Modifying the common marmoset monkey (*Callithrix jacchus*) genome: transgenesis and targeted gene modification in vivo and in vitro

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I. Abstract

The most widely used animal model for biomedical research is the mouse. Yet, the physiological differences between mice and humans sometimes result in findings of questionable translational value. The common marmoset monkey (*Callithrix jacchus*) is a non-human primate, which is gaining more and more interest as a translational model organism. However, methods for the genetic modification, which have been well-established in the mouse for decades, have only recently become available for the common marmoset.

In this study I applied different tools for transgenesis and targeted gene modification using the common marmoset as a model organism. The initial part of the study was performed at the Central Institute for Experimental Animals in Kawasaki, Japan, where I was successful in generating two transgenic common marmoset monkeys using the Early Transposon promoter and *Oct4* and *Sox2* enhancers (EOS) lentiviral vector. The EOS vector mediates pluripotency-associated expression of enhanced Green Fluorescent Protein (eGFP) and, once introduced as a transgene, can be utilized as a fluorescence marker of pluripotent cells. The other parts of my work were accomplished at the German Primate Center in Goettingen. To test EOS activity in the common marmoset, fibroblasts of the EOS transgenic monkeys were reprogrammed to induced pluripotent stem (iPS) cells by non-viral means using the *piggyBac* transposon system. Notably, I was able to reprogram primary fibroblasts making use of only the four classical reprogramming factors SOX2, OCT4, KLF4 and c-MYC, omitting LIN28, which was reported to be essential for common marmoset monkey cell reprogramming. In the end I used a recent approach for targeted gene modification, the CRISPR/Cas9 system, to modify the Parkinson's disease associated gene *LRRK2* in common marmoset primary fibroblasts. In addition to that, I generated immortalized common marmoset monkey fibroblast cell lines by stably introducing the human telomerase reverse transcriptase (*hTERT*) transgene again using the *piggyBac* transposon.

In conclusion, I successfully applied different strategies for the genetic modification of genes in the common marmoset genome. My study will hopefully contribute to an expanded application of the common marmoset in biomedical and translational research.

II. Table of contents

III. Abbreviations

°C	Degree Celsius
µ	micro (10^{-6})
A	adenine
ATP	adenosine triphosphate
bp	base pairs
BSA	Bovine Serum Albumin
C	cytosine
cDNA	complementary DNA
CO_2	Carbon dioxide
CRISPR	Clustered, regularly interspaced, short palindromic repeat
CRISPR/Cas9	CRISPR/CRISPR associated nuclease 9
crRNA	CRISPR RNA
Ctrl	Control
DEPC	diethylpyrocarbonate
dH_2O	distilled Water
ddH_2O	bidistilled Water
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
dNTP	deoxyribonucleotide triphosphate
ds	Double-strand
DSB	Double-strand break
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetatraacetic acid

eGFP	Enhanced green fluorescent protein
EOS	Early transposon promoter and *Oct-4* and *Sox2* enhancers
EOS-EiP	Early transposon promoter and *Oct-4* and *Sox2* enhancers – eGFP- IRES-Puro^r
ES	Embryonic stem (cell(s))
ESC	Embryonic stem cell
ET	Embryo transfer
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
g	gram
G	Guanine
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gly	Glycine
gRNA	Guide RNA
h	Hour(s)
HDR	Homology directed repair
HEK	Human Embryonic Kidney
H_2O	Water
hTERT	Human Telomerase reverse transcriptase
ICM	Inner cell mass
IF	immunofluorescence
Indels	Insertions/deletions
iPS	Induced pluripotent stem (cell(s))
iPSC	Induced pluripotent stem cell
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IRES	Internal ribosomal entry site
kb	kilobase
KH_2PO_4	Monopotassium phosphate

l	liter
LIG4	DNA Ligase4
LRRK2	Leucine-rich repeat kinase 2
LTR	Long terminal repeat
m	milli (10^{-3})
M	molar (mol per liter)
MEFs	Mouse embryonic fibroblasts
$MgCl_2$	Magnesium chloride
min	Minute(s)
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
N	nano (10^{-9})
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NHEJ	Non-homologous end joining
NHP	Non-human primates
NT	Nucleotides
O_2	Oxygen
OPU	Oocyte pick-up
p53	Tumor protein p53
PAM	Protospacer adjacent motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
pH	Power of Hydrogen
POM	Porcine oocyte medium
p.t.	Post transfection
Puro	Puromycin
$Puro^r$	Puromycin resistance
qPCR	Quantitative PCR

RB	Retinoblastoma
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
sec	second(s)
Ser	Serine
sgRNA	Single-guide RNA
SOKM	SOX2, OCT4, KLF4, c-MYC
SOKMLN	SOX2, OCT4, KLF4, c-MYC, LIN28, NANOG
SSEA-4	Stage specific embryonic antigen 4
ssODN(s)	Single-stranded oligodeoxynucleotide(s)
SV40T	Simian virus 40 large T antigen
T	Thymine
T7EN1	T7 endo-nuclease 1
TAE	Tris-acetate-EDTA
TALENs	Transcription activator-like effector nucleases
TERT	Telomerase reverse transcriptase
tg	Transgenic
TRA-1-60	Tumor rejection antigen-1-60
tracrRNA	Trans-activating crRNA
tris	Tris(hydroxymethyl)aminomeethane
T_m	Melting temperature
U	Enzyme Unit
UV	Ultraviolet
V	Volt
v/v	Volume by volume
wt	Wild type
w/v	Weight by volume
ZFNs	Zinc finger nucleases

1. Introduction

The following chapters will give an overview of the model organisms in biomedical research in general and the used common marmoset in detail. I will also give an overview of the main tools for genetic modification applicable in whole organisms or cells. Additionally, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, both highly relevant cell types for biomedical applications and translational research, will be introduced. Finally, possible applications of these tools and techniques for genetic modifications in (cells of) the common marmoset monkey will be described.

1.1 Model organisms in biomedical research

During the history of biomedical research many model organisms contributed significantly to today's biomedical knowledge. Among the most widely used organisms are the fruit fly *Drosophila melanogaster* (Prüßing et al., 2013; Smith et al., 2014) the nematode *Caenorhabditis elegans* (Alexander et al., 2014), and the zebra fish *danio rerio* (Newman et al., 2014; Santoro, 2014). The most commonly used mammalian models by far are rodents. Mainly the mouse model contributed essentially to today's insight into developmental, physiological and molecular processes in mammals. One of the reasons for that is the relative ease to alter the mouse genome. In 1976, the first transgenic mouse with integrated virus DNA and germline transmission was reported (Jaenisch, 1976). In 1980 the first study about transgenesis by DNA microinjection into early mouse embryos was published (Gordon et al., 1980) and one year later also germline transmission of a microinjected transgene was reported (Gordon and Ruddle, 1981).Targeted gene modification, enabled by homologous recombination, in mouse embryonic stem (ES) cells was first reported in 1989. Injection of these gene modified ES cells into mouse blastocysts resulted in chimeric mice, which were bred to obtain mice heterozygous for the modified gene (Capecchi, 1989a; 1989b). The early development of these methods for transgenesis and targeted gene modification had an immense impact on biomedical research. However, due to the phylogenetic distance of the mouse and primates, biomedical research with the mouse as a model organism often reaches its limits regarding the genetical, developmental, behavioral, and physiological comparability to humans (Adams et al., 2003; Seok et al., 2013). These differences sometimes result in questionable translational value of

findings, e.g. in research regarding neurodegenerative diseases (Markou et al., 2009; Nestler and Hyman, 2010) or infectious diseases (Roep et al., 2012).

1.1.1 Non-human primates in biomedical research

Due to their relatively recent common ancestry, non-human primates (NHP) are more similar to humans than rodents. Therefore they represent excellent model organisms for translational research aiming at humans. The similarities in anatomy, morphology, reproduction, development, cognition, social complexity and physiology between humans and NHP indicate the reasonability and validity of translational research with NHP as models (Phillips et al., 2014; Roep et al., 2012; Sasaki, 2015; t Hart et al., 2015).

In the last decade(s) the interest in research with NHP increased and important findings and developments with NHP were made, including transgenesis in rhesus macaques (Chan et al., 2001) and common marmosets (Sasaki et al., 2009) as well as directed gene-modification in rhesus and cynomolgus monkeys (Liu et al., 2014; Niu et al., 2014). This expanded set of experimental tools further promoted the attractiveness and possibly also the relevance of NHP models, especially in research areas like neuroscience, stem cell biology, and reproductive biology as well as for preclinical studies of novel therapeutic treatments.

1.1.2 The common marmoset as a model organism

Old World monkeys, primarily the rhesus macaque (*Macaca mulatta*) and the cynomolgus monkey (*Macaca fascicularis*), have been the main NHP models in biomedical research for decades. The reasons for that are mainly the evolutionary proximity to humans and the ease of access to these species. However, New World monkeys, especially the common marmoset (*Callithrix jacchus)*, gain more and more interest in biomedical research. Among the advantages of the common marmoset over other NHP like the rhesus macaque, are the smaller size, the shorter generation time, and easier colony management (Mansfield, 2003; Ward and Vallender, 2012). Additionally, colony health can be sustained at a high level (Ludlage and Mansfield, 2003), and biosafety issues like herpes B virus infection in rhesus macaques, are no concern in the common marmoset (McCarthy and Tosolini, 1975).

The common marmoset is a non-endangered species naturally living in northeastern Brazil. Common marmosets reach sexual maturity at the age of 1.5 to 2, give birth to approximately 3 to 5 offspring a year and have a bodyweight of about 350 to 450g (Abbott et al., 2003; Tardif

et al., 2003). The large number of possible offspring (rhesus macaques have only one offspring a year), together with the early onset of sexual maturation, is a great advantage of the common marmoset for the establishment of transgenic colonies (Okano et al., 2012).

Because marmosets are susceptible to a multitude of human diseases (Carrion and Patterson, 2012), the common marmoset represents an ideal model for infectious disease research, drug development, and toxicology screening (Mansfield, 2003; Smith et al., 2001; t Hart et al., 2015; Ward and Vallender, 2012). The common marmoset is a valuable and well-established model for specific purposes and already widely used in biomedical research (t Hart et al., 2012). It has been used as a model in infectious disease research, e.g. for Epstein-Barr-virus (Felton et al., 1984), and hepatitis A virus (Pinto et al., 2002), in reproductive biology (Eildermann et al., 2012; Lin et al., 2012), neuroscience (Ando et al., 2008; Jenner et al., 1984), or regenerative medicine (Bernemann et al., 2011; Kitamura et al., 2011; Pluchino et al., 2009). Furthermore, common marmoset embryonic stem (ES) cells (Müller et al., 2009; Sasaki et al., 2005) as well as induced pluripotent stem (iPS) cells (Debowski et al., 2015; Tomioka et al., 2010; Wiedemann et al., 2012) have been derived. The development of basic research resources and tools like whole genome sequencing (Worley et al., 2014), magnetic resonance imaging (MRI) techniques (Hikishima et al., 2011; Yamada et al., 2008), or the development of transgenesis in the common marmoset (Sasaki et al., 2009) further increases the value of the common marmoset as a biomedical model. All these achievements and characteristics of the common marmoset make it an ideal model organism for biomedical research. However, in the common marmoset, applications that allow transgenesis and targeted gene modification have only recently been established.

1.2 Tools for genetic modification

Advancements in research led to improved and new methods that offer new possibilities for biomedical research. The next chapters will give an insight into gene modification methods also applicable in NHPs like the common marmoset.

1.2.1 Lentiviral vectors

Viral vectors in general can be used to transfer DNA into cells. Several different types of viruses, like retroviruses and adenoviruses, are used in research. The most known and used vi-

ruses for gene delivery are retroviruses. Retroviruses can infect cells and integrate a transgene into a host cells' genome through reverse transcriptase activity.

A well-known family of retroviruses are lentiviruses. The main difference between standard retroviruses and lentiviruses is the inability of standard retroviruses to infect non-dividing cells, whereas lentiviruses can efficiently infect actively dividing and non-dividing cell types (Naldini et al., 1996; Vigna and Naldini, 2000).

Lentiviral vectors have been derived from different types of lentiviruses including Human Immunodeficiency Virus Type 1 (HIV-1), Human Immunodeficiency Virus Type 2 (HIV-2) and Simian Immunodeficiency Virus (SIV). Characterized best are the HIV-1 based lentiviral vectors and are therefore mainly used in research. Lentiviral vectors are regarded to offer long-term stable expression of inserted transgenes in vitro and in vivo (Vigna and Naldini, 2000). However, other reports indicate possible silencing of lentiviral vectors due to DNA methylation or histone modification (Antoniou et al., 2013; Ellis, 2005).

1.2.2 The *piggyBac* transposon system

A promising alternative to viral vector based gene transfer into cells are DNA transposons. Transposons are mobile DNA elements that can be utilized for gene integration into a host cell genome. The best-known and established transposon systems are the *Sleeping Beauty* and the *piggyBac* transposon system.

The *piggyBac* transposon system is derived from the cabbage looper moth *Trichoplusia ni* and is a highly efficient transposon system for gene integration (Li et al., 2013; Wilson et al., 2007). The *piggyBac* system consists of two components, a donor plasmid containing the DNA sequence of interest flanked by 5' and 3' terminal repeats, and a plasmid expressing the *piggyBac* transposase enzyme that catalyzes the integration. DNA integration into the host genome occurs at TTAA nucleotide sites (Bauser et al., 1999).

The *piggyBac* system offers virus free integration into a target genome and shows stable expression of the integrated sequences. In contrast to all other known transposon-based systems, it is completely excisable not leaving any residual modifications or mutations in the host genome. This is of particular interest if only transient expression of a transgene and genomic integrity after terminated expression is required. DNA sequences of up to 14kb can be integrated via *piggyBac,* which exceeds the sizes of lentiviral gene transfer (Kim and Pyykko,

2011). Furthermore, in contrast to lentiviral based gene integration, no transgene silencing in *piggyBac* was reported yet.

1.2.3 CRISPR/Cas9 System

A relatively new method for genome modification is the clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR associated nuclease 9 (Cas9) system. The CRISPR/Cas9 system facilitates easy and fast targeted gene modification in virtually all organisms and cell types. Previously, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been developed as tools to modify mammalian genomes (Bibikova et al., 2001; Boch et al., 2009). The CRISPR system, however, has several advantages: i) overall it is faster, easier, and cheaper to use ii) for a new target it is sufficient to change a short DNA stretch without the need to change a whole construct / protein iii) it is possible to target multiple gene loci in one experiment (Cong et al., 2013; Hsu et al., 2014; Wang et al., 2013).

CRISPR/Cas9 originally evolved as an adaptive defense mechanism in archea and bacteria for protection against invading virus or plasmid DNA (Wiedenheft et al., 2012). The system consists of two RNAs, the CRISPR RNA (crRNA), and the trans-activating crRNA (tracrRNA). These two RNAs form a sequence-specific duplex that guides the Cas9 nuclease to an invading foreign DNA, which the nuclease can cleave to protect the host organism. The crRNA contains a sequence of 20 base pairs (bp) that is complementary to the invading DNA and is inserted into the host genome from a previous invasion event. The CRISPR system in bacteria and archaea contains multiple such foreign DNA stretches in the cells genomes and therefore, it can recognize and cleave different invading DNAs.

The CRISPR/Cas9 system used as a tool in biology is derived from the bacteria *Streptococcus pyogenes*. It was shown that the mentioned crRNA and tracrRNA of *Streptococcus pyogenes* can be fused to form a single RNA sufficient for guiding the Cas9 to its target (Jinek et al., 2012). The crRNA:tracrRNA combination is usually referred to as guideRNA (gRNA) (Cong et al., 2013; Jinek et al., 2012; Mali et al., 2013b) or single-guide RNA (sgRNA) (Platt et al., 2014; Ran et al., 2013). The sequence-specific part of the gRNA can be easily exchanged to target virtually any DNA sequence. The Cas9 nuclease can cleave the double stranded (ds) DNA and induces a double-strand break (DSB) (Figure 1.1). DSBs are usually repaired by one of two cell repair mechanisms: i) non-homologous end joining (NHEJ) or ii) homology directed repair (HDR). Both mechanisms can be utilized to modify the target genome. With-

out a repair template present cleaved dsDNA is religated by the NHEJ mechanism, which frequently is inaccurate and introduces mutations in the target region in form of insertions or deletions (indels). These indels can cause frameshift mutations and premature stop codons resulting in a functional gene knock out (Cong et al., 2013; Mali et al., 2013b; Ran et al., 2013). Furthermore, multiple induced DSBs introduced in a larger target region can be used to generate extended deletions in the target genome (Cong et al., 2013). When a homologous repair template is provided together with the CRISPR/Cas9 components, HDR can be utilized to generate precise gene modifications. Possible applications include introduction of point mutations and insertion of a DNA sequence into the target genome.

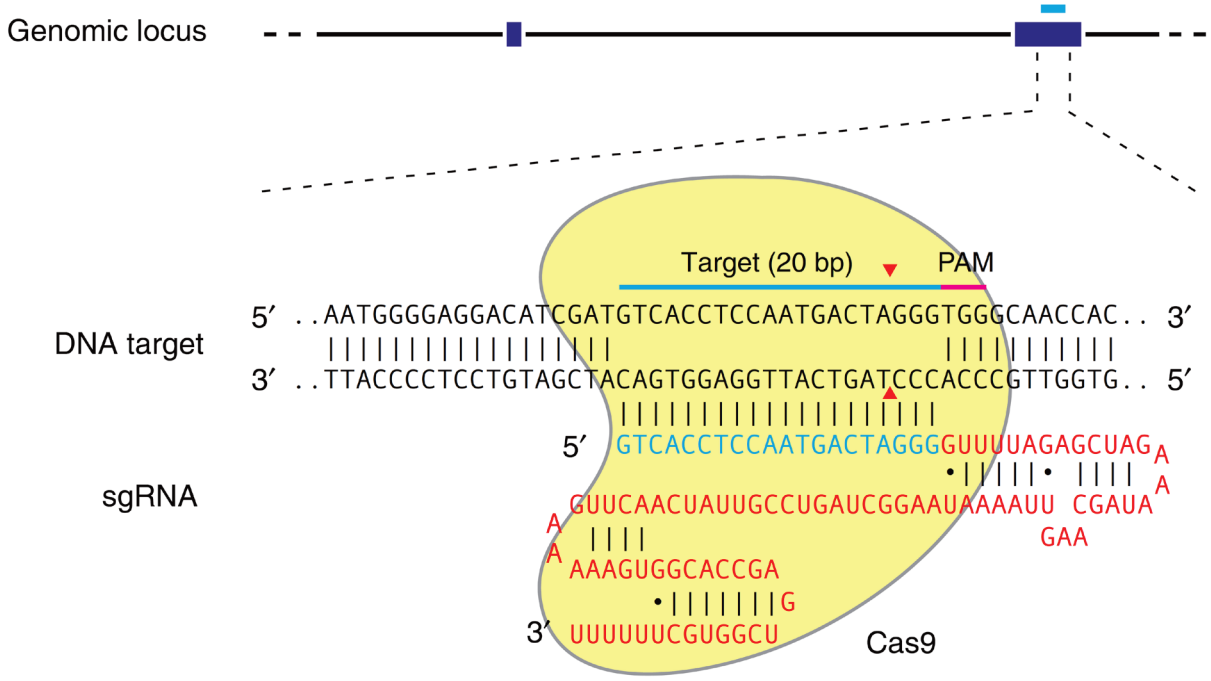

Figure 1.1: Overview of the RNA-guided Cas9 nuclease. The Cas9 nuclease from *Streptococcus pyogenes* is shown in yellow. Cas9 is guided to the genomic DNA ("DNA target") by the guide RNA ("sgRNA") consisting of a 20-nt guide sequence (marked in blue) and the sgRNA scaffold (marked in red). The 20-nt guide sequence pairs with the DNA target sequence (also called protospacer; blue bar), directly upstream of the essential 5′-NGG protospacer adjacent motif (PAM; pink bar). Cas9 introduces a DSB (red triangle) ~3 bp upstream of the PAM. (Modified from Ran et al. 2013)

The only restrictions for the use of the CRISPR/Cas9 system are the following: i) the target region, also called protospacer (Figure 1.1), needs to be of the length of 20bp, and ii) the target/protospacer need to be directly followed by a protospacer adjacent motif (PAM) consisting of NGG (Jinek et al., 2012) (Figure 1.1), where N stands for any nucleotide. This means that any sequence of the form 5' NNNNNNNNNNNNNNNNNNNGG 3' can be targeted. The PAM sequence is important for Cas9 nuclease target recognition (Jinek et al., 2012; Wiedenheft et al., 2012). However, the PAM is only present in the target sequence and not

included in the gRNA sequence. Possible target sites can be found approximately every 8-12bp in the human genome and therefore targeted gene modification is possible at virtually any locus in the genome (Cong et al., 2013; Hsu et al., 2013; Ran et al., 2013).

