Marshall Plan Scholarship

Final Report

by

Susanne WAIZENAUER

Testing the conversion of GFP to BFP in human pluripotent stem cell line using CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated proteins) mediated genome editing

Internal Supervisor: Dr. Barbara Entler

External Supervisor: Laurence Daheron, PhD

Submitted on: 26.03.2018

Acknowledgements

Firstly, I would like to acknowledge my supervisor at the iPS Core Facility, Laurence. Thank you for being so patient with me; for trusting me and showing me, how amazing science can be. Thank you for being so excited every time I came with results, this made me feel as though my work was acknowledged and cheered up every hour spent at the lab. My deepest appreciation for your excellent supervision. Danke!

I would also like to thank my lab colleagues. My sincere gratitude to you all for showing me all the fun stuff around Harvard. Thank you for all the hours we spent together, for all the funny moments where we nearly fell off our chairs. No words can describe how great it was to be a part of this international group, with so many different characters.

Moreover, I would like to express my gratitude to my internal supervisor Dr. Barbara Entler for her excellent support and supervision. She always had an open ear for all the (un)important questions I had.

Furthermore, I would like to thank the Austrian Marshall Plan Foundation for their financial support. Thank you for encouraging young people to pursue their dreams.

Finally, I would like to say thank you to my family who supported me through my whole life and always lent a hand when I needed it. I am sincerely grateful for the financial support, which made it possible to stay six months in the USA. Thank you for always being there with open arms and never-ending love.

Abstract

With the discovery of human pluripotent stem cells, a new type of disease model was established. With the extraction of embryonic stem cells, Thomson et al. (1998) revolutionized the field of medical research and took a first glimpse at the capability of stem cells. When Takahashi and Yamanaka (2006) published a protocol for induced pluripotent stem cells, the highly controversial topic of stem cells got a new taste.

These discoveries made it possible to study in vitro models of human diseases, which have never been studied before. This was especially the case for disease-specific cell types which could be generated and studied without interacting with patients or in vivo animal models.

During the same time, genome editing experienced a rise in interest, as techniques like zinc finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR) were available for daily experiments.

However, the targeting efficiency remains low in human pluripotent stem cell lines and hundreds of colonies need to be screened to detect clones with the desired modification.

The focus of this paper will be on the improvement of genome editing with CRISPR/Cas9. Therefore, a GFP expressing HPSC line was generated and the conversion of GFP to BFP was tested with CRISPR Cas9. As a result, it was possible to test different transfection systems and parameters to optimize the process of genome editing. Within 6 months it was possible to establish a protocol which illustrates an above average efficiency in CRISPR/Cas9 mediated genome editing.

Furthermore, the differentiation of HPSC into motor neurons was accomplished.

All experiments were conducted in the iPS Core Facility, in the Harvard Stem Cell Institute.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AB	Antibody
AS	Antisense Direction
BFP	Blue Fluorescence Protein
Cas9	CRISPR associated Protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
DAPI	4,6-Diamidino-2-phenylindole
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
dNTP	Deoxynucleotide Triphosphate
DPBS	Dulbeccos's Phosphate-Buffered Saline
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal Bovine Serum
FokI	Restriction Endonuclease
GFP	Green Fluorescence Protein
gRNA	guide RNA
Hb9	Homeobox Gene 9
HDR	Homology Direct Repair
HPSC	Human Pluripotent Stem Cells
ICC	Immunocytochemistry Staining
ISL-1^{+}	Insulin Gene Enhancer Protein 1
KI	Knock-In
KO	Knock-Out
Neg	Negative
NHEJ	Non-Homologous End Joining
PAM	Protospacer Adjacent Motif
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
Pos	positive
PPP1R12C	Protein Phosphatase 1 Regulatory Subunit 12C
rpm	Rounds per Minute
RT	Room Temperature

RVD	Repeat Variable Diresidue
S	Sense Direction
ssODN	Single-stranded Oligo Donor Nucleotides
T	Thymidine
TALE	Transcription Activator-like Effectors
TALEN	Transcription Activator-like Effector Nuclease
TDP-43	TAR DNA Binding Protein 43
tracrRNA	trans-activating crRNA
ZFN	Zinc-finger Nucleases

1 Introduction

1.1 Marshall Plan Scholarship Proposal

In the proposal from March 2017, the one of the main goals was to develop a rapid assay for genome editing in pluripotent stem cell lines which should in turn improve current protocols and hence increase the reliability of the genome editing service. Furthermore, the improved protocol, would help train other researchers in the field of genome editing. With the help of the GFP to BFP conversion, the process of genome edititng could easily be visualized.

In March 2017 the methodological consideration was to produce a GFP expressing pluripotent stem cell line and to further work on the conversion of GFP to BFP.

To generate the GFP expressing cell line, the choice was between two different approaches: to either transduce a lentiviral vector carrying a GFP or to use the so-called TALEN method. At the beginning of August, the second approach was chosen.

After generating a GFP expressing cell line, it was planned to study the conversion of GFP to BFP according to the publication of Glaser, McColl, and Vadolas (2016). The optimal result would be to improve targeting efficiency as well as homology direct repair (HDR) events. The efficiency of GFP to BFP conversion was evaluated with fluorescent-activated cell sorting (FACS).

The expected results were to develop a rapid assay to quantify genome editing efficiency in pluripotent stem cell lines, improve operation protocols, increase throughput and to overall improve efficiency.

Overall it can be said, that it was possible to successfully accomplish the set goals. Additionally, I had enough time to start a motor neuron differentiation from human pluripotent stem cells.

1.2 Human Pluripotent Stem Cells

Before the development of induced pluripotent stem cells, human blastocyst-derived pluripotent stem cells were considered to be state of the art. In 1998 Thomson et. al describes how embryonic stem cells can be isolated and show normal karyotypes (Thomson et al., 1998).

Due to the ethical difficulties of this technique, the time had come to develop more morally permissible methods.

Takahashi and Yamanaka (2006) showed that adult fibroblast cultures could be induced into an embryonic- like state. By introducing four factors, Oct3/4, Sox2, c-Myc and Klf4, the cells were reprogrammed (Takahashi & Yamanaka, 2006).

To reprogram human somatic cells, best suited methods have been developed. Therefore, several characteristics, such as adequate efficiency, capablilty to produce iPSC from skin fibroblasts and blood and overall simplicity of the technique must be met (Malik & Rao, 2013).

Several reprogramming methods are available for therapeutic use as well as academic research. In the following paragraph, different methods will briefly be discussed.

One method is single cassette reprogramming with lentivirus. It can infect nondividing and proliferating cells. There are several existing concerns of this method, such as the suboptimal stoichiometry due to reprogramming with four or more vectors (Malik & Rao, 2013).

Another widely used method is the reprogramming by nonintegrating viruses. Two techniques belong to this method, adenovirus and sendai virus. For this method, sendai virus shows several advantages as it can yield higher efficiency, as well as a higher production of proteins. Furthermore, it can reprogram blood cells and fibroblasts, however it has some disadvantages of its own. The sendai virus is more difficult to handle than the lentivirus. Moreover, it takes up to 10 passages to elute the virus (Malik & Rao, 2013).

Another group of transfection methods exist, namely the nonviral reprogramming methods. It consists of mRNA transfection, miRNA infection, PiggyBac, minicircle vectors and episomal plasmids. Unfortunately, not all methods are reliable or convenient to work with. mRNA transfection yields high efficiency, but is labor intensive and can only be used in fibroblasts (Malik & Rao, 2013).

1.3 Genome Editing

1.3.1 Zinc-Finger Nucleases (ZFN)

The ZFNs consist out of DNA-binding and DNA-cleavage domains, which enable to target specific gene loci (Carroll, 2011).

Kim and Chandrasegaran (1994) showed, that FokI endonuclease recognizes the nonpalindromic pentadeoxyribonucleotide 5'-GGATG-3'.5'-CATCC-3' double helical DNA and cleaves 9 and 13 nt apart from the recognition site. Furthermore they show, that by substituting the recognition domains of FokI cutting can be redirected (Kim & Chandrasegaran, 1994).

In Figure 1 a simplified approach of ZFN can be seen.

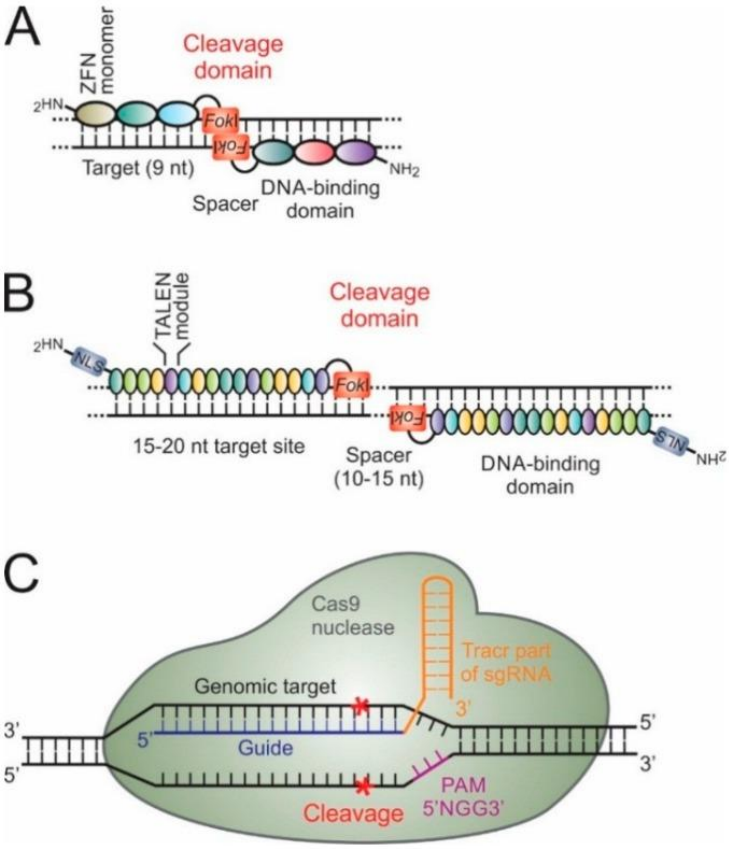


Figure 1: **Three Genome Editing Techniques: Zinc-finger Nucleases, TALEN and CRISPR/Cas9**

(A) Represents the method of the zinc-finger nucleases. The regions of DNA-binding and DNA-cleavage can be seen. Cleavage of DNA happens when FokI dimerization occurs. (B) TALEN also consists out of a right and left pair, which binds to the DNA. Again, the FokI nuclease cleaves the DNA when dimerization performs. (C) For CRISPR/Cas9 mediated genome editing a gRNA needs to be present. After binding of the gRNA, the Cas9 Nuclease domains get activated and introduce a double strand break (DSB) (Maepa, Roelofse, Ely, & Arbuthnot, 2015).
Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4581210/fi

ZFNs often consist of three DNA-binding units. Each unit includes one atom of zinc, which is surrounded by approximately 30 amino acids. With the investigation of the crystal structure, it was shown, that each unit contacts 3 bp of DNA. The three units form one finger, which binds to the recognition site. When both fingers have attached to their specific sequence, FokI dimerization occurs and cleavage is achieved. One of the advantages for this method is that the FokI domains remain inactive until dimerization (Carroll, 2011).

1.3.2 Transcription Activator-like Effector Nuclease (TALEN)

TALENs are a fusion product of transcription activator-like effectors (TALE) and the catalytic domain of FokI endonuclease. Plant pathogens produce TALEs, which effect the plant cells by invasion. There, the protein moves into the nucleus and activates gene expression of effector-specific sequences. Within the protein, the amino acid repeat changes, except for two adjacent amino acids, also called repeat variable diresidue (RVD). The RVDs form the recognition site of different DNA base pairs (Christian et al., 2010).

TALEN revolutionized the field of genome engineering due to the simplicity of the method. With the adjustment of RVDs, it is possible to target any DNA sequence. Nevertheless, the method has its limitation. By choosing the TALEN nuclease sites, it must be considered that a T is needed before the 5' end at the target sequence (Nemudryi, Valetdinova, Medvedev, & Zakian, 2014).

In Figure 1 the mechanism of TALEN is visualized. By binding to the sequence specific nucleotides, FokI can dimerize and introduce a DSB.

Both, ZFN and TALEN notice the correct DNA sequence through protein-DNA interactions (Hsu, Lander, & Zhang, 2014).

1.3.3 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

CRISPR is composed of a guide RNA (gRNA) and the endonuclease Cas9. The gRNA can be easily designed via online tools and enables the CRISPR/Cas9 system to target any sequence in the genome.

In 2005 Mojica et al showed that the CRISPR system evolved from prokaryotes to defend their genome against foreign genetic elements (Mojica, Diez-Villasenor, Garcia-Martinez, & Soria, 2005).

After many years of research and exploring, the CRISPR/Cas9 system is mature enough to be used in eukaryotes with nearly no limitations. The only drawback is the complex eukaryotic genome, which contains billions of nucleotides.

The gRNA can detect the target DNA via Watson-Crick base pairing. By remodeling the gRNA to a sequence of interest, any position in the genome can be targeted. The advantage compared to ZFN and TALENs is that short gRNAs are easier to introduce rather than big proteins (Hsu et al., 2014).

