips.

All stainings were visualized with Olympus IX51 Fluorescence Microscope (Olympus, Tokyo, Japan) using 10x or 20x objective. All images were edited using Adobe Photoshop CS4 and ImageJ. The images of cytospin samples were analyzed and the protein expression results were

used to calculate the number of positive cells. The images of end point samples were used to see how much cells there were in certain condition and to calculate the amount of positive cells.

Table 2. *Used primary antibodies.*
Abbreviations: ABCG2: ATP-binding cassette sub-family G member 2, CK: Cytokeratin, PAX6: Paired box gene-6.

Antibody	***Dilutions***	***Host species***	***Manufacturer***
anti-ABCG2 (IgG)	1:200	mouse	Merck Millipore, Darmstadt, Germany
anti-CK12 (IgG)	1:200	goat	Santa Cruz Biotechnology, Inc., Dallas, Texas, USA
anti-CK15 (IgG)	1:200	mouse	Thermo Fisher Scientific, Waltham, Massachusetts, USA
anti-CK3 (IgG)	1:300	mouse	Abcam plc., Cambridge, UK
anti-Ki67 (IgG)	1:500	rabbit	Merck Millipore, Darmstadt, Germany
anti-p63 (IgG)	1:200	rabbit	Cell Signaling Technology, Danvers, Massachusetts. USA
anti-PAX6 (IgG)	1:200	rabbit	Sigma-Aldrich, St. Louis, Missouri, USA

Table 3. *Used secondary antibodies.*
Abbreviations: A568: Alexa Fluor 568, A488: Alexa Fluor 488.

Antibody	***Dilutions***	***Host species***	***Manufacturer***
Anti-goat A568	1:800	donkey	Molecular probes, Thermo Fisher Scientific, Waltham, Massachusetts, USA
Anti-mouse A488	1:800	donkey	
Anti-mouse A568	1:800	donkey	
Anti-rabbit A488	1:800	donkey	

5. RESULTS

5.1 CELL MORPHOLOGY

5.1.1. Induction

Undifferentiated hiPSCs were detached from feeder cells using two methods: mechanical or enzymatic dissociation and the resulting EBs were cultured in induction medium. During induction, the EBs were observed and imaged with phase contrast microscope. The SCED-EBs were smaller in size and fewer in number in the first experiment compared to the following studies using smaller well plate, in which the size remained more constant between experiments (Figures 5 and 6). With time, the EBs tended to cluster and fuse in both dissociation methods.

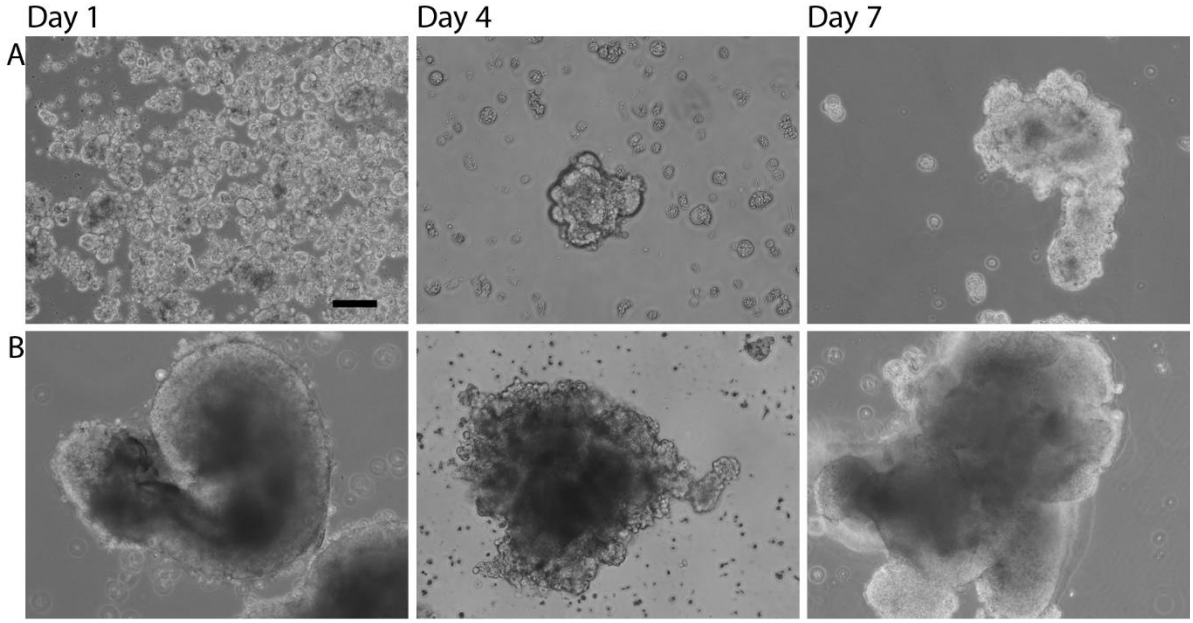

Figure 5. *Embryoid bodies (EBs) during induction stage in experiment 1.*
EB formation in suspension cultured in 6-well plate in induction medium. A: enzymatic dissociation, B: mechanical dissociation. Magnification 10x, scale bar 100 µm.

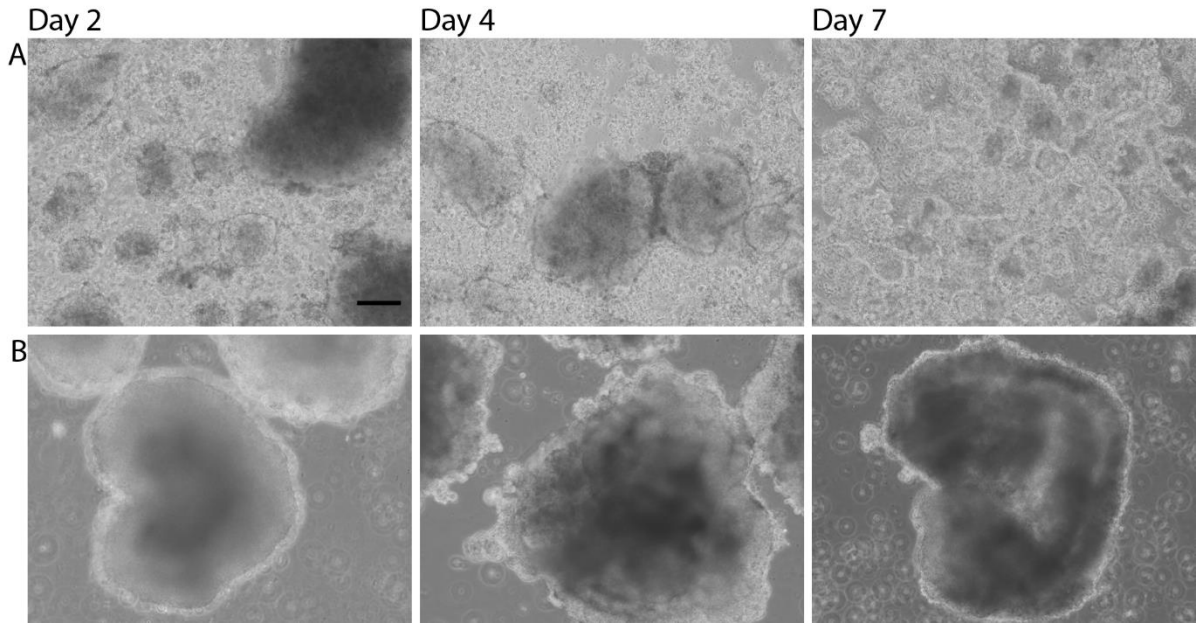

Figure 6. *Embryoid bodies (EBs) during induction period in experiment 6.*
EB formation in suspension culture in 24-well plate in induction medium. A: enzymatically dissociated EBs, B: mechanically dissociated EBs. Magnification 10x, scale bar 100 µm.

5.1.2 Adherent cell culture

After seven-day induction period the EBs were plated into two different conditions: collagen IV coating or surface x into 48- or 24- well plate. Mechanically dissociated EBs were dissected into smaller pieces for plating and the amount of plated pieces varied between 10 and 15 per well. SCED-EBs were plated 1:2 to prepared wells. The EBs, especially SCED-EBs, attached well to the bottom of the plate and started to grow immediately. Next day after plating, cell outgrowths started to appear around the EBs in every culture condition. Cell growth was initially faster in wells coated with collagen IV, but during the second week of adherent culture, the differences between conditions were less prominent. Cell proliferation continued until second to third week when the cultures were becoming confluent. Cell outgrowths had rounded shape and some of them had polygonal epithelial-like morphology at the beginning but at second or third week fibroblast-like cells started to appear (Figure 7). After reaching confluence, the cells started to detach and die.

In experiment 1 (Table 1), which lasted a total of 22 days, cells started to detach after 8 days of adherent culturing (Figure 8). After that, cells continued to detach constantly and in the last week only few cells remained adherent. Cell survival was slightly better in mechanically

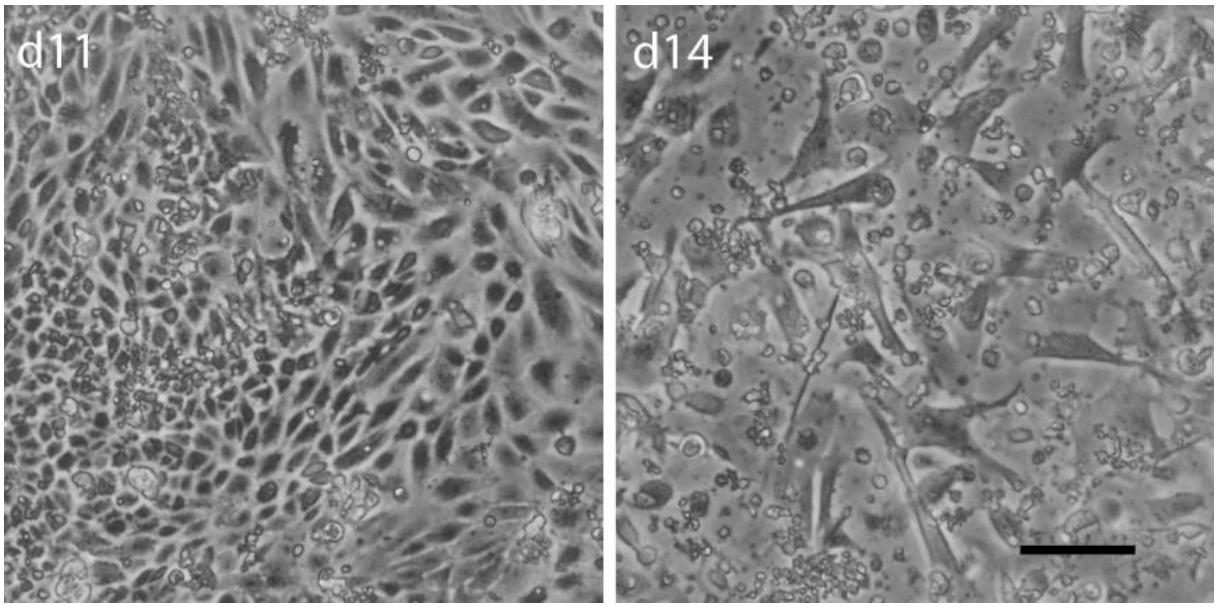

Figure 7. *Cell morphology of mechanically detached cells in adherent cell culture After 11 days (left) from initiation of the experiment, cells had round and polygonal epithelial-like morphology and at d14 (right) they had fibroblast-like morphology. Magnification 4x, scale bar 100 µm*

dissociated cells, compared to SCED-cells. In addition, there were slightly more cells in wells with surface x than in wells coated with collagen IV. At the end point, most of the cells possessed fibroblast-like morphology.