Targeted gene modification with the CRISPR/Cas9 system has already been reported for numerous mammalian species including human (Jinek et al., 2013), mice (Wang et al., 2013), the cynomolgus monkey (Niu et al., 2014), and pig (Whitworth et al., 2014), but is also applicable to non-mammalian species like the zebrafish (Hwang et al., 2013). Moreover, a recent controversially discussed publication reported the use of CRISPR/Cas9 in human embryos (Liang et al., 2015).

A concern that needs to be addressed when working with the CRISPR system is the possibility of off-target effects (Fu et al., 2013; Pattanayak et al., 2013). Previous reports suggested that for proper target recognition the gRNA and target DNA need to be 100% complimentary in the PAM sequence and the 7-12 base pairs adjacent to the PAM (gRNA 3' end). Mismatches at the 5' end of the gRNA, however, are tolerated and can lead to off-target effects (Cong et al., 2013; Jiang et al., 2013; Jinek et al., 2012).

So far, no studies have been reported that show the functionality of CRISPR/Cas9 in the common marmoset.

1.3 Pluripotent stem cells

Pluripotent stem cells play an important role in biomedical research. They have a high potential with regard to disease modeling and are promising candidates for cell replacement therapies.

1.3.1 Embryonic stem (ES) cells

In mammalian fertilization fusion of both the male and female gametes results in the formation of the zygote. During early development the zygote undergoes several cleavage divisions and gives rise to the blastocyst. The blastocyst consists of an outer single layer of cells, the trophoblast, and an inner accumulation of cells, called the inner cell mass (ICM) or embryoblast. The ICM finally gives rise to the embryo proper. If cultured in vitro under appropriate conditions, the isolated ICM can give rise to embryonic stem (ES) cells.

The first ES cells were generated independently by two groups in 1981 (Evans and Kaufman, 1981; Martin, 1981) from mouse embryos, the first human ES cells were generated in 1998 (Thomson et al., 1998). ES cells from marmosets were first reported in 1996 (Thomson et al., 1996) and later also others successfully generated marmoset ES cells (Müller et al., 2009; Sasaki et al., 2005).

Embryonic stem cells have the potential to proliferate indefinitely in vitro and they are pluripotent meaning that they can differentiate into any cell type of the three embryonic germ layers (ectoderm, mesoderm, endoderm). These characteristics made them interesting for studying early development and cell differentiation. Furthermore, ES cells have great potential for possible cell replacement therapies (Smith, 2001; Solter, 2006) of so called degenerative diseases.

In mouse genetically modified ES cells, when injected into embryos, eventually give rise to chimeric mice, which subsequently are bred and used as in vivo disease models.

ES cells form colonies with a tightly packed morphology, are usually cultured on mouse embryonic fibroblasts (MEFs) as feeder layers, and express several characteristic marker genes, e.g. OCT-4, TRA-1-60, SSEA-4 (Boiani and Schöler, 2005), indicative for the pluripotent, undifferentiated state of the cells; so called pluripotency markers.

Due to their embryo origin, especially human ES cells are highly controversial in the public. New methods nowadays allow the generation of ES cell like cells from somatic cells, thereby circumventing the usage of embryos.

1.3.2 Induced pluripotent stem (iPS) cells

A revolutionary development for biomedical research was the “reprogramming” of somatic cells via ectopic expression of pluripotency-associated factors by Takahashi and Yamanaka in 2006.

Takahashi and Yamanaka screened 24 candidate genes that are highly expressed in ES cells for their potential to induce pluripotency in somatic cells. They found that four transcription factors, SOX2, OCT4, KLF4 and c-MYC (SOKM) are sufficient to reprogram murine fibroblasts into so-called induced pluripotent stem (iPS) cells. This method to generate pluripotent cells without the necessity of destroying embryos was later also successfully applied by several others (Okita et al., 2007; Wernig et al., 2008). Other reprogramming factors, LIN28 and

NANOG, were sometimes used in exchange for KLF4 and/or c-MYC (Haase et al., 2009; Yu et al., 2009; 2007). Especially the exclusion of c-MYC is pursued (Wernig et al., 2008) as it is a proto-oncogene and enhances the tumorigenicity of iPS cells (Okita et al., 2007).

Induced PS cells show highly similar properties to ES cells: morphology, growth features, maintenance of pluripotency and expression of specific marker genes (Nakagawa et al., 2008; Okita et al., 2007; Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). Yet, iPS cells are not identical to ES cells, which is shown by the slightly different gene-expression signatures and DNA methylation status (Chin et al., 2009; Kim et al., 2010; Takahashi and Yamanaka, 2006). Nonetheless, the possibility to generate iPS cells from human somatic cells offers great opportunities for research and clinical applications as cell replacement therapies (An et al., 2012; Shaer et al., 2014; Stern and Temple, 2011; Zeng and Couture, 2013). Induced PS cells can already be used for pharmacological studies and to generate disease models by derivation of disease-specific stem cells. For instance, research with iPS cells from Parkinson's disease patients is ongoing (Loewenbrück and Storch, 2011; Soldner et al., 2009)

The first iPS cells were generated by retroviral/lentiviral based gene integration of the reprogramming factors. Use of viruses limits the clinical application of iPS cells because of the high tumorigenicity of the iPS cells due to genome modification resulting from random proviral genomic integration and ectopic expression of potential oncogenes. To obtain transgene free iPS cells, several alternatives to retroviral transduction can be applied. Reprogramming was also achieved employing adenoviral or episomal non-integrating vectors (Stadtfeld et al., 2008; Yu et al., 2009) and integrating but excisable vectors like the cre-recombinase excisable virus (Soldner et al., 2009), or the *piggyBac* transposon system (Kaji et al., 2009; Woltjen et al., 2009). Moreover, DNA free reprogramming through consecutive transfection of recombinant proteins (Kim et al., 2009) or in vitro transcribed mRNA (Plews et al., 2010; Tavernier et al., 2011; Warren et al., 2010; Yakubov et al., 2010) was realized. However, reprogramming using proteins circumventing any nucleic acid could not be repeated for far. Additionally, methods relying on repeated transfection of episomal vectors or recombinant proteins show very low reprogramming efficiencies (Kim et al., 2009; Yu et al., 2009).

Induced PS cells were also generated from the common marmoset monkey (Debowski et al., 2015; Tomioka et al., 2010; Wiedemann et al., 2012). However, iPS cell generation from common marmoset fibroblasts proofed to be more challenging as the derivation of iPS cells from mouse or human fibroblasts. Tomioka et al. reported that the four factors SOKM initial-

ly used for reprogramming were not sufficient for the reprogramming of common marmoset cells. The two additional reprogramming factors LIN28 and NANOG were shown to be necessary for the generation of stably reprogrammed common marmoset iPS cells (Tomioka et al., 2010). However, some groups reported a successful reprogramming of marmoset somatic cells with just the classical four factors, but with a morphology less comparable to marmoset ES cells then in six factor reprogrammed iPS cells (Wiedemann et al., 2012; Wu et al., 2010). Research with iPS cells in the common marmoset allows modeling of diseases and offer ideal prerequisites for preclinical testing of cell replacement therapies.

1.4 Possible applications for gene modification in the common marmoset

1.4.1 EOS-EiP transgenic common marmosets

A major obstacle in disease modeling in the common marmoset was the lack of protocols for the genetic modification of marmosets. In 2009, Sasaki et al. were the first to generate transgenic common marmosets. This study was at the same time the first that showed germline transmission of a transgene in NHPs. This achievement opened the possibility to mimic a subset of human diseases in NHP. Such models would be of great value to study disease progression as well as efficacy of new therapeutic drugs. Furthermore, in validated disease models new treatment methods like cell replacement therapy and gene therapy can be tested (Chan and Yang, 2009; Sasaki et al., 2009).

For the integration of a transgene Sasaki et al. used lenti-viral vector based gene delivery. Lenti-viral vectors proofed to be an ideal tool for transgenesis as shown for several different species like e.g. pigs and bovine (Hofmann et al., 2003), mice (Lois et al., 2002) and rhesus macaques (Yang et al., 2008). This efficient gene delivery method allows a minimal number of animals used for transgenesis experiments, thereby reducing ethical concerns associated with NHP research (Chan and Yang, 2009).

Ideal for the generation of transgenic marmosets are one-cell stage embryos, generated via in vitro fertilization (IVF) (Tomioka et al., 2012). To obtain a high number of oocytes necessary for IVF, animals are superovulated by treatment with follicle stimulating hormone (FSH), as it increases oocyte generation (Marshall et al., 2003; Sasaki et al., 2009). Oocytes are then colleted from ovaries by aspiration with a needle and syringe. The one-cell stage embryos

obtained by IVF are subsequently injected with a virus to generate transgenic animals. The advantage of one-cell stage embryos in comparison to e.g. blastocyst stage embryos is a higher possibility for virus integration into all germ layers and therefore a lower possibility for a genetic mosaic in the developing offspring. For an efficient IVF several oocyte/embryo handling techniques like in vitro maturation (IVM) and in vitro culture (IVC) are of great importance (Tomioka et al., 2012). With the help of IVM collected, immature, not yet fertilization competent oocytes can be matured for a highly efficient IVF. After IVF, IVC of the fertilized oocyte (one cell-stage embryo) is used to assure efficient development of embryos for embryo transfer into surrogate mothers. The mentioned methods are basically essential for the generation of genetically modified common marmosets, which may represent an advanced biomedical research model.

The establishment of a pluripotency reporter system in the common marmoset, already utilized in mice and in human cell lines, would be highly beneficial for biomedical research.

In human and mouse cells the *Oct4*-promoter driven expression of Green fluorescent protein (GFP) is widely used as a reporter for pluripotent cells (Boiani et al., 2002; Gerrard et al., 2005; Szabó et al., 2002). The homozygous OG2 mouse strain is used for crossbreeding, for example with disease model strains, to obtain experimental mice heterozygous for *Oct4*-GFP (Boiani et al., 2002; Szabó et al., 2002). In 2009, Hotta et al. developed an alternative pluripotency reporter to the *Oct4*-GFP reporter system: the **E**arly Transposon promoter and ***O**ct4* and ***S**ox2* enhancers (EOS) lentiviral vector containing an enhanced GFP (eGFP)-IRES-Puro^r (EiP) cassette (Figure 1.2).

Figure 1.2. EOS lentiviral vector. Ψ$^+$: Enhanced packaging signal, RRE: Rev response element, *Oct4* (red diamond) and *Sox2* (green triangle) binding sites inside the enhancer element, the Early Transposon promoter is located upstream of eGFP and indicated in red, IRES: Internal ribosomal entry site, Puro^r: Puromycin resistance, SIN: Self inactivating deletion. (Figure from Hotta et al., 2009)

The EOS-EiP reporter mediates enhanced GFP (eGFP) expression through an Early Transposon (ETn) promoter that showed high activity in mouse ES cells. To circumvent viral vector silencing and to achieve pluripotent cell specific expression OCT4 and SOX2 binding motifs from ES cell-specific enhancers were added (Figure 1.2). A major advantage of this system is the possibility not only to visually identify pluripotent cells but also to select them using Puromycin (Hotta et al., 2009). In experiments aiming at the induction of pluripotency in

mouse and human fibroblasts, it was possible to enrich emerging reprogrammed cells for the isolation of iPS cell lines using this EOS-EiP reporter. Additionally, after differentiation of iPS cells, EOS-eGFP expression is extinguished again due to *Oct4* and *Sox2* silencing. Hence, with regard to a potential application of the cells, residual pluripotent and potentially teratoma forming cells are marked by eGFP expression.

In summary, murine and human iPS cells can be monitored regarding their developmental state in real time. However, visualization of the differentiation status of common marmoset cells has not been achieved yet.

1.4.2 Establishment of CRISPR/Cas9 on Parkinson's disease related gene LRRK2

Parkinson's disease (PD), after Alzheimer's disease, is the second most common progressive neurodegenerative disease. PD affects approximately 0.5 to 1% of the population at 65 years of age, increasing to 1 to 4% in the population over 80 years of age (Nussbaum and Ellis, 2003). Furthermore, PD has an increasing prevalence due to the increasing life expectancy in todays societies (de Lau and Breteler, 2006).

For a long time PD was regarded to be a solely sporadic disease caused by environmental factors until first studies showed possible hereditary causes linking mutations in the gene α-synuclein to familial cases of PD (Polymeropoulos et al., 1996; 1997). Today it is known that approximately 90% of all PD cases are sporadic and 10% are hereditary (Thomas and Beal, 2007). The identified genes involved in hereditary cases of PD include α-synuclein and leucine-rich repeat kinase 2 (LRRK2) (Klein and Westenberger, 2012; Trinh and Farrer, 2013).

An important way to better understand PD is the use of PD animal models. The most promising Parkinson models so far were induced with the chemical reagent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Forno et al., 1993) or with 6-OHDA (Schwarting and Huston, 1996). Especially in non human primates like common marmoset and rhesus macaque these chemicals cause clinical symptoms highly similar to PD in humans (Porras et al., 2012). These models contributed to a better understanding of PD and also helped to test new therapies for treating PD symptoms (Betarbet et al., 2002; Porras et al., 2012). However, Parkinson models based on MPTP can only model some acute clinical and pathological attributes of PD (Betarbet et al., 2002). Furthermore, these models fail to display the progressive nature of PD (Betarbet et al., 2002; Porras et al., 2012).

In contrast to the immediate induction of the PD symptoms in chemically induced models, genetic models (most likely will) show a development and progression of the disease symptoms better reflecting the clinical progression seen in patients. Different transgenic mouse models for PD were established by overexpressing human α-synuclein gene. These models display different PD symptoms like age dependent loss of dopaminergic neurons (Betarbet et al., 2002; Janezic et al., 2013; Masliah et al., 2000; Porras et al., 2012) motor deficits (Janezic et al., 2013; Masliah et al., 2000; van der Putten et al., 2000) and α-synuclein positive inclusions (Masliah et al., 2000; van der Putten et al., 2000). However, the short life span of mice limits the investigation of the progressive features of PD. In addition, brain development and behavior are not entirely comparable to humans resulting in a relatively low translational value of findings made in mice (Belmonte et al., 2015; Phillips et al., 2014). Another possible target for transgenic animal models is the LRRK2 gene. Mutations in LRRK2 account for 4% of hereditary cases of PD and 1% of sporadic cases of PD which make them the most common genetic cause for PD (Healy et al., 2008; Kett and Dauer, 2012). The most common of the LRRK2 mutations is the Gly2019Ser mutation.

In recent years several groups reported LRRK2 Gly2019Ser transgenic and knockin mice (Li et al., 2010; Lin et al., 2009; Melrose et al., 2010; Ramonet et al., 2011). However, these mice models only show a limited number of PD symptoms.

A LRRK2 Gly2019Ser primate model is highly desired because it might exhibit more similar symptoms to humans. It could help to better understand the progressive nature of PD on the organismic level in a close relative of humans. Moreover, a LRRK2 transgenic NHP would allow the testing of new experimental treatments of PD. Especially the similarities between humans and common marmosets in social behavior and brain development make common marmosets an ideal model organism for studying PD (Dell'Mour et al., 2009; Okano et al., 2012).

1.4.3 Generation of immortalized common marmoset fibroblast cells

Human somatic cells, like fibroblasts, can only undergo a limited number of cell divisions in vitro before they enter a state of growth arrest termed “senescence” (Hayflick, 1965). Cells in senescence by definition cannot further divide. In contrast, cancer cells were found to be able to circumvent senescence and to continue proliferation in cell culture beyond their normal lifespan (Mathon and Lloyd, 2001). However, after some additional cell divisions cancer cells still proliferate, but also display high rates of apoptosis, a state that is named “crisis”. Crisis

results from major abnormalities in chromosomal structure (chromosomal shortening) (Mathon and Lloyd, 2001). However, some cells are able to also circumvent crisis; these cells are immortal.

The mechanism behind senescence and crisis were found to be based on telomere shortening (Wright and Shay, 1992). Telomeres are the ends of the chromosomes that are shortened with every cell cycle (Harley et al., 1990). When the telomeres reach a critical length, cells enter senescence (Wright and Shay, 1992). By inactivation of tumor suppressor genes like *p53* and retinoblastoma (*RB*), cells can overcome senescence (Mathon and Lloyd, 2001). However, the telomeres continue to shorten and result in chromosomal instability that causes crisis. The enzyme telomerase is a ribonucleoprotein enzyme that elongates telomeres and its activity allows cells to overcome crisis and become immortal. Telomerase consist of two subunits, a template RNA and the catalytic protein telomerase reverse transcriptase (TERT), which uses the RNA template to elongate the telomere DNA (Kilian et al., 1997; Meyerson et al., 1997). The template RNA was found to be expressed in all tissues irrespective of telomerase activity (Avilion et al., 1996). In contrast, *TERT* is inactive in most somatic cells, but active in the germline, pluripotent stem cells, some types of adult stem cells, cancer cells and in vitro immortalized cells. Therefore, *TERT* is regarded as the determinant of telomerase enzyme activity (Meyerson et al., 1997; Nakamura et al., 1997).

