CRISPR/Cas9 gene-editing in an *in vitro* model of a familial form of ALS

Student: Fredrik Nilsson
Supervisor: Olof Torper
Examiner: Johan Bonde Svensson
2018-06-18

Abstract

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disease characterized by the degeneration and death of both upper and lower motor neurons for which there is no cure currently available. The degeneration of motor neurons leads to the inability to carry out voluntary movement and death usually occurs 3-5 years after disease onset by respiratory failure. The most common cause of familial ALS is associated with mutations in the superoxide dismutase gene (SOD1). The SOD1 gene codes for an enzyme that is ubiquitously expressed and is involved in neutralizing reactive oxygen species. Mutations in the SOD1 gene lead to a toxic gain-of-function and astrocytes with mutated SOD1 has been shown to kill motor neurons by releasing toxic factors. In this project the gene-editing tool CRISPR/Cas9 was used to knock down SOD1 in forebrain neural progenitor cells (FB NPCs) using single guide RNAs (sgRNAs) to target the SOD1 gene specifically. The embryonic stem cell line RC17 and the induced pluripotent and patient-specific stem cell line $SOD1^{L144P}$ were differentiated to forebrain neural progenitor cells (FB NPCs) using a 16-day protocol. Lentivirus vectors were used to deliver the Cas9 protein, a sgRNA targeting either exon 2 (sgRNA:E2) or exon 3 (sgRNA:E3) and the green-fluorescent protein (GFP) as a reporter gene into the cells. The success of the differentiation was validated based on immunocytochemistry staining for the expression of FOXG1 and nestin, and the transduction on the presence of GFP. Staining for SOD1 was carried out to study the SOD1 knock-down on a protein level. The knock-down of SOD1 was also analysed based on mRNA expression using qPCR with a SOD1-specific primer. The results from the qPCR showed that the cells transduced with sgRNA:E2 and sgRNA:E3 had a reduced expression of SOD1 in both cell lines. In the case of sgRNA:E3 the reduction was more than four-fold in both cell lines.

Preface

This master thesis project was carried out between 2018-01-15 and 2018-06-18 at the biomedical center in Lund, at the department of developmental and regenerative medicine, in Malin Parmar's lab. The ambition with the project was to develop an *in vitro* model for screening for effective sgRNAs in a neural stem cell system and to use CRISPR/Cas9 to disrupt a gene associated with a familial form of amyotrophic lateral sclerosis.

I would like to thank my supervisor Olof Torper for guiding me through this project and always offering a helping hand whenever needed. I would also like to thank Professor Malin Parmar and everybody in the Malin Parmar lab for all the help and inspiration I have received throughout my master thesis project.

Table of Contents

Aims of the project

The aims of the project were divided into three parts:

1. Design two sgRNAs specific for the SOD1 gene.
2. Differentiate the RC17 and patient-specific SOD1 stem cell lines to forebrain neural progenitor cells (FB NPCs).
3. Test both sgRNAs in both cell lines and validate knock-down on an mRNA and protein level.

Background

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neuromuscular disease characterized by the death and degeneration of both upper and lower motor neurons. The function of motor neurons is to convey the signals from the brain and spinal cord to the muscles and control their movements. When motor neurons die, signals from the brain to initiate voluntary movement are lost, which leads to paralysis. Upper motor neurons relay signals between the brain and spinal cord and lower motor neurons relay the signals between the spinal cord and muscles (Hardiman et al., 2017). Extending from the neuronal cell body are axons and dendrites, collectively called neurites. The axon conducts nerve impulses over large distances and function as wires, carrying the information further within the nervous system. Usually, only one axon extends from the neuronal cell body. Surrounding the axons are layers of myelin, a lipid-rich substance that insulates it and speeds up the electrical impulses (Stryer et al., 2012). The dendrites are shorter but plentiful, working as antennas, receiving incoming signals from neighbouring axons. The information is transferred between neurons and ultimately to muscles at the synapse. The synapse is a structure where the cells come in close proximately and allows the information to be passed on, using either ions or neurotransmitters in electrical- and chemical synapses, respectively. Most commonly, neurotransmitters, such as glutamate, gamma-aminobutyric acid (GABA), acetylcholine and dopamine are used at the synapse. These are then converted to electrical signals that travel down the neurons to the next synapse where they are converted back to a chemical one, and so on until it reaches the muscles. Finally, motor neurons at the neuromuscular junction will release acetylcholine, causing the muscle fibres to contract (Bear et al., 2016). Independent of the underlying mechanism of ALS, the end result is that the motor neuron is unable to maintain its axonal projections which leads to axonal retraction and ultimately the denervation of the target cell. The signals and information cannot be transferred further, and the muscles will not be able to contract and perform voluntary movements (Hardiman et al., 2017).

ALS is the most common motor neuron disease and affects around 3 people per 100 000 per year. Most symptoms start to develop between the ages of 55 and 75 (Learn About ALS, ALS Foundation). The primary symptoms of ALS are muscle weakness, muscle atrophy, muscle cramps, spasticity, dysphagia (difficulty swallowing) and dysartheria (difficulty speaking). Furthermore, about half of the people suffering from ALS also develop cognitive and/or behavioural impairment. There is currently no cure for ALS and death usually occurs 3-5 years after disease onset by respiratory failure (Armon et al., 2017). Moreover, there is no

definitive diagnostics test which makes it sometimes difficult to distinguish ALS from other motor neuron diseases, and most drugs on the market target the individual symptoms instead the disease as a whole (Hardiman et al., 2017).

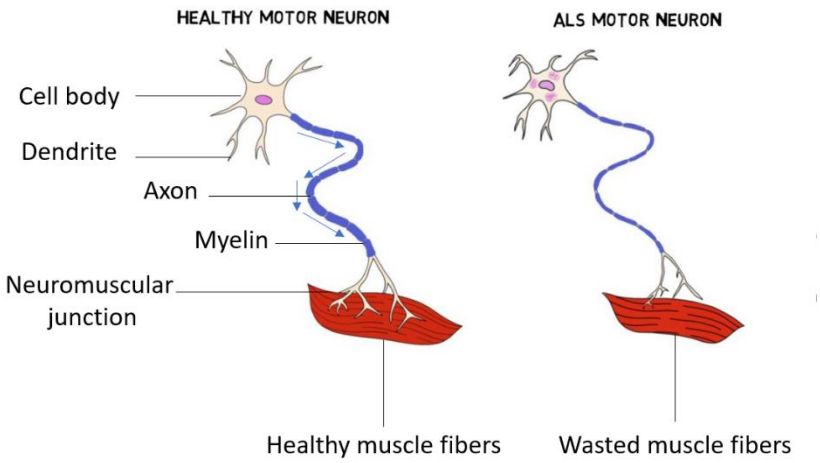

Figure 1. A healthy motor neuron passing information via its axon down to the neuromuscular junction, causing the muscle to contract. In ALS the motor neurons degenerate, leading to atrophic and wasted muscle fibres.

The Involvement of SOD1 in ALS

As with many diseases, the causes of ALS can be familial or sporadic. The familial cases are associated with a genetic predisposition to develop the disease, whereas it is absent in the sporadic ones. Approximately 10% of all the ALS cases are familial where the disease is caused by inheritable factors that are passed down from generation to generation. The remaining 90% of cases are sporadic and are not linked to distinct inheritable factors (Bosco, 2015). The most common form of familial ALS (fALS), accounting for about 20% of all cases is provoked by mutations in the superoxide dismutase (SOD1) gene (Rosen et al.,1993). Superoxide dismutase is an enzyme that is involved in neutralizing the harmful reactive oxygen species superoxide anion ($O_2^{-}\cdot$). It catalyses the breakdown of superoxide anion to oxygen (O_2) and hydrogen peroxide (H_2O_2). There are numerous mutations in the SOD1 gene that are associated with fALS, some of which impede the function and activity of the enzyme, while others do not (Borchelt et al., 1994). In either case, they lead to a toxic gain-of-function, where it seems as if these mutations turn this protein from a protective one into a cytotoxic one. Knock-out mice lacking the SOD1 gene have shown no symptoms of ALS (Reaume et al., 1996). The mechanisms behind ALS are not fully understood but they seem to involve toxic protein aggregates (Ross et al., 2005).

The fact that mutations in SOD1 is associated with fALS has been known for many years and there have been several attempts to knock down the SOD1 mRNA in cells. For instance, viruses have been used to deliver small interference RNA (siRNAs) and short hairpin RNA that hybridizes with the SOD1 mRNA and gets degraded by the cell's own machinery and prevents protein expression (Thomsen et al., 2014) (Raoul et al., 2005). Although these attempts have shown to be able to delay disease onset, the time between disease onset and

death remains the same and their success have been quite modest. Also, they do not induce permanent changes. Thus, a gene therapy treatment that can make precise and permanent changes in the SOD1 gene on a DNA level is needed.

CRISPR/Cas9

CRISPR/Cas9 is a recently discovered genome editing tool that has revolutionized the genome engineering field and has quickly become the most popular approach to edit DNA. The reason for this excitement is that CRISPR is faster and cheaper to use than previous gene editing methods while also being more accurate and efficient. It is a system found naturally in bacteria as a means to destroy invading virus, which has been exploited in the gene editing field. In bacteria it functions as an adaptive immune system by cutting up viral DNA into short segments and inserting them into their own DNA. Upon next infection the bacterium will compare the new viral DNA with the segments saved from the previous infection. If there is a match the endonuclease protein Cas9 will make a blunt ended double stranded break and destroy the virus. (Gupta & Musunuru, 2014). CRISPR/Cas9 stands for Clustered Regularly Interspaced Short Palindromic Repeat/CRISPR-Associated protein and consists of two components. The first component is called a single guide RNA (sgRNA or gRNA) which is a segment of RNA composed of a scaffold sequence necessary for attachment to the Cas9 protein and a target sequence of 20 bases that determines where in the DNA the cut will be made. The target sequence is complementary to the locus of interest and will bind to it via Watson-Crick base-pairing, activating the Cas9 endonuclease. Therefore, by only changing the target sequence the Cas9 protein can be directed to make several cuts at desired loci in the genome. This opens up the possibility of multiplexing and edit multiple genes simultaneously by simple introducing multiple sgRNAs once (Hsu et al., 2014). This is a promising aspect in tackling polygenic diseases, such as diabetes, heart disease and autism. Upon designing the target sequence of 20 bp it is paramount that this sequence is only found in the gene of interest and not anywhere else in the genome. Otherwise cuts will be made at undesired loci, resulting in off-target activity. Ideally, the Cas9 will only cut at the target locus but in realty there is always some off-target activity, raising some concerns for using CRISPR/Cas9 in clinical trials and gene therapy.

The second component is the Cas9 protein, which is an enzyme capable of making accurate cuts in the genome. It consists of two nuclease domains that both make a single stranded cut in the DNA, which in turn leads to a double stranded break. This double stranded break will be repaired by one out of two repair systems; the highly efficient but rarer homology-directed repair (HDR) system or the more common but error-prone non-homologous end joining (NHEJ) system. The NHEJ repair system often makes wrongful insertions or deletions (called indels) at the site of the break which leads to frameshift mutations, premature stop codons and ultimately disruption of the gene (Ran et al., 2013). Modifications have been made to the Cas9 protein that have enabled it to be used for more purposes than gene disruption and creating knock-out cells and animals. For example, by attaching transcription factors or fluorophores to a deactivated Cas9 (engineered Cas9 that can bind DNA but not cut it) genes can be activated, repressed, and visualized. Moreover, by creating double stranded breaks while supplying a DNA segment with complementary sequences on the flanks to the targeted DNA, new genes can be inserted using HDR (Sander et al., 2014).

Different species of bacteria have different Cas9 proteins and they can vary greatly in size, ranging from 900-1600 amino acids (Hsu et al., 2014). The size of the Cas protein is important, as too large genomic sizes may be difficult to pack into vectors without creating

genomic instability (CRISPR Guide, Addgene). The most commonly used Cas9 protein is from *Streptococcus pyogenes* (SpCas9), which has a relatively large size (~4kb coding sequence). The nucleotides immediately 5'-3' downstream of the target sequence are of great importance in order for the Cas9 protein to bind it. Different Cas9 protein require different sequences, which vary in length from Cas9 to Cas9. These short sequences are called PAM, short for Protospacer Adjacent Motif. In the case of spCas9 it requires NGG, or it will not bind Cas9. The PAM sequence serves two purposes. Firstly, it makes sure that the Cas9 protein does not make cuts in its own DNA as the matching sequences are not adjacent to PAM sequences. Secondly, it helps expediate the search for target sequences as the Cas9 protein will focus its search for target sequences by first zooming in on the correct PAM sites (Vidyasagar, 2018). Studies have shown that virus with mutations in their PAM sites are not recognized by the Cas9 protein and circumvents CRISPR interference (Hsu et al., 2014). Thus, when planning to use CRISPR/Cas9 it is not only crucial that the target sequence is specific to the gene of interest but also that it is immediately adjacent to a PAM site. If that is not the case another Cas9 protein which uses another PAM sequence can be used instead.