The following experiments lasted 28 days. In experiment 2, cell outgrowths appeared early and the wells were confluent at the second week of adherent culture. The cells started to detach after 10 days of adherent culturing (Figure 9) and in the end of culturing only a fraction of the cells were left. Remaining SCED-cells in collagen IV coating were the worst at the end point and the other conditions were almost equal. At the end point, among the remaining cells, there was only little fibroblast-like morphology and more polygonal epithelial morphology.

In experiments 3 and 4, the cell proliferation was similar between experiments. Cell outgrowths reached confluency during the second week and cell growth appeared faster in wells with surface x compared to collagen IV (Figure 10). Cells started to detach after 10 to 12 days of adherent culturing. In the end of these both experiments, there were varying amounts of cells still adherent but in some wells, the amount was quite promising. SCED-cells cultured in wells coated with collagen IV detached the most. Cell survival was best in mechanically detached cells. At the end point among the remaining cells, there was some polygonal morphology but there was quite a variation between technical replicates.

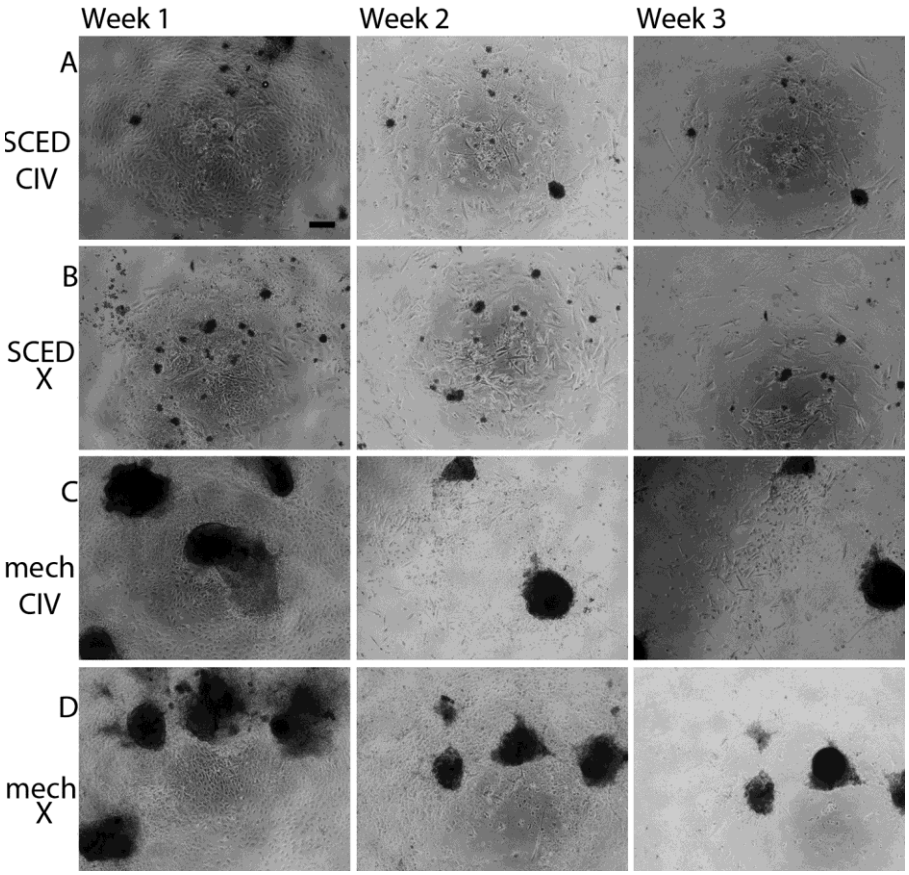

Figure 8. *Adherent CnT-30 cell culture in experiment 1 from first week to last week. Abbreviations: SCED: Enzymatically dissociated EBs, mech: mechanically dissociated EBs, CIV: collagen IV, X: surface x. Magnification 4x, scale bar 200 µm*

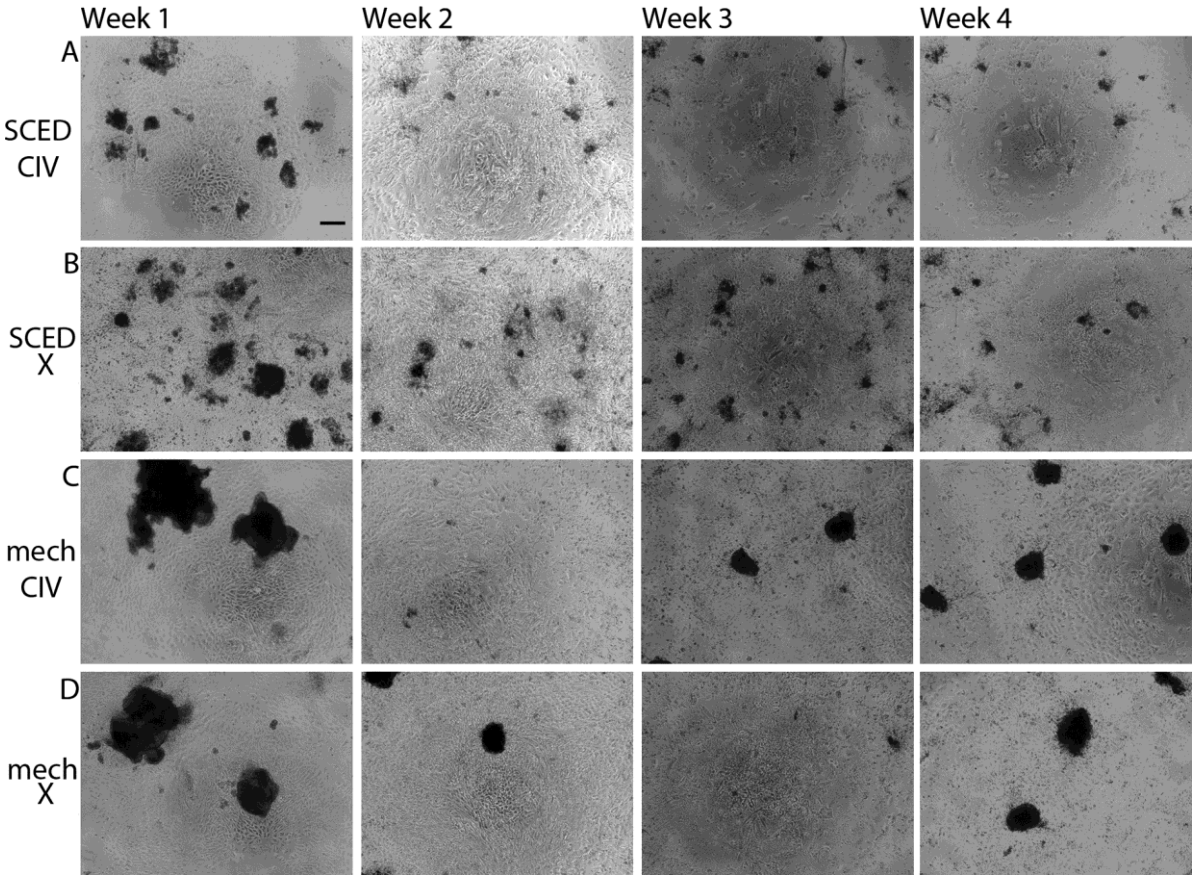

Figure 9. *Adherent CnT-30 culture in experiment 2 from first week to last week.* Magnification 4x, scale bar 200 µm.

