Formation of Induced Pluripotent Stem Cells and Ocular Regenerative Medicine

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By Episomal and Viral Reprogramming

Andreas Rasmussen
Master Project
Medicine with Industrial Specialization
Department of Health Science and Technology
Aalborg University
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Writer: Andreas Rasmussen

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Vladimir Zachar, MD & Ph.D.

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Abstract

Background:
Cornea is continues renewed by the limbal epithelial stem cells (LESCs), but due to damage of the limbus, this function can be lost and can lead to limbal stem cell deficiency (LSCD). Treatments of LSCD are either by cultivated limbal epithelial transplantation or collateral transplantation of LESCs. Induced pluripotent stem cell (iPSCs) shows great potential in treating single cell defects like Parkinsons, Diabetes and LSCD.

Objective:
The current work will focus on the reprogramming of limbal fibroblasts to iPSCs by the Epi5™Episomal iPSC Reprogramming Kit (Invitrogen), and both episomal and viral plasmids obtained from Addgene.org

Methods/Results:
Results from previous experiments using the Vivid Color™ pLenti6.2-GW/EmGFP Expression Control Vector was used for optimization experiments by electroporation it into human limbal fibroblasts, resulting in two experiments, concluding that previously using a narrower cuvette could halve electroporation voltage. Optimal parameters was 125 V, 0% modulation and 10 pulses.
In addition, these results were used for the purpose of reprogramming limbal fibroblast to iPSCs, by the Epi5™ Episomal iPSC Reprogramming Kit (Invitrogen) and the episomal reprogramming vector from Addgene.org. Futhermore viral production and viral transfection of limbal fibroblasts were also tried.

Conclusion:
The current project has further optimization the electroporation setup for electroporation of limbal fibroblasts for the usage in formation of iPSCs. Plasmids obtained from Addgene.org were verified by restriction digests and electrophoreses. Viral production was done by transfecting HeLa cells with a viral plasmid and a viral packaging system and harvesting the medium. Even though cell morphology changes during the experiments, it was not succeed to form iPSCs

Preface

Dear Reader
This report was conducted by group 14gr1022 during the 9th and 10th Semester at Medicine with Industrial Specialization at the department of Health Science and Technology at Aalborg University. The report is primarily targeted for readers with familiarity with science, bioengineering and basic medical knowledge.
The main topic of this project was to try different laboratory techniques for use in future work.
The report/project was written under supervision of Professor Vladimir Zachar. I would like to thank Ole Jensen and Helle S. Møller for their guidance and flexibility through the whole period.
I have been asked to inform the reader of this project, that I am dyslexic, meaning that there may be faulty sentences.
The Nature citation style was used to handle references in to report. The Nature citation style commonly used reference method in health science. The references were done by sequential numbers raised after a sentence or a section of text, e.g. [1]. If more than one reference has been used for the same text section, the numbers are listed after each, e.g. [1 2]. The number of the reference in the text is equal to the number of the reference in the reference list

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Introduction

Epidemiology

Vision is the most important sense because more than 80% of informations from the external world are received through the eyes. In 2012, the World Health Organization (WHO) estimated that 285 million people worldwide suffer from visual impairment and 39 million people suffer from blindness [1]. Of these approximately 4% suffers from corneal blindness [2].
One example of a disease involving defects of the cornea is Limbal Stem Cell Deficiency (LSCD). In this pathological condition, depletion of the limbal stem cells occur due to chemical burns, radiation injuries or underlying disease. The consequence of this is an invasion of conjunctival epithelium into the cornea, which induces neovascularization and leads to loss of corneal transparency [3]. Sufficient treatment of LSCD would be by limbal restoration, traditionally achieved through keratoplasty and/or limbal tissue transplantation. However, both of these treatments are complicated procedures [2 4].

What is a Stem Cell

Stem cells (SCs) are functionally defined as having a high capacity to self-renew and the ability to generate differentiated cells, meaning that they can generate daughter cells identical to the mother cell and progeny with a more limited potential. Yet, this definition of stem cells only applies for embryonic or fetal stem cells that do not persist throughout the lifetime of the organism. Just as there are many different types of specialized or differentiated cells in the body, there are many different types of stem cells in the body. Hierarchically, stem cells are classified as totipotent, pluripotent, multipotent, and unipotent, referring to their ability to generate types of differentiated cells, with more distinct morphologies and gene expression [5,6]. Another parameter, in which stem cells are special, is their ability to self-renew. They are often described as immortal and unlimited, but this criterion is not be tested, because this would outlast an investigator. Investigation of somatic cells cultured in vitro shows only a finite number of doublings, due to shortening of the telomeres [7]. Like other cells, stem cells have distinct characteristics such as morphology, high nuclear-cytoplasmatic ratio, low granularity, high expression of specific stem cell markers such as Oct-4, Alkaline phosphatase, and SEAA-1 [8-10].

The Limbal Epithelial Stem Cell

The corneal epithelium is renewed throughout life by the limbal epithelial stem cells (LESCs). LESCs are believed to reside in the transition zone between the sclera and cornea, a region called the limbus [11]. This stem cell niche or microenvironment consists of cellular and extracellular components, which maintain the slow cell turnover and keeps the stem cells from differentiating during homeostasis. Nevertheless, in the event of an injury they can become highly proliferative [12]. Thoft and Friend hypothesized that corneal epithelial maintenance follows an X, Y, Z proliferation system. Where cells in the basal layer (X) and the inward centripetal migration (Y) of the limbus equals the continuous loss of cells to the surroundings (Z), which is crucial for the corneal regeneration [13]. This centripetal migration pattern was supported by clinical observations on donors after keratoplasty [14].
The *Palisades of Voigt* support the location of limbal stem cells. To increase the contact of limbal stem cells with their microenvironment, the basement membrane of the limbus undulates, which increase the surface area, giving better access to nutrients and signaling factors. In addition, the *Palisades of Voigt* are providing a protective structure for the LESCs, protecting from wear and tear, whereas melanocytes protect them from ultraviolet radiation[15]. In the past years researchers have found evidence of a deeper epithelial ingrowth into the limbal stroma, which is

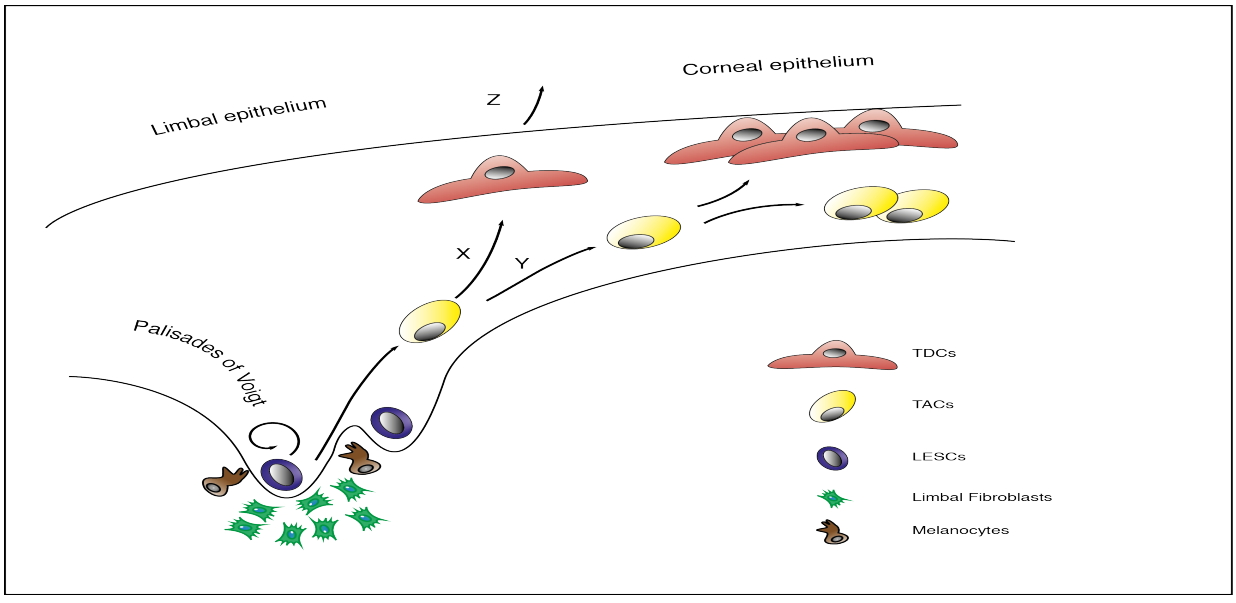


Figure 1 Limbal epithelial stem cells reside in Palisades of Voigt, which undulates at the limbus. Daughter transient amplifying cells (TACs) divide and migrate towards the central cornea, to replenish the epithelium, which correspond with the X,Y,Z hypothesis[13]. The stroma of the limbal epithelial stem cell niche is populated with fibroblasts and melanocytes.

postulated to be the actual location of the LESCs [16]. It has also been shown that hypoxia is a key regulator in maintaining the LESCs phenotype *in vitro* [17], which might also be the case for the SC niche, where a lower oxygen tension keeps the LESCs undifferentiated.
Phenotypically, LESCs appear like other stem cells with a high nuclear-cytoplasmatic ratio, a low granularity, high expression of LESCs markers such as p63α and ABCG2 [18] and low expression of differentiation marker cytokeratin 3 (CK3) [19].
LESCs have the ability to divide symmetrical or asymmetrical, producing daughter cells which follows the previously mentioned X, Y, Z proliferation and migration toward the central cornea, while differentiating into transient accelerating cells (TACs) and, finally, differentiate into the superficial terminal differentiated cells (TDCs) [13,20].
The immediate progeny are sometimes termed transient cells, as these cells are believed by some authors to have the capacity to undergo dedifferentiation [20].
For the past decades, *in vivo* animal studies have demonstrated the circumferentially and centripetally migrations toward the central cornea when re-epithelialization, compelling evidence of the limbal epithelial stem cells [21-24].

Where is LESCs located (The niche)

LESCs are believed to reside in a stem cell niche located at the corneoscleral junction, in a band, which encircles the periphery of the cornea. The cornea at the front of the eye is an avascular, transparent structure that covers the anterior surface of the eye. It is responsible for protecting the eye against insults such as injury and infections [25]. In addition, the cornea and associated tear film are responsible for almost two-thirds of the total refractive power of the eye, which make it an important part of the optical system [26].
The cornea is a lamellar-structured tissue composed of three distinct cellular layers. The anterior surface consists of non-keratinized squamous epithelial cells, a collagenous stroma sparsely populated with keratocytes and a monolayer of endothelial cells, each separated by specialized basement membranes, Bowman's Layer and Descemet's Membrane [27,28]. The corneal epithelium can be subdivided into three layers: a basal layer secreting matrix molecules, wing and squamous cells that differentiate from the basal cells. The squamous cells form tight junctions, protecting the cornea from the external environment, and wing cells participate in wound healing [25]. The limbal epithelium forms a natural barrier that separate the corneal epithelium from the conjunctival epithelium, hereby creating the unique and appropriate microenvironment to support SCs with self-renewal and multi potential activity [29].
In the aspect of the limbal niche, the *Palisades of Voigt* support molecular crosstalk from surrounding cells and soluble signals from the adjacent limbal vasculature. The

Palisades of Voigt can be seen clinically on the surface of the limbus, giving it a corrugated appearance and are more prominent at the superior and inferior limbus where the upper and lower eyelids. Among other cells it is also possible to identify antigen-presenting cells like Langerhans' cells and lymphocytes [25,30-32].