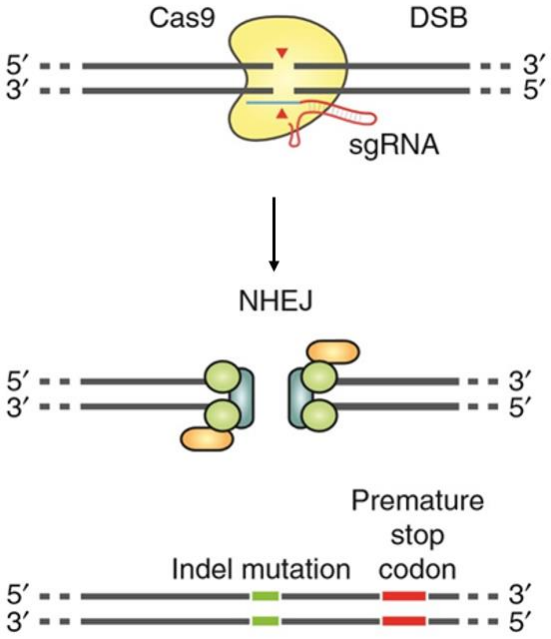

Figure 2. Schematic of how CRISPR/Cas9 works. The Cas9 protein is directed to the gene by the sgRNA and makes a double stranded break (DSB). Most often the break is repaired via non-homologous end joining (NHEJ) which frequently creates insertions or deletions (indels) that cause frameshift mutations, premature stop codons and ultimately gene disruption. Modified from Hsu et al., 2014.

Stem Cells and Differentiation

Stem cells are unspecialized cells that are essential to our development and survival. They possess the properties of being able to both self-renew as well as generate differentiated cells that have specialized functions, such as muscle cells, brain cells and blood cells. The immediate progeny of a stem cell is called a transit-amplifying cell (also called progenitor cell) which divides a finite number of times before eventually differentiating to the

specialized cell (Slack, 2016). The ability to differentiate into different type of cells with specialized function is called potency. Stem cells that are capable of giving rise all cells in the body are called pluripotent. Embryonic stem cells donated from fertilized eggs from *in vitro* fertilization clinics are pluripotent. The term multipotent is used to describe the stem cells that can generate several specialized cells but only to related cells within a certain tissue or organ. For instance, stem cells in the bone marrow give rise to all the cells in the blood (red blood cell, white blood cells and platelets). In the same fashion oligopotent stem cells differentiate into a few different progeny cells (such as lymphoid or myeloid stem cells) and unipotent stem cells differentiate into only one (such as muscle stem cells) (Hima Bindu & Srilatha, 2011).

There are several types of stem cells that are classified from where they can be found. Embryonic stem cells (ES cells) are found in the embryo, fetal stem cells in the fetus, cord blood stem cells in the umbilical cord and adult stem cells in tissues of developed organisms. The activity of different stem cells varies greatly. Some stem cells in tissues of the skin and gut divide often in order to regenerate, repair and replace worn out, damaged or dead cells. In other tissues such as the heart and pancreas the stem cells only divide in response to injury and disease (Knoepfler, 2015). In 2006 it was shown that mouse fibroblast cells which play a role in wound healing and collagen production could be reprogrammed to an embryonic state with pluripotent properties (Takahashi & Yamanaka, 2006). These cells showed ES cell morphology, growth properties and expressed protein markers characteristic for ES cells and are called induced pluripotent stem cells (iPS cells). This was achieved by transducing (infecting) the cells with virus carrying four factors that force the cells to express genes associated with embryonic stem cells. It was later shown human cells could also be reprogrammed in this fashion (Takahashi et al., 2007). By being able to create iPS cells from non-stem cells, a vast supply of ES cell like cells are available. Not only can they be patient specific and not evoke an immune response when transplanted, but they also circumvent the ethical aspects of using embryonic, fetal or umbilical cord stem cells. Treatments based on stem cell therapy provide a strategy and hope to treat many neurodegenerative diseases.

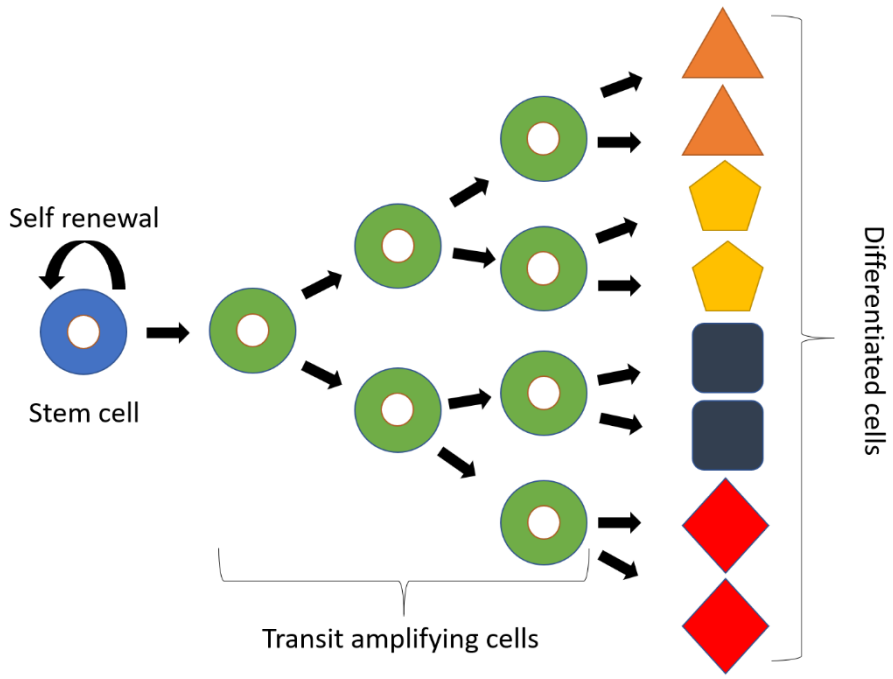

Figure 3. Illustration showing the concept of a stem cell. The stem cell renews itself and also gives rise to transit amplifying cells (progenitors) that divide a finite amount of times before differentiating. In this schematic the stem cell generates four kinds of differentiated cells.

The Roles of Adeno Associated Virus and Lentivirus as Vectors for Gene Delivery

Paramount to the success of a gene therapy treatment is the delivery method. The method of choice is often to use virus to deliver the genes of interest. Different viruses are suitable for different experiments and choosing the most appropriate viral vector depends on several factors. These include the targeted cell, if it is *in vivo* or *in vitro* and the size of the genes to be delivered (Vigene Biosciences, 2016). Another important factor to consider is the multiplicity of infection (MOI). This is defined as the ratio between virus particles and cells. Too high MOI can result in cell death while too low MOI results in too few transduced (infected) cells. The two most commonly used viral vectors are lentivirus (LV) and adeno-associated virus (AAV).

Lentivirus is a retrovirus, meaning its genetic material is in the form of RNA but upon infection it uses an enzyme called reverse transcriptase that converts the RNA to DNA. This DNA is then integrated randomly into the host cell's genome. Lentivirus has the potential to integrate its genes into both dividing and non-diving cells. Integration leads to permanent and stable insertions in the cell's DNA, which are passed on to its progenies. However, too much integration of DNA can be toxic to the cells as it leads to genomic instability. Therefore, it is important to consider the MOI. Lentiviruses are constructed without the genes for their pathogenic factors, making them safe to handle. Moreover, they can infect nearly all mammalian cell types (Davis, 2017). Also, lentivirus has a packaging capacity of 6 kb, making it possible to insert spCas9 and sgRNAs without creating genomic instability. However, there are concerns regarding lentivirus eliciting an immune response when used *in vivo* as well as integrating the genes into oncogenes causing insertional mutagenesis and disrupt genes important for cancer prevention (Follenzi et al., 2007). This is however a rare occurrence and numerous clinical trials using LV are underway.

When it comes to delivering genes *in vivo*, AAV is usually the preferable method. AAV vectors are easy to produce in high titers, infect a broad range of cells, show good diffusion *in vivo* and are safe to use. They cause very little damage to the organism as they do not integrate into the host genome and elicit a mild or no immune response, making AAV an ideal vector for *in vivo* gene therapy (Naso et al., 2017). Moreover, AAV has been used to deliver siRNA to target SOD1 in fALS in gene therapy studies (Stoica & Sena-Esteves, 2016). Like LV, AAV can infect both dividing and non-dividing cells and different serotypes of AAV can infect different tissues, showing specific tropisms. Upon infection the AAV vector with the gene of interest will be present in cell as multiple copies of the DNA linked together in large molecules called concatemers. Since the AAVs do not integrate their DNA in the host's genome there is no risk for insertional mutagenesis, however the concatemer will not be present in the daughter cells. Therefore, the number of transduced cell will be diluted over time as the cells divide (Naso et al., 2017). While having the characteristics of an ideal vector *in vivo* AAV usually show moderate to low expression *in vitro*. The biggest drawback with AAV is its small packaging capacity which presents some challenges for certain applications. Packaging spCas9 along with a sgRNA leaves barely any room for regulatory elements. Varies strategies have been employed to circumvent this problem. For instance, spCas9 and the sgRNAs can be delivered on separate AAVs but requires that both AAVs infect the target cell. Also, smaller Cas9 from other species can be used. Cas9 from *Staphylococcus aureus* (saCas9) is 30% shorter and can easily fit in an AAV along with several sgRNAs (Ran, et al., 2015).

Methods

Bioinformatics

In order to disrupt the expression of SOD1, sgRNAs targeting the SOD1 gene had to be chosen. Different bioinformatics websites use different algorithms to score and rank sgRNAs. Some base the scores of the sgRNAs solely on their on-target efficiency without any consideration of off-target activity. Other websites have the opposite approach and score the sgRNAs only on minimal off-target activity alone. Therefore, finding the most suitable sgRNA is challenging, as the different website usually do not recommend the same sgRNAs since they use different algorithms. However, using sgRNAs that have high scores on multiple websites is a good strategy. The SOD1 gene has five exons and the two sgRNAs chosen were directed at exon 2 (sgRNA:E2) and exon 3 (sgRNA:E3). The sgRNA targeting exon 2 was chosen from http://crispr.mit.edu/ and the sgRNA targeting exon 3 was chosen from https://www.crispr.ml/. The two sgRNAs can be seen below.

- Exon 2: GGATTCCATGTTCATGAGTT
- Exon 3: CTCTATCCAGAAAACACGGT

Both sgRNAs were run through BLAST protein at NCBI to make sure that the target sequences were unique to the SOD1 gene and cannot be found anywhere else in the genome.

Cloning and Agarose Gel Electrophoresis

The two sgRNAs were ordered from a company and inserted into plasmids that were later used to produce virus. A third plasmid was created with LacZ instead of sgRNA which was used a negative control throughout the project. The plasmids (52961 LentiCrispr v2 plasmids) were first treated with the enzyme BsmBI in order to make a cut in the plasmid to allow for the insertion of the sgRNAs. The plasmid backbone and the cut-out smaller segments were separated using agarose gel electrophoresis. This technique separates DNA molecules based on size. The negatively charged DNA will travel down the gel towards to positive electrode when voltage is applied. The smaller molecules will travel further down through the pores in gel. By staining the gel with ethidium bromide the bands of DNA can be visualized under UV-light and isolated. The plasmid backbone did not travel as far down the gel as the smaller segments and could easily be isolated. In order to prevent the plasmid backbone to self-ligate, it was treated with alkaline phosphatase. The sgRNAs were ordered as single-stranded oligos which needed to be annealed and phosphorylated before inserted into the backbone using DNA ligase enzyme.

The fully complete plasmid constructs containing the sgRNAs were mixed with bacteria to allow transformation to occur. The plasmids were taken up by the bacteria and were allowed to proliferate for a short time before being plated and grown on agar plates with ampicillin overnight. The plasmid construct had the gene coding for ampicillin resistance which ensured that only bacteria with the correct plasmid would be able to grow on the plate. A single colony per plate was picked and transferred to growth media to make sure all bacteria had identical plasmid constructs. After incubating the bacteria overnight, plasmid DNA was extracted from some of the bacteria, using a plasmid miniprep kit and sent for sequencing. Once the sequencing showed that the sgRNAs had been correctly inserted into the plasmid the remaining of the bacteria were allowed to grow for a day in order to amplify the number of bacteria and thus the number of plasmids. Finally, the bacteria were lysed and the plasmids were extracted and ready to be used to produce lentivirus.