Different studies showed that exogenous expression of human *TERT* (*hTERT*) alone was sufficient to immortalize cells of several species. Human *TERT*-based immortalization was, among others, reported for different human cell types (Böcker et al., 2008; Darimont et al., 2003; Salmon et al., 2000), sheep fibroblasts (Cui et al., 2002), different canine cells (Techangamsuwan et al., 2009), several types of porcine cells (Oh et al., 2007), and for rhesus macaque mesenchymal stem cells (Gao et al., 2008). Even though multiple groups reported a normal phenotype for their immortalized cells, other groups showed that *hTERT* immortalized cells can exhibit cancer-associated changes and neoplastic transformation (Belgiovine et al., 2008; Harley, 2002; Mondello et al., 2003; Zongaro et al., 2005). Immortalization of common marmoset cells with *hTERT* is not reported yet. Such cells would be especially helpful for preliminary experiments before testing/experiments in vivo are carried out.

1.5 Aim of this study

The aim of this study was to increase the available application possibilities of the common marmoset as a model organism in biomedical research. Therefore, it was planed to generate transgenic common marmosets carrying the pluripotency specific EOS-EiP marker to obtain a pluripotency reporter system in the common marmoset. Additionally, the CRISPR/Cas9 system for targeted gene modification should be established in the common marmoset. As a first target for CRISPR/Cas9 based gene modification, the LRRK2 Gly2019Ser mutation of the LRRK2 gene was chosen, which is involved in hereditary and sporadic cases of PD. Furthermore, it was planned to generate immortalized common marmoset fibroblasts to obtain cells that can proliferate indefinitely in culture and would be helpful for e.g. preliminary in vitro experiments before in vivo experiments are considered.

2. Materials & Methods

In this study, chemicals of AppliChem (Darmstadt), Amersham Pharmacia Biotech (Freiburg), Biozym (Hessisch Oldendorf), Fluka (Neu-Ulm), Merck (Darmstadt) and Sigma-Aldrich (Deisenhofen) were used. Enzymes and appropriate buffers were obtained from Applied Biosystems/Ambion (Darmstadt), New England Biolabs (Frankfurt am Main) and Roche (Mannheim). For culturing and amplification of bacteria, media from Sigma-Aldrich (Deisenhofen) were used. Materials for cell culture and cell transfection were obtained from Bio Whittaker (USA), Gibco (Berlin), Invitrogen (Karlsruhe), Promega (Mannheim), Applied Biosystems/Ambion (Darmstadt) and Mirus Bio (Madison, USA). Antibiotics were used from Biochrom (Berlin) and Roche (Mannheim). All solutions were prepared in bi-distilled water (ddH_2O) or in DEPC treated ddH_2O. If necessary, solutions were sterilized by autoclaving (20 min; 121 °C). Several methods were used from: Tobias Kahland, Diploma Thesis ”Establishing methods for reprogramming differentiated cells from the Common Marmoset monkey (Callithrix jacchus) using modified mRNA” (2011).

2.1 Laboratory Equipment

Equipment	**Manufacturer**
Automated Enzyme ImmunoassayAnalyzer AIA-900	Tosoh, Tokyo, Japan
Axio Observer Z1	Carl Zeiss, Oberkochen, Germany
Biometra® T3000 Thermocycler	Labrepco, Horsham, USA
Drying Oven, MOV-112	Sanyo, Moriguchi, Japan
Eppendorf Centrifuge	Eppendorf AG, Hamburg, Germany
Femto Jet Express	Eppendorf AG, Hamburg, Germany
Gel iX Imager	Intas, Göttingen
HeatingBlockThermostat, TH 21	BioTech, Bergheim, Austria
Heraeus incubator	Thermo Scientific, Waltham, USA
Horizon® 58 Gel Electrophoresis Apparatus	LABRepCo, Horsham, England
IKA®RCT basic safety control, #3810000	IKA, Staufen, Germnay

LSRII cytometer	BD Biosciences, East Rutherford, USA
Microcentrifuge Heraeus Fresco 21, #75002425	Thermo Scientific, Waltham, USA
Multifuge Heraeus, 1 S-R	Thermo Scientific, Waltham, USA
Mupid®-ex electrophoresis system	Mupid Co, Tokyo, Japan
NanoPhotometer TM UV/Vis Spectrophotometer	Implen, München, Germany
Narishige micromanipulator	Narishige, Tokyo, Japan
4D-Nucleofector™ System Core unit AAF-1002B X unit, AAF-1002X	Lonza, Basel, Switzerland
PowerEase™500 Power Supply, #EI8700	Invitrogen, Karlsruhe, Germany
Thermomixer® comfort	Eppendorf AG, Hamburg, Germany
Water Jacket Multi Gas Incubator	Astec Co. Ltd., Fukuoka, Japan

2.2 Polymerase chain reaction (PCR)

2.2.1 PCR conditions

PCR-Reactions were carried out with KOD Hot Start Polymerase (Merck, Darmstadt) in a T3000 Thermo Cycler from Biometra (Goettingen). The used program was as follows:

Table 2.1: PCR program for KOD Hot Polymerase. (Taken from KOD Hot Start Polymerase Manual)

	Target size			
Step	**< 500 bp**	**500–1000 bp**	**1000–3000 bp**	**> 3000 bp**
1. Polymerase activation	95°C for 2 min	95°C for 2 min	95°C for 2 min	95°C for 2 min
2. Denature	95°C for 20 s	95°C for 20 s	95°C for 20 s	95°C for 20 s
3. Annealing	Lowest Primer Tm°C for 10 s			
4. Extension	70°C for 10 s/kb	70°C for 15 s/kb	70°C for 20 s/kb	70°C for 25 s/kb

For sufficient amplification, 30-40 cycles were performed.

Each PCR reaction (50 µl) contained:

~50 ng (Plasmid) DNA or 50-100 ng genomic DNA
3 µl of each 5 µM Primer
5 µl 10 x Buffer for KOD Hot Start Polymerase

5 µl 2 mM dNTPs
3 µl 25 mM $MgSO_4$
1 µl KOD Hot Start Polymerase
X µl ddH_2O

The melting temperatures (T_m) of the primers were calculated by the following equation:

T_m = (G/C) x 4°C + (A/T) x 2°C - (Base-mismatches) x 4°C - 4°C

All used primer pairs are listed in 6.1.

2.3 *Escheria coli* (*E. coli*) cell culture

2.3.1 Used *E. coli* strain

The NEB 10-beta Competent *E.coli* (High Efficiency) cell strain was used for DNA amplification. The strain genotypes was: Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- ϕ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC) (NEB).

2.3.2 Culture medium for *E.coli*

LB medium was prepared by suspending 20 g of LB Broth (Sigma-Aldrich; #L3022) in 1 l of distilled water. To prepare LB-agar-plates, 35 g of "ready-to-use" LB agar (Sigma-Aldrich; #L2897) were suspended in 1 l of distilled water. Both the medium and the medium-agar suspension were autoclaved for 15 min at 121 °C. The autoclaved medium was stored at room temperature and incubated for 30 min at 37 °C prior to usage.

The liquified-agar-medium was cooled to roughly 50 °C. Ampicillin (final concentration: 100 µg/ml) was added before plating the agar-medium into sterile petri-dishes.

LB (Lysogeny-Broth)-Medium:

1%	(w/v)	Tryptone
0.5%	(w/v)	Yeast extract
0.5%	(w/v)	NaCI

2.3.3 Transformation of NEB10-beta competent *E. coli*

NEB10-beta (NEB) competent E. coli cells were thawed on ice for 10 minutes. 2 µL of the ligation reaction or 0.2µl of plasmid DNA was added, mixed carefully and incubated on ice for 30 min. The cells were heat-shocked at 42 °C for 30 sec, followed by incubation on ice for 5 min. Each transformation was diluted in 950 µL SOC-medium and incubated for 1 h, at 37 °C at 300 rpm. Afterwards, the cells were plated on a prewarmed selective agar plate containing 100µg/mL ampicillin for selection of transformed bacteria. The plates were incubated at 37 °C for 15-17h.

Super Optimal Broth with Catabolite repression (SOC) Medium:

0.4% (w/v)	**Tryptone**
0.5% (w/v)	Yeast extract
15 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4$
20 mM	Glucose

2.4 Cloning of DNA fragments

In this study the following plasmid vectors were used:

Cloning vector

pcDNA3.1(-)

Lentiviral packaging plasmids

pCAG-HIVgp

pCMV-VSV-G-RSV-Rev

Both lentiviral packaging plasmids were obtained from RIKEN BioResource Center DNA Bank (RIKEN, Japan).

Lentiviral pluripotency reporter plasmid:

PL-SIN-EOS-C(3+)-EiP

The plasmid PL-SIN-EOS-C(3+)-EiP was a gift from James Ellis (Addgene plasmid # 21313).

Reprogramming plasmids:

pTT-PB-SOKMLN-puro

pTT-PB-SOKM-puro

pcA3-PBase-Tomate

All plasmids used for reprogramming were provided by Katharina Debowski (Debowski et al., 2015).

hTERT plasmids

pBABE-neo-hTERT

The plasmid pBABE-neo-hTERT was a gift from Bob Weinberg (Addgene plasmid # 1774).

pTT-PB-hTERT-puro

CRISPR/Cas9 plasmids

pSpCas9(BB)-2A-Puro (PX459)

The plasmid PX459 was a gift from Feng Zhang (Addgene plasmid # 48139).

All Addgene plasmids are available at: http://www.addgene.org/

2.4.1 DNA restriction digestion with endonucleases

For restriction digest of DNA, restriction endonucleases (NEB) were used at a concentration of 5-10 U enzyme/µg plasmid DNA. The reaction was performed in 1 x incubation buffer (NEBuffer 1-4) and 1 x BSA, at 37 °C. In case of identical buffer requirements of the enzymes, incubation with several enzymes was performed simultaneously. Otherwise, buffer conditions were set in between the different incubations by adding appropriate salts. Restriction enzymes are stored in 50% glycerol, which has an inhibitory effect on enzyme activity. Therefore the amount of enzyme in a reaction should not exceed 10% of the total reaction

volume. Restriction analysis was performed in a total volume of 20 µl. Plasmid vectors were digested for at least 4 h. Otherwise, the reaction was incubated for 2 h followed by heat inactivation of the restriction endonucleases at 65 °C for 20 minutes.

2.4.2 Phosphatase treatment of linearized vector

Antarctic Phosphatase (NEB, #M0289S) was used to catalyze the removal of 5' phosphate groups of digested DNA plasmids to prevent re-circularization. De-phosphorylation was performed using ~2 µg digested vector DNA, 1/10 of the volume of Antarctic Phosphatase supplemented with 10 x Antarctic Phosphatase Reaction Buffer (NEB, #B0289S) to a final concentration of 1 x. The reaction was incubated for 1 h at 37 °C. The enzyme was heat inactivated at 65 °C for 20 min.

1 x Antarctic Phosphatase Reaction Buffer (pH 6,0):

50 mM	**Bis-Tris-Propane-HCl**
1 mM	$MgCl_2$
0.1 mM	$ZnCl_2$

2.4.3 Plasmid-safe treatment

To remove residual single stranded DNA from ligation reactions, ligations were treated with Plasmid-Safe™ ATP-Dependent DNase (Epicentre). Therefore 0.3 µl Plasmid-Safe™ ATP-Dependent DNase (Epicentre), 1.7 µl Plasmid-Safe 10x Reaction Buffer, 1.5 µl ATP (10 mM) and 3.5 µl ddH_2O were added to a 10 µl ligation reaction mixture and incubated for 30 minutes at 37 °C.

2.4.4 Ligation

For general ligation, up to 1 µg of insert DNA and ~50 ng of digested and de-phosphorylated vector DNA were incubated with 1 U T4 DNA ligase (Roche) in 1 x ligation buffer (Roche). 1 µl of 25 mM $MgCl_2$ was added to the mixture. The final volume was set to 10 µl. Ligation reactions were incubated at room temperature for at least 1 h. T4 DNA ligase was heat inactivated at 65°C for 20 min.

1x T4 DNA Ligase Buffer
50 mM Tris-HCl
10 mM $MgCl_2$
1 mM ATP
10 mM Dithiothreitol
pH 7.5 at 25°C

2.4.5 Generation of the pTT-PB-hTERT-puro plasmid for cell immortlization

The pBABE-neo-hTERT plasmid was a gift from Bob Weinberg (Addgene plasmid # 1774) and was the source of the *hTERT* cDNA. The pTT-PB-SOKMLN-puro plasmid (Debowski et al., 2015) was provided by Katharina Debowski and was used as the backbone for *piggyBac* based transposition activity. pBABE-neo-hTERT and pTT-PB-SOKMLN-puro were both digested with *Eco*RI (NEB) and *Sal*I (NEB) as described in 2.4.1. Both fragments of interest were segregated on an agarose gel (2.7.1) and extracted from the gel as described in 2.7.2. The *hTERT* cDNA was ligated into the pTT-PB-puro (without SOKMLN) backbone as described in 2.4.4. The resulting plasmid pTT-PB-hTERT-puro was transformed into NEB10-beta competent E.coli cells (2.3.3). Afterwards, DNA was isolated using Maxi preparation (2.5.2).

2.5 Plasmid DNA preparation from *E. coli* cells

2.5.1 Mini preparations of plasmid DNA

For small-scale plasmid isolation, 5ml LB medium were inoculated with a single colony and incubated over night at 37°C and permanent shaking. The plasmid DNA was isolated with the Qiaprep Spin Miniprep Kit (Qiagen), or the NucleoSpin Plasmid Kit (MACHEREY-NAGEL). Both kits were used according to manufacturer's instructions.

2.5.2 Maxi preparation of plasmid DNA

For large-scale plasmid isolation, 100ml LB medium were inoculated with a single colony and incubated over night at 37°C and permanent shaking. DNA plasmid Maxi preparations were carried out with the Qiagen Plasmid Maxi Kit (Qiagen) according to manufacturer's instructions for high copy plasmids.

2.6 Isolation of genomic DNA

Genomic DNA (gDNA) was isolated with the DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer's instructions. For gDNA isolation, either fresh cells, or cells stored at -150 °C were used.

2.7 Nucleic acid fragment segregation

2.7.1 Agarose gel electrophoresis

For analytical and preparative purposes, nucleic acids can be separated according to their size with the help of agarose gel electrophoresis. Large fragments in the range of 500-10.000 bp were separated in 0.75-1% (w/v) agarose gels. For separation of small DNA fragments, the agarose concentration was increased (1.5-2% (w/v)). The agarose was boiled in 1 x TAE buffer (Roth) until it was completely dissolved. After cooling to 60 °C, ethidium bromide was added to a final concentration of 1 µg/ml. The gel was poured into a gel tray and an appropriate comb was inserted. DNA samples were prepared by adding 1/6 volume of 6 x loading dye (Fermentas, # R0611), then loaded onto the gel. As a size standard the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas, #SM1343) was used. Electrophoresis was performed in 1 x TAE buffer (Roth) at either 200V for 25 min in a Horizon® 58 Horizontal gel electrophoresis apparatus or at 135 V for 45 min in a Mupid®-ex electrophoresis system. DNA bands were visualized under UV light (302 nm).

TAE (50 x):
2 M Tris/Acetat pH 7.5
50 mM EDTA

2.7.2 DNA fragment extraction from agarose gels

To obtain clean DNA from agarose gels, DNA bands were cut out of the agarose gel and were extracted with the Qiaquick gel extraction kit (Qiagen), or the Nucleospin Gel and PCR clean up kit (MACHEREY-NAGEL). Both kits were used according to manufacturer's instructions.

2.8 Photometric quantification of nucleic acids

Photometric quantification of nucleic acids was carried out with the Nano Photometer™ Pearl (IMPLEN, München) according to the manufacturer's instructions.

2.9 cDNA generation

For cDNA generation first RNA was isolated from cells, either with the NucleoSpin® RNA Plus Kit (MACHEREY-NAGEL), or the RNeasy Mini Kit (Qiagen). Both kits were used according to manufacturer's instructions. To generate cDNA from isolated RNA, the Omniscript RT Kit (Qiagen, #205113) was used according to manufacturer's instructions. For the reverse transcriptase (RT) reaction 1-2µg of RNA was used. For control purposes, –RT reactions were performed.

2.10 Real-time quantitative PCR

For real-time quantitative-PCR (qPCR) cDNA was generated as described in 2.9. Real-time qPCR was performed on the StepOnePlus System (Applied Biosystems). For each qPCR reaction, 10ng of template cDNA was mixed with Power SYBR PCR master mix (Applied Biosystems) and primers with a final concentration of 600 nM. All qPCR reactions were measured in technical triplicates to assure accuracy. The obtained data were normalized against the housekeeping gene 18S rRNA. All data are shown as means with the standard error of measurement. All used primers are listed in 6.1.

2.11 DNA Sequencing

To verify nucleotide sequences, sequencing of purified PCR products or plasmid DNA was carried out by LGC Genomics (https://shop.lgcgenomics.com/). For sequencing of plasmid DNA, 4 µl primer (5 pMol/µl) were added to 10 µl template DNA at a concentration of 100 ng/µl. Sequence analysis was performed with the programs 4 Peaks (http://nucleobytes.com/index.php/4peaks) and Serial Cloner 2.6 (http://serialbasics.free.fr/Serial_Cloner.html). All used primer pairs are listed in 6.1.

2.12 Animal procedures and related techniques

All mentioned methods under animal procedures were performed at the Central Institute for Experimental Animals (CIEA, Kawasaki, Japan). The methods describe the generation of transgenic common marmosets, starting with the collection of oocytes, over generation of in vitro fertilized one-cell stage embryos, to the embryo transfer into surrogate mothers.

2.12.1 Animals and animal housing

The common marmoset monkeys (*Callithrix jacchus*) used at the CIEA were obtained from the commercial breeder CLEA Japan, Inc. (Tokyo, Japan) or were bred at the CIEA.

The animals were pair-housed in vertically orientated stainless steel cages with a size of 158 cm x 40 cm x 61cm containing wooden branches and shelves for environmental enrichment. The facility temperature was 24-27°C with a relative air humidity of 40-60%. Illumination was provided through artificial lighting on a 12:12 hour light:dark cycle. The animals were fed ad libitum with foamed pellets CMS-1M (CLEA Japan Inc., Tokyo, Japan) specially designed for marmoset diet, supplemented with L (+)-ascorbic acid (Nacali Tesque, Tokyo, Japan), vitamins A, D3 and E (Duphasol AE3D; Kyoritsu Seiyaku Co., Ltd., Tokyo, Japan) and honey (Nihonhatimitsu Co., Ltd., Gifu, Japan). Additionally, chicken liver (DBF Pet, Niigata, Japan) boiled in water was fed once a week as supporting nutrition. Drinking water was available ad libitum from feed valves. Health and wellbeing of the animals were controlled daily by vetenerians and animal care attendants.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Central Institute for Experimental Animals (CIEA) (CIEA approval no: 11028, 14029) and were performed in accordance with the Basic Policies on Animal Experiments conducted in Research Institutions (Notice No. 71 of the Ministry of Education, Culture, Sports, Science and Technology of Japan, June 2006) and the Guidelines for Proper Conduct of Animal Experiments by the Science Council of Japan (2006). The laboratory animal facility of CIEA has been certificated based on the assessment by the Center for Accreditation of Laboratory Animal Care and Use, Japan Health Sciences Foundation (Certification number: 12-025).

