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Generation of keratinocyte-derived induced pluripotent stem cells using a novel hair sampling platform

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Meiner Geliebten und meinen Kindern gewidmet.

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%	Percentage
°C	Degree Celsius
CA	Cancer antigen
CDL	Chemically defined lipids
cm	Centimeter
CO_2	Carbon dioxide
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DP	Dermal papilla
e.g.	For example (Exempli gratia)
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FTDA	FGF2, TGFβ1, Dorsomorphin, Activin A
g	Gravitational acceleration ~ 9.806 m/s^2

G-CSF	Granulocyte colony-stimulating factor
h	hour
hESC	Human embryonic stem cell
hiPSC	Human induced pluripotent stem cell
hSC	Human stem cell
HKGS	Human keratinocyte growth supplement kit
HSA	Human serum albumin
iPSC	Induced pluripotent stem cell
IRS	Inner root sheath
ITS	Insulin-transferrin-selenium
K-SFM	Keratinocyte serum-free medium
KO	Knock out
MEF	Mouse embryonic fibroblasts
MG	Matrigel
$MgCl_2$	Magnesiumdichloride
mRNA	Messenger RNA
miRNA	Micro RNA
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
MOI	Molecules of infection
Mx	Matrix

µl	Microliter
µm	Micrometer
µM	Micromolar
NaCl	Sodiumchloride
NEAA	Non-essential amino acids
O_2	Oxygen
ORS	Outer root sheath
PCR	Polymerase chain reaction
PBS	Phosphate-buffered saline
PFA	Polyformaldehyde
qPCR	Quantitative polymerase chain reaction
REF	Rat embryonic fibroblasts
RNA	Ribonucleic acid
s	Second(s)
SKG	Super Keratinocyte Gel
S.E.M.	Standard error of the mean
TGF	Transforming growth factor
TS SeV	Temperature sensitive Sendai virus
U	Turns
UV	Ultraviolett

1 Introduction

1.1 Motivation, background and main aim of this project

Our latest research has been focused on the development of tools for disease modeling like the human retina-on-a-chip platform (Achberger et al. 2019), the use of a 3D embryoid body to create a stable microvascular network (Pars et al. 2021) or the immunocompetent choroid-on-chip platform (Cipriano et al. 2022), as well as personalized medicine approaches like individual gene vector efficiacy testing (Achberger et al. 2021). In this context we faced, as well as many other research groups, the problems associated with sample collection. Not only finding donors is one of these problems but also the method to get the corresponding tissue and the transport are some of the key problems. The motivation for this project was to try to overcome some of these problems by choosing the Super Keratinocyte Gel (SKG) prototype, developed 2017 by Markus Stoll of Techno Plastic Products, and use it as platform for hair sample collection, storage and keratinocyte culturing as well as adapting the reprogramming protocol of Aasen and Belmonte 2010 to reprogram the keratinocytes growing out of the collected and stored hair into human induced pluripotent stem cells (hiPSC). We referred to the adapted reprogramming protocol of Aasen and Belmonte as SKG protocol. Keratinocytes were chosen because of their great potential and our advanced experience with them (Wüstner et al. 2022).

The main idea was to develop an easy to use sample platform for a sampling procedure with minimal invasiveness and time consumption to allow sampling in various different nations and settings. We furthermore wanted to find an easy way for sample transport with minimal negative effects on the samples. Additionally, we wanted to create the SKG reprogramming protocol by modifying the well established reprogramming protocol of Aasen and Belmonte 2010. In this cause we chose to use the Sendai virus as delivering vector system (Li et al. 2000). We wanted to optimize the SKG protocol in terms of time

consumption and efficiacy.

1.2 Stem cell types and their characteristics

Stem cells are a crucial part of most organisms and tissues as their main characteristic is their ability to differentiate into various functional cell types (Till and McCulloch 1961; Becker et al. 1963; Wu et al. 1968). This allows stem cells, when being exposed to a certain microenvironment and stimulating factors, to form, maintain and even regenerate different tissues (Siminovitch et al. 1963; Visser et al. 1984). In humans mainly two types of stem cells are distinguished. The embryonic stem cells (ESC) were first isolated in 1998 and are pluripotent which means they can form all three germ layers, thereby having the ability to form the whole organism (Thomson et al. 1998; Doetschman et al. 1985). The adult stem cells were found shortly after the ESC and they are multipotent which means they can form a specific subset of cells but not all cell types of the organism. Due to this specification adult stem cells can be found in various tissues where they are essential for maintenance and repair of the corresponding tissue such as mesenchymal stem cells (e.g. repairing and reinforcing bone and muscle tissue), hematopoetic stem cells (e.g. constantly replacing erythrocytes, leukocytes and platelets), gastrointestinal stem cells (e.g. constantly replacing the epithelial cells to keep up the barrier function) and adipose stem cells (Ferrari et al 1998; Dezawa et al. 2005; Bruder et al. 1998; Benayahu et al. 1989; Zuk et al. 2001; Zuk et al. 2002; Barker et al. 2007; Mills and Gordon 2001). The latest research has revealed a third fraction, the induced pluripotent stem cells (iPSC) which exhibit the pluripotency of ESC without the ethically questionable origin (Takahashi and Yamanaka 2006). The third fraction is also the youngest and can be obtained by changing the gene expression profile of a somatic cell towards an ESC like profile. Induced pluripotent stem cells (iPSC) were generated for the first time in 2006 (Takahashi and Yamanaka 2006). The first major advantage of the iPSC compared to the ESC origins in their generation: Many adult somatic cells from

a human can serve as initial cell population which will subsequently be transformed into human iPSC (hiPSC) (Zhou et al. 2012; Ramme et al. 2021; Perriot et al. 2022). Therefore, these cells circumvent the ethical questions which arise in the course of ESC extraction from living embryos. Furthermore, it is a lot easier to isolate possible host cells for the reprogramming from the (adult) donor (Ramme et al 2019; Shi et al. 2022; Okita et al. 2011; Lichtner et al. 2015).

1.3 Applications of stem cells in research and medicine

The main target of stem cell research is the idea to have the possibility to differentiate these cells into any cell type necessary for the patient. Figure 1 shows the principal approach of hiPSC generation and their ability to re-differentiate into cells of all three germ layers. Thereby patient specific tissues and even organoids can be cultured to allow more individual research and treatment of patients and their diseases (Sato et al. 2021; Fortune et al. 2022; Imamura et al. 2021; Morizane et al. 2015; Lancaster et al. 2013). Of course such possibilities must not lead to an exclusion of the ethical justification for any approach or application (Bredenoord et al. 2017; Boers and Bredenoord 2018).

The first cells to be obtained by induced differentiation of human embryonic stem cells were neural progenitor cells (Zhang et al. 2001; Reubinoff et al. 2001). Also, embryonic stem cells were used to create hepatocytes (Touboul et al. 2010), pancreatic cells (Kelly et al. 2011) as well as endothelial cells (James et al. 2010).

Another crucial point, especially for basic medical and also clinical research, can be seen in the genetic expression profile. As the generated hiPSC carry the same genome as the person they are taken from, the impairments causing genetic diseases and disabilities are present in the hiPSC generated from cell fractions of affected individuals. This application has led to new insights in the research of hereditary diseases like Parkinson's disease (Reinhardt et al. 2013) or Niemann Pick Type C (Maetzel et al. 2014) and might offer another approach

for an individual therapy of patients suffering from hereditary diseases.

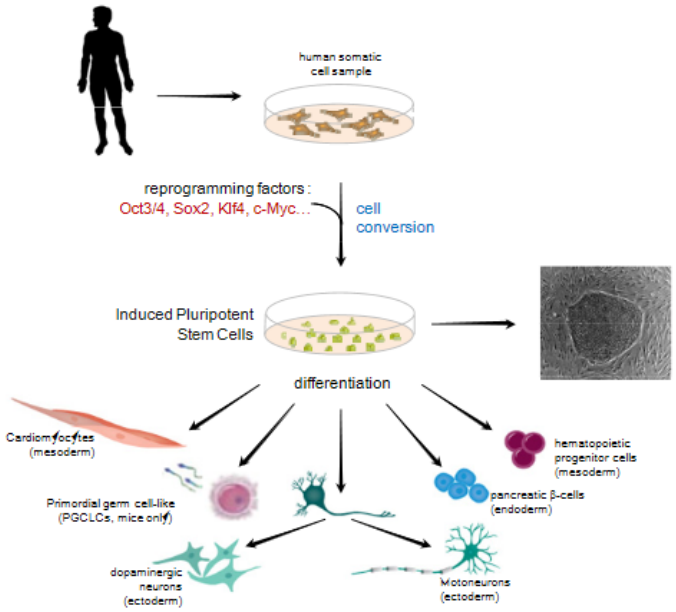

Figure 1: Overview of hiPSC generation and their differentiation potential. The arising hiPSC can differentiate into various cell types. Taken and adapted from Bayart and Cohen-Haguenauer 2013.

The downside of all pluripotent cells remains their tumorigenicity. The first hiPSC were generated using four transcription factors carried by a virus: SOX2, OCT4, c-MYC and KLF4 (Takahashi et al. 2007). The oncogenic potential of c-MYC is already well established, clearly outlining its involvement in tumor genesis. c-MYC itself is a transcription factor and able to regulate all three human RNA polymerases (Gomez-Roman et al. 2006). Furthermore it controls expression levels of ribonucleases and nucleolar proteins essential for ribosome synthesis (Schlosser et al. 2003). It was shown that about 20% of neuroblastomas show increased levels of c-MYC and MYCN and that elevated levels of these are associated with tumor progression and poor overall survival (Westermann et al. 2008; Huang et al. 2011). In the case of KLF4 a higher rate of esophageal and colon cancers has been reported (Lambertini et al. 2010; Rageul et al. 2009; Tian et al. 2010). KLF4 is a transcription factor which is essential for differentiation and cell specific functions, e.g. it was shown to be

necessary for establishing the skins barrier function (Segre et al. 1999). SOX2 (along with SOX1 and SOX3) is a transcription factor which was shown to prevent neural progenitor cells from differentiation in the process of neurogenesis (Bylund et al. 2003). It is also associated with adenocarcinoma of the lung as well as cervical cancer as the overexpression of SOX2 is associated with increased proliferation and clonogenicity (Sholl et al. 2010; Ji and Zheng 2010). OCT4 overexpression is known to increase the rate of ovarian, gastric and breast cancer (Peng et al. 2010; Asadi et al. 2010; Wang et al. 2010). The transcription factor OCT4 is known to be essential for self renewal and pluripotency of stem cells, furthermore three different variants, depending on the splicing, can be expressed, namely OCT4A, OCT4B and OCT4B1 (Atlasi et al. 2008; Cauffman et al. 2006; Lee et al. 2006).

1.4 Possible cell sources

Nearly every somatic cell population carrying a nucleus shows the ability to be transformed into hiPSC. Interestingly, the research groups around the world use a lot of different cell populations to generate hiPSC for further projects. The archetype and most frequently used cell population are fibroblasts which can be gathered from various tissues, for example skin biopsies (Takahashi and Yamanaka 2006). Human blood can be used to generate hiPSC but of course the erythrocytes are unfavorable in this case as they lack the nucleus and therefore have lost the mitotic potential. Instead, the CD34+ stem cells within the blood, normally replenishing the white blood cells, can be used as source for the hiPSC generation (Loh et al. 2009). Others possibilities are e.g. to extract exfoliated renal tubular cells from urine samples (Zhou et al. 2011), isolate epithelial cells within human breast milk (Tang et al. 2019) or make use of human hair root- derived epidermal stem cells: the keratinocytes (Aasen et al. 2008).

1.4.1 Fibroblasts

Fibroblasts are the dominant cell fraction in the connective tissue. Their main task is the production of the components of the extracellular matrix (ECM), mainly the various types of collagen (Booth et al. 1980; Lichtenstein et al. 1975; Ujtto et al. 1980). The connective tissue itself mainly consists of ECM, so the fibroblasts are located within as solitary cells surrounded by a lot of ECM. Another major task of these cells is the generation of scar tissue in the late phase of wound healing (Driskell and Watt 2015; Thulabandu et al. 2018). After the initial damaging event and the subsequent necrotic and apoptotic deaths of the affected cells, the surrounding tissue, e.g. the adipoyctes of the dermis, mediates an inflammatory reaction, thereby recruiting macrophages and Fibroblasts to the site (Schmidt and Horsley 2013). They start the phagocytosis of the detritus (remains of the cells destroyed during the damaging event or the subsequent inflammation) (Shook et al. 2016). This leaves an area free of tissue, so the macrophages attract fibroblasts to the very same which in turn start proliferation and production of ECM to fill this gap (Gurtner et al. 2008; Schmidt and Horsley 2013). Interestingly it could be shown that the suppression of Engrailed-1 leads to fibroblast mediated wound healing without scar tissue formation (Mascharak et al. 2021). Even within the skin different subpopulations of fibroblasts can be found, e.g. the papillary and the reticular fibroblasts which differ in their proliferation capacity (Harper and Grove 1979). This understanding was pushed further by one group as the authors were able the show that the fibroblasts origin from two different lineages. The upper lineage forms the upper dermis with the dermal papilla and the arrector pili muscle as well as the hair follicle. The lower lineage builds the lower dermis with the reticular fibroblasts as producers of the ECM and the adipocytes of the hypodermis (Driskell et al. 2013). The two lineages show different gene expression profiles too (Janson et al. 2012).

1.4.2 Blood cells

The human blood is another viable cell source although numerous different cell types are addressed, such as erythrocytes, B-lymphocytes, T-lymphocytes, monocytes, neutrophils, macrophages, erythroblasts among others. The problem of isolation has been solved as numerous protocols for the extraction of a specific cell fraction have been established (Dagur and McCoy 2015). It is therefore essential to differentiate the main cell fractions and the exact corresponding source: Human umbilical cord blood where the endothelial cells of the vein and the arteries are extracted (Haase et al. 2009), CD34+ cells mobilized from peripheral blood or the bone marrow (or either taken directly from the bone marrow) via stimulation (Loh et al. 2009) and mature T-cells as well as myeloid cells (mainly neutrophile granulocytes and monocytes) taken from blood samples of adult patients (Staerk et al. 2010). Even the erythroblasts, precursor cells of erythrocytes, can be used for hiPSC generation (Perriot et al. 2022).

1.4.3 Keratinocytes

1.4.3.1 Characteristics

Their main task is to protect the viable tissues from physical or chemical irritation, evaporation of water and the invasion of possible pathogens (Madara 1998; Roop 1995).

Keratinocytes produce a certain type of intermediary filaments called Keratin. Different isoforms of Keratin control the localization of hemidesmosomes and thereby allow the formation of rigid cell layers (Seltmann et al. 2013). Futhermore these Keratin filaments also control the dynamic and stability of desmosomes (Loschke et al. 2016). Intermediary filaments are chains of proteins and belong to the cytoskeleton. Their task is to provide elasticity to the cell by linking the different opposite sides of the membrane (Al-Amoudi et al. 2004; Hatzfeld et al. 2017). If the cell now changes in shape due to external

forces, it will quickly regain its previous appearance as soon as these forces no longer apply (Garrod et al. 2005; Hatzfeld et al. 2017; Ramms et al. 2013).

To create a barrier which is able to prevent small organisms or chemicals from penetration keratinocytes use protein complexes to form close cell-cell connections, thereby reducing intercellular space to a minimum (Büchau et al. 2022; Kowalczyk et al. 1999; Thomason et al. 2010). The barrier function of the human skin is often monitored using the transdermal water loss (Alexander et al. 2018; Fluhr et al. 2006; Montero-Vilchez et al. 2021).

1.4.3.2 Location

The human skin consists of different layers of tissue, each composed of a distinct mix of cell fractions (Fuchs and Raghavan 2002). The surface layer facing the outside is the epidermis, followed by the dermis and the subcutis.

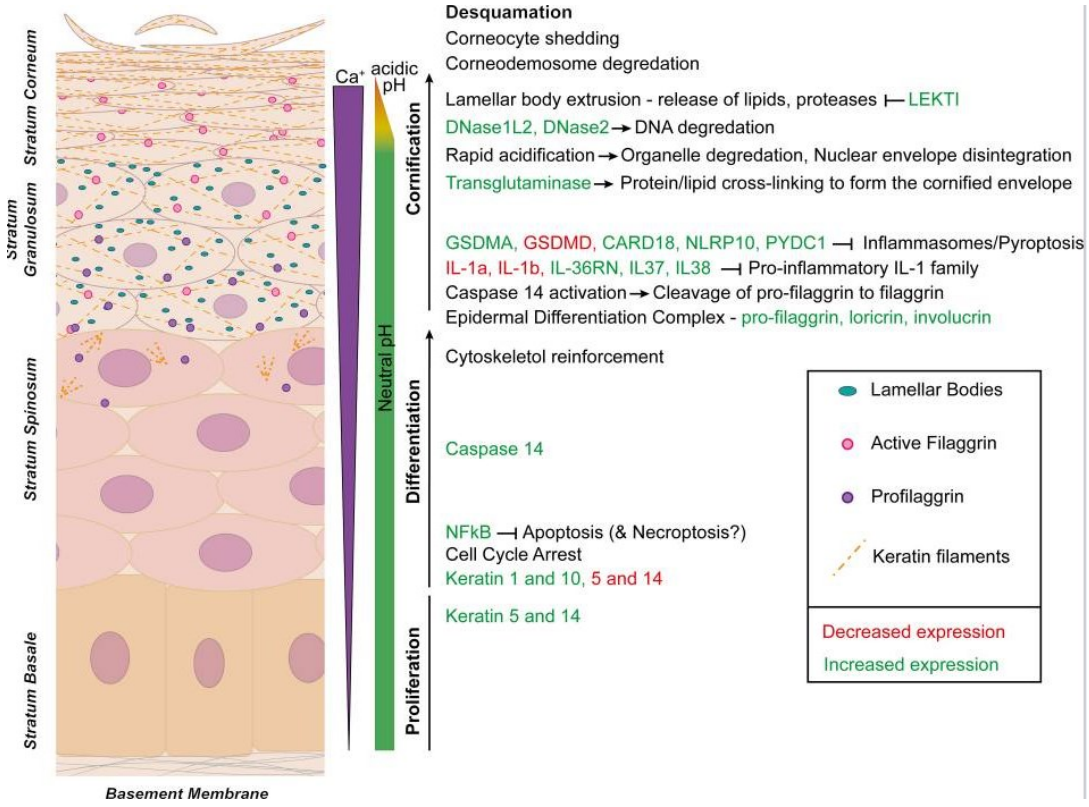

Figure 2: Depiction of epidermal structure and differentation. The epidermis consists of four different layers. The stratum basale at the bottom, connected to the basement membrane, consists of highly proliferative cells which give rise to the keratinocytes of all

other layers by asymmetrical cell division. The next layer is the stratum spinosum build up by keratinocytes closely connected via thight junctions. The name refers to the microscopic appearance of the spindle like shape of the cells. The next layer is the stratum granulosum where organelles and nuclei start to condense. The last layer is the stratum corneum which is composed of terminally diffentiated keratinocytes which are no longer viable cells, called the corneocytes. This layer is usually the thickest and forms the final barrier of the skin. Taken and adapted from Anderton and Alqudah 2022.