Cellular Maintenance and Differentiation

CRISPR/Cas9-mediated knock-down of the SOD1 gene was carried out on two stem cell lines that had been differentiated to forebrain neural progenitor cells (FB NPCs). The first cell line was an embryonic stem cell line called RC17 and the other one was a patient-specific iPS cell line with a mutation associated with fALS (L144P), $SOD1^{L144P}$. Since iPS cells should have the same properties as ES cell, they were maintained and differentiated using the same protocols. The cells were cultured in wells that had been coated with laminin in order for the cells to attach to the well. Media was changed every day and the cells were allowed to proliferate until they were 70-90% confluent (i.e. covering 70-90% of the well's surface). At this point the cells were washed to get rid of dead cells, detached from the plate and replated onto new wells. The protocol to differentiate the two stem cell lines to forebrain progenitors was 16 days (Nolbrant et al., 2017), which involved adding small molecules and neurotrophic factors to the media (see **Figure 4**). The success of the differentiation was valuated based on immunocytochemistry staining for FB NPC biomarkers.

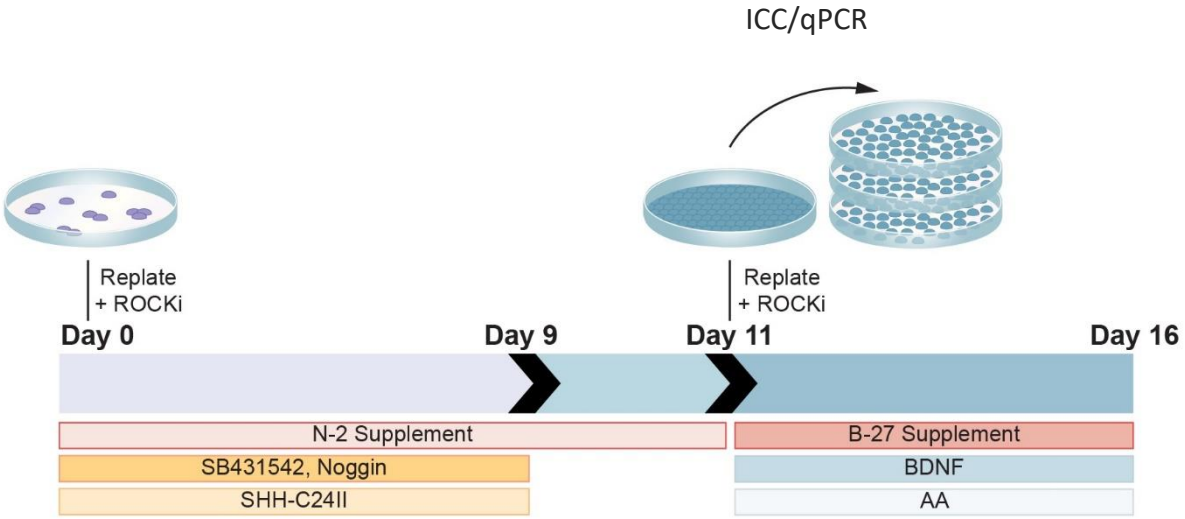

Figure 4. The 16-day differentiation protocol used to differentiate RC17 and SOD1^{L144P} stem cell lines to forebrain neural progenitor cells. The cells were plated on day 0 and N2-medium was added for the first 11 days. On day 11 the cells were replated and split to multiple wells to later be analysed by ICC and qPCR and the medium was changed to B-27 medium. The cells were transduced om day 14. SB, Noggin, SHH, BDNF and ascorbic acid (AA) were the small molecules added necessary for the differentiation.

Virus Production

The lentivirus carrying Cas9 and the sgRNA/LacZ were produced in 293T cells. The plasmid with Cas9 and a sgRNA or LacZ was mixed with three plasmids carrying the necessary genes for packaging, reverse transcriptase and envelope proteins, as well as polyethylenimine (PEI). PEI is a positively charged polymer that condenses DNA into cationic particles which bind the negatively charged surface of the cells and are transported to the cytoplasm via endocytosis. After the cells are transfected with the plasmids they start producing virus and secreting them extracellularly. Upon ultracentrifugation the cells will form a pellet and the virus will be present in the supernatant. Most of the virus particles were saved for later use while some was used to determine the virus titre (virus concentration), using qPCR. 293T cells were infected with three different concentrations of the lentivirus which integrate their genome in the host cell. After a few days the cells were lysed, the DNA extracted and the virus titre estimated using qPCR. The complete lentiviral construct can be seen in **Figure 5**.

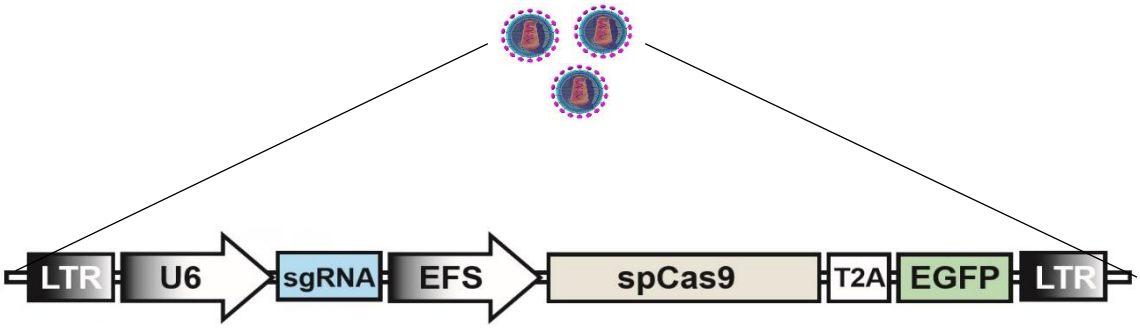

Figure 5. The lentiviral vector used for SOD1 knock-down. For the knock-down negative control the sgRNA was replaced with LacZ. From left to right: long terminal repeats (LTR), U6 promoter, sgRNA, EFS promoter, Streptococcus pyogenes Cas9 (spCas9), T2A peptide linker, enhanced green fluorescent protein (EGFP), long terminal repeat (LTR).

Immunocytochemistry

Immunocytochemistry (ICC) is a common method used to study cells and their proteins. It is based on antibodies binding to specific antigens than can either be inside the cell or located on its surface. Usually, a primary antibody, specific for the marker of interest is added to the cells and allowed to bind in. Thereafter, a fluorescent-labelled secondary antibody, specific for the primary antibody is added which allows the biomarker to be studied in a fluorescent microscope. A blocking solution should be added prior to adding the antibodies in order to prevent any unspecific binding. ICC was used to validate the differentiation, the transduction and the SOD1 knock-down on a protein level. To validate the differentiation the cells were stained for FOXG1 (forebrain progenitor marker) and nestin (early neural progenitor marker). The transduction was validated based on the GFP (present in the viral vector). The knock-down was analysed by the presence of SOD1 and GFP. Prior to the fluorescent stainings the cells were fixed with paraformaldehyde. The fixation step is carried out to immobilize antigens while at the same time retaining the cellular and protein structure. DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) was used to stain the cell nuclei in all ICC experiments.

Fluorescence-activated cell sorting (FACS)

To validate if the attempted CRISPR/Cas9 knock-down had worked the infected cells had to be isolated. The lentiviruses carried a green-fluorescent protein (GFP) reporter gene which was used to separate infected cells from uninfected cells. This was done using a fluorescence-activated cell sorter (FACS). The FACS machine dispenses each cell in a tiny droplet. It is equipped with a laser that emits coherent light of specific wavelength. The cells that pass the laser scatter the light and the fluorophores are excited and fluoresce. Scattering and fluorescence is detected by a photo multiplicator tubes (PMT) and via a computer converts the signals to analysis data. The infected cells which express GFP will be detected with the laser and the droplet will be given a charge. An electric field will then deflect the differently charged droplets to different tubes. Usually a viability marker is added to the samples to help separating dead and alive cells. In this case DRAQ7^{TM} was used, which is a DNA dye that stains dead cells. The cells expressing GFP, that is the infected cells, were collected in one tube, while the cells that did not express GFP were collected in another tube. Both GFP positive (GFP+) cells and GFP negative (GFP-) cells were collected so the SOD1 expression could be compared between the two and see if there had been a SOD1 knock-down. Also, the GFP+ Cas9/LacZ were collected as a knock-down control. Untransduced and thus GFP- cells were used as a negative control for the FACS and were also collected.

quantitative PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) is a method for quantifying DNA and RNA and was used to estimate the virus titre as well as the expression of SOD1 mRNA after attempted knock-down using CRISPR/Cas9. The method works by using an elevated temperature to denature and separate double stranded DNA into two single strands. Short primer sequences will anneal to single strands via complementary Watson-Crick base-pairing upon lowered temperature and the enzyme polymerase will add nucleotides and synthesize new double-stranded DNA molecules. This cycle is repeated several times and with each

round the number of DNA strands are doubled. The final amount of DNA will thus depend on the amount of starting DNA. In the qPCR there is a fluorescent probe that bind only double-stranded DNA and the fluorescence is measured after each cycle. Once a certain threshold of fluorescence is reached (i.e. a certain amount of dsDNA) the cycle is noted. Therefore, qPCR-results show the cycle value for when the threshold is reached. More starting DNA will require fewer cycles and will correlate to a lower cycle value. Since lentivirus integrate their DNA into the host cell's genome, a lentivirus-specific primer, absent in uninfected cells was used to estimate its amount. A higher virus titre means higher degree of transduction and more integrated viral DNA. To study the mRNA expression the mRNA was first converted to complementary DNA (cDNA) using reverse transcriptase and then analysed. The results from the qPCR does not show the exact amount of DNA molecules, but instead the relative expression compared to another gene(s). Usually, the expression of the gene of interest is compared to housekeeping genes. Housekeeping genes code for proteins that are necessary for the maintenance of the cell and is therefore usually always expressed at rather constant levels. In the virus titration albumin was used as housekeeping gene and the virus titer was determined by using a reference batch with known virus titer. For the expression analysis GAPDH and beta-actin were used as housekeeping genes. When running a qPCR experiment it is important to have a negative control that does not have the gene-specific primer sequence. The DNA of untransduced 293T cells (not having any integrated lentivirus DNA) was used as a negative control for the virus titration (called a mock) and deionized water was used in the mRNA expression analysis.

Results

Virus production

Lentiviruses carrying the genes for Cas9 and a sgRNA were produced using 293T cells. Also, virus with LacZ instead of sgRNA were produced to be used as a negative control during the transduction. All three viral vectors also contained GFP as a reporter gene. Using qPCR with primers specific for lentivirus and for the housekeeping gene albumin, as well as a mock and reference batch with known titer, the virus titres were estimated and put in **Table 1**. The cycle values from the qPCR and Excel-sheet used to convert the cycle values to titers, using the reference batch can be found in the **Appendix**.

Virus	Titer (Units/ml)
Cas9-GFP-SOD1 Exon2	$2.3 \cdot 10^8$
Cas9-GFP-SOD1 Exon3	$9.0 \cdot 10^8$
Cas9-GFP-LacZ	$2.8 \cdot 10^8$

Table 1. The virus titres (units/ml) for the vectors as determined by qPCR.

Cellular Differentiation and Transduction

CRISPR/Cas9 knock-down was attempted on the two pluripotent cell lines RC17 and $SOD1^{L144P}$. Since both cell lines should have the same properties they were differentiated to forebrain progenitor cells using the same 16-day protocol. Representative pictures were taken throughout the differentiation and pictures from day 0, 4, 10 and 16 for both cell lines can be seen in **Figure 6**.

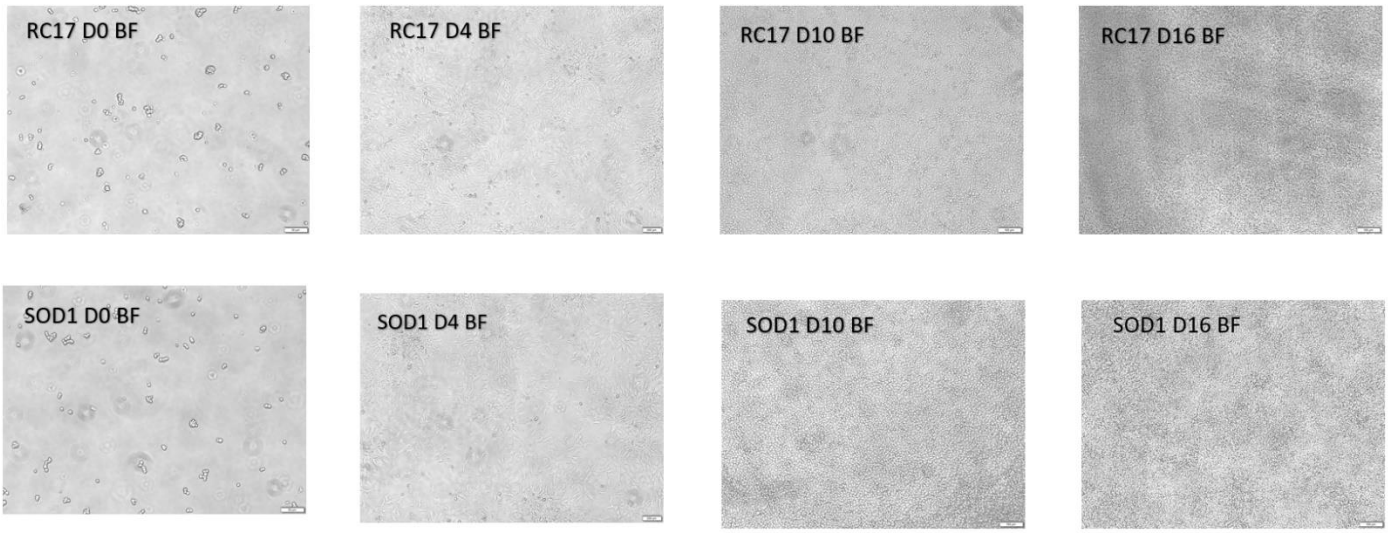

Figure 6. Representative pictures for both cell lines, for day 0, 4, 10 and 16 of the 16-day differentiation to forebrain neural progenitor cells. The pictures were taken with a brightfield (BF) microscope with 10x magnification.