In Figure 1 the schematic principle of CRISPR/Cas9 is illustrated. The gRNA can be obtained in two ways: as a single guide RNA or as two RNAs, namely crRNA and tracrRNA. After binding of the gRNA opposite of a protospacer adjacent motif (PAM) a DSB is introduced three bp upstream of the PAM.

2 Materials and Methods

2.1 Maintenance of ESC and iPSC

Depending on further use, cells were cultured on mTeSR™ 1 (Stemcell™ Technologies), Stemflex (Thermo Fisher Scientific) or Essential 8 (Thermo Fisher Scientific) media. Media were prepared according to manufacturer's manual.

ESC and iPSC were cultured on Cultrex® (Trevigen) matrix coated plates (at a concentration of 10 mg/ml). Cultrex® was plated one hour before use to allow proper surface coating.

Media for cells on mTeSR1 and Essential 8 media was changed every 24 hours. Stemflex media was changed every 48 hours.

At a confluency of 70-80 % cells were split in a ratio from 1:4 to 1:6. To dissociate the cells, they were treated with Gentle Cell Dissociation Reagent (STEMCELL Technologies) for 4 minutes at RT. The solution was aspirated and the media was added to detach cells with a cell lifter.

Spontaneous differentiation, which could occur after stress exposure, was removed under a picking microscope with the appropriate picking tools.

2.2 Freezing of ESC and iPSC

When cells reach 70-80 % confluency, they became dissociated and suspended to avoid the formation of big cell clusters. Cells were centrifuged (4 minutes at 1000 rpm) and resuspended in freezing media (10 % Dimethyl Sulfoxide (Sigma-Aldrich) and 90 % Fetal Bovine Serum (VWR)). Cell suspension was transferred into cryovials (Thermo Fisher Scientific) and stored at -80 °C in Mr. Frosty (Sigma-Aldrich) to allow a slow cool down.

2.3 Design of Cas9 Plasmid and CRISPR guide RNA

Two different Cas9 Plasmids were used during these experiments. All plasmids hadbeen obtained through Addgene.

Table 1: Used Cas9 Plasmids for the conduction of experiments.

No.	Name	Purpose	Catalog #	Reference
1	pCas9_GFP	Expression of human codon-optimized Cas9 nuclease and GFP	44719	Kiran Musunuru unpublished
2	eSPCas9(1.1)	Expression of high specificity SpCas9	71814	(Slaymaker et al., 2016)

The following gRNAs had been ordered through Integrated DNA Technologies.

Table 2: Used CRISPR gRNAs for the Conversion of GFP to BFP.

No.	Name	Direction	Sequence	Reference
1	GFP to BFP gRNA1	F	CGGCGTGCAGTGCTTCAGCC	(Glaser et al., 2016)
		R	GGCTGAAGCACTGCACGCCG	
2	GFP to BFP gRNA2	F	CCTCGTGACCACCCTGACCTA	(Glaser et al., 2016)
		R	TAGGTCAGGGTGGTCACGAGG	

gRNA 1 was ordered from Synthego for transfection with TrueCut Cas9 Protein v2 (Thermo Fisher Scientific).

2.4 Design of ssODN for GFP to BFP Conversion

The repair template for the GFP to BFP conversion was obtained from Glaser et al., 2016 and ordered through Integrated DNA Technologies. The repair template was designed as a ssODN with the following sequence:

5’ – CCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCT
CGTGACCACCCTGAGCCACGGGGTGCAGTGCTTCAGCCGCTACCCCGACCACA
TGAAGCAGCACGACTTCTTCAAGTCCGCCAT – 3’

2.5 Design of TALEN

The following TALEN pairs have been ordered through Addgene with the following characteristics:

Table 3: Used TALEN pairs for targeting the human AAVS1 locus.

No.	Name	Purpose	Catalog #	Reference
1	hAAVS1 1L TALEN	Targeting the human AAVS1 locus – Left TALEN	35431	(Sanjana et al., 2012)
2	hAAVS1 1R TALEN	Targeting the human AAVS1 locus – Right TALEN	35432	(Sanjana et al., 2012)
3	pSIN4-EF1a-TAL1-IRES-Puro	Lentiviral Vector for TAL1	61065	(Elcheva et al., 2014)

2.6 EGFP Plasmid for TALEN

The following plasmid was used to deliver the GFP sequencing into the human AAVS1 locus. The plasmid was obtained from Addgene.

Table 4: Used plasmid vector for the insertion of GFP into the human AAVS1 locus.

No.	Name	Purpose	Catalog #	Reference
1	AAV-CAGGS-EGFP	Donor vector for genomic targeting of AVVS1 locus	22212	(Hockemeyer et al., 2009)

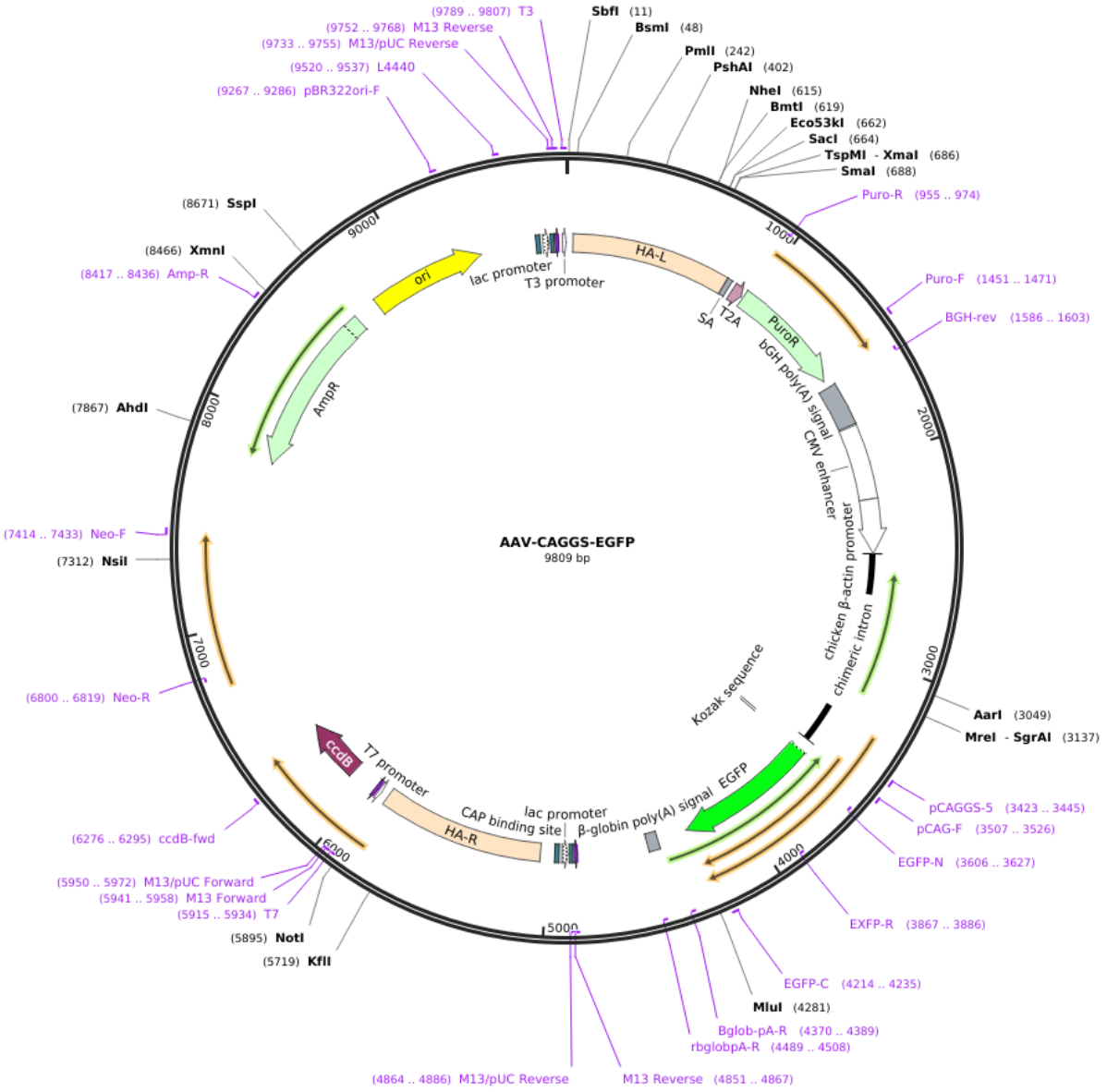


Figure 2: AAV-CAGGS-EGFP vector for targeting the human AAVS1 locus.

2.7 Oligo Annealing

Sense and antisense sequences were diluted to 100 µM with 1x TE buffer. 1.5 ml reaction tubes were prepared with 2 µl sense and 2 µl antisense sequence, 2 µl of 10X NEB restriction buffer (New England BioLabs) and were filled up to 200 µl with H_2O. The tubes were incubated for 5 minutes at 90 °C. Afterwards, the sample was cooled down to 50 °C.

2.8 Ligation

For the ligation, a ligation vector must be obtained. 15 µl of RNAse Free Water (New England BioLabs), 1 µl of 1:10 diluted cloning product (0.1 pmol/µl), 1 µl of ligation vector (0.01 pmol/µl), 2 µl of 10X T4 Ligase Buffer (New England Biolabs) and 1 µl of T4 Ligase (New England BioLabs) were mixed together and incubated for 30 minutes at RT. The following ligation vector have been obtained through Addgene:

Table 5: Ligation Vector for CRISPR/Cas9 gRNA.

No.	Name	Purpose	Catalog #	Reference
1	pSPgRNA	To express *Streptococcus pyogenes* Cas9 guide RNA in mammalian cells	47108	(Perez-Pinera et al., 2013)

2.9 Transformation into Competent *Escherichia coli* Cells

1 µl of the previously obtained ligation product was placed into a 1.5 ml tube and kept on ice until further use. To the pre-chilled 1.5 ml tubes; 5-10 µl cell solution was added, mixed and kept on ice for 30 minutes. Afterwards, the cells were heat-shocked at 42 °C for 45 seconds and incubated on ice for 45 seconds. 200 µl of the appropriate culture medium (LB medium) was added and the cells were incubated for 30 minutes at 37 °C. After the incubation, 100 µl of the culture medium was plated on antibiotic containing culture plates and incubated overnight.

2.10 Small Scale Plasmid DNA Isolation (Mini Prep)

Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions. The DNA concentration was measured with NanoVue Plus (GE). For the blank sample; RNAse free water was used.

2.11 Sequencing

Extracted DNA was sent for sequencing to Genewiz®.

2.12 Large Scale Plasmid DNA Isolation (Maxi Prep)

For large scale plasmid DNA extraction, the EndoFree Plasmid Kit (QIAGEN) was used according to the manufacturer's instructions. The DNA concentration was measured with NanoVue Plus (GE). As blank sample RNAse free water was used.

2.13 Transfection

For the comparison of the efficiency of different transfection methods the manufacturer's instructions were compared with optimized protocols of the iPS Core Facility.

2.13.1 Lipofectamine®3000 (Thermo Fisher Scientific) – Manufacturer's Instructions

The transfection was conducted according to the manufacturer's instructions. The ratios of Cas9 plasmid were kept constant with cell number to obtain comparable results.

2.13.2 Lipofectamine®Stem (Thermo Fisher Scientific) – Manufacturer's Instructions

The transfection was conducted according to the manufacturer's instructions. The ratios of Cas9 plasmid kept constant with cell number to obtain comparable results.

2.13.3 Lipofectamine®CRISPRMAX (Thermo Fisher Scientific) - Manufacturer's Instructions

The transfection was conducted according to the manufacturer's instructions. The ratios of Cas9 protein kept constant with cell number to obtain comparable results. For Cas9 protein, gRNA and ssODN concentrations see Table 6. For the manufacturer's instructions No. 2-4 have been tested.

2.13.4 Nucleofector™ (Lonza) for Cas9 Plasmid Delivery Efficiency

The transfection was conducted according to the manufacturer's instructions. The ratios of Cas9 plasmid were kept constant with cell number to obtain comparable results.

The following transfection methods followed the same cell preparation steps.
The following protocol is for culture dishes with a surface area of 78.5 cm^2. Media was aspirated from the culture dish and washed with 5 ml of DPBS (Fisher Scientific). After aspiration, 5 ml of diluted Accutase™ (Stemcell™ Technologies) (1:3 with DPBS) was added and incubated for 8 minutes at 37 °C. If detachment of cells could be observed, 5 ml of appropriate culture media was added to stop the Accutase. Cells were collected in a Falcon tube (Fisher Scientific). Cells were counted with the TC20™ Automated Cell Counter (BIO-RAD) by mixing 10 µl of cell suspension with 10 µl Trypan Blue 0.4 % (Thermo Fisher Scientific). In the mean time, cells were centrifuged at 1200 rpm for 3 minutes at RT and the supernatant was aspirated. Cells were diluted with DPBS to required density and tubes were prepared for fractionation of cells. The tubes were centrifuged at 1200 rpm for 3 minutes at RT. The last step was to wash the cells twice with 2 ml DMEM/F12 (Thermo Fisher Scientific) and centrifuge again at 1200 rpm for 3 minutes at RT and to aspirate the supernatant.