2.12.2 Progesterone measurement

In order to determine the female stage of the reproductive cycle the plasma progesterone levels were measured. Progesterone levels were checked by ELISA, using the Automated Enzyme ImmunoassayAnalyzer AIA-900 (Tosoh, Tokyo, Japan) with the enzyme immunoassay (EIA) kit (Tosoh Progesterone Kit; Tosoh, Tokyo, Japan). The kit was used according to manufacturer's instructions. The day of ovulation was defined as the day before the progesterone level exceeded 10 ng/ml.

2.12.3 Ovarian cycle synchronization and superovulation

To increase oocyte production, common marmoset females where treated with hormones as following. The hormone treatment was performed by veterinarians at the CIEA. For this study sixteen female adult common marmosets were used as oocyte donors.

To determine the time-point of the ovarian cycle, blood samples were taken from adult female common marmosets to measure the progesterone level (2.12.2). To synchronize the ovulation cycles of donor and recipient females the animals where injected intramuscular with 0,75µg prostaglandin (PG) F2α analog cloprostenol (Estrumate; Schering-Plough Animal Health, Union, USA) during their luteal phase (10 days after ovulation). This was performed to induce luteolysis and subsequently a new ovarian cycle. On day 1 after PGF2α injection another progesterone measurement was performed to verify the start of a new ovarian cycle. To achieve superovulation, the intramuscular injection of recombinant human follicle stimulating hormone (rhFSH) (FOLYRMON-P; Fuji Pharma, Tokyo, Japan) with the dosage of 25IU/day/animal was started (day 1). The FSH injection, once every morning, was continued until day 9. On day 10 at 17:00 75IU/animal of human chorionic gonadotropin (hCG) (Gonatropin; ASKA Pharmaceutical, Tokyo, Japan) were injected. On day 11 the oocyte pick-up (OPU) (2.12.5) was started at 14:00.

2.12.4 Anesthesia

The anesthesia was performed by vetenerians of the CIEA.

For induction of anesthesia, animals were injected with 0,04 mg/kg medetomidine (Dormitor; Nippon Zenyaku Kogyo, Koriyama, Japan), 0,40 mg/kg midazolam (Dormicam 10mg; Astel-

las Pharma, Tokyo, Japan) and 0,40 mg/kg butophanol (Vetorphale; Meiji Seika Pharma, Tokyo, Japan). During operation anesthesia was maintained via inhalation of 1,0-3,0% isoflurane (Forane; Abbott Japan, Tokyo, Japan) through a ventilation mask. During operation heart rate and arterial oxygen saturation was monitored. After the operation the animals were injected with 0,20 mg/kg atipamezole hydrochloride (Antisedan, Nippon Zenyaku Kogyo) for better recovery from anesthesia.

2.12.5 Oocyte pick-up (OPU)

For OPU surgery, the animals were anesthesized as described in 2.12.4. The animal abdomen was opened and the ovaries were pulled out of the abdominal cavity while still connected to the body by their oviduct and connective tissue (Figure 2.1 A). The follicles in the ovaries were punctured with a 25G needle connected to a 2.5 ml sized syringe and oocytes were collected through aspiration (Figure 2.1 A). The Oocytes were collected in Porcine Oocyte Medium (POM, Research Institute for the Functional Peptides, Yamagata, Japan). After the operation the ovaries were placed back into the abdomen and the wound was stitched. The operation was performed under sterile conditions.

Records of each animal, including photograph of the appearance of the ovaries, number of oocytes and duration of operation were documented during OPU.

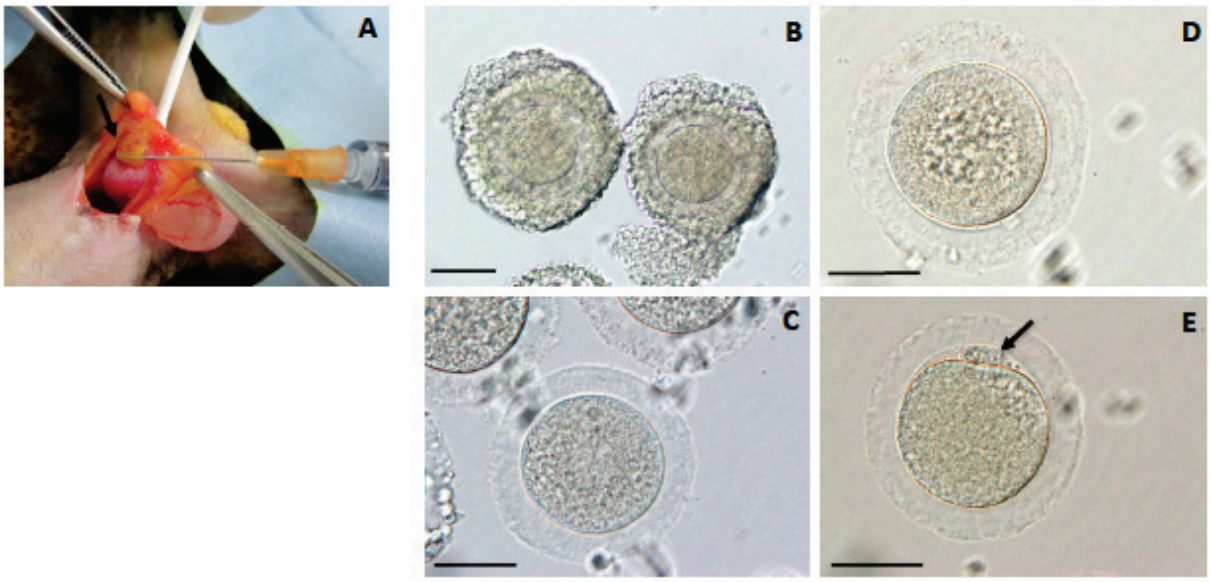

Figure 2.1. A) Aspiration of oocytes from ovaries of FSH stimulated animals. Ovaries were carefully "pulled out" of the abdomen and the oocytes were aspirated with a needle and syringe. Arrow indicates punctured ovary **B)-E)** Overview of different developmental stages of oocytes derived from FSH stimulated animals. **B-C)** Example of different possible appearances **of** Germinal vesicle (Gv) stage oocytes. **B)** Gv stage oocyte covered

with cumulus cells **C)** Gv stage oocyte without cumulus cells. **D)** Metaphase I stage (MI). **E)** Metaphase II stage (MII), arrow indicates polar body**.** Bars= ca. 50µm

2.12.6 Oocyte sorting and in vitro maturation (IVM)

After OPU, the oocytes were washed in supplemented POM and sorted by maturation stage. The different maturation stages were:

Germinal vesicle stage (Gv)
Metaphase I stage (M I)
Metaphase II stage (M II)

The different stages are described and shown in Figure 2.1 B-E. The MII stage Oocytes were directly used for in vitro fertilization (IVF) (2.12.7). The rest of the sorted Oocytes were placed in equilibrate drops of 80-100µl supplemented POM medium under mineral oil (Nacalai Tesque, Tokyo, Japan). They were incubated for in vitro maturation (IVM) at 38 °C, 5% O_2, 5% CO_2 for ~24 h. After IVM the Oocytes were sorted again. MII and MI stage Oocytes were used for IVF.

Supplemented POM:

POM
150 mIU/ml rhFSH,
20 IU/ml hCG
5% FBS

2.12.7 Sperm collection, purification and in vitro fertilization (IVF)

Fertilization competent sperm were needed to successfully fertilize oocytes. For this study a total number of seven adult male common marmosets were used. To collect sperm, common marmosets were stimulated until ejaculation with a FertiCare personal vibrator (Fertility Healthcare and Supplies, Silverado, USA). The ejaculated sperm were transferred to 500µl TYH media (LSI Medience Corporation, Tokyo, Japan). Afterwards, the sperm was centrifuged for 5 min at 400xg. The supernatant was discarded and the sperm pellet was resuspended in 500µl TYH. The sperm-TYH solution was incubated at 38°C, 5% CO2, 5% O_2 for 30 minutes. After incubation the sperm was centrifuged for 5min at 400xg. The supernatant was discarded and the sperm pellet was resuspended in 100µl TYH. The resuspended sperm was

transferred to the bottom of a conical tube containing 600µl TYH. The tube was incubated, while laying at a 30° angle**,** for 15-45 min at 38°C, 5% CO_2, 5% O_2. Afterwards, the upper 400µl, containing the so-called "swim up sperm", were collected. 6µl of the "swim up sperm" were used for sperm mobility analysis with the Sperm Mobility Analysis System (SMAS) (Ditect, Tokyo, Japan) to determine sperm concentration. 80-100µl drops of TYH containing 3.6 - 4 $x10^6$ Sperm/ml were prepared. For IVF, oocytes (2.12.6) were transferred into the drops containing the sperm and were incubated for 15-17h at 38°C, 5% CO_2, 5% O_2.

2.12.8 Pronuclei formation check

After 15-17h of incubation, the in vitro fertilized embryos were washed in ISM1 (Origio, Måløv, Denmark) to remove the sperm attached to the embryos. Next, the embryos were checked for the formation of the two pronuclei (2PN) to verify proper fertilization. Not properly fertilized embryos were discarded. In Figure 2.2 A-B an overview of possible appearances of embryos after fertilization is shown.

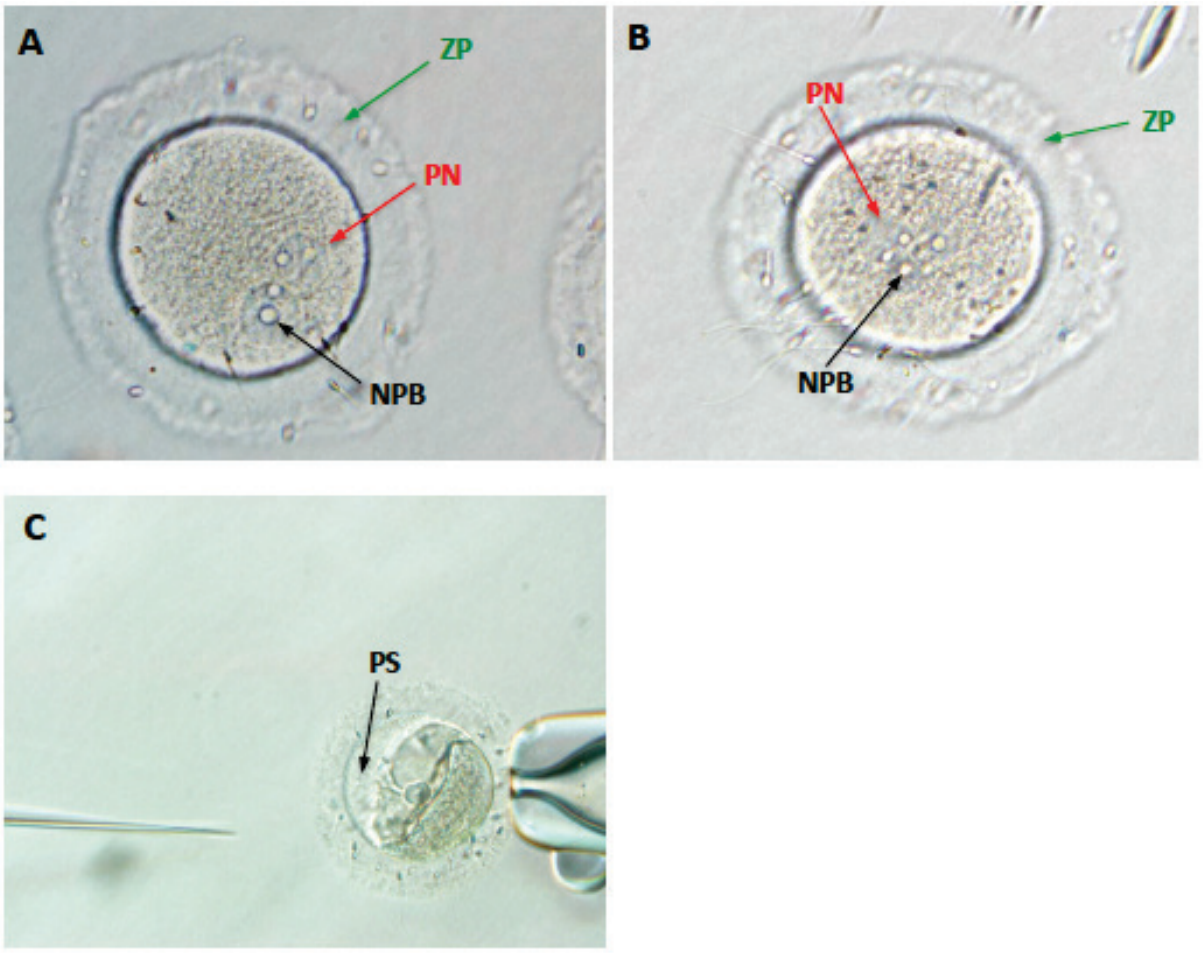

Figure 2.2. A) One-cell stage Embryo with 2PN, 17h post IVF (PN: Pronucleus NPB: Nucleolar Precursor Bodies ZP: Zona Pellucida). **B)** One-cell stage Embryo with 3PN, 17h post IVF. **C)** One-cell stage embryo in 0.25M Sucrose with enlarged perivitelline space (PS), right before virus injection into the PS.

2.12.9 Virus injection and in vitro culture (IVC)

Virus injections were performed on days 1 after IVF with the Femto Jet Express (Eppendorf) and a Narishige micromanipulator (Narishige, Tokyo, Japan) in drops of 0.25M sucrose in PB1 (LSI Medience Corporation). 0.25M sucrose in PB1 was used to extend the perivitelline space for easier virus injection. Drops of 8-10µl 0.25M sucrose in PB1 were prepared and covered with mineral oil. 2PN embryos were transferred to the drops for virus injection. After virus injection, the embryos were first washed in PB1 (3-4 times) and afterwards transferred back to drops of ISM1 medium for in vitro culture (IVC). IVC was performed at 38°C, 5% CO_2, 5% O_2. On day 4 after IVF, the Medium was changed to BlastAssist Medium (Origio), which shows better properties for blastocyst development. IVC was continued at 38°C, 5% CO_2, 5% O_2.

2.12.10 Staging and selection of embryo recipients

In this study thirteen female adult common marmosets were used as recipients for embryo transfer (ET). The recipient females used as surrogate mothers for the ET were chosen regarding to their stage in their ovulation cycle. Ideal for ET are females at day 3-5 after ovulation. The approximal time of ovulation is determined by progesterone level measurement. Progesterone was measured from blood samples as described in 2.12.2. Recipient females were paired with vasectomized males or other females.

2.12.11 Embryo Transfer (ET) and determination of pregnancy

Starting on day 4 after IVF, the embryos were checked for the number of blastomeres to determine proper and timely development, and for fluorescence, to determine proper GFP expression. Embryos were usually transferred to recipient females on day 5 after IVF (4 days after virus injection). For the embryo transfer (ET), properly developed embryos with a good fluorescence signal were used. ET was performed non-invasively through a transvaginal catheter.

After ET, the progesterone level of the recipient female was monitored (2.12.2) to check for embryo implantation. For the first measurement, on day 5-8 after ET, a progesterone level of about 20-50 ng/ml is expected. On day 7, day 14, day 21 after ET, Progesterone levels were also measured. A concentration of 40-80 ng/ml of progesterone indicates pregnancy. A progesterone level below 10 ng/ml at two consecutive weeks usually indicates the start of a new cycle, suggesting the loss of the embryo. Animals with a concentration between 10-40 ng/ml were further monitored and possible pregnancy was determined via ultrasound. Usually three weeks after ET the first ultrasound was performed to verify pregnancy.

2.13 Lentivirus preparation

2.13.1 Culture of 293T cells

293T cells, a 293 human embryonic kidney (HEK) cell line that was transduced with simian virus 40 T-antigen, was used for lentivirus packaging. 293 T cells were cultured on Poly-L-Lysine (Sigma-Aldrich) coated cell culture dishes in MEF medium. Cells were further pas-

saged when a confluency of 70-90% was reached. For passaging, cells were first washed with 5ml of 1 x PBS and then detached with 4ml Trypsin-EDTA (Gibco) for 5 min at 37 °C.

MEF medium:

	DMEM (Gibco)
10% (v/v)	FBS (Biowest)
1% (v/v)	Antibiotic-Antimycotic (Gibco)

2.13.2 Lentivirus generation with 293T cells

For generation of Lentivirus, 293T cells were transfected with the FuGENE 6 transfection kit (Promega) according to manufacturer's instructions.

For transfection, the cells were seeded on a ∅15cm dish with a density of $1x10^7$ cells per ∅15cm dish. For the transfection, 25µg pCAG-HIVgp plasmid DNA, 25µg pCMV-VSV-G-RSV-Rev plasmid DNA and 50µg PL-SIN-EOS-C(3+)-EiP plasmid DNA were diluted to a total volume of 2.5 ml in OptiMEM (Life-Technologies). 300 µl FuGENE® 6 Transfection Reagent was added to 2.2 ml OptiMEM. Afterwards, the vector OptiMEM solution and the FuGENE® 6 OptiMEM solution were gently mixed and incubated for 20 min at RT. Subsequently the solution was added dropwise to two 15cm dishes of 293T cells (2.5ml per 15cm dish). Transfected cells were incubated in 30ml MEF medium over night at 37 °C, 5% CO_2. On Day 1 post transfection the 30ml MEF medium were discarded and 30ml new medium were added. On Day 2 post transfection the 30ml medium were collected and new medium was added to the cells. On Day 3 post transfection the 30ml medium were collected and pooled with the medium from Day 2. The cells were discarded. The pooled supernatant, containing viral particles, was filtered through a 0.22µm corning filter (Corning). Afterwards, the filtered medium was ultracentrifuged for 2h at 50000g and 4°C. After centrifugation the supernatant was discarded and the virus pellet was resuspended in 60µl ISM1 (Origio). Aliquots of 12µl were stored at -80°C.

2.13.3 Lentivirus titer calculation with the Lenti-X™ qRT-PCR Titration Kit

To calculate the titer of the generated EOS-EiP lentivirus, the Lenti-X™ qRT-PCR Titration Kit (Clontech, USA) was used according to manufacturer's instructions.