Limbal Stem Cell Deficiency

As described above the corneal epithelium is continuously renewed throughout life, but in some conditions, the LESC pool can decrease as a result of hereditary or acquired injury leading to partial or total limbal stem cell deficiency (LSCD). The consequence of this LESC depletion is that the corneal epithelial healing fails, and an invasion of the conjunctival epithelium may occur [12,33]. The invasion results in neovascularization of the normally avascular cornea, leading to corneal opacification and decreased visual acuity, and prevention of limbal restoration through transplantation of grafts [34-37]. The symptoms of LSCD include photophobia, pain, tearing, and chronic inflammation [20]. Besides the symptoms of LSCD the clinical picture features loss of limbal anatomy, little to no, function of hemidesmosomes, corneal vascularization, ingrowth of fibrovascular pannus [20]. Treatment of LSCD requires therefore LESC transplantation, where it is important to transplant limbal tissue, whereas conventional keratoplasty would not be able to restore LESC pool, and would not restore the epithelial integrity.

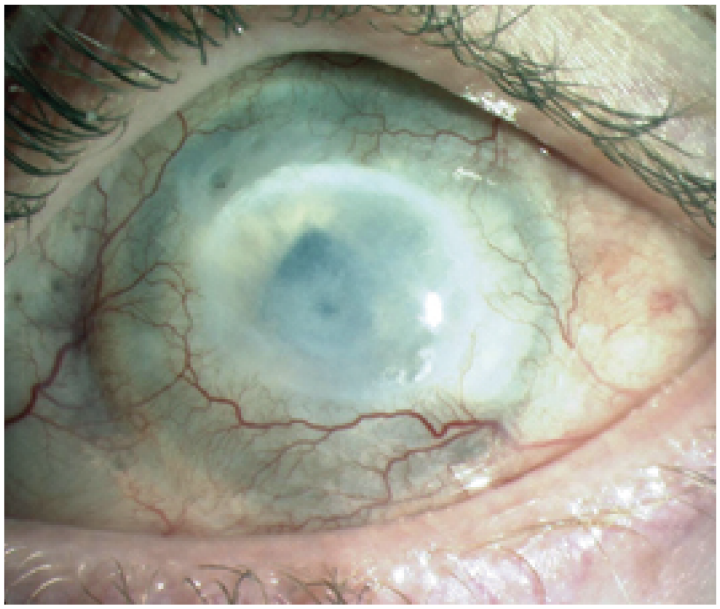


Figure 2 A case of total LSCD, where the cornea have lost its transparency. In the case corneal vascularization can also be seen. (modified from [38]111).

Current problems with treatment of LSCD

Chemical and thermal burns, inflammation, ocular damage, tear film impairment and the severity of these factors can all indeed lead to clinical failures in the treatment of LSCD. Selection and preparation of the receiving patient is therefore of the uttermost importance. Cases with severe damage of ocular structures and, alteration of the microenvironment might hamper the engraftment of cultured stem cells, and it may be necessary to do reconstruction of other ocular structures before cell transplantation. In the absence of negative systemic or genetic stimuli, the transplantation of autologous cultures of limbal cells can itself partially or totally restore the macroenvironment or microenvironment [36], resulting in the production of laminin, proteoglycans, and collagen, which might rebuild the extracellur matrix. This is able to support the cells during growth and facilitate cellular crosstalk by autocrine and paracrine secretion of growth factors.
Other causes to epithelial detachment include autoimmunity and, drug response, which are multifactorial and multigenic processes depending on complex interactions between multiple proteins and the environment. Due to these factors a precise diagnosis and grading of the LSCD are needed to choose the appropriated LESC therapy. Currently, autologous cultures of LESCs have been successful in treating chemical and thermal burn, where complete LSCD have been diagnosed [3,36,39]. For the engrafted transplant to regenerate the cornea, the LESCs must relocalize in the limbal niche to maintain self-renewal capacity. The cultured LESCs used for transplantations should contain an appropriate number of holoclones [40,41], which are able to form meroclones and paraclones. These have the properties like TAC progenitors and the ability to regenerate the cornea [42].
Additionally, cultivation of cells involves an appropriate culture medium, which plays an important role in preserving the cell characteristics; this also applies for the LESCs. Furthermore, the culture medium contains different xenogenic components, like fetal calf serum (FCS), which is inapplicable for clinical use with good manufacturing practices (GMP). It has been proposed that human autologous serum could work as a potential substitute for FCS, others use medium without serum. However, the variability of hormones and growth factors, content in human serum due to individual genetic background could be detrimental for the reproducibility of the culture [3]. While LESCs for corneal engineering seems promising, the clinical use due to the lack of autologous donor tissue and the risk of causing iatrogenic LSCD by taking an explant from otherwise healthy eye. For these reasons, it is very difficult to treat LSCD with the current options available [43].

Induced pluripotent stem cells; potential treatment for LSCD?

In 2006, Yamanaka et al. presented pluripotent stem cells (termed induced pluripotent stem cells (iPSCs)) derived from mouse and later human somatic cells by inserting a defined combination of transcription factors [44,45]. Human iPSCs has been shown to be similar to human ESCs, in terms of morphology, proliferation capacity, surface antigens, gene expression, telomerase activity and in addition be differentiated in vitro into cells from all three primary germ layers and in vivo generate teratomas [45]. Furthermore, iPSCs do not have the same ethical issues as obtaining human ESCs, because they do not originates from embryos. As an additional advantage, it is possible to form patient specific cells [45,46].
When Yamanaka et al. started to reprogram somatic cells into pluripotent cells, they identified 24 genes associated with pluripotenc. Through a series of experiment they found that using a combination of the four genes, Octamer-binding transcription factor (Oct) 3/4, Sex determining region Y box 2 (Sox2), Krüpel-like factor 4 (Klf4) and c-Myc, they observed that the somatic cells formed colonies and changed morphology similar to ESC [44,45]. Oct3/4 and Sox2 are believed to be the genes driving the reprogramming and Klf4 and c-Myc supporting the transformation [44,45].
Okita et al. independently published that by using Nanog and Lin28 it also was possible to reprogram somatic cells to pluripotent cells [47]. With the replacement of c-Myc and Klf4 by Nanog and Lin28, the reprogramming efficiency was reduced. To compensate the reduced reprogramming efficiency further examinations was done, revealing that a combination of Oct3/4, Sox2, Klf4, Lin28 and, Nanog had a higher reprogramming efficiency than Oct3/4, Sox2 either combined with c-Myc and Klf4 or Lin28 and Nanog [48].
By using a combination of inhibitors, it has been shown by direct differentiation that human iPSCs can be differentiated into corneal epithelial-like cells and further differentiated into mature cornea, confirmed by positive expression of initially p63 followed by the expression of CK3 and CK12 and ABCG2 [49].

Limbal fibroblast

The limbal fibroblast or corneal fibroblasts are specialized fibroblast residing in the limbal and corneal stroma, which is a mesenchymal tissue derived from the neural crest [50,51]. They play a crucial role in maintaining the cornea transparent and supporting the LESCs. After an injury activation of LESCs is initiated, but the limbal fibroblasts is also activated to produce interleukin 1α and interleukin 1β together with other cytokines that aid corneal epithelial wound healing [52-54]. Perrella et al. actual suggests, that like the LESCs replenish of corneal epithelium, as well does the limbal fibroblast replenish the corneal fibroblast. Meaning, that corneal fibroblasts represent another maturation state of limbal fibroblasts[55]. This supports the general though of limbal fibroblasts supporting the LESCs in the limbal niche. This could also support the different stages of maturation of fibroblast in the limbal and corneal structures[55]. They have been shown to produce the secreted protein, acidic and rich in cysteine (SPARC), which also is expressed by corneal epithelial cells, and they are believed to be involved in the wound healing process of both the epithelium and stroma of the cornea. SPARC has also been proposed to be involved in corneal epithelial migration and stratification following mechanical ablation [56,57]. The limbal fibroblasts secret higher levels of SPARC compared to central corneal fibroblasts *in vitro* without stimulation by serum or cytokines, and also *in vivo* without any wound-healing stimuli. Therefore, SPARC seems to have key role in homeostasis of the limbus and the limbal stem cell niche [58].

Why limbal fibroblasts?

While knowing that the cornea and supporting structures arises from ectoderm during embryogenesis, then using cells derived from either mesoderm or endoderm would not make sense. support the use of exactly limbal fibroblasts [38]. Every cell type or tissue has a unique DNA methylation profile comprising at least thousands of tissue-dependent differentially methylated regions, suggesting that the epigenetic changes at resides in the underlie cellular differentiation [59].
When comparing the corneal and conjunctival basement membrane to the limbal basement membrane several components were homogenously distributed in basement membranes ex. laminin-1, laminin-5, laminin (a3, a5, b3, g1, g2, and g3) chains and collagen type IV (a5 and a6) chains, collagen types VII, XV, XVII, and XVIII, and several others, suggesting that the basement membrane homogeneity is why limbal and corneal fibroblast are similar in supporting the corneal regeneration[60]. Signaling molecules, such as growth factors and cytokines, are likely to be involved in the maintenance of homeostasis of the limbal niche [61], resulting in when the cornea is injured it is not just the LESCs that start proliferating, the limbal fibroblast

also start a cascade of proliferations and signaling pathways, suggesting that these components might play a crucial role for both the limbal fibroblasts and LESCs [58]. Thus, it could be hypothesized, by using limbal fibroblasts for iPSCs and further differentiation into LESCs, will possess some kind of epigenetic memory of their origin and hence be more suitable for differentiation into LESCs than other kind of tissue specific cells. Furthermore, isolation of limbal fibroblasts are not as complicated as isolation of LESCs, where there are risk of coursing damage to a healthy eye [24], and can be done by taking a biopsy and cultivate it in a culture dish.

Plasmid presentations

Transfection was done with multiple plasmids, where some was used alone others were used in a combination of 3 and 5 plasmids. In the following section the plasmids will be presented:
The Epi5™ Reprogramming Vectors contain an optimized mixture of three episomal vectors consisting of 6 - 11 base pairs (bp) and contains an origin of replication (oriP) and Ebstein-Barr Nuclear antigen 1 (EBNA-1) backbone for delivering the reprogramming genes, Oct4, Sox2, Lin28, L-Myc, and Klf4. High transfection efficiency, constitutive expression from the oriP/EBNA-1 and CAG promoter and for episomal expression, which also means the vector only replicate extrachromosomally once every cell cycle [48]. Downstream the CAG promoter the first of two EcoRI cloning sites, containing the reprogramming genes. The ampicillin resistance gene for selection in prokaryotic cells. At this replication rate, the episomes are lost at a rate of approximately 5% per cell generation. This system shows enhanced iPSC generation through p53 suppression, and the inclusion of L-Myc has been shown to be more potent and specific then c-Myc during human iPSC generation. Epi5™ p53 & EBNA Vectors provide additional improvements to the reprogramming system. The p53 protein is known to be highly involved in cell cycle regulation and tumor suppression. p53 expression results in cell cycle arrest or cell death, so by knockdown of p53 reprogramming efficiencies have been improved as well as to prevent differentiation via the introduction of a variety of knockdown agents [62,63]. Supplemental expression of the EBNA-1 gene from a vector solely dedicated to this purpose allows for high expression of plasmids containing the origin of replication present on the reprogramming plasmids.

