Marlene Geyer

Evaluation of Novel Reprogramming Methods to Generate iPSC Lines from Blood Cells

Marshall Plan Scholarship- Research Paper

IMC FH Krems and
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Harvard Stem Cell Institute
Harvard University

Supervision:
Laurence Daheron, PhD
Mag. Dana Mezricky

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Date: 29.10.2019 Marlene Geyer

Abstract

Induced pluripotent stem cells (iPSCs) have a tremendous potential to be used in the treatment of various diseases, such as Alzheimer`s, Parkinson`s and diabetes, or for regenerative medicine and drug screening approaches to replace the need for animal models. The aim of this thesis was to test novel methods to reprogram human cells into iPSCs and compare them to already established reprogramming techniques, such as episomal vector and Sendai virus mediated reprogramming. These new methods include non-modified mRNAs, self-replicative RNAs and CRISPRa-based (clustered regularly interspaced short palindromic repeats) reprogramming. All of these approaches utilize the Yamanaka factors, which were shown to be sufficient for direct reprogramming of human somatic cells by Shinya Yamanaka in 2007. Blood cells, like erythroblasts and fibrocytes were used as starting material due to their ease of accessibility. In addition, fibroblasts, which require only a minimally invasive surgical isolation procedure, were also reprogrammed with these approaches to determine reprogramming efficiency. Erythroblasts could only be reprogrammed with the Sendai virus method but reprogramming of fibroblasts was successful using all of these methods, providing a variety of effective approaches for future study. After successful reprogramming, the newly generated iPS cells were tested for their stem cell characteristics using qRT-PCR and immunocytochemistry staining for pluripotency markers. After embryoid body formation, qRT-PCR for differentiation markers was performed to determine their ability to differentiate into the three germ layers. Characterization experiments assured that all generated iPS cells express both pluripotency and differentiation potential. However, reprogramming efficiency varied amongst those methods, requiring further experiments to improve efficiency, quality and safety of the iPS cells.

Moreover, the role of the miRNA 302/367 cluster, which is only expressed in stem cells was examined. By activating the expression of the miRNA 302/367, we showed that the expression of some of the key pluripotency markers were activated.

Key words: CRISPRa-based system, differentiation, iPSC, miR-302/367 cluster, RNA reprogramming, pluripotency, reprogramming

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Special thanks to the Austrian Marshall Plan Foundation for the financial support during my stay in the United States and therefore providing me with the chance to conduct research at the Harvard Stem Cell Institute.

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Amp^R	Ampicillin (resistance)
AP	Alkaline phosphatase
bFGF	Basic Fibroblast Growth Factor
(k)bp	(kilo) base pairs
BSA	Bovine serum albumin
Cas9	CRISPR-associated protein 9
cDNA	Complementary DNA
c-Myc	Cellular Myc
CRISPR	Clustered regularly interspaced short palindromic repeats
DMEM	Dulbecco´s modified eagle medium
DMEM/F12	Dulbecco's modified eagle medium, nutrient mixture F12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DPBS	Dulbeccos's Phosphate-Buffered Saline
DSB	Double strand break
EB	Embryoid body
EMT	Epithelial-to-mesenchymal transition
EpiSC	Epiblast stem cell
(h)ES or (h)ESC	(human) embryonic stem cell
E8	Essential 8
hPSC	Human pluripotent stem cell
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
ICM	Inner cell mass
iPS(C)	Induced pluripotent stem (cell)
Klf4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
LIN-28	Human protein encoded by LIN28 gene

MEF	Mouse embryonic fibroblasts (feeder cells)
MET	Mesenchymal-to-epithelial transition
miRNA	MicroRNA
mg/µg	Milligram/microgram
ml/µl	Milliliter/microliter
mm/µm	Millimeter/micrometer
MOI	Multiplicity of infection
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
Myc	Myelocytomatosis oncogen
NANOG	Homeobox protein, transcription factor
nt	Nucleotides
Oct4	Octamer-binding transcription factor 4
OSKM	Yamanaka Factors: Combination of Oct4, Sox2, Klf4, c-Myc
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
(RT)-qRT-PCR	(real time) quantitative PCR
SeV	Sendai virus
SFEM	Serum-free expansion media
Sox2	SRY-Box containing gene 2
srRNA	Self replicative RNA
TALEN	Transcription Activator-like Effector Nuclease
TF	Transcription factor
TSS	Transcription start site
WT	Wild type

1. Introduction

1.1. Historical background

The term “stem cell” originates from the Russian histologist Alexander Maksimov in 1908. Stem cell research and the application of stem cells in many therapeutic treatments has revolutionized medicine for the last several decades. For instance, the identification of hematopoietic stem cells has been instrumental in the development of bone marrow transplants to treat patients with leukemia. In 1962, John B. Gurdon´s experiments of somatic cell nuclear transfer into an enucleated frog oocyte led to the reversion of somatic cells into pluripotent cells. The beginning of the field of pluripotent stem cell research can be traced back to this experiment and to 1981 when the first successful isolation of mouse ESCs was described by Martin Evans. In 1998, the first human ESCs were isolated from preimplantation human embryos (Thomson, 1998), giving hope to millions of patients with degenerative diseases who could benefit from this unlimited source of cells for transplantation.

Reprogramming evolved from studies where the nucleus of an adult cell was transferred into an unfertilized enucleated oocyte, resulting in the generation of new offspring. Furthermore, cloning of mammals, such as the sheep Dolly by Ian Wilmut in 1997, laid the foundation for reprogramming. However, the major breakthrough in the field was the discovery made by Takahashi and Yamanaka in 2006. Their work involved reprogramming mouse fibroblasts into induced pluripotent stem cells via transfection of four transcription factors now termed “Yamanaka factors”: OCT4, KLF4, SOX2 and c-MYC (Rodolfa et al., 2006). iPS cells are almost identical to embryonic stem cells in pluripotency and self-renewal potential. In addition, they show similar morphology, proliferation, teratoma formation and transcription profiles, only differing in methylation and epigenetic modification pattern. In 2007, Yamanaka showed the same results in human cells utilizing the same factors. In the same year, Thomson used a slightly different reprogramming cocktail containing OCT4, SOX2, NANOG and LIN28 to reprogram human cells with lentivirus vectors, which added yet another way to reprogram cells. Capecchi, Evans and Smithies

received the Nobel Prize on their work with mouse ESC upon generating knockouts in 2007. In 2012, John B. Gurdon and Shinya Yamanaka shared the Nobel Prize in Physiology or Medicine.

1.2. Waddington`s landscape model

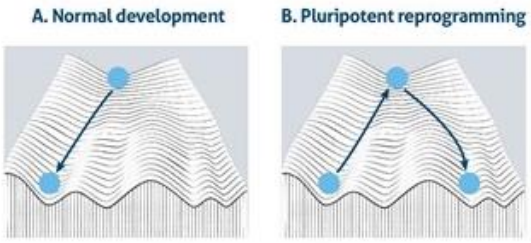


*Figure 1: **The landscape model by Waddington**: A ball rolling down a hill and not being able to go back to the top on its own can be used to describe somatic cell development because differentiated cells cannot reprogram themselves into stem cells on their own. Figure adapted from proteintech.*

Waddington describes the nature of cell fate as a ball running down from the top to the bottom of a mountain: it cannot go back on its own. This model (figure 1) represents the unidirectionality of cell development and the ability of stem cells to give rise to somatic cells, but not vice versa. Upon reprogramming, when a somatic cell type is converted to an induced pluripotent stem cell, the unidirectional trajectory of cell development is reversed as a differentiated cell can now become a stem cell again.

1.3. Adult stem cells versus embryonic stem cells

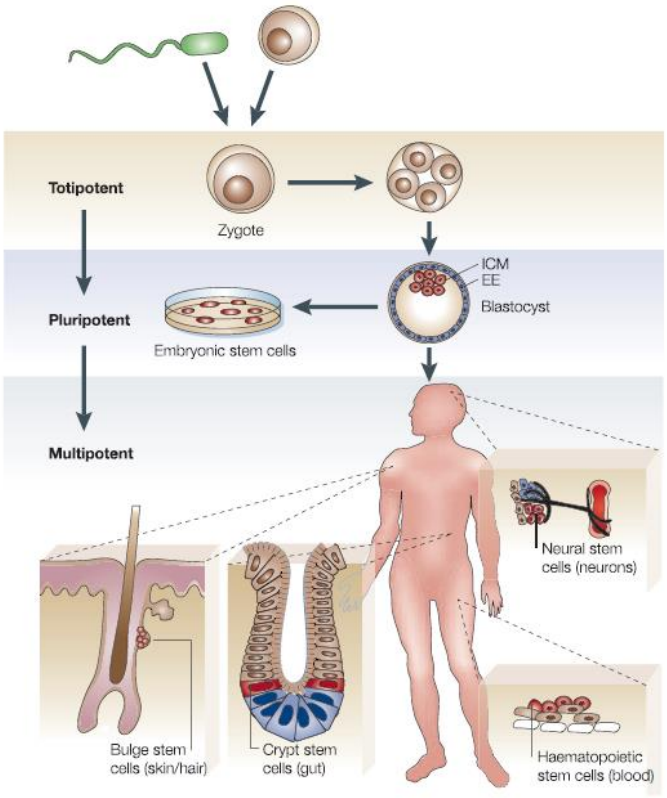


*Figure 2: **Derivation of stem cells**: Upon fertilization, a zygote forms, divides and develops into the blastocyst. The zygote is totipotent and can give rise to all cell types including extraembryonic cells. Pluripotent embryonic stem cells are derived from the inner cell mass of a blastocyst and can give rise to any cell type in the body. Cells like adult stem cells and cord blood cells are considered to be multipotent, giving rise to various cell types of specific lineages. Figure adapted from Eckfeldt et al., 2005.*

Development starts with two sex gametes: the haploid egg and the haploid sperm (figure 2). After fertilization, the zygote undergoes mitosis and forms the morula, which then assembles to the blastocyst. Cleavage to the blastocyst does not lead to an increase in size but, instead, several asymmetric divisions of the cells. The blastocyst consists of an outer layer called the trophoblast and an inner cell mass composed of stem cells, forming three germ layers upon gastrulation. The ectoderm gives rise to the skin and nerves, the mesoderm develops into the blood, muscle and some organs, and the endoderm becomes the inside skin and gut lining. After gastrulation, neurulation leads to the formation of the neural tube, giving rise to the brain and spinal cord (Nüsslein-Volhard, 2006).

Embryonic stem cells are capable of self-renewal through mitotic cell division, giving rise to new cells, and of pluripotency, being able to differentiate into various kinds of cells from the three germ layers. Embryonic stem cells are derived from the inner cell mass of a blastocyst five days post fertilization whereas adult or somatic stem cells originate from the bone marrow, blood, eye, skeletal muscle, lining of the gastrointestinal tract and nervous system. Adult stem cells are considered multipotent as they cannot differentiate into any type of tissue. They can simply self-renew and differentiate into some specialized cells, like hematopoietic stem cells, giving rise to various blood cells such as, lymphocytes, monocytes, red blood cells and granulocytes. Other adult stem cells are unipotent, such as muscle stem cells which can only generate one cell type. Adult stem cells are vital for repairing damaged muscle tissue, maintaining the gut wall, renewing blood, supporting the immune system and growing the nervous system (Kalra et al., 2014).

1.4. Embryonic stem cells versus induced pluripotent stem cells

In 2007, Yamanaka used the reprogramming cocktail of OCT4, SOX2, c-MYC and Klf4 on human fibroblasts to generate the first human iPSCs. Induced pluripotent stem cells are reprogrammed adult cells. They are similar to embryonic stem cells: they have an indefinite capability to self-replicate and they can develop into any tissue type. Induced pluripotent stem cells can be generated from somatic cells from patients and therefore provide a unique tool for disease modelling. These iPSC lines carrying mutations can be used to study diseases and genotype-phenotype correlations. Using the CRISPR/Cas9 or transcription activator-like effector nuclease (TALEN) technology, genes can be targeted to correct mutations, providing isogenic control lines for the research. As human ESCs are isolated from the inner cell mass of a blastocyst, which requires the use of in-vitro fertilization embryos, ethical and religious concerns might lead to restrictions of their use, making iPSC studies preferable. That does not absolve iPSCs of their limitations, as incomplete reprogramming or random mutations can generate imperfect iPSCs. Reprogramming methods still need to be further optimized to limit the use of viral gene delivery, which alters the genome, and to determine the whole mechanism of interplaying reprogramming factors.

1.5. Importance of stem cell research

Embryonic stem cells differ from differentiated cells in molecular signaling, morphology, gene activation, metabolism, epigenetic and transcriptional profiles and self-renewing ability.

They are valuable due to their potential applications in regenerative medicine, gene therapy, and drug screening. As these cells can differentiate into any type of cells, they could provide an unlimited source of cells for transplantation to treat diseases such as Parkinson`s disease, diabetes or macular degeneration. However, ethical issues preclude the handling of human embryonic stem cells for experiments in many countries. An alternative is to reactivate the pluripotency in human somatic cells by ectopic expression of several transcription factors, such as OCT4, SOX2, KLF4, LIN28 and NANOG.

With iPSCs, the persons own cells could be used for transplants, thereby reducing the risk of immune rejection to zero because of their histocompatibility. Besides being able to model function and development of diseases, iPSCs can also be used in personalized medicine (Takahashi et al., 2006). Understanding genotype-phenotype relationships and toxicity tests can also be achieved using iPSCs.

The methods to generate iPSCs are still very time-consuming, expensive and often limited to a specific cell type. Fibroblasts have been extensively used as a source of cells for iPSCs derivation due to their ease of maintenance. In addition, skin biopsies are minimally invasive, requiring only local anaesthesia. However, blood cells remain the optimal cell source for reprogramming as the method for sample collection is even less invasive than a skin biopsy.

1.6. Reprogramming approaches

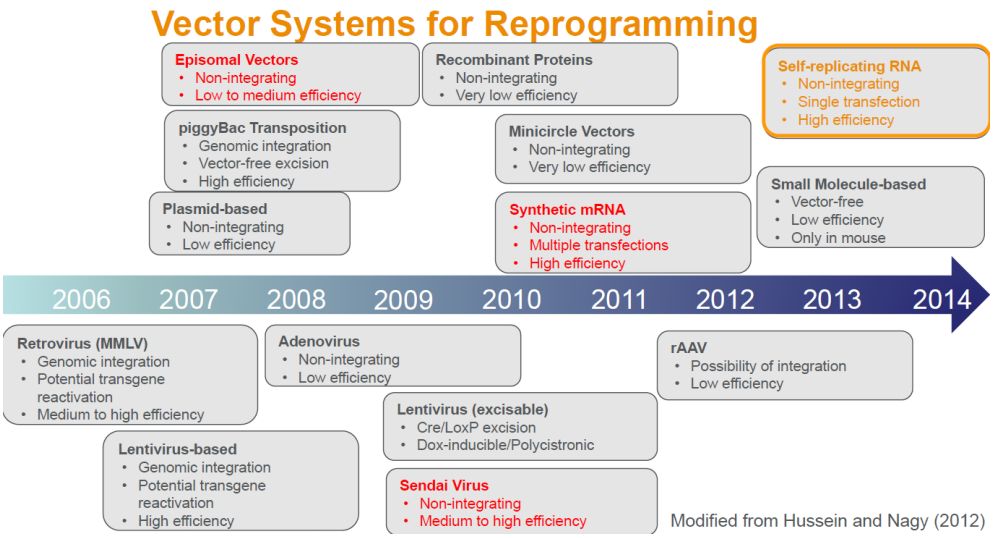


*Figure 3: **Different reprogramming techniques timeline**: Different vector systems such as retrovirus and lentivirus integrate into the genome and have been replaced by Sendai virus. Episomal vectors have a small chance of integration, making the use of RNAs and protein systems for reprogramming of somatic cells into iPSCs preferable. In my thesis, I will focus on non-modified mRNAs and self-replicating RNAs, as well as CRISPR-based reprogramming, which was first done in 2018 and therefore not shown on this timeline. In addition, I worked with episomal vectors and Sendai virus. Figure adapted from Stemcell Technologies.*

There are three different reprogramming approaches, however this thesis only covers direct reprogramming. The first approach is nuclear transfer (nt), where the nucleus of a somatic cell is isolated and transferred into an enucleated oocyte or zygote. These ntES cells are indistinguishable from hESC derived from embryos but their derivation is technically challenging and requires a large number of human oocytes.

The second approach, cell fusion of a somatic cell with an ES cell, leads to the generation of a tetraploid cell. While the use of tetraploid cells is very restricted, the method shows that there are some proteins in hESCs that can revert the state of the somatic cell nucleus.

The final approach, direct reprogramming shown by Yamanaka in 2006, occurs through the overexpression of various factors into somatic cells that result in the generation of an iPS cell.

Yamanaka and Takahashi identified 24 genes involved in pluripotency and tested those factors for their reprogramming potential. They narrowed down the number of necessary genes to four: OCT4, SOX2, KLF4 and c-MYC, which are now known as the Yamanaka factors. Octamer-binding transcription factor 4, OCT3/4 or POU5f1 forms a heterodimer with Sox2 and maintains stem cell characteristics. It is involved in germline formation and can be detected in the inner cell mass of the blastocyst. Upon gastrulation, which is the beginning of differentiation, OCT4 is downregulated and inhibits both the tumor suppressor factor p53 and human chorionic gonadotropin (Zhang et al., 2014). Only the isoform OCT4-IA is required for stemness.

Krüppel-like factor 4 (KLF4) is a downstream target of OCT4 through LIF signaling and therefore also important in pluripotency (Zeineddine et al., 2014). KLF4 regulates NANOG expression upon binding to its promoter. KLF4 also functions in Tert regulation via β-catenin, leading to telomere length control (Hoffmeyer et al. 2012).

SOX2, known as sex determining region Y-box 2 (SRY-box2), together with KLF4 and NANOG, control pluripotency gene expression. SOX2 can also be found in the ICM and deletion of SOX2 in embryos is lethal. OCT4, SOX2 and NANOG are the core transcriptional factors for maintaining self-renewal characteristics of pluripotent stem cells. SOX2 and OCT4 co-occupy many promoters and enhancers, thereby activating OCT4, SOX2 and NANOG genes and maintaining pluripotency. NANOG and OCT4 were shown to be involved in hypomethylation and formation of high-quality iPSCs (Zhang et al. 2014).

c-MYC dimerizes with the Myc-associated factor X via its helix-loop-helix leucine zipper motif and activates transcription. It is also a downstream target of the JAK/STAT3 pathway and is involved in controlling stem cell characteristics. c-MYC has been shown to be dispensable for reprogramming but does increase the reprogramming efficiency. Studies revealed that exogenous expression of c-MYC and KLF4 can be replaced with LIN28 and NANOG expression and that small molecules like BIX can further increase reprogramming efficiency.

BIX is an inhibitor of G9a histone methyltransferase and it has been shown to reduce H3K9me2 levels. Since transcriptional repression of Oct4 in somatic cells is linked

to H3K9me2 marks, adding BIX might open the chromatin to facilitate the reactivation of the endogenous pluripotency markers. (Shi et al., 2008). Other factors that are known to improve efficiency are p53 siRNA and UTF1. KLF4 functions upon p53 repression/knockout (Zhao et al., 2008). LIN28 binds RNAs and plays a vital role in pluripotency by blocking a hairpin structure of miRNA let-7 and influencing translation to prevent premature differentiation in the blastocyst. LIN28a is highly upregulated in stem cells and contributes to self-renewal ability (Shyh-Chang et al., 2013).

To deliver the Yamanaka factors into somatic cells, different methods have been described, mainly divided into integrative (retrovirus or lentivirus) and non-integrative approaches. For this project, only non-integrative methods were tested. Well established methods such as the episomal vector and Sendai virus were compared to newer methods like RNA and CRISPRa-mediated reprogramming.

1.*6.1. Episomal vector reprogramming*

Episomal vector reprogramming is a non-integrating vector transfection approach based on the Epstein-Barr virus. The vector comprises an oriP and a DNA binding protein EBNA1, which are necessary for retention of the vector in mammalian cells by extrachromosomal replication. However, plasmid retention is not perfect and it results in a loss of the vector after some time. The Epi5 kit from ThermoFisher® comprises five episomal vectors containing OCT4, SOX2, KLF4, L-MYC, LIN28, mp53DD and EBNA1 (Okita et al., 2011). A major limitation to the episomal vector method for the generation of clinical grade iPS lines is due to both the insertional mutagenic and latent gene activation potential.

1.6.2. Sendai virus mediated reprogramming

The Sendai virus mediated reprogramming utilizes the respiratory virus of mouse and rat from the *Paramyxoviridae* family. It was isolated in Sendai, Japan in the 1950s. The virus contains a single-stranded negative-sense RNA, which is released into the infected cell following attachment of the virus to the cell and fusion to the plasma membrane. After RNA replication, positive-sense RNAs are generated allowing a very high transgene expression level. As the virus does not undergo a

DNA phase, there is zero footprint, an advantage over the episomal vector which could integrate into the host genome. The CytoTune 2.0 Sendai reprogramming kit (Life Technologies, Cat#A16517) includes a polycistronic KLF4-OCT4-SOX2 vector, a c-MYC and a KLF4 vector (Fusaki et al, 2009). Advantages of Sendai virus mediated reprogramming include a wide range of targets, zero footprint, no infectious virus particle production and a high transduction efficiency. In addition, only one transduction is necessary for efficient reprogramming.

For the Sendai virus method, the iPS lines need to be screened to confirm the clearance of the transgenes as the RNA virus could linger in the cells. Therefore, RNA-based approaches such as mRNA-based reprogramming were developed to generate transgene free iPS lines which would be safer for clinical applications.