2.13.5 Lipofectamine3000 (Thermo Fisher Scientific) for Cas9 Plasmid Knock-In (KI), Knock-Out (KO) and Plasmid Delivery Efficiency

The following amounts were for a reaction of 800 000 cells per tube. During the centrifugation steps of the cell mass, the following transfection reagents were prepared:

- Solution 1:
  - Opti-MEM™ (Thermo Fisher Scientific) 125 µl
  - Lipofectamine 3000 reagent 7.5 µl

➔ Mix well

- Solution 2:
    - Opti-MEM™ 125 µl
    - Plasmid DNA Cas9 (KI, KO / Efficiency) 1.5 / 2 µg
    - gRNA (for KI and KO only)
    - ssODN (for KI only)
    - P3000 reagent 2 µl / µg DNA

After preparing the individual solutions, solution 2 was added to solution 1, which results in a final volume of ~250 µl. The transfection reagents were incubated for 5 minutes at RT. After incubation, the DNA-lipid complex was added to the cells drop-wise. The cell pellet was gently loosened by tapping the tube and then was incubated for 12 minutes. Caution was needed in order to not go over 15 minutes. After the transfection, cells were plated in a 6-well plate coated with appropriate culture system and media. At the end, 4 µl of ROCK inhibitor (EMD Millipore) was added per well and incubated at 37 °C 5 % CO_2 until analysis.

2.13.6 Lipofectamine®3000 (Thermo Fisher Scientific) for TALEN

The following amounts were for a reaction of 800 000 cells per tube. During the centrifugation steps of the cell mass, the following transfection reagents were prepared:

- Solution 1:
    - Opti-MEM™ (Thermo Fisher Scientific) 125 µl
    - Lipofectamine 3000 reagent 3.75 µl

➔ Mix well

- Solution 2:
    - Opti-MEM™ 125 µl
    - Left TALEN 1 µg
    - Right TALEN 1 µg
    - EGFP Plasmid 1.6 µg
    - P3000 reagent (2 µl/µg DNA) 7.2 µl

After preparing the individual solutions, solution 2 was added to solution 1, which results in a final volume of ~250 µl. The transfection reagents were incubated for 5 minutes at RT. After incubation, the DNA-lipid complex was added to the cells drop-wise The cell pellet was gently loosened by tapping the tube and then was incubated for 12 minutes. Caution was needed in order to not go over 15 minutes. After the transfection, cells were plated in a 6-well plate coated with appropriate culture system and media. At the end, 4 µl of ROCK inhibitor (EMD Millipore) were added per well and incubated at 37 °C 5 % CO_2 until analysis.

2.13.7 Lipofectamine®Stem for Cas 9 Plasmid Delivery Efficiency

The following amounts were for a reaction of 800 000 cells per tube. During the centrifugation steps of the cell mass, the following transfection reagents were prepared:

- Solution 1:
  - Opti-MEM™ (Thermo Fisher Scientific) 125 µl
  - Lipofectamine Stem Reagent 3.75 µl

➔ Mix well

- Solution 2:
  - Opti-MEM™ 125 µl
  - Cas9 Plasmid DNA 2 µg

➔ Add solution 2 to solution 1, which results in a final volume of ~250 µl.

➔ Incubate the transfection reagents for 5 minutes at RT.

After preparing the individual solutions, solution 2 was added to solution 1, which results in a final volume of ~250 µl. The transfection reagents were incubated for 5 minutes at RT. After incubation, the DNA-lipid complex was added to the cells drop-wise. The cell pellet was gently loosened by tapping the tube and then was incubated for 12 minutes. Caution was needed in order to not go over 15 minutes.. After the transfection, cells were plated in a 6-well plate coated with appropriate culture system and media. At the end, 4 µl of ROCK inhibitor (EMD Millipore) were added per well and incubated at 37 °C 5 % CO_2 until analysis.

2.13.8 Lipofectamine®CRISPRMAX (Thermo Fisher Scientific) for Cas9 Protein KO and KI

The following amounts are for a reaction of 500 000 cells per tube.

The first optimization step was to find the optimal Cas9 Protein (TrueCut™ Cas9 Protein v2 (Thermo Fisher Scientific)) to gRNA (Synthego) ratio for best KO efficiency. The following concentrations were tested:

Table 6: Different ratios of Cas9 Protein to gRNA for the optimization of Knock-Out efficiency.

No.	Cas9 Protein (pmol/ µl)	gRNA (pmol/ µl)	Ratio Cas9:gRNA
1	20	20	1:1
2	20	60	1:3
3	20	120	1:6
4	20	180	1:9

After determination of the most efficient Cas9 to gRNA ratio the KI efficiency had been tested by applying different ssODN concentrations:

Table 7: After analyzing the optimal Cas9:gRNA ratio, the concentration of ssODN was tested with the following set-up.

No.	Cas9 Protein (pmol/ µl)	gRNA 1 (pmol/ µl)	ssODN (µg)	Ratio Cas9:gRNA
1	20	120	0.33	1:6
2	20	120	0.5	1:6
3	20	120	1	1:6
4	20	120	1.5	1:6
5	20	120	2	1:6

During the centrifugation steps of the cell mass, the following transfection reagents were prepared:

- Solution 1:
  - Opti-MEM™ 125 µl
  - Cas9 Protein
  - gRNA 1
  - Lipofectamine Cas9 Plus Reagent 5 µl

➔ Mix well

- Solution 2:
  - Opti-MEM™ 125 µl
  - ssODN (for KI only)
  - Lipofectamine CRISPRMAX regent 7.5 µl

The solutions were incubated separately for 5 minutes at RT. After preparing the individual solutions, solution 2 was added to solution 1, which results in a final volume of ~250 µl. The transfection reagents were incubated for 5 minutes at RT. After incubation, the DNA-lipid complex was added to the cells drop-wise The cell pellet was gently loosened by tapping the tube and then was incubated for 12 minutes. Caution was needed in order to not go over 15 minutes. After the transfection, cells were plated in a 6-well plate coated with appropriate culture system and media. At the end, 4 µl of ROCK inhibitor (EMD Millipore) were added per well and incubated at 37 °C 5 % CO_2 until analysis.

2.13.9 Neon® (Thermo Fisher Scientific) for Cas9 Protein KO and KI

For the Neon® two different operations set-ups were used:

Table 8: Parameter set-up for Electroporation with Neon®

No.	Electric Potential [V]	Pulse Length [ms]	No. of Pulses	Number of cells	
1	1100	20	1	$1x10^5$	$1.4x10^5$
2	1200	30	1	$1x10^5$	-

During the preparation of the cells, the Neon® systems were set up according to the manufacturer's manual. For the RNP formation in Resuspension Buffer R (Volumes for three reactions) the following components were prepared:

- TrueCut™ Cas9 Protein v2 20 pmol/µl
- gRNA 1 120 pmol/µl
- Resuspension Buffer R 17.5 µl

The mixture was incubated for 5-20 minutes at RT. Afterwards, the cell pellet was resuspended in 17.5 µl of Resuspension Buffer R and transfered to a 1.5 ml tube. The previously prepared solution with the RNP was added to the cell suspension and mixed gently. The electroporation of the cells followed the manufacturer's manual. After transfection, the cells were plated in a 6-well plate coated with appropriate culture system and media. At the end, 4 µl of ROCK inhibitor (EMD Millipore) were added per well and incubated at 37 °C 5 % CO_2 until analysis.

2.14 Fluorescence-activated Cell Sorting (FACS)

Cells were analyzed for GFP and BFP expression via FACS. Cells were prepared by aspirating media and washing with DPBS. Further, the cells were treated with 1:3 diluted Accutase™ with DPBS for 8 minutes at 37 °C. After cell dissociation media was added and cells were collected in Falcon tubes. Cells were centrifuged at 1200 rpm for 3 minutes and the supernatant was aspirated. Cells were washed twice with DPBS. Afterwards, the cells were fixed with 500 µl of 4 % PFA and incubated at RT for 15 minutes. Cells were centrifuged and washed with PBS. After washing, cells were resuspended in 200 µl of 1 % FBS/PBS. The cell suspension was filtered through FACS tubes (Fisher Scientific) and stored on ice and covered with aluminum foil until use.

2.15 Polymerase Chain Reaction (PCR)

For the following PCR reactions, the term "Primer Mix" is used in the composition for the master mix. The term refers to the mix of sense (S) and antisense (AS) primer sequence of a concentration of 5 µM each. The primers were diluted with RNAse Free Water (New England BioLabs).

2.15.1 Intact AAVS1 Locus

With this PCR, the wildtype human AAVS1 was reviewed. The PCR might lead to some conclusion of homozygosity and heterozygosity.

Table 9: Primer Sequence for wildtype human AAVS1 locus

No.	Name	Direction	Sequence	Product Size	Company
1	Human AAVS1 locus	S	CTCACTCCTTTCATTTGGGC	536 bp	Integrated DNA Technologies
		AS	AGGAGACTAGGAAGGAGGAG		

Table 10 shows the individual components and amount for the PCR master mix to evaluate the AAVS1 locus.

Table 10: Master Mix for PCR for intact AAVS1 locus

Component	Amount [µl] / reaction
5X Phusion® GC Buffer (New England BioLabs)	4
Primer Mix	2
dNTP (New England BioLabs)	0.5
DMSO (Thermo Fisher Scientific)	1
Phusion® High Fidelity DNA Polymerase (New England BioLabs)	0.2
Sample DNA [50 µg/µl]	2
RNAse Free Water (New England BioLabs)	10.3

Table 11 shows the PCR Reaction Conditions for an intact AAVS1 locus. The Denaturation, Annealing and Extension phases were repeated 30 times.

Table 11: PCR Reaction Conditions for intact AAVS1 locus

Phase	Temperature [°C]	Time
Initialization	95	1 minute
Denaturation	96	10 seconds
Annealing	61	10 seconds
Extension	68	30 seconds

Final Elongation	68	5 minutes
Final Hold	4	∞

2.15.2 Random Insertion of EGFP Plasmid

With this PCR, the random insertion of the EGFP plasmid into the human genome was shown.

Table 12: Primer Sequence for Random Integration of EGFP Plasmid

No.	Name	Direction	Sequence	Product Size	Company
2	Random Insertion of EGFP Plasmid	S	AAAGAGTCCCCAGTGCTATC	246 bp	Integrated DNA Technologies
		AS	AGGGCGAATTGAATTTAGCG		

Table 13 shows the individual components and amount for the PCR master mix to evaluate the random integration of EGFP Plasmid.

Table 13: Master Mix for Random Integration of EGFP Plasmid

Component	Amount [µl] / reaction
10X Standard Taq Reaction Buffer (New England BioLabs)	2
Primer Mix	2
dNTP (New England BioLabs)	0.5
DMSO (Thermo Fisher Scientific)	1
Taq DNA Polymerase (New England BioLabs)	0.2
Sample DNA [50 µg/µl]	2
RNAse Free Water (New England BioLabs)	12.3

Table 14 shows the PCR Reaction Conditions for the random integration of the EGFP plasmid into the human genome. The Denaturation, Annealing and Extension phases were repeated 30 times.

Table 14: PCR Reaction Conditions for the Random Integration of EGFP Plasmid

Phase	Temperature [°C]	Time
Initialization	95	1 minute
Denaturation	96	10 seconds
Annealing	61	10 seconds
Extension	68	30 seconds
Final Elongation	68	5 minutes
Final Hold	4	∞

2.15.3 3’ Arm of EGFP Plasmid

With this PCR, the right insertion of the EGFP plasmid into the AAVS1 locus was verified. The PCR primers were designed in a way to amplify a part of the 3’ arm of the plasmid and part of the nucleotide sequence of the AAVS1 locus.

Table 15: Primer Sequence for 3‘ Arm of EGFP Plasmid

No.	Name	Direction	Sequence	Product Size	Company
3	3’ Arm EGFP Plasmid	F	CCTGGGGTGCCTAATGAGTG	1083 bp	Integrated DNA Technologies
		R	CCAAAAGGCAGCCTGGTAGA		

Table 16 shows the individual components and amount for the PCR master mix to evaluate the 3’ Arm of the EGFP plasmid.

Table 16: Master Mix for 3’ Arm of EGFP Plasmid

Component	Amount [µl] / reaction
5X Phusion® GC Buffer (New England BioLabs)	2.5
Primer Mix	2
dNTP (New England BioLabs)	0.5
DMSO (Thermo Fisher Scientific)	1

Phusion® High Fidelity DNA Polymerase (New England BioLabs)	0.2
Sample DNA [50 µg/µl]	2
RNAse Free Water (New England BioLabs)	11.8

Table 17 shows the PCR Reaction Conditions for the 3' Arm of the EGFP plasmid. The Denaturation, Annealing and Extension phases were repeated 35 times.

Table 17: PCR Reaction Conditions for the 3' Arm of EGFP Plasmid

Phase	Temperature [°C]	Time
Initialization	95	1 minute
Denaturation	96	10 seconds
Annealing	63	10 seconds
Extension	68	1 minute
Final Elongation	68	5 minutes
Final Hold	4	∞

2.15.4 5' Arm of EGFP Plasmid

With this PCR, the right insertion of the EGFP plasmid into the AAVS1 locus was verified. The PCR primers were designed in a way to amplify a part of the 5' arm of the plasmid and part of the nucleotide sequence of the AAVS1 locus.