The epidermis is mainly built up by keratinocytes, with a few melanocytes located at the basal layer, and is further divided into four layers (Selby 1955). All layers consist of keratinocytes with very little ECM and reflect the proliferation steps of the keratinocytes in the epidermis during their journey towards the top layer (Selby 1955). Figure 2 displays the different layers, the differentiation steps of the keratinocytes as well as the contributing factors. The bottom layer is the *stratum basale*, located on top of the basal membrane, where the basal epidermal cells are constantly asymmetrically dividing to not only create committed keratinocytes but also maintain the number of basal epidermal cells (Lechler and Fuchs 2005). Interestingly in the embryonic development of the epidermis in mice it was shown that from the beginning of stratification until keratinization about 75-85% of the mitotic figures were horizontally oriented in the basal layer in contrast to 75-85% of the mitotic figures in the suprabasal layer being vertically oriented. It was concluded that during this time the suprabasal layer recruits daughter cells via mitosis of suprabasal cells while in the established epidermis the basal cells give rise to new daughter cells for the suprabasal layer (Smart 1970). This is the only layer in which melanocytes can be found, they are located solely between the keratinocytes and, in contrast to them, keep their position (Hashimoto et al. 1966; Kropp 1957). The *stratum spinosum* is the next layer and consists of the keratinocytes produced by the *stratum basale*. The name refers to the spindle-like shape of the keratinocytes. Here the keratinocytes form close connections using tight junctions (Brandner et al. 2002; Kirschner and Brandner 2012). Now they reach the *stratum granulosum* where they start to condense their organelles and nuclei in

intracellular vesicles (Odland 1960). These are visible through the microscope and have given this layer its name. The last layer is also usually the thickest one among these four with about 10 to 20 µm, the *stratum corneum* (Menon et al. 2012). The keratinocytes in here have finished proliferating and are no longer viable cells (Lippens et al. 2005). It was shown that the Keratin filaments are arranged in a cubic-like rod packing geometry to allow dehydration and reduced cell volume of the corneocytes (Norlén and Al- Amoudi 2004). The cell volume is reduced by roughly 50% (Al-Amoudi et al. 2004). This is achieved mainly by dehydration as the intracellular water decreases from about 70% to about 40% (Caspers et al. 2001). About 85% of the intracellular protein mass of corneocytes is build up by Keratins, in contrast to about 30% in viable Keratinocytes (Sun and Green 1978). The corneocytes keep tight connections via corneodesmosomes (Robinson et al. 1997; Simpson et al. 2011). They also have lost their organelles and nuclei (Wier et al. 1971).

The second place to find keratinocytes is human hair, where their main task is to produce keratin for the growth of hair. Three main types of hair can be distinguished: Lanugo hair, vellus hair and terminal hair. While lanugo hair is a special type only expressed during the first stages of life and therefore can usually only be found on newborns (Gareri and Koren 2010), vellus hair is the first type of hair to arise during childhood. The vellus hair then transforms androgen dependent into terminal hair (Stohr 1904).
A hair consists of the shaft, being the greatest part of it, the root and its' surrounding cell layers (Birbeck and Mercer 1957a). These layers all together are called the hair follicle which is the center of hair growth and regeneration (Birbeck and Mercer 1957b). Figure 3 shows the anatomy of the hair as well as the embryologic development. One of these layers is the outer root sheath (ORS) as it not only contains matrix keratinocytes to promote hair growth but also stem cells to replenish the matrix keratinocyte population (Amoh et al. 2009). Interestingly, these stem cells can contribute to wound healing but do not influence the homeostasis of the epidermis (Ito et al. 2005). The differentiation

of the outer root sheath and the stem cell compartment is highly dependent on various factors such as SOX9 expression or β-Catenin expression (Vidal et al. 2005; Huelsken et al. 2001). As hair is composed of keratinocytes and keratin, it belongs to the epidermis, even though the follicle and the root are located deeper within the skin (Cotsarelis 2006).

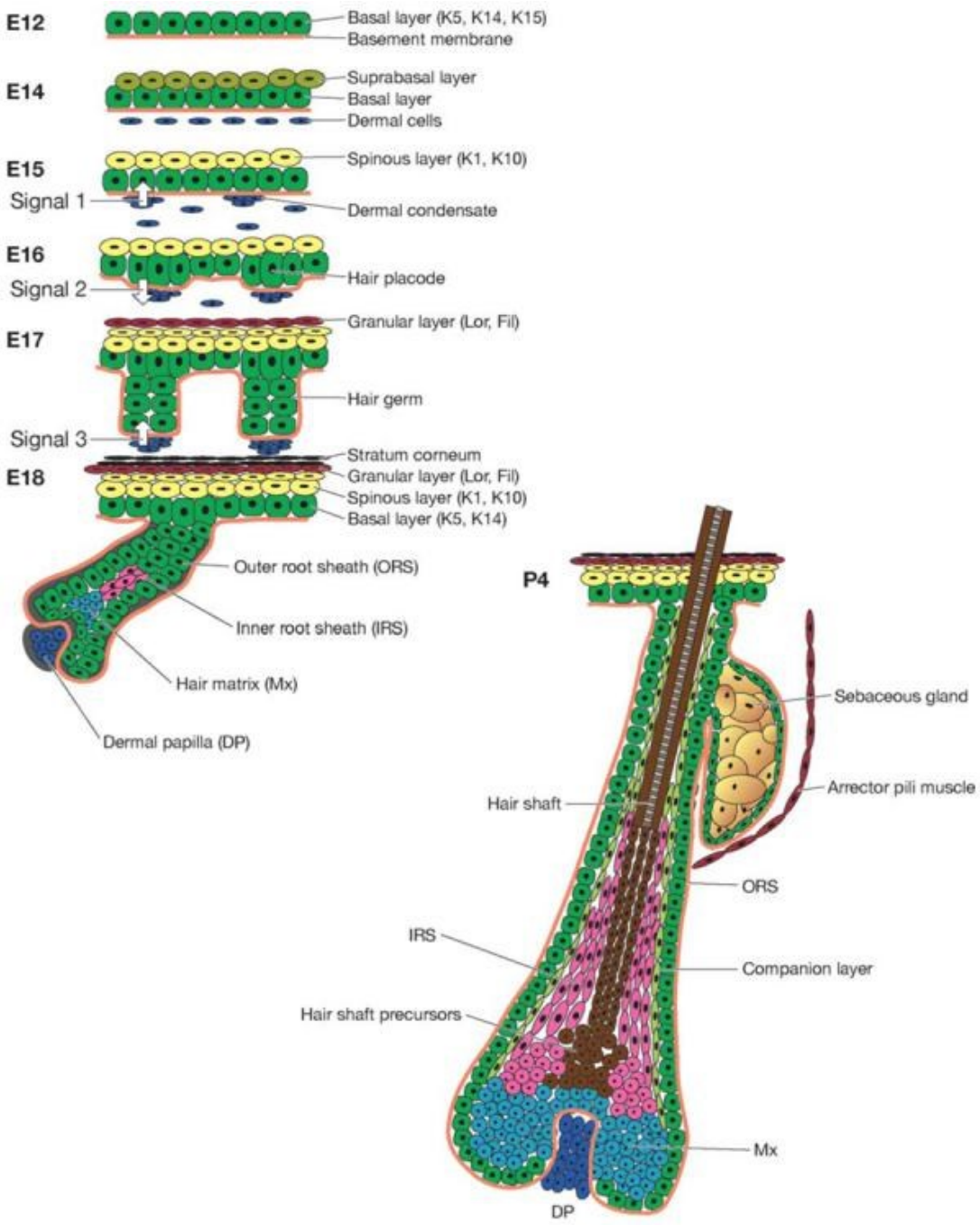

Figure 3: Hair follicle development and final structure. Skin and hair arise from the ectodermal layer. During embryogenesis while the different layers of the epidermis are formed, some cells get the signal to differentiate into hair follicles (Signal 1). In turn a signal is send to the underlying dermis (Signal 2) to form the dermal papilla (DP). With the progress of the development a signal is sent from the DP to the hair follicle to promote the differentiation of the different cell lines of the resulting hair follicle (Signal 3). The final hair follicle is composed of the hair shaft at its center, surrounded by layers

of cells forming the inner root sheath (IRS). The IRS is enclosed by the companion layer, followed by the the basal cell layer and the basement membrane, together called the outer root sheath (ORS), to which additive structures like sebaceous glands (Lubrication) or the arrector pili muscle (Movement) are linked. At the base of the follicle a pool of cells is located, refered to as the matrix (Mx), which regenerate the different cell fractions, especially the hair shaft and the IRS. Taken and adapted from Blainpain and Fuchs 2006.

Like most other tissues in the body, human hair is a rather dynamic tissue, reflected by different stages through which hair cycles. These stages are the anagen stage, the catagen stage, the telogen stage and the exogen stage. Together they form the so-called hair follicle cycle as visible in figure 4 (Cotsarelis et al. 1990; Lee and Tumbar 2012; Blanpain and Fuchs 2006).

The anagen stage is the growth phase in which the matrix keratinocytes proliferate fast and produce keratin to elongate the hair shaft. These matrix keratinocytes reside in the bulb of the hair. The duration of this phase not only varies between species but also between hair of different length as human eyebrow hair usually stays in this stage for 2-4 weeks whereas scalp hair can prolong this stage for a couple of years (Cotsarelis 2006).

Finally, the matrix keratinocytes stop their proliferation and end the growth of the hair shaft. This is the catagen stage, also defined by a regression of the hair follicle and the bulb (Kligman 1959).

This leads to the telogen stage, the resting phase, characterized by a degenerated root sheath and no growth of the hair shaft (Blanpain et al. 2004).

The exogen stage describes a phase combining the regeneration of the hair follicle, the subsequent formation of a new hair shaft and the necessary removal of the previously formed hair by this newly formed hair shaft by simply pushing the older hair shaft through the tunnel it used to reach the surface (Milner et al. 2002). The regenerative capacity of the dermal papilla depends on Wnt signaling (Kishimoto et al. 2000).

This stage is followed by the anagen stage. It could be shown that hair follicle growth and regeneration depend on epithelial Wnt ligand secretion, Noggin as well as Notch1 (Myung et al. 2013; Botchkarev et al. 2001; Vauclair et al. 2005).

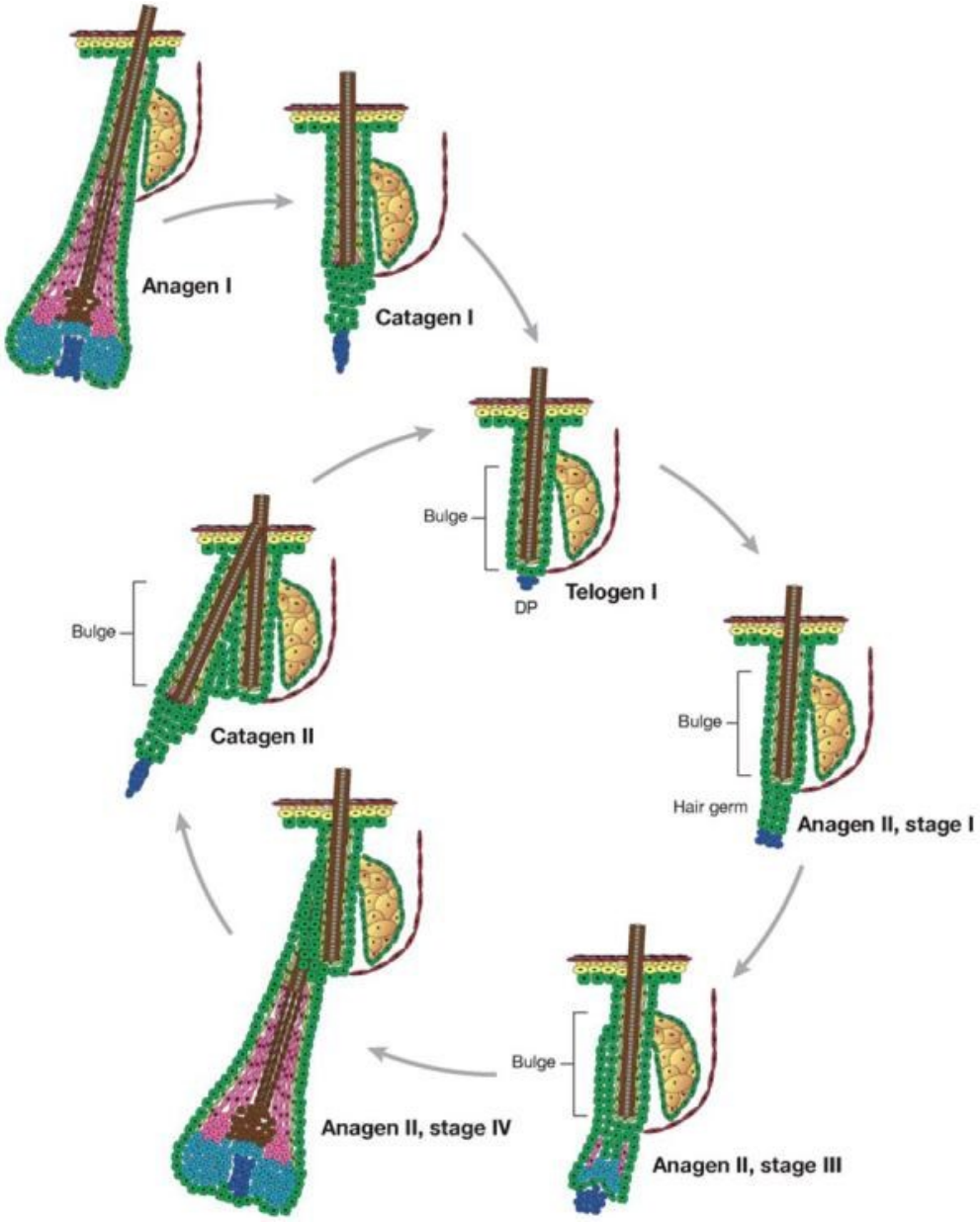

Figure 4: Depiction of the hair follicle cycle with its different stages. The hair follicle is located in a pocket called the bulge. Within this bulge the hair follicle stem cells are located which can give rise to a new follicle. In the anagen stage the hair grows and actively regenerates. After some time the hair will enter catagen stage, which leads to the degeneration of the hair, especially the lower two thirds. In this stage the old hair shaft rearranges into a club shape. The next phase is the telogen stage which is a period of rest with little cell activity. Taken and adapted from Blanpain and Fuchs 2006.

1.5 Reprogramming

The process of reprogramming can be achieved by various routes as seen in table 1. These different ways to reprogram the cells compete with each other in terms of efficiency, vector integration, oncogenic potential and work load.

In this contest the long-favored ways were the retroviral and lentiviral transduction (Takahashi et al. 2007; Novak et al. 2010). Other approaches involve modified mRNA (Warren et al. 2010), plasmids (Okita et al. 2008) or small molecules (Huangfu et. al. 2008; Shi et al. 2008). Another breakthrough was achieved by the employment of the Sendai virus as transfection vector (Fusaki et al. 2009).

The process of reprogramming keratinocytes takes 3 to 4 weeks if a lentiviral system is used (Streckfuss-Bömeke et al. 2013), whereas the initiation of the Sendai virus has reduced this time frame to 1 to 2 weeks (as visible in our results).

One of the Lentivirus vectors currently in use is named STEMCCA and shows a reprogramming efficiency of 0,1-1,5% (Somers et al. 2010). The reprogramming efficiency of the Sendai virus differs in the literature and seems to be cell dependent. Using fibroblasts, it is reported to be 0,077% (Schlaeger et al. 2015), others could yield 1% for fibroblasts and 0,1% for terminally differentiated T-cells in peripheral blood (Fusaki et al. 2009; Seki et al. 2010; Ban et al. 2011).

The transformation of keratinocytes into hiPSC has been successfully performed even with hair from donors older than 70 years (Streckfuss-Bömeke et al. 2013).

1.5.1 Reprogramming factors

To reprogram a cell means to change this cell type into another. By extraction of an oocyte nucleus and injection of a somatic cell nucleus instead it could be shown that the emerging cell has regained pluripotency as the oocyte cytoplasm changed the gene expression profile of the somatic cell nucleus (Gurdon et al. 1958; French et al. 2008). This led to an increased research

activity to uncover the transcription factors involved promoting the pluripotency of stem cells. Among others, four transcription factors were discovered, namely OCT4, KLF4, SOX2 and c-MYC (Niwa et al. 2000; Nichols et al. 1998; Li et al. 2005; Avilion et al. 2003; Cartwright et al. 2005). Based on these four transcription factors, the first hiPSC were generated by reprogramming fibroblasts (Takahasi et al. 2007). One year earlier the same group successfully reprogrammed mouse fibroblasts identically (Takahashi and Yamanaka 2006). Since then this mixture of transcription factors is known as the Yamanaka mix or the OSKM system and widely used in the scientific society. More transcription factors have been established such as LIN28 or NANOG during the course of implementing new reprogramming techniques (Yu et al. 2007). Table 1 illustrates the various reprogramming systems and the reprogramming factors used by each.