1.6.3. Non-modified messenger RNA reprogramming

After the mRNA is delivered into the cells, it is transcribed into proteins. The reprogramming factors are thereby synthesized, and the mRNA is degraded afterwards, leaving zero footprint. In 2010, Rossi used synthetic pseudouridine and 5-methylcytosine modified mRNAs for reprogramming, which required daily transfections for 17 days. The methods yielded a high reprogramming efficiency of 1-2%. The reprogramming mRNA mix contains the four Yamanaka factors, Lin28 and GFP for better detection of the transfection efficiency. However, the approach is technically challenging and very toxic for the cells if not used in combination with B18R, which reduces innate immune response by interfering with the interferon receptor. Moreover, this method does not work for blood cells. In 2015, an optimized version of this method, using non-modified mRNA (nm-mRNA) was published (Poleganov, 2015). This new method requires only 4 transfections for the reprogramming of fibroblasts.

Without modification, messenger RNAs are immunogenic and toxic, as they inhibit mRNA translation, cause cytoskeletal rearrangements and apoptosis. To overcome this problem, Poleganov et al. added three immune evasive mRNAs to the cocktail. Therefore, the nm-mRNA reprogramming utilizes the transfection of cells with OCT4, SOX2, KLF4, cMYC, NANOG, LIN28A (OSKMNL), the immune evasive mRNAs E3, K3, B18R (EKB) and green fluorescent protein (GFP). During this

research project, StemRNA^{TM}- NM Reprogramming by Stemgent® using Non-Modified RNAs was used, whereby mRNA is combined with pluripotency-inducing 302a-d/367 microRNAs (miRNAs), as it was confirmed to be one of the most effective non-integrating methods with the lowest aneuploidy rates. In order to overcome immunogenicity, synthetic non-modified mRNAs without 5-methylcytidine and pseudo uridine are used. These mRNAs differ in 5´phosphate and are recognized by both cytoplasmic and membrane-bound receptors upon molecular patterns. Interestingly, they alter gene expression by type I interferon (IFN) secretion, leading to protein kinase R activation and phosphorylation of eukaryotic initiation factor 2 alpha, inhibiting translation. E3 and K3 isolated from vaccinia virus are hypothesized to stimulate protein translation and to decrease cytotoxicity that is induced upon IFN beta response. In addition, the combination of E3, K3 and B18R is sufficient to prevent IFN response gene 2´5´oligoadenylate synthetase initiation. Furthermore, the addition of miRNAs to the previously mentioned factors improves reprogramming by acting synergistically with the transcription factors. Transfection of the Ψ^{-}/5me^{-} mRNAs also leads to elevated Toll-like receptor- mediated signaling, promoting reprogramming upon introduction of epigenetic changes. Therefore, non-modified mRNAs provide advantages in terms of immunotoxicity and cytotoxicity as well as reprogramming efficiency compared to Ψ/5me-modified mRNAs (Poleganov et al, 2015 and Schlager et al., 2015).

1.6.4. Self-replicative RNA reprogramming

This method avoids the usage of viruses and only requires a single transfection, therefore making it a very safe and time efficient protocol. One transfection of the non-infectious self-replicating Venezuelan equine encephalitis (VEE) virus RNA replicon containing OCT4, KLF4, SOX2 and GLIS and c-MYC is sufficient. The VEE replicate is a positive single stranded RNA, which does not integrate as it does not undergo a DNA phase. In addition to the four reprogramming factors it also contains a puromycin selection cassette for positive selection of transfected cells and it requires treatment with B18R, which decoys the interferon receptor to ensure transfected-cell survival (Yoshioka et al., 2013).

1.6.5. CRISPR/Cas9 system and CRISPR-based reprogramming

The clustered regularly interspaced short palindromic repeats (CRISPR) together with the CRISPR associated protein 9 (Cas9) were identified as a part of the prokaryotic immune system that provides resistance to foreign DNA and RNA of plasmids and viruses by cutting the genetic elements, working analogous to RNA interference in eukaryotes. A few years ago, the bacterial CRISPR/Cas9 system was modified to allow gene editing in eukaryotic cells. The system has advantages over other gene editing tools like transcription activator-like effectors (TALEN) and RNA interference in specificity, time-efficiency and simplicity in design (Garneau et al, 2010). The most commonly used CRISPR/Cas9 system originates from *Streptococcus pyogenes* and it consists of a CRISPR-associated (Cas) protein 9 and CRISPR guide RNAs (gRNAs). Its natural function can be seen in figure 4. The Cas9 nuclease can introduce double strand breaks (DSB) at specific loci in the DNA, thereby enabling Knock-ins and Knock-outs. The DSBs trigger cellular repair processes like non-homologous end-joining (NHEJ) or homology directed repair (HDR). NHEJ can generate small deletions or insertions at the target site, which can abolish gene function. HDR is less error-prone and requires a repair template.

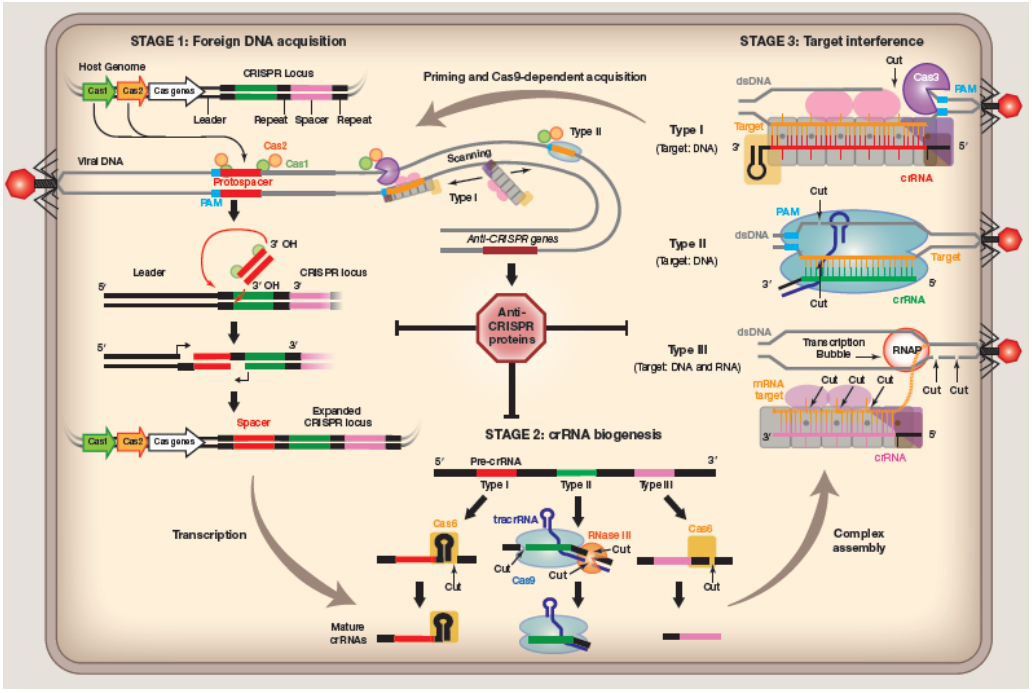


Figure 4: ***Three Stages of CRISPR/Cas9 function****: In stage one of the process, a new protospacer sequence is acquired from foreign invading DNA, which leads to the architecture of CRISPR loci. Those loci are defined by a series of short repeats with a length of around 20-50 bp separated by protospacers and flanked by several Cas genes (Wiedenheft et al., 2012). The protospacers are surrounded by a protospacer adjacent motif. The long CRISPR transcripts are then, in step two, processed to short crRNAs. The mechanism responsible for this differs in the different subtypes. In type II a tracrRNA, which is complimentary to the repeat sequence, forms an RNA duplex that is recognized and cleaved by RNase III. This structure is further processed leading to a mature crRNA which then associates with one or more cas proteins to form surveillance complexes. The final recognition and cleavage of foreign invading DNA is step three (Carter et al., 2015).*

Additional modifications of the CRISPR/Cas9 system have been developed to expand the repertoire of genome engineering tools, including: gene regulation, epigenome editing or chromatin imaging. CRISPRa- reprogramming is based on the CRISPR activator (CRISPRa) system, which is able to activate gene expression via an activator domain fused to the catalytically inactive dCas9 protein. This protein is recruited to gRNAs, which specifically bind to the complementary DNA sequence of interest. Thus, the genes at these loci can be activated or repressed depending on the domain fused to dCas9, for instance an activator like VP64/VP192/VPH or a repressor domain like KRAB (figure 5). Normal Cas9 protein generates a blunt double-strand break in the DNA used to generate knock-outs or knock-ins thereby altering the genotype. Dead Cas9 (dCas9) controls gene expression upon physical interference with the mRNA and the transcription complex or via functioning as

fusion protein, leading to activation by targeting the promoter. Thus, activation of genes that characterize stem cells (such as the Yamanaka factors) can lead to reprogramming of cells into iPSCs.

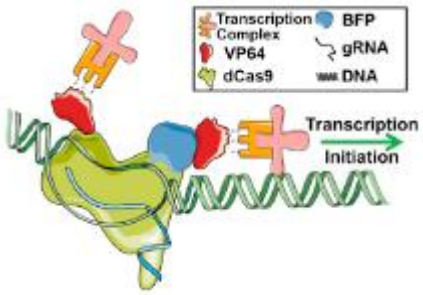


Figure 5: ***Functional complex necessary for reprogramming****: It consists of dCas9 activator targeted to the promoter of the Yamanaka factors. VP64 is fused to the transcription complex, which acts as an activator domain containing several transcription factors. The gRNAs guide the dCas9 to the right loci on the DNA. Figure adapted from Chakraborty et al., 2014.*

One approach to enhance reprogramming efficiency of CRISPRa systems is to combine gRNAs, which target the promoter of reprogramming factors, and regulatory elements such as Alu-motifs (EEA-motifs) (Weltner et al., 2018). Embryo genome-activation (EEA)-motif targeting increases the activation of NANOG and REX1. Those Alu-motifs are enriched in the promoter region of the genes expressed during human embryo genome activation. Weltner et al. hypothesized that EEA motif targeting could change the chromatin structure, making pluripotency genes more accessible. This combination is more powerful then targeting the six reprogramming factors alone, but still needs to be further improved. In addition, re-modelling of the promoter, enhancer and H3K27 histone acetylation modification was proven to be crucial in generating a pluripotency network (Liu et al., 2018). Moreover, a study showed that an increased number of transactivator domains fused to dCas9 enhanced gene activation to the level necessary to reprogram cell phenotype (Chakraborty et al., 2014).

1.7. miRNA 302/367 cluster and miRNA reprogramming

Coding mRNAs as well as non-coding RNAs play a vital role in cellular reprogramming. Yamanaka and Takahashi showed that replacement of c-MYC, which decreases the risk of tumour formation but negatively affects reprogramming, with miR-302d also leads to fully reprogrammed cells. Overexpression of the miRNA 302/367 was shown to be sufficient for human fibroblast reprogramming in presence of valproic acid. A number of miRNAs have emerged as regulators of the maintenance of stem cell potential: self-renewal and pluripotency. For instance, the miR-302/367 and the miR-371/372/373 are specifically expressed in pluripotent stem cells and are downregulated upon differentiation. They are scattered across the whole genome and they are also important for cell cycle regulation, metabolic pathway regulation, apoptosis and differentiation. They regulate chromatin assembly and transcription factor expression. Due to their small size of 18 to 24nt efficient knock-in and knock-out approaches remain difficult. Upon complementary base pairing with mRNAs they lead to degradation of the mRNA or translational repression (Zhang et al., 2013). Intergenic miRNAs are thought to contain the regions necessary for their own transcription, whereas intronic miRNAs, associated with Alu elements are transcribed by their host genes. Moreover, a shorter G1 phase in the cell cycle can be observed in ES cells compared to somatic cells. The miR-302 family plays a role in cell cycle regulation upon targeting Cyclin D1 which regulates G1 phase. In addition, they indirectly regulate OCT4 and TGFβ/Nodal during gastrulation for pluripotency maintenance. The miRNA biogenesis is depicted in figure 6.

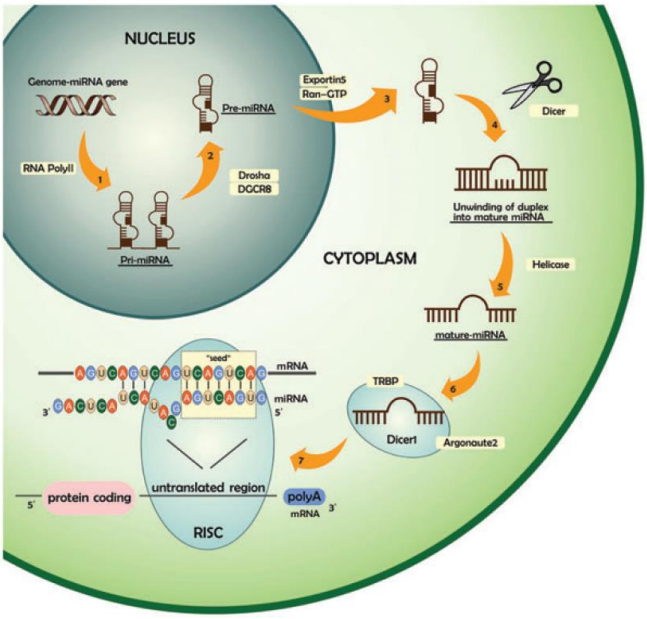


*Figure 6: **miRNA biogenesis**: Within the nucleus pri-miRNA (capped and polyadenylated) is generated upon transcription of the gene with RNA PolII. Those hairpin structures are then cleaved by Drosha (RNase III) to generate pre-miRNA. The pre-miRNA is exported into the cytoplasm and cleaved by Dicer (RNaseIII) into 20-22nt duplexes. The pieces get unwound by Helicase and the mature single stranded miRNA is loaded onto the RISC complex containing the proteins Ago-2 and GW182. The miRNAs typically bind at the 3´UTR (untranslated region) of mRNAs leading to degradation or translational repression of mRNAs. Figure from Gan et al, 2015.*

The intronic miRNA 302/367 cluster comprises -302b/b*/c/c*/a/a*/d and -367 that are co-transcribed polycistronically (figure 7). It is located in the chromosome 4q25 region and gets transcribed by RNA PolII. The 302 family members share similar sequences, just differing in 3´hexanucleotides.The genes targeted by the miRNA 302/367 are involved in cell cycle progression, mesenchymal-to-epithelial transition (MET) and epigenetic modulation. Overexpression of the cluster downregulates TGFβR2 protein in fibroblasts and increases E-cadherin expression involved in MET and reprogramming (Liao et al., 2011). Balancing G1-to-S transition of the cell cycle, genome-wide demethylation and regulation of Nodal and TGF-β pathway through BMP signaling are only a few functions of the miR-302/367 cluster.

The phylogenetically conserved cluster was shown to be only expressed in stem cells and not in fully differentiated cells.

Reprogramming factors like SOX2, OCT3/4, NANOG and REX-1 serve as regulators of the cluster leading to the stem cell properties of cells (Gao et al, 2015).

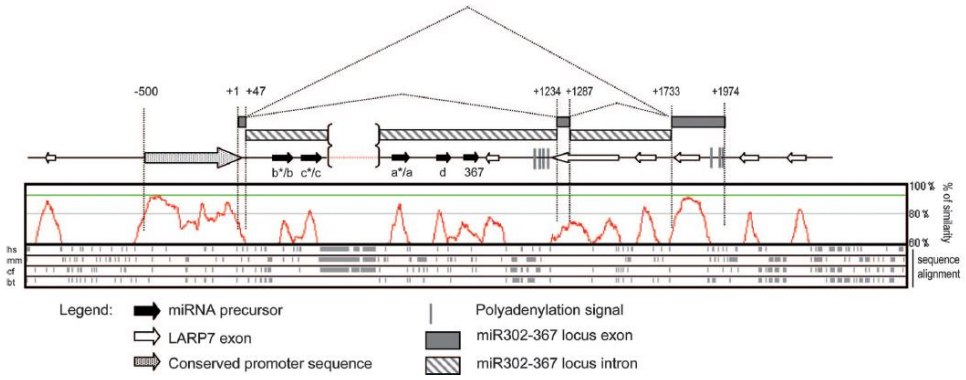


*Figure 7: **miRNA 302/367 cluster genomic region**: +1 represents the TSS and the black errors show the miRNA precursors, whereas the LARP7 gene directly follows the 367 locus and therefore partly overlaps the lncRNA region downstream of the miR-302/367 cluster. Alternative splicing is also indicated resulting into two different splice variants. Also, a sequence alignment between H. sapiens, M. musculus, C. familiaris and B. Taurus and therefore phylogenetical conservation of the miRNAs is shown. Figure from Barroso-delJesus et al, 2008.*

Overexpression of the miR-302/367 in combination with the Yamanaka factors led to an increased number of iPSC colonies. Moreover, overexpression of the cluster together with a histone deacetylase inhibitor is sufficient to reprogram cells (Sandmaier et al, 2015). The miRNA cluster is found in intron 1 of a non-coding gene consisting of 3 exons with alternative splicing properties leading to two different products. The core proximal promoter is 525bp in size with a TATA box in position -50bp. The miR-302/367 gene lies within intron 8 of *LARP7* gene but is not regulated by the LARP7 promoter.

Experiments showed that transduction of the miR-302/367 cluster into cancer cells led to gene expression profiles and OCT4 methylation patterns similar to human embryonic stem cells (Leonardo et al, 2012).

Several studies suggested that reprogramming of cells can be exclusively done upon transfection of miRNAs, while other protocols utilize miRNA in addition to the Yamanaka factors to achieve complete reprogramming (Lee et al., 2016). As

already mentioned, the miR-302/367 cluster is only expressed in stem cells and is believed to target MBD2, which normally binds to the NANOG promoter and inhibits reprogramming. As a result, when overexpressing the miR-302/367, NANOG expression increases dramatically and the efficiency of reprogramming is increased. In addition to downregulation of MBD2 and DNMT1, the miR-302/367 cluster regulates OCT4, SOX2 and SSEA3 (Lee et al, 2013). It also facilitates the epithelial to mesenchymal transition, one of the first step of the reprogramming process. Moreover, a full knockout of the miR-302/367 cluster by TALEN showed a complete block of the reprogramming of somatic cells into iPSCs (Zhang et al, 2013). One group showed that the overexpression of the miR-302-367 was sufficient to reprogram human fibroblasts in presence of valproic acid (VPA), a histone deacetylases inhibitor (Anoyke-Danso et al., 2011).

1.8. Diverse cell types used

In order to choose the right source of somatic cells for reprogramming the following things have to be taken into consideration: The cells should be easy to collect, such as blood samples, urine or minimally invasive skin biopsies. They should be safe to use with little or no mutations caused by age and UV exposure. In addition, the cells should be easy to reprogram such as stem or progenitor cells compared to fully differentiated cells.

1.8.1. Fibroblasts

The spindle shaped cells play an important role in the connective tissue and wound healing process. Upon hyperproliferation of fibroblasts scar tissue is formed. Juvenile foreskin fibroblasts show a mesenchymal stem cell phenotype and differentiation potential. They express vimentin, collagen and fibronectin and have a higher proliferation rate compared to MSCs, which provides an advantage in regenerative medicine. Similar to mesenchymal stem cells, they can suppress T-cell proliferation (Ichim et al., 2018). Fibroblasts play a role in wound repair as they produce the extracellular matrix. Foreskin fibroblasts have the advantage of low protein carbonyl levels and low oxidative stress due to high antioxidant activity

compared to other types of fibroblasts. In addition, they show slow telomere shortening, which is favored in tissue culture (Lorenz et al., 2001).

1.8.2. Fibrocytes

Fibrocytes are circulating spindle-shaped progenitor cells, that are derived from monocytes upon entering the tissue instead of becoming macrophages. They express a variety of markers such as CD45+, collagen1+/3+ and CD34+ and have fibroblast as well as blood (macrophage) and mesenchymal cell characteristics (Iqbal et al., 2012). They have a unique set of markers: CD45RO, 25F9 and S100A8/A8, but not PM-2K. Their differentiation can be inhibited by serum-free media and proliferation is inhibited by blood plasma protein serum amyloid P, which is the reason for cultivating them in serum-free media. Fibrocytes contribute to ECM formation and tissue repair upon enhancing leukocyte adhesion. They also release vascular endothelial growth factor (VEGF) and play a role in antigen presentation. They can differentiate into osteoblasts, adipocytes and myofibroblasts. Their cell number is increased upon inflammation, whereas they migrate to injuries and differentiate, thereby changing the expression of markers. Higher levels of fibrocytes are associated with heart failure, end stage kidney disease and pulmonary fibrosis (Cao et al., 2016).

1.8.3. Peripheral Blood Mononuclear Cells

Lymphocytes (B cells, T cells and Natural Killer cells), monocytes and macrophages are peripheral blood mononuclear cells, which can be isolated using a Ficoll gradient. Erythroblasts, which are nucleated precursor cells to erythrocytes are commonly used for reprogramming with the Sendai virus method. A proerythroblast undergoes morphological changes due to chromatin condensation, which is mainly driven by Epo. An interaction of the erythroblast with a macrophage is necessary to drive differentiation and proliferation of erythroblasts (Moras et al., 2017).

1.8.4. HEK293T cells

HEK293 cells are human embryonic kidney 293 cells and were exposed to human adenovirus type 5 DNA. HEK293T cells express T-antigen from SV40, which bind

enhancers to increase protein expression. They are neomycin resistant. These cells grow very fast and are easy to transfect. Therefore, they are often used in the laboratory (Thomas et al, 2005).

1.8.5. iPS culture conditions

Human iPSCs are quite compact colony-forming cells, that appear flat and not dome-like as mouse pluripotent stem cells. In addition, they are roundly packed cells and have a large nucleus to cytoplasm ratio (Thomson et al, 1998). Often, a multi-layering of cells can be seen in the center of the colonies with well-defined borders. They show a high telomerase activity, which is linked to immortality as the enzyme adds nucleotides to the telomeres to prevent cells from dying due to the Hayflick limit. They exist in a primed state and require basic FGF, whereas mouse SCs are still naïve and require LIF (Ludwig et al., 2006). iPS cells can either be grown on mouse embryonic fibroblasts (MEF), or on feeder-free culture where they are plated onto a layer of extra cellular matrix proteins such as Cultrex (isolated from Engelbreth-Holm-Swarm mouse fibrosarcoma cell line which includes laminin, collagen, heparan sulfate proteoglycan and entactin), Matrigel, Laminin 521 and recombinant human protein matrix Vitronectin. MEFs are mitotically arrested upon irradiation or treatment with mitomycin C to prevent them from growing. They release important factors that are necessary for hESC culture. iPSC colonies on feeder cells have a distinct border, whereas iPSCs on a matrix material appear to be spiky at the border. The elimination of an undefined compound, saving time in quality control, elimination of feeder batch-to-batch variation, better downstream applications, easier removal of spontaneous differentiation in the culture and the simpler scale-up are advantages of feeder-free systems.