Table 18: Primer Sequence for 5' Arm of EGFP Plasmid

No.	Name	Direction	Sequence	Product Size	Company
4	5' Arm EGFP Plasmid	S	TCGACTTCCCCTCTTCCGAT	1200 bp	Integrated DNA Technologies
		AS	GGATTCTCCTCCACGTCACC		

Table 19 shows the individual components and amount for the PCR master mix to evaluate the 5' Arm of the EGFP plasmid.

Table 19: Master Mix for 5' Arm of EGFP Plasmid

Component	Amount [µl] / reaction
10X Standard Taq Reaction Buffer (New England BioLabs)	2
Primer Mix	2
dNTP (New England BioLabs)	0.5
DMSO (Thermo Fisher Scientific)	1
Taq DNA Polymerase (New England BioLabs)	0.2
Sample DNA [50 µg/µl]	2
RNAse Free Water (New England BioLabs)	12.3

Table 20 shows the PCR Reaction Conditions for the 5' Arm of the EGFP plasmid. The Denaturation, Annealing and Extension phases were repeated 30 times.

Table 20: PCR Reaction Conditions for the 5' Arm of EGFP Plasmid

Phase	Temperature [°C]	Time
Initialization	95	1 minute
Denaturation	96	10 seconds
Annealing	60	10 seconds
Extension	68	1 minute
Final Elongation	68	5 minutes
Final Hold	4	∞

2.16 Agarose Gel Electrophoresis

PCR products were run on a 1.5 % agarose gel (Bio-Rad) containing 0.2 µg/ml Ethidium Bromide (Sigma Life Science). The agarose gel was prepared with 1X TAE buffer (Fisher Scientific). PCR samples with Taq DNA Polymerase were dyed with 6X Gel Loading Dye, Purple (New England BioLabs). Appropriate DNA ladders were used to identify band size. Samples were run with 155 V and 18 mA.

2.17 Immunocytochemistry Staining (ICC)

Cells were cultured in 0.95 cm^2 culture dishes and cultured to reach around 50 % confluency. After washing the cells three times with 500 µl DPBS, they were fixed with 150 µl 4 % PFA for 20 minutes at room RT. After fixing, cells were washed three times with 500 µl DPBS containing 0.05 % Tween® 20 (Sigma-Aldrich). To permeabilize the cells, they were incubated for 15 minutes with 500 µl DPBS containing 0.1 % Triton® X-100 (Sigma-Aldrich) at RT. Cells were washed again three times with 500 µl DPBS containing 0.05 % Tween® 20 (Sigma-Aldrich). Cells were blocked with 500 µl DPBS containing 4 % Donkey Serum (Sigma-Aldrich) at 4 °C overnight. After blocking, cells were washed with 500 µl DPBS and 100 µl of primary antibody (AB) diluted in 4 % Donkey Serum/DPBS) was added (see Table 21). After one hour incubation at RT, cells were washed tree times with 500 µl DPBS containing 0.05 % Tween® 20. Afterwards, 100 µl of 1:500 diluted secondary AB (Invitrogen) was added and incubated for one hour at RT in the dark. After removal of the secondary AB, cells were washed three times with 500 µl DPBS containing 0.05 % Tween® 20. Cells were stained with 1:10000 diluted DAPI (Thermo Fisher Scientific) for 20 seconds or left on.

Table 21: Used Antibodies for ICC Staining for characterization of human pluripotent stem cells.

No.	Primary AB	Dilution Ratio	Secondary Antibody	Provider	Catalog #
1	Oct4	1:100	Rabbit IgG	Abcam	ab19857
2	Nanog	1:50	Rabbit IgG	Abcam	ab21624
3	SSEA3	1:200	Rat IgM	Millipore	MAB4303
4	SSEA4	1:200	Mouse IgG	Millipore	MAB4304

2.18 Karyotyping

For karyotyping of human pluripotent stem cells, cells were grown in a flask with 25 cm^2 surface area (CELLSTAR® VWR). Cells were sent to WiCell® for further analysis.

2.19 DNA Extraction

DNA was extracted using the QIAmp® DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The DNA concentration was measured with NanoVue Plus (GE). For the blank sample, RNAse free water was used.

2.20 RNA Extraction

RNA was extracted using the RNeasy® Mini Kit (QIAGEN) according to the manufacturer's instructions. The RNA concentration was measured with NanoVue Plus (GE). For the blank sample, RNAse free water was used.

2.21 cDNA Synthesis

1 µg of extracted RNA was mixed with 4 µl of 5X qScript cDNA Super Mix (Quanta Bio) and filled up to 20 µl with RNase Free Water (Quanta Bio). cDNA was synthesized by the following incubation times: 5 minutes at 25 °C, 30 minutes at 42 °C, 5 minutes at 85 °C and hold at 4 °C. Afterwards cDNA was diluted 1:20 with RNase Free Water.

2.22 Real Time PCR

2.22.1 Pluripotency Markers

Previously obtained cDNA was diluted 1:20 and a TaqMan® Gene Expression Assay (Applied Biosystems) master mix was prepared. The master mix included 10 µl of TaqMan® master mix solution, 1 µl of primer, 4 µl of RNAse free water (Thermo Fisher). 5 µl of cDNA were added to the master mix and qPCR was conducted according to manufacturer's protocol.

Table 22: Used Primers for TaqMan qPCR for Pluripotency Markers

No.	Primer	Provider	Catalog #
1	Oct4	Thermo Fisher	Hs04260367_gH
2	Lin28	Thermo Fisher	Hs00702808_s1
3	SOX2	Thermo Fisher	Hs01053049_s1

4	GAPDH	Thermo Fisher	Hs04420566_g1

2.22.2 GFP Expression Level

The GFP expression level were quantified with SybrGreen® (Quantabio). The qPCR was conducted with a 10 µl reaction volume. The PCR was run according to the manufacturer's manual.

Table 23: Primer Sequence for qPCR Expression Levels of GFP

No.	Name	Direction	Sequence	Product Size	Company
5	GFP expression level - qPCR	S	TCAAGGAGGACGGCAACATC	84 bp	Integrated DNA Technologies
		AS	TTCTGCTTGTCGGCCATGAT		

2.23 Differentiation of Motor Neurons

Unfortunately, the used protocol is not yet published, which restricts the paper for further details on the differentiation process.

2.24 Staining Motor Neurons

The cells were prepared according to the ICC protocol. Different AB (see Table 24) have been used to stain the motor neurons.

Table 24: Used Antibodies for ICC motor neuron staining.

No.	Primary AB	Concentration [µg/ml]	Secondary Antibody	Provider	Catalog #
1	Beta III Tubulin	5	Goat Anti Rabbit IgG	Abcam	Ab18207
2	Islet 1 & 2	5	Rat Anti Mouse IgG	University of Iowa	39.4D5

3 Results

3.1 Transfection Efficiency of Cas9 Plasmid

A variety of transfections methods are available, offered by different companies. Due to the special culture conditions of human pluripotent stem cells, transfection methods which obtain high efficiency in mammalian cells, might not be suitable for human pluripotent stem cells.

For the conduction of this experiment, two different transfection principle had been used. Firstly the lipid-mediated transfection principle and secondly the electroporation principle were used. The following products were tested:

- Lipofectamine® 3000 (Thermo Fisher Scientific)
- Lipofectamine® Stem (Thermo Fisher Scientific)
- Nucleofector™ (Lonza)

For the culture of human pluripotent stem cells in feeder-free conditions, mainly two culture media were used:

- mTeSR™1 (Stemcell™ Technologies)
- StemFlex™ (Thermo Fisher Scientific)

To see if the culture media effects the transfection, efficiency experiments were conducted with cells growing on each media.

Furthermore, a comparison between the manufacturer's manual of Lipofectamin®3000 and Lipofectamine®Stem (referred to as attached) and protocols from the lab (referred to as suspension) were compared by transfection efficiency and cell viability. For the Nucleofector™ two different version of the machine (referred to as old and new) have been compared.

To measure the efficiency of transfection, a Cas9 and GFP expressing plasmid was transfected and analyzed by FACS.

3.1.1 Cell Viability

Figure 3 A to C show the results of cell viability post- transfection. A difference in cell viability can be seen in number of fold increase. To compare results, the doubling time was calculated to compare cell number at the same day. It can be said that cells which were transfected according to laboratory protocol have a decrease in cell viability

compared to the manufacturer's manual. Overall it can be seen that Nucleofection™ has the highest impact on cell viability. With a fold increase of less than 2, it shows that cells undergo stress and leads to a decrease in viability.

In Figure 3 D, the cell viability of the most efficient methods, were compared and so according to the comparison, the most effective method was selected.

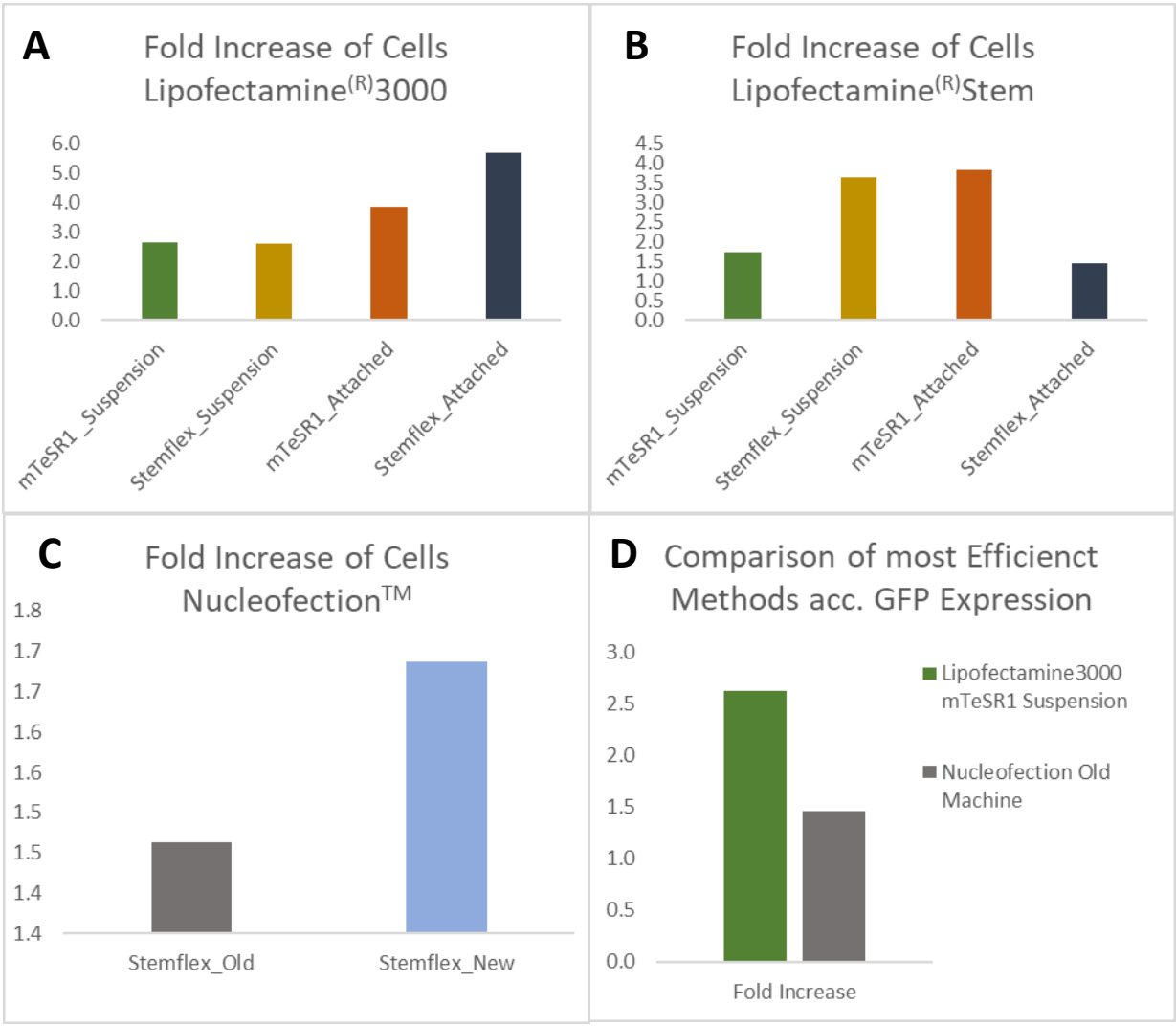


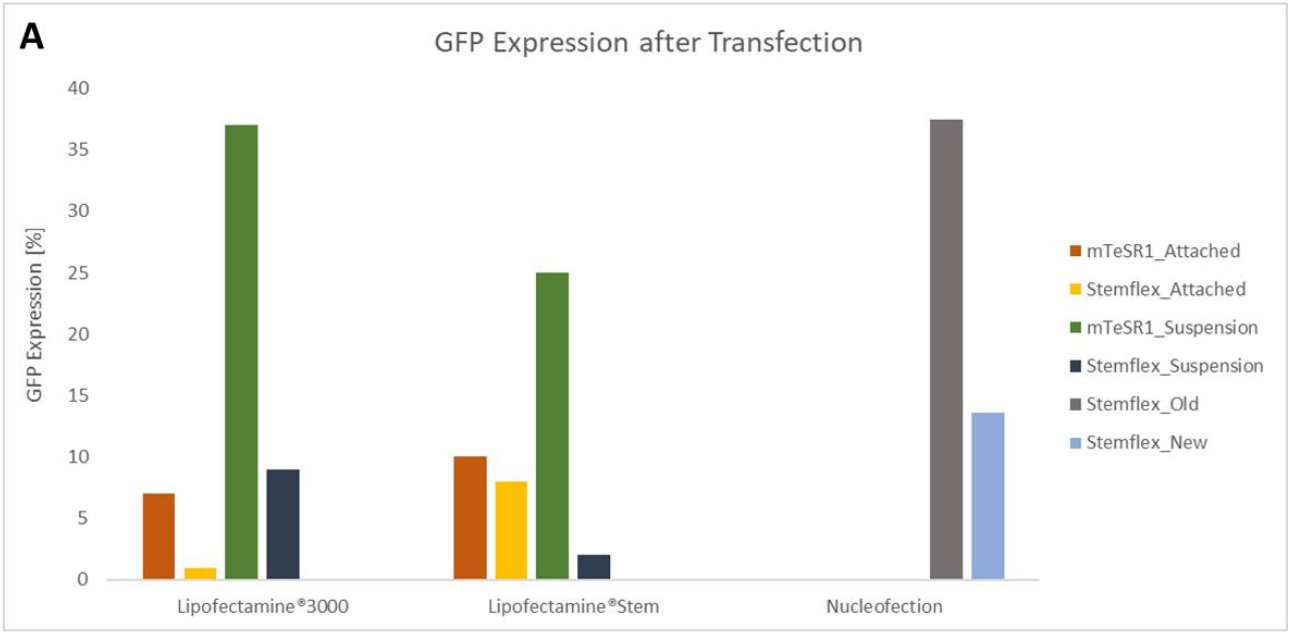
Figure 3: **Cell Viability Post-Transfection**