Table 1: Summary of reprogramming approaches.

This table lists the different possible reprogramming approaches regarding delivery method, reprogramming factor combination, with possible addition of boosting chemicals, with the corresponding cell type used. The last column refers to key papers describing this approach. O = OCT4, S = SOX2, K = hKFL4, M = c-MYC, L = LIN28, N = NANOG. Taken and adapted from Bayart and Cohen-Haguenauer 2013.

Delivery Method		Transgene	Chemicals	Cell type	PMID
Integrating Vectors	Retrovirus	OSKM	-	Fibroblast-like synoviocytes Fibroblasts, fibroblasts (primary: BJ)	18035408
	Polycistronic retrovirus	OSKM/OSK	-	Fibroblasts (foreskin, xeno-free primary culture)	19890879
	Lentivirus	OSLN	-	Fibroblasts (IMR90) Fibroblasts (neonatal foreskin)	18029452
	Inducible Lentivirus	OSKM/OSK	-	Bone marrow mesenchymal stem cells (diseased patients), fibroblasts (diseased patients)	18691744
		OSKM	-	Fibroblasts (fetal dermal) Fibroblasts (fetal lung)	20572011
		OSKMN/OSKM	-	Fibroblasts (differentiated from iPS), Fibroblasts (neonatal foreskin), keratinocytes	18786420
		OSKM	-	Peripheral blood myeloid cells peripheral blood T cells	20621045
		OSKMN		Fibroblasts Fibroblasts (secondary)	20569691
	Polycistronic lentivirus	OSKM	-	Fibroblasts	20682452
	Inducible polycistronic lentivirus	OSKM	-	Keratinocytes (foreskin)	19109433
	Inducible Plasmid	mir-302	-	Hair follicle cells	20870751
Excisable integrating vector	Excisable (LoxP) lentivirus	OSKM/OSK	-	Fibroblasts (skin of patients suffering from Parkinson disease)	19269371
	Excisable (LoxP) polycistronic lentivirus	OSK	-	Fibroblasts (adult humanized sickle cell anemia mouse)	19415770
	Excisable (FRT) polycistronic lentivirus	OSKM	-	Murine fibroblasts SC1	20385817
	PiggyBack Transposon	OSKM	-	Fibroblasts (embryonic)	19252477
			Butyrate	Fibroblasts (fetal lung, IMR90)	18511599
	Inducible PiggyBack Transposon	OSKM	-	Fibroblasts (embryonic)	19252478
	Sleeping Beauty	OSKM	-		Izsvak *et al.* 2011
Non-integrating vectors / pathways	Adenovirus	OSKM	-	Fibroblasts (IMR90)	19697349
	Sendaï virus	OSKM	-	Neonatal foreskin fibroblasts BJ Dermal fibroblasts	19838014
		OSKM	-	Terminally differentiated circulating T cell	20621043
	Lentivector (plasmid)	OSLN	MEK inhibitor	Fibroblasts (foreskin)	20682060
	EBV based plasmid	OSKMNL + TSV40 + shRNAp53	-	Fibroblasts (neonatal foreskin)	19325077
		OSKMNL + TSV40	-	Neonatal cord blood, adult peripheral blood mononuclear cells	21243013
		OSKL+L-Myc+ shRNAp53	-	Dermal fibroblasts, Dental pulp cell line	21460823
	Polycistronic plasmid	OSKM	poly(β-amino esters)	Fibroblasts (foreskin)	21285354
	Minicircles	OSLN	-	Adipose stem cells	20139967/ 21212777
	RNA	OSLN	-	Fibroblasts (foreskin)	20188704
		OSKML/OSKM	-	Fibroblasts (fetal lung), fibroblasts (fetal skin), Fibroblasts (foreskin) Fibroblasts (neonatal foreskin) Fibroblasts (skin from cystic fibrosis patient)	20888316
	Proteins	ES cell extracts	-	Mouse fibroblasts: primary cardiac and primary skin	20439621
		OSK/OSKM	VPA	Mouse fibroblasts	19398399
		OSKM		Neonatal fibroblasts	19481515

1.5.2 Reprogramming techniques

The first successful generation of hiPSC, employed fibroblasts and a retrovirus targeting the four factors SOX2, OCT4, KLF4 and c-MYC, yielded an efficiency of ~0,02% and took 30 days of transfection (Takahashi et al. 2007). Since this first advance, numerous reprogramming techniques have been researched and protocols have successfully been established, as visible in table 1. These methods can be further divided into three groups: Viral, genome integrating vector; viral, non-genome integrating vector; non-viral vector. Figure 5 gives an overview of the different technological options for iPSC induction.

The first group consists of retroviral and lentiviral transfection systems.

The second group composes of adenoviral systems and the Sendai virus.

The third division gathers around protein systems, mRNA and miRNA systems, PiggyBac, minicircle vectors and episomal plasmids.

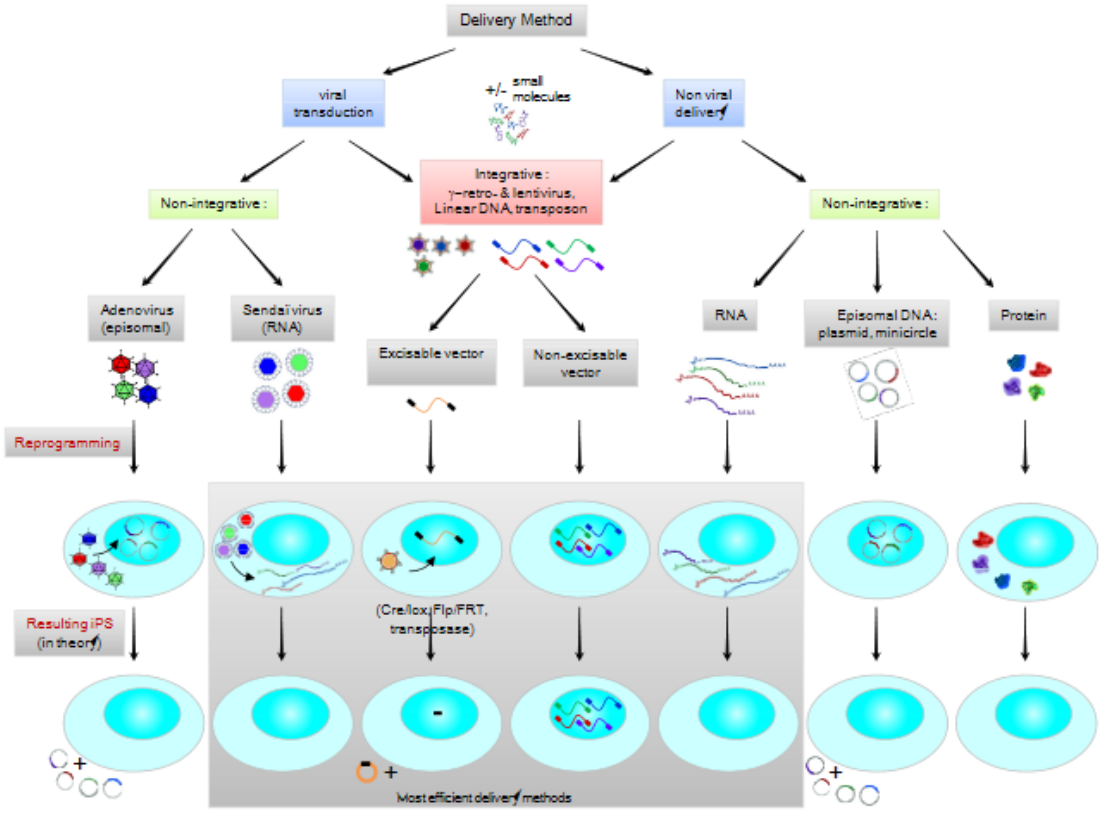

Figure 5: Overview of reprogramming techniques. This figure recapitulates the various

delivery methods for the iPSC generation. It also shows if there is a persistence of the genomic material used for the reprogramming. Taken and adapted from Bayart and Cohen-Haguenauer 2013.

1.5.2.1 Retroviral systems

A gamma-retrovirus was the first vector to be chosen for establishing a reprogramming system for the generation of hiPSC out of somatic cells (Takahashi et al. 2007). The name of this virus family arises from their ability to transcript their genome, which they transport as RNA, into DNA. This is possible because they express the reverse transcriptase enzyme which was discovered in 1970 (Baltimore 1970; Temin and Mizutani 1970).

Advantages of retroviral systems include a small genome which can easily be modified, various commercially available and cheap retroviral vectors and the vector itself being already specialized in infiltrating certain human somatic cells (Milone and O'Doherty 2018; Omole and Fakoya 2018; Rodríguez-Pizà et al. 2010; Stewart et al. 1982).

Drawbacks on the other side include a high oncogenic potential, insertion of tumor DNA into the host genome, the instability of the RNA genome and the limitation of the infiltration potential to dividing cells (Okita et al. 2007; Takahashi et al. 2007; Wernig et al. 2008). Also these viruses can only target dividing cells (Bukrinsky et al. 1993; Roe et al. 1993).

1.5.2.2 Lentiviral systems

The first lentiviral reprogramming system was applied in 2007 to fetal MRC5 lung fibroblasts and newborn BJ-1 foreskin fibroblasts. It relied on NANOG, LIN28, OCT4 and SOX2 as reprogramming factors and had an efficiency of 0,03-0,05% for fetal and 0,01% for newborn fibroblasts after 20 days of transfection (Yu et al. 2007). Lentiviral vectors are a subclass of retroviruses as they are derived from some retroviruses such as HIV-1 or HIV-2 (Naldini et al. 1996).

Therefore, lentiviruses exhibit the same advantages as retroviruses except they can target dividing as well as non-dividng cells, thereby greatly increasing the number of possible target cell types to be reprogrammed (Naldini et al. 1996; Omole and Fakoya 2018).The drawbacks still include the high oncogenic potential, the possible integration of the viral genome into the host genome and the instability of the RNA genome (Kafri et al. 1997; Carey et al. 2009; Sommer et al. 2009; Woods et al. 2003). Table 2 summarizes the comparison of this reprogramming technique towards others.

After these first steps concerns arose regarding security and applicability of lentiviral systems (Zhong et al. 2022). The applicability is hindered by the unbalanced stoichiometry coming from the four or more single reprogramming factors used in this protocol (Papapetrou et al. 2009). To overcome this disadvantage, a single cassette reprogramming vector was designed in which all factors are integrated, separated only by a self-cleavage peptide signal (Carey et al. 2009; Chang et al. 2009; Sommer et al. 2009). The issue of security refers to the integration of the lentiviral genome into the host genome leading to a genomic alteration along with an oncogenic potential. To address this concern some of the vectors were modified with LoxP sites for subsequent Cre-LoxP excision of the integrated vectors (Chang et al. 2009; Sommer et al. 2009). One of these vectors currently in use is named STEMCCA and shows a reprogramming efficiency of 0,1-1,5% (Somers et al. 2010). Furthermore safe harbours for integration of DNA have been described (Sadelain et al. 2011). The vector can also be safely deleted after reprogramming (Chang et al. 2009). However, it could be shown that the viral DNA must be expressed for at least 12 days to generate iPSC (Brambrink et al. 2008).

1.5.2.3 Adenoviral systems

Although they were used very early it was shown that delivered genes and their resulting products were only expressed for a short time, e.g. for 2-3 days,

before these cells were removed by cellular and humoral defense mechanisms (Dai et al. 1995; Tripathy et al. 1996). The capacity of the first generation vectors to carry DNA were usually limited to 8,3 kb (Bett et al. 1994). The elimination of the viral genes led to an increased capacity of these second generation vectors up to 35 kb of DNA and the transferred DNA showed a stable expression (Kochanek et al. 1996; Kumar-Singh and Chamberlain 1996). The main advantage of adenoviruses made them the next logical choice as they usually do not integrate their genome into the genome of the host cell. Furthermore, they use DNA to store information which is much more stable than RNA. One group could yield an infection efficiacy of 40-50% and iPSC colonies grew after 24 to 30 days in culture (Stadtfeld et al. 2008).

The use limiting drawback remains the unearthly low reprogramming efficiency of 0,0002% after a truckload of transfection cycles at a MOI of 250 (Zhou et al. 2009). In addition, an integration of the adenovirus genome into the host genome, preferentially into genes, at a very low likelihood was discovered (Harui et al. 1999; Stephen et al. 2009).

1.5.2.4 Sendai Virus Transfection

The Sendai virus (SeV) belongs to the *Paramyxovirdae* family and transports its genetic information as a roughly 15kb long, non-segmented, single-stranded, negative-sense RNA, protected by an envelope (Lamb and Kolakofsky 1996).

A major advantage of this method remains the reproduction cycle of the Sendai virus (SeV): The virus does not integrate into the host genome of the infected cell but replicates simply in the cytoplasm, thereby causing little to no DNA alteration or mutation (Lamb and Kolakofsky 1996). But as the cytoplasmic replication continues in the generated iPSC these cells must consequently still be seen as a potential threat with an active viral infection (Fusaki et al. 2009). One way is to eliminate the cells in the colony with an active viral replication by applying an antibody (anti-SeV-HN antibody), although there still could be working viral particles left in the remaining cells (Fusaki et al. 2009). Such

measures also greatly decrease the number of the just generated, valuable hiPSC. Other groups relied on the constant degeneration of RNA and their instability by simply passaging the generated hiPSC cells and discovered a total loss of the Sendai vectors after about 10 passages (Ban et al. 2011; Macarthur et al. 2012). The downside of this approach is the time consuming passaging of the cells.

To overcome the threat of this virus in the generated iPSC one group tried to generate temperature-sensitive Sendai virus vectors (TS SeV). The SeV uses an RNA polymerase which is composed of the phosphoprotein and the large protein. They finally produced three temperature sensitive virus vectors, one with mutations in the large protein gene and two with mutations in the large protein gene and the phosphoprotein. Newly formed iPSC colonies which were afterwards treated with 38°C for 3 to 5 days could be shown to be virus free even after four passages of recloning. This result clearly shows the possibility of virus elimination after the successful iPSC generation (Ban et al 2011).

The reprogramming efficiency of the Sendai virus differs in the literature and seems to be cell type dependent. Using fibroblasts, it is reported to be 0,077% (Schlaeger et al. 2015), others could yield 1% for fibroblasts and 0,1% for terminally differentiated T-cells in peripheral blood (Fusaki et al. 2009; Seki et al. 2010; Ban et al. 2011). It was also shown that the reprogramming efficiacy depends on the MOI, ranging from 0,01% with a MOI of 3 to 0,1% with a MOI of 20 (Seki et al. 2010). Table 2 summarizes the comparison of this reprogramming technique towards others.

1.5.2.5 Protein-based systems

The idea seems to be both simple and effective: Instead of transferring genetic information into the target cells, the product of these genes is inserted. In 1988 it could be demonstrated that the tat protein of the HI virus was able to access the cell membrane due to a concentration of basic amino acids at positions 48 to 60 (Frankel and Pabo 1988; Frankel et al. 1988). The obvious problem of

limited access of proteins to the cytoplasm via the membrane has therefore been overcome by tailoring the proteins with basic amino acids, e.g. a poly-arginine tail (Wender et al. 2000). Using this modification and a HEK293 cell line to express the recombinant reprogramming factor one research team was able to transform neonatal fibroblasts into hiPSC by repetitive exposition of the fibroblasts to a solution with this protein (Kim et al. 2009).
The striking advantage of this approach is the complete absence of genomic material, thereby preventing oncogenic potential.
Drawbacks of this system include the very slow transformation process, taking eight hours of exposure peer week for 6 weeks, as well as a very low efficiency of 0,001% (Kim et al. 2009).

1.5.2.6 mRNA-based systems

Taking one step back in the process of proteinbiosynthesis reveals mRNA as another alternative vector for delivering reprogramming factors to target cells. Like the cell derived mRNA, the synthetic mRNA needed modifications such as the cap at the 5'-end and the poly-A tail at the 3'-end (Sridhar et al. 2016; Yakubov et al. 2010).
Furthermore, in the course of establishing this technique strong initiation signals were placed ahead of the gene transcripts in the 5' untranslated region (UTR) and to counter possible antiviral responses of the target cells the generation of the mRNA was performed using pseudo-uridine instead of uridine and 5'methylcitidine instead of cytidine. These mRNA's in combination with cationic shuttles and interferon inhibitors yielded a reprogramming efficiency of about 1,4% using human fibroblasts and took 20 days (Warren et al. 2010). Table 2 summarizes the comparison of this reprogramming technique towards others.
This reprogramming efficiency has leaped to 4,4% after improvements of this protocol were made by adding the reprogramming factor LIN28, incubating the cells with 5% oxygen and adding valproic acid to the culture medium (Warren et al. 2010). Other studies showed a reprogramming efficiacy comparable to

retroviral systems (Sridhar et al. 2016).

Main advantages of this reprogramming strategy include the absence of genomic alteration, the high reprogramming efficiency and the fast growth of hiPSC colonies (Sridhar et al. 2016; Warren et al. 2010; Yakubov et al. 2010).

Drawbacks on the other hand are labor intensity, as the target cells need to be treated daily on 7 consecutive days, the fragility of the mRNA, as RNAses are all over the skin of the people working with it, and the sample dependency, as some patient samples could not be reprogrammed using this approach (Schlaeger et al. 2015; Warren et al. 2010).