2.14 CRISPR/Cas9 associated procedures

2.14.1 Identifying potential gRNA sequences

For CRISPR-Cas9 gRNA the potential target sites have the sequence of 5'-NNNNNNNNNNNNNNNNN-NGG on the leading strand or. The common marmoset DNA sequences of interest were obtained from Ensemble *(http://www.ensembl.org/)*. Possible target sites in these sequences were analyzed with the CRISPR design tool *(http://crispr.mit.edu/)*, provided by the Zhang Lab. For a given sequence the CRISPR design tool provides a list of all potential target sites and grades these regarding to their potential off-target effects (potential of targeting other (unintended) sequences in the target genome). The CRISPR design tool does not contain the common marmoset genome, therefore the human genome was used as reference and potential target sites were subsequently compared to the marmoset genome.

2.14.2 gRNA design and assembly

Suitable target sequences, identified as mentioned in 2.14.1, have been ordered from Sigma-Aldrich as top and bottom oligos (Figure 2.3). Therefore nucleotides necessary for later processing were added to the 20bp long target sequences as described in Figure 2.3. The top and bottom guide oligos were annealed and phosphorylated. Therefore, 1 µl of the top and 1µl of the bottom gRNA oligo (100 µM), 1 µl of 10x T4 Polynucleotide Kinase Reaction Buffer (NEB), 0.5 µl T4 Polynucleotide Kinase, and 6.5 µl ddH_2O were mixed. The reaction was incubated at 37 °C for 30 minutes, followed by 5 minutes at 95 °C and ramped down to 25 °C at a speed of 5 °C/minute. For the ligation of the annealed top and bottom oligos into the PX459 plasmid (2.14.3), the annealed oligos were diluted 1:200 in ddH_2O. All used oligos are listed in 6.1.

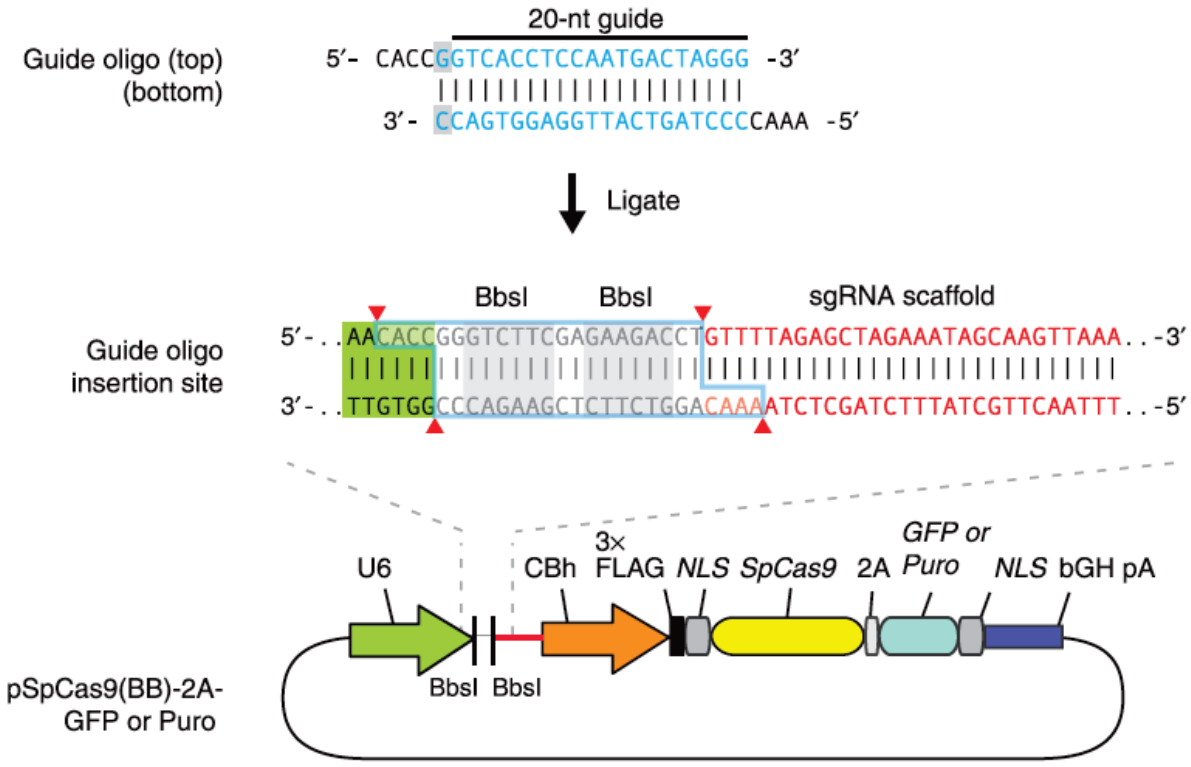

Figure 2.3. Overview for cloning of the guide sequence oligos into the plasmid containing Cas9 and the sgRNA scaffold (pSpCas9(BB)). The guide oligos (sequence marked in blue) contain overhangs for the ligation into the pair of BbsI sites in pSpCas9(BB). Digestion of pSpCas9(BB) with BbsI (excised fragment outlined in blue) allows the insertion of the annealed guide oligos. To aid the selection of transfected cells versions of pSpCas9(BB) containing GFP or a puromycin resistance gene are available. Here the plasmid pSpCas9(BB)-2A-Puro (PX459) was used. (From Ran et al. 2013)

2.14.3 Cloning gRNA into pSpCas9(BB)-2A-Puro (PX459) plasmid

The annealed gRNA was cloned into the PX459 plasmid as shown in Figure 2.3. The PX459 plasmid was a gift from Feng Zhang (Addgene plasmid # 48139). 1 µg PX459 plasmid was digested with 10 U BbsI (NEB, #R0539S) in NEB Buffer 2 at 37°C for 30min. Next the enzyme was heat inactivated at 65°C for 20 min. Afterwards, the digested PX459 plasmid was dephosphorylated as described in 2.4.2. The digested and dephosphorylated plasmid was eluted from a 1% agarose gel (2.7.1) as described in 2.7.2. Next, the annealed guide oligos (2.14.2) were ligated into the plasmid (Figure 2.3) as follows: 50ng PX459 (BbsI digested and dephosphorylated), 1µl diluted gRNA oligos (1:200), 1µl Ligase buffer and 1µl T4 Ligase (2.4.4) were mixed and filled up with ddH_2O to a total 10µl. The reaction was incubated for 1h at room temperature. Afterwards the plasmid was treated with Plasmid-Safe™ ATP-Dependent DNase (Epicentre) as described in 2.4.3. Next the plasmid was transformed into E.coli cells (2.3.3), colonies were picked and a mini preparation was performed (2.5.1). The prepared plasmid DNA was send in for sequencing as described in 2.11. Sequence verified

plasmids were transformed into E.coli cells (2.3.3) and afterwards used for Maxi-preparation as described in 2.5.2. After Maxi-preparation plasmids were ready for transfection (2.15.6.1) into cells.

2.14.4 ssODN design

To achieve CRISPR/Cas9 based homology-directed repair (HDR), homologous templates have to be present in the cell. As homologous templates, single stranded DNA oligo nucleotides (ssODNs) were designed using Serial Cloner 2.6 (http://serialbasics.free.fr/Serial_Cloner.html). The ssODNs used in this study had a total length of 92 and 162 nucleotides, respectively, with two homology arms flanking the sequence containing the mutation of interest. The ssODNs were ordered from Sigma Aldrich. The used ssODNs are shown in Figure 3.7 and are listed in 6.1.

2.14.5 T7 endonuclease I assay

T7 endonuclease I was used to check CRISPR/Cas 9 treated cells for induced mutations. T7 endonuclease I (T7EN1) recognizes mismatches resulting from hybridization of wt DNA and mutated DNA and digests the DNA at these mismatches (Figure 2.4). The DNA target of interest was PCR amplified as described in 2.2.1. For the hybridization reaction, 2 µl NEBuffer 2 (NEB), 200 ng of purified PCR product, and ddH_2O were added to a total volume of 19,3µl. The hybridization reaction was incubated for 5 minutes at 95 °C, ramp down to 85 °C at 2 °C/s and ramp down to 25 °C at 0.1 °C/s, followed by a hold at 4 °C. Following the hybridization reaction, 0.7 µl (10U) T7 endonuclease I were added and the solution was incubated for 15 minutes at 37 °C for digestion. Afterwards, the reaction was stopped by adding 1 µl 0.5 M EDTA. Subsequently, the sample was loaded onto a 1 % agarose gel for gelelectrophoresis (2.7.1) to investigate resulting DNA fragment lengths (Figure 2.4). The used primer pairs are listed in 6.1.

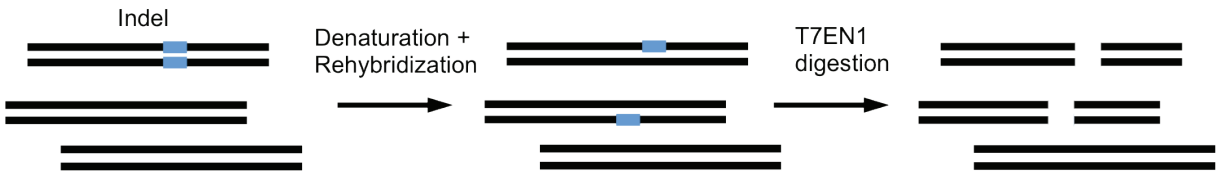

Fig. 2.4. Simplification of T7EN1 digestion for CRISPR/Cas 9 activity control. Heterogenous population of DNA is denaturated and rehybridized. Rehybridized DNA fragments contain sequence mismatches. T7EN1

recognizes these mismatches and digests DNA at these positions. The resulting different fragment lengths are proof of CRISPR/Cas9 activity

2.14.6 Single clone sequencing analysis

To be able to sequence single clone DNA, PCR amplified DNA from gRNA+ssODN nucleofections was ligated into pcDNA3.1(-). PCR primers containing restriction sites for *Nhe*I and *Xho*I were used to PCR amplify the region of interest. PCR products and pcDNA 3.1 plasmid were both digested with *Nhe*I and *Xho*I as described in 2.4.1. Next the PCR products were ligated into pcDNA 3.1(-) as described in 2.4.4. The pcDNA3.1(-) plasmids containing the sequence of interest were transformed into NEB10-beta *E.coli* cells as described in 2.3.3. Single clones were picked and DNA was prepared (2.5.1). The clones were digested with *Sca*I (2.4.1) analysed on an agarose gel (2.7.1) to prescreen for CRISPR/Cas9 mediated HDR. Putative positive clones were sequenced as described in 2.11. The used primer pairs are listed in 6.1.

2.15 Cell culture

The following cells were used in this study:

Fibroblast cells:

Primary fibroblasts from the common marmoset

Primary fibroblasts from the putative transgenic common marmosets I780tgM, I779tgM and I664tgF

Pluripotent cells:

cjes001 common marmoset ES cell line (Gift from Erika Saski (Müller et al., 2009))

iPSC#3.5 iPS cell line from the common marmoset provided by Katharina Debowski (Debowski et al., 2015)

Cells for lentivirus generation

293T cells first described by (DuBridge et al., 1987)

2.15.1 Culture conditions for primary fibroblasts (*Callithrix jacchus*)

Primary fibroblasts were isolated from skin biopsies of the common marmoset. The skin was washed in 1 x PBS (Life-Technologies) and remaining hair was removed from the skin by use of razor blades. The skin was cut into pieces of 1 x 1 - 2 x 2 mm. Single pieces of skin were transferred to a tube, containing 950 µL DMEM. Collagenase IV (Gibco) was dissolved in 1 x PBS to a final concentration of 5 mg/ml. Collagenase IV (50 µl) was added to the skin pieces. Skin was incubated (37 °C; 800 rpm) for at least 1 h. Cells were centrifuged at 300 x g for 5 min at room temperature. The supernatant was discarded. Two cell pellets were seeded per culture dish (∅ 5 cm), in 3 ml M10. The cells were incubated at 37 °C, 5% CO_2. M10 medium was changed twice a week. At approximately 90% confluence, cells were detached and distributed to new dishes. Therefore cells were first washed with 3 ml of 1 x PBS and then detached with 1ml TrypLE Express (Gibco; 6 min; 37 °C). For long-term storage, cells were kept in freezing medium. Aliquots of 2 x 10^6 cells/ml were stored at -150 °C.

Freezing medium:

	DMEM, GlutaMAX™
25%	FBS
10% (v/v)	DMSO

M10 medium:

	DMEM, GlutaMAX™
10% (v/v)	FBS (Gibco)
2 mM	L-Glutamine (Gibco)
1%	Penicillin/Streptomycin (Gibco)
1%	non-essential amino acids (Gibco)

PBS (pH 7.4):

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4

2.15.2 Mouse embryonic fibroblasts (MEFs)

To generate MEFs, embryos from CD1 mice at stage E12.5 were used. The embryos were washed in 1xPBS and the head as well as the hematopoietic organs of each embryo were removed. The rest of the embryos were minced and transferred into fresh 1x PBS. Next, the minced embryos were centrifuged for 20 min at 400 x g at room temperature. The supernatant was discarded and the pellet was resuspended in 0.05 % Trypsin/EDTA and incubated for 20 min at 37 °C. To stop the Trypsin/EDTA reaction M10, 5% (v/v) FBS and DNaseI (Roche, 100Kunitz units/ml), were added. Cells were Incubated for 10 min at 37°C and afterwards pelleted at 300 x g at room temperature. Afterwards, the cells were seeded in fresh M10 medium. For γ irradiation, the cells needed to be in passage 0-4 and were irradiated with 30 Gy

(5Gy/min). After irradiation cells were centrifuged at 300 x g (room temperature) for 15 min. For cryo-preservation, cells were resuspended in 90% FBS/10% DMSO and frozen at -150°C and a density of 6 x 10^6 cells/ml.

For usage as feeder cell layer, the MEFs were thawed and seeded on gelatin (0,5 % w/v; Fluka, #48720-100G-F) coated cell culture dishes. The cells were seeded at a density of 50.000 to 100.000 cells per cm^2 in M10, usually 1-3 days prior to use.

M10:

	DMEM, GlutaMAX™
10% (v/v)	FBS (Gibco/BRL)
2 mM	L-Glutamine (Gibco/BRL)
1%	Penicillin/Streptomycin (Gibco/BRL)
1%	non-essential amino acids (Gibco/BRL)

2.15.3 Culture conditions of pluripotent cells

Pluripotent cells were cultured on MEFs in ESM medium. Cells were incubated at 37 °C, 8 % CO_2, 5 % O_2. Medium was changed every second day. When close to confluency, cells were washed with 1x PBS and incubated with Stem Pro Accutase (Gibco) for 4 min at 37 °C, to detach the cells. Cells were pelleted for 5 min at 200 x g, the supernatant was discarded and cells were resuspended in ESM medium. The desired amount of cells was plated on new feeder cells.

Aliquots of frozen pluripotent cells were rapidly thawed at 37 °C and incubated in ESM medium. For cryopreservation, cells were harvested at 60-70 % confluency. Cells were detached as described above. The cell suspension was then pelleted for 10 min at 200 x g, resuspended in freezing medium (2.15.2) and frozen. Cells were stored at -150°C

ESM:

	KNOCKOUT-DMEM (Gibco)
20%	Knockout Serum Replacement (Gibco)
1 mM	MEM-NEAA (Gibco)
2mM	GlutaMAX™-I (Gibco)
50µM	2-Mercaptoethanol (Gibco)
1%(v/v)	Pen Strep (Gibco)
0.25µg/ml	Amphotericin B (Sigma)

2.15.4 Derivation of clonal cell lines

Cells were detached from culture dishes as described in 2.15.1. Afterwards single cells were transferred to 24 well multi well plate (MWP) to obtain monoclonal cell lines, using a fine glass pipette connected to a plastic tube.

2.15.5 Population-doubling level (PDL) calculation

Fibroblasts were passaged in Petri dishes (∅10cm) as described in 2.15.1. For each passage $2{,}5x10^5$ cells /dish were seeded. When reaching confluency, cells were harvested and counted before they were seeded again at $2{,}5x10^5$ cells /dish for the next passage. Outgoing from consecutive counts the PDL was calculated as follows: $PDL_{(n/n-1)}$ = log (Nf/N0)/log 2, where n=passage number, Nf=final number of cells, N0 = number of cells seeded at passage (Bischoff et al., 2012).

2.15.6 Cell transfection

2.15.6.1 Transfection with Amaxa™ 4D Nucleofector™ (Lonza)

Common marmoset primary fibroblasts were transfected with the Amaxa™ 4D Nucleofector™ (Lonza) with the P2 Kit for primary mammalian fibroblasts (Lonza). Cells were detached using TrypLE Express (2.15.1) (Gibco) and counted. For each nucleofection 1 x 10_6 cells were centrifuged for 10 min, at 90g at room temperature. The pelleted cells were resuspended in 100µL nucleofection solution (buffer 2), plasmid DNA was added and the solution was transferred to a nucleofection cuvette. For nucleofection the programme CA-137 was used. Afterwards, cells were incubated at room temperature for 10 minutes and were resuspended in 500 µL prewarmed M10. Cells were plated on dishes or multi well plates (MWP).

2.16 Reprogramming of fibroblasts to induced pluripotent stem (iPS) cells

Fibroblasts were nucleofected with one of the *piggyBac* reprogramming constructs and the transposase, PBase, catalyzing the integration of the reprogramming factors (2.4) (Figure 3.2). The Amaxa™ 4D Nucleofector™ (Lonza) (2.15.6.1) was used for the transfections. After nucleofection, cells were cultured in M10 medium and selected with 1-2µg/ml puromycin for

five days on cell culture dishes. Cells were transferred onto MEF coated culture dishes (2-6 × 10^4 cells per Ø10cm culture dish) and cultured in ESM (2.15.3) at 37°C, 8% CO_2, 5% O_2. For the first six days of culture, ESM was supplemented with 2mM valproic acid (Calbiochem). Developing colonies were picked manually from day 28 on. Picked colonies were transferred to fresh MEF feeder layers for further culture. Cells were further passaged with StemPro Accutase as described in 2.15.3.

2.17 Immunofluorescence staining of cultivated cells

Fibroblasts were detached with TrypLE (2.15.1) and seeded on a 48well MWP. Pluripotent cells were detached with StemPro Accutase (2.15.3). After the cells reach an appropriate density the medium was removed and cells were washed twice with PBS. Cells were fixed in 2-4% PFA (in PBS) for 10-30 min. After PFA fixation the cells were washed twice with PBS. Afterwards, cells were treated with 0.1% TritonX-100 for 10-15 min at RT followed by washing twice with PBS. Primary antibodies were diluted in PBS + 5% BSA pipetted onto the cells and incubated over night at 4°C. Afterwards the cells were washed twice with PBS. The secondary antibody, diluted in PBS + 5% BSA, was added to the cells and incubated for 20 min at room temperature in the dark. The cells were washed twice with PBS and subsequently stained with 5% DAPI in PBS for 1-2 min at room temperature. Afterwards, cells were washed twice with PBS and mounted with Citifluor mountant medium (CITIFLUOR). Microscopy images were taken with a Zeiss Observer Z1 (Zeiss). The used primary antibodies and their dilutions were: TERT (abcam, #ab32020, 1:200), OCT4 (Cell Signalling, #2890, 1:100), NANOG (Cell Signaling, #4903, 1:300), LIN28 (Cell Signaling, #3978S, 1:70), TRA-1-60 (eBioscience, #14-8863, 1:50), SSEA-4 (Merck Millipore, #MAB4304, 1:50). The used secondary antibodies were: AlexaFluor488 goat anti-mouse (Life Technologies, #A10680), AlexaFluor488 donkey anti-rabbit (Life Technologies, #A21206), AlexaFluor488 donkey anti-mouse (Life Technologies, #A21202), AlexaFluor594 donkey anti-rabbit (Life Technologies, #A21207). All secondary antibodies were diluted 1:200.