(A) Shows the fold increase of cells post-transfection with Lipofectamine®3000. Cells treated in suspension show less increase compared to attached ones. (B) Shows the fold increase of cells post-transfection with Lipofectamine®Stem. (C) Shows the fold increase of cells post-transfection with Nucleofector™. Decreased cell viability with the older version of the machine can be seen. (D) Shows a comparison fold increase of cells grown on mTeSR™1 transfected with Lipofectamine®3000 and cells grown on Stemflex™ and transfected with older version of Nucleofector™.

3.1.2 Efficiency of Cas9 Plasmid Delivery

Figure 4 A shows the GFP expression post-transfection. Different transfection methods, as well as different culture media had been used. The efficiency of transfection varied with culture medium, transfection method and transfection protocol. For the transfection method Nucleofection™ only results for the culture medium of Stemflex® are available, due to the contamination of mTeSR™1 cultured cells. Overall it can be seen that mTeSR™1 has a higher efficiency in lipid-mediated transfection. Furthermore, the data shows clear differences in efficiency between Lipofectamine®3000 and Lipofectamine®Stem. Additionally, it can be identified that the older version of the Nucleofector™ yields higher efficiency than the newer version. The two transfection set-ups show comparable efficiency in transfection of Cas9 plasmid: Lipofectamine® 3000 in mTeSR™1 in suspension and the older version of the Nucleofector™ with an efficiency of 37 %. The data was obtained through FACS analysis.

Figure 4 B, shows how transfected cells with Lipofectamine® 3000 express GFP. The pictures illustrate that a high amount of cells does not express GFP. This requires further improvement of the method.



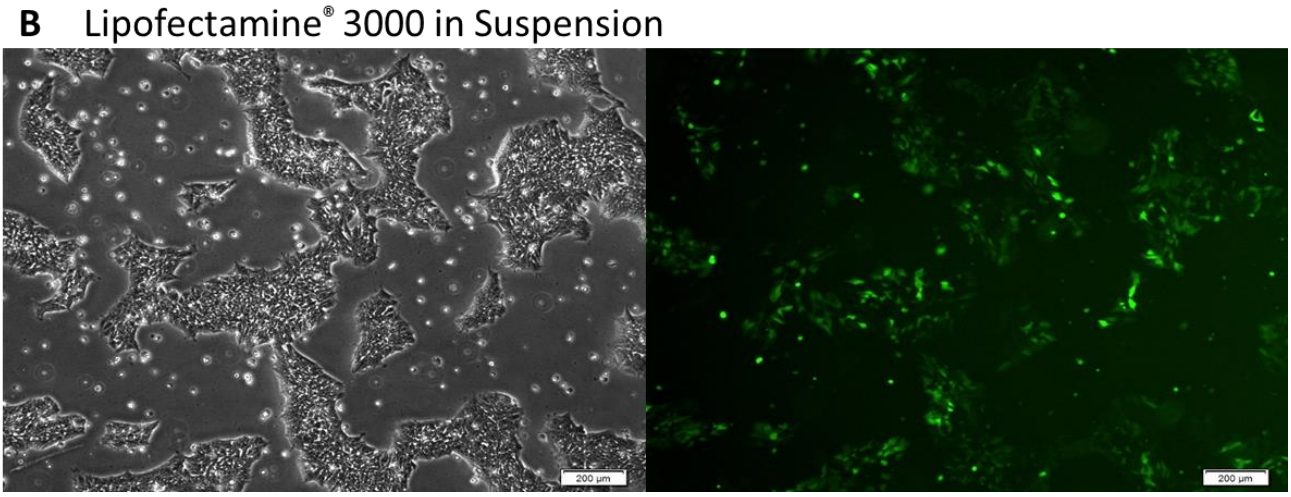


Figure 4: **GFP Expression Post-Transfection**

(A) Shows the GFP expression in % post transfection. Lipofectamine®3000 on mTeSR™1 in suspension and Nucleofector™ show the highest expression of GFP according to FACS analysis. (B) Brightfield and fluorescence pictures of cells growing in mTeSR®1 three days post-transfection with Lipofectamine®3000 according to laboratory protocol.

In consideration of cell viability and transfection efficiency one method was the preferred one for further plasmid delivery. With an efficiency of 37 % and a fold increase of more than 2.5, Lipofectamine®3000 on mTeSR™1 in suspension is the best method among the tested ones for human pluripotent stem cells.

3.2 Green Fluorescence Protein expressing HPSC

For the development of the GFP expressing HPSC, pre-designed TALEN pairs and EGFP plasmid were used. The designed TALEN pairs from Sanjana et al. had been used to target the AAVS1 locus on the PP1R12C gene (Sanjana et al., 2012).

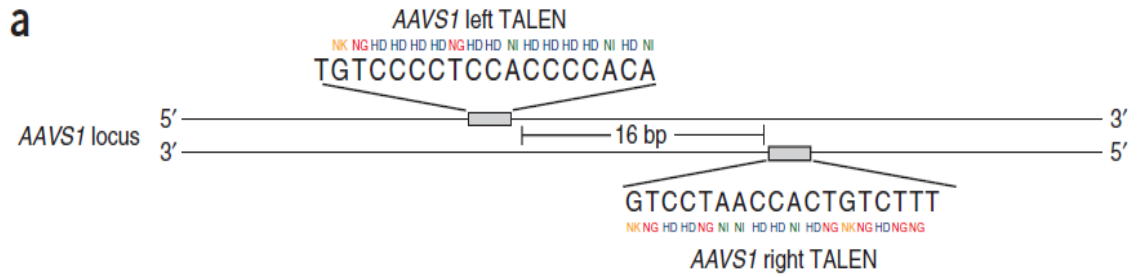


Figure 5: **Targeting the AAVS1 locus on Human Chromosome 19**

Designed TALEN pairs target the AAVS1 locus. Binding sites flank a 16 bp spacer and each recognition site begins with a T. Each TALE DNA-binding site is fused to the catalytic domain of FokI endonuclease. When FokI dimerizes, it cuts the DNA in the region between the left and right TALEN binding site. Modified after: (Sanjana et al., 2012)

The mentioned EGFP plasmid in Materials and Methods from Hockemeyer et al. was introduced into the resulting break of the HPSC line. The insertion of the plasmid lead to the following arrangement on Chromosome 19 PPP1R12C gene. The plasmid is inserted into intron1:

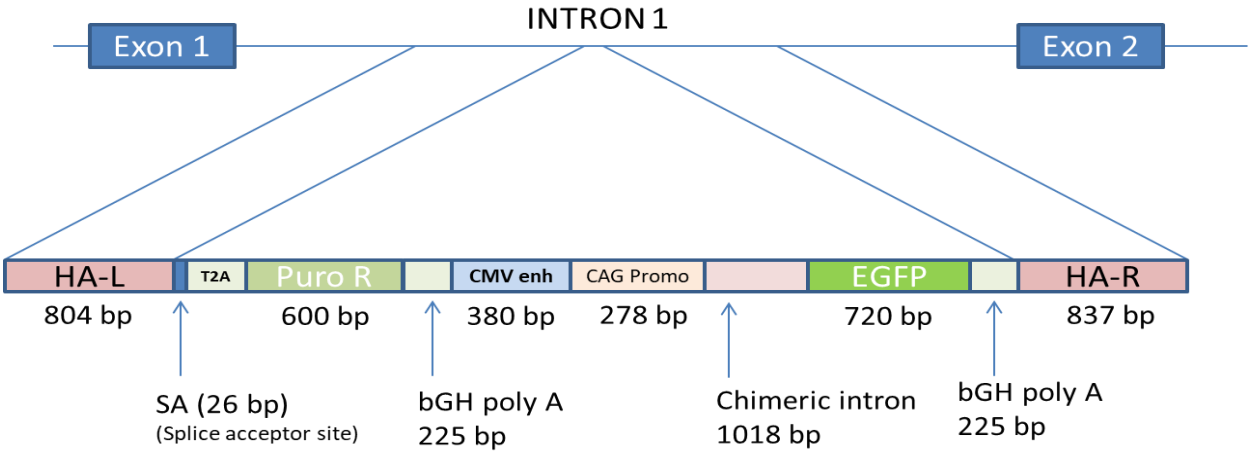


Figure 6: **Insertion of EGFP plasmid into Human AAVS1 locus**

The inserted EGFP plasmid from Hockemeyer et al. is introduced into intron 1 of the PPP1R12C gene on human chromosome 19. The insertion includes a puromycin resistance gene which allows treatment with puromycin. Furthermore, the plasmid has a CAG promoter and a EGFP gene.

HPSC were transfected according to Lipofectamine®3000 (Thermo Fisher Scientific) for TALEN. After recovery, the cells were plated at low density in 10 cm^2 culturing dishes and treated with 0.5 µg/µl Puromycin (Invivogene) for 4 days. After selection, clones were picked and expanded. After expansion, the DNA was extracted and selected clones were expanded and frozen.

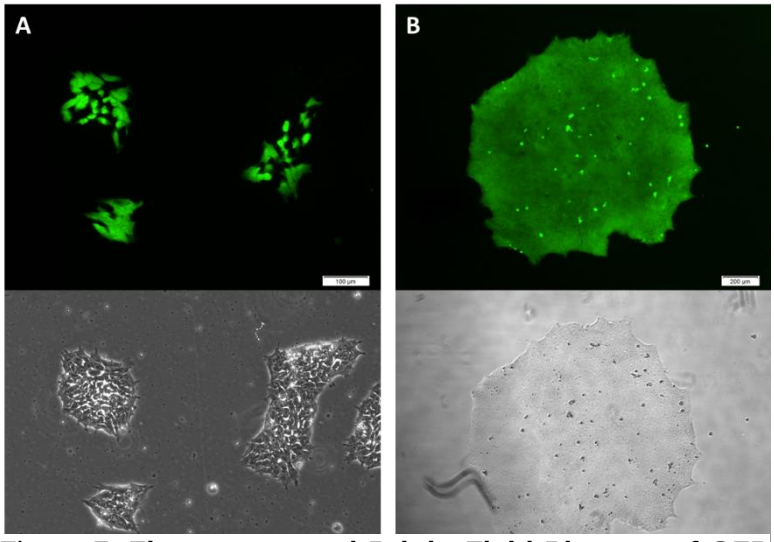


Figure 7: **Fluorescent and Bright Field Pictures of GFP expressing HPSC**

(A) Shows cells after puromycin selection. Non-GFP expressing cells can still be found within some colonies. (B) Expansion of a picked clone, which expresses GFP.

To confirm the right insertion of the EGFP plasmid; several approaches were conducted. The following parameters were tested with PCR: Intact or disrupted AAVS1 locus, 3' arm of plasmid, 5' arm of plasmid, random integration of plasmid. The following parameter was tested with q-PCR: EGFP amplification with SybrGreen.

Two different clones were expanded, one was a homozygous GFP HPSC clone and the other a heterozygous GFP HPSC clone.

Furthermore, one HPSC line from the Berlin Institute of Health with a heterozygous GFP, was used to conduct some of the experiment. The cell line was obtained with the same methods as described and was confirmed to have a normal karyotype. Furthermore, the cell line was confirmed as pluripotent through ICC staining and qPCR for pluripotency markers.

3.2.1 Homozygous HPSC GFP Line

Cells were tested with PCR for the right integration of the EGFP plasmid. The clone HUES8 GFP002 was expanded for the homozygous HPSC GFP line. The clone did not have an intact AAVS1 locus, which together with the qPCR SybrGreen results, lead to the assumption of a homozygous EGFP insertion. Furthermore, the right insertion of the plasmid was confirmed by evaluating the 3' arm and 5' arm. Additionally, there was a low risk of a random integration of the plasmid into the genome verified by PCR.