1.5.2.7 miRNA-based systems

Certain microRNA's (miRNA) are highly expressed in ESC and therefore were identified as possible tools as well (Bartel 2009). First described in 1993, many miRNA's function as silencers by binding to the mRNA product of a certain gene, thereby preventing the translation of this mRNA without stopping the degradation of it (Lee et al. 1993). miRNA can serve as both a booster when being combined with other reprogramming techniques, as well as a reprogramming platform itself. Fibroblasts were successfully reprogrammed with a lentiviral system boosted by addition of miRNA-302b or miRNA-372, this increased reprogramming efficiency 10 to 15 times (Wilson et al. 2009).

Another approach combining only miRNA-200c, miRNA-302s and miRNA-369s yielded a reprogramming efficiency of 0,002% after 20 days using fibroblasts and adipose stromal cells (Miyoshi et al. 2011).

Advantages of this method include the possibility to increase efficacy of other reprogramming techniques and the reduced tumorigenecity of the arising hiPSC (Lin et al. 2010; Wilson et al. 2009).

Drawbacks are mainly the reduced cell cycle rate as the miRNA greatly slow down this process, the low reprogramming efficacy and the genomic alterations as only 83,3% of the arising hiPSC showed a normal karyotype with 46 chromosomes (Lin et al. 2010; Miyoshi et al. 2011).

1.5.2.8 Transposon-based systems

Discovered in 1950, Transposons are DNA sequences which can change their position in the genome, they are often referred to as mobile or jumping DNA sequences (McClintock 1950). A transposon-like delivery system has been developed by a research group, the Piggyback. It is composed of two plasmids, one carrying the reprogramming genes, flanked by inverted terminal repeats (ITR), the other one carrying the transposase enzyme gene. The enzyme is necessary to mediate the integration or excision of the transposon into the DNA or out of it (Cary et al. 1989; Ding et al. 2005; Fraser et al. 1996). It was shown that fibroblasts could be reprogrammed into hiPSC using the PiggyBac and subsequently the transposons were fully eliminated without altering the DNA or leaving any measurable protein products in the target cells (Kaji et al. 2009; Woltjen et al. 2009).

Reprogramming human mesenchymal stem cells yielded an efficiency of 0,02% (Mali et al. 2010).

This approach, especially using PiggyBac, seems advantageous regarding the delivery of genes with the subsequent removal of these transgenes, thereby creating genetically unaltered hiPSC. Also, the very small DNA molecule used in this method increases the handling and makes the vector generation easier, this in turn saves time and resources (Kaji et al. 2009; Mali et al. 2010; Woltjen et al 2009).

The main drawback is the transposase enzyme itself as it allows excision and integration of any transposon at identical efficiency. This leads to concerns whether there is truly no alteration in the host genome as transposons are present in the host genome as well. Moreover, this approach depends on the DNA delivery methods which in general suffer limitations regarding cytotoxicity or resistance of the target cells (Kaji et al. 2009; Mali et al. 2010; Woltjen et al 2009).

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1.5.2.9 Episomal plasmid systems

Famous for being the source of certain antibiotic drug resistances of some bacteria, plasmids may as well serve human research. Composed of a DNA sequence which forms a circle structure, plasmids may contain a number of genes, non-coding regions, promoters and silencers. They do not integrate into the host genome but instead serve as a small, additional genome (Yu et al. 2009). Table 2 summarizes the comparison of this reprogramming technique towards others.

Very early in the course of constructing a plasmid-based vector it was discovered that these vectors were deleted after a few passages in the target cells, making the reprogramming process impossible. Repetitive transfections for seven or more consecutive days were necessary to create hiPSC (Montserrat et al. 2011).

One of the latest constructs uses two origin-of-replication/Ebstein-Barr-nuclear-antigen 1-based (ori/EBNA) vectors which show the ability of autonomous replication. One vector contains the three Yamanaka factors OCT4, SOX2, KLF4 and additionally LIN28 as well as NANOG. The other one carried the SV40 large T antigen. These two vectors where used in combination to reprogram CD34+ mononuclear cells of the peripheral blood as well as the bone marrow. In combination with sodium butyrate added to the medium the research group used a single transfection and achieved a reprogramming efficiency of 0,009% (peripheral blood) and 0,005% (bone marrow) after 14 days (Chou et al. 2011). Further improvements of the transfection led to a reprogramming efficiency of 0,14% using epidermal keratinocytes and lipid-mediated transfection (Piao et al. 2014).

The advantages of this method include the low workload necessary to create hiPSC due to the self replication, the loss of the plasmids after a number of passages and the absence of DNA integration in the host cell (Okita et al. 2011; Piao et al. 2014; Yu et al. 2009).

Drawbacks remain the low reprogramming efficiency combined with the above mentioned limitations of DNA delivery methods and a higher aneuploidy rate in

the emerging hiPSC as well as increased need for reprogramming factors as attempts to generate hiPSC out of keratinocytes using the OSKM mix failed (Chou et al. 2011; Piao et al. 2014; Schlaeger et al. 2015).

1.5.2.10 Minicircle systems

Reducing a plasmid vector down to its' essential parts yield a minicircle. They simply consist of a eukaryotic promoter followed by the cDNA sequences of the necessary genes. This lack of possible methylation sites to silence gene expression have made minicircles superior to plasmids regarding both reprogramming efficiency and expression time (Chen et al. 2005; Jia et al. 2010). They usually carry no origin-of-replication or antibiotic resistance gene.

A minicircle vector composed of LIN28, GFP, NANOG, SOX2 and OCT4 successfully reprogrammed human adipose stromal cells with a reprogramming efficiency of 0,005% after 28 days (Jia et al. 2010; Narsinh et al. 2011).

Advantages and drawbacks are pretty much equal to those of plasmids, but few publications exist showing the successful reprogramming of other cell types (Chen et al. 2005; Jia et al. 2010).

Table 2: Comparison of the different reprogramming techniques regarding key characteristics. Taken and adapted from Schlaeger et al. 2015.

Feature	RNA Stemgent	SeV Life Technologies	Epi Yamanaka/Addgene	Lenti Mostoslavsky/BU
Efficiency (fibroblasts)	~1%	~0.1%	~0.01%	~0.5%
Reliability (fibroblasts)	<50% (miRNA + mRNA: >70%)	>90%	>90%	>90%
Reprogramming workload	High	Low	Low	Very high (excision)
Aneuploidy rate	2.3%	4.6%	11.5%	4.5%
Input cell requirement (fibroblasts)	~50 k	~500 k	~500–800 k	~100 k
Time to colony emergence	Fast	Moderate	Moderate	Moderate
Adoption rate (fibroblasts)	46%	62%	33%	19%
Efficiency (blood)	Not yet reported	High	High	High
Reliability (blood)	Not yet reported	High	High	High
Adoption rate (blood)	Not yet reported	61%	44%	40%
Special equipment needs	5% O_2 incubator	None	Transfection device, and O_2 incubator (blood)	None
Lines free of reprogramming agents by passage 5	100%	0% (Cytotune 1)	60.9%	0%
Lines free of reprogramming agents by passage 9–11	100%	78.8%	66.7%	0%

1.5.3 Genomic stability

A direct comparison of integrating and non-integrating reprogramming methods regarding caused genomic alterations revealed a significant difference between the two. The group compared 2 human somatic cell lines (hSC) as reference, 5 human embryonic stem cell lines (hESC) and 6 hiPSC lines reprogrammed with either a lentiviral system (integrating) or episomal vectors as well as Sendai virus vectors (non-integrating). When looking at copy number variations in terms of gain mutations, hSC showed a total number of 7, hESC 13, non-integrating hiPSC 29 and integrating hiPSC 120. Not only the number but also the size differed significant with a maximum size of 567,5kb in hSC, 857kb in hESC, 1277,3kb in non-integrating and 23116,3kb in integrating hiPSC. The Figure is equal when changing the focus on copy number variations resulting in loss mutations. There were 5 identified in hSC, 3 in hESC, 5 in non-integrating and 83 in integrating hiPSC. The maximum length of these fragments was 147,9kb for hSC, 139,9kb for hESC, 709,3kb for non-integrating and 26586,3kb for integrating hiPSC. Overall a clearly higher genomic alteration of the host genome could be shown when employing integrating reprogramming techniques in contrast to non-integrating approaches (Kang et al. 2015).

1.6 Recapitulation of the main aim

The main idea was to develop an easy to use sample platform for a sampling procedure with minimal invasiveness and time consumption to allow sampling in various different nations and settings. We furthermore wanted to find an easy way for sample transport with minimal negative effects on the samples. Additionally, we wanted to create the SKG reprogramming protocol by modifying the well established reprogramming protocol of Aasen and Belmonte 2010. In this cause we chose to use the Sendai virus as delivering vector system (Li et al. 2000). We wanted to optimize the SKG protocol in terms of time consumption and efficiacy.

2 Material and Methods

2.1 Material

2.1.1 Stationary laboratory equipment

Table 3: List of stationary laboratory equipment

Laboratory equipment	**Company**
Heraeus™ Megafuge™ 16 centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Heraeus™ Fresco 17 centrifuge	Thermo Fisher Scientific, Waltham, MA, USA
Cryostat, Microm HM 560	Thermo Fisher Scientific, Waltham, MA, USA
Fridge (4°C)	Liebherr, Biberach, Germany
Freezer (-20°C)	Liebherr, Biberach, Germany
Freezer (-80°C)	Thermo Fisher Scientific, Waltham, MA, USA
Ice machine AF103	Scotsman, Great Blakenham, UK
Heracell™ 240i incubator (37°C)	Thermo Fisher Scientific, Waltham, MA, USA
Axioskop 2 mot plus, Primo Vert microscope	Zeiss, Oberkochen, Germany
CryoPlus™ 2 nitrogen tank	Thermo Fisher Scientific, Waltham, MA, USA
MSC Advantage sterile bench	Thermo Fisher Scientific, Waltham, MA,

	USA
Vortexer	Bender+Hobein, Zürich, Switzerland

2.1.2 Tools and small laboratory equipment

Table 4: List of tools and small laboratory equipment

Laboratory equipment	**Company**
6- and 12-well plates (Tissue treated)	TPP Technologies, Trasadingen, Switzerland
Gloves Peha-Soft® nitrile	Hartmann, Heidenheim, Germany
Petri dishes (6cm, 10cm)	Greiner Bio-One, Frickenhausen, Germany
Pipette tips (1µl, 10µl, 100µl, 200µl, 1ml, 5ml)	Eppendorf, Hamburg, Germany
Pipettes (1µl, 10µl, 100µl, 200µl, 1ml, 5ml)	Eppendorf, Hamburg, Germany
Reaction tubes (15ml, 50ml)	Becton Dickinson, New York, NY, USA
Small reaction tubes (0,5ml, 1,5ml, 2ml	Sarstedt, Nümbrecht, Germany
Sterile filters (0,22µm, 0,45µm)	Merk Millipore, Darmstadt, Germany
T25 culture flasks (culture treated)	Greiner Bio-One, Frickenhausen, Germany
Pipette boy	Hirschmann Laborgeräte, Eberstadt, Germany
Serological Pipette tips	Sarstedt, Nümbrecht, Germany

2.1.3 Cell culture media and supplements

Table 5: List of cell culture media and supplements

Cell culture media and supplements	Supplier
Antibiotic-Antimycotic 100x liquid	Thermo Fisher Scientific, Waltham, MA, USA
Cryostem™	Biological Industries, Beit Haemek, Israel
DMEM, high glucose	Thermo Fisher Scientific, Waltham, MA, USA
Epilife® + HKGS supplement	Thermo Fisher Scientific, Waltham, MA, USA
Fetal bovine serum (FBS)	Thermo Fisher Scientific, Waltham, MA, USA
GlutaMax™ 100x liquid	Thermo Fisher Scientific, Waltham, MA, USA
Knock-Out™-DMEM	Thermo Fisher Scientific, Waltham, MA, USA
Knock-Out™-serum replacement	Thermo Fisher Scientific, Waltham, MA, USA
Non-essential amino acids	Thermo Fisher Scientific, Waltham, MA, USA
Phosphate-buffered saline (PBS) w/o magnesium and calcium	Thermo Fisher Scientific, Waltham, MA, USA
Synth-a-Freeze® cryopreservation medium	Thermo Fisher Scientific, Waltham, MA, USA
ReproTESR	StemCell Technologies, Vancouver, Canada

2.1.4 Chemicals and Enzymes

Table 6: List of chemicals and enzymes

Chemicals	**Manufacturer**
Ethanol	Serva, Heidelberg, Germany
Human recombinant FGF-2	Cell Guidance Systems LLC, St. Louis, MO, USA
ITS	BD Bioscience, San Jose, CA, USA
Dorsomorphin dihydrochloride	Tocris Bio-Techne, Wiesbaden-Nordenstadt, Germany
Activin A	Cell Guidance Systems LLC, St. Louis, MO, USA
Human serum albumin	Biological Industries, Cromwell, CT, USA
Isopropanol	VWR, Radnor, PA, USA
L-Ascorbic Acid	Sigma-Aldrich, St. Louis, MO, USA
ROCK-Inhibitor Y-27632	Ascent Scientific, Avonmouth, UK
TGFβ1	Cell Guidance Systems LLC, St. Louis, MO, USA
Collagen IV	Sigma-Aldrich, St. Louis, MO, USA
Matrigel ®	Corning, New York, NY, USA
Vitronectin	
TrypLE™ Express	Thermo Fisher Scientific, Waltham, MA, USA
B-Mercaptoethanol	Thermo Fisher Scientific, Waltham, MA, USA

2.1.5 Cells

Table 7: List of cells

Cell type	Source
Rat embryonic fibroblasts (REF's)	Obtained as described in Linta L, Stockmann M, Kleinhans KN, Böckers A, Storch A, Zaehres H, et al. Rat Embryonic Fibroblasts Improve Reprogramming of Human keratinocytes into Induced Pluripotent Stem Cells. Stem Cells Dev. 2012;21(6):965–76.
Cultured keratinocytes from donors K1, K3, K5, CM Plucked hair from donors K1, K3, K5, CM	Healthy individuals who gave informed consent

2.1.6 Viruses and Reprogramming Kits

Table 8: List of viruses and reprogramming kits

Virus	Source
Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit Cataloguenumber: A16517	Thermo Fisher Scientific, Waltham, MA, USA
Lentivirus pRRL.PPT.SF.hOKSMco.idTom.preFRT	Warlich E, Kuehle J, Cantz T, Brugman MH, Maetzig T, Galla M, et al. Lentiviral Vector Design and Imaging Approaches to Visualize the Early Stages of Cellular Reprogramming. Mol Ther. 2011 Apr;19(4):782–9.

2.1.7 Prototypes

SKG hair culture prototypes were self-manufactured in collaboration with Markus Stoll of Techno Plastic Products in Switzerland.

2.1.8 Software

Table 9: List of software

Software	**Company, Website**
OpenOffice Writer	The Apache Software Foundation, www.openoffice.org
OpenOffice Impress	The Apache Software Foundation, www.openoffice.org
Adobe Illustrator	Adobe Corporation, San José, CA, USA
Graph Pad (Prism)	Graphpad Software, La Jolla, CA, USA
Microsoft Office Word 2010	Microsoft, Redmond, WA, USA

2.1.9 Stock solutions for SeV transfection of keratinocyte cell culture

E8:

DMEM, high glucose

2mM GlutaMAX™

1x Non-essential amino acids (100x)

1x Antibiotic-antimycotic (100x)

100µM Sodiumbutyrat

1:1000 ITS

10 ng/ml FGF2
0,5 ng/ml TGFβ1
50µg/ml L-ascorbic acid
10µM DAPT

hiPS:
KnockOut™ DMEM
20 % KO-Serum Replacement
100µMβ-Mercaptoethanol
2mM GlutaMAX™
1x Non-essential amino acids (100x)
1x Antibiotic-antimycotic (100x)
50µg/ml L-ascorbic acid
10 ng/ml FGF2
10 mM Y27632 (ROCK-Inhibitor).

ReproTESR: Commercially available mixture.
1x Addition (20x)
1x Addition (500x)

MEF:
DMEM, high glucose
15% FBS
1x Non-essential amino acids (100x)
1x Antibiotic-antimycotic (100x)
10 ng/ml FGF2
10 mM Y27632 (ROCK-Inhibitor).

Epilife® + HKGS Supplement: Commercially available mixture.

FTDA:

DMEM

2mM GlutaMAX™

1:1000 ITS

1:100 Human serum albumin (HSA)

1:100 Chemically defined lipids (CDL)

10 ng/ml FGF2

0.5 ng/ml TGFβ1

50 nM Dorsomorphin

5 ng/ml Activin A

1x Antibiotic-Antimycotic (100x)

NTM buffer for AP staining:

ddH_2O

1x NaCl (50M)

1x $MgCl_2$ (1M)

1x TRIS (1M)

2.2 Methods

2.2.1 Cell culture

The cell culture experiments were performed under sterile conditions using a sterile bench. Incubation took place in an incubator at 37°C with a water-saturated atmosphere of 5% CO_2 and 5% O_2. All media were heated in a 37°C water bath prior to use.

The use of human material was approved by the ethical committee of the Tübingen University (678/2017BO2) and in compliance with the guidelines of

the Federal Government of Germany and the Declaration of Helsinki concerning Ethical Principles for Medical Research Involving Human Subjects. All hair donors gave informed consent.

2.2.2 Coating

Matrigel was diluted in Epilife to a final concentration of 1%. Vitronectin was diluted in PBS to a final concentration of 1%. Collagen IV was diluted in PBS to a final concentration of 1% collagen IV. These mixtures were filled into culture flasks or petri dishes or 6-well plates and incubated at 37°C for 1 hour.

2.2.3 Plucking hair

A pincer was used to grab a small number of hairs and quickly pull them out. Efficiency was increased when grabbing the hair close to the root at the base and using one swift and strong movement to extract them. Right after this the plucked hair should be inspected as the ORS is visible as a white cover on the surface of the hair root. Only hair with such an ORS were used and immediately transferred to a small amount of DMEM until further use.