The cells were stained with fluorescent antibodies one week after transduction. The success of the differentiation to forebrain neural progenitor cells was validated based on the expression of FOXG1 and nestin. FOXG1 is marker for forebrain progenitors and nestin is an early neural progenitor cell marker and both should be expressed after the differentiation. Pictures from the staining of differentiated untransduced RC17 cells can be seen in **Figure 7**.

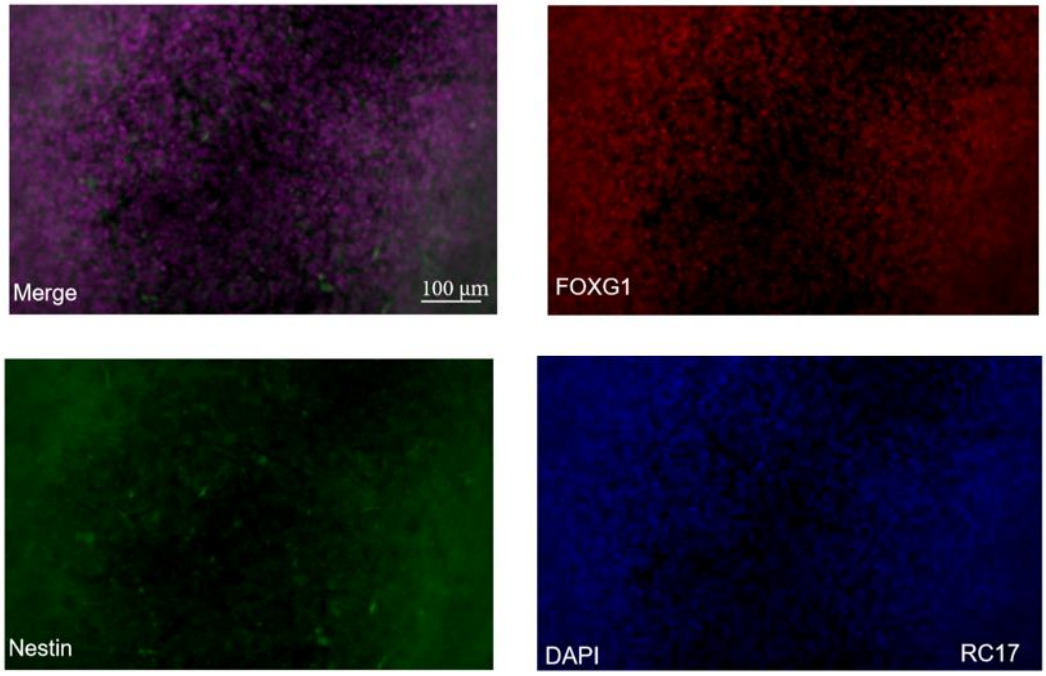

Figure 7. Pictures taken with a fluorescent microscope of untransduced RC17, 7 days after differentiation. FOXG1, a forebrain progenitor marker was stained in red and nestin, a neural progenitor marker was stained in green. DAPI was used to stain all cell nuclei. The merged picture shows the channels overlayed on top of each other with FOXG1+/Nestin+ cells in purple. Scale bar: 100 µm.

The cells were transduced with virus on day 14 of the differentiation and FACS-sorted for RNA analysis as well as fixed for immunocytochemistry on day 21. The cells for the mRNA analysis received an MOI of 20 and the cells for the immunocytochemistry staining received an MOI of 10. For instance, a well containing 600 000 cells was prior to RNA analysis transduced with 600 000 x 20 = 1 200 000 virus particles. Upon infection the transduced cells showed decreased homeostasis and more cell death as compared to the untransduced cells. Representative pictures were taken of transduced and untransduced cells for both cell lines on day 17 (three days after transduction) and can be seen in **Figure 8**.

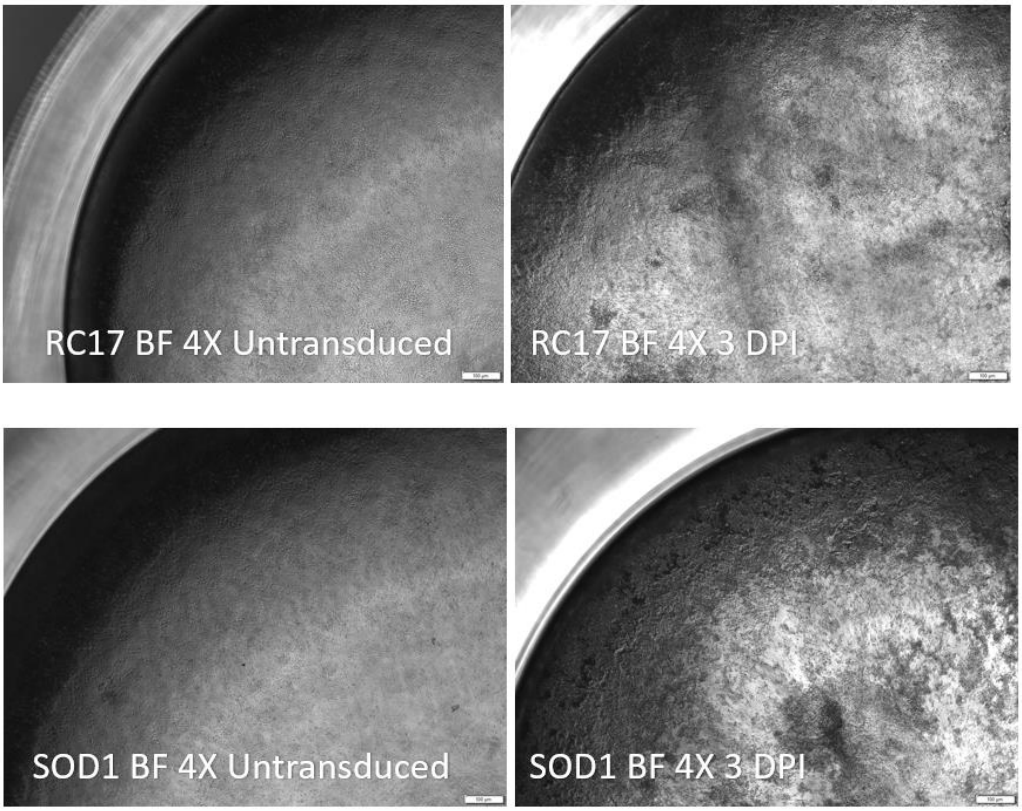

Figure 8. Pictures taken with brightfield (BF) microscope 3 days post infection (3 DPI) showing increased cell death and decreased homeostasis in transduced cells compared to untransduced cells.

All transduced and untranduced cells from both cell lines were stained for GFP and nestin. GFP acted as a reported gene which was present in the viral vector and should only be expressed in transduced cells. Thus, GFP+ cells are transduced cells. The untransduced RC17 and SOD1 cells were used as a negative control for GFP. **Figure 9** and **Figure 10** show representative pictures taken of the GFP and nestin stainings for untransduced and transduced RC17 and $SOD1^{L144P}$ cells, respectively.

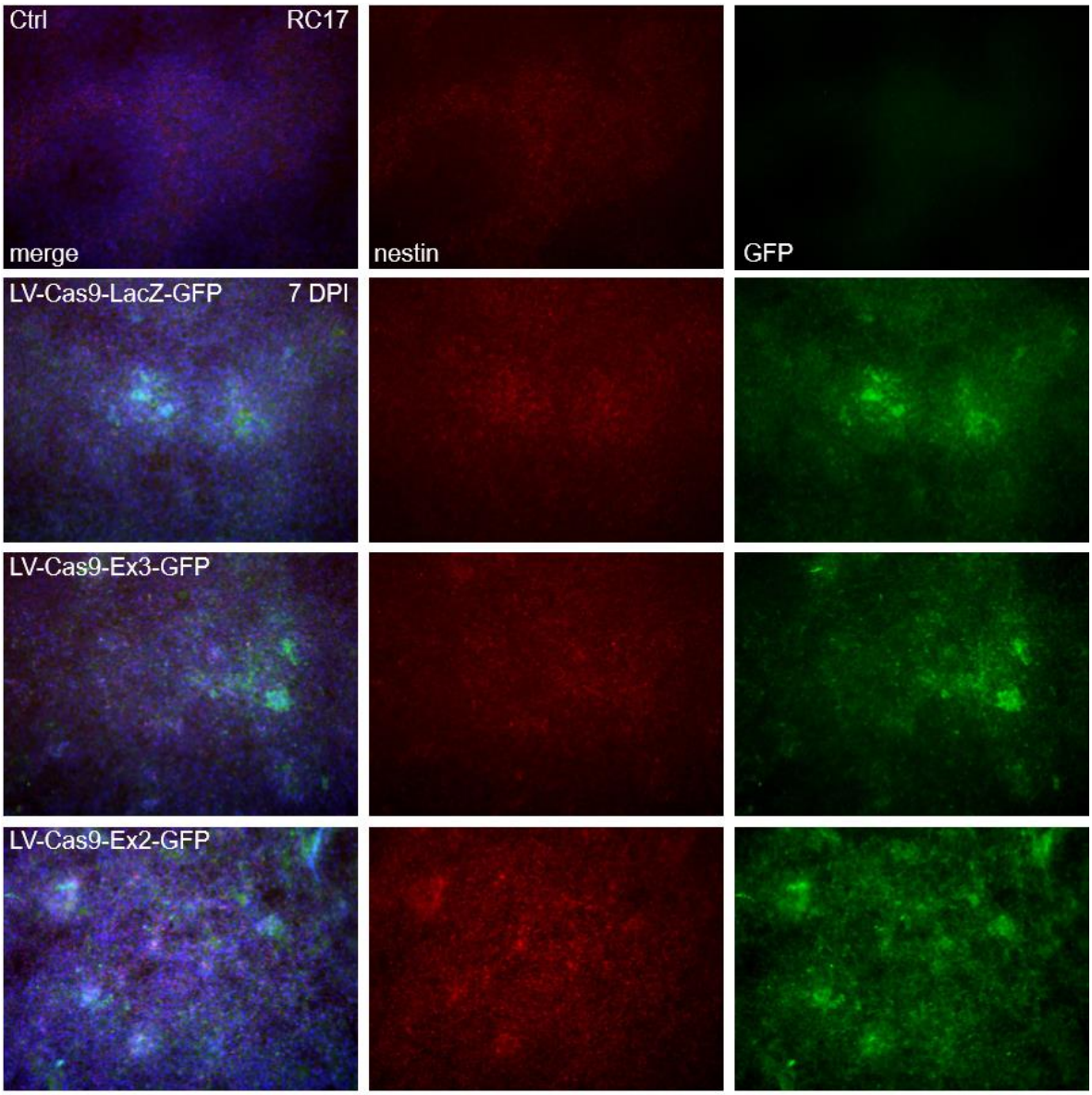

Figure 9. Pictures taken of untransduced (Ctrl) RC17 and transduced RC17 with sgRNA:E2, sgRNA:E3 and LacZ, 7 days post infection (7 DPI). GFP was stained in green, nestin in red and DAPI in blue (not shown individually). The merged pictures show all stainings overlayed.