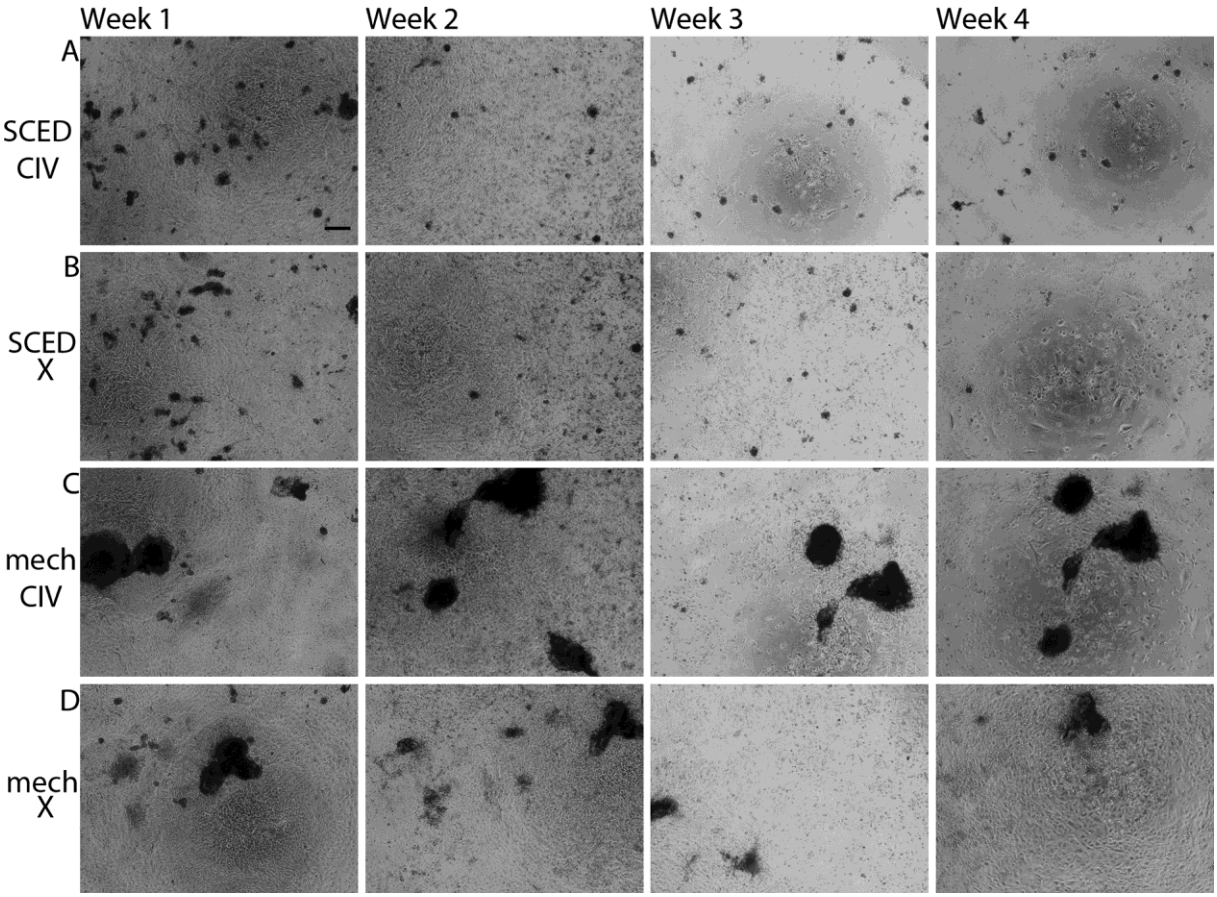

Figure 10. *Adherent CnT-30 cell culture in experiment 4 from first week to last week. Cell proliferation and detaching was similar to experiment 3. Magnification 4x, scale bar 200 µm.*

Between experiment 5 and 6, cell growth was similar. Cell outgrowths reached confluency by second week of adherent culturing and proliferation was fastest in wells with surface x (Figure 11). The amount of growing SCED-cells was greater than mechanically dissociated cells in the two last week of cell culturing. Fibroblast-like cells started to appear during the third week of adherent culture but in the end, there were more polygonal epithelial-like cells than fibroblast-like cells. There was cell detachment at the second week and it continued through the experiments but the cell loss was not so visible as in previous experiments and cell population stayed constant in these both experiments. At the end point of experiments, the cell attachment was better than in previous experiments and the best cell amount was observed in wells with surface x.

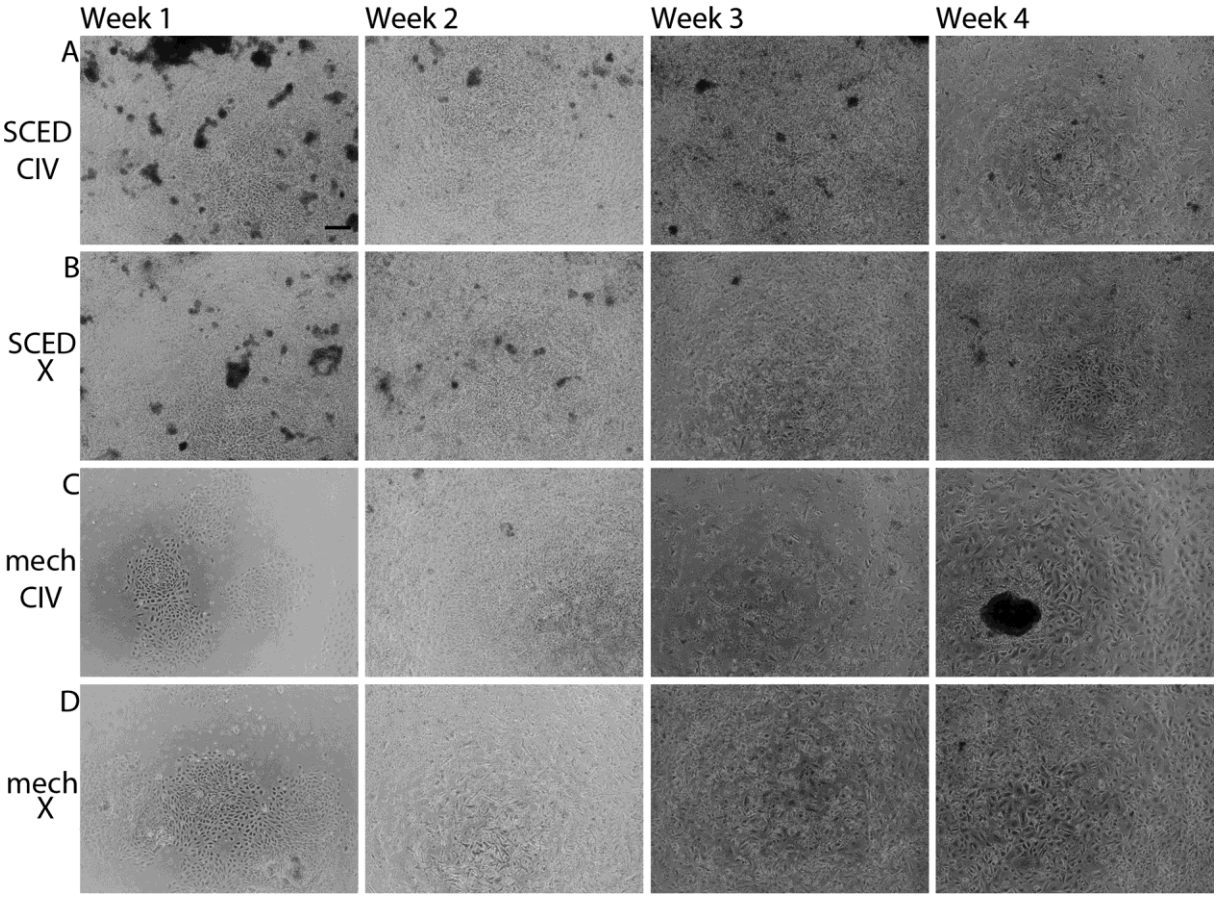

Figure 11. *Adherent CnT-30 cell culture in experiment 6 from first week to last week. Cell proliferation and detaching was similar also in experiment 5. Magnification 4x, scale bar 200 µm.*

5.3 ANALYSIS OF CELL NUMBER

The cell number was analyzed by counting the cells stained with DAPI at the end point of the study (day 22 in experiment 1, and d28 in experiments 2-6). The cell number from experiment 1 is presented in Figure 12A. Cell numbers were very low in SCED conditions, and only slightly better with mechanically dissociated cells, although there was large variation between technical replicates. The cell number was highest in mechanically dissociated cells cultured on wells with surface x. In experiment 2, SCED-cell numbers were also low and mechanically dissociated cells were still varying greatly between technical replicates (Figure 12B). Cell numbers between different conditions in experiments 3 and 4 were similar with each other but there were more cells in experiment 3, cell amounts are presented in Figure 12C and 12D. The SCED-cell number was low in both experiments and mechanically dissociated cell numbers were higher but varying between growth surface materials.

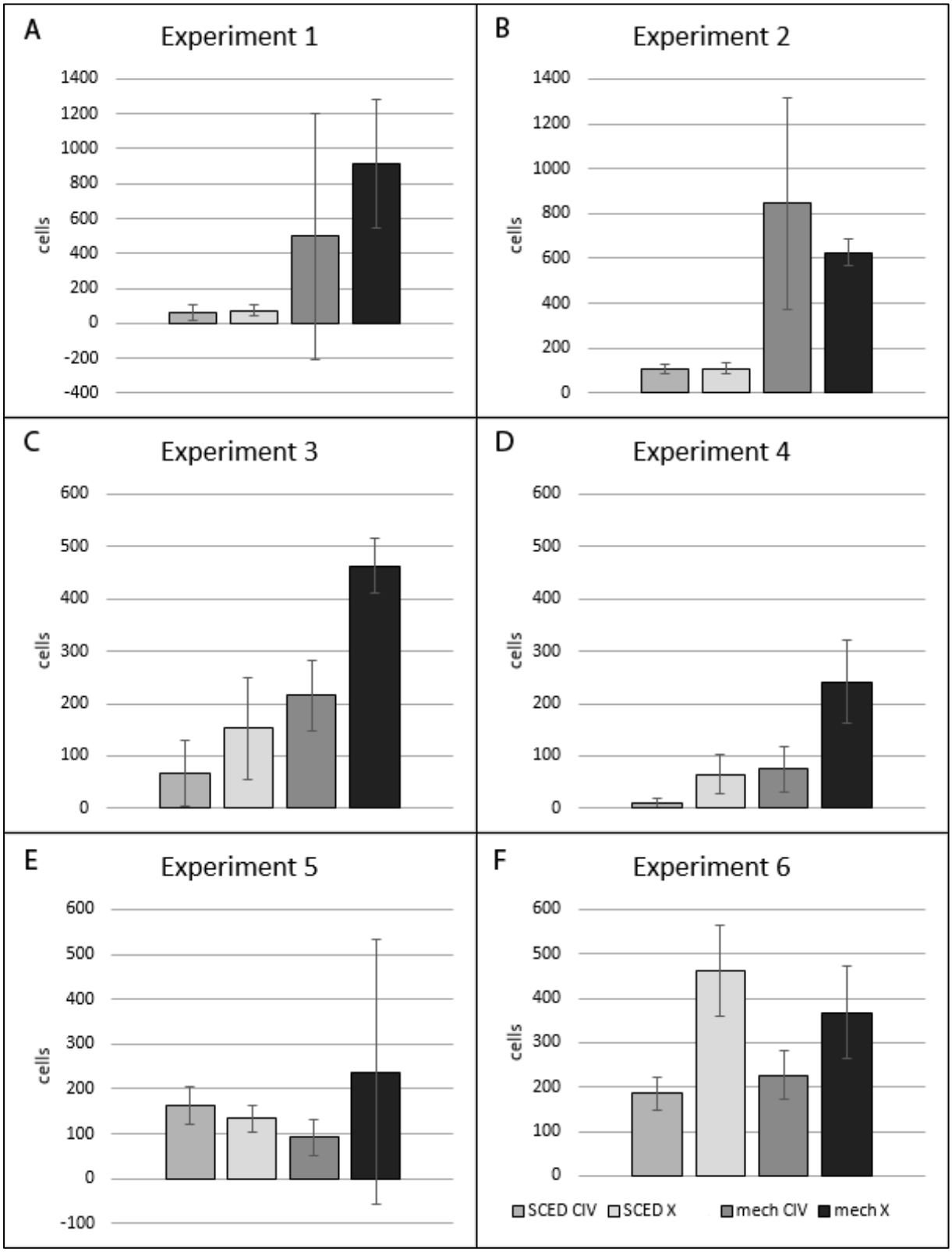

Figure 12. *The total amounts of cells in experiments 1-6, determined via nuclear staining with DAPI.*
All columns represent calculated averages between technical replicates (n=2-4), the error bars are standard deviations of the cell amounts. Abbreviations: SCED: Enzymatically dissociated EBs, mech: mechanically dissociated EBs, CIV: collagen IV, X: surface x.