The pSIN4-CMV-K2M lentiviral vector [48], is a plasmid consisting of 8496 base pairs (bps), and with a lentiviral backbone of pSin4-EF2-IRES-Pur. Under the expression of the cytomegalovirus (CMV) promoter and internal ribosome entry site (IRES) intron are the Klf4 and c-Myc gene coexpressed. High transfection efficiency, constitutive expression is secured from the CMV promoter and the integration of the lentivirus. This vector is used in combination with pSIN4-EF2-O2S and pSIN4-EF2-

N2L [48]. The pSIN4-EF2-O2S lentiviral vector and pSIN4-EF2-N2L lentiviral [48], are consisting of 9030 bps and 8626 bps with the same lentiviral backbone as the pSIN4-CMV-K2M. Both pSIN4-EF2-O2S and pSIN4-EF2-N2L have SpeI as a cloning site and the pSIN4-CMV-K2M have NheI, where the reprogramming genes are placed. These three vectors all have a ampicillin resistance gene for selection in prokaryotic cells.

The OKSIM lentiviral vector [64], consisting of 12419 bps and with the pSin4-EF2-IRES-Pur. The Human elongation factor-1 alpha (EF-1α) promoter ensures the expression of the four reprogramming genes. The WHP Posttransccriptional Regulatory Element / Locus of X-over P1 (WPre/LoxP) is placed downstream of the reprogramming genes for increased expression delivered by the vector.

pEP E02S CK2M EN2L episomal vector [48], consist of 19949 bps and contains an origin of replication and Ebstein-Barr Nuclear antigen 1 (EBNA-1) backbone for high-level, constitutive expression from the CMV promoter and for episomal expression, which also means the vector only replicate extrachromosomally once every cell cycle. Klf4 and Myc are coexpressed under the strong CMV promoter. A SV40 polyadenylation (PA) signal secures proper processing of the 3' end of the MCS in mammalian cells. The ampicillin resistance gene and hygromycin resistence gene allow for the selection of properly transformed mammalian and prokaryotic cells. Oct4 and Sox2 are coexpressed under a strong EF-1α promoter. Nanog and Lin28 are coexpressed under a separate (but identical) EF-1α promoter [48].

Hypothesis

Limbal stem cell deficiency is an extremely debilitating eye disease and while much research has been done in order to treat the damage, the options are still very limited. The use of iPSCs seems promising for single cell deficiency, though much work still remains before it can be used for clinical purposes. Various different methods of forming iPSCs are available, each associated with advantages and disadvantages. Even though iPSCs show similar gene expression, surface antigens, morphology and proliferation capacity as ESCs, they might retain epigenetic memory of the tissue from which they were isolated. Thus it could be hypothesized that cells originating from the eye would be more suitable for forming iPSCs that could be used for corneal engineering.
Thus, the objective of this project was to optimize a procedure and form iPSCs for future cornea-limbal tissue engineering.

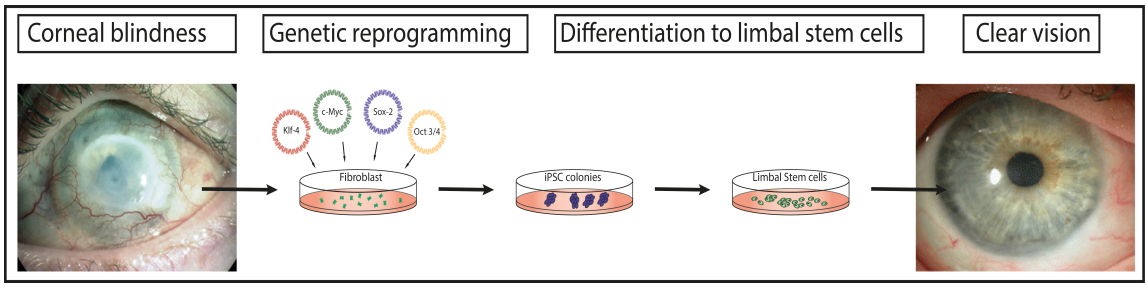


Figure 3 A schematic of the hypothesis, where corneal blindness could be treated by differentiating iPSCs generate from the patient.

Methodology and Materials

Isolation and cultivation of human limbal fibroblasts

Human limbal fibroblasts were isolated from corneoscleral rings obtained from the Danish Cornea Bank, Department of Ophthalmology, Aarhus University Hospital, Denmark. These were cleared from excessive debris by scraping followed by washing three times in s-PBS. Digestion of the corneoscleral rings was performed in s-PBS containing 200 U/mL collagenase IV (Gibco, Cat. No. 17104-019) and incubated for 20 hours in a 37°C incubator with 5 % CO_2. After digestion, the epithelium was gently scraped off and the corneoscleral rings were cut in pieces of 2 – 4 mm in thickness, seeded in T25 flasks (Greiner Bio-One) and checked every third and fourth day for limbal fibroblast outgrowth. The limbal fibroblasts were cultured in complete growth medium, consisting of Dulbecco's Modified Eagle Medium (DMEM) with high glucose, pyrovat, GlutaMAX^{tm}-1 (Invitrogen, Nærum, Denmark), 10 % Fetal Bovine Serum (FBS, Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), which was changed every third day. The limbal fibroblasts were expanded for as few passages as possible to minimize morphological changes.

Isolation of Addgene lenti- and episomal vectors

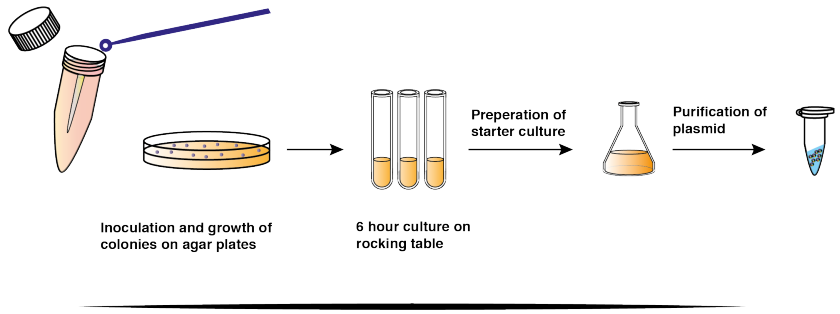
Bacterial stabs (addgene.org) were transformed with the following different vectors;

- pSIN4-CMV-K2M lentiviral vector coding human Klf4 and c-Myc (addgene.org, plasmid no. 21164),
- pSIN4-EF2-N2L lentiviral vector coding human Nanog and Lin28 (addgene.org, plasmid no. 21163),
- pSIN4-EF2-O2S lentiviral vector coding human Oct4 and Sox2 (addgene.org, plasmid no. 21162),
- OKSIM lentiviral vector coding human Oct4, Klf4, Sox2 and c-Myc (addgene.org, plasmid no. 24603),
- pEP4 E02S CK2M EN2L episomal vector coding human Oct4, Klf4, Sox2, Lin28 and Nanog (addgene.org, plasmid no. 20924),
- psPAX2, a 2nd generation packaging plasmid (addgene.org, plasmid no. 12260)
- pMD2.G a 2nd generation envelope plasmid (addgene.org, plasmid no. 12259)

The transformed bacteria were streaked on agar plates containing 100 µg/mL ampicillin (Sigma-Aldrich, cat. no. A-0166) and incubated overnight at 37 °C (Figure

4). One colony per agar plate was transferred to 6 mL S.O.C. medium supplemented with 100 µg/mL ampicillin and incubated at 37 °C on a 225 rpm rocking table for 6 hours, and subsequently plasmid DNA was extracted using the Purelink® HiPure Plasmid Midiprep Kit (Life Technologies, Cat. No K2100-04). Briefly, bacteria were harvested by centrifugation at 4000 x g for 10 min and resuspended in 50 mM Tris-HCl buffer containing 10mM EDTA and 20 mg/mL RNase A. Subsequently, the bacteria were lysed in lysis buffer containing 0.2 M NaOH with 1 % SDS for 5 minutes, followed resuspension in 3.1 M potassium acetate. The samples were loaded on to a filter membrane spin column and centrifuged at 9000 x g to bind the plasmid DNA, followed by washing in buffer containing 0.1 M sodium acetate, pH 5.0 and 825 mM NaCl. The plasmid DNA was eluted in elution buffer consisting of Tris-HCl with a pH of 8.5 and 1.25 M NaCl. An alcohol precipitation step was performed to desalt the plasmid DNA. First, the plasmid DNA was mixed with isopropanol followed by centrifugation at 9000 x G for 45 min at 4 °C. Afterwards, the pellet was washed in 70 % ethanol and centrifugated at 9000 x G for 10 min at 4 °C. The pellets were air-dried for 10 min before resuspension in TE buffer. The plasmid DNA concentration and purity were measured on a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden).

1.



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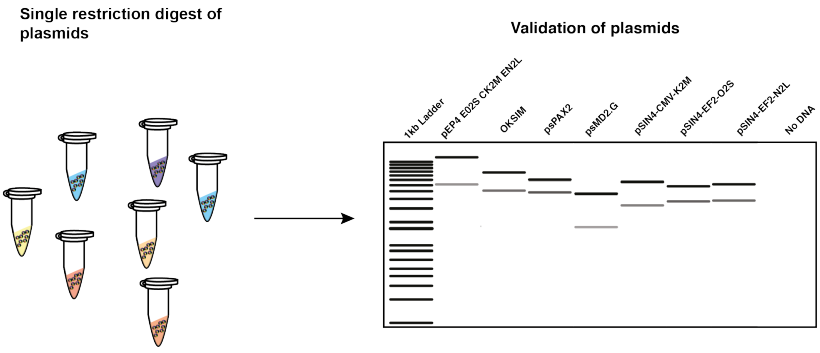

Figure 4 (1) Bacterial stabs containing the plasmids, where inoculated and grown on agar plates, for then to be culture overnight. The following day, a bacterial colony was picked for a starter culture. Followed by the purification of the plasmids. (2) Shows how the validations of the obtained plasmid were done. By using a restriction digest of each plasmid and a gel electrophoresis the plasmids were validated.

Plasmid validation

In order to validate the different plasmids obtained from the Midipreps, a restriction digest was done followed by an agarose gel electrophoresis. Single digests were performed using the restriction enzymes shown in table 1, to produce linearised sequences of each plasmid.
A restriction reaction mix was prepared containing 20 ng/µL plasmid, 1 % bovine serum albumin, 10 % NEBuffer and 10 X digestion enzyme (both from New England BioLabs) in nuclease free water (Life Technologies). Each digestion was performed for one hour at 37 ^{0}C in a Block heater (Stuart Equipment, Slangerup, Denmark) and the results were visualized using agarose gel electrophoresis.
For agarose gel electrophoresis, 0.8 % w/v agarose (Sigma-Aldric) was dissolved in Tris-acetate-EDTA (TAE) buffer, by microwave heating. 0.2 µg/mL ethidum bromide (Sigma-Aldrich) was added and the solution was transferred to a casting plate with wells. Electrophoresis samples were prepared by mixing 10µL plasmid digest solution with 2 µL 6x DNA loading dye (cat#R0611, Thermo Scientific, Slangerup, Denmark). After solidification, the agarose gel was placed in an Electrophoresis System Gel AGT2 (VWR, Leicestershire, United Kingdom) containing TAE buffer. 5 µL GeneRuler^{TM} 1 kb ladder, ranging from 250-10.000 bp (Thermo-Scientific) was added to the first well, while the restriction digests were added to their appropriate well. The gels were run for approximately 20 minutes at 100V and visualized on a Kodak Image Station 4000MM PRO.