2.2.4 Hair culture prototype

The hair culture prototype was made of plastic material, had a four corner shape and is flat. In each corner a spacer is formed by a small plastic foot to maintain the optimal distance between the down-facing side of the prototype and the underlying surface. The prototype consisted of two different areas, the greater part belongs to the main area with a constant height and many perforations forming channels from the top to the bottom of the prototype. The smaller part has a triangular shape in the side view as it has a decreasing height towards the end.

2.2.5 Prototype sterilization

Prototypes were manually cleaned with PBS, dried and incubated in ethanol (~96%) for one hour. After this they were incubated for 24 hours in PBS. Finally, they were placed under the sterile bench for one hour of UV-radiation.

2.2.6 Classical hair root culture (Aasen and Belmonte 2010)

As classical hair culture method, the well established protocol of Aasen and Belmonte (2010) was used where the hair is plucked, subsequently the roots were cut and transferred onto a Matrigel coated surface. Finally, they are fixed with another drop of Matrigel (1:5 dilution in Epilife) and the well is filled with MEF medium and incubated at 37°C.

2.2.7 SKG culture

The prototype was loaded with freshly plucked hair which has been shortened by cutting it almost directly behind the root and placing the root over one of many holes of the prototype. The prototype was then positioned in one Vitronectin or Matrigel covered well of a 6-well plate, the hairs facing down and therefore being close to the Vitronectin or Matrigel beneath. Sterile tips were used to press the prototype into the plate to ensure a minimum of distance between hair and surface beneath. Following this the well was filled with MEF medium and the plate incubated at 37°C.

2.2.8 Thawing of keratinocytes

Cryo tubes with cells stored at -80°C were incubated in a water bath at 37°C until the phase of the suspension changed to liquid again. At this stage, each one was mixed with 500µl Epilife + HKGS and a total volume of 1ml was transferred to one collection tube for all cryo tubes. Once again, 500µl Epilife + HKGS was added and mixed inside the cryo tubes. The resulting mixture was

transferred to the same collection tube. Having collected all the keratinocytes from all cryo tubes in one tube now this collection tube was centrifuged at 1500 turns per minute for 2 minutes. Following this, the supernatant got removed and the pellet got re-suspended in Epilife + HKGS. At this point the solution containing the keratinocytes was equally divided between the wells of the 6-well plate covered with collagen IV and incubated at 37°C.

2.2.9 Thawing of Rat embryonic fibroblasts (REF's)

Inactivated REF's in cryo tubes stored at -80°C were heated in a water bath at 37°C. Once liquid again the cell suspensions were collected in one tube and mixed with MEF medium. To continue this procedure the tube was centrifuged at 1500U/min for 2 minutes at 20°C and the superanatant was removed afterwards. The pellet got re-suspended in MEF medium and 2ml of the resulting cell suspension was used per well to fill the wells of a 6-well plate. Now the incubation at 37°C followed.

2.2.10 Hair resistance against evaporation

Hair was plucked from two subjects and exposed to air under the sterile bench for either 5 seconds, 1 minute, 3 minutes or 5 minutes. After the time frame passed DMEM was gently poured on the hairs and the hairs were pressed under the surface with a sterile tip to ensure a full covering of the hairs. Next, they were transferred onto the prototypes which in turn were placed in the wells (each containing 2ml MEF-medium) of a 6-well plate and incubated at 37°C. MEF medium was changed to K-SFM after seven days.
Medium was changed every day (without changing the type of medium) to prevent accumulation of metabolic products and keep level of nutrients high.

2.2.11 Replating

Once keratinocytes grew out of the hairs which were loaded on the prototype

these colonies were given time to grow large enough to hopefully resist the extraction. Another well of the plate was covered with Vitronectin. Once ready the prototype was carefully removed from the well where the outgrowth was successfully and transferred into the newly covered well. It was covered with MEF medium and incubated at 37°C with daily changes of the medium for three consecutive days and switched to Epilife on the fourth.

2.2.12 Freezing and thawing of the loaded prototypes

Prototypes were loaded with freshly plucked hair from one donor (K5 line) and transferred in 6-well plates. These were filled with freezing medium. Now they were placed in a styropor box and stored at -80°C for 1 day.

On the next day the styropor box was opened and the plates thawed using warmed MEF medium (37°C) and room temperature. A small amount of warm MEF medium was added to the wells and as soon as the thawing process was sufficient the liquid phase was removed (composed of warm MEF medium and thawed freeze medium) and new warm MEF medium was added. The plate was then incubated at 37°C with daily medium changes until the keratinocytes reached a sufficient confluence. At this point the medium was changed to K-SFM.

2.2.13 Feeder-assisted and feeder-free reprogramming using the SEV transfection kit

To reprogram keratinocytes, the commercially available Sendai transfection kit was used and the enclosed protocol with alterations was executed. The first step was to estimate the number of cells per well using the Neubauer counting chamber. Next, the three viral components were mixed with Epilife depending on the chosen MOI, which was 1 for KOS (polycistronic hKLF4, OCT3/4 and SOX2) and hc-MYC as well as 0,6 for hKLF4. This reprogramming mixture was

now added to the wells. Consequently, a spin infection was performed using 1000g for 30 minutes at 37°C. After the spin infection the plates were incubated at 37°C for 24 hours.

As 24 hours have passed the virus was removed and the transfected cells were relocated either to coated wells or onto the feeder layer built up by the REF's. Therefore the keratinocytes were washed three times with PBS. Then 500µl TrypLE was added per well and incubated for 20 minutes with repeated cycles of light percussion to increase the desorption effect of the TrypLE .

After 20 minutes Epilife medium was added. All resulting cell suspensions were now centrifuged at 1500 U/min for 2 minutes. The resulting supernatant was removed while the pellets were re-suspended in the reprogramming medium (or different reprogramming media). Now cells were either placed in coated wells or the old medium of the REF's was replaced with these newly acquired cell suspensions. The plates were gently moved to dispense the cells and incubated at 37°C. Epilife medium was used initially and got replaced by ReproTESR medium stepwise until complete replacement on day 4 took place.

In a second approach after the initial cell counting the keratinocytes were detached as described above and a specific number was seeded into every well of a 12-well plate. Six different numbers of cells were chosen (250, 1000, 2000, 5000, 10000, 30000), each of them in two wells as half of the wells contained REF's. Now the spin infection as described above took place followed by the incubation for 24 hours at 37°C. After this the virus was removed and new Epilife medium was added and again stepwise replaced by ReproTESR as described above.

2.2.14 IPSC Culture

The hiPSC were cultivated in FTDA medium in the 6-well plates while being incubated at 37°C in a water-saturated atmosphere of 5% O_2 and 5% CO_2. Once they reached a sufficient confluence they were either fixed and immunostained or used for RNA isolation.

Fixation of cells

The cells were first washed with PBS subsequently fixed with 4% PFA/20% sucrose for 20 minutes at room temperature. Afterwards the cells were washed with PBS twice. The fixed cells were kept in PBS.

Immunostaining

Samples were blocked with 10 % normal donkey serum + 0.2 % Triton®-X in PBS for 1 hour at room temperature. Then the primary antibodies were added to the blocking buffer solution overnight at 4 °C. Afterwards three washing steps with PBS were performed. The secondary antibodies were added in 1:1 diluted blocking buffer in PBS for 1 hour at room temperature. After this the secondary antibodies were removed and the samples were washed again three times with PBS. Now they were mounted in Prolong™ antifade reagents containing DAPI.

Microscopy

Microscopy was performed using an Axiokope 2 mot plus microscope.

2.2.15 Statistical tests

Student's t-test was used for statistical analysis employing Microsoft Excel 2016 and Open Office Calc. Results are provided as mean ± standard error of the mean (S.E.M.).

3 Results

3.1 Comparison of the Classical System (Aasen and Belmonte 2010) versus the SKG system

Research is not only driven by the passion of the scientific community but also by the applied methods. Increasing their efficiency and decreasing their difficulty is therefore one major field of research. To establish new methods in the research community it is essential to show their superiority regarding time consumption, decreased difficulty or efficacy.

To illustrate the improvement of the SKG protocol, a direct comparison between the very well implemented as well as highly efficient protocol derived by Aasen and Belmonte 2010 (Figure 6a) and the SKG system (Figures 6b and 6c) was performed. To increase comparability four repetitions were made, each with hair from a different donor.

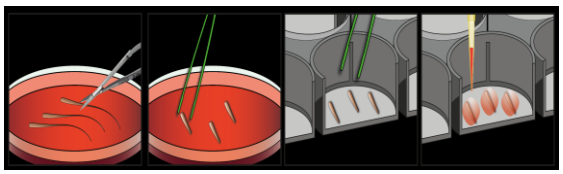
For the Classical System (Aasen and Belmonte 2010), the well established protocol of Aasen and Belmonte (2010) was used where the hair is plucked, subsequently the roots are cut and transferred onto a Matrigel coated surface. Finally, the roots were fixed with another drop of Matrigel, the well was filled with MEF medium and incubated at 37°C. For the SKG system, the prototype was loaded with freshly plucked hair which has been shortened by cutting it almost directly behind the root and placing the root over one of many holes this prototype also has. The prototype was then positioned in one Vitronectin or Matrigel covered well of a 6-well plate, the hairs facing down and therefore being close to the Vitronectin or Matrigel beneath. Sterile tips were used to press the prototype into the plate to ensure a minimum of distance between hair and surface beneath. Following this the well was filled with MEF medium and the plate incubated at 37°C.

This cultivation was performed for three days in total with a consecutive medium change on the fourth day to Epilife. The cultivation was finished after 7 days but most of the outgrowth was seen until day 4.

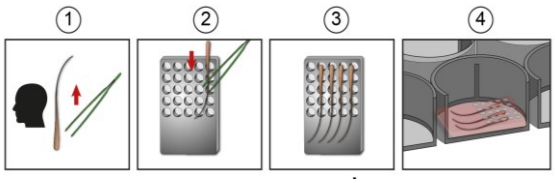
At first, the question of time consumption of each protocol was addressed (Figure 6d). While the experimental procedure of the Classical System (Aasen and Belmonte 2010) took about 4 hours, the SKG system protocol was finished after about 1 hour and 15 minutes.
The second step was to analyze the efficacy in terms of outgrowth rates of the placed hairs (Figure 6e, Table 10). For the SKG system, furthermore two different coatings for the wells were tested: Matrigel and Vitronectin. Overall, the Vitronectin coated wells yielded an efficiency of 50% outgrowth (9 of 18 hairs) while the Matrigel coating reached 35% outgrowth (7 of 20 hairs). The Classical system reached 26% outgrowth (5 of 19 hairs).

In summary, the SKG system needed less time to be executed and yielded higher outgrowth rates.

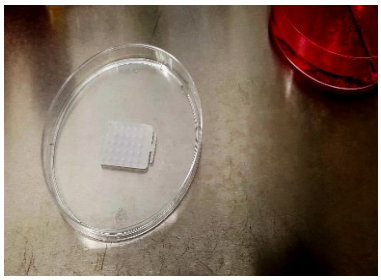
a



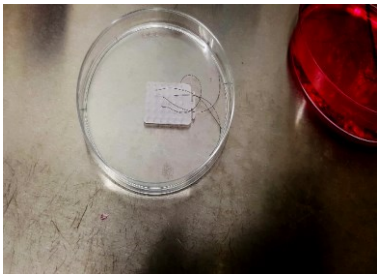
b



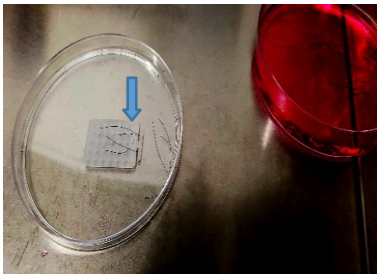
c



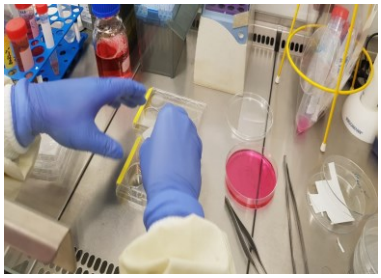
Place SKG



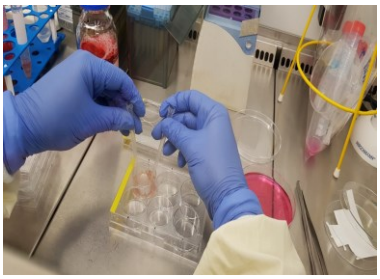
Add Hair



Place adhesive foil on the hair shaft



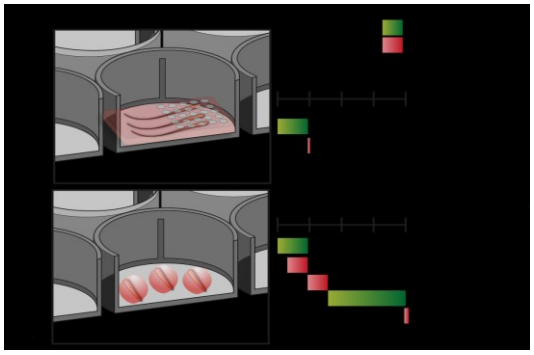
Place SKG



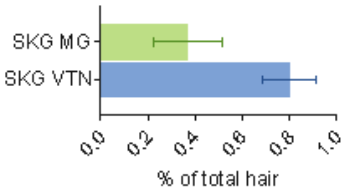
Press SKG on surface

SKG culture

d



e



f

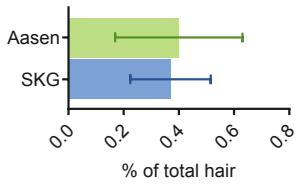


Figure 6: Comparison of the Classical System (Aasen and Belmonte 2010) versus the SKG system. (a) Principle way to cultivate keratinocytes outgrown of hair using the Classical System (Aasen and Belmonte 2010). The hair is plucked, subsequently the roots are cut and transferred onto a Matrigel coated surface. Finally they are fixed with another drop of Matrigel, the well is filled with MEF medium and incubated at 37°C. (b) Principle way to culture keratinocytes outgrown out of hair using the SKG system. Step 1: Fresh hair was plucked. Step 2: The prototypes were loaded with freshly plucked hair which has been shortened by cutting it almost directly behind the root and placing the root over one of many holes this prototype also has. Step 3: The hair was fixed with an adhesive foil placed at the shaft. Step 4: The prototype was then positioned in one Vitronectin or Matrigel covered well of a 6-well plate, the hair facing down and therefore being close to the Vitronectin or Matrigel beneath. Sterile tips were used to press the prototype into the plate to ensure a minimum of distance between hair and surface beneath. Following this the well was filled with MEF/REF medium and the plate incubated at 37°C. (c) Practical handling of the prototype. (d) Workload comparison of the Classical System (Aasen and Belmonte 2010) versus the SKG system. (e) Comparison of outgrowth efficiency achieved by the SKG system using either Matrigel or Vitronectin as coating. After 7 days the number of hair showing a keratinocyte outgrowth was examined using a regular microscope. Overall the Vitronectin coated wells yielded an efficiency of 50% outgrowth (9 of 18 hairs) while the Matrigel coating reached 35% outgrowth (7 of 20 hairs). The Classical system reached 26% outgrowth (5 of 19 hairs; data not shown). (f) Comparison of the Classical system and the SKG system using Matrigel as coating. The Classical system, according to Aasen and Belmonte (2010), reached an outgrowth efficiency of 40% while the SKG system reached an outgrowth efficiency of 35%. Error bars: S.E.M.

Table 10: Attributes and results of the experimental comparison of the Classical System (Aasen and Belmonte 2010) versus the SKG system

Run	Keratinocyte donor	System	Coating	Number of hair used	Number of outgrown hair (%)
1	K5	Classical	Matrigel	5	2 (40)
1	K5	SKG	Matrigel	4	1 (25)
1	K5	SKG	Vitronectin	5	4 (80)
2	K3	Classical	Matrigel	6	0 (0)
2	K3	SKG	Matrigel	5	1 (20)
2	K3	SKG	Vitronectin	5	3 (60)
3	CM	Classical	Matrigel	5	0 (0)
3	CM	SKG	Matrigel	5	0 (0)
3	CM	SKG	Vitronectin	5	0 (0)
4	K1	Classical	Matrigel	3	3 (100)
4	K1	SKG	Matrigel	6	5 (83,3)
4	K1	SKG	Vitronectin	3	2 (66,7)

3.2 Freezing and thawing the loaded prototypes

The next issue to address was the transport of the loaded prototypes to the laboratories. One of the most common options is to cool biological samples down in order to reduce metabolism to a minimum. To evaluate this option, prototypes were loaded with freshly plucked hair from one donor (K5 line) and transferred in 6-well plates. These were filled with MEF medium containing 10% DMSO as freezing medium. Afterwards, they were stored in cryo boxes at -80°C for 24h. Once the time had passed they were thawed by removing the thawing medium and replacing it with MEF medium. This plate was consecutively

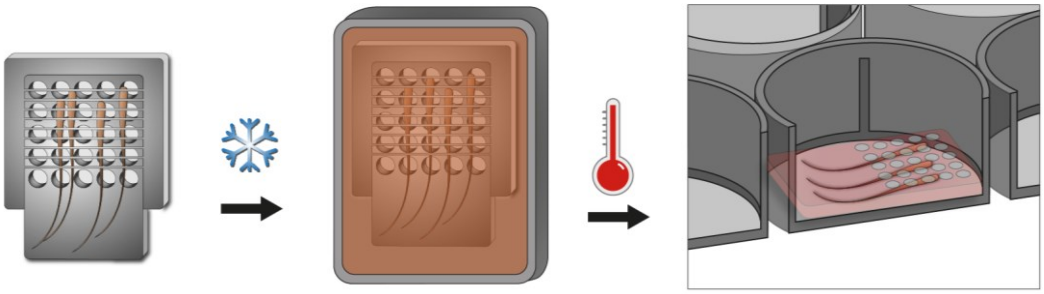
incubated at 37°C with daily medium changes until the keratinocytes reached a sufficient confluence. At this point, the medium was changed to K-SFM containing 0,1% ROCK-Inhibitor and 1% AA. Finally the number of hair showing a keratinocyte outgrowth was analyzed with a microscope. Two repetitions with the same donor and a Vitronectin coating were performed. The runs were terminated after 14 days but outgrowth was already seen after 7 days.