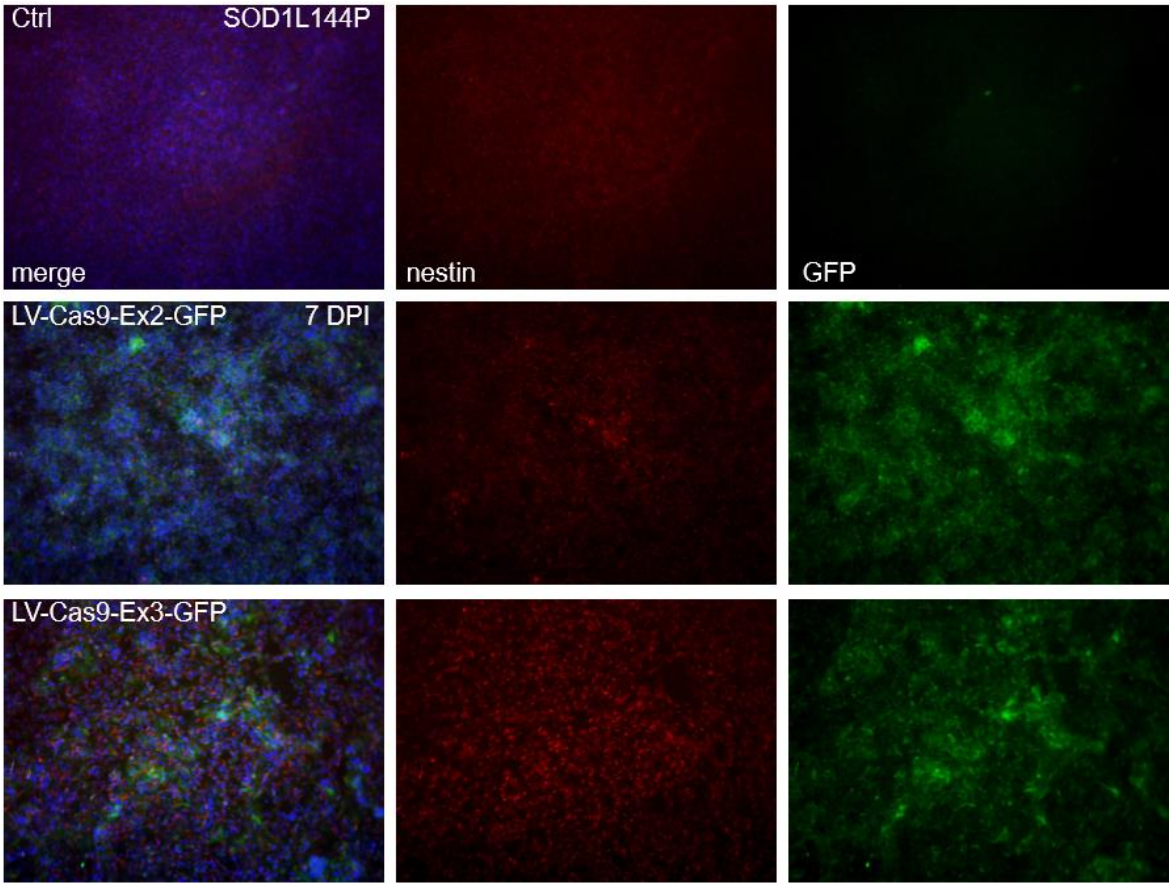

Figure 10. Pictures taken of untransduced (Ctrl) SOD1^{L144P} and SOD1^{L144P} transduced with sgRNA:E2 and sgRNA:E3, 7 days post infection (7 DPI). GFP was stained in green, nestin in red and DAPI in blue (not shown individually). The merged pictures show all stainings overlayed.

Fluorescent-Activated Cell Sorter (FACS)

A week after the transduction the cells were sorted based on the presence of GFP. Infected cells expressed GFP and were sorted into one tube for RNA analysis while the GFP-negative cells were sorted into another tube, also to be analysed later with qPCR. Cells that had not been transduced, that is, cells from the wells where virus had not been added, would not express GFP and were used as a negative control to help sort out the GFP-positive cells. For the cells that had been infected with virus carrying Cas9/sgRNA, 10 000 GFP+ and 10 000 GFP- cells were sorted out, while for the cells infected with Cas9/LacZ only 10 000 GFP+ cells were sorted out. Also, 10 000 untransduced cells were collected. This was carried out with both cell lines, yielding 12 samples in total. Before adding the cells to the FACS machine, the cells were run through a filter to yield a cell suspension of single cells, as to not clog up the machine. Furthermore, the viability marker DRAQ7^{TM} was added, to make sure only living cells were collected. It is a DNA dye that stains the nuclei of dead cells, while being impermeable to living cells. Therefore, all living cells would be DRAQ7-negative (DRAQ7-). The untransduced cells that were used as a negative control and collected, functioned to gate the DRAQ7-/GFP- cells. Using this gate as a reference mark, the effectively transduced living cells (DRAQ7-/GFP+) and the noninfected living cells (DRAQ7-/GFP-) could be sorted out. **Figure 11** and **Figure 12** show the gated untransduced cell for the RC17-and SOD1^{L144P} cell line, respectively. Using the untransduced cells as a negative control, the transduced cells were gated as DRAQ7-/GFP+. **Figure 13** and **Figure 14**

show the gated DRAQ7-/GFP+ cells for sgRNA:E2 for the RC17-and SOD1 cell line, respectively. The complete FACS analysis can be found in the **Appendix**.

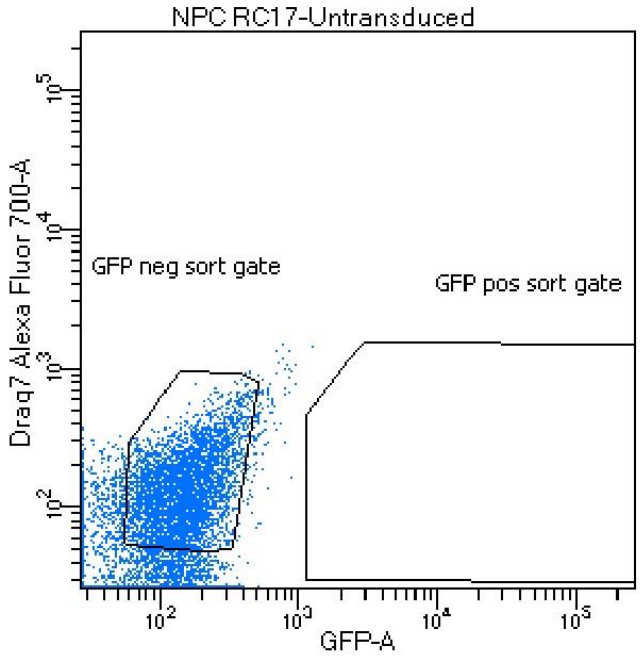

Figure 11. Plot from the FACS for untransduced RC17 with GFP intensity on the x-axis and DRAQ7 intensity on the y-axis. The untransduced cells were used as a negative control to gate the GFP- and GFP+ cells. DRAQ7 was used as a viability marker to sort away dead cells.

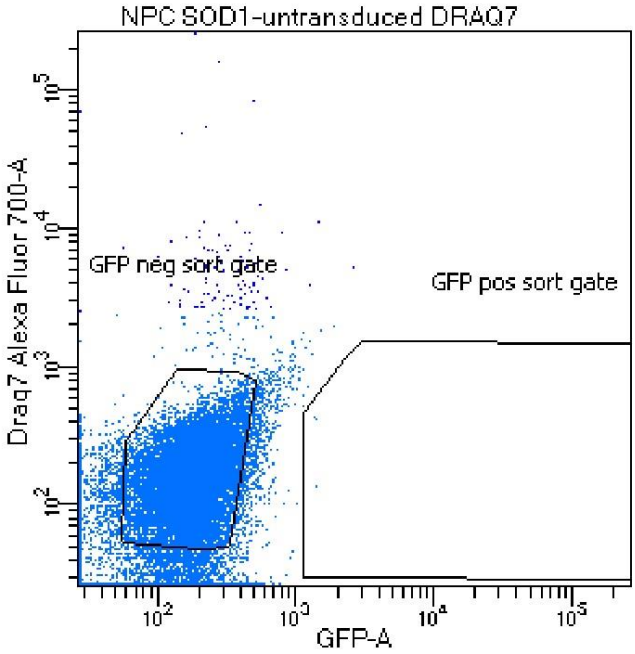

Figure 12. Plot from the FACS for untransduced SOD1^{L144P} with GFP intensity on the x-axis and DRAQ7 intensity on the y-axis. The untransduced cells were used as a negative control to gate the GFP- and GFP+ cells. DRAQ7 was used as a viability marker to sort away dead cells.

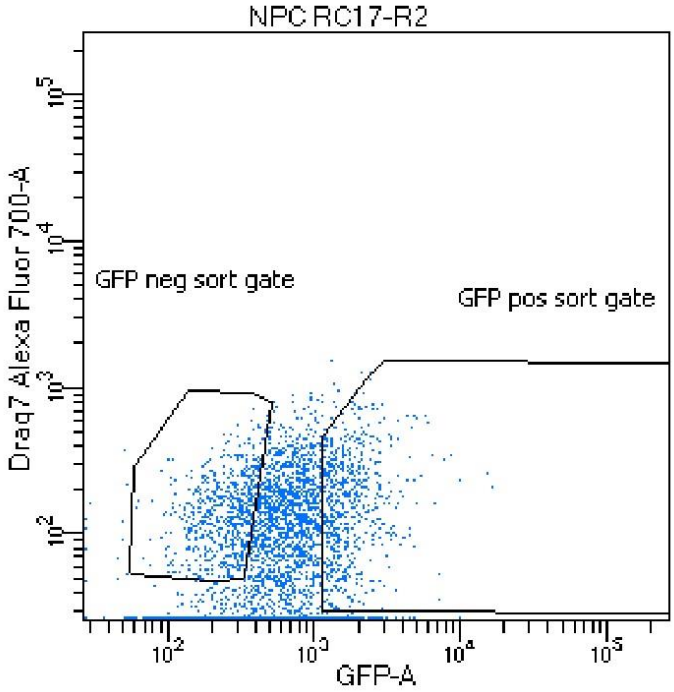

Figure 13. Plot from the FACS analysis with untransduced and GFP- RC17 cells in left gate and transduced (sgRNA:E2) and GFP+ RC17 cells gated to the right.

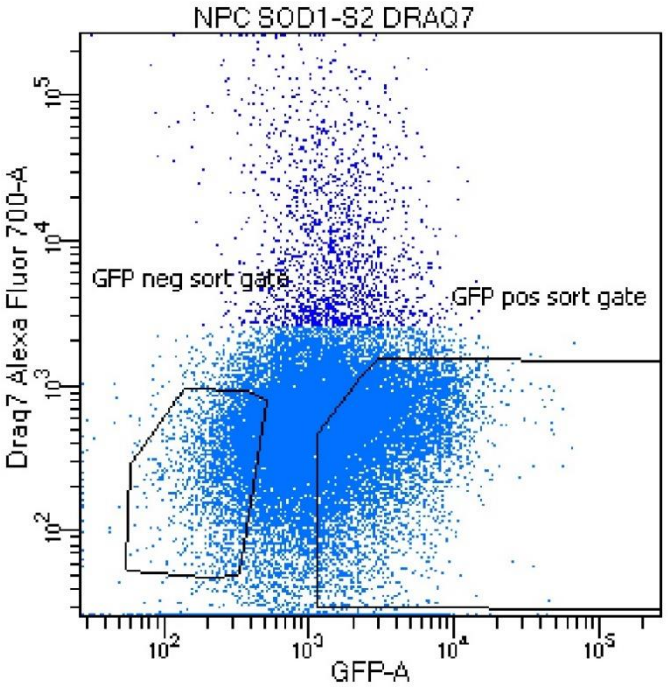

Figure 14. Plot from the FACS analysis with untransduced and GFP- SOD1^{L144P} cells in left gate and transduced (sgRNA:E2) and GFP+ SOD1^{L144P} cells gated to the right.

Immunocytochemistry

SOD1^{L144P} cells transduced with sgRNA:E2 were stained for GFP and SOD1 to look for SOD1 knock-down on a protein level. A complete knock-down would result in GFP positive but SOD1 negative cells. **Figure 15** show representative pictures from the staining, with potentially GFP+/SOD1- cells marked by arrows.