Restriction enzymes used for plasmid validation

Plasmid \ Restriction enzyme	EcoR V	Sph 1	Age 1	Kpn 1	Not 1
pEP4 E02S CK2M EN2L	X				
OKSIM		X			
psPAX2			X		
pMD2.G				X	
pSIN4-CMV-K2M		X			
pSIN4-EF2-O2S					X
pSIN4-EF2-N2L					X
No DNA	X				

Table 1 An overview of the restriction enzymes used in the validation of the plasmids obtaining from Addgene.org

Optimization of electroporation with 0.2 cm and 0.4 cm cuvettes

Human limbal fibroblasts were cultured in T175 culture flasks (Greiner, Bio-One) until 70-90 % confluence. By using Trypsin/EDTA, the cells were detached and resuspended in PB – Sucrose. To ensure the right amount of cells needed to perform the experiment, a cell count was done. The electroporation suspension consisted of 1 x 10^5 cells/mL and 10 µg/mL Vivid ColorsTM pLenti6.2-GW/EmGFP Expression Control Vector in PB – Sucrose. An initial experiment concluded that a 0.4 cm Gene Pulser Cuvette (Bio-Rad Laboratories, Copenhagen, Denmark) containing 400 µL electroporation suspension and exposed to the following parameters 250V, 10 pulses and 0 % modulation, gave the highest transfection efficiency [65], however, it was rationalized that a cuvette half the size would conserve materials and would not change the outcome if parameters were halved, like the size of the cuvette. Using the Gene Pulser II Electroporation System with the RF Module attached, the cells were exposed to the following parameters: 125 and 250 volts, 10 bursts and 0% modulation. Preheated fibroblast growth medium was added immediately after electroporation, and the cells were seeded in tissue culture plates. Medium was changed every day. After 24, 48 and 72 hours phase contrast and fluorescent images were captured with an inverted fluorescence/brightfield/phase contrast microscope (Axiovert 200M, Carl Zeiss, Göttingen, Germany) attached to an AxiocamMR camera (Carl Zeiss) at 10x magnification. Together with the images, a viability test was done. Mixing 10 µL of cells from the electroporation suspension with 10 µL Trypan Blue. After 3 min incubation at room temperature viability was assessed by counting the number of dead cells vs. the number of living cells before electroporation. Monitoring the transfected cells for 72 hours the transfection efficiency was tested. For a better monitoring the cells were live stained with Hoechst 33342 (Invitrogen, Cat. No H1399) and incubated for 30 minutes at room temperature, followed by washing in fresh medium (Figure 5).

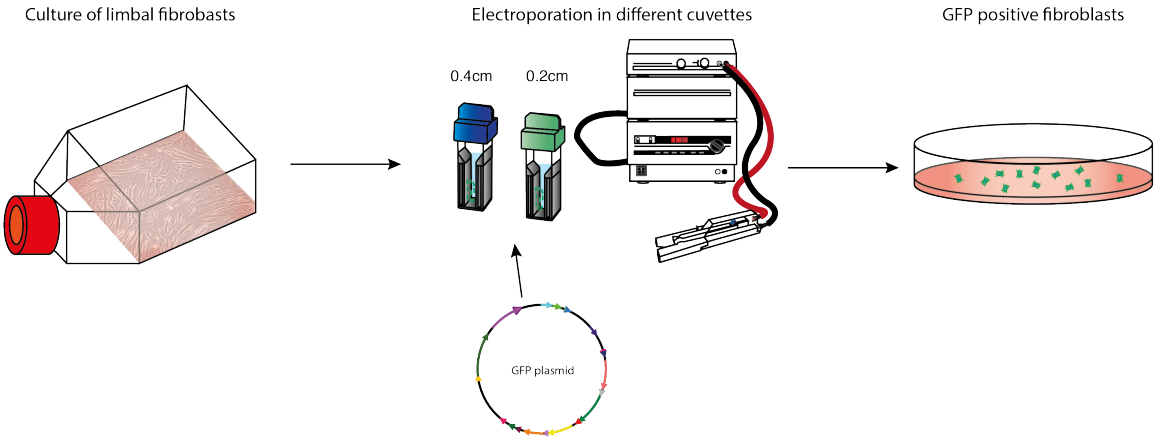

Figure 5 Human limbal fibroblasts cultured to 70-80 % confluence. A 0.2 and a 0.4 cm cuvette containing the electroporation suspension, consisting of cells, GFP plasmid and PB-sucrose were electroporated, followed by vitality testing and seeding of the fibroblasts. Monitoring for GFP expression was done for the next 48 hours.

Production of lentiviral particles

Lentiviral particles were produced by transfecting HeLa cells with plasmids coding for the Yamanaka and James Thomson factors Oct4, Sox2, C-Myc, Klf4, Lin28 and Nanog (addgene.org, Plasmid numbers: 24603, 21164, 21163 and 21162).
Initially, HeLa cells were grown in 12-well plate dishes until 90% confluence in HeLa medium containing DMEM medium with High-Glu + L-Glutamine, 10 % FCS and 1 mL non-essential amino acids.
At the day of transduction 1.5 μg of the each individual plasmids were mixed with 1.5μg pMD2.G, 1.5µg psPAX (both available from addgene.org, plasmid numbers: 12259 and 12260), 600µL DMEM and 30µL ipofectamine® 2000 transfection reagent (Life Technologies, Cat. No. 11668-027, Nærum, Denmark) in Eppendorf tubes and incubated for 25 min. Each mixture were split in three and added to three wells. 24, 48 and 72 hours post-transduction, viral particles were harvested by collecting the HeLa medium (Figure 6).

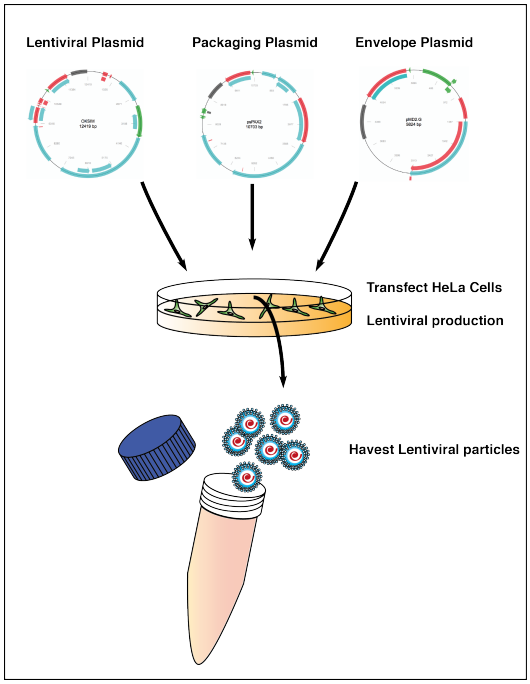

Figure 6 Simple schematic of the viral production. Transfection of HeLa cells with a lentiviral- and packaging plasmids, which after 24 hours starts to produce virus particles. The virus-containing medium are harvested every day for three days.

Forming iPSCs using lentiviral particles

The day before viral transfection, 6-well tissue culture plates were coated with human recombinant vitronectin (Gibco®, Cat. No. A14700, Nærum, Denmark) and human fibroblasts were seeded at a density of 5400 cells/cm^2 in iPSC medium containing DMEM with High-Glu + L-Glutamine, 10 % FCS and 1 mL non-essential amino acids. Transfection of fibroblasts was performed by replacing the fibroblast medium with the HeLa medium containing the viral particles and supplemented with 6µg/mL polyprene. The following day medium was replaced with human iPSC medium consisting of DMEM/F12 (1:1) supplemented with 20 % KnockOut^{TM} Serum Replacement, 100 µM MEM non-essential Amino Acids solution (Invitrogen, Cat. No. 11140-050), 55μM β-mercaptoethanol (Sigma Aldrich, Product No. M6250, Brøndby, Denmark) and immediately prior to use, supplemented with 0.5 mM valproic acid (Sigma Aldrich, Product No. P6273) and 12 ng/mL basic Fibroblast Growth Factor (bFGF) (Invitrogen, Cat. No. PHG0261). The medium was replaced every second day and cells were monitored for emergence of colonies resembling human embryonic stem cells. Representative phase contrast images were taken at 5x and 10x magnification using a Zeiss Axio Observer (Zeiss Mikroskopi, Birkerød, Denmark).

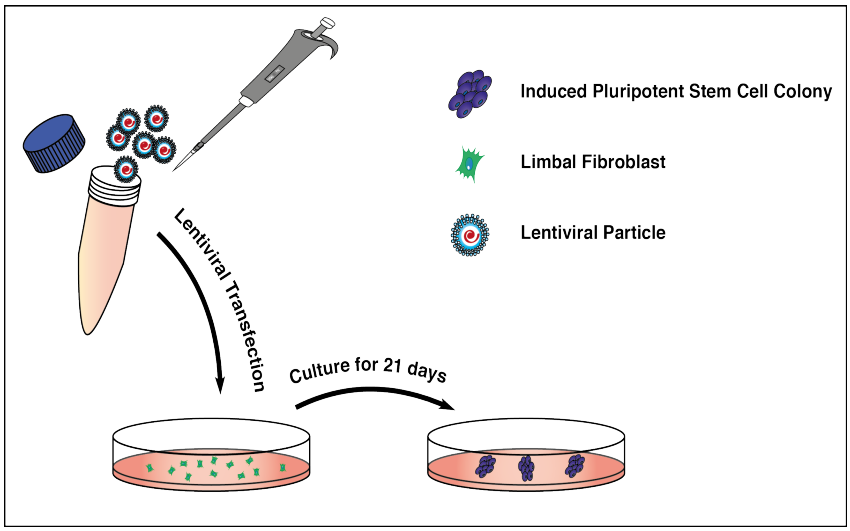

Figure 7 Schematic of the formation of iPSCs using lentiviral particles. After harvesting the lentiviral particles, it was used to transfect the limbal fibroblasts. After the transfection the cells where cultured for 21 days and had medium changed every second day as well observed for emerging iPSC colonies.