The theoretical approach is shown in figure 7a while figure 7b illustrates the practical steps as well as the observed successful outgrowth of keratinocytes. Figure 7c displays the successfully outgrown keratinocytes at a higher magnitude.

The first repetition yielded an outgrowth rate of 50% (1 of 2 hairs), but the second repetition yielded an outgrowth rate of 0% (0 of 6 hairs), as shown in table 11.

Overall the outgrowth rate in our experiments of frozen and thawed hair loaded on the prototype was 1 out of 8 or 12,5%.

a



b

SKG culture in Freezing Medium Frozen SKG + HAIR Outgrowth after thawing

c

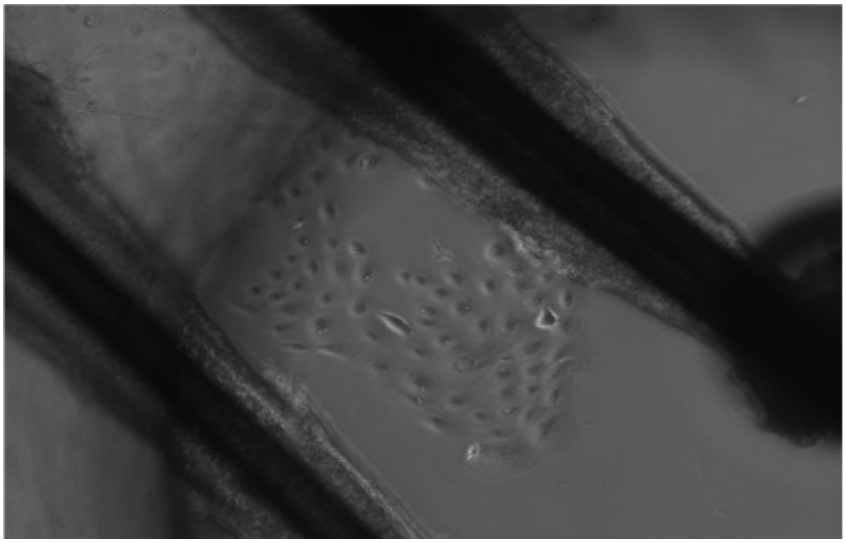


Figure 7: Freezing and thawing of the prototypes. (a) Illustration of freezing and thawing

hair loaded onto the prototypes step by step. Left: Prototypes were loaded with freshly plucked hair from one donor (K5 line) and transferred in 6-well plates. Afterwards they were filled with freezing medium (MEF medium containing 10% DMSO) and stored in cryo boxes at -80°C for 24h. Middle: Once the time had passed they were thawed by removing the thawing medium and replacing it with MEF medium. Right: This plate was consecutively incubated at 37°C with daily medium changes until the keratinocytes reached a sufficient confluence. At this point the medium was changed to K-SFM containing 0,1% ROCK-Inhibitor and 1% AA. Finally the number of hair showing a keratinocyte outgrowth was analyzed with a microscope. (b) Pictures of the first repetition showing the loaded prototype in the 6-well plate right before addition of freezing medium. The next picture shows the frozen prototype prior to the thawing process. After freezing and thawing the hairs were cultured and keratinocytes grew out of them as visualized in the last picture on the right side. (c) Observed outgrowth of keratinocytes between two hairs.

Table 11: Results of the experimental runs of freezing of loaded prototypes

Run	Keratinocyte donor	System	Freeze medium	Coating	Number of hair used	Number of outgrown hair (%)
1	K5	SKG	MEF with 10%DMSO	Vitronectin	2	1 (50)
2	K5	SKG	MEF with 10%DMSO	Vitronectin	3	0 (0)
2	K5	SKG	MEF with 10%DMSO	Vitronectin	3	0 (0)

3.3 Hair resistance against evaporation

One major obstacle in the research with samples is their possible alteration by external factors. In the case of the SKG system, one main external factor is the evaporation time the hair samples have to face in the process of plucking until

their transfer to the prototype and the addition of cell culture medium.

To assess the time frame the plucked hairs can be handled an experiment was designed where plucked hairs were exposed to evaporation for a defined time span of either 5 seconds, 60 seconds, 180 seconds or 300 seconds. Three repetitions were done using hair from a different donor each time. In every case the prototype was used to install the hair and Vitronectin-coated 6-well-plates for the outgrowth phase. For the K5 line, two samples were used for 5 seconds and 60 seconds evaporation time each. The runs were terminated after 13 days but no outgrowth was seen after 9 days. Using a microscope the number of hairs showing a keratinocyte outgrowth was assessed.

Overall an evaporation time of 5 seconds yielded an outgrowth rate of 47,6% (10 of 21 hairs), while an evaporation time of 60 seconds yielded an outgrowth rate of 70% (14 of 20 hairs, Figure 8 and table 12). An evaporation time of 180 seconds yielded an outgrowth rate of 40% (6 of 15 hairs) and an evaporation time of 300 seconds yielded an outgrowth rate of 0% (0 of 14 hairs).

In summary an evaporation time of 60 seconds yielded the highest outgrowth rate of 70%. After 300 seconds of evaporation time no outgrowth could be observed.

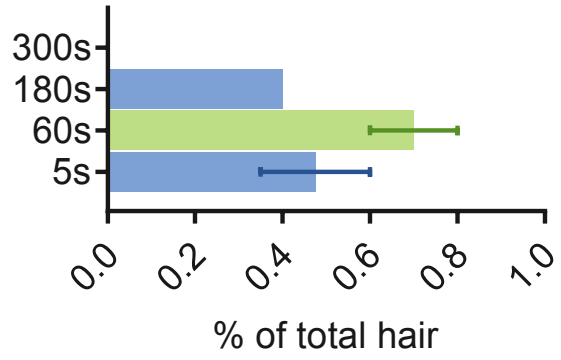


Figure 8: Overall outgrowth of hair in reference to the evaporation time faced. Hair was plucked, exposed to air for a specific amount of time and afterwards cultured using the prototype. 13 Days later the number of hair showing an outgrowth was assessed using microscopy. Overall an exposition of roughly 60 seconds to air yielded the highest outgrowth rate of about 70%. No outgrowth could be observed after an evaporation time of 300 seconds.

Table 12: Results of the hair resistance against evaporation experiments

Run	Evaporation time in seconds	Keratinocyte donor	Coating	Number of hair used	Number of outgrown hair (%)
1	5	K1	Vitronectin	6	3 (50)
1	60	K1	Vitronectin	5	4 (80)
1	180	K1	Vitronectin	5	2 (40)
1	300	K1	Vitronectin	4	0 (0)
2	5	K3	Vitronectin	5	2 (40)
2	60	K3	Vitronectin	5	4 (80)
2	180	K3	Vitronectin	5	2 (40)
2	300	K3	Vitronectin	5	0 (0)
3	5	K5	Vitronectin	5	4 (80)
3	5	K5	Vitronectin	5	1 (20)
3	60	K5	Vitronectin	5	2 (40)
3	60	K5	Vitronectin	5	4 (80)
3	180	K5	Vitronectin	5	2 (40)
3	300	K5	Vitronectin	5	0 (0)

3.4 Replating

To further decrease the amount of time the keratinocyte culturing takes replating experiments were performed: In the course of culturing the keratinocytes, instead of discarding the hair they grew out of, a second outgrowth approach was initialized. Therefore, the whole prototype was taken with all the loaded hair and placed in the next well of a 6-well plate with a Vitronectin covered surface. Cultivation protocol was identical to the first run. Overall four repetitions were

made using three different hair donors with a first and second replating approach.

In summary, the first replating run yielded an outgrowth rate of 55,56% (10 of 18 hairs) while the second run yielded an outgrowth rate of 11,11% (2 of 18 hairs, see table 13).

Table 13: Results of the replating experiment

Replating	Keratinocyte donor	System	Coating	Number of hair used	Number of outgrown hair (%)
1. Time	K5	SKG	Vitronectin	5	2 (40)
1. Time	K1	SKG	Vitronectin	2	1 (50)
1. Time	K1	SKG	Matrigel	6	4 (66,7)
1. Time	K3	SKG	Vitronectin	5	3 (60)
2. Time	K5	SKG	Vitronectin	5	0 (0)
2. Time	K1	SKG	Vitronectin	2	0 (0)
2. Time	K1	SKG	Matrigel	6	0 (0)
2. Time	K3	SKG	Vitronectin	5	2 (40)

3.5 Feeder-assisted and feeder-free reprogramming using the SEV transfection kit

As already described, hair was taken from a distinct donor (K5), attached to the prototypes and set into Vitronectin-covered wells. After a sufficient incubation, keratinocytes grew out of the hair. These keratinocytes were harvested, cultured and finally reprogrammed into hiPSC using the commercially available Sendai virus transfection kit and the corresponding protocol. The target factors of the SEV transfection were KOS (polycistronic hKLF4, OCT3/4 and SOX2), hKLF4 and c-MYC. In case of the cell number specific reprogramming the

keratinocytes were counted and a distinct number was added to every well for consecutive transfection. The experiment was repeated four times: The first run as feeder-assisted and feeder-free reprogramming of a chosen hair line with a MOI of 3 for KOS and hKLF4 as well as 2 for c-MYC. The second and third run as feeder-free reprogramming with a reduced number of cells (250, 1000, 2000, 5000) and a reduced MOI of 1 for KOS and hKLF4 as well as 0,6 for c-MYC. The fourth run as direct reprogramming using the outgrown keratinocytes without counting them. The wells for the reprogramming were coated with collagen IV in the first three runs but with Vitronectin in the fourth run as the optimized surface of the SKG system was used directly.

In case of feeder-assisted reprogramming the cells were plated on REF feeder cells and cultured in Epilife medium for three days with an increasing addition of ReproTESR medium up to the total exchange on the fourth day. Successful reprogramming took about 14 days.

In case of feeder-free reprogramming the cells were solely cultured in Epilife medium for three days with an increasing addition of ReproTESR medium up to the total exchange on the fourth day. Successful reprogramming took about 14-28 days.

Overall the wells with 250 keratinocytes showed no arising hiPSC for both the feeder-assisted as well as feeder-free reprogramming in any of the three runs. All other wells, regardless of feeder-assisted or feeder-free, showed high numbers of hiPSC arising. No difference could be observed when the reduced MOI of 1 for KOS and hKLF4 as well as 0,6 for c-MYC was applied.

In summary, the reduced MOI for the reprogramming factors KOS, hKLF4 and c-MYC and a number of about 1000 keratinocytes yielded a sufficient number of hiPSC with no major difference if the keratinocytes were assisted by REF's (Table 14).

Table 14: Results of the feeder-assisted and feeder-free cell number specific reprogramming experiments

Run	Keratinocyte donor	Coating	Number of cells	Trans-fection virus	MOI KOS and hKLF4	MOI hc-MYC	hiPSC arised
1	K5 + REF	Collagen IV	250	Sendai	3	2	No
1	K5	Collagen IV	250	Sendai	3	2	No
1	K5 + REF	Collagen IV	1000	Sendai	3	2	Yes
1	K5	Collagen IV	1000	Sendai	3	2	Yes
1	K5 + REF	Collagen IV	2000	Sendai	3	2	Yes
1	K5	Collagen IV	2000	Sendai	3	2	Yes
1	K5 + REF	Collagen IV	5000	Sendai	3	2	Yes
1	K5	Collagen IV	5000	Sendai	3	2	Yes
1	K5 + REF	Collagen IV	10000	Sendai	3	2	Yes
1	K5	Collagen IV	10000	Sendai	3	2	Yes
1	K5 + REF	Collagen IV	30000	Sendai	3	2	Yes
1	K5	Collagen IV	30000	Sendai	3	2	Yes
2	K5	Collagen IV	250	Sendai	1	0,6	No
2	K5	Collagen IV	250	Sendai	1	0,6	No
2	K5	Collagen IV	1000	Sendai	1	0,6	Yes
2	K5	Collagen IV	1000	Sendai	1	0,6	Yes
2	K5	Collagen IV	2000	Sendai	1	0,6	Yes
2	K5	Collagen IV	2000	Sendai	1	0,6	Yes
2	K5	Collagen IV	5000	Sendai	1	0,6	Yes
2	K5	Collagen IV	5000	Sendai	1	0,6	Yes

3	K5	Collagen IV	250	Sendai	1	0,6	No
3	K5	Collagen IV	250	Sendai	1	0,6	No
3	K5	Collagen IV	1000	Sendai	1	0,6	Yes
3	K5	Collagen IV	1000	Sendai	1	0,6	Yes
3	K5	Collagen IV	2000	Sendai	1	0,6	Yes
3	K5	Collagen IV	2000	Sendai	1	0,6	Yes
3	K5	Collagen IV	5000	Sendai	1	0,6	Yes
3	K5	Collagen IV	5000	Sendai	1	0,6	Yes

IPSC Characterization

The reprogrammed cells were checked for expression of the pluripotency markers OCT4, SOX2, NANOG, TRA-1-80, TRA-1-81 and SSEA4 by immunofluorescence staining. All those pluripotency markers were found in the stained cells. These cells therefore have an expression profile which is common for human induced pluripotent stem cells.

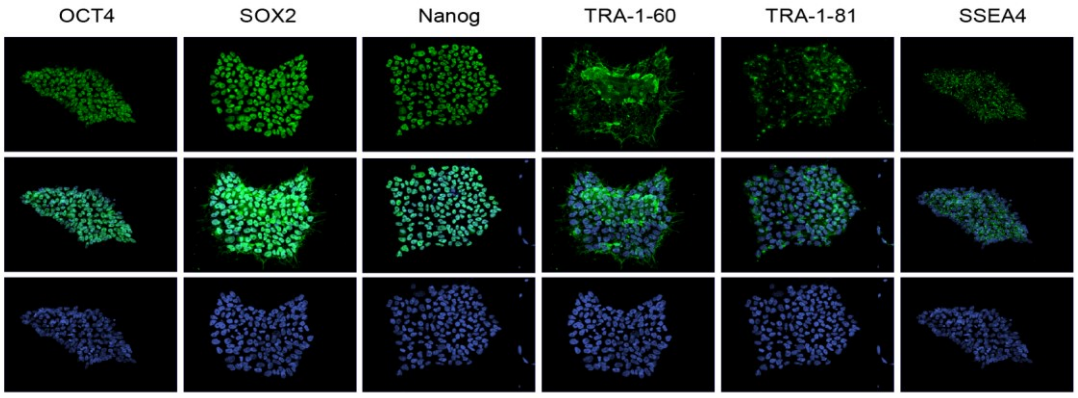


Figure 9: Depiction of reprogrammed keratinocytes stained for pluripotency markers OCT4, SOX2, Nanog, TRA-1-60, TRA-1-81 and SSEA4.

4 Discussion

The main achievements of this study are: I) The establishment of the SKG system as a valid sampling platform for acquisition, transport and culture of keratinocytes. II) Further reduction of time and workload for generation of hiPSC by replating and III) reprogramming of Keratinocytes using the Sendai Virus and the optimized SKG protocol.

The SKG system was designed for easy and effective sample collection, transport and culture (Figure 9). It exhibits several advantages in comparison with the Classical System (Aasen and Belmonte 2010). The first major advantage is the time saving aspect: From sample collection to culture takes about 1 hour and 15 minutes using the SKG system compared to about 4 hours when the Classical System (Aasen and Belmonte 2010) is employed. The second major advantage is the more than equal outgrowth efficacy of the SKG system, in our experiments 35% for the SKG system and 26% for the Classical System (Aasen and Belmonte 2010) with the Matrigel coating but the SKG system could be pushed to 50% outgrowth efficacy if the Vitronectin coating was utilized. The third major advantage is the optimized clinical application, namely the sample collection: Having pre-coated wells the SKG system allows one sample collector to pluck the hairs, cut them, lay them on the prototype, fix them with adhesive foil, push the loaded prototype into one of the pre-coated wells and add medium. This allows one person to execute the whole process from sample collection to begin culturing immediately without any interruptions such as waiting periods or preparation measures for further steps. Even for an untrained person the whole process hardly takes 10 minutes. The SKG system therefore enables a large scale sampling collection as minimal personnel and material resources are required not only for sampling collection but even starting the cell culture, which in turn will reduce the necessary time again as the transportation time is no lost time but a part of the keratinocyte outgrowth and culture time. The fourth major advantage is the sample stability: Once fixed

on the prototype and installed in the well the hairs remained in position despite any fast movement or shaking of the plate. Even fast extraction or addition of medium could not dislocate any of the hairs in our experiments. In contrast to this the Classical System (Aasen and Belmonte 2010) lacks such a stability: Even after being fixed with the second drop of Matrigel there is a major chance for the hairs to lose their attachment to the surface and dislocate due to fast movement, shaking or medium addition/extraction.

In summary the SKG system is a platform for a fast and large scale sample collection with a high sample stability, requiring minimal personnel and material resources in combination with a high outgrowth rate of keratinocytes out of the collected hairs.
The prototypes are meant to be sterilized and reused, therefore the important ecological issue of recycling has been addressed as well.