1.9. Characterization of iPSCs

The need of high-throughput and cost-effective iPSC characterization is highly desired to ensure mutation-free high-quality cells and patient safety. Teratoma assay is the injection of iPSCs into immunodeficient mice and the subsequent study of the formed teratoma. A teratoma is a tumor that includes cells and tissue from all three germ layers. Nowadays, this labor-intensive method is replaced by embryoid

body (EB) formation, where the ability of iPSCs to develop into ectoderm, mesoderm and endoderm is observed. It is a very cheap and easily scalable approach, as no additional growth factors need to be added. The differentiation potential of newly derived iPS lines can be evaluated by EBs, RNA isolation, cDNA synthesis and qRT-PCR for differentiation markers (EN1, MAP2, NR2F2, SNAI2, RGS4, HAND2, SST, KLF5 and AFP). In addition, TaqMan Scorecard Assay helps identifying differentiation potential. Flow cytometry provides an assessment of pluripotent marker expression. Expression of pluripotency genes is determined by qRT-PCR for pluripotency genes (Actin as housekeeping gene, OCT4, NANOG, REX1, DNMT3B, hTERT and SOX2). Pluripotency marker (OCT4, NANOG, TRA160, SSEA-4) expression is also determined via immunocytochemistry (Antonio et al., 2017).

1.10. Aim of the project

The aim of this research project is to evaluate the reprogramming potential of easily-accessible human blood cells (such as erythroblasts and fibrocytes) and fibroblasts into iPSCs using non-modified mRNAs, self-replicative RNAs and CRISPR-based chromatin remodelling approaches as they would be the best methods to produce xeno-free and feeder-free iPSCs that can be used in clinical applications. All methods should be appraised to ensure a robust and reproducible generation of high-quality iPSCs. The goal is to compare these methods with more conventional methods such as Sendai virus and episomal vector. In addition, the role of the miRNA 302/367 cluster in reprogramming will be studied.

2. Materials and Methods

2.1. Cell culture

2.1.1. Media Preparation

MEF Media

450 ml DMEM (Gibco, Cat#11995-065)

50 ml Fetal Bovine Serum (VWR, Cat#97068-100)

5 ml Penicillin-Streptomycin (10000 U/ml) (optionally) (Life Technologies, Cat# 15140-122)

5ml L-Glutamine (Gibco, Cat#25030081)

hES Media

400 ml DMEM/F-12 (Gibco, Cat#11330-032)

100 ml KnockOut Serum Replacement (Life Technologies, Cat#10828-028)

5 ml L-Glutamine (Gibco, Cat#25030081)

5 ml Pen/Strep (10000 U/ml)

5 ml MEM Non-Essential Amino Acid Solution (100x) (Gibco, Cat#1140-050)

500 µl 2-Mercaptoethanol (55mM) (Life Technologies, Cat#21985-023)

10ng/ml Basic FGF (Life Technologies, Cat#PHG0261) were added to the daily media aliquots prior to feeding the cells.

mTESR1

400 ml mTeSR1 (STEMCELL Technologies, Cat#AD16384286)

100 ml mTeSR1 supplement was thawed at 4°C overnight.

5 ml Pen/Strep (optionally)

The media was aliquoted and frozen or used within two weeks.

Erythroblast media

StemSpan™ SFEM II media with erythroblast supplements (Stemcell Technologies, Cat#09655)

Freezing Media

9 ml Fetal Bovine Serum (Sigma Aldrich, Cat# A7159-50mL)

1 ml Dimethyl Sulfoxide (Sigma, Cat# D-2650)

Fibrocyte media

500 ml RPMI 1640 (Corning, Cat#10-040-CV)

10 mM HEPES (Gibco, Cat#15360-080)

1% 100x NEAA (Gibco, Cat#1140-050)

1 mM sodium pyruvate (Gibco, Cat#11360-070)

2 mM glutamine (Gibco, Cat#25030081)

100 U/ml Pen/Strep (Gibco, Cat#15140-122)

1% ITS+3 (Sigma-Aldrich, Cat#I1884)

EB Media

45 ml DMEM/F12

5 ml Knockout Serum Replacement

0.5 ml Pen/Strep (100x)

2.1.2. Feeder-dependent Cell Culture

Plating MEFs

6-Well plates with a cell density of 170 000 cells per well were used. The plates were coated with 0.1% gelatine for 15-20 minutes at RT. MEF cryovials were put into a water bath at 37°C for thawing. The MEFs were diluted with pre-warmed MEF media dropwise to prevent an osmotic shock. The samples were centrifuged at 200xg for 4 minutes and the supernatant was removed. The pellet was resuspended in MEF media and the cells were plated in 2 ml media per 6-well plate. The cells were evenly distributed in the plate and incubated for at least 8 hours to let the cells settle. The cells were used within 48 hours.

Feeding feeder-dependent hPSCs

Daily microscopic control of the cells was done to ensure absence of differentiated cells. Differentiated cells were removed by scraping using a dissection microscope

placed into a Biosafety Cabinet. The cells were fed daily with 2 ml of pre-warmed hES media with 10ng/ml Basic-FGF (added fresh before use). Over the weekend, cells were fed with 3 ml media instead of 2 ml to skip one day.

Passaging

The cells were usually split in a 1:1 to 1:3 ratio depending on cell density. Firstly, the media was removed and the plates were washed with DPBS (Life Technologies, Cat# 14190-250) once. 1 ml collagenase IV (StemCell Technologies, Cat# 07923) was added and the cells were incubated for 10 min at 37°C. At this stage, the cells are still attached to the plate, but the edges of the colonies are curly. The collagenase was removed and the plate was washed with DPBS once. Trypsin was not used since human PSCs have a poor survival after full dissociation. A milder enzyme should be used to keep the cells in clumps. hES media without FGF was added to the plates and the cells were detached using a cell scraper (VWR, Cat# 29442-200). The solution was pipetted into a Falcon tube and centrifuged at 200xg for 4 min. The supernatant was aspirated and the cells were resuspended in hES media with FGF. The MEF media from the plates was removed and the plates were washed with DPBS once, allowing the cells to be plated in a final volume of 2 ml per well. The cells were then incubated at 37°C.

Thawing

Matrigel or Vitronectin coated plates were prepared. The cryovials were partially thawed in a water bath at 37°C and 1 ml pre-warmed hES media was transferred to the vials dropwise. The suspension was transferred into a 15 ml Falcon tube and filled with media to around 8 ml. Centrifugation at 200xg for 4 minutes and aspiration of the supernatant was carried out, followed by resuspension of the cells in hES media with 10 ng/ml Basic-FGF and 4 µl Y-27632 ROCK inhibitor (10 µM) to increase survival. The MEF medium from the MEF-coated plates was removed, rinsed with DPBS and the cells were plated in the above-mentioned media and incubated at 37°C.

Freezing

Cryovials (VWR, Cat# 82050-180) were prepared with appropriate labelling and placed on ice. Mr. Frosty (VWR, Cat# 55710-200) was pre-chilled at 4°C. Passaging of the cells was carried out until the colonies were collected in a Falcon tube. A suspension was created upon pipetting up and down, followed by centrifugation at 200xg for 4 min. The pellet was then resuspended in 1 ml ice-cold freezing media per cryovial. The cells were immediately transferred to the cryovials and placed in -80°C with Mr. Frosty, enabling a freezing procedure of 1°C decrease per minute. After approximately 4 hours, the vials were placed in the liquid nitrogen tank.

2.1.3. Feeder-free culture

Plating Matrigel/Cultrex/Vitronectin/ Laminin-521

Matrigel aliquots were thawed at 4°C overnight, as Matrigel would otherwise solidify above 10°C. 500 µl ice-cold DMEM/F12 and 500 µl Matrigel media were mixed at the time of use. The Matrigel mix was added to 25 ml DMEM/F12 media and 1 ml of the diluted Matrigel was plated per well of a 6-well plate. The plates were incubated at RT for 1 h or in the fridge at 4°C sealed with Parafilm for a week.

Vitronectin was thawed at RT and diluted with PBS to 5 µg/ml (60 µl Vitronectin into 6 ml DPBS). The solution was gently mixed and the Vitronectin was coated immediately.

rhLaminin-521 (Gibco, Cat#80153) was thawed at RT and the 60 µl (100ug/ml) vial was mixed with 6 ml DPBS with Ca^{2+} and Mg^{2+} and incubated for at least an hour at 37°C.

Feeding

Daily feeding with RT-warm mTeSR1/mTeSR+/NutriStem/hES media was performed after removing the old media.

Passaging human iPSCs

Cells were passaged at least every 7 days to ensure fresh substrate. In general, cells were split after reaching 70-80% confluency, or upon appearance of

spontaneous differentiation or cell death. Plating too sparsely increases the risk of spontaneous differentiation, so a 1:1 to 1:4 splitting ratio every 4 days was chosen. The same procedure was carried out with feeder-dependent cells with the following changes: 1 ml Gentle Dissociation Mix, instead of collagenase, with an incubation time of 3-5 min. mTeSR1 media without ROCK inhibitor was used for dissolving the cell pellet after centrifugation and for incubation. Single cell suspension was avoided, instead aiming for small clumps of colonies upon resuspension.

Removing spontaneous differentiation

A picking tool (pipette tips or needles) under the picking microscope was used to remove the differentiated cells by scraping. The plate was then rinsed with DPBS and incubated with new media. If differentiation was too extensive, one MEF or Matrigel/Vitronectin well of a 6-well plate was prepared. The old plate was rinsed with DPBS and fresh media was added. The colonies were cut around the edges, broken into pieces, released into fresh media and plated on a new well. 10 µM Y-27632 was added for 24 h.

Transfer between feeder-dependent and feeder-free culture

One Cultrex-coated well of a 6-well plate was prepared and the cells on MEF were rinsed with DPBS. mTeSR medium was added and a picking tool under the picking microscope was used to cut around the edges of the colonies. The colonies were then broken into pieces and pipetted into fresh mTeSR media on re-plated Cultrex wells. 10 µM Y-27632 was added for 24 h and cells were subsequently cultivated using the normal procedure.

2.1.4. Cultivation of somatic cells

Cultivation of PBMC

SFEM II media was used for cultivation of PBMCs. PBMCs contain a mixture of different blood cells such as lymphocytes, monocytes, natural killer cells and dendritic cells. Some of them grow in suspension, whereas fibrocytes derived from monocytes grow as adherent cells.

Cultivation of fibroblasts

Fibroblasts were cultivated in non-coated T25 flasks with 7 ml MEF media. The media was changed every second day. 10% trypsin was used to passage the cells.

Cultivation of fibrocytes

Fibrocytes were cultivated in non-coated 6-well plates with 2 ml fibrocyte media. The media was changed every second day.

Passaging Fibroblast or HEK293 cells

Media was removed from the 6-well plates and the cells were washed once with PBS. Then the cells were incubated with 1 ml 10% trypsin for 3 min. 2 ml fresh media was added and the suspension was spun down for 4 min at 1000 rpm. The supernatant was removed, and the cells were resuspended in new media, followed by distribution to new wells in a desired splitting ratio.

Passaging suspension blood cells

Cells were collected and placed into a Falcon tube. The cells were spun down for 4 minutes at 1000xg and the pellet was resuspended in fresh media. The resuspended cell mix was then plated on new plates.

Counting cells

The cells were counted by spinning the cells down within a Falcon tube, resuspending the pellet in 1 ml fresh media. 10 µl of the cell suspension was mixed with 10 µl Trypan blue Stain (0.4%) (Gibco, Cat#2023039). 10 µl of the mix was placed on Counting Slides Dual Chamber for Cell Counter (BIO-RAD, Cat#145-0011) and counted with a TC10™ Automated Cell Counter (BIO-RAD).

2.1.5. Diverse transfection systems

Transfection using Lipofectamine (Thermo Fisher)

Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, Cat#L3000008), Lipofectamine™ MessengerMAX™ Transfection Reagent (Thermo Fisher, Cat#LMRNA003) and Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent

(Thermo Fisher, Cat#CMAX00001) were used for different transfections types. All three kits were used according to the manufacturer´s protocol.
For mRNA transfection of one well of a 24-well plate, 100 000 cells were used. 25 µl Opti-mem I media and 1.5 µl Lipofectamine MessengerMAX reagent were mixed and incubated for 10 min at RT. 25 µl Opti-mem I media and 5 µl mRNA (500 ng) were mixed and added to the other Eppendorf tube containing the reagent. The mix was incubated at RT for 5 min and 50 µl of the complex was added dropwise to the well. Analysis of transfection was done under the microscope and by FACS. For determining the correct RNA concentration and transfection conditions, GFP mRNA (Stemgent, Cat#05-0020) was used.

For transfection of plasmids (containing the CRISPR transactivator domains), Lipofectamine 3000 was used. 80 000 cells were seeded on the day prior to transfection. 25 µl Opti-mem I media and 1.5 µl Lipofectamine 3000 reagent were mixed per sample. 25 µl Opti-mem I media, 1 µl P3000 reagent and 1 µg DNA were combined and the mix was added to the first one. Incubation for 10-15 min at RT was carried out and the mix was added dropwise to the cells.

Transfection using Neon™ transfection system (Invitrogen)

For NEON transfection (Thermo Fisher, Cat#MPK1025), 100 000 cells per well of a 24-well plate were used and the cells were harvested, spun down at 1000 rpm for 4 min and the pellet was resuspended in PBS before spinning down once again. PBS was removed and the cell pellet was resuspended in buffer T or R (depending on the cell type), together with DNA (500 ng-1 µg), to a total volume of 10 µl, making sure the DNA volume did not exceed 10% of the total volume. The Neon® Tube was filled with 3 mL Electrolytic Buffer (Buffer E for 10 µL Neon® Tip and Buffer E2 for 100 µL Neon® Tip) and the desired voltage, time and number of pulses were set. Meanwhile a 24-well plate was prepared and the cells were replated into the media with the Neon tip after the transfection. 1300 V, 20 ms and 3 pulses were used for Neon transfection of blood cells.

Transfection using Amaxa™ 4D-Nucleofector Kit (Lonza, Cat#V4XP-2012)

500 000 cells were harvested and centrifuged for 10 min at 90xg. The supernatant was removed and the cells were resuspended in a solution containing 82 µl Nucleofector Solution and 18 µl supplement. 1-5 µg plasmid DNA (at a total of 10% of solution volume) was added and the mix was transferred into Nucleocuvette vessels. The samples were put into the instrument for electroporation. The cells were incubated after the transfection for 10 min at RT before transferring them to a 6-well plate with media.

FACS Analysis

2% FBS in PBS was prepared and 5 ml was aliquoted. Cells were collected from the wells into labelled Eppendorf tubes. 1 ml PBS was added to the cells and they were centrifuged for 5 min at 400 rcf. The supernatant was removed and the pellet was resuspended in 500 µl of 2% FBS solution. The cell suspension was filtered through filter-cap FACS tubes (FisherScientific, Cat#08-771-23) and added to the BD CSampler™ Plus. Linear FSC-A to logarithmic SSC-A plots, linear FSC-A to linear FSC-H and GFP-A to APC-A scatter plots were used to gate the cells. For determining cell viability, 10 µl propidium iodide was added to the cells. PI would just bind to dead cells, allowing for the determination of cell viability.

2.1.6. Reprogramming of cells

non-modified mRNA reprogramming

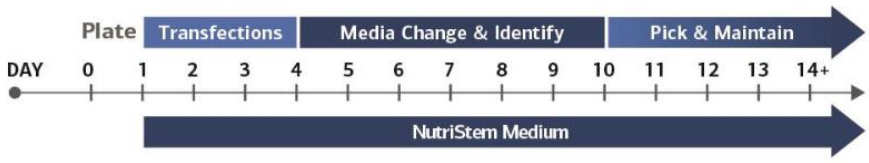


*Figure 8: **nm-mRNA reprogramming**: Timeline for reprogramming of cells with the non-modified mRNA method. On day 0, fibroblasts were plated in MEF media. On the following four days, media was changed in the morning and the cells were transfected in the afternoon to increase the survival rate of cells. Daily NutriStem media changes followed and colonies were picked after two to three weeks. Figure adapted from Reprocell.*

The Stemgent® StemRNA™ 3rd Gen Reprogramming Kit for Reprogramming Adult and Neonatal Human Fibroblasts (Reprocell, Cat#00-0076) was used for

reprogramming of fibroblasts. Instead of the RNAiMAX transfection reagent, 5.4 µl Lipofectamine MessengerMAX Reagent (Thermo Fisher Scientific, Cat#LMRNA001) and 90 µl Opti-Mem I Reduced Serum Medium (Thermo Fisher Scientific, Cat# 31985062) were mixed and incubated for 10 min. 90 µl Opti-Mem I media and 1.8 µg RNA (15.4µl premixed OSKMNL NM-RNA + EKB + nm-microRNA) were added to the mix and incubated for another 5 min at RT. Five hours prior to adding the Lipofectamine mix, media was changed to increase survival of the cells, as RNAs are very toxic for the cells. The mix was added dropwise to the cells and they were grown with a daily media change of NutriStem media for approximately two weeks until the first colonies could be picked (figure 8). Transfections were carried out on four days in a row.

self-replicative RNA reprogramming

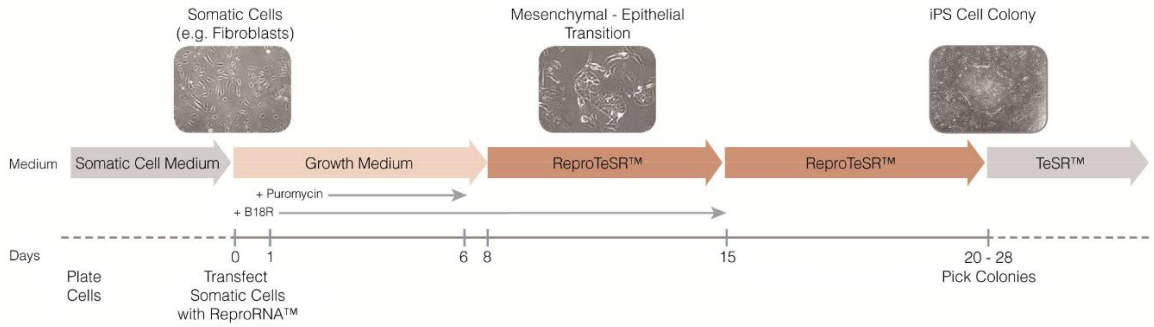


*Figure 9: **srRNA reprogramming**: Timeline for reprogramming cells with srRNAs. Only one transfection is required and Puromycin selection is needed for negative selection, whereas B18R is used for positive selection, so transfected cells do not die due to RNA toxicity. On day 0, cells were transfected and fed for one day in growth media plus B18R. Cells were grown in different media conditions for approximately 24 days until colonies could be picked. Figure adapted from Stemcell Technologies.*

The ReproRNA-OKSGM Kit (Stemcell Technologies, Cat#05930) and Erythroid Progenitor Reprogramming Kit (Stemcell Technologies, Cat#05924) were used and adjusted (figure 9):

The following media were prepared:

- Growth media: 1 ml SFEMII and MEF+ 0.35 µl 175 ng/ml B18R (0.5 mg/ml stock)
- ReproRNA Cocktail per well (2 wells): 3 µl/6 µl MessengerMAX reagent+ 50µl/100µl Opti-MEM. The mix was incubated at RT for 10 min and

50µl/100µl Opti-MEM were mixed with 1 µl/2 µl ReproRNA-OKSGM. The two tubes were mixed and incubated at RT for 5 min

- Growth media + Puromycin (prepared daily): Puromycin (final concentration=0.8 µg/ml and for erythroblasts 0.3 µg/ml) plus 1.5 ml/3 ml Growth media
- ReproTeSR (prepared at end of week one): 4.08 µl/12.25 µl 0.5 mg/ml Recombinant B18R (0.5 mg/ml stock) Protein plus 11.6 ml/35 ml complete ReproTeSR medium

On day 0, plates were coated with the appropriate matrix. Cells were counted and 100 000 cells per 6-well plate were seeded (adherent cells were plated the day before to let them attach). The media was removed, and the cells were washed with DPBS twice. Growth media with B18R was then added and the cells were incubated for 20 min at 37°C. Meanwhile, the ReproRNA cocktail was prepared and added dropwise to the cells. NEON transfection (9 µl buffer T and 1 µl ReproOKSGM RNA) was also carried out to determine the better transfection method.

On day 1, the media was removed from attached cells and 1.5 ml Growth media + B18R + Puromycin were added. Media was added to suspension cells without removing the old media. On days 2-5, media of attached cells was replaced with new media. On days 3 and 5, media of the cells in suspension was replaced by 1 ml ReproTeSR + B18R + Puromycin without removing the old media. On days 6 and 7, media of the attached cells was removed and replaced with 1.5 ml Growth media + B18R.

On days 8 to 14, media was removed and replaced by 1.5 ml ReproTeSR with B18R (for both attached and suspension cells). On days 15 to 28, media was removed and cells were incubated with ReproTeSR without B18R. The iPS colonies were manually isolated and plated on Cultrex with mTeSR1.