2.18 Flow cytometry of iPS cells

For flow cytometry, iPS cells were first detached with Stem Pro Accutase as described in 2.15.3. The cells were incubated in ESM for 2h on gelatin-coated petri dishes. After this step the majority of remaining MEFs were attached to the petri dish and the iPS cells were still in

suspension. Induced PS cells in suspension were pelleted at 200 x g for 5 min. Cell pellets were resuspended in Stem Pro Accutase and incubated for 30 min at 37°C (under constant rotation). Cells were centrifuged at 200 x g for 5 min and resuspended in PBS. To obtain mainly single cells, the cells were pipetted through a 40 µm cell strainer. Cells were centrifuged at 200 x g for 5 min and fixated in 0.37% PFA (in PBS). The flow cytrometric measurement was performed with a LSRII cytometer (BD Biosciences). Flow cytometry data analysis was performed with the FlowJo 9.6 (TreeStar) software.

3. Results

3.1 Transgenesis in the common marmoset

The experiments for the generation of transgenic common marmosets were performed in the labratory of Prof. Erika Sasaki at the Central Institute for Experimental Animals (CIEA) in Kawasaki, Japan. The goal of my work at the CIEA was to generate transgenic common marmosets carrying the EOS-EiP transgene. For the successful generation of a transgenic marmoset it was crucial to learn and perform the different required methods in perfection. The used methods are listed in 2.12.

3.1.1 EOS-EiP virus injection and embryo check

For the generation of transgenic common marmosets, overall 16 superovulated animals (2.12.3) were used as oocyte donors for oocyte collection (2.12.5). Collected oocytes were in vitro matured (2.12.6) and used for in vitro fertilization (IVF) (2.12.7). IVF embryos showing proper development, i.e. the formation of the maternal and paternal pronuclei (2.12.8), were used for virus injection. To integrate the EOS-EiP (Figure 1.2) transgene into the marmoset embryo genome, EOS-EiP self-inactivating lentivirus was generated as described in 2.13.2. The titer of the used virus was $2.8x10^9$ infectious units/ml (IFU/ml) (2.13.3). In order to prepare the embryos for virus injection they were transferred to drops of 0.25 M sucrose in PB1 medium. The hyperosmotic sucrose solution enlarges the perivitelline space and allows easier virus injection (Figure 2.2 C) (2.12.9). Overall, 72 one-cell stage embryos were injected with the EOS-EiP virus (Table 1). After injection, embryos were incubated in ISM1 medium for in vitro culture (IVC) (2.12.9). The IVC embryos were checked for proper development and eGFP signal (indicating successful EOS-EiP integration) starting 72 hours after injection.

3.1.2 Embryo transfer (ET) resulted in three newborn common marmosets

Seventy-two embryos were injected with the lentivirus. Twenty-five (34.7%) of them showed timely development combined with a clear eGFP signal (Table 1). These embryos were used for embryo transfer (ET) (2.12.11). Figure 3.1 A+D show examples of embryos used for ET. Overall, 25 EOS-EiP virus injected embryos were transferred into overall 13 surrogate moth-

ers (Table 1). Five to eight days after ET the first blood samples from surrogate mothers were taken for progesterone measurement in order to determine pregnancy status. Starting around three weeks after ET, the putative pregnant females were also monitored using ultrasound. Figure 3.1 C+G show the ultrasonographic pictures of the implantation site and the embryo. The Transfer of the 25 embryos resulted in three pregnancies from four transferred embryos (16% of the overall 25 transferred embryos): two singletons and one twin pregnancy. One of the singleton pregnancies resulted in an abortion 71 days after ET (Table1).

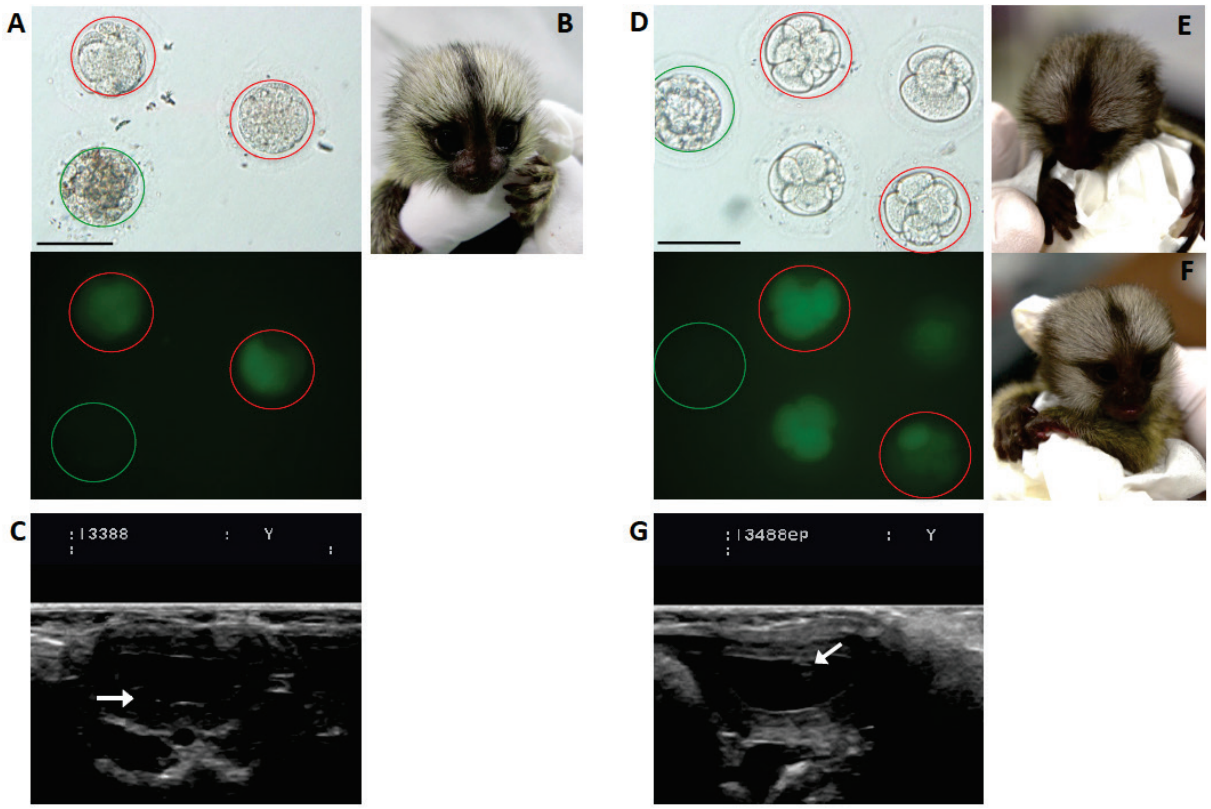

Figure 3.1. Transgenic common marmosets. A) Brightfield and Fluorescence pictures of EOS-EiP virus injected embryos. The green circle marks an uninjected wildtype control embryo, red circles the eGFP-positive embryos used for retransfer. One resulted in the birth of newborn I664tgF. Bar= ca. 100µm **B)** Newborn I664tgF (♀). **C)** Ultrasound picture of surrogate mother the I3388F, 48 days post retransfer of the embryos shown in A). White arrow indicates implanted embryo. **D)** Brightfield and Fluorescence pictures of EOS-EiP virus injected embryos. The green circle marks an uninjected wildtype control embryo, red circles the embryos used for retransfer and resulting in the birth of newborns I779tgM and I780tgM. Bar= ca. 100µm **E)** Newborn I780tgM (♂). **F)** Newborn I779tgM (♂). **G)** Ultrasound picture of the surrogate mother I3488epF, 50 days post retransfer of embryos shown in D). White arrow indicates implanted embryo.

Two of the surrogate mothers successfully delivered offspring with a total number of three newborns (12% of the total 25 transferred embryos) (Table 1). The first putative EOS-EiP transgenic common marmoset (I664tgF) was born on November 12th 2013, two more (I779tgM, I780tgM) were born on November 24th 2013. Figure 3.1 B+E+F show the neonatal common marmosets. Animal I780tgM was euthanized eight days after birth due to a develop-

ing sepsis. Due to time constrains, the genotyping of the transgenic animals was not performed at the CIEA but later at the German Primate Center in Goettingen using fibroblasts of the animals.

Table 1. Overview of number of used animals, virus injected embryos, retransferred embryos, pregnancies from retransfers and newborns from retransfers

	Oocyte donors	Surrogate mothers	Virus injected embryos	Retransfered embryos	Pregn. from retransfers	Newb. from retransfers
EOS-EiP	16	13	72	25 (34.7%)	4*(16%)	3**(12%)

*: 1 abort
**: 1 newborn euthanized

3.2 Generating iPS cells from EOS-EiP transgenic common marmoset fibroblasts

After we generated putatively transgenic common marmosets carrying the EOS-EiP transgene, we wanted to reprogram fibroblasts of these animals. The reprogramming experiments were performed at the Stem Cell Biology Unit of the German Primate Center in Goettingen.

3.2.1 Analysis of fibroblasts from EOS-EiP positive marmosets

Skin biopsies of the juvenile animals I664tgF and I779tgM were taken to obtain fibroblast cells for reprogramming experiments. From the animal I780tgM, skin samples were taken after euthanization. The skin biopsies and subsequent fibroblast cell culture was performed by employees of the CIEA. The fibroblast cells of the animals I664tgF and I779tgM, together with the fibroblast cells of the euthanized animal I780tgM were provided by the CIEA for further use at the German Primate Center. The cells of the putatively EOS-EiP transgenic marmosets were further analyzed and used to generate induced pluripotent stem (iPS) cells from these animals.

Genomic DNA (gDNA) of the three different fibroblast cultures was prepared (2.6). The gDNA was used for genotyping by PCR (2.2) using primers specific for the EOS-EiP cassette. As shown in Figure 3.2 E, the genotyping PCR revealed the presence of an EOS-EiP specific DNA sequence in the samples of the animals I779tgM and I780tgM. The animal I664tgF was

negative for EOS-EiP. The fibroblasts of animal I664tgF were not further included in this study.

3.2.2 Reprogramming of EOS-EiP positive fibroblasts using the *piggyBac* transposon system

EOS-EiP should drive eGFP expression in toti- and pluripotent cells, i.e. cells of the early embryo and pluripotent stem cells. To show the EOS-EiP activity in pluripotent stem cells (Hotta et al., 2009), I reprogrammed the EOS-EiP transgenic fibroblast using the *piggyBac* transposon system. For this work two different *piggyBac* (1.2.2) constructs were used: One

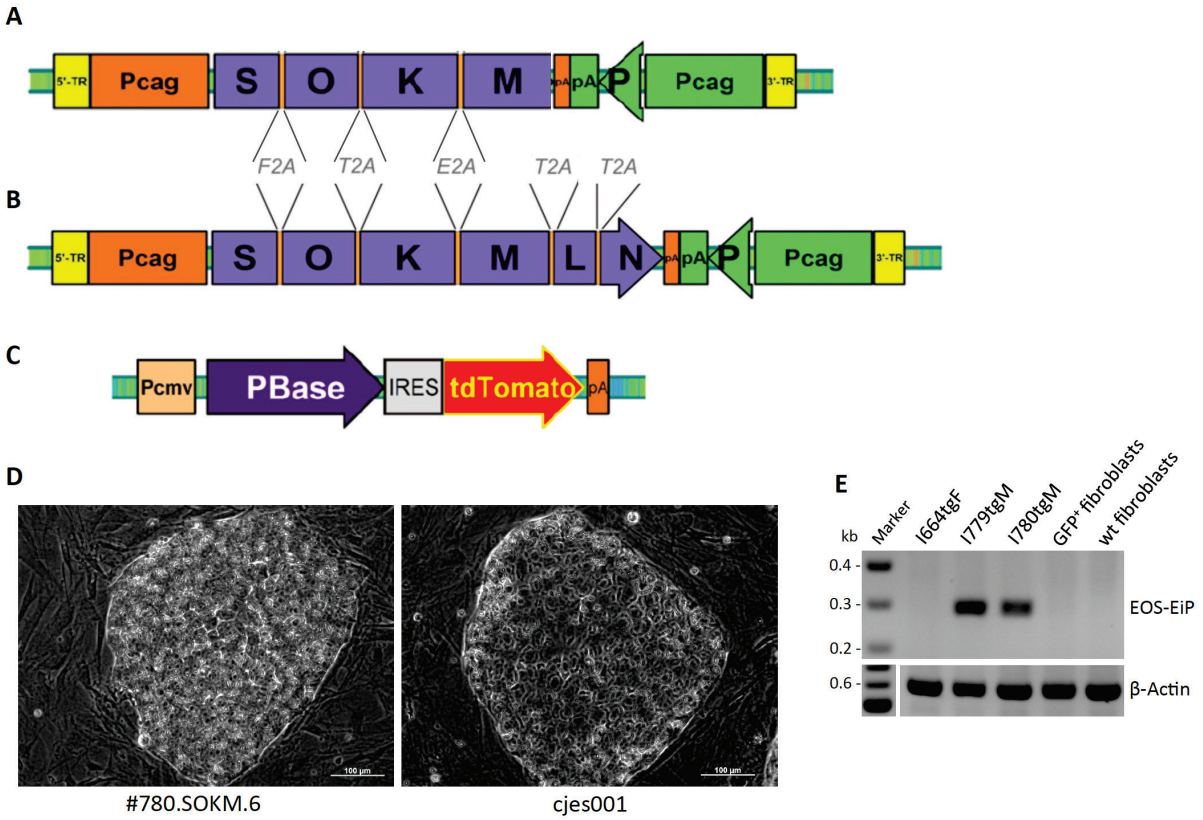

Figure 3.2. A)-B) Constructs used for reprogramming. Reprogramming factors are under control of the CAG promoter (Pcag). pA: poly A signal; 5'-TR: 5'-terminal repeat; 3'-TR: 3'-terminal repeat; S: SOX2; O: OCT4; K: KLF4; M: c-MYC; L: LIN28; N: NANOG; P: puromycin resistance gene. The stop codons of the first three (**A**) or five (**B**) reprogramming factors are substituted with coding sequences for 2A peptides (F2A, T2A, E2A) **C)** Expression of the Transposase (PBase) is driven by the Cytomegalovirus promoter (Pcmv); IRES: internal ribosomal entry site; tdTomato: red fluorescence protein used for transfection control. **D)** Morphology comparison of iPS cells and ES cells. The generated iPS cell line 780.SOKM.6 and the ES cell line cjes001 show similar morphology. **E)** Genotyping PCR results. gDNA of fibroblasts of the three putative EOS-EiP transgenic animals was used for PCR with EOS-EiP specific primers, β-Actin was amplified as a loading control. Bars = 100µm (A, B, C Modified from Debowski et al. 2015)

construct consisting of the four classical reprogramming factors *SOX2, OCT4, KLF4* and *c-MYC* (SOKM) (four factor construct) and one with the classical factors plus the two additional factors *LIN28* and *NANOG* (SOKMLN) (six factor construct) (3.2 A-B) previously shown to be necessary for reprogramming of common marmoset monkey cells (Tomioka et al., 2010) (1.3.2). Both constructs were generated by Katharina Debowski, the six factor construct was published in Debowski et al. 2015. Fibroblasts of the two EOS-EiP positive animals (I779tgM, I780tgM) were used for reprogramming with the two *piggyBac* constructs. Fibroblasts of both animals were nucleofected with either the six factor construct or the four factor construct and the transposase, called PBase, catalyzing the genomic integration of the reprograming cassette (2.16) (Figure 3.2 C).

After transfection the I779tgM and I780tgM fibroblasts were treated and cultured as described in 2.16. For the cells transfected with the six factor construct the first cell colonies showing morphology similar to ES cells, were picked after 28 days of culture on MEFs. Overall 51 colonies from six factor transfected cells were picked. The first colonies resulting from cells transfected with the four factor construct were picked after 91 days of culture on MEFs. Overall six colonies from four factor transfected cells were picked. From the picked colonies the two putative iPS cell lines 780.SOKM.5 and 780.SOKM.6 were generated. Both cell lines resulted from cells transfected with the four factor construct. Both showed a morphology comparable to ES cells and were passaged more then 30 times. A typical colony of the iPS cell line 780.SOKM.6 is shown in Figure 3.2 D. Further reprogramming experiments with the EOS-EiP positive fibroblasts resulted in multiple putative iPS cell lines generated with the six factor construct (data not shown). Generation of common marmoset iPS cells with the classical four factors was reported previously (Wiedemann et al., 2012; Wu et al., 2010). The following analysis was only performed for the four factor iPS cell lines 780.SOKM.5 and 780.SOKM.6.