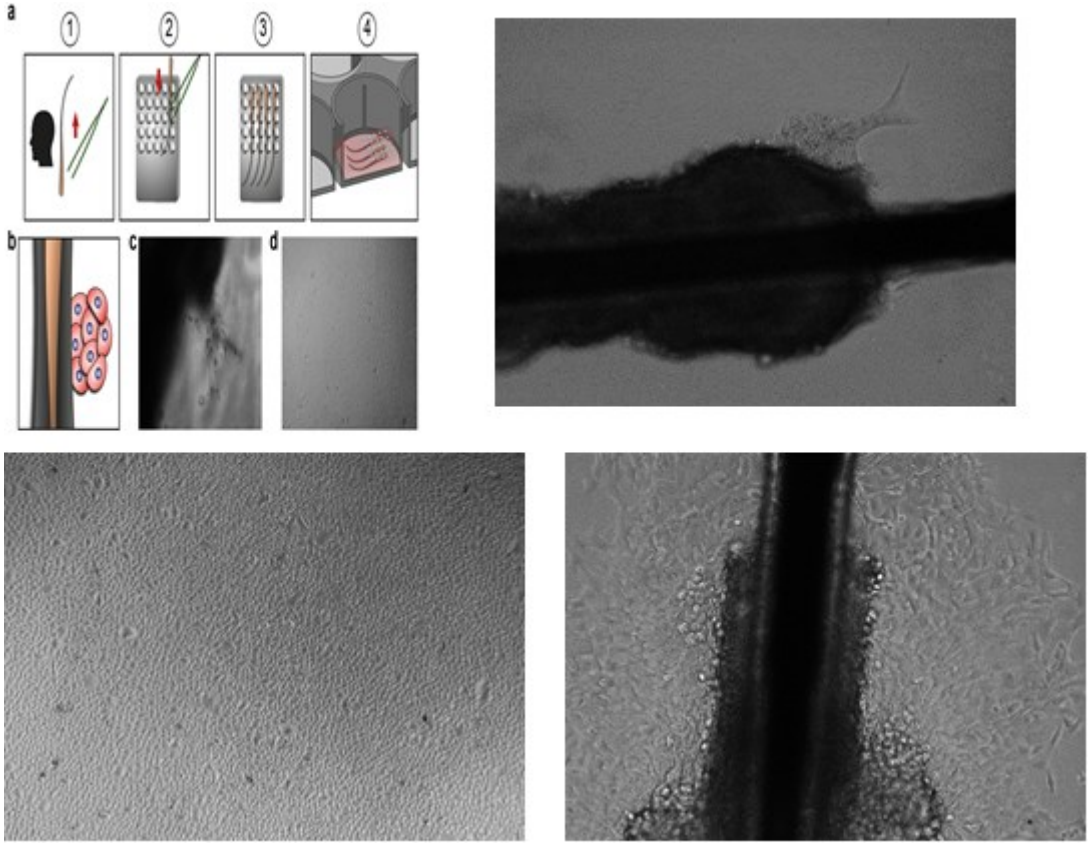


Figure 10: Depiction of successful outgrowth of keratinocytes. The main aim of the cultivation is depicted in the upper left image, the other three show examples of the successful cultivation attempts in different magnitudes.

4.1 Cell sources and established protocols

The first iPSC that were generated used both embryonic and adult mouse fibroblasts (Takahashi and Yamanaka 2006). One year later a protocol for transforming human fibroblasts into hiPSC was published (Takahashi et al. 2007). Fibroblasts are a well-known cell fraction to the research community as they have been intensively investigated. Because they show a certain resistance, these cells are quite easy to handle compared to other more vulnerable and therefore more demanding cell populations (Streckfuss-Bömeke et al. 2013).

Another advantage is their availability as established cell lines can be purchased which makes research and reproduction of experiments a lot easier. These purchasable fibroblast lines origin from many different tissues such as the lung (Salazar et al. 2009), the dermis (Lowry et al. 2008), periodontal ligaments (Kumada and Zhang 2010) or the heart (Saxena et al. 2008). Very convenient for research purposes are the purchasable fibroblast cell lines from patients suffering from common diseases like Parkinson's Disease (Byers et al. 2011), familial Alzheimer's Disease (Israel et al. 2012), Type 1 Diabetes mellitus (Maehr et al. 2009), primary ovarian insufficiency (Leng et al. 2015), frontotemporal dementia (Almeida et al. 2013), asthma bronchiale, cystic fibrosis, psoriasis or COPD (Somers et al. 2010). Even more convenient, an increasing number of fibroblast-derived hiPSC lines have been established using fibroblasts of people with rare diseases like Marfan Syndrome (Quarto et al. 2012), Retinitis pigmentosa (Schwarz et al. 2015), β-Thalassemia (Ye et al. 2009a), Duchenne muscular dystrophy (Shoji et al. 2015), Spinocerebellar ataxia Type 2 (Xia et al. 2013), Gaucher disease (Panicker et al. 2012), Friedreich ataxia (Liu et al. 2011), Menke's disease (Kim et al. 2015), arrhythmogenic right ventricular dysplasia (Kim et al. 2013; Caspi et al. 2013) and rett syndrome (Djuric et al. 2015).

In comparison established keratinocyte cell lines and corresponding hiPSC from healthy individuals are available (Shrestha et al. 2019) but keratinocyte-derived hiPSC lines for disease modeling are rare, like attention-deficit hyperactivity

syndrome (Re et al. 2018).

In case of blood cells hiPSC lines for disease modeling are available e.g. for myelodysplastic syndrome (Kotini et al 2015), myeloproliferative disorder (Ye et al. 2009b) or craniometaphyseal dysplasia (Chen et al. 2013).

4.2 Sampling

Keratinocytes can be recovered simply from plucked hair as it does not only circumvent the invasive aspect but also can be done by virtually any person with a short instruction and pain is reduced to a minimum. Besides the three already mentioned advantages another major upside here is that plucking hair is not defined as invasive enabling the sampler to explain the process with the possible risks and get the informed consent of the participants. Any hair on the human body can be used as long as it is in the anagen stage. Unfortunately, the stage of the corresponding hair can only be determined after plucking it as hair in the anagen stage with a sufficient ORS show this as a white coverage on the surface of the root, a hockey stick shaped bulb and a darker pigmentation. In contrast, telogen hair has no such covering, a club shaped bulb and is less pigmented (Cotsarelis 1997). After the isolation the plucked hair needs to be transferred to a cell culture medium (like DMEM) immediately to prevent evaporation. This medium and hair containing tube can now be stored for several days at room temperature (Raab et al. 2014).

Fibroblasts are the dominant fraction in the connective tissue, therefore they are only accessible via a biopsy. Most samples are taken from the human skin using a punch biopsy or removed human foreskin (Schlaeger et al 2015; Trokovic et al. 2015a). Beneficial in this case is the connective tissue can be found in nearly any organ of the human body allowing various sampling sites. Several disadvantages have to be reported: The first remains the very painful biopsy (a small part of the skin is ripped out) not only with the consecutive bleeding but also with possible risks such as infection, compromised wound healing, growth of scar tissue or need for surgical wound treatment (Alguire and Mathes 1998).

This first disadvantage directly leads to the second: Invasiveness. A physician is required for the biopsy, this deceases the potential samplers to the comparably low number of physicians. Being defined as an invasive process the third disadvantage arises: Informed consent needs to be gathered by a physician. It is essential to have a physician to explain the whole process with all aspects and possible risks, finally an informed consent has to be given (in most cases in a written form). Another time intensive aspect which will reduce the number of participants is the result.

Endothelial progenitor cells can be found in both arteries and veins as well as lymphatic vessels and can be very effectively harvested from the umbilical cord blood (Murohara et al. 2000). Possibly the biggest advantage of these cells is that endothelial progenitor cells show the true individual genetic configuration because they have accumulated few mutations, thereby serving as a quiet healthy platform for storage to be used later (Haase et al. 2009). The acquisition seems very convenient at first because the umbilical vessels have to be cut off during birth, so the raw material is readily available. On second thoughts though a blood sample is considered an invasive process coming along with the same disadvantages as mentioned above except for the first one. Another drawback remains the limited time of accessibility during or directly after birth which in turn only enables this fraction to serve as source in personalized medicine in case a disease is known prior to birth.

In case of mature T-cells and myeloid cells, a sample of peripheral blood is necessary. Advantageous in this case is the fact that blood sample collection is a well established process in hospitals and medical centers. As already mentioned blood sampling is considered an invasive process, therefore the three disadvantages depicted for the fibroblast sampling also have to be named here.

CD34+ hematopoetic stem cells and progenitor cells are a stem cell fraction mainly in the bone marrow but also in the peripheral blood which are very potent in terms of reprogramming (Eminli et al. 2009). The acquisition was a major problem for a long time; These cells could either be gathered by direct

bone marrow aspiration or by sampling blood after stimulating their release into the peripheral blood. Both protocols are established for stem cell donation. Direct bone marrow aspiration is very painful, highly invasive, time consuming and expensive regarding required personnel and material. The other way starts by stimulation of the CD34+ cells in the bone marrow by controlled injections of granulocyte colony-stimulating factor (G-CSF) for five days (Souza et al. 1986). This mobilizes a greater number of CD34+ cells into the blood stream where they can be separated from the plasma and the cellular fraction of the blood in a process called apheresis. Comparable to a donation of thrombocytes, the donor's blood circulation system is connected to a machine which separates the CD34+ cells from the rest of the blood, stores them and transfers the remaining blood back into the donor's circulation system. Depending on the number of harvested cells, the apheresis might be repeated the following days (Szczepiorkowski et al. 2010). Both ways exhibit the same five disadvantages already described for fibroblast sampling but are furthermore very time consuming as well as personnel and material requirements are high.

Thanks to the efforts of many research groups things have changed lately, starting with the sample size. While some research groups diminished the amount of blood necessary for the isolation of enough CD34+ cells to 2-6ml (Merling et al. 2013, Ye et al. 2013), others pushed this even further to the volume of a single blood drop (10µl), easily obtainable by a finger prick commonly used to measure the level of glucose in the blood (Tan et al. 2014). Another breakthrough has been achieved in 2019 when a protocol for harvesting a sufficient number of CD34+ hematopoetic stem and progenitor cells simply from peripheral blood without previous stimulation or any subsequent treatment. On top both the reprogramming efficacy has been increased and the time frame got reduced, although this group used higher sample volumes ranging from 22 to 40 ml (Okumura et al. 2019).

The sampling method remains the major weak point: As described above, blood sampling is considered an invasive process that comes along with the three disadvantages already pointed out for the fibroblast sampling.

In summary keratinocytes are the only fraction which can be sampled by a non-invasive approach, thereby circumventing all the disadvantages of invasive sampling such as extensive pain, possible side effects, extensive personnel and material requirements (Figure 10). Keratinocytes can be easily gathered by plucking hairs which exhibits not only the smallest pain and fewest possible side effects but also can be done by virtually any person with a short instruction. Without the need for physicians and extensive material requirements but with the employment of the SKG system, large scale keratinocyte sampling is possible in contrast to the other cell sources depicted here.

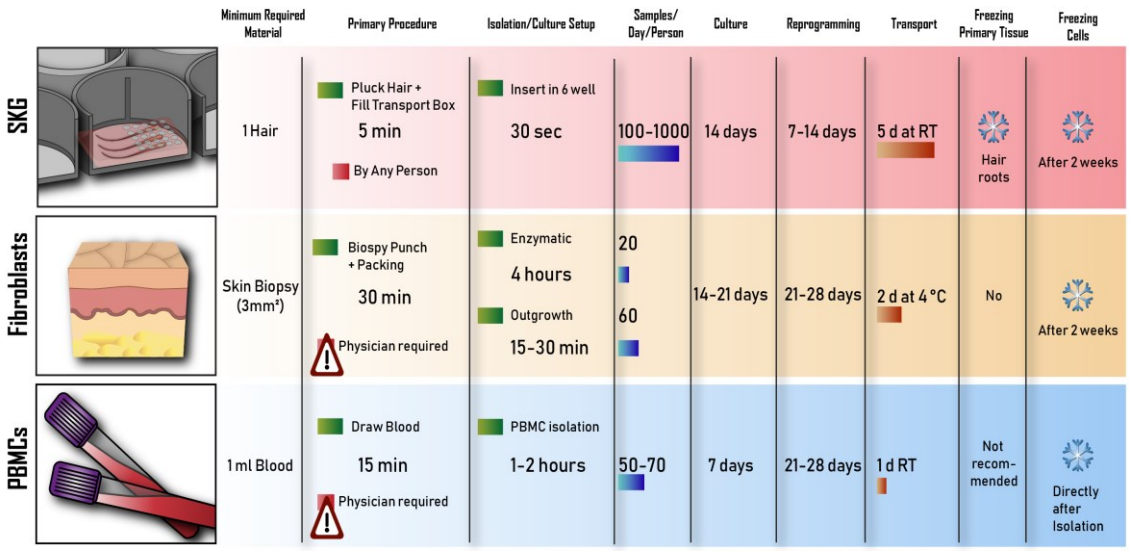


Figure 11: Comparison of needed resources for sample collection, culture and reprogramming of fibroblasts, peripheral blood cells or keratinocytes (using the SKG system).

4.3 Freezing and thawing

The idea of using low temperatures to preserve food is very old and can be traced back even to the period 2000 BC as historical sources report ice houses throughout Mesopotamia as storage rooms for food (Love 2009).

Effective cryopreservation is vital to scientific research and medicine. In case of medicine e.g. the field of organ transplantation extremely depends on

cryopreservation as the time frame to find a suitable recipient for a donated organ is limited by the organ decay. Still today a significant number of donated organs are discarded because the time frame expires before a suitable recipient is found, e.g. about 20% of all donated kidneys in the USA (Ibrahim et al. 2020; Reese et al. 2016) and approximately 67% of all donated hearts world wide (Ardehali 2015). These numbers could be reduced by implementing more effective cryopreservation methods (Israni et al. 2017). Stem cell-derived tissues and organoids play an emerging role in scientific research as they not only allow drug testing and side effect testing under realistic conditions (Liu et al. 2016; Truskey 2018) but also patient specific drug testing (Sandow et al. 2015). Long term effective cryopreservation methods therefore bring up three advantages. First they enable the establishment of bio banks. The second point is the reduction of invested resources and produced waste because if the organoids can be preserved there is no need to execute the previous steps to reprogram cells to hiPSC and to further differentiate them to organoids. The exclusion of animal testing is the third advantage.

In this case the SKG system benefits from a sufficient cryopreservation by allowing long distance transportation of samples with only minimal metabolic activity and therefore few mutations of the DNA and a sufficient viability of the cells.

An optimized freezing and thawing protocol has been shown to be essential to prevent the formation of ice crystals as they damage the cell membrane, thereby causing the death of numerous cells in the process of cryopreservation (Acker and McGann 2003). Various chemicals have been tested regarding their ability to prevent water from forming ice crystals. Such cryoprotectants must show the ability to pass through the cell membrane without any cytotoxic effect (Lawson et al. 2011). Some of the first cryoprotectants to be discovered were dimethyl sulphoxide (DMSO) and glycerol which are both still frequently used (Lovelock and Bishop 1959; Lovelock 1953). The different positive effects of cryoprotectants are not fully understood yet.

The major drawback of cryopreservation is the possible damage dealt to the

cells during the process of freezing that might cause cell death. This leads to a significant reduction of viable cells in the sample. Such damage can be inflicted by dehydration (Mazur 1963) or extracellular ice crystal formation: They can either directly damage the cell membrane by physical puncture or cause osmotic stress due to water depletion in the extracellular environment (Takamatsu and Zawlodzka 2006; Lovelock 1953). Intracellular ice crystals have an even higher damage potential by destroying intracellular structures or hindering metabolism (Persidsky 1971; Yu et al. 2017). A very good recovery of cryopreserved hiPSC could be shown if a cooling rate of -1°C to -3°C was used (Li et al. 2018). Recently the problem of the contradictory cooling rates, namely a slow cooling rate to prevent ice crystal formation versus a fast cooling rate to prevent cellular dehydration, has been addressed. The cooling process (and vice versa the warming process) was divided into three zones: First the dehydration zone, second the intracellular ice formation zone and third the further cooling zone. This work yielded the fast-slow-fast freezing protocol for optimized hiPSC cryopreservation with enhanced cell survival (Hayashi et al. 2020; Hayashi et al. 2021).
Overall cryopreservation is huge scientific topic itself, but the difficulty level of the SKG system had to be kept at a low level to prevent the exclusion of various possible cooperative laboratories and clinics due to limited resources. The decision was made to take these results into account as far as possible.
Improvements of cryopreservation protocols have been pushed further as it was shown that the addition of oxidative stress inhibitors to a solution using DMSO as cryoprotectant, such as N-acetyl cystine or resveratrol, increased the overall viability of cells after thawing by about 20% (Baust et al. 2022).

In summary a simple protocol has been used in this project reflecting the slow cooling rates and using DMSO as cryoprotectant. This simple protocol was implemented to once again decrease the overall difficulty of the SKG system and to increase its applicability. While freezing and thawing hair yielded satisfying results in the past, the attempts to freeze and thaw the loaded

prototypes failed in all attempts but one. This was most likely due to the entrance of Isopropanol into the freezing chambers with the consecutive elimination of the cells. More elaborate freezing chambers have to be developed to serve all the needs for a successful freezing and thawing of the loaded prototypes.

4.4 Evaporation

The SKG system was designed in order to have people with little to no training collect the samples. In this course the critical issue of cellular stress inflicted by the evaporation had been addressed.

A very interesting observation was the increased outgrowth rate of hair after 60 seconds of exposure of the root to air. While the highest outgrowth was seen after 60 seconds a still more than sufficient outgrowth rate after 180 seconds of exposure of the hair root to air was visible.

These findings are consistent with the results of research groups in the field of wound healing. One group prepared allogenic cultured epidermis using keratinocytes from the patient's skin. One allogenic cultured epidermis was dried containing no viable cells afterwards, the other one was used fresh. It could not only be shown that both contained an increased and similar level of growth factors such as basic fibroblast growth factor, platelet-derived growth fractor-BB, vascular endothelial growth factor and epidermal growth factor, but also that these factors even in the dried epidermis are functional and can stimulate keratinocyte proliferation (Sakamoto et al. 2020; Sakamoto et al. 2022).

These properties are not unique for keratinocytes, other groups could show the high wound healing potential of dried human amnion/chorion membrane grafts which contain fibroblasts and trophoblasts (Koob et al. 2013). The use of these grafts led to the closure of chronic wounds in four patients suffering from diabetes while conservative and even advanced measures failed (Sheikh et al. 2014). To underline the clinical value and usability of these membranes their

resistance has been pointed out as they can be exposed for up to 72 hours to temperatures ranging from -78,7°C to 73,5°C without impairments to their regenerative ability (Koob et al. 2014).