Formation of iPSCs using the Epi5 Episomal Reprogramming Kit

One hour prior to the experiment, 6-well tissue culture plates were coated with 0.5 µg/cm^2 human recombinant vitronectin (Gibco®, Cat. No. A14700) in s-PBS and incubated at room temperature for one hour. Human limbal fibroblasts were cultured in T175 culture flasks (Greiner, Bio-One) until reaching 70-90 % confluence. Using Trypsin/EDTA, the cells were detached and resuspended in PB – Sucrose. Immediately prior to the experiment, the vitronectin solution was removed and 2 mL supplemented fibroblast medium, consisting of DMEM with high glucose, pyrovat and GlutaMAX™-I, 10 % Fetal Bovine Serum and 0.1 mM MEM Non-essential Amino Acids (Invitrogen, Cat. No. 11140-050), without P/S. The electroporation suspension, which consisted of 1 x 10^5 cells/mL and 200 µg/mL Epi5™Episomal iPSC Reprogramming vectors and 150µg/mL Epi5™ p53 & EBNA vectors (Life Technologies, Cat. No. A15960) in PB – Sucrose was added to a Gene Pulser Cuvette with a 0.2 cm gap, and the cells were allowed to rest for 5 minutes before electroporation with the Gene Pulser II Electroporation System with the RF Module attached. The electroporation parameters found during the optimization was used to transfer the vectors to the cells. The electroporated cell suspension was transferred to one of the wells in a 6-well tissue culture plate containing 2 mL of prewarmed supplemented fibroblast medium and placed a hypoxic cell culture system (Xvivo System, BioSpherix, Redfield, NY) at 5 % O_2, 5 % CO_2 and 37 ^{0}C for the rest of the experiment.
The day after electroporation, the supplemented fibroblast medium was replaced with N2B27 medium consisting of DMEM/F12 with HEPES, 1 % 100x N-2 supplement (Life Technologies), 2 % 50x B-27® supplement (Life Technologies), 0.1mM MEM Non- essential Amino Acids, 0.5 % 100x GlutaMAX™-I and 0.18 % 55 mM β- Mercaptoethanol (Sigma Aldrich, Brøndby, Denmark) and freshly supplemented with bFGF (Invitrogen). The N2B27 medium was changed every second day for the next 14 days. At day 15 post-transfection, medium was changed from N2B27 medium to Essential™ 8 Medium consisting of DMEM/HAM-F12 1:1, with 2 % 50x Essential 8™ supplement (Life Technologies, Cat. No. A14666SA) for the rest of the experiment. Figure 8, can help visualized the strategy. During the reprogramming, when the cells had medium change, the cells were examined under a stereo microscope and phase contrast images was taken, to follow the development of forming iPSCs.

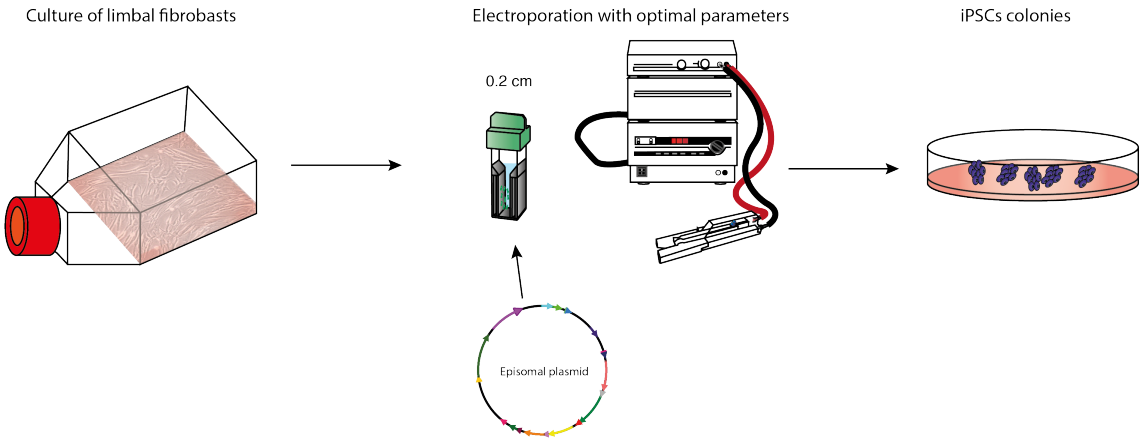

Figure 8 Simple schematic of the procedure to transfer the episomal plasmid into the limbal fibroblasts. Human limbal fibroblasts cultured to 70-80 % confluence were detached using trypsin-EDTA, centrifugated and resuspended in PB-sucrose. A 0.2 cuvette containing the electroporation suspension, consisting of cells, episomal reprogramming plasmid and PB-sucrose were electroporated, followed by vitality testing and seeding of the cells. Monitoring for iPSC colony formation for the next 21 days.

Formation of iPSCs using the pEP4 E02S CK2M EN2L Vector

One hour prior to the experiment, 6-well tissue culture plates were coated with 0.5 µg/cm^2 human recombinant vitronectin (Gibco®, Cat. No. A14700) in S-PBS and incubated at room temperature for one hour. Human limbal fibroblasts were cultured in T175 culture flasks (Greiner, Bio-One) until reaching 70-90 % confluence. Using Trypsin/EDTA, the cells were detached and resuspended in PB – Sucrose. Immediately prior to the experiment, the vitronectin solution was removed and 2 mL supplemented fibroblast medium, consisting of DMEM with high glucose, pyrovat and GlutaMAX™-I, 10 % Fetal Bovine Serum and 0.1 mM MEM Non-essential Amino Acids (Invitrogen, Cat. No. 11140-050), without P/S). The electroporation suspension, which consisted of 1 x 10^5 cells/mL and 1 mg/mL pEP4 E02S CK2M EN2L episomal vector (addgene.org, Plasmid No.20924) in PB – Sucrose was added to a Gene Pulser Cuvette with a 0.2 cm gap, and the cells were allowed to rest for 5 minutes before electroporation with the Gene Pulser II Electroporation System with the RF Module attached. The electroporation parameters found during the optimization was used to transfer the vectors to the cells. The electroporated cellssuspension was transferred to one of the wells in a 6-well tissue culture plate containing 2 mL of prewarmed supplemented fibroblast medium and placed a hypoxic cell culture system (Xvivo System, BioSpherix, Redfield, NY) at 5 % O_2, 5 % CO_2 and 37 ^{0}C for the rest of the experiment.

The day after electroporation, the supplemented fibroblast medium was replaced with N2B27 medium consisting of DMEM/F12 with HEPES, 1 % 100x N-2 supplement (Life Technologies), 2 % 50x B-27® supplement (Life Technologies), 0.1 mM MEM Non- essential Amino Acids, 0.5 % 100x GlutaMAX™-1 and 0.18 % 55 mM β- Mercaptoethanol (Sigma Aldrich, Brøndby, Denmark) and freshly supplemented with bFGF (Invitrogen) and CHALP molecule cocktail consisting of 0.5 µM Mitogen-activated Protein Kinase Kinase (MEK) inhibitor (Stemgent, Bergisch Gladbach, Germany), 3 µM Glycogen synthase kinase (GSK3β) inhibitor (Stemgent), 0.5 µM Transforming growth factor (TGF)-β inhibitor (Stemgent), 10 ng/mL human Leukemia inhibitory factor (hLIF, Life Technologies) and 10 µM ROCK inhibitor. The N2B27 medium was changed every second day for the next 14 days. At day 15 post-transfection, medium was changed from N2B27 medium to Essential™ 8 Medium consisting of DMEM/HAM-F12 1:1, with 2 % 50x Essential 8™ supplement (Life Technologies, Cat. No. A14666SA) for the rest of the experiment. Figure 8, can help visualized the strategy. During the reprogramming, when the cells had medium change, the cells was examined under a stereo microscope and brightfield images was taken, to follow the development of forming iPSCs.

Results

Validation of plasmids

To validate the viral episomal vectors obtained from Addgene.org a restriction digests analysis was performed and, the outcome can be seen in figure 9. The digests were compared with a 1 kbp Generuler ladder (New England Biolabs, Cat. No. N3232S). The single digest of each plasmid produced 2 clear bands, corresponding to the size of the predicted digest products form the selected restriction enzyme. A negative control was also made to show that the bands were not produced, due to the restriction enzyme.

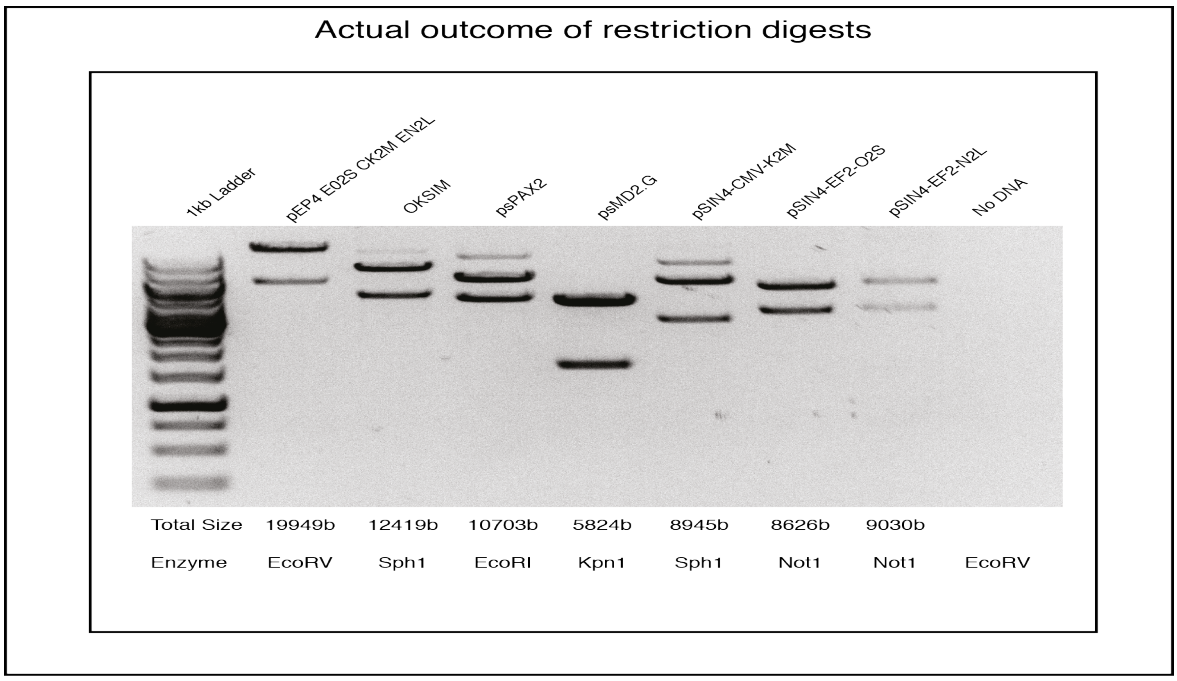

Figure 9 Restriction digest of the 6 plasmid obtained from Addgene.org at 0.8 % agarose gel. Ladder was a 1 kbp GeneRuler. Each plasmid can be seen above is lane, below the lane the total size of the plasmid, and restriction enzyme for each digest.

Optimization of electroporation

The hypothesized setup with a small sized cuvette led to a series of experiments based upon the earlier results, where it was determined that the optimal electroporation parameters were 250 V, 10 pulses and 0% modulation.
The electroporation experiments were used to determine the size of the cuvette, being a 0.2 cm or 0.4 cm cuvette, and the voltage being 125 V and 250V, with 10 pulses. The electroporation time was kept constant at 100ms together with Radio Frequency at 40Hz and pulse duration at 2 msec.