Sendai virus transduction of erythroblasts

100 000 cells were put into a 15 ml conical tube and spun down for 5 min at 1000 rpm. The cells were resuspended in 1 ml StemSpan™ SFEM II media with erythroblast supplement. The cells were transduced with the Sendai virus CytoTune 2 kit (Life Technologies Cat#A16517) in 1 well of a 12-well plate. A MOI of 5 for OSK (Oct4-Sox2 and Klf4) and for c-MYC and a MOI of 3 for KLF4 was used as recommended by the Life Technologies. A spinoculation was performed by spinning the plate at 2250 rpm for 30 min. After 24 h, the cells were collected in a 15 ml tube, spun down at 1000 rpm for 10 min and resuspended in 2 ml of fresh SFEM II media. On day 2, 6 wells of irradiated MEF were plated per transduced sample. On day 3, the cells were again collected, spun down and resuspended in 12 ml hES media with FGF. 2 ml of resuspended cells were seeded into each well of the plated MEF. On day 5, the media was changed by collecting the cells in a 15 ml tube and 1 ml hES media was added into the aspirated wells immediately to prevent attached cells from drying out. The tube was spun down at 1200 rpm for 5 min and the cells were resuspended in 6 ml hES media before being re-plated. On days 7 and 9, media was changed following the same procedure as day 5. On day 11, the media was aspirated and the cells were fed with hES plus FGF every day. The cells were grown until day 19-20 and iPSC colonies were picked and expanded.

Episomal vector reprogramming using LONZA Amaxa transfection

500 000 fibroblasts were transferred to a 15 ml tube and centrifuged at 1000 rpm for 4 min. The cell pellet was resuspended in 100 µl P2 Nucleofection solution (82 µl of solution plus 18 µl supplement, Lonza, Cat#V4XP-2012) containing 1 µg of each Epi5 plasmid (pCXLE-hOCT3/4-shp53, Addgene, Cat#27077; pCXLE-hSK, Addgene, Cat#27078; pCXLE-hUL, Addgene, Cat#27080) or using the Epi5™ Episomal iPSC reprogramming kit (Life Technologies, Cat#A15960). The solution was transferred to the Nucleofector vessel and the NHDF program was used to transfect cells. The cuvette was incubated for 10 min at RT and then transferred to a 10 cm dish with fibroblast media. The fibroblasts were then transferred onto MEF coated plates after 7 days and the media was changed to hES plus FGF at day 8. Media was changed every other day until colonies appeared and then changed

every day. At days 25-30, colonies were picked and further analyzed for their stem cell characteristics.

2.1.7. Cloning and gRNA design

For the activation of the miR-302/367 cluster, guide sequences were designed using the crispor online tool (http://crispor.tefor.net/crispor.py?batchId=fSJahSYaceT2zZpMdNMe#lists212). 4 gRNAs close to the TSS were chosen with a high specificity score (figure 10). For each gRNA, a sense sequence CACCGsequence(N19) and an antisense sequence Csequence(N19)CAAA were purchased to be cloned in a plasmid vector.

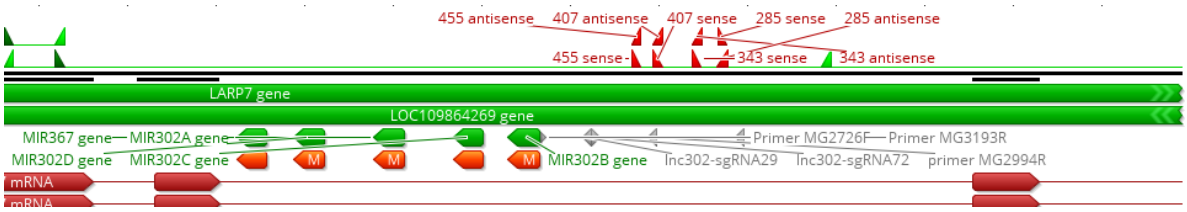


*Figure 10: **miR-302/367 cluster with all used primers and guide RNAs**: The green bars represent the miRNA genes. The red triangles are the self-designed guide RNAs (455, 407, 343 and 285) that are used to activate the miRNA cluster. The grey triangles are the primers used for PCR and sequencing (primer 2726, 2994 and 3193).*

Table 1: Oligo RNA sequences

Oligo RNA	Sequence
407 Sense	CACCG TCTCAGGGAATGTATGATCC
407AntiSense	C GGATCATACATTCCCTGAGA CAAA
285 S	CACCG ATGCCATCAAACAAGCAGAT
285 AS	C ATCTGCTTGTTTGATGGCAT CAAA
455 S	CACCG ATCTCAGAGAATCATTACAA
455 AS	C TTGTAATGATTCTCTGAGAT CAAA
343 S	CACCG AGGAAGATATCTTGTGGTAA
343AS	C TTACCACAAGATATCTTCCT CAAA

For oligo annealing, the heat block was turned to 90°C and both the oligo sense and antisense were diluted to 100 µM with TE buffer or water. To make the linker 2 µl oligo sense, 2 µl antisense, 2 µl NEB restriction buffer and 194 µl water were mixed

and placed onto the heating block for 5 min. The heat block was turned off and allowed to cool down to 50°C. Then the heat block was turned on to 42°C. The obtained oligo linker was diluted 1:10 with water. For ligation, 1 µl 0.1 pmol/µl vector, 15 µl water, 1 µl diluted oligo linker, 2 µl T4 ligase buffer, 1 µl T4 ligase and 1 µl T4 PNK (for the 5´Phosphate) were mixed and incubated at RT for 30 min. For transformation, 1 µl of ligation product was placed into an empty Eppendorf tube and stored on ice for several minutes. The competent cells (One Shot™ TOP10 Chemically Competent E. coli, Thermo Fisher, Cat#C404010) were thawed on ice for 5 min and 5-10 µl cell solution was added to the ligation product. The mix was kept on ice for 30 min, followed by heat-shock for 45 sec at 42°C. The resulting mix was incubated on ice for 2 min and 200 µl S.O.C media (Thermo Fisher, Cat#15544034) was added to each tube, followed by a 30 min incubation at 37°C. 50 µl were plated onto LB plus Ampicillin plates and incubated overnight. 3 colonies were picked the next day and incubated in 2 ml LB plus Amp overnight at 37°C. Minipreps were then carried out according to ZYPPY Plasmid Miniprep Kit (Zymo Research, Cat#D4100).

Maxiprep with EndoFree Plasmid Maxi Kit (Qiagen, Cat#12362)

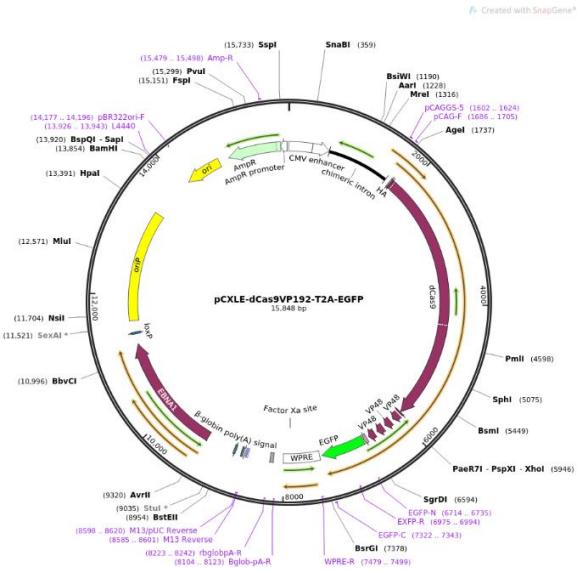
For the CRISPRa reprogramming the plasmids were obtained from Addgene (https://www.addgene.org/). The bacterial stab culture was scraped with a sterile pipet tip and streaked onto a LB plus Amp^R agar plate containing 100 µg/ml ampicillin. Colonies were picked and incubated with 2 ml LB plus ampicillin at 37°C overnight. The 2 ml were poured into a 200 ml flask and incubated overnight while shaking. The bacteria were harvested by centrifugation at 6000xg for 15 min at 4°C. Then the protocol provided by the manufacturer was followed. The DNA pellet was then resuspended in 200 µl buffer TE.

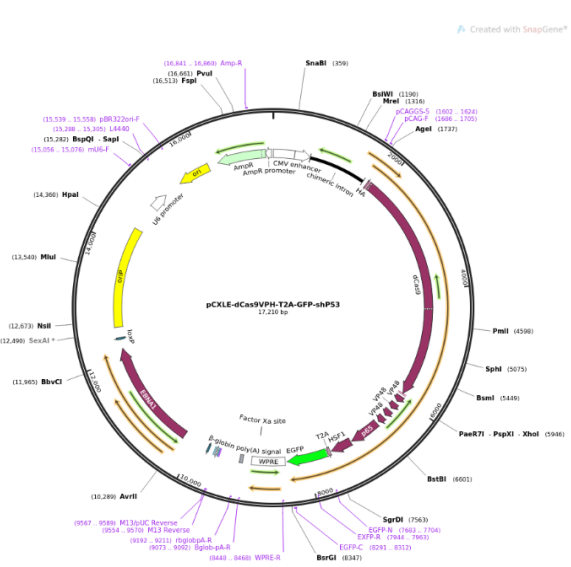
Miniprep with ZYPPY Plasmid Miniprep Kit (Zymo Research, Cat#D4100)

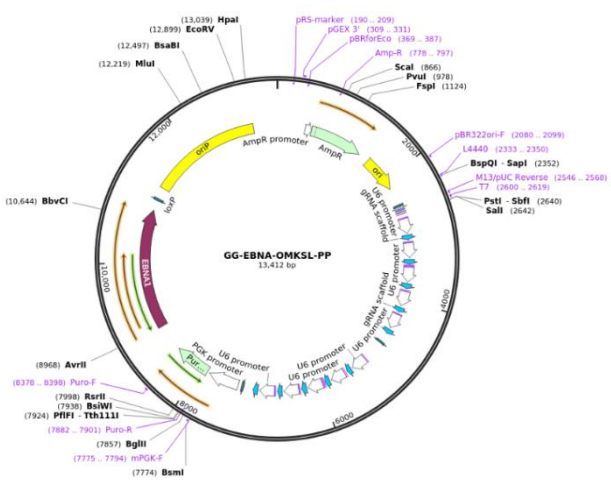
The manufacturer´s instructions were followed. DNA concentrations were measured with NanoVue Plus (GE Healthcare Life Sciences, Cat#28956057) and 500 ng plasmid DNA in 10 µl water was sent out for sequencing with M13F primer. The

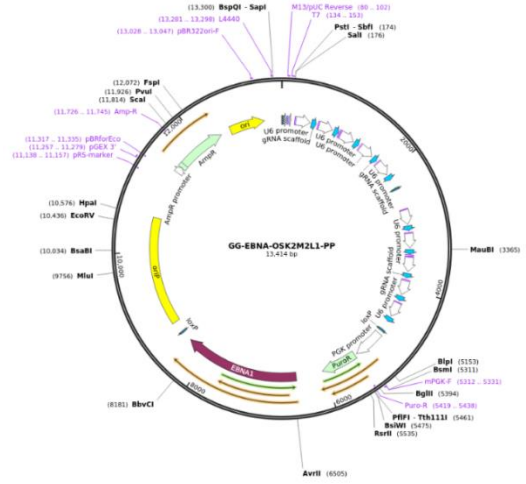
leftover of the positive gRNA stock was stored at -80°C in glycerol (700 µl sample in 500 µl glycerol).

Restriction digest









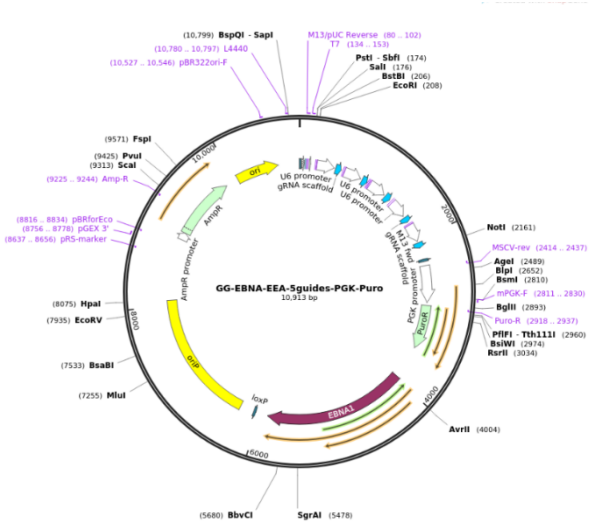


Figure 11: ***Plasmids containing transactivator domains and stem cell specific gRNAs****: The upper two plasmids contain VP192 (Addgene plasmid # 69536) and VPH (Addgene plasmid # 102895) transactivator domains, which were co-transfected with the GG-EBNA-OMKSL (GG-EBNA/GG) (Addgene plasmid # 102899) plasmid, the GG-EBNA-EEA-5guides-PGK-Puro (EEA) (Addgene plasmid#102898) or the GG-EBNA-OSK2M2L1-PP (OSK2M2L) (Addgene plasmid#102902) that contain gRNAs that target genes for stem cell characteristics. In addition to the GG-EBNA-OMKSL plasmid, the KM plasmid (not shown here, from Weltner et al.) was co-transfected to increase the expression of c-MYC and KLF4, as those two genes were not properly expressed upon transfection of the first plasmid alone. Upon transfection, the genes should be activated leading to reprogramming of somatic cells into iPSCs. Figures adapted from Addgene.*

In order to test the quality of the plasmids containing the transactivators and guides (figure 11), a restriction digest of the plasmid DNA was done by mixing 2 µl buffer (either NEB 1.1, 2.1, 3.1, CutSmart), 0.4 µl enzyme I (in case of VPH and VP192: MluI and for GG-EBNA: BglII), 0.4 µl enzyme II (VPH and VP192: PvuI and for GG-EBNA: PstI) and 0.4 µg DNA. The mixture was filled with water up to 20 µl and incubated at 37°C for 1 hour. The restriction digest was then diluted with 4 µl Gel Loading Dye, Purple (6X) (New England Biolabs, Cat# B7024S) and then loaded onto a 2% agarose gel.

RNA extraction

The RNeasy Mini Kit (Qiagen, Cat#74106) was used to isolate RNA. The cells were collected (normal passaging protocol) and spun down to remove the supernatant. 350 µl Buffer RLT was added to the cells and they were vortexed for 1 minute. 350 µl of 70% ethanol was then added to the cells and mixed. 700 µl of the sample was

transferred to a RNeasy spin column and centrifuged for 15 seconds at 10 000 rpm. The flow-through was discarded and 350 µl of Buffer RW1 was added to the RNeasy spin column. Centrifugation for 15 seconds and discarding of the flow-through was carried out. In a separate Eppendorf tube, 10 µl DNase was mixed (by inverting tube) with 70 µl Buffer RDD and spun down to collect liquid from the sides of the tube. 80 µl of DNase mix was added directly to the column membrane. The column was incubated for 15 minutes at RT. 350 µl of Buffer RW1 was added to the column and centrifuged for 15 seconds. The flow-through was discarded and 500 µl Buffer RPE was added. The column was centrifuged for 15 seconds at 10 000 rpm and the flow-through discarded. 500 µl Buffer RPE was added to the column, centrifuged for 2 min and the collection tube with the flow through was removed. The column was placed in a new 2 ml collection tube and spun down for 1 minute at 12000 rpm. The column was placed into a new 1.5 ml tube and 30-50 µl RNase-free water was added to the column and centrifuged for 1 min at 10000 rpm. The column was discarded, and RNA concentration and quality were determined with NanoVue.

cDNA synthesis

The qScript™ cDNA SuperMix (QuantaBio, Cat#95048-100) was used. The cDNA was subsequently diluted with ddH_2O in a 1:20 ratio and used for PCR. 4 µl qScript cDNA SuperMix (5x) and 1 µg RNA template were used, adding the appropriate volume of water to bring the total reaction volume to 20 µl. The reaction was run for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The 20 µl cDNA was then diluted with 380 µl water before running the qRT-PCR.

DNA extraction

The DNeasy Blood and Tissue Kit (Qiagen, Cat#69504) was used.

Phusion PCR

4 µl 5x Phusion HF buffer, 0.5 µl dNTPs (10 mM), 1 µl (10 mM) forward primer, 1 µl (10 mM) reverse primer and 0.2 µl Phusion DNA polymerase were used for a 20 µl reaction. The PCR was performed according to table 3.

Table 2: PCR protocol

Phase	Temperature	Time
Denaturation	98°C	1 min
Denaturation	98°C	10s
Primer annealing	58°C-62°C	30 s
Elongation	72°C	30 s
Final elongation step	72°C	5 min

Gel electrophoresis

According to the expected size of the PCR product, a 1%, 1.5% or 2% agarose gel was used. 100 ml 1x TAE was warmed with agarose powder (Fisher Scientific, Cat. No. BP1356-500) until the agarose melted. The solution was cooled for 10 min and the gel was stained with 10 µl EtBr. The PCR products were run on the gel in parallel with either a 100 bp ladder (NEB, Cat#N3231S) or a 1 kb DNA Ladder (New England Biolabs, Cat#N3232L) to determine the size of the resulting band (figure 12). The gel was run at 100 volts for 40 min and analyzed under ultraviolet light.

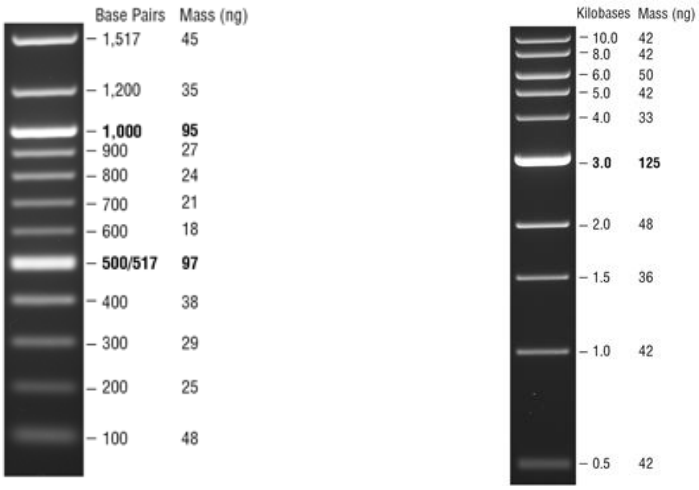


*Figure 12: **100bp and 1kb ladder from NEB** to determine the size of the bands on an agarose gel. Figure adapted from NEB.*

2.1.8. iPS characterization

Embryoid body formation: in-vitro differentiation

To determine the differentiation potential of stem cells, iPS cells were cultivated in 6-well plates until 80% confluence. The media was aspirated and the plate was washed with 1 ml DPBS. After aspirating the DPBS, 1 ml Gentle Cell Reagent was added to the cells and allowed to sit for 5 min at RT. The reagent was then removed and the cells were once again washed with DPBS. 1 ml EB formation media was added per well and the cells were scraped into small clumps containing around 20 cells. The clumps were transferred into a 15 ml conical tube and allowed to sit for 10 min. The media was aspirated and the clumps were carefully resuspended in 12 ml EB media containing 4 µl Rock inhibitor per 2 ml media. The cells were then plated into an ultra-low attachment 6-well plate (Corning, Cat# 3471). After 24 hours, the cells were collected in a 15 ml conical tube and allowed to sit for 10 min. The supernatant was removed and fresh media without Rock inhibitor was used to re-plate them. The media was changed every other day until day 8. Next, cells were re-plated on gelatin-coated plates with DMEM 10% FBS media and cultured for 7 days until day 15.

For determining expression levels, cells were collected on different days and the pellet was frozen at -80°C until RNA extraction, cDNA synthesis and qRT-PCR were completed.

Scorecard analysis

The TaqMan™ hPSC Scorecard™ Panel 384-well (Thermo Fisher, Cat# A15870) was used for qRT-PCRs to determine the differentiation potential of iPSCs into the three germ layers. The 20 µl cDNA product was diluted in 550 µl nuclease-free water and 570 µl Gene Expression Master Mix was added. 140 µl of the mix was loaded into each tube of an 8-tube strip. 10 µl of that was added into the Scorecard panel with a multichannel pipette. The Scorecard template Viia7 was used to run the Scorecard in the qRT-PCR machine. The results were analyzed with software provided by the manufacturer.

qRT-PCR

The samples were prepared in triplicates for better reproducibility and 2 µl water, 5 µl 2x Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, Cat#4385610), 0.5 µl forward primer and 0.5 µl Reverse primer were mixed and added to 2 µl cDNA in 384-well plates. The plates were spun down for 2 min at 1500xg and run in the QuantStudio 12K Flex (applied biosystems by life technologies) with a hold stage for 20 s at 95°C, a PCR stage for 1 s at 95°C and 20 s at 60°C for 40 repeats and a melt curve stage for 15 s at 90°C and 1 min at 60°C.

qRT-PCR for pluripotency markers used SYBR Fast with primers for Actin or RPLPO, NANOG, SOX2, REX1, Dnmt3b, hTERT and OCT4. qRT-PCR for differentiation markers was carried out using 5 µl TaqMan Mastermix, 0.5 µl primer, 2 µl water and 2.5 µl cDNA. The samples were tested for the following primers: RPLPO for gene expression control, EN1, MAP2, and NR2F2 for ectoderm formation, SNAIL2, RGS4, and HAND2 for mesoderm formation, and SST, KLF5 and AFP for endoderm formation.