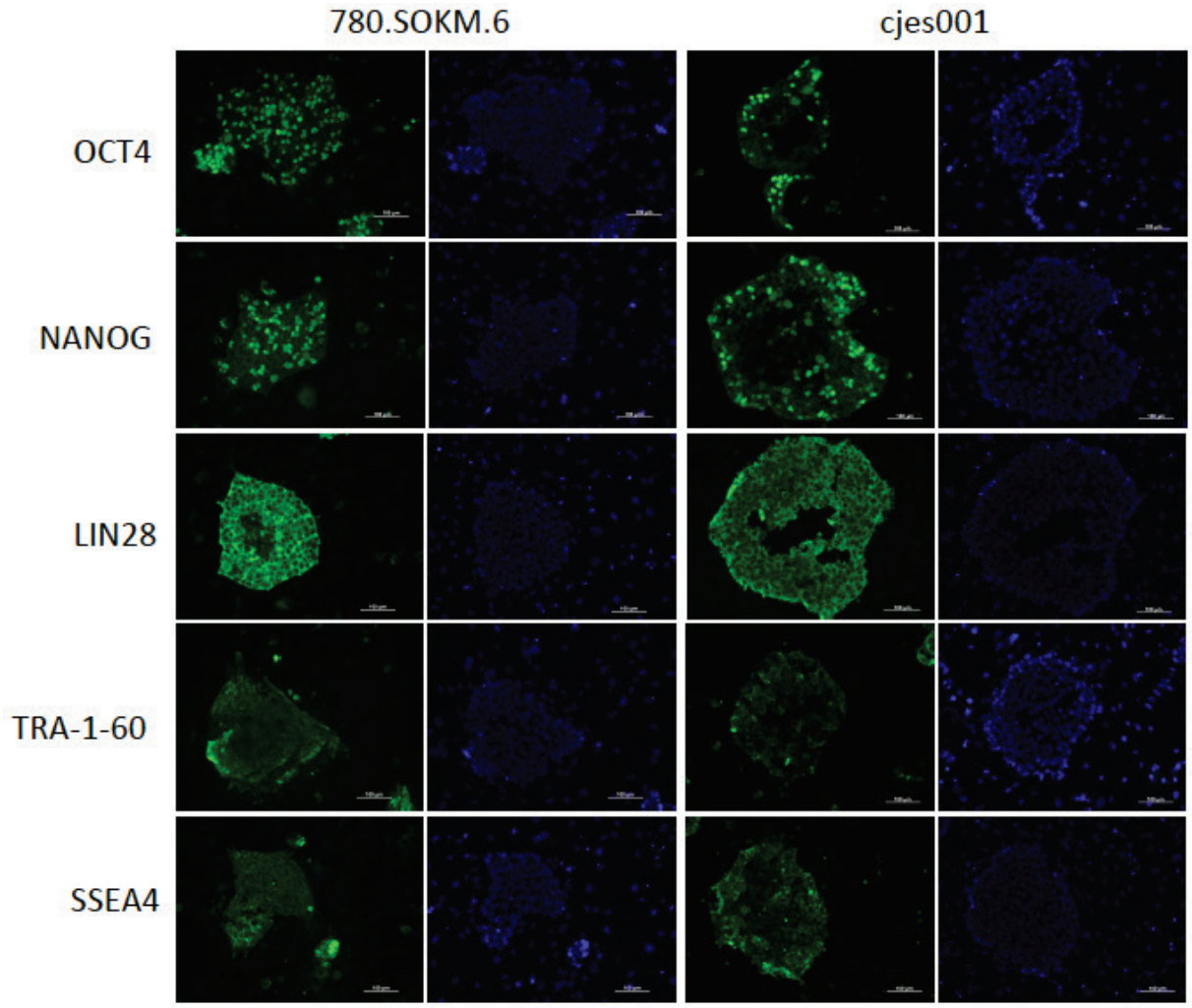

Figure 3.3. Immunofluorescence staining of the iPS cell line 780.SOKM.6 and the ES cell line cjes001 (positive control). As negative control, wildtype fibroblasts were stained (pictures not shown). The staining shows similar expression of the key pluripotency factors OCT4, NANOG, and LIN28 and of the pluripotency markers TRA-1-60 and SSEA-4 in the iPS cell line 780.SOKM.6 and the ES cell line cjes001. This indicates pluripotency of the 780.SOKM.6 iPSCs.

3.2.3 Common marmoset iPS cell analysis indicates pluripotency

To show expression of reprogramming factors and surface pluripotency markers, immunofluorescence (IF) staining of the iPS cells and the ES cell line cjes001 as positive control were performed. IF staining showed expression of the used reprograming factors OCT4, NANOG and LIN28 (Figure 3.3). The cells were also positive for the surface pluripotency markers stage specific embryonic antigen 4 (SSEA-4) and tumor rejection antigen-1-60 (TRA-1-60) (Figure 3.3). The stainings of the iPS cells and the ES cells (positive control) showed comparable staining patterns and intensities (Figure 3.3). This marker expression pattern indicates reprogramming of the fibroblasts to pluripotency (compare with Debowski et al., 2015). As

negative controls for IF staining common marmoset wildtype fibroblasts were used. In the negative controls no expression was detectable.

The cells nucleofected with the reprogramming vectors contain two versions of the factors present in the reprogramming cassette: an endogenous gene and the exogenous cDNA provided by the introduced vector. To check for the origin of the expressed pluripotency factors in the iPS cells, we performed several RT-PCRs (2.9, 2.2) either with primers specific for the reprogramming factors present in the reprogramming cassette or with primers specific for the endogenous reprogramming factors. The primers specific for the endogenous reprogramming

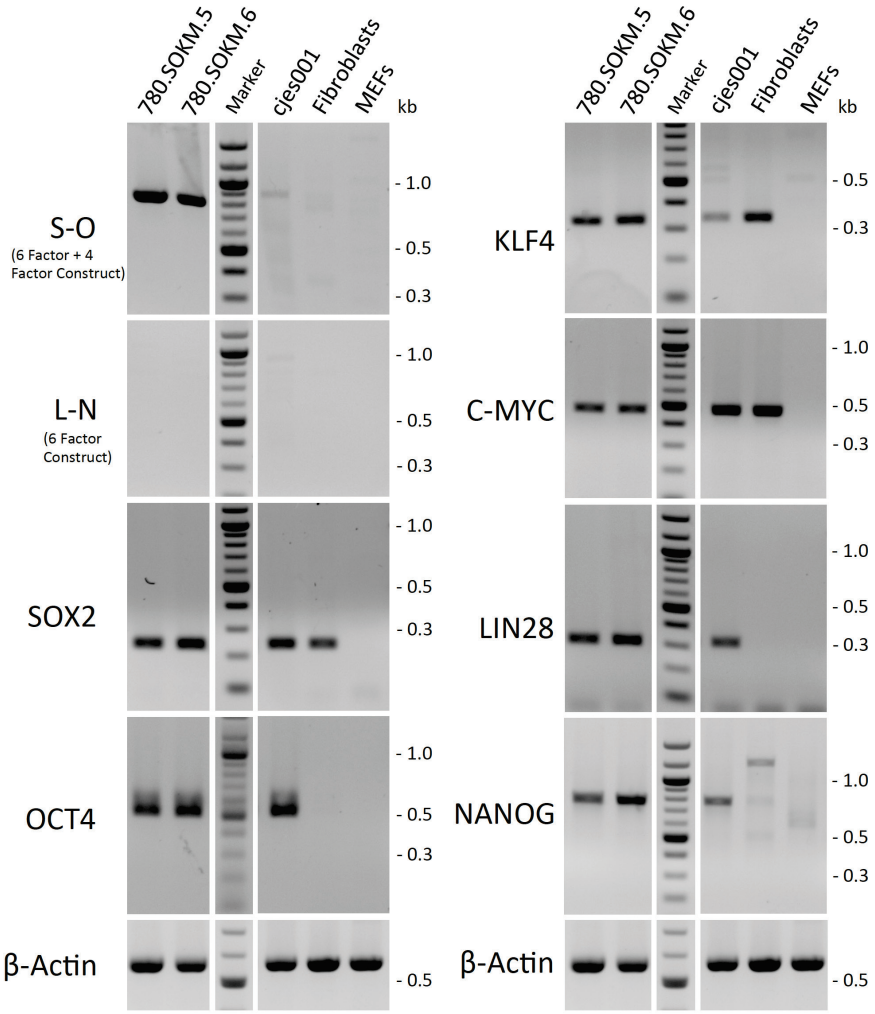

Figure 3.4. Characterization of pluripotency factor expression by RT-PCR. cDNA from the iPS cell lines (780.SOKM.5, 780.SOKM.6), the ES cell line cjes001, common marmoset fibroblasts and MEFs was used as template DNA. Primer pairs specific for the endogenous factors (*SOX2, OCT4, KLF4, C-MYC, LIN28, NANOG*) or the reprogramming cassette (S-O, L-N) were used. As a loading control, β-Actin was amplified. For all samples –RT controls were used. SOX2, KLF4 c-MYC were also detected in cDNA from common marmoset fibroblasts. Only transcripts derived from the four factor reprogramming cassette were detected in the iPSCs, but no transcripts specific for the six factor reprogramming cassette. This further proves successful four factor reprogramming.

factors annealed to untranslated regions of the mRNA, which are not present in the reprogramming cassette. As controls we used the ES cell line cjes001 and common marmoset fibroblasts. As shown in Figure 3.4, all generated iPS cell lines express the reprogramming factors *SOX2, OCT4, KLF4, c-MYC, LIN28* and *NANOG* endogenously. The RT-PCR specific for the exogenous reprogramming factors present in the *piggyBac* cassette showed that in the 780.SOKM.5 and 780.SOKM.6 line only *SOX2-OCT4* (S-O) were expressed, *LIN28-NANOG* (L-N) was not detected (Figure 3.4). These results further indicate that the cells were reprogrammed by only the four factor construct.

3.2.4 EOS-EiP transgenic iPS cell lines lack detectable GFP fluorescence

The EOS-EiP transgene is supposed to be active in pluripotent cells. Its activity is reported by enhanced Green Fluorescence Protein (eGFP) expression. Primary iPS cell colonies on MEFs (before picking) as well as the two putative four factor iPS cell lines were frequently checked for eGFP activity. Unfortunately no eGFP signal was detectable. By fluorescence microscopy of the iPS cell lines only green and red auto fluorescence was detectable. Similar autofluorescence was detectable in other common marmoset iPS cells lacking GFP (Figure 3.5 A). In order to find out on which level the eGFP reporter system failed, the iPS cells were checked for eGFP mRNA expression by RT-PCR (2.9, 2.2). The RT-PCR results indicate eGFP expression in both iPS cell lines (Figure 3.5 B). Because no fluorescence was detectable by fluorescence microscopy, we performed flow cytometry analysis (2.18) to determine if a small fraction of cells exhibit eGFP fluorescence. Moreover, flow cytometry is more sensitive than visual fluorescence microscopy. However, the flow cytometry analysis showed no significant eGFP signal (Figure 3.5 C) above the background fluorescence present in the negative control ES cell line cjes001. Finally, the mRNA expression level of eGFP was analyzed by real-time quantitative PCR (2.10) to determine the relative eGFP expression level in the iPS cells. GFP-expressing fibroblasts were included as positive control, and the ES cell line cjes001 as negative control. Compared to the negative control, GFP expression was slightly increased in the iPS cell lines. However, GFP expression in the iPS cells was only approximately 1/100 of the GFP expression in the positive control (Figure 3.5 D). These results indicate an expression level of GFP in the iPS cell lines below the detection limit of the microscopic fluorescence detection system, equipped with a highly sensitive camera, as well as below the detection limit of the flow cytometer.

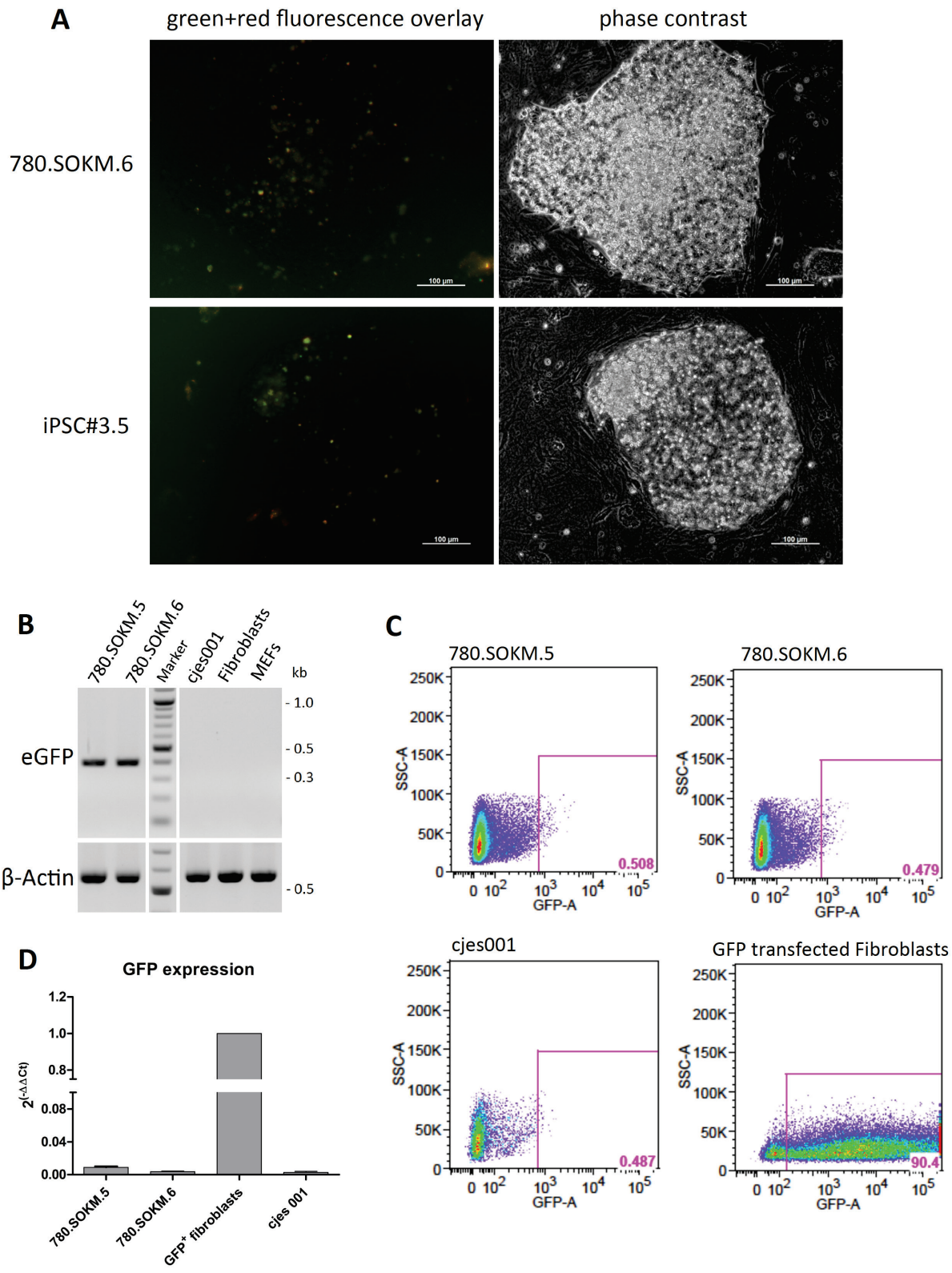

Figure 3.5. A) Autofluorescence in the iPS cell line 780.SOKM.6 and the iPS cell line iPSC#3.5. An overlay of detected green and red fluorescence is shown as well as the respective phase contrast images. **B)** GFP RT-PCR. cDNA from the iPS cell lines (780.SOKM.5, 780.SOKM.6), the ES cell line cjes001, common marmoset fibroblasts and MEFs was used as template DNA. For amplification primer pairs for eGFP were used. As a loading control β-Actin was amplified. For all samples –RT controls were used. In both iPS cell lines eGFP was detected**)** Flow cytometry analysis of the generated iPS cell lines. Shown is the site scatter (SSC) on the y-axis against GFP intensity on the x-axis. **D)** Real-time quantitative PCR for GFP. GFP expression levels in the iPS cell lines

were compared to levels in GFP positive fibroblasts and the GFP negative ES cell line cjes001. The standard error of measurement is shown for the iPS cells and ES cells.

To determine if sequence re-arrangements in the integrated EOS-EiP cassette may cause the low levels of eGFP expression, relevant parts of the expression construct integrated into the iPS cells´ genome were sequenced. The EOS promoter region (early transposon promoter + OCT4 and SOX2 enhancer regions) and the eGFP open reading frame were PCR amplified (2.2), using gDNA samples of the iPS cell lines, and subsequently sequenced (2.11). The results showed the expected sequence, i.e. no sequence rearrangements were detectable (Data not shown).

In summary of this part, it was shown that EOS-EiP transgene is present in the iPS cell lines. However, eGFP mRNA expression is severely reduced in the iPS cell lines compared to fibroblasts with detectable GFP activity. The reduced eGFP mRNA expression leads to undetectable eGFP activity. The reason for the low eGFP mRNA expression in the iPS cell lines is currently not known.

3.3 Targeted gene modification in common marmoset fibroblasts

Another new and very promising method for targeted gene modification is the CRISPR/Cas 9 system. It allows gene modification in basically all organisms, and was applied in this study to the common marmoset monkey. The target of interest in this study was the gene Leucine-rich repeat kinase 2 (*LRRK2*), a gene associated with Parkinson's disease (PD). Mutations in *LRRK2* are the most common genetic cause for PD, with the point mutation Gly2019Ser being the most prevalent among them (Healy et al., 2008; Kett and Dauer, 2012) (1.4.2).

3.3.1 CRISPR/Cas9 LRRK2 gRNA efficiently generates gene mutation in common marmoset fibroblasts

For the use of the CRISPR/Cas9 system first the guideRNAs (gRNA) guiding the Cas9 nuclease to the target of interest were generated (2.14.1, 2.14.2). We designed two gRNAs, one at position 86 (po86) of the *LRRK2* exon 41 and one at position 113 (po113) of exon 41 (Figure 3.6 A). The position of the gRNAs was defined as the starting point (nucleotide number) of the gRNAs in the exon 41. As described in detail in chapter 2.14.3, the gRNAs were subsequently cloned into the pSpCas9(BB)-2A-Puro (PX459) plasmid (gift from Feng Zhang

(Addgene plasmid # 48139)). The PX459 Plasmid contains the Cas9 nuclease as well as the gRNA scaffold and, after cloning, the gRNA (Figure 2.3). Therefore a single plasmid is sufficient for CRISPR/Cas9 mediated gene targeting.