In experiments 3 and 4, the cell numbers in wells with surface x were higher in both dissociation methods (Figures 12C and 12D). The difference between mechanically dissociated cells cultured on collagen IV coating, compared with surface x, was high and cell number was higher in surface x (Figure 12C and D). In experiment 4, the SCED-cell number varied much and average number was too low for further analysis (Figure 12D).

In experiment 5, SCED-cell amount was promising and more consistent between technical replicates and cell number higher compared to previous experiments. Mechanically dissociated cells cultured on wells coated with collagen IV were lower in number than SCED-cells. On the other hand, mechanically dissociated cells cultured on wells with surface x were varying in number between technical replicates (Figure 12E). In experiment 6, cell numbers were different compared to previous experiments and highest cell number was in SCED-cells cultured on surface x, although the mechanically dissociated cells cultured on the surface x also looked promising (Figure 12F). Also in this last experiment, the overall cell numbers were more consistent between conditions and technical replicates than in other experiments. In this experiment, cells cultured on collagen coating were similar in numbers, regardless of dissociation method. Overall results indicate that mechanical dissociation combined with adherent culture on surface x yielded the highest cell numbers.

5.3 CHARACTERIZATION OF DIFFERENTIATION

The cell differentiation was analyzed with immunofluorescence at d14 and d28 from initiation. The d14 analysis was done using immunostained cytospin samples obtained from dissociated cells. The d28 immunostainings were done directly to adherent cells in the well plates.

In order to evaluate the differentiation of hiPSCs with immunofluorescence, markers for early eye development, corneal epithelial progenitors, and mature corneal epithelium were used and the number of positive cells calculated. The used markers were LESC markers: p63, ABCG2, CK15, mature corneal epithelial markers: CK3, CK12, PAX6 and proliferating cell marker Ki67.

5.3.1 Marker expression at d14

The cytospin samples from d14 time point were used to calculate the amount of positive cells compared to the total cell number. The degree of cell differentiation can be estimated from these percentages. Total amount of cells was acquired by counting DAPI-stained cells, as described above.

No results were obtained at d14 from experiment 1. In experiments 2 and 3, cell numbers were not high enough to get reliable results. In experiment 4, immunostaining was done directly in the well plate and marker protein expression is presented in Figure 13. The amount of positive markers is promising; there is mainly over 50 % positive cells for PAX6, and about 30 % CK3 and CK12 positive cells in every condition. Positive cells for p63 vary between SCED and mechanically dissociated cells and there are over 30 % positive cells for mechanical cell and below 20 % for SCED-cells.

In experiment 5, the positivity varied between samples (Figure 14). In experiment 5, no results were obtained for PAX6 and CK12 staining from mechanically dissociated cells cultured

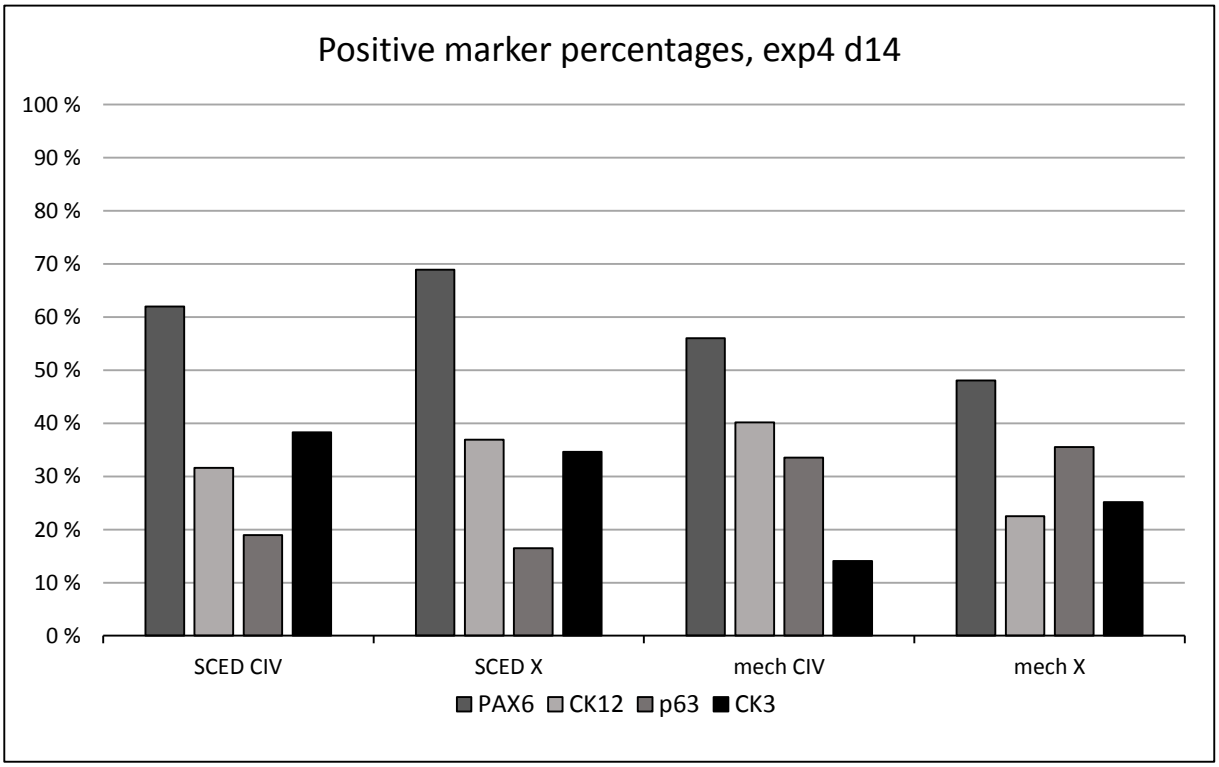

***Figure 13**. Protein expression of key markers at d14 in experiment 4.*
Expression in different culture conditions was evaluated by comparing positively stained cells to the total cell number.

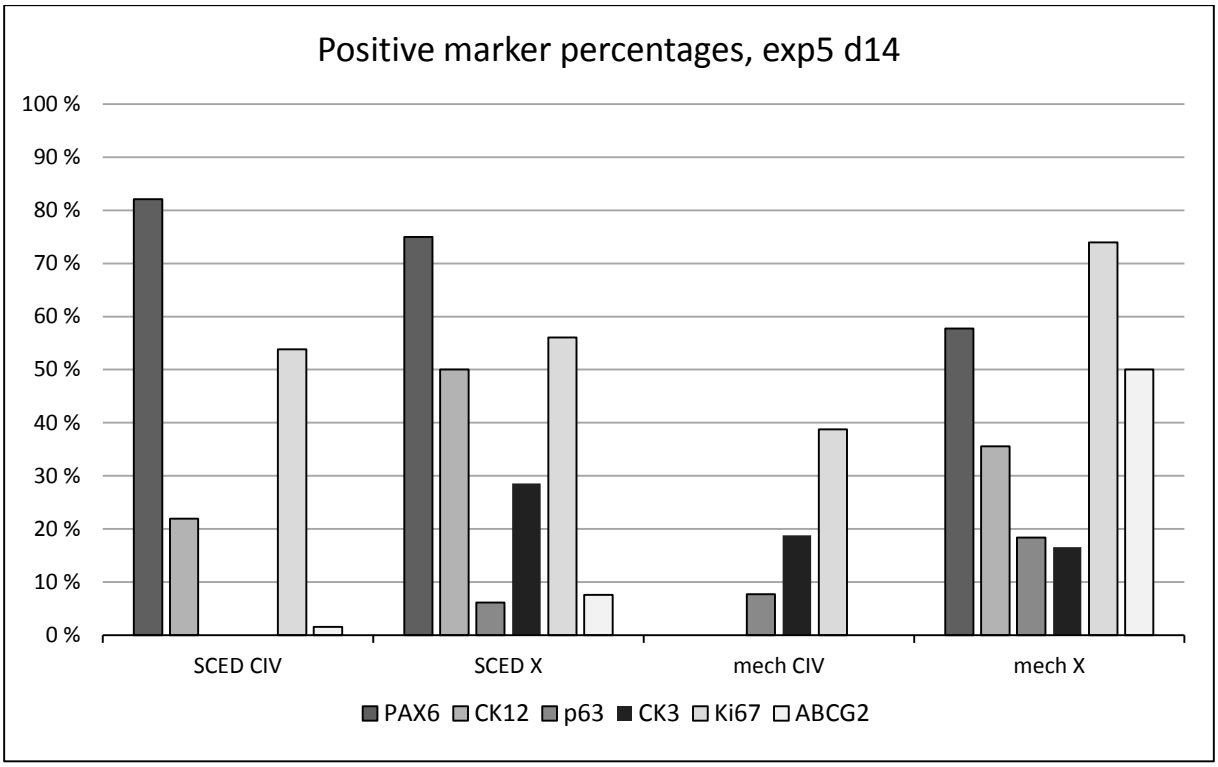

Figure 14*. Protein expression of key markers at d14 cytospin sample in experiment 5. Expression in different culture conditions was evaluated by comparing positively stained cells to the total cell number. PAX6 and CK12 stainings were not performed for mechanically dissociated cells on CIV coating.*