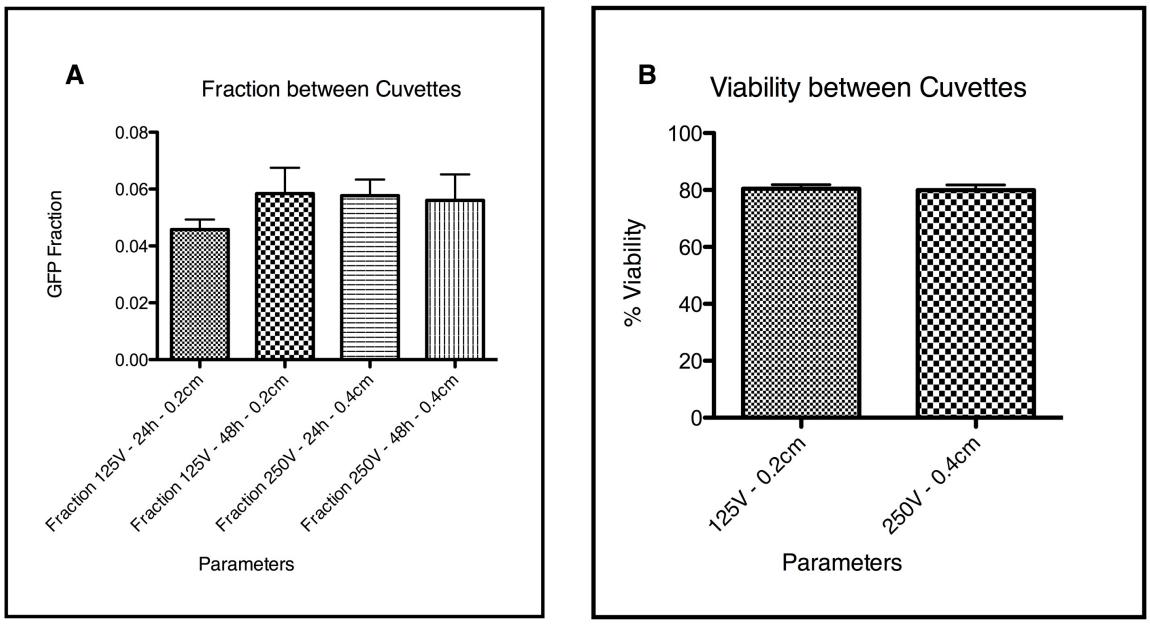

Figure 10 Results of the optimization between electroporation cuvettes. A) Shows the GFP positive fraction between the cuvettes. B) Shows the viability after the electroporation.

As seen in figure 10, the combination of voltage and viability, correspond to the cuvette size. As well does the GFP fraction, where the 0.2 cm cuvette at 24 hours have a smaller amount of GFP positive cells, however after 48 hours the number of GFP positive cells are merely the same between the cuvettes and, no significance can be seen. From this a conclusion was made, that with no statistical significance the 0.2 cm cuvette would be as good as the 0.4 cm cuvette.

Generation of iPSCs using the episomal and lentiviral vectors

Human limbal fibroblasts were cultured to approximately 70-90 % confluence, followed by either reseeding in a 6 well culture plate or by being exposed to the electroporation parameters which were 125V, 10 pulses and 0 % in a 0.2 cm cuvette. The electroporation should ensure the transfection with the episomal reprogramming vectors. After the electroporation, the now transfected fibroblasts were placed in a hypoxic incubator overnight. The day after a substantial amount of the cells had attached and medium was changed as for every second day for the next 21 days. The fibroblasts that were reseeded in the culture plates were left overnight for then to be transfected with viral particles overnight. The day after transfection medium was changed to iPSC medium and this was changed every second day. Table 2 is an overview of reprogramming experiments that have been done during this project. During the reprogramming, the cells undergo morphology changes, where they become more round and start cluster together like small colonies [66]. Both during the viral and the episomal transfection the cells seemed first started changing morphology after 5 – 7 days and single clusters become visible short here after, figure 11. To test for pluripotency, an alkaline phosphatase staining was done, albeit the staining did not seem be located just to the clusters. Until day 21 the cultures were observed for further development of the cells clusters. At the end of the experiments the clusters have not developed further and formation of iPSCs were not succeeded.

Experiment	Vector	Parameter	Medium	Supplements	Effect
Episomal Reprogramming	Epi 5 Episomal Vectors from Invitrogen	Electroparation w. 125V	N2B27 / Essential 8 Medium	bFGF	Cell Death, High β-mercaptoethanol
Episomal Reprogramming	Epi 5 Episomal Vectors from Invitrogen	Electroparation w. 125V	N2B27 / Essential 8 Medium	bFGF	Cell Death, High β-mercaptoethanol
Episomal Reprogramming	Epi 5 Episomal Vectors from Invitrogen	Electroparation w. 125V	N2B27 / Essential 8 Medium	bFGF	No morphology changes, No cell death.
Episomal Reprogramming	Epi 5 Episomal Vectors from Invitrogen	Double amount of DNA, Electroparation w. 125V	N2B27 / Essential 8 Medium	bFGF	No morphology changes, No cell death.
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Electroparation w. 125V	N2B27 / iPSCs medium	Small molecules*	Fibroblast Clusters
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Electroparation w. 250V	N2B27 / iPSCs medium	Small molecules*	Fibroblast Clusters
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Lipofectamine 2000	N2B27 / iPSCs medium	Small molecules*	No changes.
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Electroparation w. 125V	N2B27 Medium	Small molecules*	Cells detached
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Electroparation w. 250V	N2B27 Medium	Small molecules*	Cells detached
Episomal Reprogramming	pEP4 E02S CK2M EN2L	Lipofectamine 2000	N2B27 Medium	Small molecules*	Cells detached
Viral Reprogramming	pSIN4-CMV-K2M, pSIN4-EF2-N2L and pSIN4-EF2-O2S	Viral transduction	iPSC Medium	bFGF + Valproic acid	Fibroblast Clusters
Viral Reprogramming	OKSIM	Viral transduction	iPSC Medium	bFGF + Valproic acid	No changes.
Viral Reprogramming	pSIN4-CMV-K2M, pSIN4-EF2-N2L and pSIN4-EF2-O2S	Viral transduction	iPSC Medium	bFGF + Valproic acid	Cells detached
Viral Reprogramming	OKSIM	Viral transduction	iPSC Medium	bFGF + Valproic acid	Cells detached

Table 2 A combined list of reprogramming experiments done during this project.

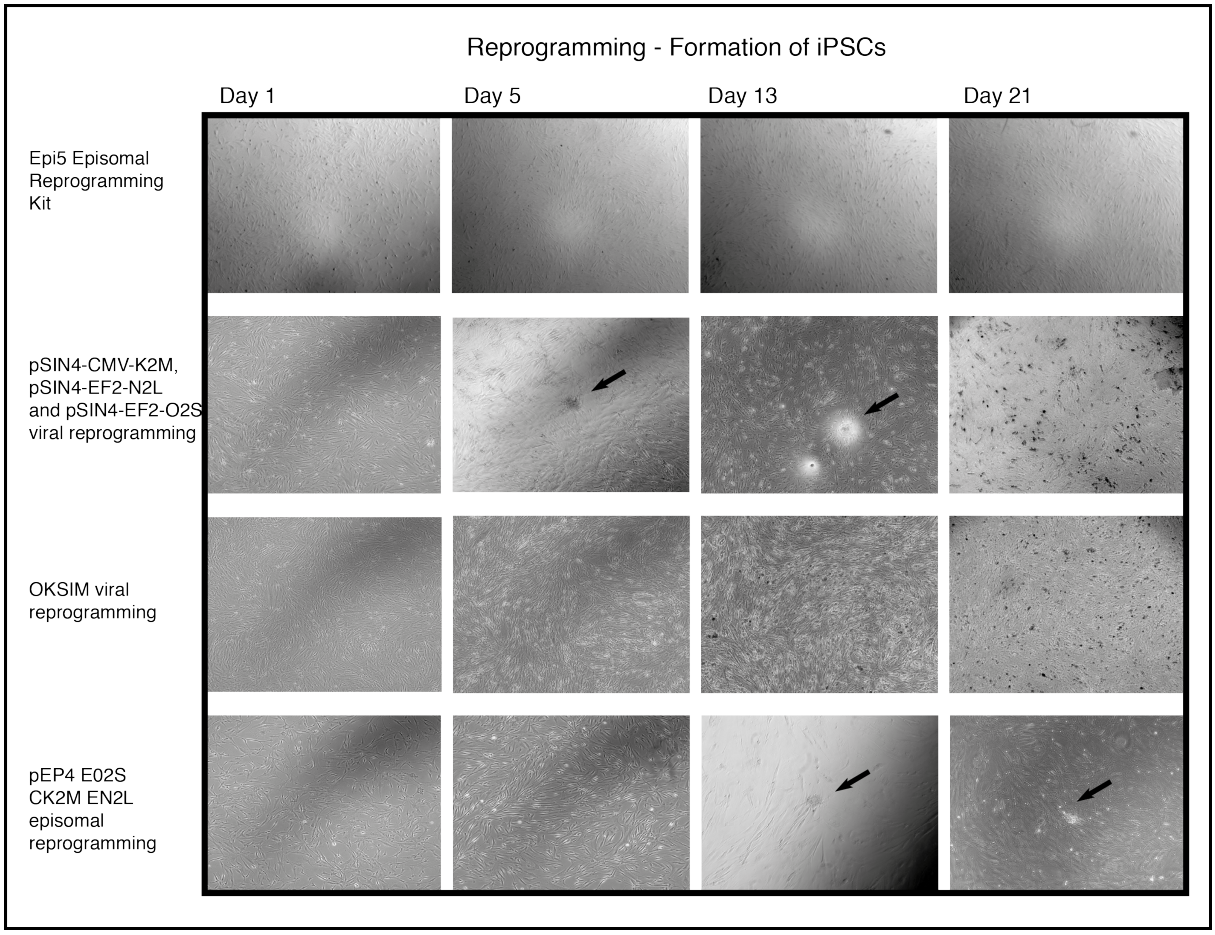

Figure 11 **Reprogramming human limbal fibroblasts using the Epi5 episomal iPSC reprogramming kit, a 3 lentiviral setup, the OKSIM viral vector, and the pEP4 E02S CK2M EN2L episomal vector. At day 1 following electroporation, the cells looked like fibroblasts. During the first 5 days the cells become more confluent, especial the cells where the Epi5 kit was used. In the setup containing the 3 lentiviral vectors, cluster of cells appeared. 13 days, cells starts to cluster in the pEP4 E02S CK2M EN2L setup. These clusters were followed until day 21. At day 21, no changes were seen in setups where clusters were formed and cells seemed to be necrotic. Arrows shows the cell clusters.**

Discussion

Plasmid validation

When validating the plasmids obtained from Addgene.org, all digests products were analyzed by agarose gel electrophoresis. Based on the each single plasmid DNA, it was determined that a single digest, cutting at a restriction site placed twice on each plasmid, created two bands of each plasmid on the gel, as would be expected (Figure 12). To determine each fragment of DNA, a 1 kbp ladder was used. An unequal migration of the ladders was observed, creating a smiling effect within each lane. This made it difficult to determine the exact location of the bands, since the bands representing 10 kbp, 8 kbp and 6 kbp were overlapping.