Table 3: Primers used for qRT-PCR

Primer	Forward sequence	Reverse sequence
Actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGGGTACAG
DNMT3b	ATAAGTCGAAGGTGGGTCGT	GGCAACATCTGAAGCCATTT
hTERT	TGTGCACCAACATCTACAAG	GCGTTCTTGGCTTTCAGGAA
NANOG	CAGTCTGGACACTGGCTGAA	CTCGCTGATTAGGCTCCAAC
OCT4	TGTACTCCTCGGTCCCTTTC	TCCAGGTTTTCTTTCCATAGC
REX1	TGGACACGTCTGTGCTCTTC	ATAAGTCGAAGGTGCGTCGT
SOX2	GCTAGTCTCCAAGCGACGAA	GCAAGAAGGCTCTCCTTGAA
KLF4	CTGATCGGGCAGGAAGGATG	ACCCTGGGTCTTGAGGAAGT
LIN28	AGGAGACAGGTGCTACAACTG	TCTTGGGCTGCGGTGGCAG
c-MYC	AGCGACTCTGAGGAGGAACA	CTCTGACCTTTTGCCAGGAG
EX (for miRNA)	TCTGGAGGAGAACACGAATCT	TGAAGTCAGAACAGGCAAGAG

Immunocytochemistry

Table 4: Primary and secondary antibodies used for immunocytochemistry staining

Primary antibody	Dilution factor of primary antibody	Secondary antibody
Oct4 (Abcam, Cat#ab19857)	1:20	Alexa Fluor® 488 donkey anti-rabbit IgG (Invitrogen, Cat#A21206)
Nanog (Abcam, Cat#ab21624)	1:50	Alexa Fluor® 488 donkey anti-rabbit IgG (Invitrogen, Cat#A21206)
SSEA4 (Millipore, Cat#MAB4304)	1:200	Alexa Fluor® 488 goat anti-mouse IgG (Invitrogen, Cat#A21121)
TRA-1-60 (Millipore, Cat#MAB4360)	1:200	Alexa Fluor® 555 goat anti-mouse IgM (Invitrogen, Cat#A21426)

5 wells of a 48-well plate were seeded with cells. The plate was incubated for 4-5 days. The cells were then washed with 500 µl PBS three times and fixed with 150 µl 4% PFA for 20 min at RT. Following adequate fixation, the cells were washed with 500 µl PBS/0.05% Tween 20 three times. Subsequent permeabilization with 500 µl PBS/0.1% Triton X-100 for 15 min at RT and three washing steps with 500 µl PBS/0.05% Tween 20 were carried out. The reactions were blocked with 500 µl 4% Donkey Serum in PBS at 4°C for 1 h wrapped in parafilm. The cells were then washed once with 500 µl PBS. The primary antibodies (1:20 Oct4, 1:50 Nanog, 1:200 SSEA 4 and 1:200 TRA-1-60) were diluted with 4% Donkey Serum/PBS and 200 µl were incubated with the cells at 4°C overnight. The cells were washed again three times with 500 µl PBS/0.05% Tween 20 and the secondary antibodies (rabbit IgG, rabbit IgG, mouse IgG and mouse IgM) were diluted in PBS in a 1:1000 ratio to a total volume of 200 µl. The plate was covered with tinfoil and incubated for 1 h at RT in the dark. Three washing steps with 500 µl PBS/0.05% Tween 20 and

staining with DAPI (1 µl in 10 ml PBS) was carried out. The plate was analyzed under the fluorescent microscope.

Sequencing

PCR products were sent out for sequencing to Genewiz®. The unpurified products were diluted 1:3 and sent together with sequencing primers to the company. The results were analyzed with Geneious Prime and ICE (Synthego).

3. Results

3.1. Reprogramming of somatic cells using nm-mRNAs and srRNAs

For these reprogramming approaches, blood cells (such as erythroblasts and fibrocytes) and fibroblasts were used. Before reprogramming, the most efficient transfection methods had to be determined. Therefore, erythroblasts and fibrocytes were transfected with eGFP mRNA (Stemgent, Cat#05-0020) using Lipofectamine MessengerMAX and NEON transfection (1300V, 20ms, 3x).

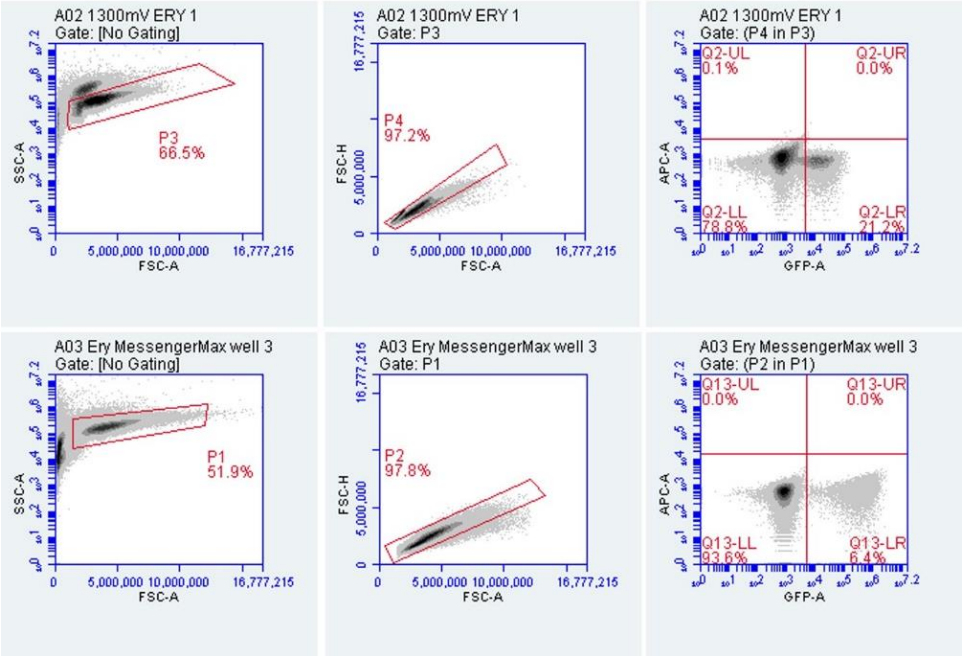


Figure 13: ***FACS image of erythroblast transfection:*** *NEON transfection lead to 21% transfection efficiency, whereas Lipofectamine Messenger Max only lead to 6% transfection efficiency. Cells were gated to exclude very tiny cells and doublets.*

Based on FACS analysis (figure 13), the efficiency for erythroblast transfection with the GFP mRNA was 21.2% using the Neon system and 6.4% using the Lipofectamine. The efficiency is overall low. Next, the transfection of fibrocytes derived from blood was tested.

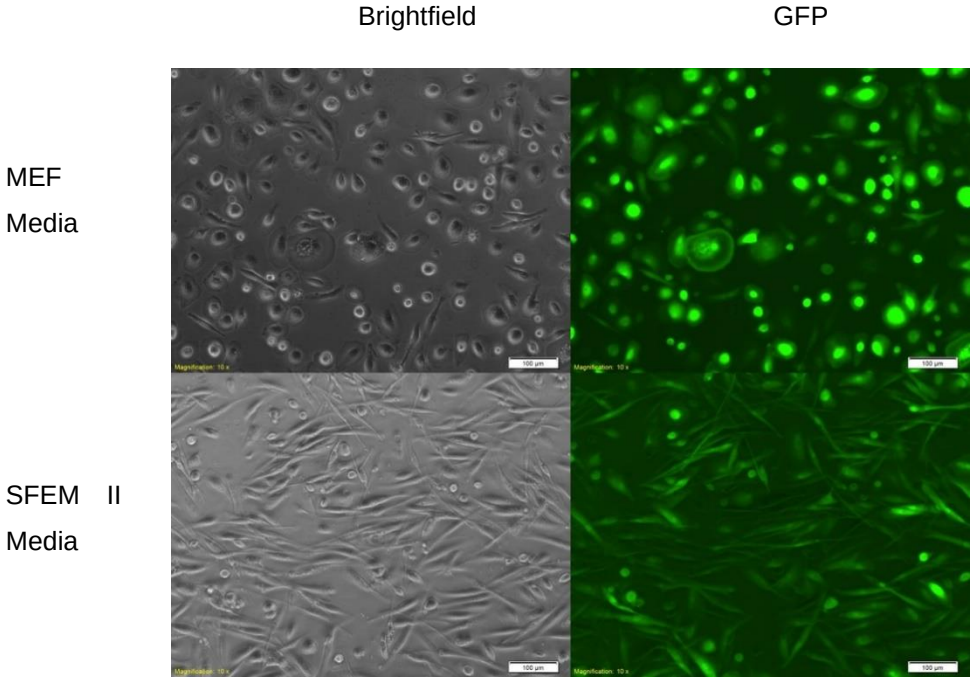


Figure 14: ***Microscopic images of fibrocytes 24 h post-Lipofectamine MessengerMAX transfection*** *(10x magnification; 100 µm scale bar)****:*** *Pictures in the top row show the transfection efficiency of cells grown in MEF media, whereas the bottom row shows the transfection efficiency of cells grown in SFEM II media. A morphological difference can be seen between cells grown in SFEM II and MEF media, suggesting that the different media support the growth of different cell types. The big round cells in the top row could be immature dendritic cells of the immune system. Fibrocyte (fibroblast-looking cells) growth is better in SFEM II media. A high transfection efficiency can be seen, which correlates to the FACS data.*

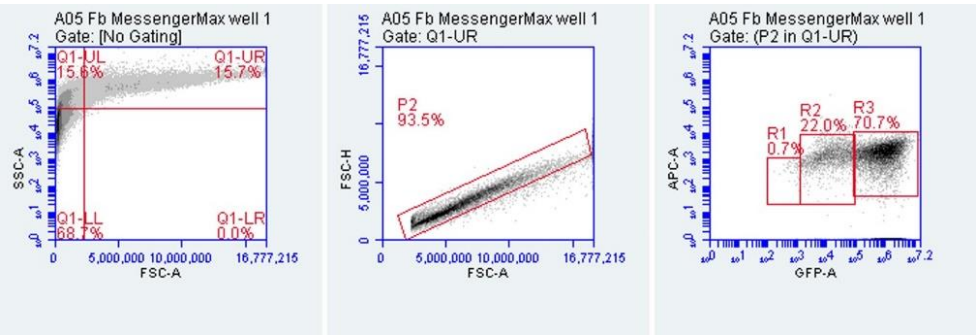


Figure 15: ***FACS analysis of fibrocyte transfection:*** *Fibrocyte transfection lead to 0% efficiency using the NEON system (not shown here) and to 70% transfection efficiency using Lipofectamine MessengerMAX. As different cell types appeared in the fibrocyte culture, the small round cells were transfected with an efficiency of 22% and the fibrocytes with an efficiency of 70%, since this would correspond to the GFP images taken.*

Figures 14 and 15 demonstrate the high transfection efficiency of fibrocytes using Lipofectamine MessengerMAX, as around 70% of the cells were GFP positive while the cells could not be transfected with the Neon system (data not shown).

Based on these transfection data, fibrocytes seemed to be more suitable for the reprogramming, however these cells are extremely difficult to grow. Their proliferation is slow and their survival is low after splitting. To expand the fibrocytes, different matrices such as laminin 521, vitronectin and Cultrex, and different media such as MEF, SFEMII and fibrocyte media were tested, but the cells started dying in all of those conditions after 12-15 days in culture. Fibrocyte growth was best on uncoated plates or on Cultrex coated plates.

Nevertheless, both cell types (erythroblasts and fibrocytes) were used for the nm-mRNA reprogramming in parallel with fibroblasts as a control. The cells were transfected 4 days in a row with the nm-mRNA using the most efficient transfection method for each cell type. The cells were analyzed daily under the microscope. Rapidly, the erythroblasts and fibrocytes underwent apoptosis and no cells could be recovered. In contrast, iPS colonies emerged from the fibroblast reprogramming within ~10 days. A total of 30 iPS colonies were generated. 2 colonies were picked for further characterization. Since the successive transfections of the nm-mRNA are too toxic for the erythroblasts and fibrocytes, the srRNA reprogramming on these cells was tested next since it requires only one transfection. The srRNA is a large RNA of around 15kb. The transfection efficiency was optimized using a GFP mRNA which is small. Therefore, the transfection efficiency was expected to be lower for the srRNA. A lot of cell death was observed following the transfection of the erythroblasts and fibrocytes, while the skin fibroblasts survived well. After approximately 14 days post transfection of the fibroblasts, 2 colonies emerged. They were picked for characterization.

Figure 16 shows the morphological changes of fibroblasts using the srRNA reprogramming approach during a time course of 23 days.

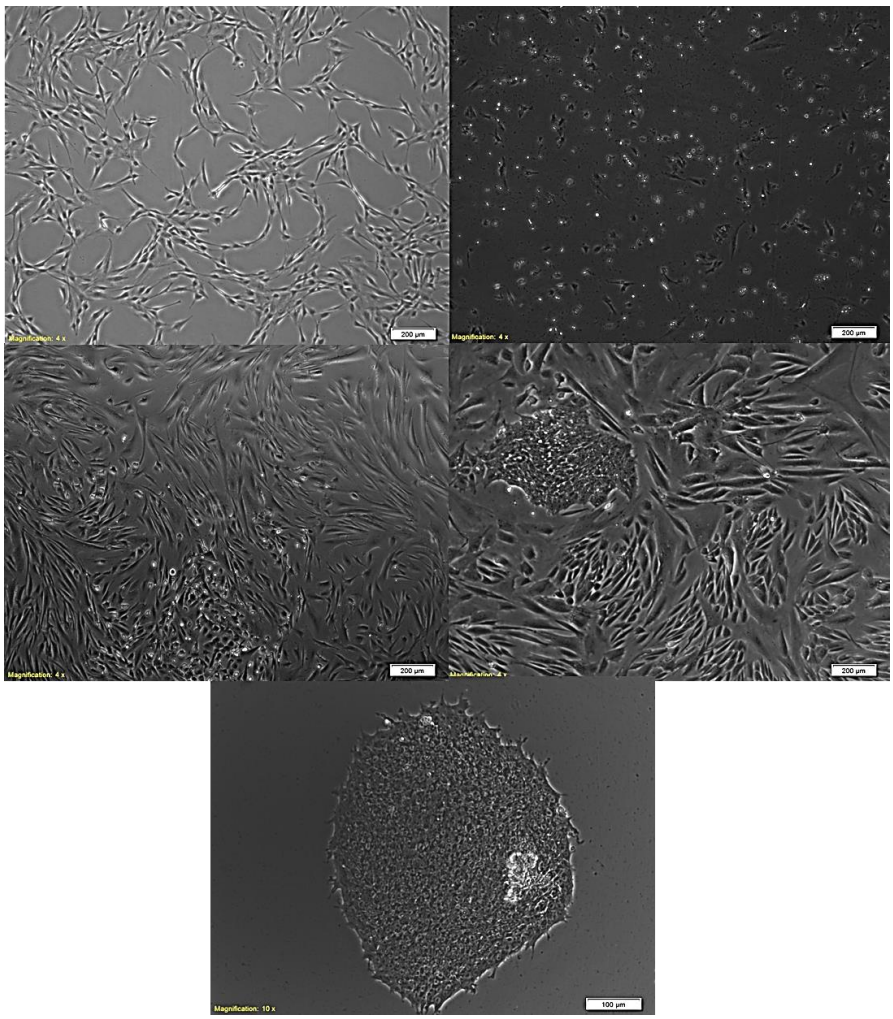


Figure 16: ***Sr-RNA reprogramming of fibroblasts****: Pictures were captured every day during a 23-day time course. It took approximately 14 days until the first colony appeared. Unfortunately, this method proved to be inefficient with only two emerged colonies compared to the nm-mRNA reprogramming, where over 30 colonies appeared within the same time course. The picture shows the reprogramming of fibroblasts at day 0, 4, 13, 18, 26, whereas the picture from day 26 represents the colony after picking it into a new well. There is a change in density between day 0 and 4, which is due to the negative selection with Puromycin, to make sure only transfected cells survive. At first, cells died because of the selection and toxicity of the RNA and then they started to grow, reassemble and form colonies.*

The edges of the iPS colonies are not sharp, which is due to the Cultrex matrix (figure 16). Only when using feeder systems, the edges of colonies are clearly distinguishable from the other cells and they appear white. There are also differently shaped cells around the colony, which could be partially reprogrammed cells. The colonies were expanded and passaged four times before further analysis. First,

qRT-PCR for pluripotency markers DNMT3B, hTERT, SOX2, REX1, OCT4 and NANOG was performed to ensure stem cell characteristics.

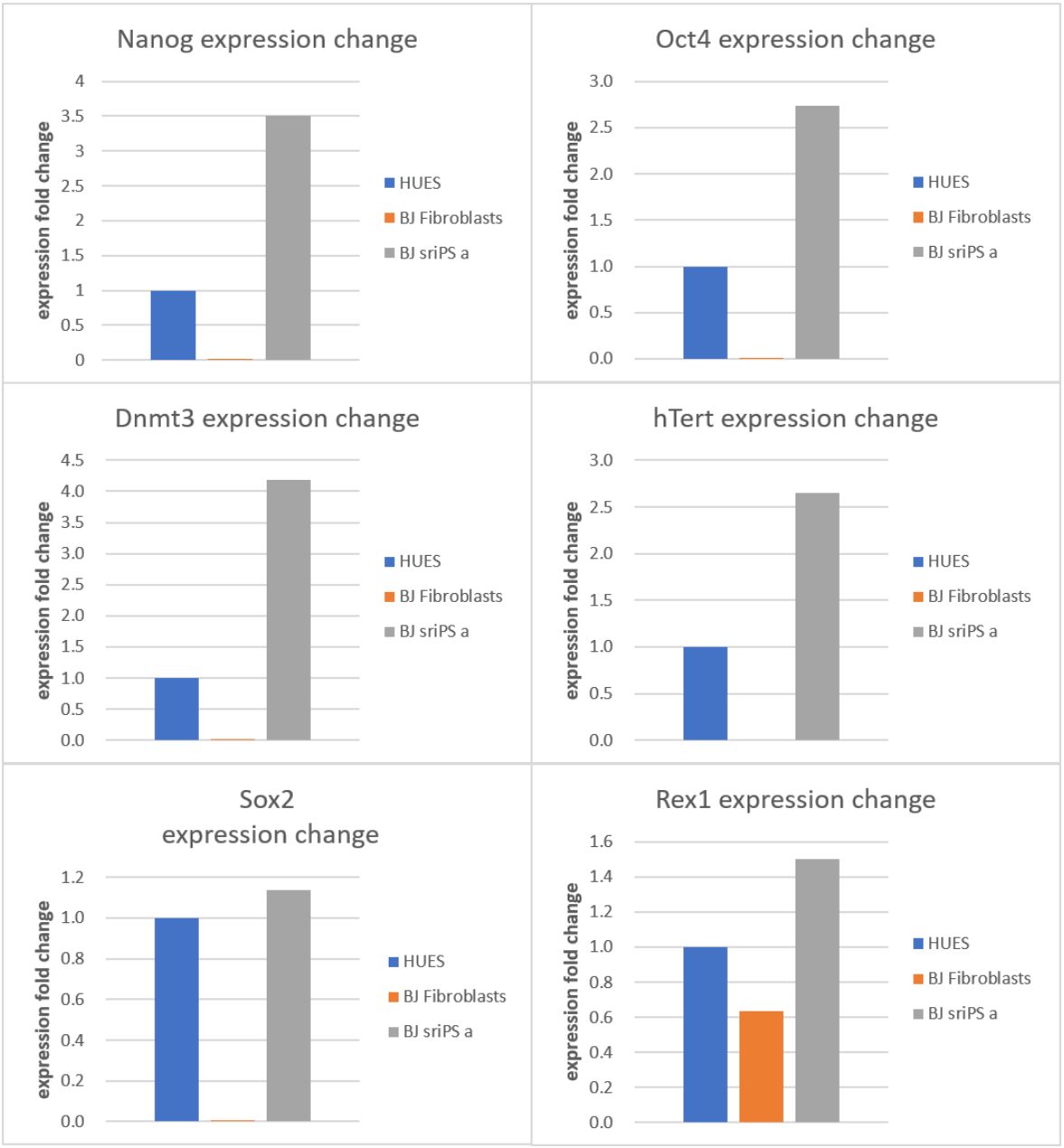


Figure 17: ***qRT-PCR results to determine the expression of pluripotency markers****: Expression fold change was calculated with untransfected fibroblasts as the reference sample and actin as the endogenous control. DNMT3b, hTERT, SOX2, REX1, OCT4 and NANOG marker expression were determined. All markers were increased compared to the negative control. The HUES mix represents a positive control as it is a mix of various ES cell lines.*

Figure 17 shows the pluripotency marker expression for the BJ sriPS-a, a colony which arose from the srRNA reprogramming. It depicts that expression of pluripotency genes is increased in the BJ sriPS-a line compared to fibroblasts

(negative control) and is more similar to the level of expression in a mix of embryonic stem cell lines (HUES, positive control). REX1 is the only marker that is also expressed in fibroblasts, so the data fully confirms the reactivation of the pluripotency markers in the cells. To confirm pluripotency, cells were tested for their differentiation capacity using the embryoid body assay.

Embryoid body formation was carried out for 15 days. RNA was isolated on day 0, 7 and 15 and Scorecard analysis and qRT-PCR for differentiation markers were performed. Also, pictures of the differentiation of the newly generated iPSCs into diverse cell types were taken (figure 18).

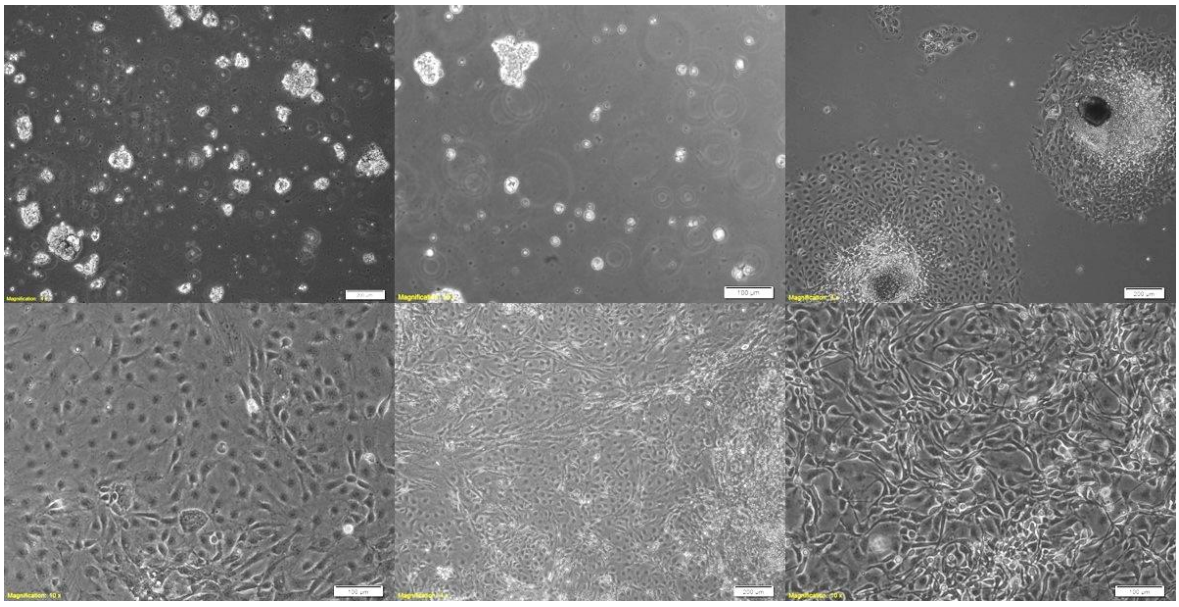


Figure 18: ***Pictures of embryoid body formation of newly derived iPS cells****: iPS cells are pluripotent and should therefore be able to develop into any kind of cells. By EB formation cells can spontaneously differentiate into any cell type. iPS cells often differentiate into their original cell type, they are believed to have an epigenetic memory. That is the reason, why a lot of fibroblasts can be seen in the pictures. However, EBs tend to have a lot of fibroblasts regardless of the reprogrammed cell type. It is because fibroblasts are a fast lineage to form and they are fast-growing. EB formation is carried out on ultralow attachment plates until day 7, which is the reason why the cells appear in clumps on the first two pictures (day 1 and 5). On day 9 cells were already attached to gelatin coated plates and they began to differentiate (top right picture). The bottom picture row shows EBs at day 11, 14 and 15.*

Embryoid body formation, formation of diverse cell types after differentiation confirms pluripotency of the generated iPS cell line BJ sriPS-a (figure 18). Cells were harvested at day 0, 7 and 15 and a qRT-PCR and Scorecard analysis for differentiation potential was carried out.