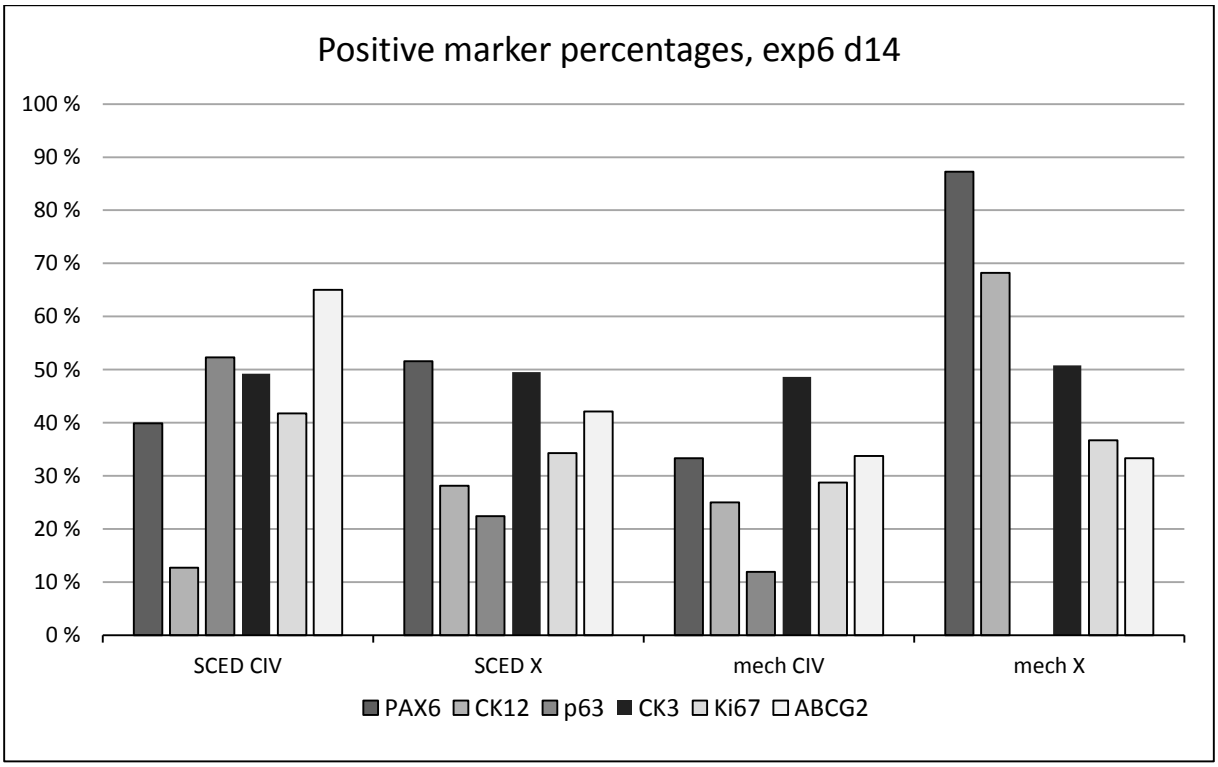

Figure 15*. Protein expression of key markers at d14 cytospin sample in experiment 6. Expression different culture conditions was evaluated by comparing positively stained cells to the total cell number.*

on wells coated with collagen IV due to low cell numbers. Cytospin samples had over 50 % positive cells for markers PAX6 and Ki67 in every studied condition. Other markers had varying expression, mainly lower than 30 %, except for CK12 in cells cultured on surface x; where over 30 % of cells expressed CK12. In this stage of experiment 5, some of the cells seem to be proliferating and in differentiated stage towards corneal epithelium based on the protein expression.

The results from experiment 6 are presented in Figure 15. In this experiment, the cytospin sample results are slightly different from experiment 5 but there are still some similarities. In every culture condition, PAX6 is expressed in about 40 % of cells. As an exception, almost 90 % of mechanically dissociated cells cultured on surface x express PAX6. Similarly, CK12 was expressed by an average of 20 % of cells in all conditions except mechanically dissociated cells cultured on surface x, where CK12 was expressed in about 70 % of cells. CK3, Ki67 and ABCG2 have almost constant positive percentages average 50 %, 30 % and 40 % in every culture condition.

5.3.2 Marker expression at d28

At the end of each experiment, differentiation was analyzed with immunofluorescence. Expression of marker protein is represented in charts and later with immunofluorescent images to visualize the results. In experiments 1-4, the number of the positive cells could not be calculated reliably due to low cell numbers and high variation between technical replicates.

In experiments 5 and 6, total cell numbers were higher and the marker expressions could be calculated. The percentages of positive cells from experiments 5 and 6 are presented in Figure 16. In experiment 5, there are over 20 % p63 and over 70 % ABCG2 positive cells in SCED-cells. In mechanically dissociated cells the positive cells for p63 were under 15 % and for ABCG2 lowest was under 70 %. Overall, in experiment 5, the expression of all studied proteins were high. In every cell condition, there are high numbers of cells positive for PAX6 (average 90 %), CK12 (average 90 %) and ABCG2 (average 75 %). There are also almost constant number of positive cells for CK3 and Ki67 (average 50 %). The CK15 stain was not performed to mechanically dissociated cells so it has positive results only from SCED-cells.

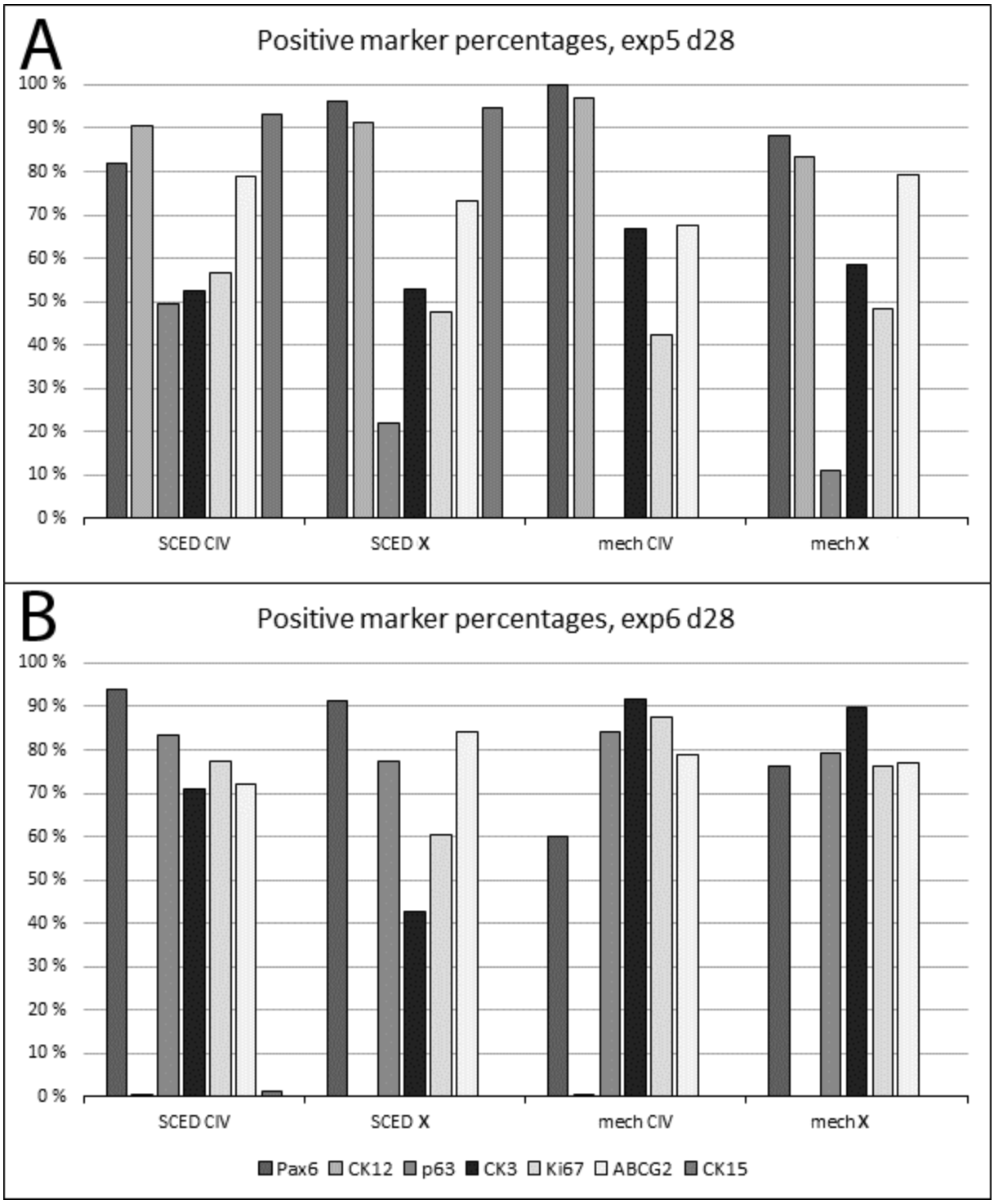

Figure 16. *Protein expression of key markers at d28 in experiment 5 (A) and 6 (B). CK15 stainings were not performed for mechanically dissociated cells*

In experiment 6, there are more varying amounts of positive cells but there are still both LESC markers and mature corneal epithelial markers expressed (Figure 16B). In d28 immunostainings from experiment 6, the results are also slightly different from experiment 5, but there are similarities with the amounts of ABCG2 and CK3 positive cells. The biggest difference is that in experiment 6, there were almost no CK12 or CK15 positive cells but p63 is highly positive in all conditions (average 80 %).

From immunofluorescence images, the success of stainings can be seen well and compared with each other. The above-mentioned percentages were calculated from these immunofluorescence images for experiments 5 and 6 (Figures 17 and 18). Appropriate localization of each protein can also be seen from immunofluorescence images.