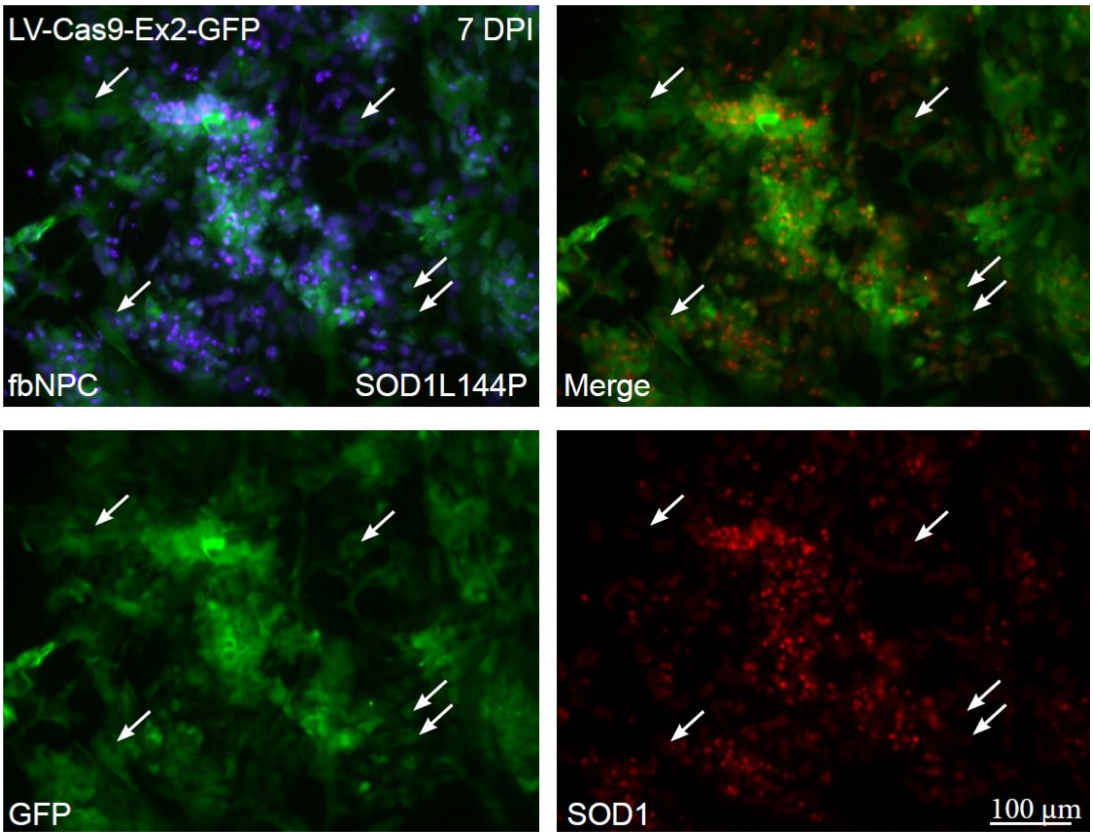

Figure 15. Pictures taken of SOD1^{L144P} cells 7 days post infection (7 DPI) with sgRNA:E2. SOD1 was stained in red, GFP in green and DAPI in blue (not shown separately). Cells appearing to be GFP+/SOD1- are marked by white arrows.

qPCR Expression Analysis

The knock-down of SOD1 on an mRNA expressional level was determined with qPCR. A SOD1-specific primer was used to compare the SOD1 expression relative to all the other samples. The expression of SOD1 for all samples were compared to the first sample to get the fold change. Therefore, the SOD1 expression of the first sample was normalized and set to 1. All samples were also individually normalized to the housekeeping genes GAPDH and beta-actin to compensate if there were changes in amount of starting DNA. The relative SOD1 expression values for RC17 and SOD1 were put in **Figure 16** and **Figure 17**, respectively. All relative values and qPCR cycle values can be seen in the **Appendix**.

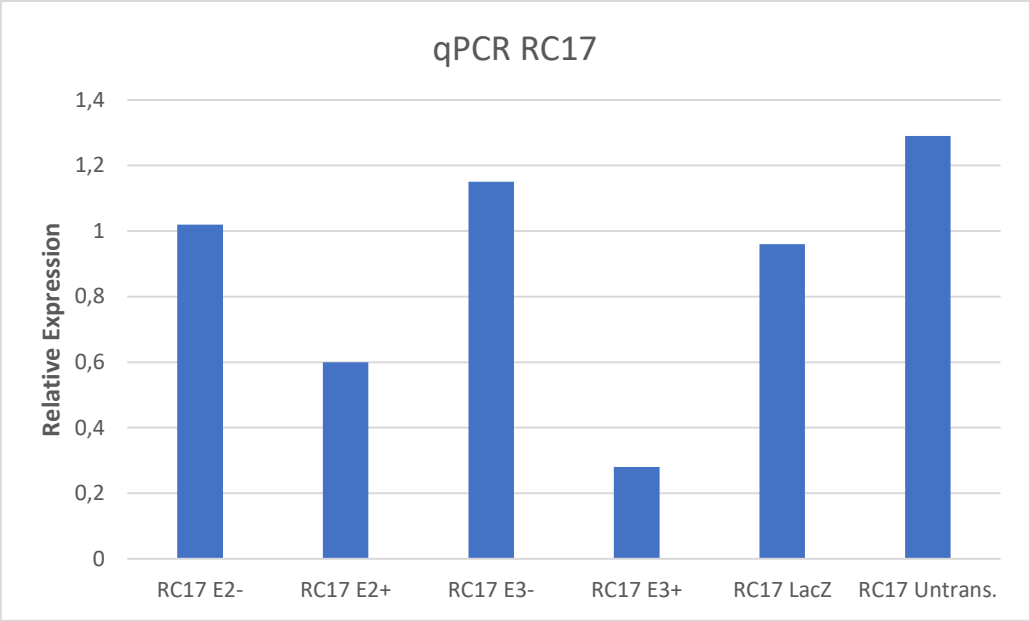

Figure 16. Relative values of SOD1 expression for RC17 cells analysed with qPCR with SOD1-specific primer. The minus and plus signs refer to if the samples were sorted as GFP-/GFP+.

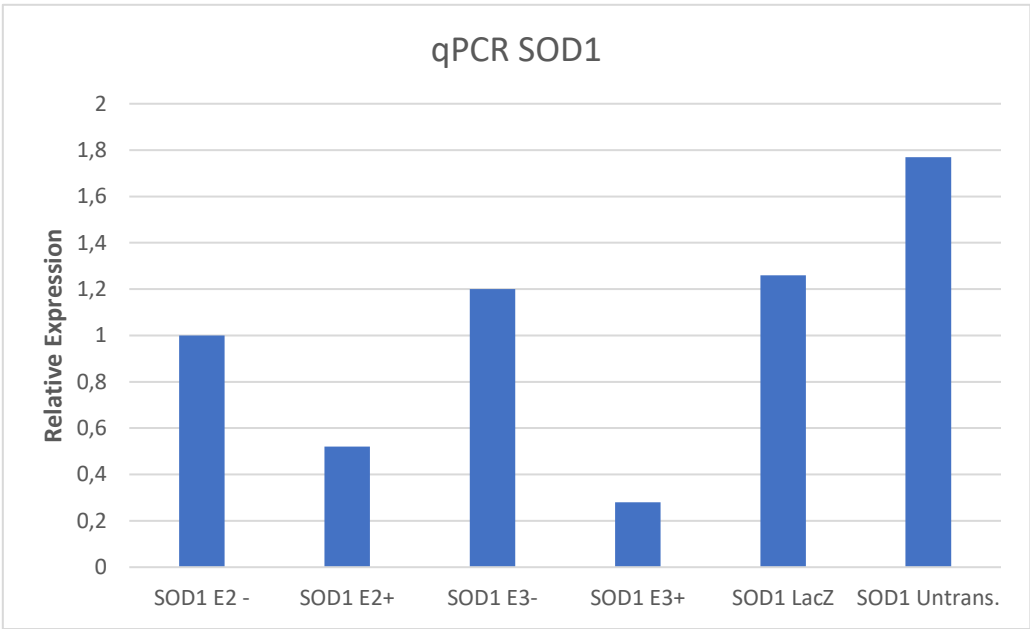

Figure 17. Relative values of SOD1 expression for $SOD1^{L144P}$ cells analysed with qPCR with SOD1-specific primer. The minus and plus signs refer to if the samples were sorted as GFP-/GFP+.

To compare the SOD1 expression between GFP+ and GFP- cells from the same samples, the ratio of the relative values from **Figure 16** and **Figure 17** were taken and put in **Figure 18** and **Figure 19**. The value for GFP- cells were divided by the value for GFP+ cells to get a number for the fold reduction of SOD1. Thus, values over 1 indicates a reduction in SOD1 expression.

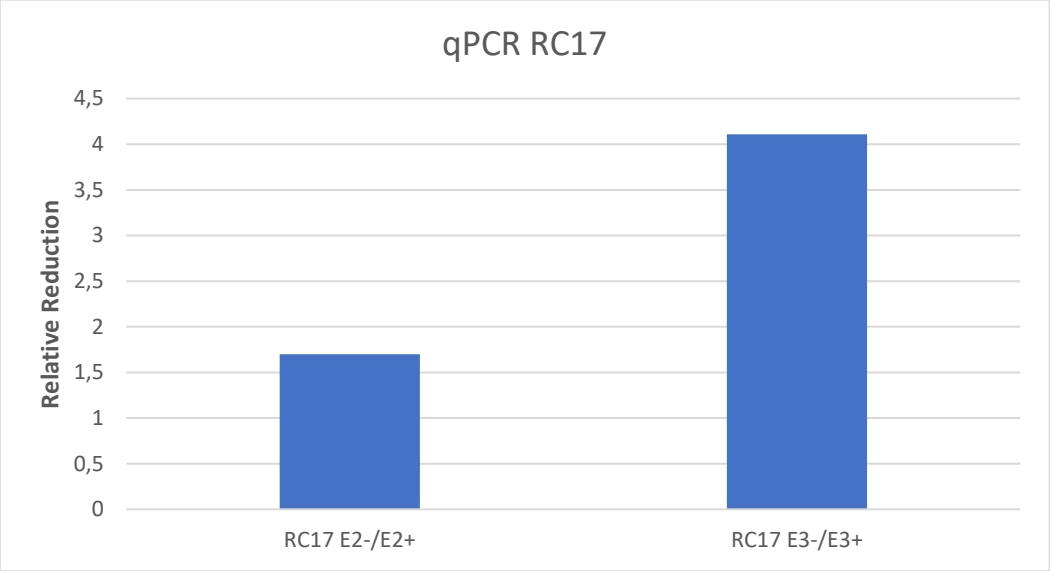

Figure 18. The fold reduction of SOD1 in RC17 cells that between cells sorted out in the FACS as GFP-/GFP+. Values of 1 indicate a SOD1 reduction.

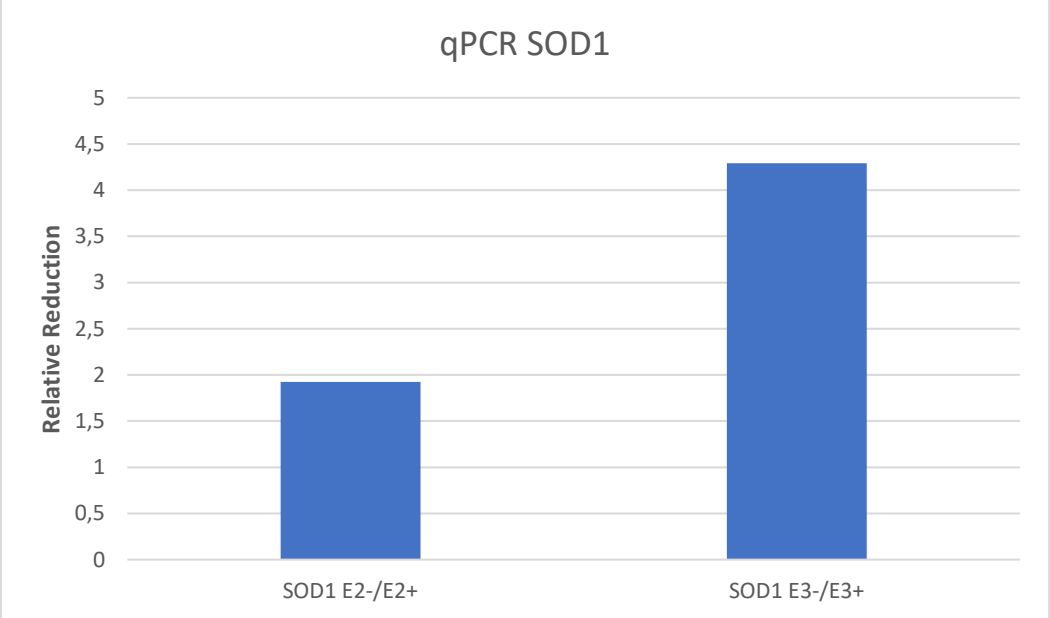

Figure 19. The fold reduction of SOD1 in $SOD1^{L44P}$ cells that between cells sorted out in the FACS as GFP-/GFP+. Values of 1 indicate a SOD1 reduction.

Discussion

Virus Production

The virus titers for the three different vectors can be seen in **Table 1**. The titers are all in the magnitude of 10^8 units/ml which is a good yield, especially considering the size of the cassette with Cas9 that was inserted. There is an inverted correlation between cassette size and virus titer yield, and it is likely that the yield would have been higher if a smaller Cas9 protein had been used.