In summary a short exposure of the hair root to air led to an increased outgrowth rate and even a medium exposure of 180 seconds still led to more than sufficient outgrowth rates. This might be the result of the ability of keratinocytes to produce various growth factors at high levels and the fact that these factors are still functional after the death of the producing keratinocyte.
Taken together with the above mentioned easy and quick sample collection as well as installation in the prototype, the SKG system is a useful tool for large scale sample collection. Even little trained persons could pluck the hair and would have up to 3 minutes to place them in medium or directly on the prototype. Therefore no high speed working and no highly trained personnel is necessary when working with the SKG system.

4.5 Reprogramming

One main aim of this thesis was to expand the principles guiding design and development of the SKG system also to the reprogramming part in the course of generating hiPSC. Therefore we not only had to choose a reliable and effective reprogramming system but also one with few steps and lacking the need for rare and special laboratory equipment. As it has been pointed out earlier the choice of the cell fraction and the reprogramming system are the two main aspects of generating hiPSC. Keratinocytes for example showed a 100 times higher reprogramming efficacy (~0,8 %) and were reprogrammed two times faster than skin fibroblasts under the same conditions (Aasen et al. 2008).
Reprogramming of human fibroblasts takes about 4 weeks using the Sendai virus (Ban et al. 2011) and 4 to 5 weeks when employing a lentiviral system (Streckfuss-Bömeke et al. 2013). As it is the case for many cells, the reprogramming efficacy inversely correlates with the number of passages.

Fibroblasts should be reprogrammed not later than passage 5 (Streckfuss-Bömeke et al. 2013) Reprogramming efficacy varies in different studies and ranges from 0,05% (Streckfuss-Bömeke et al. 2013) to 0,8% (Elanzew et al. 2020). Many variants of reprogramming protocols exist, for example skin fibroblasts can be reprogrammed without c- Myc. This decreases the risk of tumorigenesis, which is beneficial for therapeutic purposes, but exclusion of c-MYC also decreases reprogramming efficiency to about ~0,0004 % (Nakagawa et al 2008).
Recently a fully automatic system has been developed, the StemCellFactory, which is able to execute the full process from culturing fibroblasts to Sendai Virus transfection as well as subsequent detection, harvest and propagation of emerging hiPSC. The reprogramming takes 21 days and yields an efficacy of 0,6% to 0,8% with a MOI of 5 (Elanzew et al. 2020).
Fibroblasts have a moderate methylation status while keratinocytes have a high methylation status of the promoter region of OCT4 and NANOG (Streckfuss-Bömeke et al. 2013), both factors which were identified as highly important for pluripotency (Tanaka et al. 2015; Karwacki-Neisius et al. 2013; Takahashi et al. 2007).

Starting from 0,02% (Trokovic et al. 2014) the reprogramming efficacy of blood cells has improved as well as reprogramming peripheral blood CD34+ cells in about 8 to 14 days yielded an efficacy of 2,55% lately, although this group used higher sample volumes from 22 to 40 ml (Okumura et al. 2019).
T cells show a ten to fifty times lower reprogramming efficacy compared to fibroblasts (Staerk et al. 2010). One research group could even outline a higher risk for T-cell lymphomas when using mature T-cells due to their V(D)J-rearrangement (Serwold 2007).
Nevertheless, these cells show a potency as reprogramming them yields cells with identical characteristics as hESC or other hiPSC (Loh et al. 2009).
Another cell fraction for hiPSC generation that can be sampled without an invasive process are human nasal epithelial cells, however culturing them takes

about 2 weeks and the reprogramming using the Sendai Virus takes further 20 days until hiPSC arise. The reprogramming efficacy was reported to be 0,08% to 0,1% with a MOI of 3 to 4 which is clearly inferior to our keratinocyte approach (Ono et al. 2012).

Reprogramming efficacy is negatively affected by cellular senescence (Banito et al. 2009). This term describes the late stage of cellular aging with a cell division cycle arrest caused by the stepwise up-regulation of the p53/p21 and p16 pathways (Li et al. 2013; Jackson and Pereira-Smith 2006).
To overcome these negative effects it was shown that the knockdown of p21 increased reprogramming efficacy in both donor cells from old donors as well as donor cells with a high passage number, in this experiment passage 25 and donor ages 81 and 83 (Trokovic et al 2015b). This clearly links reprogramming efficacy to cellular senescence as it has been previously described that cellular senescence could be reversed by knockdown of the p21 gene in human fibroblasts (Brown et al. 1997). Conclusively if hiPSC have to be generated from old patients and the keratinocytes yield an insufficient reprogramming efficacy, one possible solution might be a temporary knockdown of p21 to partially restore reprogramming efficacy.
Keratinocytes show a tendency to reach a state of senescence in the presence of calcium, therefore low calcium media can increase the time frame before this state is reached (Aasen and Belmonte 2010). Also the c-MYC induced apoptosis can be significantly reduced by addition of the ROCK-Inhibitor Y-27632 (Dakic et al. 2016). The combination of a low calcium medium and the ROCK-Inhibitor Y27632 has been shown to increase the proliferation rate and prevent cellular senescence without negatively affecting their differentiation competence (Strudwick et al. 2015).
Although the differentiation stage has been shown to have a great influence on the reprogramming efficacy, in this experiment for blood cells (Eminli et al. 2009), our results clearly underline the high reprogramming efficacy of terminally differentiated human keratinocytes in the anagen stage of hair follicle

cycle.

In summary we modified a very well established protocol for keratinocyte sampling and culturing (Aasen and Belmonte 2010) and chose the Sendai virus for transfection. Once again the main aim was to keep the protocol simple and fast, using a commercially available virus mixture for a simple spin transfection without many washing, purification or clearing steps seemed advantageous. Furthermore we quickly eliminated the feeder-assisted reprogramming for possible negative effects and additional resources needed. Our results point out reprogramming of keratinocytes to hiPSC using the SKG system can be achieved in about 21 days.

4.6 Genomic stability

Keratinocytes and fibroblasts, especially skin fibroblasts, show an increasing risk of accumulation of DNA mutations due to their continuous exposure to external effects like UV radiation and chemicals. In case of fibroblasts numerous mutations in the coding regions were shown in the used fibroblasts as well as in the reprogrammed hiPSC which have arisen (Gore et al 2011). Regarding genomic stability in terms of accumulated mutations blood cells tend to show a higher stability with the endothelial progenitor cells have by far the lowest number of somatic mutations leading to one of the highest genomic stabilities of all possible cell sources (Haase et al. 2009).

4.7 Possible applications

Besides the mentioned use of the SKG system for creating hiPSC by reprogramming of keratinocytes there are multiple other scientific and clinical areas which could benefit form employing the SKG system.

For cell culture experiments, the SKG system provides an easy tool for sampling and initial culturing of keratinocytes. Not only can these keratinocytes be used directly for cell culture experiments e.g. for psoriasis research, where a

decreased calcium influx into keratinocytes of affected people due to a downregulation of all transient receptor potential canonical channels in comparison to those of healthy individuals could be shown (Leuner et al. 2011). There is also the option to expand the keratinocytes in culture e.g. to create allogenic cultured epidermis to treat severe burn injuries or chronic wounds (Sakamoto et al. 2020; Sakamoto et al. 2022).

Even diagnostic approaches can benefit from using the SKG system as keratinocytes can be easily sampled and cultured. Containing the whole genome of the corresponding person these cells might serve as deliverers for DNA, RNA and even mitochondrial DNA and RNA. hiPSC still show DNA methylation signatures which are characteristic form the cells they were derived from. Additionally it could be demonstrated that hiPSC differentiate better into cells belonging to the same germ layer than those of a different germ layer (Kim et al. 2010). Because of their ectodermal origin, hiPSC derived from keratinocytes differentiate more readily into neuroectodermal cell lines compared with fibroblast-derived ones, although the basic characteristics of the two hiPSC showed no significant difference (Chlebanowska et al 2020). Therefore keratinocytes and the keratinocyte-derived hiPSC are ideal candidates for the research of neuronal diseases such as Parkinson's disease or neurodegenerative diseases.

4.8 Personalized medicine

Hippocrates already took the individual needs and characteristics of the patients into account when treating their diseases (Sykiotis et al. 2005; Pray 2008). Various different definitions of personalized medicine exist. The term refers to the concept of setting the patients individual desires, characteristics and genetic information as key aspects to increase therapeutic drug delivery and preventive medical care to the patient (Savoia et al. 2017; Ginsburg and McCarthy 2001; Jain 2002).

Delivering the best possible treatment is one of the main aims of personalized

medicine. For example, one study could illustrate a very beneficial effect of medication depending on the individual genotype. Treating patients suffering from colorectal cancer which exhibit a somatic mutation in the PIK3CA gene with acetylsalicylic acid after the surgery not only increased their overall survival but also reduced for cancer-specific deaths (Liao et al 2012). In this context the SKG system allows easy sampling and direct diagnostic search for genomic alterations in the DNA of the arising keratinocytes of the corresponding patient to evaluate the best possible treatment options. This underlines the use of the SKG system for an optimal individual drug therapy as one key aspect of personalized medicine.

Besides the single mutation diseases personalized medicine also focuses on risk and likelihood to develop diseases out of various genes and mutations. In this context polygenic risk scores have been developed to assess the individual risk of a person for certain illnesses which were shown to have good detection rate (Shieh et al. 2016; Khera et al. 2018). One group has given the scientific community access to over 100.000 fully sequenced genomes of patients and their family members with rare diseases or who are suffering from cancer (Turnbull et al. 2018). All these information were made accessible for the scientific community in different biobanks (Landrum et al. 2014; Rehm et al. 2015). These biobanks with numerous genomes allow large scale analysis and therefore enables more individual therapeutic approaches (Lek et al. 2016). One study could outline reduced morbidity and costs of hospitalization of inpatient infants if a quick whole genome sequencing was performed (Farnaes et al. 2018). Given this background, the SKG system could serve as an easy to use tool for large scale sample acquisition for subsequent whole genome sequencing which can be performed literally everywhere around the world (due to the simple protocol and the low requirements regarding material and personnel resources) and is even suitable for children (due to the non-invasive aspect and the low risk along with it).

Not only beneficial effects but also individual adverse drug reactions are subject to personalized medicine, e.g. one study could outline the risk of developing

colorectal cancer when constantly taking acetylsalicylic acid depends on the genotype and varies from low to high (Nan et al. 2015). Other groups aim to establish drug screening platforms using hiPSC derived from affected cell fractions of the patients such as sporadic amyotrphic lateral sclerosis (Fujimori et al. 2018). Successful organoid generation from hiPSC has already been shown for kidney (Taguchi et al. 2014), midbrain for modeling Parkinson's disease (Nickels et al. 2020), forebrain from patients suffering from Rett Syndrome (Gomes et al. 2020), cerebral cortex (Lancaster et al. 2013), intestine (Spence et al. 2011) and liver (Takebe et al. 2013). For testing individual drug effects the SKG system could serve as an easy and quick sampling platform, the arising keratinocytes would be reprogrammed into hiPSC. These cells would be used to create patient specific organoids as testing platforms for individual possible beneficial drug effects as wells as adverse drug reactions, a key aspect of personalized medicine.

Of course the SKG system has its limitations and therefore can't cover every aspect of personalized medicine. Individual environment exposure, personal thresholds, nutrition among others cannot be substituted by the SKG system and need to be monitored separately. One research group was able to point out the advantage of using personal thresholds instead of population thresholds. Personal thresholds in this case mean monitoring the person's individual values over time and observe changes always in context to the individual baselines. This group could show that the use of such personal thresholds when monitoring the value of the cancer marker CA125 led to the detection of ovarian cancer in women approximately one year earlier compared to the application of population thresholds (Drescher et al. 2013).

In summary, the SKG system is a great tool for non-invasive large scale sampling with very low requirements regarding material or personnel resources. The protocol established in this thesis allows quick and easy culturing of the keratinocytes as well as fast and effective reprogramming them into hiPSC, with

the possibility of direct reprogramming pushing the time saving aspect to the peak. Of course there is still potential for improvements, especially the direct reprogramming and the possibility of culturing the keratinocytes already during transport could further increase the efficiency of the SKG system and the protocol.

5 Summary/Zusammenfassung

5.1 Summary

The main idea was to develop an easy to use sample platform for a sampling procedure with minimal invasiveness and time consumption to allow sampling in various different nations and settings. We furthermore wanted to find an easy way for sample transport with minimal negative effects on the samples. Additionally, we wanted to create the Super Keratinocyte Gel (SKG) reprogramming protocol by modifying the well established reprogramming protocol of Aasen and Belmonte 2010. In this cause we chose to use the Sendai virus as delivering vector system (Li et al. 2000). We wanted to optimize the SKG protocol in terms of time consumption and efficiacy.

To address the idea of the modified protocol, we collected hair samples and compared the classical protocol and the SKG prototype as platform for culturing keratinocytes. Next we tested freezing and thawing the loaded prototypes as a possible transportation and storage option. As we aimed to apply a protocol empowering almost anyone as sample aquisitor, we examined the effect of evaporation on the outgrowth rate of the hair during the loading on the prototype. As another time saving possibility, we tried to replate the prototypes with the loaded hair after the outgrowth of keratinocytes was achieved. Finally we compared feeder assisted and feeder free of the keratinocytes into hiPSC using our developed protocol regarding time consumption and efficacy.
The main achievements of this study are: I) The establishment of the SKG system as a valid sampling platform for acquisition, transport and culture of keratinocytes. II) Further reduction of time and workload for generation of hiPSC by replating and reprogramming using the Sendai Virus and the optimized SKG protocol.

Altogether the SKG system is a great tool for non-invasive large scale sampling

with very low requirements regarding material or personnel resources. The protocol established in this thesis allows quick and easy culturing of the keratinocytes as well as fast and effective reprogramming them into hiPSC, with the possibility of direct reprogramming pushing the time saving aspect to the peak. This large scale sampling and effective reprogramming would enhance diagnostic and therapeutic research of various diseases.

5.2 Zusammenfassung

Die Hauptidee war einen Probenträger zu entwickeln für eine Probensammlung mit minimaler Invasivität und Zeitanforderung, um eine Probensammlung in verschiedensten Nationen und Umgebungen zu ermöglichen. Wir wollten außerdem einen einfachen Weg zum Probentransport finden mit minimalen negativen Einflüssen auf die Proben. Zusätzlich wollten wir das Super Keratinocyte Gel (SKG) Protokoll erstellen durch Modifizierung des gut etablierten Reprogrammierungsprotokolls von Aasen und Belmonte 2010. Zu diesem Zweck wählten wir den Sendai Virus als Übertragungsvektorsystem (Li et al. 2000). We wollten das SKG Protokoll hinsichtlich Zeitbedarf und Effektivität optimieren.

Um das modifizierte Protokoll zu erstellen sammelten wir Haarproben und verglichen das klassische Protokoll und den SKG Protoyp als Plattform zur Kultivierung von Keratinozyten. Als nächstes testeten wir das Einfrieren und Auftauen der beladenen Prototypen als mögliche Transport- und Lagerungsmöglichkeit. Da wir darauf abzielten, ein Protokoll anzuwenden, welches nahezu jede Person in die Lage des möglichen Probensammlers versetzt, untersuchten wir den Effekt der Evaporation auf die Auswachsrate der Haare während es Beladungsprozesses des Prototypen. Als weitere zeitsparende Möglichkeit versuchten wir die mit Haaren beladenen Prototypen erneut auszuplatieren und anzuzüchten nachdem das erste Auswachsen der Keratinozyten erreicht war. Schlussendlich verglichen wir die

ammenzellassistierte und die ammenzellfreie Reprogrammierung der Keratinozyten in humane induzierte pluripotente Stammzellen unter Nutzung unseres entwickelten Protokolls hinsichtlich Zeitaufwand und Effektivität.
Die Haupterfolge dieses Forschungsprojekts sind: I) Die Etablierung des SKG Systems als eine valide Probenplattform für Sammlung, Transport und Kulturierung von Keratinozyten. II) Die weitere Reduzierung von Zeit und Arbeitsaufwand zur Erstellung von humanen induzierten pluripotenten Stammzellen durch erneutes Ausplatieren und Reprogrammieren unter Nutzung des Sendai Virus und des optimierten SKG Protokolls.

Alles in allem ist das SKG System ein großartiges Werkzeug zur nicht invasiven, massenhaften Probensammlung mit sehr geringen Anforderungen hinsichtlich materieller oder personeller Voraussetzungen. Das in dieser Arbeit etablierte Protokoll erlaubt eine schnelle und einfache Kultivierung der Keratinozyten sowie eine schnelle und effektive Reprogrammierung derselben zu humanen induzierten pluripotenten Stammzellen, wobei die Möglichkeit der direkten Reprogrammierung den zeitsparenden Aspekt auf die Spitze treibt. Diese massenhafte Probennahme und effektive Reprogrammierung würde die diagnostische wie auch therapeutische Forschung diverser Erkrankungen vorantreiben.

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7 Erklärung zum Eigenanteil/Statement of contribution

Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde im Institut für Neuroanatomie und Entwicklungsbiologie unter Betreuung von Prof. Dr. Stefan Liebau durchgeführt.

Die Konzeption der Studie erfolgte durch Prof. Dr. Stefan Liebau und Dr. Kevin Achberger.

Die Versuche wurden nach Einarbeitung durch Dr. Kevin Achberger von mir mit Unterstützung durch Dr. Kevin Achberger durchgeführt.
Die qPCRs für die iPSC Charakterisierung sowie Keimblattfärbung wurden von Dr. Kevin Achberger durchgeführt.

Die statistische Auswertung erfolgte maßgeblich durch Dr. Kevin Achberger und durch mich.

Ich versichere, das Manuskript selbständig nach Anleitung durch Dr. Kevin Achberger verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 24.08.2024

Michael Wagner

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