The expression of marker proteins of p63, CK3, PAX6 and CK12 obtained in experiment 5 are presented in Figure 17. The same immunostainings as in experiment 5, of experiment 6 are presented in Figure 18. From Figures 17 and 18 can also be seen that there are more cells in wells with surface x than in wells coated with collagen IV.

5.4 ANALYSIS OF GENE EXPRESSION

Gene expression was meant to be analyzed using quantitative polymerase chain reaction (qPCR). The undifferentiated hiPSC samples were collected from experiments 2, 3, 5 and 6 and differentiated end point cell samples from experiments 5 and 6. However, amounts of total RNA obtained from the cell samples was too low for cDNA synthesis. Therefore, the qPCR could not be performed.

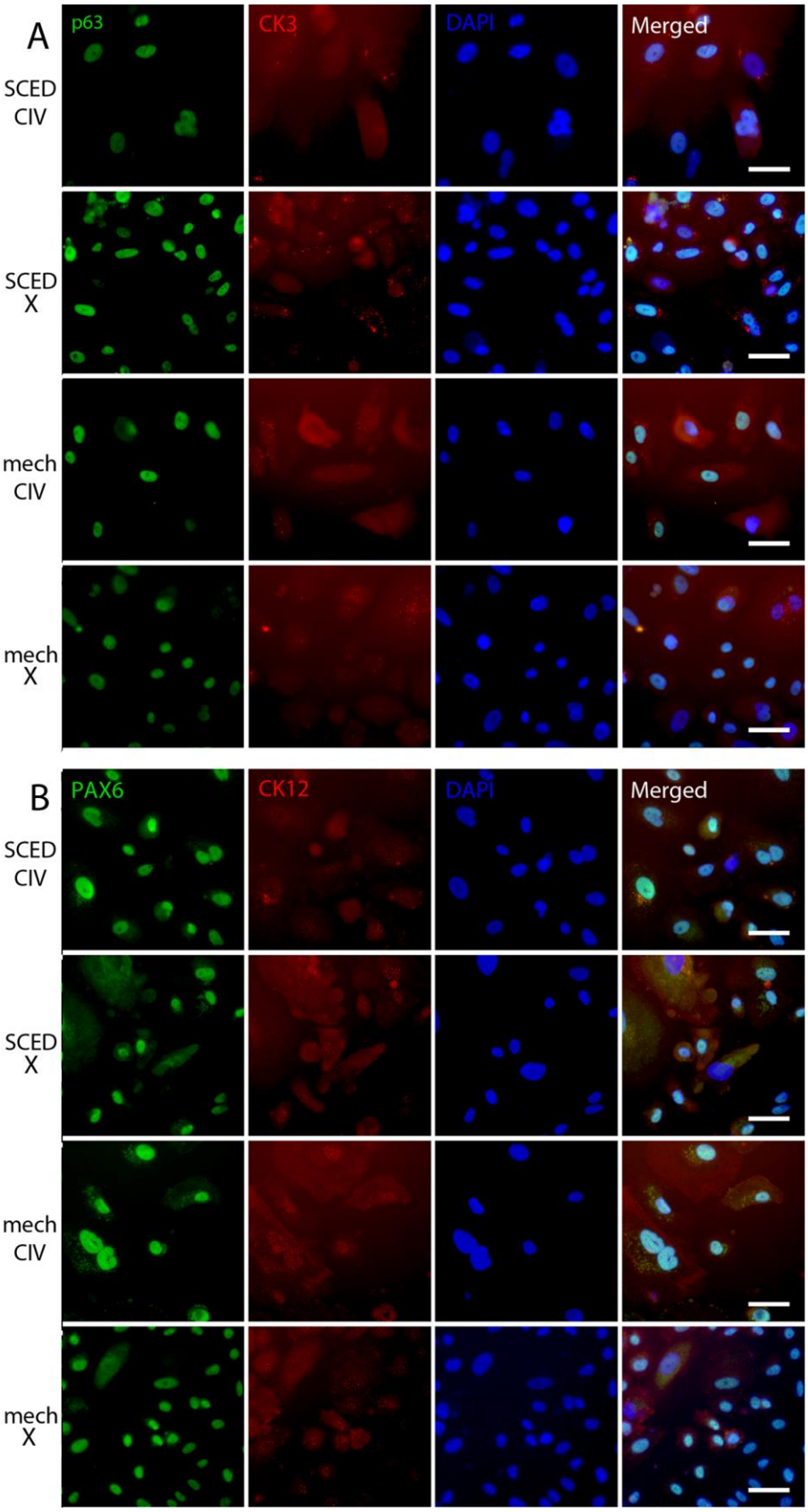

Figure 17. *Protein expression and localization at d28 in experiment 5. Immunofluorescence results for markers A: p63 (green), CK3 (red) and DAPI (blue), B: PAX6 (green), CK 12 (red) and DAPI (blue). Magnification 20x, scale bar 50 µm.*

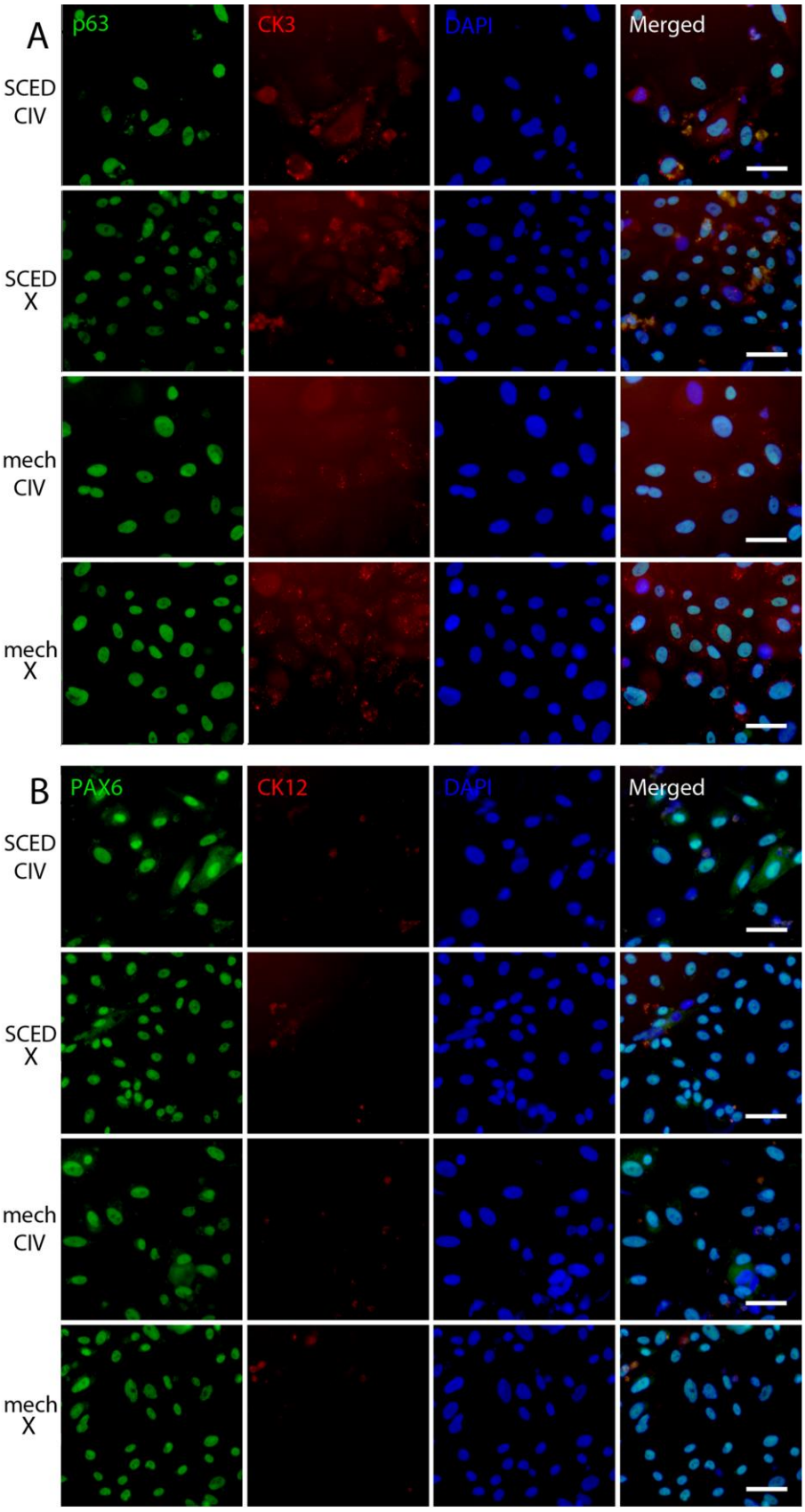

Figure 18. *Protein expression and localization at d28 in experiment 6. Immunofluorescence results for markers A: p63 (green), CK3 (red) and DAPI (blue), B: PAX6 (green), CK 12 (red) and DAPI (blue). Magnification 20x, scale bar 50 µm.*

6. DISCUSSION

The aim of this study was to optimize corneal epithelial differentiation of hiPSCs to improve the differentiation protocol. Two cell dissociation methods were tested: the standard mechanical dissection method, used by Mikhailova et al. (2014), and SCED-method with aid of blebbistatin. The differentiation protocol was tested six times and some parameters varied between experiments. The changes were minor in order to keep the experiments comparable. Through the whole study, both dissociation methods were used and compared.

The analyses for gained results were done only with qualitative immunofluorescence because cell samples did not yield enough RNA to perform quantitative PCR analysis. However, from the immunofluorescence stainings cell number and the amount of the positive cells could be calculated and quantified. The immunofluorescence was performed to assess protein expression of several putative LESC markers as well as terminally differentiated corneal epithelial markers.

6.1 EVALUATION OF CELL ATTACHMENT AND CELL NUMBER

In this study, cell culturing consisted of two main stages: surface ectoderm induction culture and corneal epithelium differentiation culture. In order to simplify and speed up the differentiation method, the already existing corneal epithelial differentiation method was optimized in this study by testing SCED-method. The induction culture was done in suspension and corneal epithelium differentiation culture was adherent cell culture. The aim of induction was to cultivate cells in inhibitor and growth factor supplemented medium to mimic corneal development and initiate cell differentiation in seven days. In the adherent cell culture, cells were maintained in commercial corneal epithelium medium CnT-30 and this stage lasted three weeks. One aim of this study was to be able to control the cell numbers better in induction culture and later in adherent culture. The optimization of these stages was done by altering culture conditions between experiments and evaluating cell growth and morphology with light microscopy. One important aspect was also to see does the surface x enhance differentiation better than collagen IV.