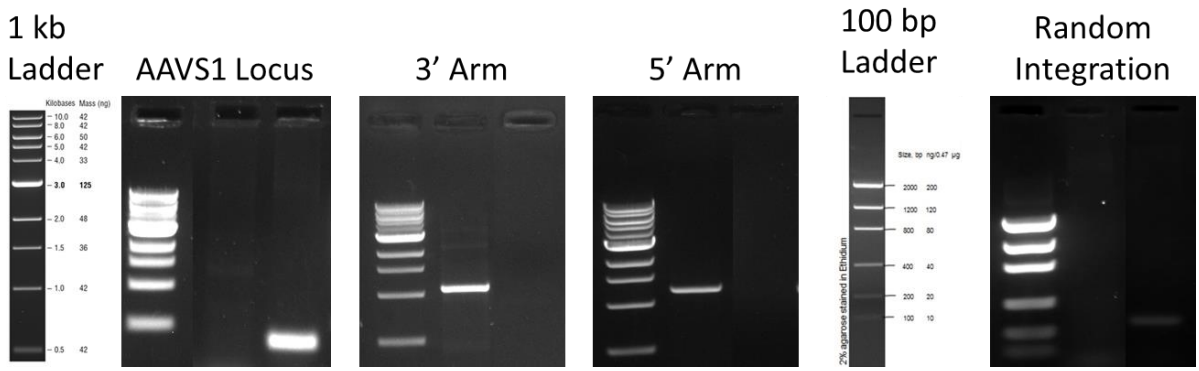


Figure 8: **Gel Electrophoresis results of PCR of different regions in homozygous clone**

The first figure shows 1 kB Ladder (New England BioLabs). The first gel shows the amplification of the AAVS1 locus. First column shows the 1 kb ladder, the second shows the AAVS1 amplification of the GFP clone and the last one shows the amplification of a non-edited HPSC. It can be seen, that no intact AAVS1 locus exists. The second gel shows the amplification of the 3' arm, which includes the 1 kb ladder. The second column shows the 3' arm amplification of the GFP clone and the last one shows the amplification of a non-edited HPSC. The same applies for the third gel referring to the 5' arm. The fifth figure shows a Low DNA Mass Ladder (Thermo Fisher). The last figure shows the random integration of the EGFP plasmid including the 100 bp ladder. The second column shows the amplification in the GFP clone. The third column shows the amplification of the EGFP plasmid as a positive control.

Furthermore, the amplification of a part of the GFP gene by SybrGreen qPCR was conducted to obtain insight into the expression levels. For this purpose, the ΔCt of the gene TDP-43 and GFP were used. With the obtained data, it was possible to exclude clones with too high or too low expression levels. In Table 25 all AAVS1 negative clones are compared and according to the ΔCt value, clone number GFP002 was picked for expansion. Clones with a ΔCt lower than three and higher than four were excluded, as well as clones which did not have the right insertion of the plasmid or had a random integration of the EGFP plasmid.

Several clones were acceptable for clonal expansion, according to the table. However, clone 002 was chosen according to morphology and GFP expression level, evaluated by eyesight.

Table 25: Homozygous clones in comparison after evaluation of defined parameters.

Sample Name	GFP	3' Arm	5' Arm	Random Integration	AAVS1	qPCR	Use
Optimal	**pos**	**pos**	**pos**	**neg**	**neg**		
HUES8 GFP001	pos	pos	pos	neg	neg	3.6	**Yes**
HUES8 GFP002	pos	pos	pos	neg	neg	3.15	**Yes**
HUES8 GFP003	pos	pos	pos	neg	neg	3.47	**Yes**
HUES8 GFP004	pos	pos	pos	neg	neg	3.68	**Yes**
HUES8 GFP005	pos	pos	pos	neg	neg	3.37	**Yes**
HUES8 GFP006	pos	pos	pos	neg	neg	3.7	**Yes**
HUES8 GFP007	pos	pos	pos	pos	neg	7.19	**No**
HUES8 GFP008	pos	pos	pos	pos	neg	3.33	**Yes**
HUES8 GFP009	pos	neg	pos	pos	neg	2.18	**No**
HUES8 GFP010	pos	pos	pos	neg	neg	2.88	**No**
HUES8 GFP011	pos	pos	pos	pos	neg	1.28	**No**

After clonal expansion, the cell line was sent for karyotyping to confirm that no chromosomal abnormalities in the genome occurred. The karyotype was confirmed as normal.

3.2.2 Heterozygous HPSC GFP Cell Line

For the heterozygous HUES8 GFP cell line, cells were tested with the same method, except for the SybrGreen qPCR. According to the results one clone F was expanded. Figure 9 shows the PCR and gel electrophoreses results of clone F. The figure shows a band at the AAVS1 locus, meaning that at least one allele was intact. Furthermore, the 3’ arm and the 5’ arm of the inserted plasmid were checked. Additionally, the clones were screened for a random integration of the EGFP plasmid into the genome.

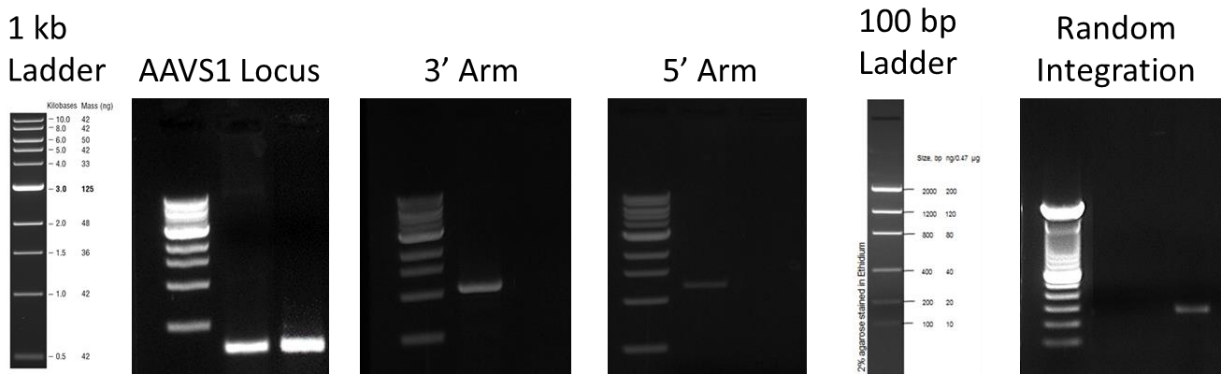


Figure 9: **Gel Electrophoresis results of PCR of different regions in heterozygous clone.**

The first figure shows 1 kB Ladder (New England BioLabs). The first gel shows the amplification of the AAVS1 locus. First column shows the 1 kb ladder, the second shows the AAVS1 amplification of the GFP clone and the last one shows the amplification of a non-edited HPSC. It can be seen, that an intact AAVS1 locus exists. The second gel shows the amplification of the 3' arm, which includes the 1 kb ladder. The second column shows the 3' arm amplification of the GFP clone and the last one shows the amplification of a non-edited HPSC. The same applies for the third gel referring to the 5' arm. The fifth figure shows a Low DNA Mass Ladder (Thermo Fisher). The last figure shows the random integration of the EGFP plasmid including the 100 bp ladder. The second column shows the amplification in the GFP clone. The third column shows the amplification of the EGFP plasmid as a positive control.

Table 26 summarizes the PCR and gel electrophoresis results of all clones with a positive AVVS1 locus. Clones with a negative locus were excluded and not further tested.

Additionally, ΔCt values were omitted as the results did not yield sufficient evidence in the homozygous clones.

Table 26: Heterozygous clones in comparison after evaluation of defined parameters.

Sample Name	GFP	3' Arm	5' Arm	Random Integration	AAVS1	Use
Optimal	**pos**	**pos**	**pos**	**neg**	**pos**	
HUES8 GFP A	pos	pos	neg	pos	**pos**	**No**
HUES8 GFP F	pos	pos	pos	neg	**pos**	**Yes**
HUES8 GFP I	pos	pos	pos	neg	**pos**	**Yes**
HUES8 GFP J	pos	pos	pos	pos	**pos**	**No**
HUES8 GFP K	pos	pos	pos	pos	**pos**	**No**
HUES8 GFP L	pos	neg	pos	neg	**pos**	**No**
HUES8 GFP O	pos	pos	pos	pos	**pos**	**No**
HUES8 GFP Q	pos	pos	pos	pos	**pos**	**No**
HUES8 GFP R	pos	pos	pos	neg	**pos**	**Yes**

HUES8 GFP U	pos	neg	pos	pos	**pos**	**No**
HUES8 GFP V	pos	neg	pos	pos	**pos**	**No**
HUES8 GFP W	pos	pos	pos	pos	**pos**	**No**

According to the evaluated parameters, three clones were acceptable for clonal expansion. However, clone F was chosen according to morphology and GFP expression level, evaluated by eyesight.
After clonal expansion, the cell line was sent for karyotyping to confirm that no abnormalities in the genome of the cells occurred. The karyotype was confirmed to be normal.

3.3 GFP to BFP

3.3.1 Cas9 delivered as Plasmid

All experiments, for which Cas9 was delivered as aplasmid, the German GFP HPSC line was used. For the first trials of the conversion GFP to BFP, the knock-out efficiency of the two gRNA was evaluated. After transfection with Lipofectamine®3000, the cells were kept in culture and analyzed with FACS analysis. The obtained data shows that gRNA 1 yields 8 % KO and gRNA2 yields 0.7 % KO. With this data, all further experiments were performed with gRNA1 only.

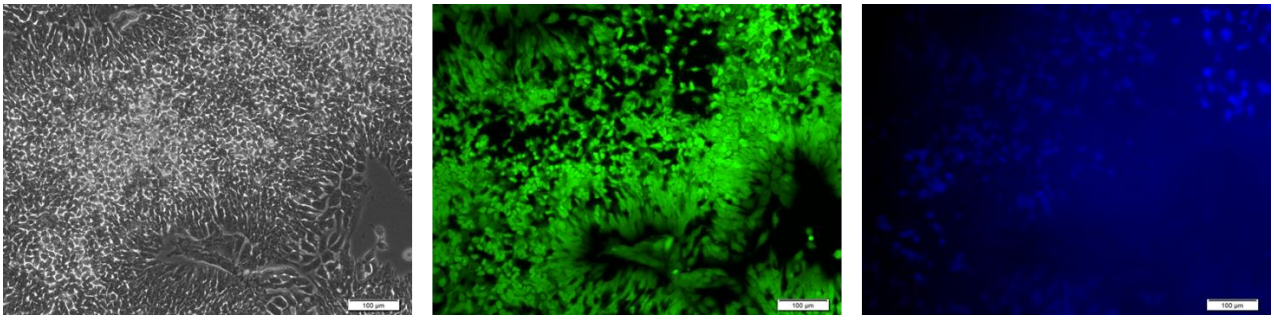


Figure 10: **Conversion of GFP to BFP with Cas9 delivered as Plasmid**

Pictures were taken of male German HPSC GFP expressing cell line 6 days post-transfection. First picture shows a brightfield photograph of the selected area. Picture two shows the area with a green fluorescence filter and picture three shows the same area with a DAPI filter.

The obtained data after FACS analysis shows that 14.1 % of the cells were repaired by non-homologous end joining (NHEJ), which resulted in a KO of GFP. 5.8 % were repaired with homology direct results (HDR), which results in a KI of BFP.

3.3.2 Cas9 delivered as Protein

As there were no reference values for the ration of Cas9 to gRNA, the first experiment was a KO of GFP to detect the optimal parameters. The first experiment was conducted with the Lipofectamine© CRISPRMAX system. In total four different ratios were tested to determine the highest efficiency. The following ratios of Cas9:gRNA1: 1:1, 1:3, 1:6, 1:9, which were applied to a cell number of 500 000. Furthermore, the manufacturer's protocol was compared with the protocol developed in laboratory. The experiment was conducted with the German GFP HPSC line (heterozygous). In Table 27 the manufacturer's protocol is referred to "Attached" and the lab protocol is referred to "Suspension".

A difference in transfection protocol can be seen in number of KO. Furthermore, it can be seen that higher Cas9:gRNA ratio often leads to higher NHEJ events, seen in % KO. An important fact to consider is the high cell death in higher Cas9:gRNA ratios. With this fact in mind, all further experiments were conducted with protocol in suspension and a ratio of Cas9:gRNA of 1:6.

Table 27: Results of FACS analysis of NHEJ in different ratios of Cas9:gRNA.

Attached/Suspension	Ration Cas9:gRNA	% GFP	% KO
Attached	1:3	91.1	8.9
Attached	1:6	88.5	11.5
Attached	1:9	85.5	14.5
Suspension	1:1	84.5	15.5
Suspension	1:3	72.3	27.7
Suspension	1:6	64.6	35.4
Suspension	1:9	67.0	33.0

To get more insight into the KO of GFP a TIDE analysis (Brinkman, Chen, Amendola, & van Steensel, 2014) of two timepoints has been conducted. For attached cells the timepoints were 48 hours and 6 days post-transfection. For suspension cells, the timepoints were 72 hours and 6 days post-transfection. The difference in timepoints occured due to bad recovery of cells in suspension. Overall the results indicate that after 48/72 hours still editing process occurring due to the higher percentage of KO.