Cellular Differentiation and Transduction

After the 16-day protocol, the differentiation from stem cells to forebrain neural progenitor cells (FB NPCs) was validated with immunocytochemistry. The cells were fixed with paraformaldehyde and stained with FOXG1 and nestin, two markers that should be expressed by FB NPCs but not by undifferentiated cells. As can be seen in **Figure 7**, the cells are expressing both FOXG1 and nestin, suggesting a successful differentiation to FB NPCs.

In **Figure 8** the activity and toxicity of the lentivirus is clearly visible, as patches of detached, dead cells can be seen in the wells with transduced cells. This is what is to be expected since too much lentiviral infection is toxic to cells and the cells were infected with a lot of virus (MOI of 10-20). If a lower MOI had been used there would have been less cell death and more cells for analysis, however, it would increase the risk of too low degree of infection and the difficulty of being able to distinguish GFP+ cells from GFP- cells in the FACS analysis.

Figure 9 and **Figure 10** show that the transduction was successful. As can be seen in these figures, the GFP intensity is significantly higher in the transduced cells as compared to the untransduced cells. Although the intensity of GFP is significantly lower in the untransduced samples there still is some fluorescent light coming from the GFP channel. However, this is the result of unspecific binding. Since the untransduced cells were never infected with virus carrying GFP there should not be any GFP+ cells in that well. The untransduced cells were still differentiated and therefore the cells are nestin+.

Immunocytochemistry

The SOD1 staining was carried out to study the potential SOD1 knock-down on a translational level. The cells were stained with anti-SOD1 and anti-GFP antibodies to look for GFP+/SOD1- cells, which would indicate an effective knock-down. The arrows in **Figure 15** show possible candidates. Due to the confluency of the cells in the wells it was difficult to distinguish individual cells. Another possibility why it was difficult to find GFP+/SOD1- cells is that a partial knock-down still would result in expression of some SOD1. To confirm and quantify reduced protein levels a Western blot could have been done. However, there was not enough cells to carry out such an experiment.

FACS

The fluorescent-activated cell sorter was used separate the GFP+ cells from the GFP- cells, that is, the infected cells from the uninfected cells, respectively. The DNA dye and viability marker DRAQ7 was added to all samples to help sort out only alive cells. DRAQ7 will stain the DNA of all dead cells which were gated as DRAQ7+ in the FACS analysis. Also, no

doublet cells were collected, only single cells. Doublet cells have the characteristics of having the same intensity as single cells but have twice the size. The untransduced cells were used as a negative control and to gate all GFP- cells. As can be seen in **Figure 13** and **Figure 14**, there is not a clear line between GFP- and GFP+ cell, but rather a big smear of cells with a GFP gradient. Using the untransduced cells as a negative control, a gate was set for the GFP+ cells. However, considering that the samples show a big smear instead of two separate cell populations as well as the high MOI (20) used for the transduction it is not impossible that all cells have been transduced but simply to various degrees. Nevertheless, the cells show high GFP intensity with many cells still alive (DRAQ7-), further indicating a successful transduction.

qPCR Expression Analysis

The results from the qPCR SOD1 expression analysis can be seen in **Figures 16-17** and show positive results. Comparing the SOD1 expression with the first sample (sgRNA:E2-) as a baseline, the CRISPR/Cas9 treated cells show a lower mRNA expression for both sgRNAs and for both cell lines. **Figure 18** and **Figure 19** show the fold reduction for RC17 and SOD1 respectively. For the RC17 cell line the SOD1 expression was reduced 1.7 times with sgRNA:E2 and 4.1 times with sgRNA:E3. Also, for the $SOD1^{L144P}$ cell line the sgRNA:E3 showed higher knock-down efficiency, reducing the SOD1 expression 4.3 times. The results from the qPCR analysis thus suggest that sgRNA:E3 is the better of the two sgRNAs. Although the virus titer was higher for sgRNA:E3 (see **Table 1**) this should not matter since the same MOI was used to infect the cells.

Interestingly, the samples showing the highest SOD1 mRNA expression were the two untransduced ones. For both cell lines the SOD1 expression is higher for the untransduced cells than for the samples considered GFP- by the FACS analysis. This could hint at the cells that were considered uninfected actually were transduced but to a smaller extent, as discussed above in the FACS analysis.

Unfortunately, in the FACS only GFP+ cells were collected for the Cas9/LacZ samples. It would have been interesting to look at the ratio of SOD1 expression between LacZ- and LacZ+ and have it as an additional control. Since the transduced cells do not have sgRNA there should not have been any knock-down and the ratio between LacZ- and LacZ+ should have been 1.

Although there was only one well of cells for the qPCR analysis per sample, undeniably limiting the statistical power, the results show great promise and the two sgRNAs deserve further investigation. This includes increasing the number of replicates to increase the statistical power, complement ICC with Western blot to confirm and quantify reduced protein levels and use DNA sequencing to analyze indels and off-target cuts.

Conclusions

The two stem cell lines RC17 and SOD1^{L144P} were both successfully differentiated into forebrain neural progenitor cells and expressed FOXG1 and nestin after the 16-day protocol. The sgRNA, targeting exon 2 or exon 3 of the SOD1 gene were inserted into lentiviral vectors along with GFP as a reporter gene, and the cells were transduced on day 14 of the differentiation protocol. Immunocytochemistry staining for the reporter gene GFP showed a successful transduction. Moreover, a possible knock-down could be observed on a protein level. Finally, qPCR analysis with a SOD1-specific primer showed a knock-down of SOD1 with both sgRNAs in both cell lines. The sgRNA targeting exon 3 was particularly efficient and resulted in a four-fold reduction of SOD1 mRNA in both cell lines. Despite the low statistical power, the project showed positive results that deserves further exploration.

The project resulted in the development of an in vitro model for screening for effective sgRNAs in a neural stem cell system. The model is not limited to ALS and can be translated to other diseases with the ambition to use CRISPR/Cas9 to disrupt a gene.

References

Berg, J. M., Tymoczko, J. L., & Stryer, L. Biochemistry, 7th ed. New York, NY: W. H. Freeman, 2012.

Hardiman, O. et al. Amyotrophic lateral sclerosis. Nat. Rev. Dis. Primers 3, 17071 (2017).

Armon, C. et al. Amyotrophic Lateral Sclerosis. (2017, May 22). Retrieved from https://emedicine.medscape.com/article/1170097-overview

Bear, M. F., Connors, B. W., & Paradiso, M. A. (2016). Neuroscience: Exploring the brain. Philadelphia: Wolters Kluwer.

Learn About ALS. (n.d.). Retrieved February 23, 2018, from http://www.alsfoundation.org/learn/facts.htm

Bosco, D. A. (2015) The Role of SOD1 in Amyotrophic Lateral Sclerosis. Nature Education 8(3):4

Rosen, D. R. et al. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62 (1993). doi: 10.1038/362059a0

Borchelt, D. R. et al. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral sclerosis possesses significant activity. PNAS 91, 8292–8296 (1994).

Nagai, M., Re, D. B., Nagata, T., Chalazonitis, A., Jessell, T. M., Wichterle, H., & Przedborski, S. (2007). Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. Nature Neuroscience, 10(5), 615-622. doi:10.1038/nn1876

Ross, C. A., & Poirier, M. A. (2005). What is the role of protein aggregation in neurodegeneration? Nature Reviews Molecular Cell Biology, 6(11), 891-898. DOI: 10.1038/nrm1742

A.B. Reaume, J.L. Elliott, E.K. Hoffman, N.W. Kowall, R.J. Ferrante, et al. Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury Nat. Genet., 13 (1996), pp. 43-47

Gurney, M. E. et al. Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. Science 264, 1772–1775 (1994). doi: 10.1126/science.8209258

Ralph, G. S. et al. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. Nature Medicine 11, 429–433 (2005). doi: 10.1038/nm1205

Raoul, C. et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. Nature Medicine 11, 423–428 (2005). doi: 10.1038/nm1207
(Raoul, C. et al., 2005)

Thomsen, G. M. et al. (2014). Delayed Disease Onset and Extended Survival in the SOD1G93A Rat Model of Amyotrophic Lateral Sclerosis after Suppression of Mutant SOD1 in the Motor Cortex. Journal of Neuroscience, 34(47), 15587-15600. doi:10.1523/jneurosci.2037-14.2014

CRISPR Guide. (n.d.). Retrieved from https://www.addgene.org/crispr/guide/?gclid=Cj0KCQjwuYTYBRDsARIsAJnrUXC6q2_Kvwv4pL-n13i3Gcdb6didI78Q-zi3DQU6Ad_yR0QqS7bqioIaAqXBEALw_wcB

What are genome editing and CRISPR-Cas9? - Genetics Home Reference. (2018). Retrieved from https://ghr.nlm.nih.gov/primer/genomicresearch/genomeediting

Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. J Clin Invest. 2014 Oct;124(10):4154-61. doi: 10.1172/JCI72992. Epub 2014 Oct 1.

Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. Cell. 2014 Jun 5;157(6):1262-78. doi:10.1016/j.cell.2014.05.010. Review.

Sander, J. D., & Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nature Biotechnology, 32(4), 347-355. doi:10.1038/nbt.2842

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nature Protocols, 8(11), 2281-2308. doi:10.1038/nprot.2013.143

Vidyasagar, A. (2018, April 20). What Is CRISPR? Retrieved from https://www.livescience.com/58790-crispr-explained.html

Slack, J. M. (2016). Stem cells: A very short introduction. Oxford: Oxford University Press.

Knoepfler, P. (2015). Stem cells: An insider's guide. Hackensack, NJ: World Scientific Publishing Company Pte.

A, H. B., & B, S. (2011). Potency of Various Types of Stem Cells and their Transplantation. Journal of Stem Cell Research & Therapy, 01(03). doi:10.4172/2157-7633.1000115

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

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell, 131(5), 861-872. doi:10.1016/j.cell.2007.11.019

Naso, M. F., Tomkowicz, B., Perry, W. L., & Strohl, W. R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. BioDrugs, 31(4), 317-334. doi:10.1007/s40259-017-0234-5

Stoica, L., & Sena-Esteves, M. (2016). Adeno Associated Viral Vector Delivered RNAi for Gene Therapy of SOD1 Amyotrophic Lateral Sclerosis. Frontiers in Molecular Neuroscience, 9. doi:10.3389/fnmol.2016.00056

Davis, E., (2017) Choosing Between Lentivirus and Adeno-associated Virus For DNA Delivery Retreieved (2018-05-20) http://www.genecopoeia.com/wp-content/uploads/2017/09/GeneCopoeia-Technical-Note-Lentivirus-vs.-AAV-03-2017.pdf

Vigene Biosciences, 2016. AAV, Adenovirus and Lentivirus-based Gene Delivery Handbook. Retrieved (2018/05/20) at https://www.vigenebio.com/uploads/AAV_Adenvirus_and_Lentivirus_based_Gene_Delivery_Handbook_Vigene.pdf

Ran, et al. (2015). In vivo genome editing using Staphylococcus aureus Cas9. Nature 526, 186.

Follenzi, A., Santambrogio, L., & Annoni, A. (2007). Immune Responses to Lentiviral Vectors. Current Gene Therapy, 7(5), 306-315. doi:10.2174/156652307782151515

Nolbrant, S., Heuer, A., Parmar, M., & Kirkeby, A. (2017). Generation of high-purity human ventral midbrain dopaminergic progenitors for in vitro maturation and intracerebral transplantation. Nature Protocols, 12(9), 1962-1979. doi:10.1038/nprot.2017.078