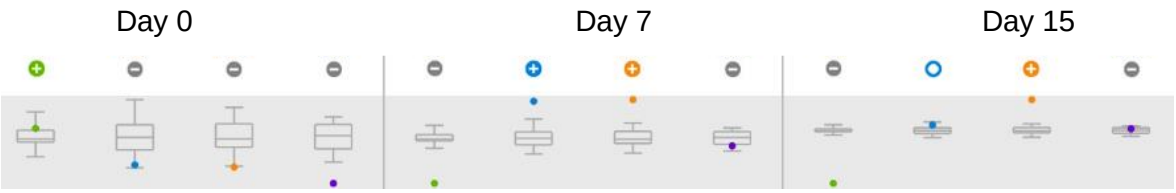


Figure 19: ***Scorecard (ThermoFisher) analysis for determination of pluripotency potential*** *and development into the three germ layers ectoderm, mesoderm and endoderm. With this method several different markers representing the germ layers were analyzed. On the left side the generated iPSCs (negative control) are shown. The two boxes on the right show expression of markers after 7 and 15 days of embryoid body formation.*

Figure 19 depicts differentiation marker expression determined via Scorecard analysis. After 7 and 15 days, the expression of the self-renewal markers is downregulated while the markers for the three germ layers, ectoderm, mesoderm and endoderm are elevated. Ectodermal marker expression of EB 15 is lower than EB 7, but the expression in each category is measured relative to the total number of cells, so mesodermal/endodermal cells could have grown more during the second week, making the ectodermal cells a relatively smaller part of the population. This result demonstrates that this iPS line is pluripotent.

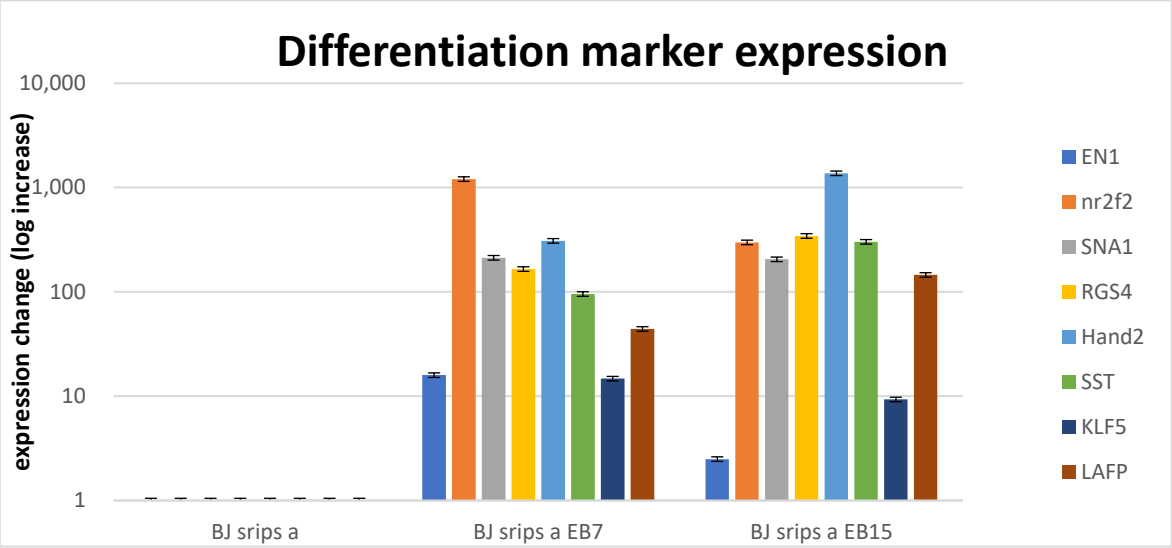


Figure 20: ***qRT-PCR result of differentiation marker expression after harvesting cells*** *after 0, 7 and 15 days of EB formation. Expression fold change was calculated with the newly generated BJ sriPS-a cells as the reference sample and RPLP0 as the endogenous control. EN1 and NR2F2 represent ectoderm makers, SNAI2, RGS4 and HAND2 represent mesoderm and SST, KLF5 and AFP represent endoderm marker expression. The graph is depicted in logarithmic scale.*

Besides the scorecard analysis, a qRT-PCR for three markers of the ectoderm, mesoderm and endoderm is commonly performed at the HSCI iPS core. The BJ sriPS-a was then tested by this method (figure 20).

Except for EN1 and HAND2 expression, all marker expressions were highly increased compared to undifferentiated iPS cells.

Immunocytochemistry staining for pluripotent markers using NANOG, OCT4 and the surface markers SSEA-4 and TRA-1-60 was also performed (figure 21).

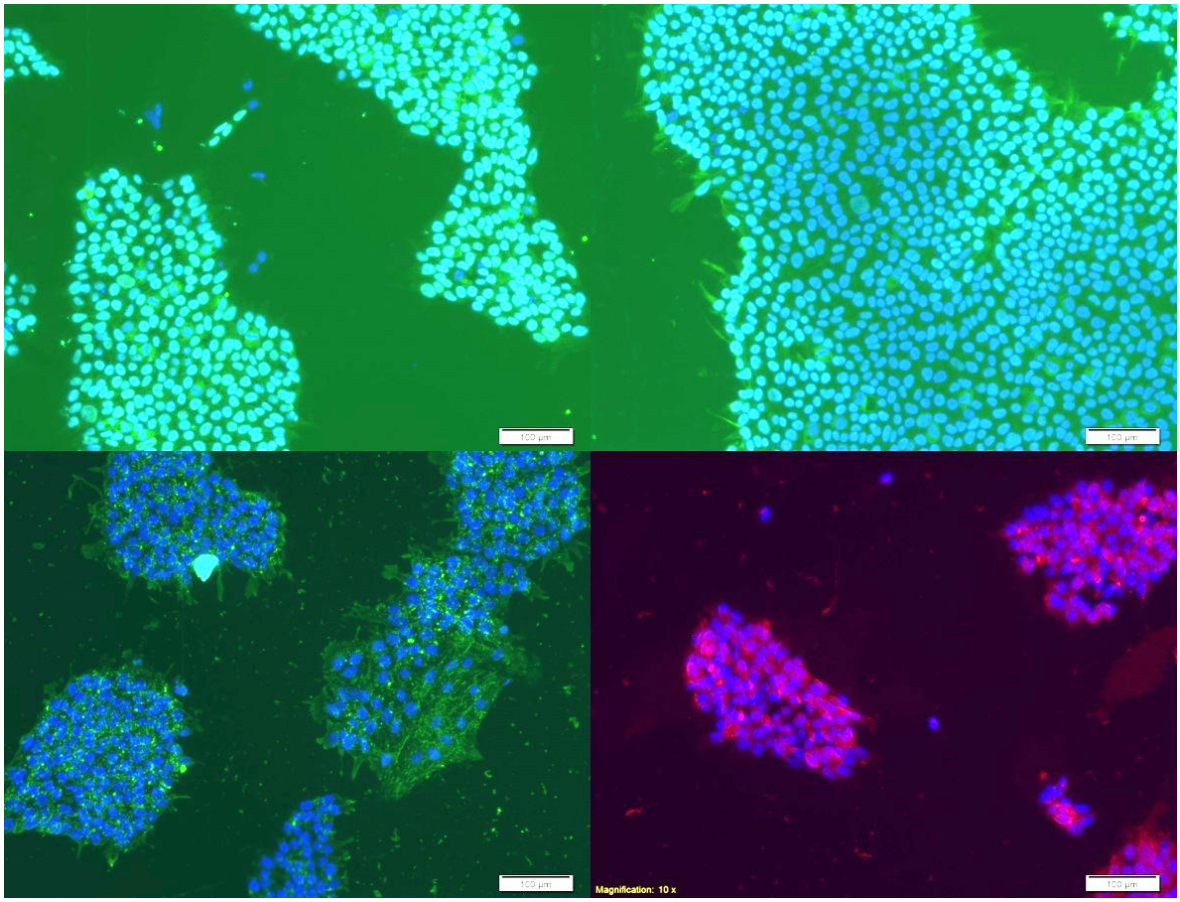


Figure 21: ***Pictures after immunocytochemistry staining of the cells*** *(100 µm scale bar): OCT4 and NANOG in the top row, SSEA-4 and TRA-1-60 in the bottom row: The staining shows that iPS cell derivation was successful. Cells were analyzed at 10x magnification under the microscope using the following channels: GFP and DAPI channels were applied for OCT4, NANOG and SSEA-4. DAPI and TXRED were used for TRA-1-60.*

OCT4 and NANOG are transcription factors expressed in the nucleus. The immunostaining showed the nuclear localization of these markers. In contrast, SSEA-4 and TRA-1-60 are surface markers. As all cells expressed all four pluripotency markers, this result confirmed stem cell-like properties.

Fibroblasts were also successfully reprogrammed with Sendai virus and episomal vector (data not shown due to similar characterization results).

3.2. CRISPR-based reprogramming

Last year, two groups described the derivation of human iPS lines using the CRISPRa system to directly activate the Yamanaka factors in somatic cells. The HSCI iPS core was interested in evaluating this new method. The plasmids for the dCas9a and gRNAs were obtained through Addgene or directly through the investigator who reported this work. It consists of two dCas9a (fused to VPH or VP192) and different combinations of gRNAs cloned into episomal vectors. The gRNAs can recruit the transactivators to the target sequences. After binding to the target promoters, the transactivators should activate the pluripotency genes in somatic cells, so that they can be reprogrammed into iPSCs.

First, the goal was to test whether pluripotency marker expression was increased upon transfection of the transactivators (plasmid VPH/VP192) and the guide RNA-containing plasmid (GG-EBNA).

VPH is a 17 kb episomal plasmid containing dCas9 fused to four VP48 transactivators and p65-HSF1 under the CAG promoter. It also contains a p53 shRNA expression cassette to boost reprogramming efficiency. VP192 is a 15.8 kb episomal plasmid containing dCas9 fused to four VP48 domains under the CAG promoter. The GG-EBNA plasmid comprises 10 gRNAs targeting OCT4 (3), KLF4 (1), LIN28 (3), SOX2 (2) and c-MYC (1) under the U6 promoter. In addition, a puromycin selection cassette can be found. The GG-EBNA-OSK2M2L1-PP (OSK2M2L) plasmid has an U6 promoter including ten gRNAs which target OCT4, SOX2, KLF4, c-MYC and LIN28A promoters. It also includes a Puromycin selection cassette. The GG-EBNA-EEA-5guides-PGK-Puro (EEA) targets EEA with 5 gRNAs and contains an U6 promoter and a Puromycin selection cassette. After maxi-prep, plasmid quality was determined upon cutting the plasmids with restriction enzymes and performing an agarose gel electrophoresis. The VP192 plasmid was digested with *Mlu*I and *Pvu*I giving two fragments at 2728 bp and 13120 bp. The VPH plasmid was digested with the same enzymes leading to a 3121 bp and a 14089 bp fragment. GG-EBNA was digested with *Bgl*II and *Pst*I to 5215 bp and 8197 bp fragments. The OSK2M2L-PP plasmid was cut with *Bgl*II and *Pvu*I resulting in a 5382 bp and an 8031 bp band. The KM plasmid from Weltner et al. (2018) comprises one guide RNA for KLF4 and c-MYC and was digested with the same enzymes, however, as no

map was available, plasmid quality was assumed to be high. The GG-EBNA-EEA-5 guides-PGK-Puro plasmid was digested with *Pvu*I and *Bgl*II showing a 6532 bp and a 4381 bp fragment.

To test whether these plasmids can activate the expression of the pluripotent markers in somatic cells, HEK293T cells, which are very easy to transfect, were used. Lipofectamine 3000 (Thermo Fisher) was used to introduce the plasmids into HEK293T cells.

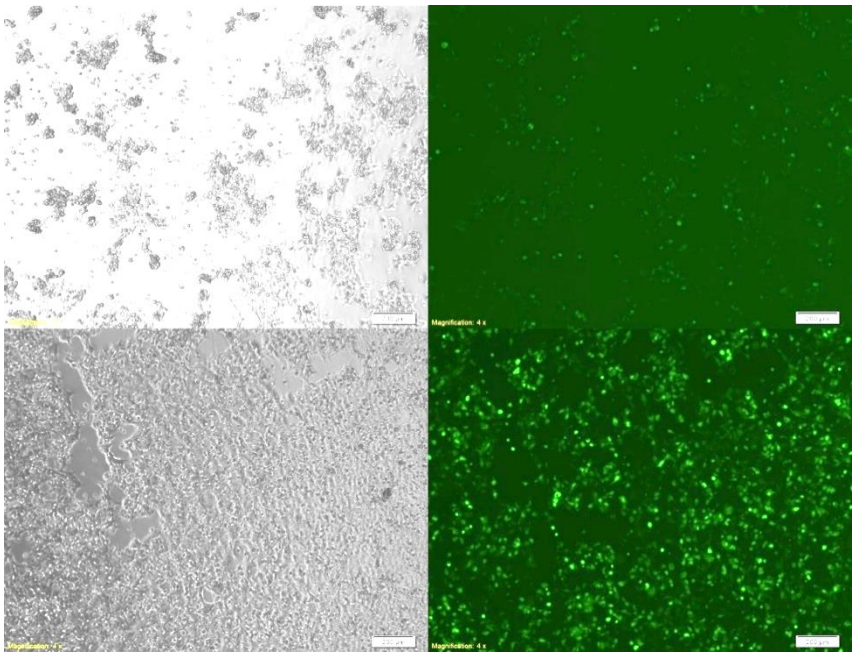


Figure 22: ***Microscopic image*** *(4x magnification; 200 µm scale bar)* ***of HEK293T cells 72 h post transfection*** *with VPH and GG-EBNA plasmid in the top row and VP192 and GG-EBNA in the bottom row. The brightfield images are on the left and GFP expression of successfully transfected HEK293T cells is shown on the right.*

Figure 22 shows the transfection efficiency of HEK293T cells using Lipofectamine 3000. Although cells transfected with VP192 were brighter than those transfected with VPH, the transfection efficiency was very similar. HEK293T cells were harvested 72 hours post transfection, RNA was isolated and a qRT-PCR for OCT4 and SOX2 marker expression was performed.

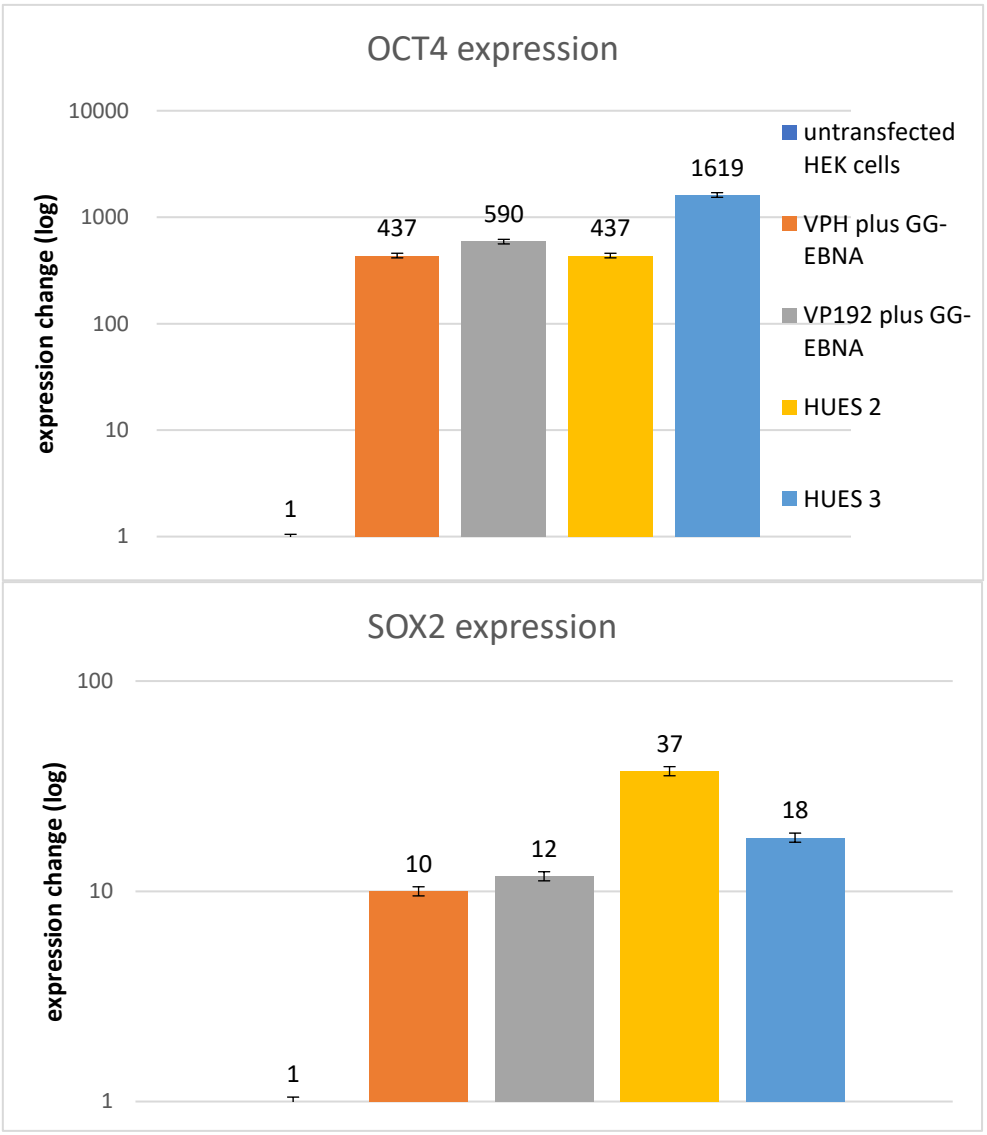


Figure 23: ***Fold expression change of OCT4 and SOX2 after CRISPRa based reprogramming of HEK293T cells:*** *Cells were harvested 72 h after transfection of VPH/VP192 in combination with GG-EBNA. Subsequent RNA isolation, cDNA synthesis and qRT-PCR were performed. Expression fold change was calculated with untransfected HEK293T cells as the reference sample and actin as the endogenous control. OCT4 and SOX2 expression levels after CRISPRa based reprogramming compared to different iPS cell lines are shown. HUES 2, 3 represent different ES cell lines and therefore act as a positive control of OCT4 and SOX2 expression. The graphs are depicted in log-scale.*

Transfection of the HEK293T cells with the transactivator and guide RNA-containing plasmids showed that OCT4 and SOX2 expression increased compared to the untransfected control cells (figure 23). SOX2 increased 10-fold, whereas OCT4 increased 500-fold, which correlates with the data from Weltner et al. (2018), shown in figure 24. The expression was compared to positive controls, some HUES lines (2 and 3), Harvard University Embryonic Stem cell lines. SOX2 expression is around

half and OCT4 expression is one third after transfection compared to hES cells. Therefore, expression levels for those two markers could be sufficient for reprogramming.

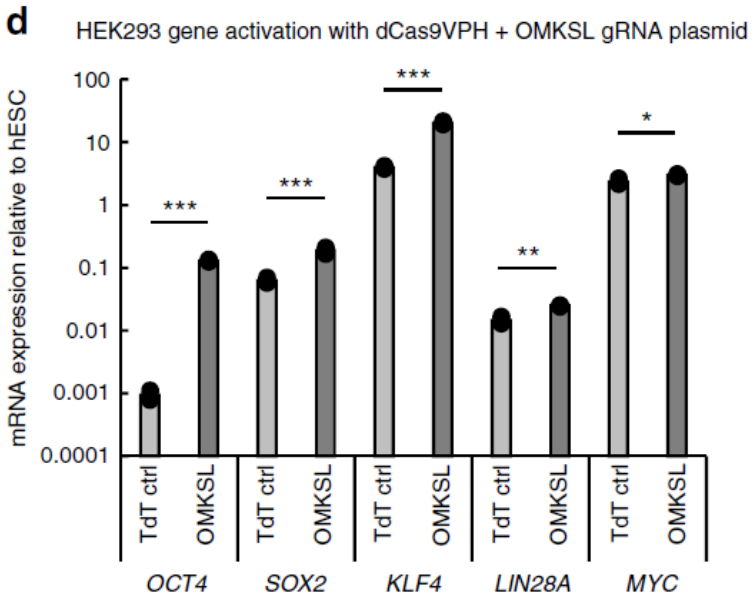


Figure 24: ***Gene expression levels after activation of genes with VPH and GG-EBNA plasmid****: The graph is depicted in logarithmic scale. Figure adapted from Weltner et al., 2018.*

Additional plasmids were tested in HEK293T cells to determine the most effective combination for the reprogramming experiment. To increase the activation of KLF4 and c-MYC, Weltner et al. (2018) recommended to use the OSK2M2L plasmid or GG-EBNA(-OSKMNL) in combination with the KM plasmid. Moreover, they showed that targeting the Alu-motif (with the EEA plasmid) was necessary to reprogram adult fibroblasts. The HEK293T cells were transfected with all the different combinations of gRNA plasmid, RNA was isolated 72 hours post transfection and qRT-PCR was performed for determination of pluripotency marker expression.