After determining the best ratio of Cas9:gRNA, the most efficient concentration of repair ssODN had to be tested. Therefore, five different concentration were tested: 0.33 µg, 0.5 µg, 1 µg, 1.5 µg and 2 µg per transfection of 500 000 cells.

The main difference can be seen in KO events. Out of the attained data, the first conclusion that can be drawn, is that a higher concentration of ssODN does not lead to more HDR events. Overall, it can be said that a concentration of 0.5 µg obtains the best KO to KI to GFP ratio with 41.4 to 6.2 to 53.4 %.

Table 28: Results of FACS analysis of HDR and NHEJ with different repair template concentrations.

ssODN [µg]	KO [%]	KI [%]	GFP [%]
0.33	40.2	4.5	55.3
0.5	41.4	6.2	53.4
1	27.2	3.4	69.4
1.5	32.1	3.7	64.2
2	32.1	4.5	63.4

For further experiments GFP to BFP experiments with Cas9 as protein the following concentrations for 500 000 cells were used:

- Cas9:gRNA 1:6
- ssODN 0.5 µg

3.3.2.1 Lipofectamine© CRISPRMAX

For the GFP to BFP conversion with Lipofectamine© CRISPRMAX three different HPSC lines were tested. Two heterozygous cell lines and one homozygous cell line were transfected according to the developed parameters:

- BiHI001A2 (heterozygous)
- HUES8 GFP F (heterozygous)
- HUES8 GFP002 (homozygous)

In HUES8 GFP002 the conversion of GFP to BFP was repeated 3 times, in BiHI001A2 was done twice and for HUES8 GFP F, the experiment was conducted once.

The results of this experiment are summarized in Figure 12.

3.3.2.2 Neon®

For the transfection with Neon® two different machine settings were used. The first parts of the testing were conducted with the German heterozygous cell line. Per reaction 100 000 cells were transfected. Additionally, two different media were compared for recovery.

Overall, condition two yields higher KI efficiency and just a minimal amount of GFP positive cells can be detected. Furthermore, the cell viability in Stemflex post-transfection is higher than in mTeSR1. A graphical summary of the transfection and targeting efficiency can be seen in Figure 11 B.

Table 29: FACS analysis of GFP to BFP post-transfection with Cas9 Protein transfected with Neon®

Media	Cell Number/Transfection	Setting	BFP [%]	KO [%]	GFP [%]
mTeSR1	100 000	1100V/20ms/1pulse	20.8	68.3	10.6
	100 000	1200V/30ms/1pulse	53.1	42.7	2.7
Stemflex	100 000	1100V/20ms/1pulse	4	57.5	37.9
	100 000	1200V/30ms/1pulse	35.2	59.5	4.3

The highest transfection efficiency can be yielded with a parameter setting of 1200V, 30 ms and 1 pulse. A slightly higher BFP percentage can be detected in cells grown on mTeSR1 with 53.1 % KI.

For the following experiments the parameters were set to 1200 V, 30 ms and 1 pulse. Cells were grown on Stemflex™, to increase cell survival.

Figure 11 A, shows how GFP and BFP cells can easily be disiguished by eyevision. Furthermore, it clearly shows the high conversion ratio of GFP to BFP.

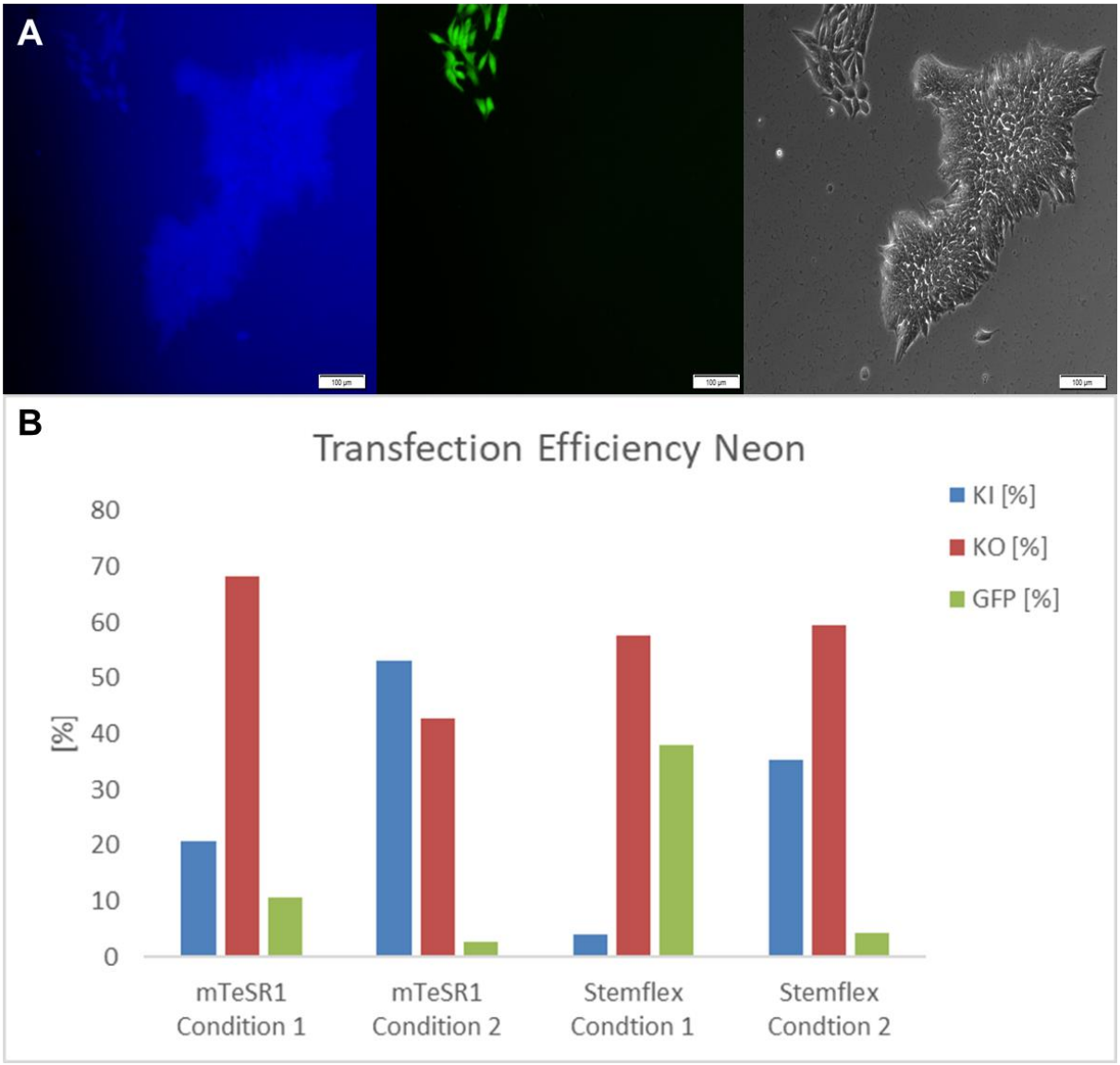


Figure 11: **Transfection Efficiency Neon®**

(A) Shows fluorescence and brightfield pictures of cells on mTeSR1 and condition 2. (B) Transfection efficiency of Neon® represented graphically. Highest % of KI can be seen in mTeSR1transfected with condition 2.

3.3.3 Summary GFP to BFP Efficiency of Cas9 as Protein

The conversion of GFP to BFP with Cas9 protein, was conducted in three different cell lines: HUES8 GFP002 (homozygous), HUES8 GFP F (heterozygous) and BIHi001 (heterozygous). For HUES8 GFP002 three repetitions were conducted, which shows high deviations. For HUES8 GFP F only one experiment was conducted due to time limitations. For the cell line BIHi001 two repetitions were accomplished. After transfection with Neon®, cells were grown on Stemflex™. Cells with the transfection with Lipofectamine© CRISPRMAX were grown on mTeSR1.

Figure 12 shows a graphical summary of the GFP to BFP conversion. For the cell line of HUES8 GFP002 it was possible to convert GFP to BFP. The results show, that the applied transfection method has an impact on the efficiency of conversion. The standard deviation clearly shows a variance in expression level. For Neon®, the GFP expression post transfection is 55.7 %, the KO is 24.9 % and the BFP expression is at 18.6 %. GFP/BFP expression is at around 1 %. For Lipofectamine© CRISPRMAX the GFP expression is at 68.2 %, the KO is like Neon® with 25.5 %. The BFP expression levels are at around 5 %. GFP/BFP expression is slightly higher with 2 %.

The cell line HUES8 GFP F is a heterozygous GFP expressing cell line, which allows only to be GFP or BFP. In this cell line high differences between Neon® and Lipofectamine© CRISPRMAX can be spotted. In Neon® the GFP expression post transfection is 6.8 %, the KO is 87.2 % and the BFP expression is at 6 %. For Lipofectamine© CRISPRMAX the GFP expression is at 33 %, the KO accounts for 53.3 %. The BFP expression levels are at around 13.4 %.

The cell line BIHi001 is again a heterozygous cell line. It is distinguished from the previous one by being an induced pluripotent stem cell line. In this cell line slightly less, differences can be seen in between the methods. Still they do not show the same distribution. In Neon® the GFP expression at the day of evaluation is 19 %, the KO is 59.7 % and the BFP expression is at 20.7 %. For Lipofectamine© CRISPRMAX the GFP expression is at 42.5 %, the KO accounts for 29.6 %. The BFP expression levels are at around 27.2 %.

The errors bars indicate the standard deviation and show a high variance of the results.

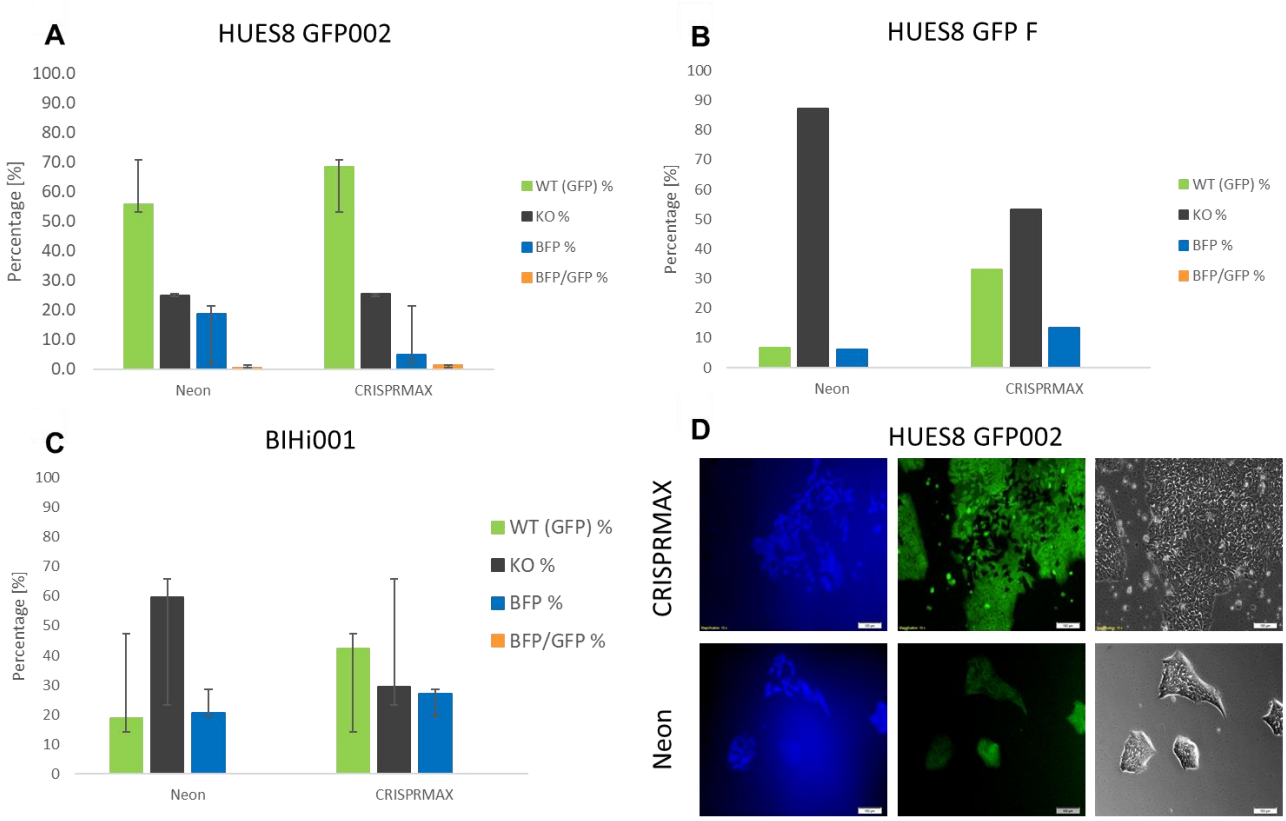


Figure 12: **Summary of GFP to BFP conversion in different cell lines**

(A) shows the expression of GFP, BFP, GFP/BFP and KO analyzed with FACS in HUES8 GFP002. Two different methods have been applied: Lipofectamine© CRISPRMAX and Neon®. Due to the homozygosity of the cell line a GFP and BFP expression is possible. A high standard deviation is noticeable. (B) Similar graph for the cell line HUES8 GFP F. No standard deviation is available. Expression levels vary with method. (C) Similar graph for the cell line BIHi001. The graph shows high a standard deviation. Furthermore, the methods show a difference in results.