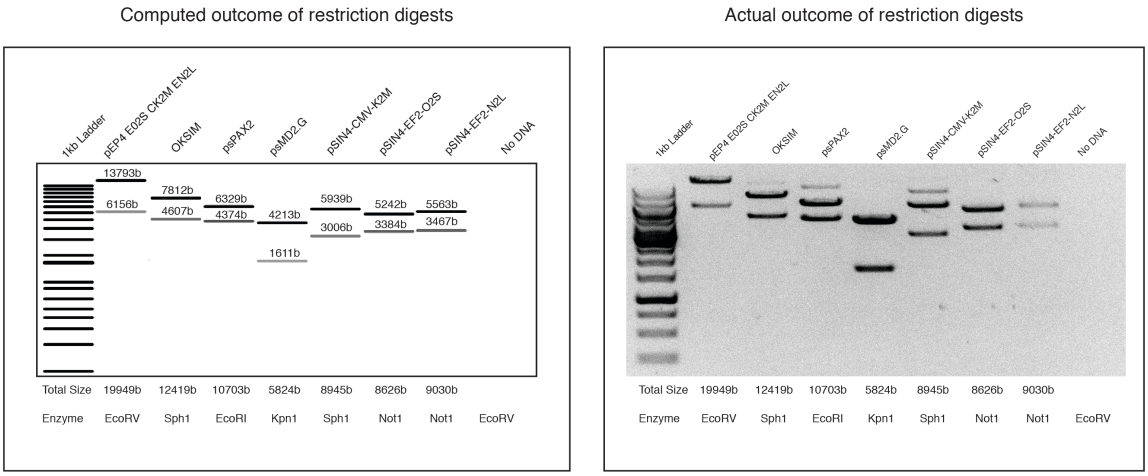

Figure 12 shows the restriction enzyme used for each plasmid, and both a computed outcome and the actual outcome of each digests. The computed outcome was created using piece of software called *"a plasmid editor"* (M. Wayne Davis, Biologylabs, Utah).

When comparing, the actual outcome and computed outcome, these were found to correspond very well to each other, although an additional band was observed in the digest of OKSIM, psPAX2 and, pSIN4-CMV-K2M.
The first band in the digest of the OKSIM plasmid was estimated to be larger than 10.000 bps, and the following two bands can be estimated to correspond with the predicted digest products of the plasmid, consisting of bands of 7812 bps and 4607 bps.
The same applies for both psPAX2 and pSIN4-CMV-K2M, where the first of the band is approximately 10.000 bps. The following bands for psPAX2 and pSIN4-CMV-K2M correspond with the digest, 6329 bps and 4374 bps and, 5939 bps and 3006 bps.
The bands that do not correspond with the expected outcome, could be due to the topography of the plasmid [67,68]. The estimated size of these first bands correspond with the size of each of the plasmids. Thus, if a portion of these plasmids has been

left undigested or just partial digested (nicked), the plasmids will sustain greater friction in the agarose gel, than a supercoiled plasmid, resulting in bands no corresponding with the digest. Therefore, it was concluded that each plasmid was matched with the digest and further usage of the plasmids could continue.

Production of viral particles

To produce viral particles, HeLa cells were transfected with a lentiviral reprogramming plasmid, together with a packaging and envelope plasmid. The virus-containing medium was harvested the following day and this procedure was done every day for the four days. The virus-containing medium, was then used for transfection of the limbal fibroblasts, however it could not be determined if the virus-containing medium did or did not contain virus, due to combination of lentiviral vectors and packaging system.
The packaging plasmid, psPAX2, is a second generation packaging plasmid, which was used together with the pMD2.G envelope plasmid that together creates a strong packaging vector system, which would function with most experiments. It could be discussed if a third generation packaging system would have fit the viral vectors better, but when using a third generation packaging system two packaging plasmids and one envelope plasmid are needed, leading to a more difficult setup. A third generation packaging system would have been safer to use, than a second generation system, but when taking the right precautions the risks are very low [69]. Thus, a second generation packaging system was chosen.
The packaging system did not support a way of determining if the medium collected from the HeLa cells contained virus particles and the viral plasmids did not contain a reporter protein, which could be detected when transfecting the limbal fibroblasts. Therefore, when using the virus-containing medium, the virus content could have been non-existing, leading to non-developing iPSC colonies. A plasmid containing both reprogramming factor and a reporter protein could ensure the detection of virus particles in the medium.

Reprogramming failures

During this project several reprogramming systems have been tried, though it has not yet been possible to generate iPSCs. Initially the Epi5™ Episomal Reprogramming kit was used, but due to a to high concentration of β-mercaptoethanol, which cause significant cell death, the reprogramming did not succeed. This was corrected and reprogramming experiments were continued, but as seen in figure 11, reprogramming of the fibroblasts did not succeed, even though manufacturers protocol were followed. This raises the question, whether the electroporation transfered the episomal plasmids to the fibroblasts. From the

electroporation optimization results, a transfection efficiency up to 9,7 % GFP-positive fibroblasts could be observed, and in theory the same efficiency and amount of positive cells should be similar. The manufacturer's protocol describes that a reprogramming efficiency from 0.04 to 0.3 % should be achieved [70], which could be interpreted such that if 9,7 % of the cells receive the reprogramming vectors only 0.04 to 0.3 % of these would continue the reprogramming and form iPSCs. This would therefore result in a very small actual reprogramming efficiency. It could also be speculated that the high cell confluence obtained in the experiments prevented reprogramming from happening. The failures in the viral reprogramming could be due the content of the virus-containing medium, as explained in the discussion section: *"Production of viral particles"*.

Other Reprogramming strategies

In this experiment, formation of iPSCs was tried with episomal vectors, since the potential clinical application of generation of iPSCs should be devoid of any viral components [48]. With further experiments, this concept could possibly produce LESCs for transplantation, but in this study, the setup did not succeed in producing iPSCs. However, as a proof of concept to show that limbal fibroblasts are superior to other cells in producing LESCs and other tissues of the eye, the viral transduction approach could still be used [44]. However, the disadvantage of this approach is that viral genetic material can be integrated into the host genome, and also cause insertional mutagenesis. This would leave iPSCs non-suitable for clinical use, though they will provide information of differentiation of iPSCs into LESCs. Multiple other induction systems have been developed since Yamanaka and colleagues first produced iPSCs.
The non-integrating Adeno- and sendaiviral vectors have been used to produce vector-free iPSCs. However it has been shown that sendaivirus reside in the cells long after reprogramming and that adenovirus has a very low reprogramming efficiency [71]. A system using Piggybac transposons has also been used to reprogram cells with a "cut and paste" mechanism [72], and also accomplished the production of protein-reprogrammed iPSCs [73,74]. Common to all of them are the problems of with low reprogramming efficiency or integration into the host cell genome. Throughout this project, the delivery of the episomal plasmids has been done by electroporation. During the optimization, a transfection efficiency of 9,7 % was reached, but this could maybe be increased either by using another electroporation system or by using a chemical reagent. By using another electroporation system, it would require a new electroporation optimization, because electroporation varies from system to system and therefore the setup on another system would have to be optimized again without the possibility of using the findings of the present study. Lipofectamine is a transfection reagent used to transfer RNA or

plasmid DNA into cells. Lipofectamine forms liposomes in aqueous enviroments, which entraps the plasmid, and allow transport over the cell membrane. This way of transfection would also require a series of optimization experiments, but it could results in a higher efficiency than electroporation.

Differentiation of iPSCs into LESCs

Ahmad et al. has shown that by using both a co-culture, conditioned medium and different coatings system, ESCs can be differentiated into corneal like cells [75]. This could probably also apply for differentiation of iPSCs. While the limbal fibroblasts grow, they secrete growth factors to the medium. The conditioned medium is then harvested and used when iPSCs differentiation takes place.
Hayashi et al. differentiated human iPSCs derived from both dermal fibroblasts and corneal limbal epithelium [51]. By using stromal-cell derived inducing activity (SDIA), which is a co-culture system, they showed that after 6 weeks of culturing the first cells started expressing markers for corneal epithelium. Like the SDIA method, co-culturing with limbal fibroblasts might also differentiate iPSCs to LESCs. The limbal fibroblast would work as the limbal niche, and supplement the medium with vital growth factors would support LESCs differentiation and growth. Further, experiments could be performed to force expression of Pax6, because this would differentiate iPSCs into LESCs. It is widely known that Pax6 plays a crucial role in eye development [76,77].

Conclusion

In the current project, it has been determined that a 0.2 cm cuvette is as sufficient in transferring plasmid DNA into cells as a 0.4 cm cuvette, as long every other electroporation parameters are halved as well.
In addition, both viral and episomal plasmids were obtained from Addgene.org. Retrieved from bacterial stabs and verified by restriction digests.
HeLa cells were transfected with the viral plasmids, for production of viral particles, which were used in order to reprogram limbal fibroblasts to pluripotent cells. Even though, changes in morphology were seen in multiple culture plate wells, no iPSCs were generated using viral vectors.
Furthermore, was the Epi5™ Episomal iPSC Reprogramming kit purchased from Invitrogen, for generation of vector- and transgene free iPSCs. However, it was not possible to obtain iPSCs using this kit, even when following the protocol provided by Invitrogen.
Using the pEP4 E02S CK2M EN2L episomal vector from Addgene.org and the determined electroporation parameters, changes in morphology were seen, during the reprogramming experiment. When stained with alkaline phosphatase the cells did not show to express this marker for pluripotency and during the rest of the reprogramming no more morphology changes were seen.

Acknowledgements

I wish to thank my supervisor, Vladimir Zachar, for his professional guidance in this project. I wish to thank Jens Isak Andersen for his invaluable support in the laboratory and during experiment setups and acknowledge. Lastly, I thank my fellow colleague, Kasper Bendix Johnsen, for his support during this project period.

References:

1. Organization, W. H. WHO | Visual impairment and blindness. *WHO* 1–4 (2014). at <http://www.who.int/mediacentre/factsheets/fs282/en/#>

2. Pascolini, D. & Mariotti, S. P. Global estimates of visual impairment: 2010. *British Journal of Ophthalmology* (2012). doi:10.1136/bjophthalmol-2011-300539

3. Pellegrini, G., Rama, P., Di Rocco, A., Panaras, A. & De Luca, M. Concise review: hurdles in a successful example of limbal stem cell-based regenerative medicine. *Stem Cells* **32,** 26–34 (2014).

4. Bath, C. *et al.* Hypoxia is a key regulator of limbal epithelial stem cell growth and differentiation. *Stem Cell Research* **10,** 349–360 (2013).

5. Mountford, J. C. Human embryonic stem cells: origins, characteristics and potential for regenerative therapy. *Transfus Med* **18,** 1–12 (2008).

6. Mitalipov, S. & Wolf, D. Totipotency, pluripotency and nuclear reprogramming. *Adv. Biochem. Eng. Biotechnol.* **114,** 185–199 (2009).

7. HAYFLICK, L. & MOORHEAD, P. S. The serial cultivation of human diploid cell strains. *Experimental Cell Research* **25,** 585–621 (1961).

8. Furue, M. *et al.* Leukemia inhibitory factor as an anti-apoptotic mitogen for pluripotent mouse embryonic stem cells in a serum-free medium without feeder cells. *In Vitro Cell.Dev.Biol.-Animal* **41,** 19–28 (2005).

9. Nagafuchi, S., Katsuta, H., Kogawa, K., Akashi, T. & Kondo, S. Establishment of an embryonic stem (ES) cell line derived from a non-obese diabetic (NOD) mouse: in vivo differentiation into lymphocytes and potential for …. *FEBS letters* (1999).