6.1.1 Induction of cell differentiation

The experiments were initiated by detaching undifferentiated hiPSCs from feeder cells, either on Monday or on Thursday. For the most part, hiPSC colonies showed undifferentiated colony morphology with well-defined edges. However, it seemed that their quality was better when the initiation was on Thursdays. The good quality of the undifferentiated hiPSCs is highly important for cells to differentiate and study to succeed. The cells had better quality on Thursdays due to favorable cell splitting time because weekend, when the cells are not fed, is not close to initiation of experiments. When dissecting the colonies mechanically, the differentiated parts in the middle of the stem cell colonies were removed and only the undifferentiated areas were detached.

In SCED, these possible differentiated areas in the colonies were not removed beforehand. The detaching was done using the marine-origin enzyme Accutase as cell dissociation reagent. Because enzymatic dissociation is rougher to the cells than mechanical dissociation and SCED-cells cannot form EBs from single-cell suspension spontaneously, blebbistatin was used to improve their survival. In the case of mechanical dissociation, the pieces were transferred to well plate containing induction medium where the pieces would form EBs spontaneously. The amount of transferred pieces was even between experiments. In SCED, the amount of the dissociated cells was counted and 300 000 cells transferred to UM supplemented with blebbistatin for 24h.

Blebbistatin is non-muscle myosin II inhibitor and it has been shown to improve the survival of single cells in suspension conditions (Walker et al., 2010). In this study, blebbistatin was used to enhance the formation and survival of SCED-EBs. SCED-EBs survived well in 24-well plate and their amount remained approximately constant throughout the induction stage in different experiments, although some of the EBs clustered together (Figure 6A). In 6-well plate, the SCED-EBs seemed to remain smaller and their survival was poorer than in smaller well size. It seemed that SCED-EBs survival was better with smaller well size when EBs lay more compactly and are able to cluster with each other.

After blebbistatin treatment, SCED-EBs were also cultivated in induction medium like mechanically dissociated EBs, until seventh day after initiation of differentiation. The medium was changed three times a week but weekends caused two-day gaps in the induction. Induction

medium contained bFGF, which is critical factor for stem cell maintenance and differentiation but its levels decrease after few hours of cell culture because of its weak stability (Lotz et al., 2013). Therefore, even with daily medium changes the bFGF levels cannot be maintained constant. New methods to keep bFGF levels high for example, using microspheres with controlled release have been reported (Lotz et al., 2013). This may explain why experiments initiated on a Monday were generally more successful, likely due to the more stable levels of bFGF. On the other hand, experiments initiated on a Thursday had the two-day gap between medium changes due to weekend early on during the induction stage.

6.1.2 Cell plating and adherent cell culture

After induction stage, the EBs were plated to either 48- or 24-well plate. At first, the 48-well plates were used but in two last experiments, CellBind-treated 24-well plates were used, in attempts to improve cell attachment. The well plates were coated with collagen IV or surface x Collagen IV is natural part of the microenvironment of limbal basement membrane (Wan et al., 2015). It has been suggested, that LESCs bind to collagen IV and that it would induce stem cells to differentiate into epithelial lineage cells (Mei et al., 2012). Surface x is believed to have properties to support cell attachment and in maintaining LECSs. The EBs were transferred to adherent cell culture in CnT-30 medium for 21 days. The most crucial points for cell survival seemed to be at the second week of adherent cell culture.

The well plates were prepared with two different concentration of surface x: low and high. The higher concentration of surface x resulted in better cell attachment. The amount of collagen was same that Mikhailova et al., (2014) had used. The SCED-EBs attached better than mechanically dissociated EBs, which partly detached in few first days during medium changing. In wells coated with collagen IV, the cell outgrowths started to appear faster after plating than in wells with surface x. However, during the second week of adherent culture cell growth seemed comparable between collagen IV and surface x. Thus, it seems that it takes somewhat longer for cells on wells with surface x to grow, but after a certain point their growth accelerates. Overall, surface x gave better cell numbers than collagen IV coating.

At the second week of differentiation culture, when the wells started to reach confluency, the cells started to detach and die. It is possible that cells reached confluence too early and did not have enough space to grow and because of that, they started to die. The results from two last

experiments where the used well size was bigger support these conclusions because the cells stayed adherent longer and cell death was not so severe. It is also possible that the material of the well plates affected cell attachment because the 48-well plates had so called Delta surface, and 24-well plates were especially plasma treated CellBind plates. Because CellBind well plates are manufactured especially for optimal cell attachment, while Delta surface plates are not said to have any special properties to improve cell attachment. The amount of plated cells was the same with 48- and 24-well plate; only the surface area was different. To optimize this further, the plated cell amount would have to be even smaller so that cells could differentiate more before reaching confluency.

6.1.3 Cell number

Along the experiment, cells were monitored using light microscopy before medium change to follow cell morphology and growth. This analysis was done to see which one of the dissociation methods was better and which surface worked better for cell attachment and growth. The cell number was analyzed at the end point of the study with immunofluorescent DAPI stain.

In the four first experiments, the cell numbers were highly variable between experiments and there was usually more SCED-cells than mechanically dissociated cells present in the wells (Figures 12A-D). In those experiments, it seemed that cells originated from SCED-EBs were under stress or they reached confluence too early and started to die due to contact inhibition. In the first experiment, cells did not reach confluency like in following experiments and it seems that the number and inadequate formation of SCED-EBs could be the most reasonable causes for the results. In the first experiment even the mechanically dissociated cells were in poor condition even though their amount was mostly good (Figure 12A). It is possible, that the first experiment did not succeed because it was the first time doing the experiment and the techniques were new. In addition, the enzymatic dissociation could have been too harsh because in the first experiment the sufficient dissociation time for Accutase was also tested. In the second experiment, results were mostly similar to first experiment but SCED-cell number was better in this experiment (Figure 12B). There was not much variation between collagen IV and surface x.

In experiments 3 and 4, the SCED-cell numbers were low and mechanically dissociated cell number higher and cell numbers in wells with surface x were better. In experiment 5 the amount

of mechanically dissociated cells cultured on wells coated with collagen IV was the lowest and mechanically dissociated cells cultured on wells with surface x had the greatest amount (Figure 12E). In experiment 6, the results were the most consistent of all experiments (Figure 12F). The highest cell number was in SCED-cells cultured on wells with surface x.

As summary, it can be said that when the culture conditions were optimal for the well size and growth surface material, as in two last experiments, the cell number was best with surface x. Therefore, it seems that surface x somehow stabilizes the ECM coating for cells so that they will grow better. Collagen is already known to be important for LESC adhesion (Li et al., 2005) but effects of surface x have not been reported before. The composition of surface x could be further optimized, possibly slightly higher concentration of both would be even better.

6.2 EVALUATION OF CELL DIFFERENTIATION

Protein expression of several key markers was quantified at two time-points (d14 and d28) using immunofluorescence, to assess the degree of cell differentiation towards corneal epithelium between different conditions and coatings. The differentiation could also be assessed by following cell morphology. It is possible that collagen IV and surface x induced different kind of cell growth or committed them towards different direction. Mei et al. (2012) introduced that collagen IV induces embryonic stem cell differentiation towards epithelial cells and limbal stem cells tend to bind to it. However, same kind of data have not been found of surface x.

In the first three experiments, the cell amounts were so low that the marker expression could not have been calculated reliably, and therefore the expression results from them were excluded. In experiment 4 at d14, the cell amount was more reliable and the expression could be calculated. However, the cells did not survive to end of the experiment. Consequently, the results from experiments 5 and 6 were the ones analyzed for protein expression also at d28.

For these d14 results from experiment 4, it appears that the rate of corneal epithelial differentiation has been affected in the cells, because the LESC marker p63 is expressed at a lower number than the markers of mature corneal epithelium cytokeratins 3 and 12. There is more p63 and less CK3 positive cells in mechanically dissociated cells than in SCED-cells.

In experiment 5 positive marker percentages from cytospin samples at d14 are more varying than at d28. At d28, LESC marker p63 is expressed at higher numbers in SCED-cells than mechanically dissociated cells. ABCG2, another LESC marker, is also expressed at high numbers. These results suggest that corneal epithelial differentiation with SCED-cells have been more efficient. However, there are also markers for more mature cells expressed in every studied condition. Therefore, in this stage, it seems that the mechanically dissociated cells have matured bit more than SCED-cells but there is high corneal epithelial differentiation markers present in all cell condition.

In d28 immunostainings from experiment 6, the results are also slightly different than in experiment 5 but there is similarities with the amounts of ABCG2 (over 70 %) and CK3 (over 70 %, except in one condition over 40 %) positive cells. The lack of positive cells for markers CK12 and CK15 could be due to unsuccessful staining or just that there is only a few of cells that are positive to them. It could be possible that growing and differentiating cells are in different stage than in experiment 5 and cells positive for those markers are not there. There is many Ki67 positive cells in every culture condition but few more in mechanically dissociated cells, which indicated that there is more proliferating cells present in mechanically dissociated cells. Overall, this experiment suggests that in conditions optimal for corneal epithelial differentiation, the results between SCED and mechanically dissociated cells are similar. However, there is fewer cells positive for CK3 in SCED-cells but more PAX6 positive cells in mechanically dissociated cells. In surface x if both dissociation methods had more CK12, CK3 and Ki67 positive cells.

In summary, the results from d28 of the experiments 5 and 6 imply that the cells have partly differentiated so that their commitment for corneal epithelial differentiation, due to protein expression of PAX6, CK12 and CK3, is clear but they still have stem cell-like properties and are positive for LESC markers p63 and ABCG2. However, because CK3 or CK12 are positive in both experiments cells cannot be considered clearly LESCs anymore (He and Yiu, 2014). Although so far no specific LESC markers have been identified, the co-localization of several markers could be considered reliable so that it could be said that the cells are growing and differentiating into right direction. When comparing the effects of different dissociation methods it could be said that SCED-method was comparable to mechanical dissociation and could be used for routine LESC differentiation. It seemed that the surface x improved cell

attachment and therefore could have positive effects to hPSC differentiation towards corneal epithelial cells.