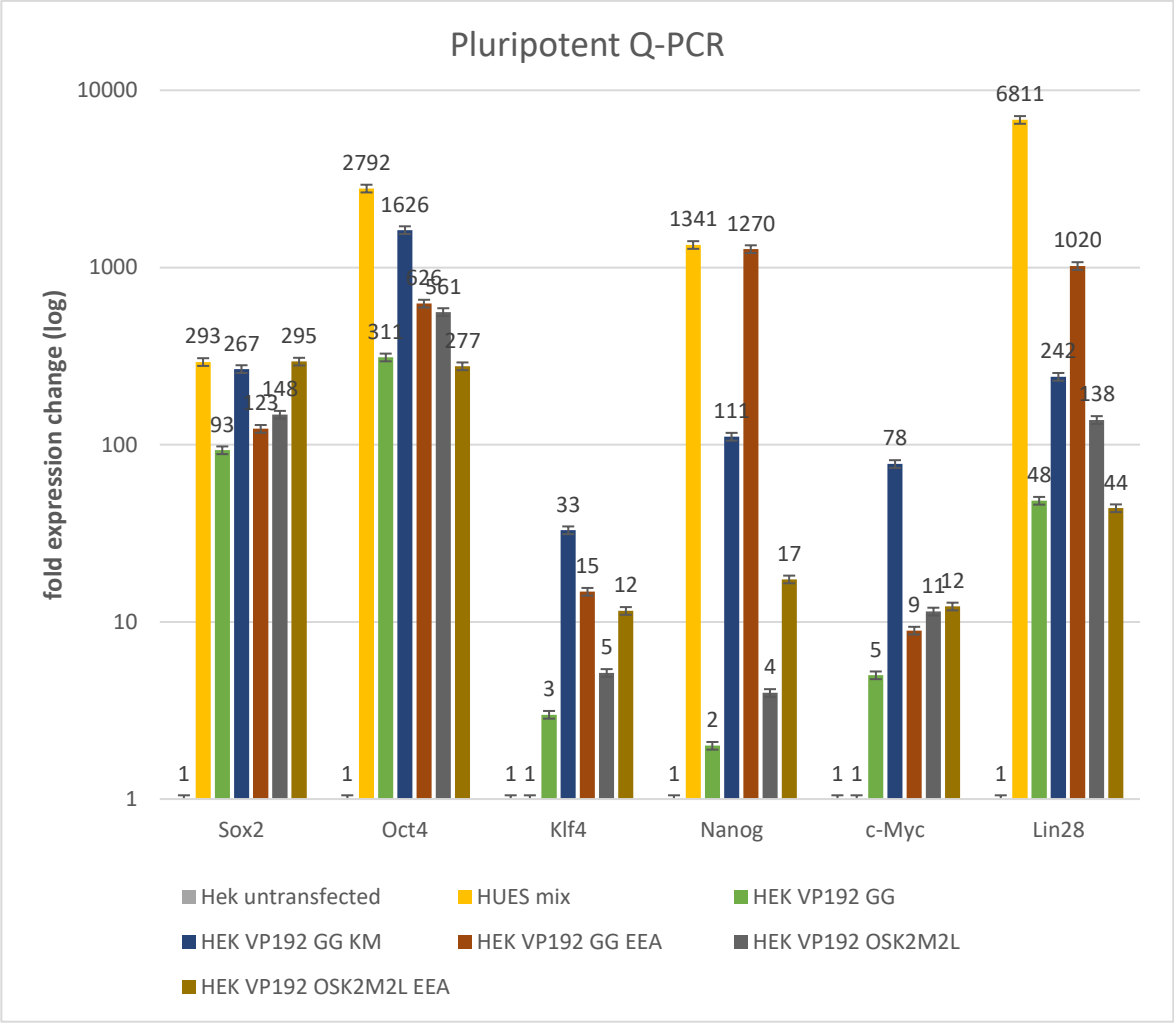


Figure 25: ***Pluripotency marker expression levels when transfecting HEK293T cells with transactivators VPH/ VP192 and GG-EBNA/KM/EEA/OSK2M2L****: qRT-PCR data shows SOX2, OCT4, KLF4, NANOG, c-MYC and LIN28 expression. Expression fold change was calculated with untransfected HEK293T cells as the reference sample and actin as the endogenous control. The HUES cell line mix serves as a positive control. The graph is depicted in log-scale.*

Figure 25 shows the qRT-PCR results of the pluripotency marker expression in HEK293T cells transfected with VPH/VP192 plus different combinations of gRNA plasmids. A strong activation of OCT4 and SOX2 was observed in all conditions. However, the activation of the other markers varied between the different plasmids. The plasmid cocktail that offers the best activation for the 6 pluripotent genes are GG-EBNA+KM or GG-EBNA+EEA. KLF4 and c-MYC activation is not as strong, but the levels are already higher than in iPSCs, as those genes are already highly expressed in HEK, so reprogramming would be possible.

To test whether it is possible to reprogram fibroblasts with the vectors containing the transactivator and the gRNA containing plasmids, two neonatal fibroblast lines, BJ and HS27 (obtained through ATCC) were used for these experiments. Fibroblasts were transfected with the LONZA Amaxa nucleofection or the NEON system.

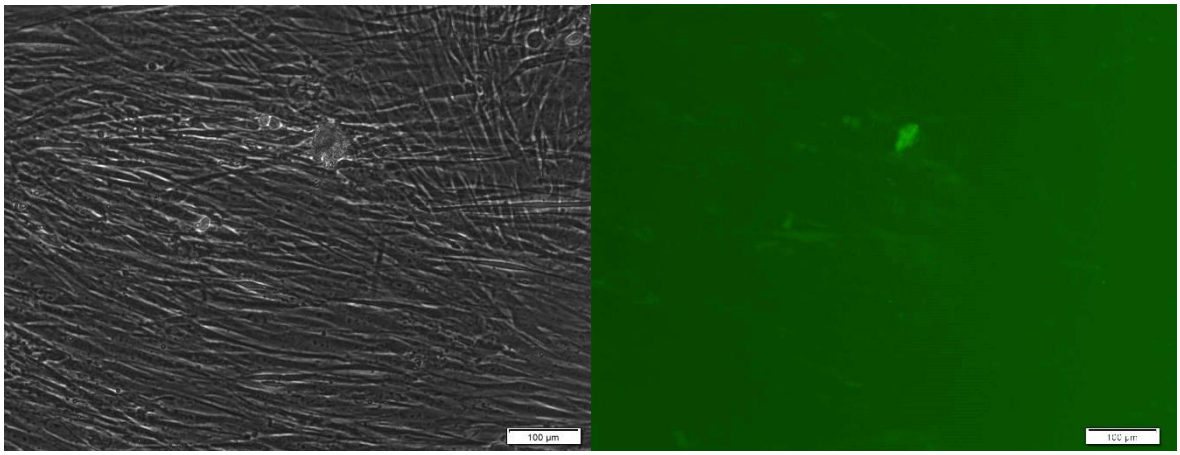


Figure 26: ***Microscopic image of fibroblasts after transfection of VP192 and GG-EBNA****: The pictures were taken 11 days post transfection and show an efficiency of only 2%, which was confirmed with FACS. The left picture shows the brightfield image and on the right the cells are visible through the GFP filter.*

Overall, a poor transfection efficiency was observed as shown by the low percentage of GFP positive cells in figure 26. This is due to the size of the vectors (>15kb). The cells were collected a few days post transfection to determine the activation of the pluripotency markers by qRT-PCR.

Morphological changes were observed, which could be partially reprogrammed cells. Transfection efficiency of fibroblasts was only 2%. Nevertheless, the expression change of the pluripotency markers was determined. The cells were harvested and qRT-PCR was done to analyze the expression.

The expression increase of pluripotency markers in HEK293T cells is only slightly comparable to those in fibroblasts, as the activation of OCT4, SOX2 and NANOG could be detected with a fold increase of only 3, 12 and 3 respectively (table 6). However, since the transfection efficiency is low, the activation of the pluripotent markers in the small percentage of GFP positive cells might be sufficient for reprogramming.

Table 5: Fold expression change of pluripotency markers when transfecting BJ fibroblasts with VP192 and GG-EBNA

	OCT4	SOX2	KLF4	c-MYC	NANOG	LIN28
Untransfected BJ fibroblasts	1	1	1	1	1	1
BJ fibroblasts transfected with VP192 and GG-EBNA	3	12	1	1	3	1

Therefore, reprogramming experiments using both BJ and HS27 fibroblasts with VP192 transactivator and different combinations of the guide RNA plasmids were performed. The cells were transfected with the Lonza Amaxa or the Neon nucleofection systems. Few days post transfection, the fibroblasts were collected and replated on MEFs or on Cultrex for feeder free conditions. Some morphological changes were observed for different conditions but iPS colonies emerged only with the following condition: VP192 + OSK2M2L + EEA (figure 27).

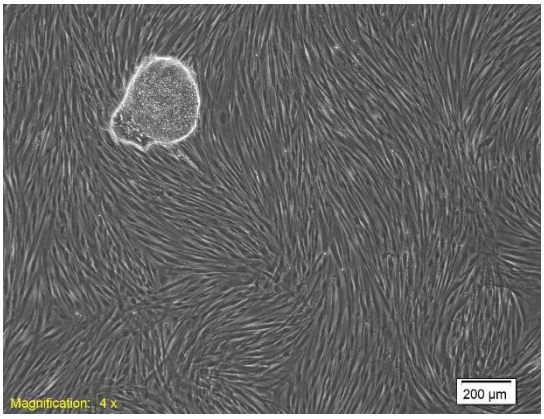


Figure 27: ***Microscopic picture of iPSC colonies that evolved through CRISPRa-based reprogramming****: The picture was taken 15 days post NEON transfection (1400 V, 20 ms, 1 pulse) with the plasmids VP192 + OSK2M2L + EEA. The cells were grown on Cultrex.*

Although this plasmid cocktail did not show the best qRT-PCR results (figure 25), it was the only combination that yielded 4 colonies (figure 27). Unfortunately, there was not enough time for the characterization of these iPS lines.

3.3. The miRNA 302/367 cluster

The goal of this experiment was to study the role of the miR-302/367 cluster and its involvement in reprogramming. Therefore, the expression of the cluster was determined first in different cell types by employing RT-PCR and qRT-PCR.

The cluster is important for maintaining pluripotency in stem cells and is therefore only expressed in undifferentiated cells. iPS cells, fibroblasts, PBMCs, HEK cells and embryoid bodies at day 5, 10 and 15 were tested for the cluster expression upon RNA isolation, cDNA synthesis and qRT-PCR using primers (called Ex) that target the cluster. The cell line Ery ED3 siPS-a p27 was used for the experiment. The expression was analysed by running the PCR products on an agarose gel.

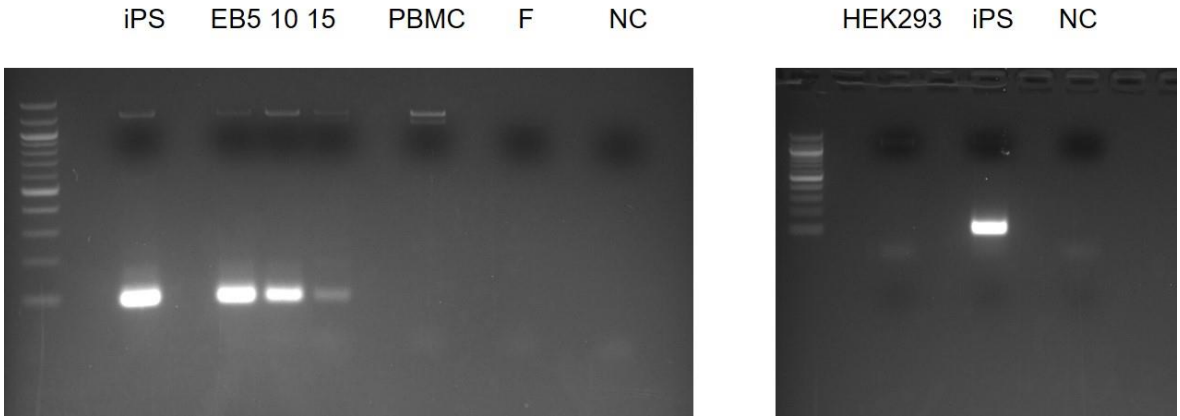


Figure 28: ***Ex primer testing to confirm that the miRNA 302/367 cluster is only expressed in undifferentiated cells****: Embryoid bodies after day 5, 10 and 10 as well as PBMC, fibroblast and HEK293T cell cDNA was analyzed on a 1.5% agarose gel. NC shows the negative control, where cDNA was replaced with water. The 100 bp ladder from NEB was used to confirm the size of the 100 bp fragment.*

Figure 28 shows that the miR-302/367 cluster was only expressed in the induced pluripotent stem cells, as only those cells showed a fragment of 100bp on the agarose gel. The fragments for the embryoid bodies are due to the fact that embryoid bodies consist of different cell types including stem cells. However, as differentiation progresses, there are fewer iPSCs over time within EBs, which is the reason why the bands (=amount of DNA) become weaker during the 15-day time course.

Next, miRNA cluster expression was analyzed with qRT-PCR to confirm the results from the gel.

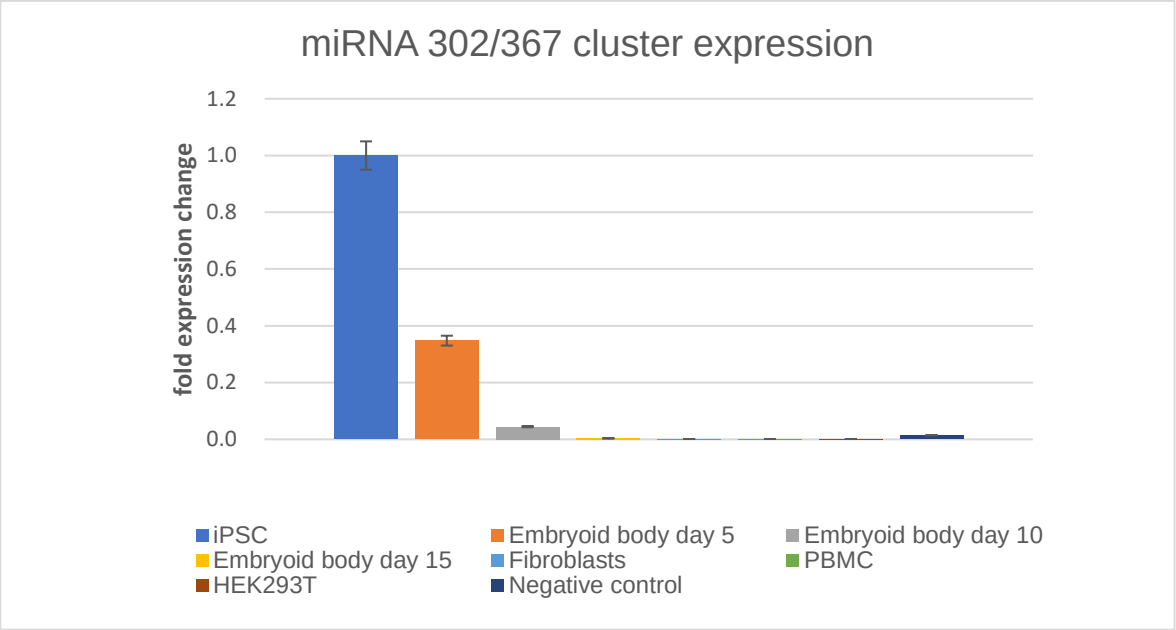


Figure 29: ***qRT-PCR result for miRNA 302/367 cluster expression levels in different cell lines:*** *Cells were harvested, and the cluster expression was determined using the Ex primer. Expression fold change was calculated with iPS cells as the reference sample and actin as the endogenous control. Water instead of cDNA was used for the negative control.*

The qRT-PCR confirmed that the cluster was only expressed in stem cells. Figure 29 shows a slight expression in EBs at day 5 and 10 as not all the cells have completely undergone differentiation at this stage.

In 2011, one group (Anokye-Danso et al.) reported that iPSCs could be generated solely through the expression of a set of miRNAs, without the Yamanaka factors. They used a lentivirus system to deliver the miR-302/367 to mouse and human fibroblast cells. They showed a rapid reactivation of the endogenous pluripotency genes with this method and a high reprogramming efficiency.

The overexpression of the miR-302/367 using the CRISPRa system is proposed in this thesis. First 4 gRNAs within the 500 nucleotides-long promoter were designed. HEK293T cells were transfected with these 4 gRNAs (285, 343, 407 and 455) individually with the transactivators VPH or VP192. Cells were collected three days post-transfection for RNA extraction and qRT-PCR.

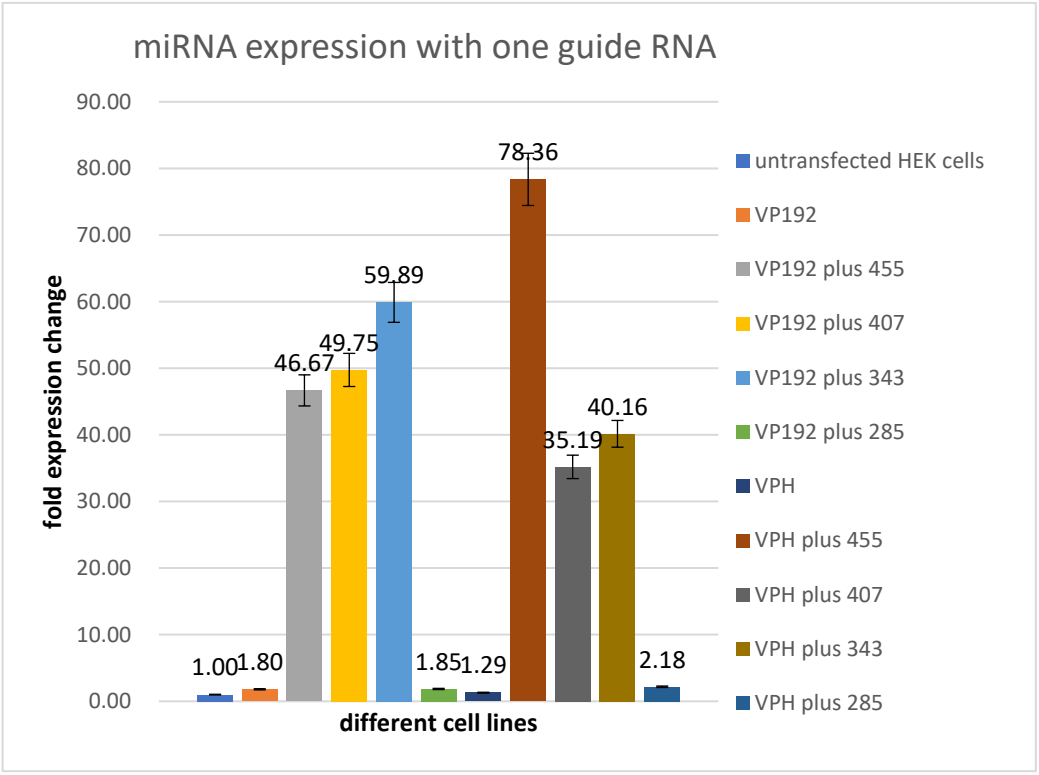


*Figure 30: **miRNA cluster expression levels determined with qRT-PCR**: HEK cells were transfected with VP192/VPH and one gRNA each. Expression fold change was calculated with untransfected HEK293T cells as the reference sample and actin as the endogenous control.*

With 3 of the 4 gRNAs (343, 407, 455) an increased expression of the miRNA cluster from 35 to 78-fold was demonstrated (figure 30). Both VPH and VP192 transactivators strength are similar. Guide 285 in combination with either transactivator led to the smallest expression increase, which suggests that guides closer to the TSS work best (figure 10).

Fold expression change was determined when transfecting three and four guides together with VP192 in HEK293T cells to test whether higher activation of the cluster by combining the gRNAs could be achieved. HEK293T cells were transfected with three or all four guides together with VP192. For all following experiments, only VP192 was used since both transactivators always led to similar results.

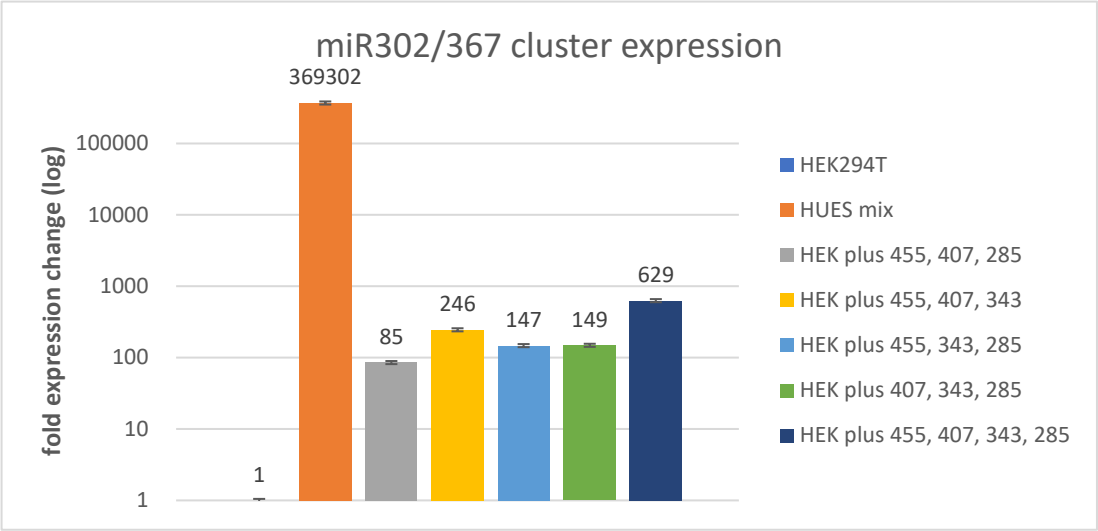


Figure 31: ***miRNA cluster expression change determined with qRT-PCR:*** *HEK cells were transfected with VP192 and four gRNAs expression fold change was calculated with untransfected HEK293T cells as the reference sample and actin as the endogenous control. The HUES cell line mix served as a positive control. The graph is depicted in logarithmic scale.*

Cells transfected with three guides in different combinations showed an expression increase of 85 to 246-fold (figure 31). However, a 629-fold expression increase of the miR-302/367 cluster was obtained upon transfection of all four guides, which target diverse locations within the promoter region of the cluster. In iPS cells the cluster expression is increased 369 302-fold compared to untransfected HEK293T cells.