10. Raman, J. D. *et al.* Decreased expression of the human stem cell marker, Rex-1 (zfp-42), in renal cell carcinoma. *Carcinogenesis* **27,** 499–507 (2006).

11. Mann, I. A study of epithelial regeneration in the living eye. *The British journal of ophthalmology* (1944).

12. Ahmad, S. Concise review: limbal stem cell deficiency, dysfunction, and distress. *Stem Cells Translational Medicine* **1,** 110–115 (2012).

13. Thoft, R. A., Thoft, R. A., Friend, J. & Friend, J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Investigative Ophthalmology & Visual Science* **24,** 1442–1443 (1983).

14. Kinoshita, S., Friend, J. & Thoft, R. A. Sex chromatin of donor corneal epithelium in rabbits. *Investigative Ophthalmology & Visual Science* (1981).

15. Higa, K., Shimmura, S., Miyashita, H., Shimazaki, J. & Tsubota, K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. *Experimental Eye Research* **81,** 218–223 (2005).

16. Dua, H. S. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *British Journal of Ophthalmology* **89,** 529–532 (2005).

17. Bath, C. Human corneal epithelial subpopulations: oxygen dependent ex vivo expansion and transcriptional profiling. *Acta Ophthalmol* **91 Thesis 4,** 1–34 (2013).

18. Chen, Z. *et al.* Characterization of Putative Stem Cell Phenotype in Human Limbal Epithelia. *Stem Cells* **22,** 355–366 (2004).

19. Schermer, A. *et al.* Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* **103,** 49–62 (1986).

20. Dua, H. S., Joseph, A., Shanmuganathan, V. A. & Jones, R. E. Stem cell differentiation and the effects of deficiency. *Eye (Lond)* **17,** 877–885 (2003).

21. Collinson, J. M., Chanas, S. A., Hill, R. E. & West, J. D. Corneal development, limbal stem cell function, and corneal epithelial cell migration in the Pax6(+/-) mouse. *Investigative Ophthalmology & Visual Science* **45,** 1101–1108 (2004).

22. Shortt, A. J., Secker, G. A., Notara, M. D. & Limb, G. A. Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. *Survey of …* (2007).

23. Espana, E. M., Grueterich, M., Ti, S. E. & Tseng, S. Phenotypic study of a case receiving a keratolimbal allograft and amniotic membrane for total limbal stem cell deficiency. *Ophthalmology* (2003).

24. Kenyon, K. R. & Tseng, S. C. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* **96,** 709–22– discussion 722–3 (1989).

25. Ordonez, P. & Di Girolamo, N. Limbal Epithelial Stem Cells: Role of the Niche Microenvironment. *Stem Cells* **30,** 100–107 (2012).

26. Ruberti, J. W. & Roy, A. S. Corneal biomechanics and biomaterials. *Annual review of …* (2011).

27. Echevarria, T. J. & Di Girolamo, N. Tissue-Regenerating, Vision-Restoring Corneal Epithelial Stem Cells. *Stem Cell Rev and Rep* **7,** 256–268 (2011).

28. DelMonte, D. W. & Kim, T. Anatomy and physiology of the cornea. *Journal of Cataract & Refractive Surgery* (2011). doi:10.1016/j.jcrs.2010.12.037

29. Schofield, R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells* (1977).

30. Davanger, M. & Evensen, A. Role of the pericorneal papillary structure in renewal of corneal epithelium. (1971).

31. Di Girolamo, N. Stem cells of the human cornea. *Br Med Bull* (2011). doi:10.1093/bmb/ldr026

32. Li, W., Hayashida, Y., Chen, Y.-T. & Tseng, S. C. Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res* **17,** 26–36 (2007).

33. Santos, M. S. *et al.* Survival Analysis of Conjunctival Limbal Grafts and Amniotic Membrane Transplantation in Eyes With Total Limbal Stem Cell Deficiency. *American Journal of Ophthalmology* **140,** 223.e1–223.e9 (2005).

34. Schwab, I. R. Cultured corneal epithelia for ocular surface disease. *Transactions of the American Ophthalmological …* (1999).

35. Rama, P., Bonini, S., Lambiase, A. & Golisano, O. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency1. *…* (2001).

36. Rama, P., Matuska, S. & Paganoni, G. Limbal stem-cell therapy and long-term corneal regeneration. *… England Journal of …* (2010). doi:10.1056/NEJMoa0905955)

37. Pellegrini, G. *et al.* Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* **349,** 990–993 (1997).

38. Secker, G. A. & Daniels, J. T. Limbal epithelial stem cells of the cornea. (2009). doi:10.3824/stembook.1.48.1

39. Sangwan, V. S., Basu, S. & Vemuganti, G. K. Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. *British Journal of …* (2011). doi:10.1136/bjophthalmol-2011-300352

40. Barrandon, Y. & Green, H. Three clonal types of keratinocyte with different capacities for multiplication. *PNAS* **84,** 2302–2306 (1987).

41. Pellegrini, G. *et al.* Location and Clonal Analysis of Stem Cells and Their Differentiated Progeny in the Human Ocular Surface. *J Cell Biol* **145,** 769–782 (1999).

42. Pellegrini, G. *et al.* Biological parameters determining the clinical outcome of autologous cultures of limbal stem cells. *Regenerative Medicine* **8,** 553–567 (2013).

43. Limb, G. A. & Daniels, J. T. Ocular regeneration by stem cells: present status and future prospects. *Br Med Bull* **85,** 47–61 (2008).

44. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **126,** 663–676 (2006).

45. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131,** 861–872 (2007).

46. Trounson, A. The Production and Directed Differentiation of Human Embryonic Stem Cells. *Endocrine Reviews* **27,** 208–219 (2006).

47. Okita, K., Ichisaka, T. & Yamanaka, S. Generation of germline-competent induced pluripotent stem cells. *Nature* (2007).

48. Yu, J. *et al.* Human induced pluripotent stem cells free of vector and transgene sequences. (2009). doi:10.1126/science.1172482

49. Mikhailova, A., Ilmarinen, T., Uusitalo, H. & Skottman, H. Small-Molecule Induction Promotes Corneal Epithelial Cell Differentiation from Human Induced Pluripotent Stem Cells. *Stem cell reports* (2014).

50. Fini, M. E. & Stramer, B. M. How the cornea heals: cornea-specific repair mechanisms affecting surgical outcomes. *Cornea* **24,** S2–S11 (2005).

51. Hayashi, R. *et al.* Generation of Corneal Epithelial Cells from Induced Pluripotent Stem Cells Derived from Human Dermal Fibroblast and Corneal Limbal Epithelium. *PLoS ONE* **7,** e45435 (2012).

52. Li, D. Q. & Tseng, S. C. Differential regulation of cytokine and receptor transcript expression in human corneal and limbal fibroblasts by epidermal growth factor, transforming growth factor- …. *Investigative Ophthalmology & Visual Science* (1996).

53. Wilson, S. E. *et al.* The corneal wound healing response: cytokine-mediated interaction of the epithelium, stroma, and inflammatory cells. *Prog Retin Eye Res* **20,** 625–637 (2001).

54. Stapleton, W. M., Chaurasia, S. S. & Medeiros, F. W. Topical interleukin-1 receptor antagonist inhibits inflammatory cell infiltration into the cornea. *Experimental eye …* (2008).

55. Perrella, G., Scott, C. A., Spelat, R., Brusini, P. & Dua, H. S. Cultured Human Keratocytes from the Limbus and Cornea Both Express Epithelial Cytokeratin 3: Possible Mesenchymal-Epithelial Transition. *International Journal of Ophthalmic Pathology* **01,** (2012).

56. LATVALA, T., PUOLAKKAINEN, P., VESALUOMA, M. & TERVO, T. Distribution of SPARC Protein (Osteonectin) in Normal and Wounded Feline Cornea. *Experimental Eye Research* **63,** 579–584 (1996).

57. Proteomic analysis of soluble factors secreted by limbal fibroblasts. (2006). at <http://www.molvis.org/molvis/v12/a55/v12a55-shimmura.pdf>

58. Wilson, S. E., Chaurasia, S. S. & Medeiros, F. W. Apoptosis in the initiation, modulation and termination of the corneal wound healing response. *Experimental Eye Research* (2007).

59. Hirosawa-Takamori, M., Lim, H. W., Yagi, S. & Shiota, K. Epigenetics for Biomedical Sciences. *Cornea* (2009). doi:10.1097/ICO.0b013e3181ae96bf

60. Daniels, J. T., Dart, J. & Tuft, S. J. Corneal stem cells in review. *Wound repair and …* (2001).

61. Kruse, F. E. & Tseng, S. C. [Differing regulation of proliferation of limbus and corneal epithelium caused by serum factors]. *Der Ophthalmologe: Zeitschrift der Deutschen …* (1993).

62. Hong, H. *et al.* Suppression of induced pluripotent stem cell generation by the p53-p21 pathway. *Nature* **460,** 1132–1135 (2009).

63. Spike, B. T. & Wahl, G. M. p53, Stem Cells, and Reprogramming Tumor Suppression beyond Guarding the Genome. **2,** 404–419 (2011).

64. Ross, P. J., Suhr, S. T. & Rodriguez, R. M. Human-induced pluripotent stem cells produced under xeno-free conditions. *Stem cells and …* (2009).

65. Rasmussen, A. *Formation of Induced Pluripotent Stem Cells and Ocular Regenerative Medicine–An Optimization Approach*. 1–48 (2013).

66. Ozgenc, A. Generation of Human Induced Pluripotent Stem Cells (hiPSCs) from Fibroblasts using Episomal Vectors. 1–15 (2012).

67. Utsuno, K., Tsuboi, M. & Katsumata, S. Viewing of complex molecules of ethidium bromide and plasmid DNA in solution by atomic force microscopy. *CHEMICAL AND …* (2001).

68. Dröge, P. & Cozzarelli, N. R. Topological structure of DNA knots and catenanes. *Meth. Enzymol.* **212,** 120–130 (1992).

69. Addgene. Lentiviral Packaging Plasmids. *Addgene.org* at <http://www.addgene.org/lentiviral/packaging/>

70. Okita, K. Stem Cell Reprogramming Tools | Life Technologies. *Lifetechnologies.com* at <http://www.lifetechnologies.com/dk/en/home/life-science/stem-cell-research/stem-cell-engineering-reprogramming.html>

71. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad Ser B Phys Biol Sci* **85,** 348–362 (2009).

72. Woltjen, K. *et al.* piggyBac transposition reprograms fibroblasts toinduced pluripotent stem cells. *Nature* **458,** 766–770 (2009).

73. Thier, M., Münst, B., Mielke, S. & Edenhofer, F. Cellular Reprogramming Employing Recombinant Sox2 Protein. *Stem Cells International* **2012,** 1–10 (2012).

74. Zhou, H. *et al.* Generation of Induced Pluripotent Stem Cells Using Recombinant Proteins. *Cell Stem Cell* (2009). doi:10.1016/j.stem.2009.04.005

75. Ahmad, 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* **25,** 1145–1155 (2007).

76. Kozmik, Z. Pax genes in eye development and evolution. *Current opinion in genetics & development* (2005).

77. Secker, G. A. & Daniels, J. T. Corneal Epithelial Stem Cells: Deficiency and Regulation. *Stem Cell Rev and Rep* **4,** 159–168 (2008).