3.4 Differentiation into Motor Neurons

For the differentiation of embryonic stem cells into motor neurons, two cell lines were used. The first cell line was HUES3 HB9 GFP, which was tagged with a GFP when expressing the homeobox gene Hb9. The second cell line was HUES8.

After the differentiation process, cells were stained with Islet 1 & 2 (green), which is a transcription factor found in the nucleus. Due to time restrictions, no further primary AB was tested, as the primary AB was too unspecific. Furthermore, they were stained for DAPI (blue) and Beta III Tubulin (red).

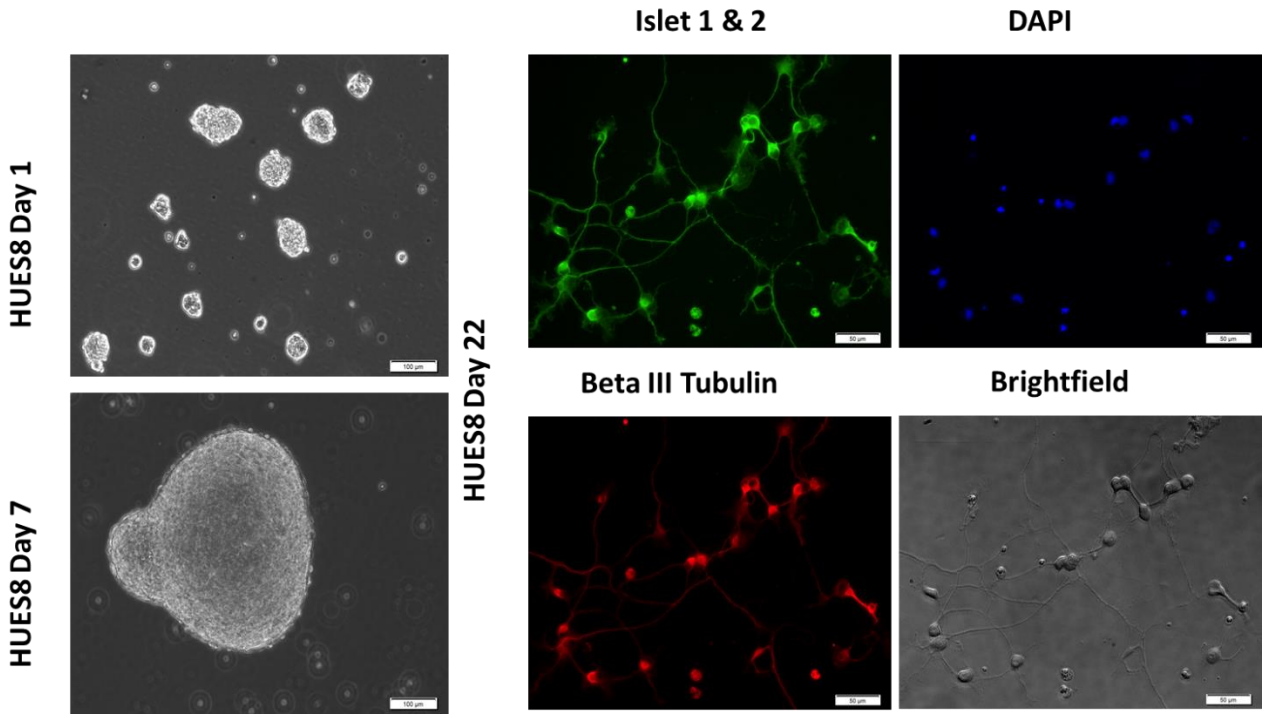


Figure 13: **Motor Neurons on Day 1, Day7 and Day 22**

The picture shows HUES8 on day 1 of motor neuron differentiation forming small embryo body-like structures. On day 7 the bodies are merging and have increased enormously in size. On day 22 the differentiation is completed and cells were stained for Islet 1 and 2, DAPI and Beta III Tubulin.

4 Discussion

4.1 Transfection Efficiency of Cas9 Plasmid

Out of the data shown in Figure 4, it is clear that Stemflex™ has a negative impact on the lipid-mediated transfection methods. Furthermore, a trend of decreasing cell viability with increasing transfection efficiency could be concluded.

Unfortunately, samples on mTeSR™1 transfected with Nucleofector™ were contaminated, which illustrates the high risk of this method. To transfect cells with Nucleofector™, cells have to be transferred into a special cuvette, which then leaves the biosafety hood and thus leads to an increased risk of contamination.

The lower cell viability with the Nucleorfator™, shows that electroporation causes higher stress levels in the cell compared to lipid-mediated transfection.

Unfortunately, these efficiency values just represent the number of cells, which successfully expressed the plasmid. They do not give any conclusion of how many cells will be effectively targeted.

4.2 Green Fluorescence Expressing HPSC

The generation of the GFP expressing HPSC line had to be conducted twice, as the first set-up yielded only homozygous cells. For the second time, more clones were picked to obtain heterozygous GFP cells. One of the main problems that could have occurred during the picking process of colonies, was that only bright GFP clones could have been picked, which could have lead to only homozygous ones.

The GFP SybrGreen qPCR did not yield sufficient evidence to conclude for homozygosity or heterozygosity. The designed primers were too unspecific, as well as the SybrGreen method. To obtain a strongerconclusion, it would be advisable to use a Taqman qPCR system, as it yields in some cases more specific results. Furthermore, a new primer pair should be designed as well.

For further clarification of the homozygous or heterozygous nature of the cell lines, a southern blot would have given more advantageous results. Unfortunately, it was not possible to conduct one, because of the radioactive probe.

There may be the question why a homozygous and heterozygous cell line is needed. One of the reasons is that in genome editing often disease models are created in lab,

with the specific requirement of just editing one allele. So far it is not really known in which ratio of modified versus wildtype, alleles occur. Therefore, it is helpful to have a homozygous GFP cell line. The purpose to develop a heterozygous cell line is mainly for the fact to repair heterozygous mutations and to obtain results for the efficiency of these events.

4.3 GFP to BFP

Unfortunately, it was not possible to repeat all experiments at least three times to attain a more significant result. To get more reliable results, the project will be carried on.

The project was started with Cas9 delivered as plasmid. Due to the low efficiency of this system, the experiment was carried on with Cas9 as a protein.

Several reasons exist why transfection as Cas9 plasmid yields lower results. Firstly, the process of expressing Cas9 and gRNA takes some time and it can also be eluted from the cell, before expression is possible. Furthermore, Cas9 as plasmid is bigger, which means that transporting the plasmid through the cell membrane takes more effort.

However, the delivery of Cas9 as a plasmid brings one crucial advantage where the plasmid can be tagged with an expression of a fluorescent protein FACS, can be applied. This enables the process of colony picking to be more efficient.

Furthermore, it is shown that cell cycle timing has an influence on the HDR efficiency in CRISPR/Cas9 systems. This would explain the variance within the same cell line (Lin, Staahl, Alla, & Doudna, 2014). Nevertheless, this phenomenon concerns both expression systems.

As the lab group had no experience with Cas9 delivered as a protein, the whole experiment set up had to be planned. Furthermore, the Neon® system was not familiar to the group and training was required.

During the test runs of Cas9 protein, different effects were noticed. Firstly, an increased concentration of gRNA lead to increased toxicity. One surprising result was that the concentration of repair ssODN does not have a high impact on HDR efficiency.

Additionally, the method of transfection has a high impact on cell survival. Cells transfected with Neon® showed, that the permeabilization of the cell wall has an influence on cell survival.

The food processing method of electroporation for sterilization illustrates the toxic effects of electric fields (Saulis, 2010).

It could be interpreted, that the condition and confluency of cells pre- transfection have an impact on the conversion efficiency. Unfortunately, this was never monitored.

The cell line of HUES8 GFP002 probably offers the most real-life scenario by representing a homozygous cell line. Due to the high variance from experiment to experiment it is difficult to draw a significant conclusion.

But, the results already show, that it is difficult and a major challenge to just target one allele. This experiment shows, that the technology of CRISPR/Cas9 needs further improvements. If this problem can be overcome, another big step for gene therapy will be made.

The overall conclusion is, that Cas9 delivered as a protein yields higher efficiency and faster read outs as plasmids. Due to the higher targeting events, less clones need to be screened. This simplifies the process, which results in cost and time saving. Different studies came to the same conclusion, as Kouranova et al. (2016).

For the decision of which transfection method to use, it must be clarified if one or both alleles (GFP/BFP or BFP only) should be targeted.

Aspects to increase GFP/BFP

Several groups worked on the goal to just target one allele and came up with several approaches:

2 Design of gRNA closer/further away from DSB (Paquet et al., 2016)

3 Add two ssODN for Repair (Paquet et al., 2016)

4 Asymmetric ssODNs enhance HDR (Richardson, Ray, DeWitt, Curie, & Corn, 2016)

5 List of References

Brinkman, E. K., Chen, T., Amendola, M., & van Steensel, B. (2014). Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic Acids Res, 42*(22), e168. doi:10.1093/nar/gku936

Carroll, D. (2011). Genome engineering with zinc-finger nucleases. *Genetics, 188*(4), 773-782. doi:10.1534/genetics.111.131433

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., . . . Voytas, D. F. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics, 186*(2), 757-761. doi:10.1534/genetics.110.120717

Elcheva, I., Brok-Volchanskaya, V., Kumar, A., Liu, P., Lee, J. H., Tong, L., . . . Slukvin, I. (2014). Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. *Nat Commun, 5*, 4372. doi:10.1038/ncomms5372

Glaser, A., McColl, B., & Vadolas, J. (2016). GFP to BFP Conversion: A Versatile Assay for the Quantification of CRISPR/Cas9-mediated Genome Editing. *Mol Ther Nucleic Acids, 5*(7), e334. doi:10.1038/mtna.2016.48

Hockemeyer, D., Soldner, F., Beard, C., Gao, Q., Mitalipova, M., DeKelver, R. C., . . . Jaenisch, R. (2009). Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol, 27*(9), 851-857. doi:10.1038/nbt.1562

Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell, 157*(6), 1262-1278. doi:10.1016/j.cell.2014.05.010

Kim, Y. G., & Chandrasegaran, S. (1994). Chimeric restriction endonuclease. *Proc Natl Acad Sci U S A, 91*(3), 883-887.

Kouranova, E., Forbes, K., Zhao, G., Warren, J., Bartels, A., Wu, Y., & Cui, X. (2016). CRISPRs for Optimal Targeting: Delivery of CRISPR Components as DNA, RNA, and Protein into Cultured Cells and Single-Cell Embryos. *Hum Gene Ther, 27*(6), 464-475. doi:10.1089/hum.2016.009

Lin, S., Staahl, B. T., Alla, R. K., & Doudna, J. A. (2014). Enhanced homology-directed human genome engineering by controlled timing of CRISPR/Cas9 delivery. *Elife, 3*, e04766. doi:10.7554/eLife.04766

Maepa, M. B., Roelofse, I., Ely, A., & Arbuthnot, P. (2015). Progress and Prospects of Anti-HBV Gene Therapy Development. *Int J Mol Sci, 16*(8), 17589-17610. doi:10.3390/ijms160817589

Malik, N., & Rao, M. S. (2013). A review of the methods for human iPSC derivation. *Methods Mol Biol, 997*, 23-33. doi:10.1007/978-1-62703-348-0_3

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., & Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *J Mol Evol, 60*(2), 174-182. doi:10.1007/s00239-004-0046-3

Nemudryi, A. A., Valetdinova, K. R., Medvedev, S. P., & Zakian, S. M. (2014). TALEN and CRISPR/Cas Genome Editing Systems: Tools of Discovery. *Acta Naturae, 6*(3), 19-40.

Paquet, D., Kwart, D., Chen, A., Sproul, A., Jacob, S., Teo, S., . . . Tessier-Lavigne, M. (2016). Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. *Nature, 533*, 125. doi:10.1038/nature17664
https://www.nature.com/articles/nature17664#supplementary-information

Perez-Pinera, P., Kocak, D. D., Vockley, C. M., Adler, A. F., Kabadi, A. M., Polstein, L. R., . . . Gersbach, C. A. (2013). RNA-guided gene activation by CRISPR-Cas9-based transcription factors. *Nat Methods, 10*(10), 973-976. doi:10.1038/nmeth.2600

Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L., & Corn, J. E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat Biotechnol, 34*(3), 339-344. doi:10.1038/nbt.3481

Sanjana, N. E., Cong, L., Zhou, Y., Cunniff, M. M., Feng, G., & Zhang, F. (2012). A transcription activator-like effector toolbox for genome engineering. *Nat Protoc, 7*(1), 171-192. doi:10.1038/nprot.2011.431

Saulis, G. (2010). Electroporation of Cell Membranes: The Fundamental Effects of Pulsed Electric Fields in Food Processing. *Food Engineering Reviews, 2*(2), 52-73. doi:10.1007/s12393-010-9023-3

Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., & Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. *Science, 351*(6268), 84-88. doi:10.1126/science.aad5227

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*(4), 663-676. doi:10.1016/j.cell.2006.07.024

Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., & Jones, J. M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science, 282*(5391), 1145-1147.