Interestingly, upon transfection and activation of the miR-302/367 cluster with the transactivator VP192, pluripotency genes like NANOG, OCT4, SOX2 and LIN28 were increased too (figure 32).

In contrast, when transfecting HEK293T cells with VP192 and GG-EBNA, which activates pluripotency genes, the increase in miR-302/367 expression is very low (12-fold).

HEK293T cells were transfected with VP192 transactivator and guides 455, 407, 343 and 285 to determine pluripotency marker expression.

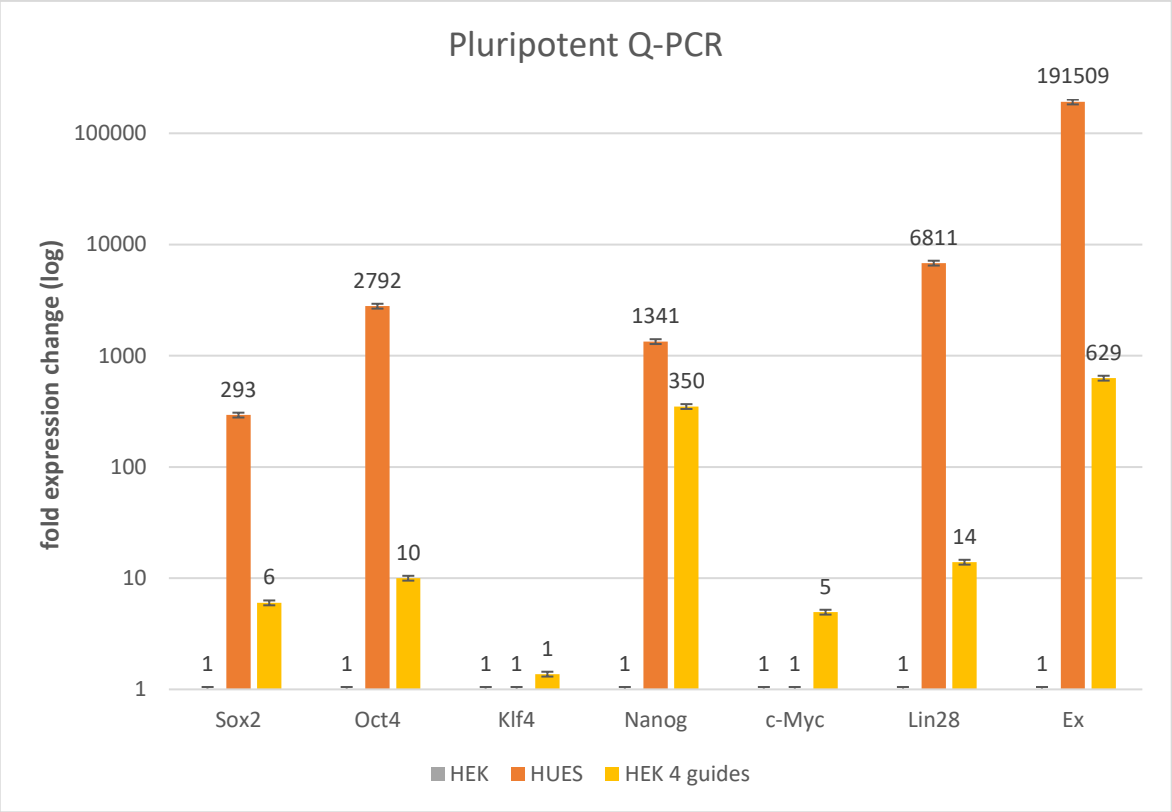


Figure 32: ***Pluripotency marker expression change when transfecting guides that target the miR-302/367 cluster:*** *RNA was isolated after transfecting the four self-designed guides 455, 407, 343 and 285. qRT-PCR evaluation for SOX2, OCT4, KLF4, NANOG, c-MYC, LIN28 and the miRNA primer EX was analyzed. Expression fold change was calculated with untransfected HEK293T cells as the reference sample and actin as the endogenous control. The HUES cell line mix served as a positive control. The graph is depicted in logarithmic scale.*

Figure 32 show a dramatic increase of 350-fold for NANOG expression, when miR-302/367 is activated, which also correlates to the literature (Lee et al., 2013). The NANOG expression in iPS cells is 4 times higher. KLF4 shows no expression change and c-MYC expression increases 5-fold. OCT4 increases by 10-fold, SOX2 by a factor of 6 and LIN28 by a factor of 14. The level of expression of the major pluripotency transcription factors after activation of miR-302/367 is much lower than in hESCs. However, these genes are known to collaborate to form regulatory circuitry of autoregulatory and feed-forward loops. Therefore, this initial activation of the endogenous 3 main (OCT4, SOX2, NANOG) pluripotent genes could be sufficient for reprogramming or could facilitate the reprogramming in combination with the Yamanaka factors.

The gRNAs (GG-EBNA, OSK2M2L and KM) used for the reprogramming experiment are cloned into episomal vectors to allow sustained expression of the gRNA for the 3 weeks needed for the reprogramming of human somatic cells. In contrast, the 4 gRNAs targeting the miR-302/367 promoter were cloned into a vector only allowing transient expression. Therefore, the kinetics of the miR-302/367 activation had to be determined over 9 days.

HEK293T cells were transfected with VP192 and the four guides targeting the miRNA cluster. RNA was harvested at different days and expression of the cluster and the 3 main pluripotent transcription factors was evaluated by qRT-PCR.

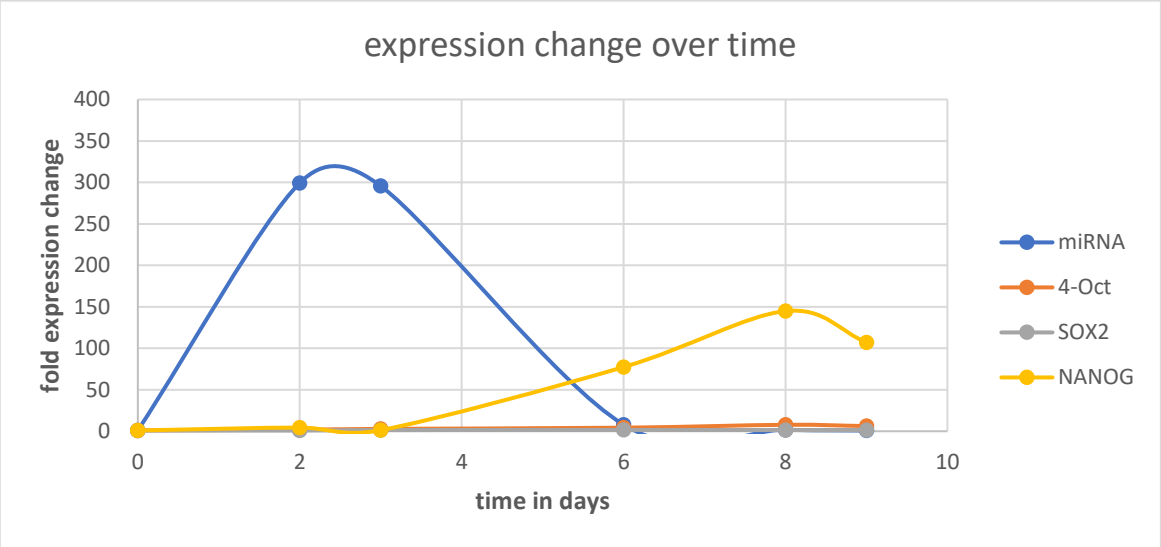


Figure 33: ***Time course of expression levels after transfecting HEK293T cells with miRNA guides****: Expression of the miR-302/367 cluster, OCT4, SOX2 and NANOG was determined via qRT-PCR after harvesting samples at different days. Expression fold change was calculated with untransfected HEK cells as the reference sample and actin as the endogenous control.*

As seen in figure 33, miR-302/367 cluster expression starts to increase on day 2 and remains highest between day 2 and 4. It decreases on day 4 and terminates on day 8. In order to keep high miRNA expression levels, the four guides plus the transactivator VP192 should be transfected every 3-4 days to improve reprogramming efficiency. OCT4 expression increased by a factor of 8 and SOX2 changed by 4-fold, which confirms the data from figure 32. NANOG expression increased over 150-fold, which also correlates with the data from figure 32. However, it takes about 3 days to activate NANOG, which is the reason for the shift between miRNA and NANOG activation.

miRNA expression change was then tested in fibroblasts by qRT-PCR after transfecting the cells with VP192 and the four gRNA plasmids targeting the miRNA cluster. It led to a 72-fold increase for the miRNA 302/367 cluster, compared to a 629-fold increase in HEK cells. Upon transfection of VP192 and GG-EBNA the miRNA cluster expression was increased by 6-fold only. However, the low transfection efficiency observed with the large plasmid containing the dCas9 VP192 could be the reason for this weaker activation.

4. Discussion

4.1. Reprogramming of fibroblasts and blood cells using nm-mRNAs and srRNAs

The first part of the project was to test the RNA reprogramming methods (nm-mRNAs and srRNA) on blood cells, in comparison to fibroblasts. At the beginning, optimal transfection conditions for fibrocytes, fibroblasts and erythroblasts were determined using a GFP mRNA. The efficiency was low for erythroblasts but >70% for fibrocytes and fibroblasts. These transfections conditions were applied to deliver the nm-mRNA or srRNA to the 3 cell types. Erythroblast reprogramming was ineffective. The reason for unsuccessful transfection in erythroblasts could be suboptimal transfection parameters like voltage and pulse number when using Neon transfection. It could also be due to higher sensitivity to RNA transfection. It is known that the transfection of RNA triggers an innate immune response in the cells leading to cell death. This response could be stronger in blood cells leading to elimination of the successfully transfected cells. In contrast, fibrocytes could easily be transfected but they started dying after a few days in culture, which could be due to inadequate culture conditions. Moreover, when fibrocytes were split, they stopped dividing and growing, showing an overall poor proliferation capacity. Therefore, fibrocyte reprogramming was impossible because of slow proliferation and toxicity post transfection.

Sendai virus mediated reprogramming is the standard method used in the iPS core facility. Therefore, the efficiencies of the new methods were compared to it. Sendai virus mediated reprogramming was the method of choice for blood cells such as erythroblasts.

Self-replicative-RNA and non-modified-mRNA reprogramming was only successful with fibroblasts. With the non-modified-mRNA reprogramming 30 colonies emerged, whereas srRNA reprogramming only led to the formation of 2 colonies. This could be due to suboptimal culture conditions, overly dense cell growth and/or inefficient transfection efficiency of the srRNA. In addition, as a lot of fibroblasts can be seen in figure 16, positive selection with puromycin did not seem to work, because puromycin should have eliminated all cells which were not transfected.

All of those methods make use of direct reprogramming and the cells were then analyzed to confirm their pluripotency state. The expression of the pluripotency markers DNMT3b, hTERT, SOX2, REX1, OCT4 and NANOG was analyzed by qRT-PCR. All pluripotency marker levels were clearly upregulated compared to somatic cells and morphology of the iPS colonies also confirmed reprogramming efficiency. Embryoid bodies were formed, which showed a variety of spontaneously differentiated cells. Embryoid bodies were analyzed by qRT-PCR for several differentiation markers: EN1, MAP2 and NR2F2 for ectoderm formation, SNAI2, RGS4 and HAND2 for mesoderm and SST, KLF5 and AFP for endoderm. Scorecard analysis confirmed the differentiation potential into cells from the three germ layers: ectoderm, mesoderm and endoderm. Immunocytochemistry staining showed that all transcription factors tested (OCT4 and NANOG in the nucleus and SSEA4 and TRA-1-60 on the cell surface) were located in their original cell compartments and that the generated cells resemble stem cell-like characteristics.

Comparing sr-RNA-, nm-mRNA- and Sendai virus mediated reprogramming of fibroblasts, Sendai virus mediated reprogramming was the best method, as over 100 colonies were formed, whereas nm-mRNA reprogramming resulted in approximately 30 colonies and sr-RNA reprogramming only gave 2 colonies. Sendai virus mediated reprogramming was also the fastest way to derive iPSCs and apart from being the most time-efficient method, it was also the least labor-intensive, as only one transfection was necessary. Non-modified-mRNA reprogramming required four transfections and sr-RNA reprogramming required one transfection, but different media had to be prepared throughout 20 days, making it the method with the biggest workload. The quality of all iPSCs generated from any of the three methods was comparable, as all of them showed sufficient pluripotency and differentiation marker expression. Non-modified-mRNA reprogramming could be a good alternative to Sendai virus mediated reprogramming, as no virus particles are used and the method is completely xeno-free and serum-free. Since both methods are non-integrative, they are safe for cell therapy, but Sendai virus reprogramming requires a few passaging cycles to eliminate the viral RNA before the cells are safe for use in patients.

4.2. CRISPR-based reprogramming

The CRISPRa system offers the opportunity to directly reactivate the pluripotency genes in somatic cells. In 2018, Weltner et al. used this approach the generate human iPS lines. To test this new method, the plasmids described in their publication were obtained.

First, HEK293T cells were transfected with the transactivators VPH or VP192 and the plasmid GG-EBNA, which contains guide RNAs to target the pluripotency genes. HEK cells showed a high transfection efficiency of 50% compared to an efficiency of 2% in fibroblasts. The low transfection efficiency is due to the size of the plasmids (>15kb). Cytotoxicity due to the plasmid transfection itself was observed when transfecting three versus two plasmids: Cells transfected with the 3 plasmids VP192, EEA and OSK2M2L showed a sigificantly lower survival rate compared to cells transfected with VP192 and OSK2M2L only. The huge plasmids generate huge holes in the cells, which consequently die. HEK293T cells were first transfected to determine OCT4 and SOX2 expression levels and the results correlated with the data from Weltner et al. (2018).

HEK293T cells transfected with the different combinations of gRNA plasmids showed elevated levels of OCT4, SOX2 and NANOG. The addition of the EEA plasmid increased the expression of NANOG and LIN28. Therefore, the expression increase should be high enough to reprogram the cells into iPSCs. Both KLF4 and c-MYC showed a very low activation which is due to an already high expression of these genes in HEK293T cells. Higher expression levels of OCT4 and SOX2 (figure 25) were achieved compared to the first experiment in HEK293T cells (figure 23), which could be due to the use of a different HEK293T cell line. In general, differences in pluripotency and actin marker expression in different HEK cell lines and passages were observed. Another explanation for the varying results is, that the transfection efficiency in the first experiment was lower and thus fewer cells contained the plasmids, which lowered the overall expression level.

The next approach was to reprogram fibroblasts: Although the plasmid combinations of VP192 with GG-EBNA plus KM/EEA gave the highest expression levels in HEK293T cells, CRISPRa based reprogramming of fibroblasts was successful only in the presence of OSK2M2L and EEA. With the other vectors, morphological

changes were observed suggesting a partial reprogramming. Vector construction and delivery could also be a reason for partially reprogrammed cells, as morphological changes were visible when tranfecting fibroblasts. Further optimization needs to be done to generate iPS lines more efficiently with the CRISPRa system.

The reason for low efficiency in fibroblasts could be that culture conditions, such as media, matrix, MEFs and time points of changing cells to different conditions were not optimal. Moreover, in some experiments the fibroblasts did not seem to grow very well, which could be due to a high passage number and/or the cell line. The health of the cells is important to ensure a good survival rate and growth after transfection.

In addition, transfection efficiency of fibroblasts was very low compared to HEK293T cells. To augment the efficiency of the transfection, the use of smaller plasmids would be helpful. The dCas9-VP192 gene is ~6Kb and the episomal vector contains many elements (such as EBNA1) that increase the size of the plasmids. Switching to a different delivery method could improve the transfection of fibroblasts. For instance, Ribonucleoprotein (RNP) delivery has been shown to be more efficient to transfect human iPS cells, cells known to be difficult to transfect. However, there is currently no dCas9-transactivator commercially avalailable in the protein form. An mRNA for the dCas9 transactivator would be an alternative too, but the main challenge for this approach would be to transfect the cells every day or every other day to ensure a sustained activation of the endogenous pluripotent stem cells.

4.3. The miRNA 302/367 cluster

The miR-302/367 cluster expression was assessed in different cell types: iPSCs, HEK293T, fibroblasts, EBs and PBMCs. RNA was extracted and cDNA was generated, which was used for RT-PCR and qRT-PCR. The miR-302/367 cluster is highly expressed in human pluripotent stem cells and downregulated upon EB differentiation. The expression level decreases from day 5 to day 10 and is undetectable at day 15 as cells are fully differentiated. EBs harvested at day 5 show higher levels than EBs from day 10, because the population from day 10 contains more differentiated cells.

The CRISPRa system was employed to overexpress the miR-302/367 cluster in somatic cells to determine if the cluster expression is sufficient to reprogram cells or if it could increase the reprogramming efficiency in combination with the overexpression of the Yamanaka factors.

The transactivators (dCas9-VPH or dCas9-VP192) together with custom-designed guides (455, 407, 343 and 285) targeting the promoter of the miR-302/367 cluster were transfected into HEK293T cells and fibroblasts. In HEK293T cells, an activation of the miR-302/367 was observed when using individual gRNAs but the expression increased drastically when the 4 gRNAs were combined, suggeting an additive effect. Also guides that are closer to the transcription start seem to be more efficient. Then the four guides were transfected together with VP192 into HEK293T cells to determine their activation kinetics over time. After four days the expression decreased, which means that the transfection was only transient and the guides were degraded or diluted after some time. Therefore, a transfection every 3-4 days would be necessary to keep a sustained miRNA expression level for reprogramming. Another option would be to clone the gRNAs into episomal vectors as Weltner et al. did in 2018.

To determine if the activation of the miR-302/367 would induce the expression of the main pluripotent markers, a qRT-PCR was performed. NANOG expression level was increased upon transfection of the 4 gRNAs and dCas9VP192. This correlates with a previous study showing that overexpression of miR-302 suppresses MBD2 (Methyl-DNA binding domain protein 2) which in turn increases Nanog expression (Lee et al., 2013).

Upon transfection of the miRNA guides pluripotency marker expression increases because the miRNAs activate transcription factors of those genes. OCT4 and SOX2 expression was increased after activation of the miR-302/367 but at a lower level. Both KLF4 and c-MYC showed a very low activation but it might be due to an already high expression of these genes in HEK293T cells.

In contrast, when pluripotency guides (GG-EBNA) and VP192 were transfected into HEK293T cells, a 12-fold increase of the miR-302/367 expression was detected.

When transfecting fibroblasts, no real changes in the expression of the pluripotency markers were observed, which is due to poor transfection efficiency, as only 1-2% of the total cell population from which RNA was isolated contains the transactivator and the guides.

In order to achieve successful reprogramming using fibroblasts, culture conditions including media, matrix and timing have to be improved. Also, transfection conditions using electroporation, nucleofection or lipofection need to be adjusted, as only a small percentage of fibroblasts were transfected. Maybe smaller plasmids could be designed to increase efficiency and to reduce cell death.

HEK293T cell transfection showed that reprogramming using VP192 and the gRNA containing plasmids targeting the miRNA cluster (455, 407, 343 and 285) would be possible, as those cells show high levels of all pluripotency gene expression necessary for reprogramming.

Also, the miRNA cluster has to be further studied and guides have to be designed that target the cluster so that they can be used in combination with already existing guides to further increase the expression of the miRNA 302/367 cluster. We showed, for the first time, that the CRISPRa technology can also be used to activate the miR-302/367 cluster. This activation could provide a boost for the reprogramming efficiency of somatic cells that are hard to reprogram.

5. Summary

In conclusion, reprogramming of blood cells was only possible with Sendai virus, and episomal vector.

Fibroblasts were successfully reprogrammed into iPSCs with the non-modified mRNA and self-replicative RNA. The resulting induced pluripotent stem cells showed all stem cell characteristics such as pluripotency and self-renewal.

Fibroblasts could also be reprogrammed with the CRISPRa system but only when the EEA was activated in addition to the pluripotency genes. These iPS lines could not be characterized as they emerged on the last month of my internship. HEK293T cells, which were used as a control for all the experiments as they are easy to transfect, showed high levels of pluripotency marker expression after transfection of the CRISPR activators. Therefore, reprogramming of human somatic cells with this system could be possible, but more research is needed to optimize transfection, dosage and cultivation conditions of the cells to guarantee a high reprogramming efficiency.

The discoveries of iPSCs and their applications in disease modelling and therapies have been of tremendous importance for stem cell research within the last decade. The RNA and CRISPRa reprogramming methods hold great potential to be used in clinics one day. The unprecedented potential of iPSCs in regenerative medicine, disease modeling, replacing animal models, drug screening and cell therapies is enormous. However, the methods are still very time-consuming, expensive and inefficient for establishing large iPS cell banks. More research needs to be done to be able to fully replace the use of ethically controversial embryonic stem cells and to use iPSCs in various medical therapies. The hurdles will hopefully be overcome soon to treat millions of patients with different diseases.

The miR-302/367 cluster seems to play an important role in maintaining stem cell characteristics, as it is only expressed in ESCs and iPSCs. Reprogramming with the new system of transactivators and gRNAs might be challenging, as pluripotency markers such as OCT4, NANOG and SOX2 are only slightly upregulated. Nevertheless, a boost of reprogramming factors by using four guides targeting the cluster was achieved, confirming the importance of the miR-302/367 cluster in reprogramming. More studies are necessary to be able to successfully use the

cluster in addition to other reprogramming techniques to make reprogramming methods less time-consuming and more effective.

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