Appendix

Virus Titration

Two batches of virus were made with the sgRNAs and the samples with the highest virus titers were used from each batch.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	**Sample**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2	Batch	**Ref**	**Ref**	**Ref**	**Mock**	**Mock**	**Mock**	**sgRNA:E2**	**sgRNA:E2**	**sgRNA:E2**	**sgRNA:E3**	**sgRNA:E3**	**sgRNA:E3**
3	**uL**	**0,3**	**1**	**3**				**0,3**	**1**	3	0,3	1	3
4													
5													
6	**LV2**	25,28	23,93	22,86	33,1	33,16	32,77	29,85	27,59	24,61	26,17	22,8	21,85
7	**LV2**	25,59	24	23,11	33,26	32,81	33,01	30,5	27,84	24,86	26,54	23	21,96
8	**LV2**	25,45	24,16	22,95	33,21	33,24	32,65	30,56	27,77	24,81	26,51	22,98	21,97
9	**alb**	27,21	27,52	28,16	27,12	27,83	28,07	28,97	27,79	25,96	28,02	25,97	25,23
10	**alb**	27,44	27,65	28,3	27,51	28,03	28,33	29,19	28,09	26,05	28,3	26,14	25,61
11	**alb**	27,32	27,82	28,24	27,48	27,84	28,2	29,05	28,08	26,11	28,29	26,23	25,56
12													
13													
14													
15													
16	**Av WPRE**	**25,44**	**24,03**	**22,97**	**33,19**	**33,07**	**32,81**	**30,30**	**27,73**	**24,76**	**26,41**	**22,93**	**21,93**
17	**Av alb**	**27,3233**	**27,6633**	**28,2333**	**27,3700**	**27,9000**	**28,2000**	**29,0700**	**27,9867**	**26,04**	**28,20**	**26,11**	**25,47**
18													
19	**delta ct**	**-1,88333**	**-3,63333**	**-5,26000**	**5,82000**	**5,17000**	**4,61000**	**1,23333**	**-0,25333**	**-1,28000**	**-1,79667**	**-3,18667**	**-3,54000**
20	**delta delta**	**3,38**	**1,63**	**0,00**	**11,08**	**10,43**	**9,87**	**6,49**	**5,01**	**3,98**	**3,46**	**2,07**	**1,72**
21		**0,0963**	**0,3238**	**1,0000**	**0,0005**	**0,0007**	**0,0011**	**0,0111**	**0,0311**	**0,0634**	**0,0907**	**0,2376**	**0,3035**
22	**TITER;**	**1,35E+09**	**1,36E+09**	**1,40E+09**	**6,47E+06**	**3,04E+06**	**1,50E+06**	**1,55E+08**	**1,31E+08**	**8,87E+07**	**1,27E+09**	**9,98E+08**	**4,25E+08**
23													
24	**ref titer**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**
25													
26						Titer			Titer				Titer
27		ref	1,37E+09		Mock	3,67E+06		sgRNA:E2	1,25E+08				8,97E+08

Figure A1. Screenshot of the qPCR cycle values for virus titration batch 1 put into an Excel-file to calculate the titer. Only the virus with sgRNA:E3 was used from this virus batch.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	**Sample**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
2	Batch	**Ref**	**Ref**	**Ref**	**Mock**	**Mock**	**Mock**	**sgRNA:E2**	**sgRNA:E2**	**sgRNA:E2**	**sgRNA:E3**	**sgRNA:E3**	**sgRNA:E3**	**LacZ**	**LacZ**	**LacZ**
3	**uL**	**0,3**	**1**	**3**				**0,3**	**1**	3	**0,3**	**1**	3	0,3	1	3
4																
5																
6	**LV2**	25,23	23,98	20,75	38,07	38,07	38,07	28,95	28,01	26,12	27,53	25,69	25,63	29,16	25,59	24,2
7	**LV2**	25,4	24,25	21	37,9	37,9	37,9	29,3	28,02	26,29	27,66	25,9	25,97	29,29	25,85	24,41
8	**LV2**	25,58	24,27	21,08	37,11	37,11	37,11	29,3	28,02	26,38	27,64	25,89	25,96	29,32	25,72	24,41
9	**alb**	27,03	27,98	26,1	28,04	28,46	29,01	28,44	29,04	28,87	26,71	26,96	28,75	27,43	27,56	27,56
10	**alb**	27,22	28,09	26,43	28,14	28,67	29,23	28,66	29,24	29,09	26,87	27,17	28,9	27,59	27,58	27,58
11	**alb**	27,07	28,09	26,26	28,12	28,62	29,18	28,62	29,09	29,1	26,82	27,19	28,69	27,5	27,67	27,67
12																
13																
14																
15																
16	**Av LV2**	**25,40**	**24,17**	**20,94**	**37,69**	**37,69**	**37,69**	**29,18**	**28,02**	**26,26**	**27,61**	**25,83**	**25,85**	**29,26**	**25,72**	**24,34**
17	**Av alb**	**27,1067**	**28,0533**	**26,2633**	**28,1000**	**28,5833**	**29,1400**	**28,5733**	**29,1233**	**29,02**	**26,8000**	**27,1067**	**28,78**	**27,51**	**27,60**	**27,60**
18																
19	**delta ct**	**-1,70333**	**-3,88667**	**-5,32000**	**9,59333**	**9,11000**	**8,55333**	**0,61000**	**-1,10667**	**-2,75667**	**0,81000**	**-1,28000**	**-2,92667**	**1,75000**	**-1,88333**	**-3,26333**
20	**delta delta**	**3,62**	**1,43**	**0,00**	**14,91**	**14,43**	**13,87**	**5,93**	**4,21**	**2,56**	**6,13**	**4,04**	**2,39**	**7,07**	**3,44**	**2,06**
21		**0,0815**	**0,3703**	**1,0000**	**0,0000**	**0,0000**	**0,0001**	**0,0164**	**0,0539**	**0,1692**	**0,0143**	**0,0608**	**0,1903**	**0,0074**	**0,0924**	**0,2404**
22	**TITER;**	**1,14E+09**	**1,56E+09**	**1,40E+09**	**4,54E+05**	**1,90E+05**	**9,33E+04**	**2,30E+08**	**2,26E+08**	**2,37E+08**	**2,00E+08**	**2,55E+08**	**2,66E+08**	**1,04E+08**	**3,88E+08**	**3,37E+08**
23																
24	**ref titer**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**	**1,40E+09**
25																
26						Titer			Titer				Titer			Titer
27		ref	1,37E+09		Mock	2,46E+05		sgRNA:E2	2,31E+08				2,41E+08			2,76E+08
28																

Figure A2. Screenshot of the qPCR cycle values for virus titration batch 2 put into an Excel-file to calculate the titer. The virus with sgRNA:E2 and LacZ were used from this virus batch.

qPCR mRNA Expression Analysis

Two runs were made with the same SOD1-specific primer and cDNA samples to make sure the values were consistent between runs.

Condition 2	SOD1 E2 -	SOD1 E2 +	SOD1 E3 -	SOD1 E3 +	SOD1 LZ +	SOD1 OT	RC17 E2 -	RC17 E2 +	RC17 E3 -	RC17 E3 +	RC17 LZ +	RC17 OT
gene	27,29	26,72	28,11	29,21	26,56	28,51	27,73	28,67	27,94	29,43	27,2	28,55
gene	27,3	26,56	28,65	28,6	26,6	27,87	27,91	28,22	28,43	28,77	26,94	28,09
gene	27,47	26,56	28,44	29,49	26,68	27,97	27,71	28,18	28,33	29,08	26,82	28,2
Actin	27,6	25,86	29,06	27,46	27,69	29,52	29,22	28,19	29,01	27,9	30,36	29,56
Actin	27,93	25,95	29,51	28,12	27,32	29,79	28,84	28,47	29,23	28,08	35	29,61
Actin	27,91	26	29,27	27,9	27,53	30,51	28,88	28,13	29,31	27,99	35	30,01
Gapdh	25,06	23,26	25,99	24,64	24,47	25,61	25,6	24,91	25,54	24,44	24,18	25,27
Gapdh	24,97	23,56	26,28	24,87	24,42	25,75	25,82	25,18	25,72	24,65	24,2	25,22
Gapdh	25,02	23,62	26,17	24,96	24,56	25,88	25,88	25,2	25,77	24,71	24,27	25,08
Av gene	27,35333333	26,6133333	28,4	29,1	26,6133333	28,1166667	27,7833333	28,3566667	28,2333333	29,0933333	26,9866667	28,28
Av Actin	27,81333333	25,9366667	29,28	27,8266667	27,5133333	29,94	28,98	28,2633333	29,1833333	27,99	33,4533333	29,7266667
Av Gapdh	25,01666667	23,48	26,1466667	24,8233333	24,4833333	25,7466667	25,7666667	25,0966667	25,6766667	24,6	24,2166667	25,19
Av HPRT	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
/Actin	-0,46	0,67666667	-0,88	1,27333333	-0,9	-1,8233333	-1,1966667	0,09333333	-0,95	1,10333333	-6,4666667	-1,4466667
dCT actin	0	1,13666667	-0,42	1,73333333	-0,44	-1,3633333	-0,7366667	0,55333333	-0,49	1,56333333	-6,0066667	-0,9866667
ddCT actin	**1**	**0,4548092**	**1,3379276**	**0,3007563**	**1,3566043**	**2,5727893**	**1,6663214**	**0,6814438**	**1,4044449**	**0,3383684**	**64,296427**	**1,9816012**
/GAPDH	2,336666667	3,13333333	2,25333333	4,27666667	2,13	2,37	2,01666667	3,26	2,55666667	4,49333333	2,77	3,09
dCT GAPDH	0	0,79666667	-0,0833333	1,94	-0,2066667	0,03333333	-0,32	0,92333333	0,22	2,15666667	0,43333333	0,75333333
ddCT GAPDH	**1**	**0,5756777**	**1,0594631**	**0,2606164**	**1,1540188**	**0,97716**	**1,2483305**	**0,5272893**	**0,8585654**	**0,2242739**	**0,7405488**	**0,5932313**
/HPRT	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
dCT GAPDH	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
ddCT HPRT	**##########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**##########**	**########**
Average	**1,00**	**0,52**	**1,20**	**0,28**	**1,26**	**1,77**	**1,46**	**0,60**	**1,13**	**0,28**	**32,52**	**1,29**

Figure A3. Screenshot of the qPCR cycle values for SOD1 mRNA expression run 1. The values from the qPCR analysis were put into an in-house Excel-file and the relative expression values were calculated.

Condition 2	SOD1 E2 -	SOD1 E2 +	SOD1 E3 -	SOD1 E3 +	SOD1 LZ +	SOD1 OT	RC17 E2 -	RC17 E2 +	RC17 E3 -	RC17 E3 +	RC17 LZ +	RC17 OT
gene	27,79	26,45	28,17	29,09	27,16	28,18	28,53	27,97	28,61	28,79	27,24	27,96
gene	27,55	26,51	27,9	29,31	26,92	28,08	28,29	28,15	28,68	28,77	27,25	28,16
gene	27,79	26,5	28,15	28,6	26,88	28,13	28,47	27,94	28,69	28,84	27,01	28,04
Actin	27,55	25,58	28,13	27,23	27,21	29,12	28,04	27,77	28,99	27,57	27,85	29,05
Actin	27,58	25,72	27,75	27,26	26,98	29,58	28,23	28,1	28,83	27,71	27,57	29,51
Actin	27,64	25,73	27,97	27,58	27,05	29,74	28,43	28,09	28,93	27,84	27,52	29,35
Gapdh	24,72	22,96	24,89	24,68	24,09	25,61	25,62	24,97	25,49	24,51	23,99	24,91
Gapdh	24,99	23,6	25,19	24,88	24,52	25,86	25,61	25,25	25,77	24,64	24,32	25,44
Gapdh	25,03	23,63	25,22	24,97	24,61	25,76	25,91	25,16	25,71	24,59	24,48	25,18
HPRT												
HPRT												
HPRT												
Av gene	27,71	26,4866667	28,0733333	29	26,9866667	28,13	28,43	28,02	28,66	28,8	27,1666667	28,0533333
Av Actin	27,59	25,6766667	27,95	27,3566667	27,08	29,48	28,2333333	27,9866667	28,9166667	27,7066667	27,6466667	29,3033333
Av Gapdh	24,91333333	23,3966667	25,1	24,8433333	24,4066667	25,7433333	25,7133333	25,1266667	25,6566667	24,58	24,2633333	25,1766667
Av HPRT	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
/Actin	0,12	0,81	0,12333333	1,64333333	-0,0933333	-1,35	0,19666667	0,03333333	-0,2566667	1,09333333	-0,48	-1,25
dCT actin	0	0,69	0,00333333	1,52333333	-0,2133333	-1,47	0,07666667	-0,0866667	-0,3766667	0,97333333	-0,6	-1,37
ddCT actin	**1**	**0,6198538**	**0,9976922**	**0,3478812**	**1,1593638**	**2,7702189**	**0,948246**	**1,0619138**	**1,2983386**	**0,5093279**	**1,5157166**	**2,5847057**
/GAPDH	2,796666667	3,09	2,97333333	4,15666667	2,58	2,38666667	2,71666667	2,89333333	3,00333333	4,22	2,90333333	2,87666667
dCT GAPDH	0	0,29333333	0,17666667	1,36	-0,2166667	-0,41	-0,08	0,09666667	0,20666667	1,42333333	0,10666667	0,08
ddCT GAPDH	**1**	**0,8160145**	**0,8847448**	**0,3895823**	**1,1620456**	**1,3286858**	**1,057018**	**0,9351912**	**0,866537**	**0,3728499**	**0,9287314**	**0,9460576**
/HPRT	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
dCT GAPDH	#DIVISION/0!	########	########	########	########	########	########	########	########	########	#DIVISION/0!	########
ddCT HPRT	**##########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**########**	**##########**	**########**
Average	**1,00**	**0,72**	**0,94**	**0,37**	**1,16**	**2,05**	**1,00**	**1,00**	**1,08**	**0,44**	**1,22**	**1,77**

Figure A4. Screenshot of the qPCR cycle values for SOD1 mRNA expression run 1. The values from the qPCR analysis were put into an in-house Excel-file and the relative expression values were calculated.