7. CONCLUSIONS

Differentiation of hPSCs towards corneal epithelial cells is challenging, as previously published studies suggest. Therefore, the aim of this study was to optimize the differentiation to make it more standardized. At first it seemed that cultured hiPSC died in the third week after initiation of differentiation. The immunofluorescence results were unreliable in the first experiments because there were not much positive cells or cells in general. However, when the cell density in well plate and well plate's coating material concentration was changed the results were more promising. With the six repeats, which were done in this study, it is clear that hiPSCs are fragile cells and many factors affect the success of their differentiation.

Overall, three main aspects were gained from this study. First of all, the enzymatic dissociation method, SCED, was a successful method for initiating the differentiation of hiPSCs as EBs. Typically, the differentiation is initiated by mechanically dissecting the undifferentiated hPSC colonies from feeder cells. On the other hand, if the dissociation is done using enzymatic dissociation methods that have been considerably rough, the EB formation has been practically impossible. In this study, Accutase in combination with blebbistatin was used to enable the EB formation and it seemed to work well and greatly enhance the EB survival. Mechanical dissociation method is not so accurate because the dissected pieces are not equal in size and cells cannot be counted, so it would be truly useful if the dissociation could be done enzymatically when the cell amount is controllable. In this study, the used enzymatic dissociation reagent Accutase does not contain mammalian or bacterial derived products by information provided by manufacturer. Manufacturers also say that Accutase is more gentle dissociation method, which does not harm cells, unlike trypsin or other reagents used for dissociation. Therefore, when using this enzymatic dissociation method with Accutase the results were promising and it worked well as a method. There were no big differences in the marker protein expressions between different dissociation methods, but SCED-cells had partially higher expression for LECS markers and lower for mature corneal epithelium markers.

Secondly, the size of EBs affected their attachment to the ECM coatings. The smaller SCED-EBs attached better than the larger and unevenly sized mechanically dissociated EBs. In addition, during medium changes in adherent cell culture, the smaller SCED-EBs remained attached while the bigger mechanically dissociated EBs detached easily.

Finally, the two different surfaces, collagen IV coating and surface x were compared. Surface x improved cell attachment. Surface x concentration was raised after two experiment but collagen IV concentration was kept constant. It seems that cells on collagen IV coating grew faster at first compared to cells on surface x. However, the cells cultured on surface x reached the growing rate at second week of adherent cell culturing. After that, the cells on surface x grew faster to confluence and stayed adherent better than collagen IV coated cells. However, it seems that surface x did not have much effect to cell differentiation. Further studies are needed to optimize the differentiation method more regarding to induction period, surface material concentrations and plating conditions.

REFERENCES

Ahmad S. Concise review: limbal stem cell deficiency, dysfunction, and distress. Stem Cells Transl Med 2012;1:110-5.

Ahmad S, Kolli S, Lako M, et al. Stem cell therapies for ocular surface disease. Drug Discov Today 2010;15:306-13.

Ahmad S, Stewart R, Yung S, et al. Differentiation of human embryonic stem cells into corneal epithelial-like cells by in vitro replication of the corneal epithelial stem cell niche. Stem Cells 2007;25:1145-55.

Baylis O, Figueiredo F, Henein C, et al. 13 Years of Cultured Limbal Epithelial Cell Therapy: a Review of the Outcomes. J Cell Biochem 2011;112:993-1002.

Casaroli-Marano RP, Nieto-Nicolau N, Martinez-Conesa EM, et al. Potential Role of Induced Pluripotent Stem Cells (IPSCs) for Cell-Based Therapy of the Ocular Surface. J Clin Med 2015;4:318-42.

Castro-Munozledo F. Review: corneal epithelial stem cells, their niche and wound healing. Mol Vis 2013;19:1600-13.

Collomb E, Yang Y, Foriel S, et al. The corneal epithelium and lens develop independently from a common pool of precursors. Dev Dyn 2013;242:401-13.

DaCosta Byfield S, Major C, Laping NJ, et al. SB-505124 is a selective inhibitor of transforming growth factor-beta type I receptors ALK4, ALK5, and ALK7. Mol Pharmacol 2004;65:744-52.

Daniels JT, Dart JKG, Tuft SJ, et al. Corneal stem cells in review. Wound Repair and Regeneration 2001;9:483-94.

Davies SB, Di Girolamo N. Corneal stem cells and their origins: significance in developmental biology. Stem Cells Dev 2010;19:1651-62.

DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg 2011;37:588-98.

Dhouailly D, Pearton DJ, Michon F. The vertebrate corneal epithelium: from early specification to constant renewal. Dev Dyn 2014;243:1226-41.

Dua HS, Azuara-Blanco A. Limbal Stem Cells of the Corneal Epithelium. Surv Ophthalmol 2000;44:415-25.

Figueira EC, Di Girolamo N, Coroneo MT, et al. The phenotype of limbal epithelial stem cells. Invest Ophthalmol Vis Sci 2007;48:144-56.

Fortier LA. Stem cells: classifications, controversies, and clinical applications. Vet Surg 2005;34:415-23.

Frank MH, Frank NY. Restoring the cornea from limbal stem cells. Regen Med 2015;10:1-4.

Fuhrmann S. Wnt signaling in eye organogenesis. Organogenesis 2008;4:60-7.

Ghezzi CE, Rnjak-Kovacina J, Kaplan DL. Corneal tissue engineering: recent advances and future perspectives. Tissue Eng Part B Rev 2015;21:278-87.

Hayashi R, Ishikawa Y, Ito M, et al. Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLoS One 2012;7:e45435.

He H, Yiu SC. Stem cell-based therapy for treating limbal stem cells deficiency: A review of different strategies. Saudi J Ophthalmol 2014;28:188-94.

Holland EJ. Management of Limbal Stem Cell Deficiency: A Historical Perspective, Past, Present, and Future. Cornea 2015;34 Suppl 10:S9-S15.

Li DQ, Chen Z, Song XJ, et al. Partial enrichment of a population of human limbal epithelial cells with putative stem cell properties based on collagen type IV adhesiveness. Exp Eye Res 2005;80:581-90.

Li Y, Inoue T, Takamatsu F, et al. Differences between niche cells and limbal stromal cells in maintenance of corneal limbal stem cells. Invest Ophthalmol Vis Sci 2014;55:1453-62.

Lotz S, Goderie S, Tokas N, et al. Sustained fs of FGF2 maintain undifferentiated stem cell cultures with biweekly feeding. PLoS One 2013;8:e56289.

Majo F, Rochat A, Nicolas M, et al. Oligopotent stem cells are distributed throughout the mammalian ocular surface. Nature 2008;456:250-4.

Mei H, Gonzalez S, Deng SX. Extracellular Matrix is an Important Component of Limbal Stem Cell Niche. J Funct Biomater 2012;3:879-94.

Menzel-Severing J, Kruse FE, Schlötzer-Schrehardt U. Stem cell-based therapy for corneal epithelial reconstruction: present and future. Can J Ophthalmol 2013;48:13-21.

Mikhailova A, Ilmarinen T, Uusitalo H, et al. Small-molecule induction promotes corneal epithelial cell differentiation from human induced pluripotent stem cells. Stem Cell Reports, published in www Feb 11.

Nakamura T, Inatomi T, Sotozono C, et al. Ocular surface reconstruction using stem cell and tissue engineering. Prog Retin Eye Res 2015.

Nakamura T, Kinoshita S. New hopes and strategies for the treatment of severe ocular surface disease. Curr Opin Ophthalmol 2011;22:274-8.

Notara M, Alatza A, Gilfillan J, et al. In sickness and in health: Corneal epithelial stem cell biology, pathology and therapy. Exp Eye Res 2010;90:188-95.

Ojala M, Prajapati C, Pölönen R, et al. Mutation-Specific Phenotypes in hiPSC-Derived Cardiomyocytes Carrying Either Myosin-Binding Protein C Or α-Tropomyosin Mutation for Hypertrophic Cardiomyopathy. Stem Cells International 2015, in press.

Osei-Bempong C, Figueiredo FC, Lako M. The limbal epithelium of the eye - A review of limbal stem cell biology, disease and treatment. Bioessays 2013;35:211-9.

Ramos T, Scott D, Ahmad S. An Update on Ocular Surface Epithelial Stem Cells: Cornea and Conjunctiva. Stem Cells Int 2015;2015:601731.

Sareen D, Saghizadeh M, Ornelas L, et al. Differentiation of human limbal-derived induced pluripotent stem cells into limbal-like epithelium. Stem Cells Transl Med 2014;3:1002-12.

Schlötzer-Schrehardt U, Dietrich T, Saito K, et al. Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. Exp Eye Res 2007;85:845-60.

Schlötzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. Exp Eye Res 2005;81:247-64.

Shalom-Feuerstein R, Serror L, Aberdam E, et al. Impaired epithelial differentiation of induced pluripotent stem cells from ectodermal dysplasia-related patients is rescued by the small compound APR-246/PRIMA-1MET. Proc Natl Acad Sci U S A 2013;110:2152-6.

Shalom-Feuerstein R, Serror L, De La Forest Divonne S, et al. Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. Stem Cells 2012;30:898-909.

Skottman H. Derivation and characterization of three new human embryonic stem cell lines in Finland. In Vitro Cell Dev Biol Anim 2010;46:206-9.

Takacs L, Toth E, Berta A, et al. Stem cells of the adult cornea: from cytometric markers to therapeutic applications. Cytometry A 2009;75:54-66.

Takahashi K, Tanabe K, Ohnuki M, et al. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell 2007;131:861-72.

Vitale AM, Matigian NA, Ravishankar S, et al. Variability in the generation of induced pluripotent stem cells: importance for disease modeling. Stem Cells Transl Med 2012;1:641-50.

Walker A, Su H, Conti MA, et al. Non-muscle myosin II regulates survival threshold of pluripotent stem cells. Nat Commun 2010;1:71.

Wan PX, Wang BW, Wang ZC. Importance of the stem cell microenvironment for ophthalmological cell-based therapy. World J Stem Cells 2015;7:448-60.

Yoon JJ, Ismail S, Sherwin T. Limbal stem cells: Central concepts of corneal epithelial homeostasis. World J Stem Cells 2014;6:391-403.

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