The two plasmids (PX459_po86, PX459_po113) containing the different gRNAs (*LRRK2* gRNA po86, *LRRK2* gRNA po113) were sequenced to verify successful and correct cloning (data not shown). To test the CRISPR/Cas9 system common marmoset fibroblasts were transfected with one of the two CRISPR/Cas9 plasmids (2.15.6.1). Preliminary experiments showed that the puromycin resistance in the PX459 plasmid was not sufficient to efficiently select for positively transfected cells. No transfected cells survived puromycin selection. To achieve efficient selection I started cotransfecting fibroblasts with a CRISPR/Cas9 plasmid and a plasmid containing a strong puromycin resistance. In a cotransfection usually both plasmids

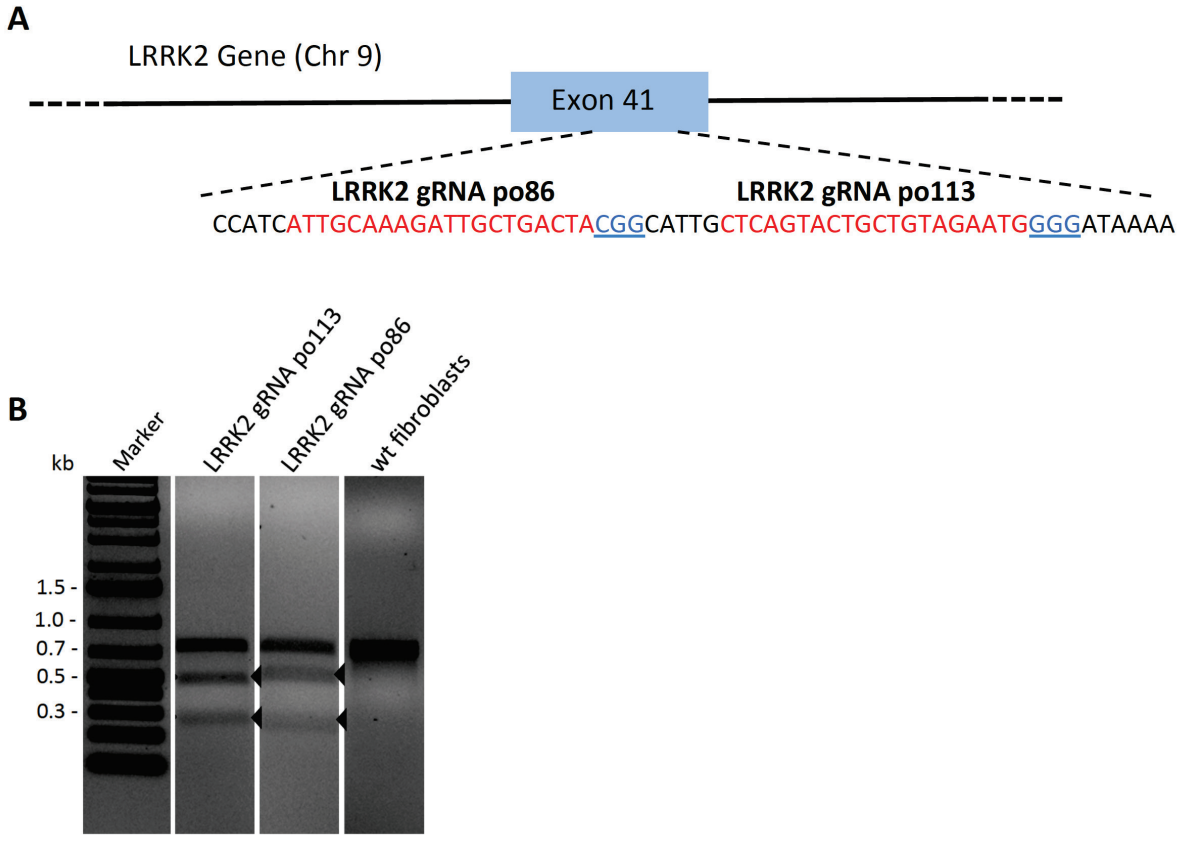

Figure 3.6. A) Schematic overview of the gRNAs targeting LRRK2 exon 41. PAM sequences are underlined and highlighted in blue. gRNA target sequences are highlighted in red. **B)** T7EN1 assay. Detection of CRISPR/Cas9 mediated gene modification by T7EN1 cleavage assay. As template gDNA of fibroblasts transfected with LRRK2 gRNA po113, LRRK2 gRNA po86 and untransfected wildtype fibroblasts (negative control) was used. Primers specific for the LRRK2 exon 41 were used for amplification.

or none are inserted into the cells, allowing to efficiently select for transfected cells even if only one plasmid contains the resistance gene.

The transfected cells were selected with puromycin for 3-4 days post transfection. Afterwards the cells were grown to confluency and harvested for genomic DNA extraction (2.6). The genomic DNA was used for PCR amplification (2.2) of the LRRK2 exon 41 region containing the gRNA target sites. The amplified DNA was analyzed using the T7 endo-nuclease 1 (T7EN1) cleavage assay to test for site-specific gene modifications (2.14.5). The cleaved DNA bands shown in Figure 3.6 B indicate the expected activity of CRISPR/Cas9 through non-homologues end joining (NHEJ) (1.2.3). This demonstrates the functionality of the CRISPR/Cas9 system in common marmoset monkey fibroblasts.

3.3.2 CRISPR/Cas9 based integration of ssODN HDR donors into the LRRK2 gene was unsuccessful in common marmoset fibroblasts

The activity and general functionality of the CRISPR/Cas9 system was shown in chapter 3.3.1. As described in the introduction (1.2.3), the CRISPR/Cas9 system can also be used to introduce point mutations or whole genes into a target genome by homology-directed repair (HDR). We designed single stranded oligodeoxynucleotides (ssODNs) as homologous for the introduction of the LRRK2 Gly2019Ser point mutation (2.14.4). The ssODNs consisted of a 12bp long sequence containing the LRRK2 point mutation as well as a point mutation knocking out a *Sca*I restriction site, flanked by sequences homologues to the target sequence (Figure 3.7 A+B). The homology arms were 75 NT (162 NT ssODN) and 40 NT (92 NT ssODN) long, respectively. The knockout of the *Sca*I restriction site was introduced to perform a prescreening of DNA clones by *Sca*I digestion, thereby reducing number of clones necessary for sequencing. To introduce the point mutations, the CRISPR/Cas9 LRRK2 gRNA plasmids were co transfected with the ssODNs and a puromycin resistance plasmid into common marmoset fibroblasts. The four combinations of LRRK2 gRNAs and ssODNS were: LRRK2 gRNA po86 + ssODN 162NT, LRRK2 gRNA po86 + ssODN92, LRRK2 gRNA po113 + ssODN 162NT, LRRK2 gRNA po113 + ssODN 92NT. After selection with Puromycin for three to four days, the cells were grown to confluency and genomic DNA was extracted (2.6). To obtain single DNA clones, the sequence potentially containing the LRRK2 point mutation was cloned into the pcDNA 3.1(-) plasmid as described in 2.14.6. The obtained clones (every one only containing one LRRK2 DNA sequence) were digested with *Sca*I for prescreening (2.14.6). Different fragment lengths were found (data not shown). Uncut clones were regarded

being putatively positive for CRISPR/Cas9 HDR because the *Sca*I restriction site was inactive, suggesting CRISPR/Cas9 mediated HDR. The putatively positive clones were sequenced

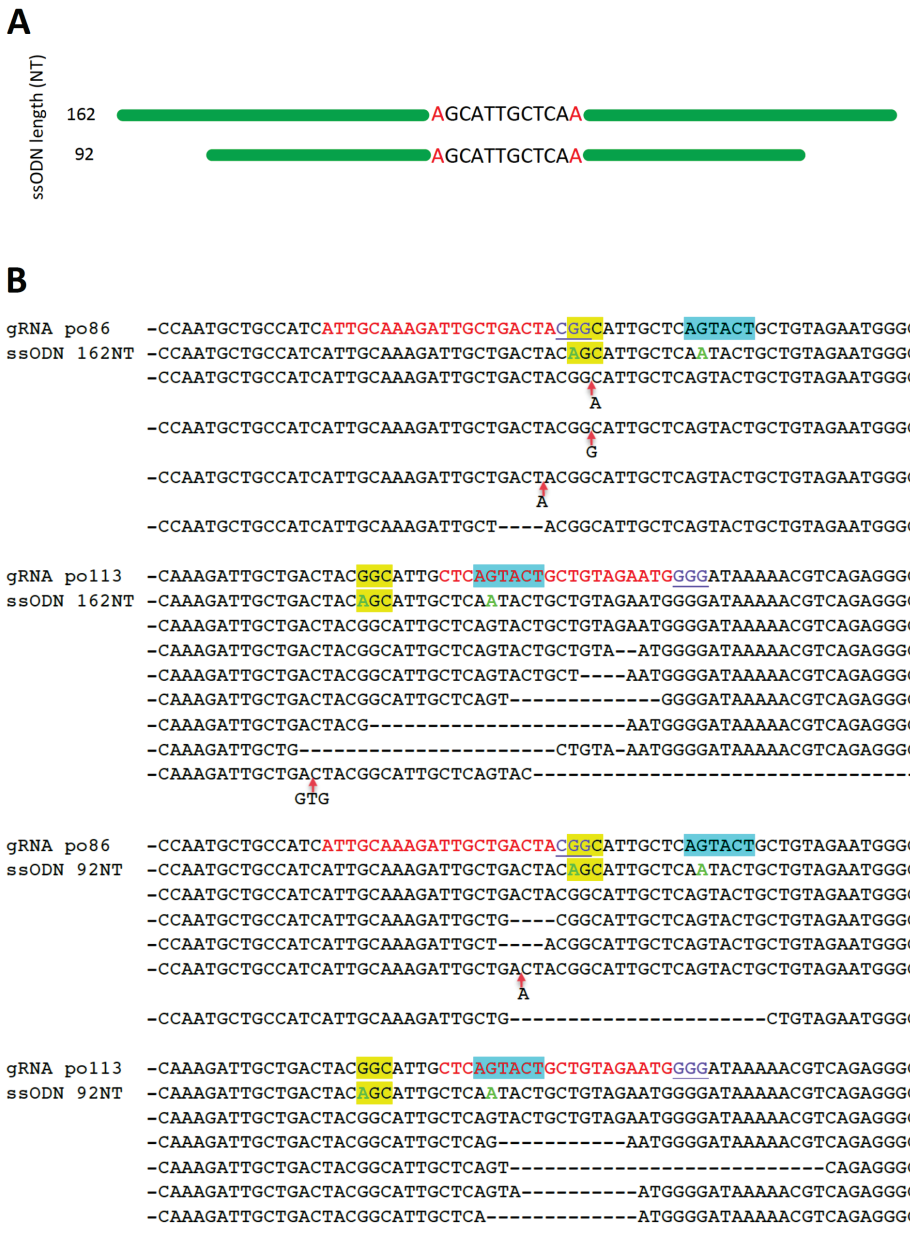

Figure 3.7. A) Used single stranded oligodeoxynucleotides (ssODNs). ssODNs consist of a 12 bp long stretch containing the mismatches against the genomic DNA for LRRK2 Gly2019Ser mutation introduction and *Sca*I restriction site knockout. The flanking homologous arms were 75 NT (162 NT ssODN) and 40 NT (92 NT ssODN) long. **B)** Sequences of common marmoset fibroblasts from LRRK2 Gly2019Ser mutation knock-in experiments. Clones of the PCR products were prescreened by *Sca*I digestion and putatively positive clones were analyzed by DNA sequencing. Additionally, some control (Ctrl) clones (putatively negative for HDR) were sequenced for comparison reasons. The PAM sequences are underlined and highlighted in blue, the gRNA targeting sequences in red, the position oft he ScaI restriction site is marked with a blue bar, the position of the Gly2019Ser mutation is marked with a yellow bar. The mutations carried by the ssODNs are highlighted in green. In brackets: deletions = (-), insertions =(+). N/N indicates colonies with same sequence out of total sequenced.

and aligned against the *LRRK2* wt sequence to check for the introduction of the *LRRK2* pointmutation. Figure 3.7 B shows the alignment of sequences of putatively positive clones and several control clone (putatively negative) sequences. The sequencing results show that no sequence changes resulting from HDR were detectable. However, different deletions and insertions were detected indicating activity of CRISPR/Cas9 through NHEJ as described in 3.3.1. Some putatively positive clones were false positive in the prescreening assay due to misligations or empty, religated plasmids. Overall 576 colonies were analyzed, 144 clones for LRRK2 gRNA po86 + ssODN 162NT, 216 clones LRRK2 gRNA po86 + ssODN 92NT, 96 clones of LRRK2 gRNA po113 + ssODN 162NT, 120 clones LRRK2 gRNA po113 + ssODN 92NT.

3.4 Immortalization of common marmoset fibroblasts using *piggyBac*-mediated *hTERT* transgenesis

As part of this study I wanted to immortalize common marmoset fibroblast by robust long-term expression of human telomerase reverse transcriptase (*hTERT*) using the *piggyBac* transposon system. Human *TERT* immortalization circumvents cell senescence and crisis and allows virtually infinite cell proliferation. Such cells would allow long-term studies with fibroblast-like cells without the potential severe effects of cell senescence likely to occur in primary fibroblasts.

3.4.1 Exogenous *hTERT* prolongs cell proliferation potential in common marmoset fibroblasts

To immortalize common marmoset fibroblasts I used human telomerase reverse transcriptase (*hTERT*). For the *hTERT* transgene insertion into the fibroblast genome we used the *piggyBac* transposon system. The *hTERT* cDNA was cloned into the *piggyBac* transposon system as described in 2.4.5.

Fibroblasts were cotransfected (2.15.6.1) with the *piggyBac* plasmid containing the *hTERT* cDNA (3.8 A) and the PBase plasmid (Figure 3.2 C) catalyzing the integration of the *piggyBac* transposon system. After transfection, the transfected cells were puromycin selected for ten passages. The first generated cells were polyclonal (derived from different cells) and were named polyclonal *hTERT* cells. Additionally the polyclonal cells were clonally expanded as described in 2.15.4, to obtain monoclonal (derived from one cell) cell lines. Two monoclonal

cell lines (#1, #4) were generated and further passaged as well as the polyclonal cell line. The cell lines were passaged in parallel to non-transfected primary fibroblasts (as control). To examine whether the *hTERT* transfected cells have an extended ability to replicate, we calculated the population-doubling level (PDL) (2.15.5) to get an estimate of their proliferation potential. The PDL is an approximation of the occurring population doublings over time (passages). The same was performed for the control cells. As expected, the *hTERT* transfected cells clearly show higher proliferation potential then primary fibroblasts (Figure 3.8 B). The *hTERT* transfected cells underwent several hundred population doublings until the experiments were terminated (Figure 3.8 B). In contrast, the primary fibroblast controls underwent a significantly

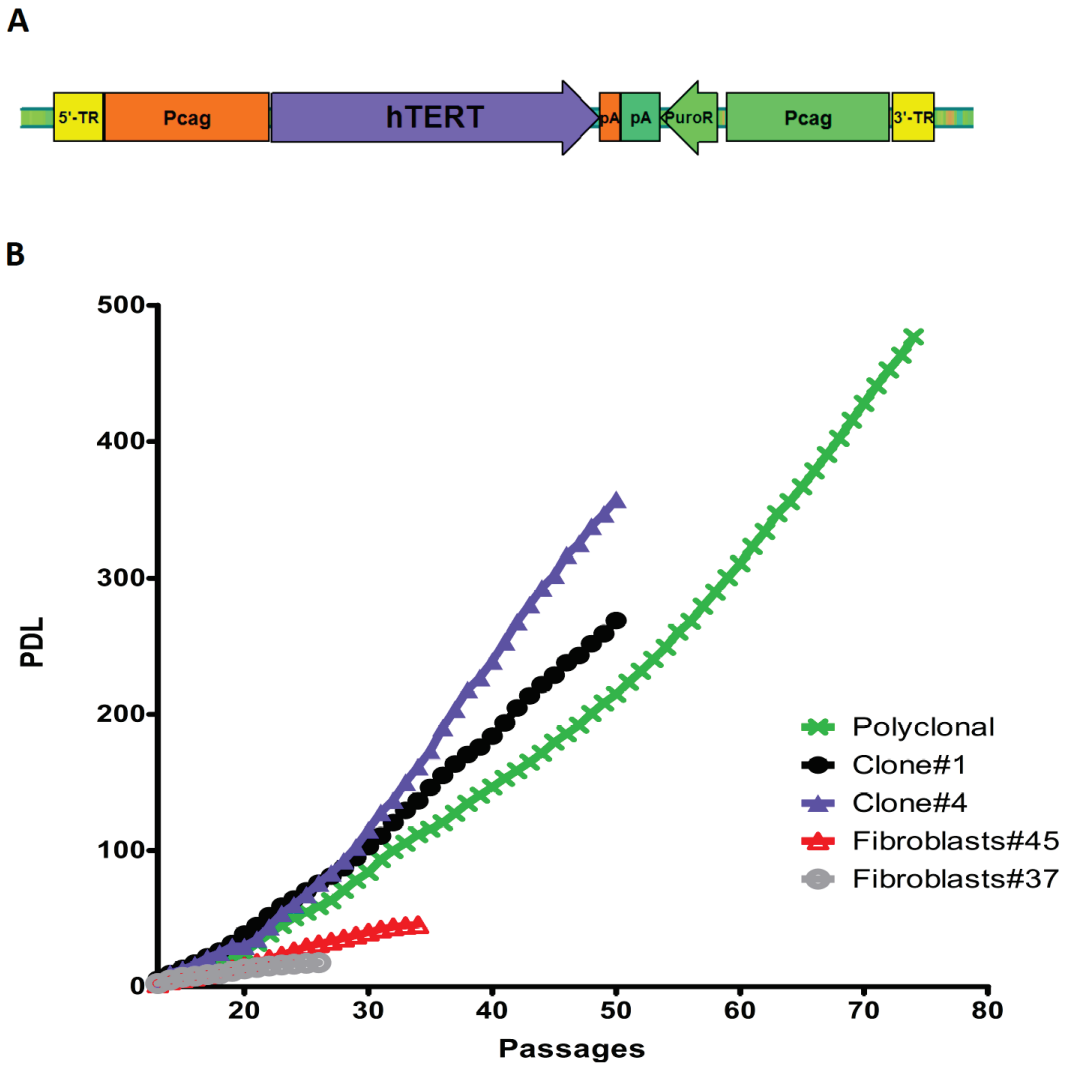

Figure 3.8. A) *piggyBac* construct used for immortalization. Human *TERT* is under control of the CAG promoter (Pcag). pA: poly A signal; 5'-TR: 5'-terminal repeat; 3'-TR: 3'-terminal repeat;; P: puromycin resistance gene. **B)** hTERT Cell Population-doubling level (PDL). PDL was determined by using cell counts and the respective number of passages. PDL was calculated with the formula: $PDL_{(n/n-1)} = \log (Nf/N0)/\log 2$; n=passage number, Nf=final number of cells, N0 = number of cells seeded at passage.

lower number of population doublings, reaching only a PDL of 46 and 18, respectively (Figure 3.8). These experiments were performed over a timeframe of more than nine months.

Phase contrast microscopy of the cells revealed a morphological change of the immortalized cells in comparison to proliferating fibroblasts. The immortalized cell lines exhibit a thin and "needle like" shape (Figure 3.9 A). Such changes in morphology could indicate changes in gene expression, as common in cancer cells. Images of non-proliferating fibroblasts in a high passage show the typical morphology of cells in senescence (Blagosklonny, 2006). They show an enlargement of the cytoplasm (cell hypertrophy), with change from the fibroblast typical narrow, drawn-out morphology to a broader, rounder morphology (Figure 3.9 A). The

immortalized cell lines still exhibit proliferation activity, even in high passages (Figure 3.9 A). To determine *hTERT* transgenesis, I performed immunofluorescence (IF) staining using a TERT specific antibody (2.17). Staining was performed using the *hTERT* immortalized cell lines as well as fibroblasts and ES cells as controls (2.17).

All three immortalized cell lines (polyclonal, #1, #4) showed expression of *TERT* (Figure 3.9 B). Additionally, it was observed that the *hTERT* immortalized cell lines have smaller sized nuclei than primary fibroblasts (Figure 3.9 B), further indicating changes associated with cancerous transformation. Furthermore, we performed RT-PCR (2.2, 2.9) to show *hTERT* mRNA expression in the immortalized cell lines (Figure 3.9 C).

These results indicate cell immortalization in common marmoset fibroblasts through *hTERT* transgene expression. However, the generated cell lines do not show the same morphology as primary fibroblasts. To our knowledge, no *hTERT* immortalized common marmoset cell lines have been reported so far.

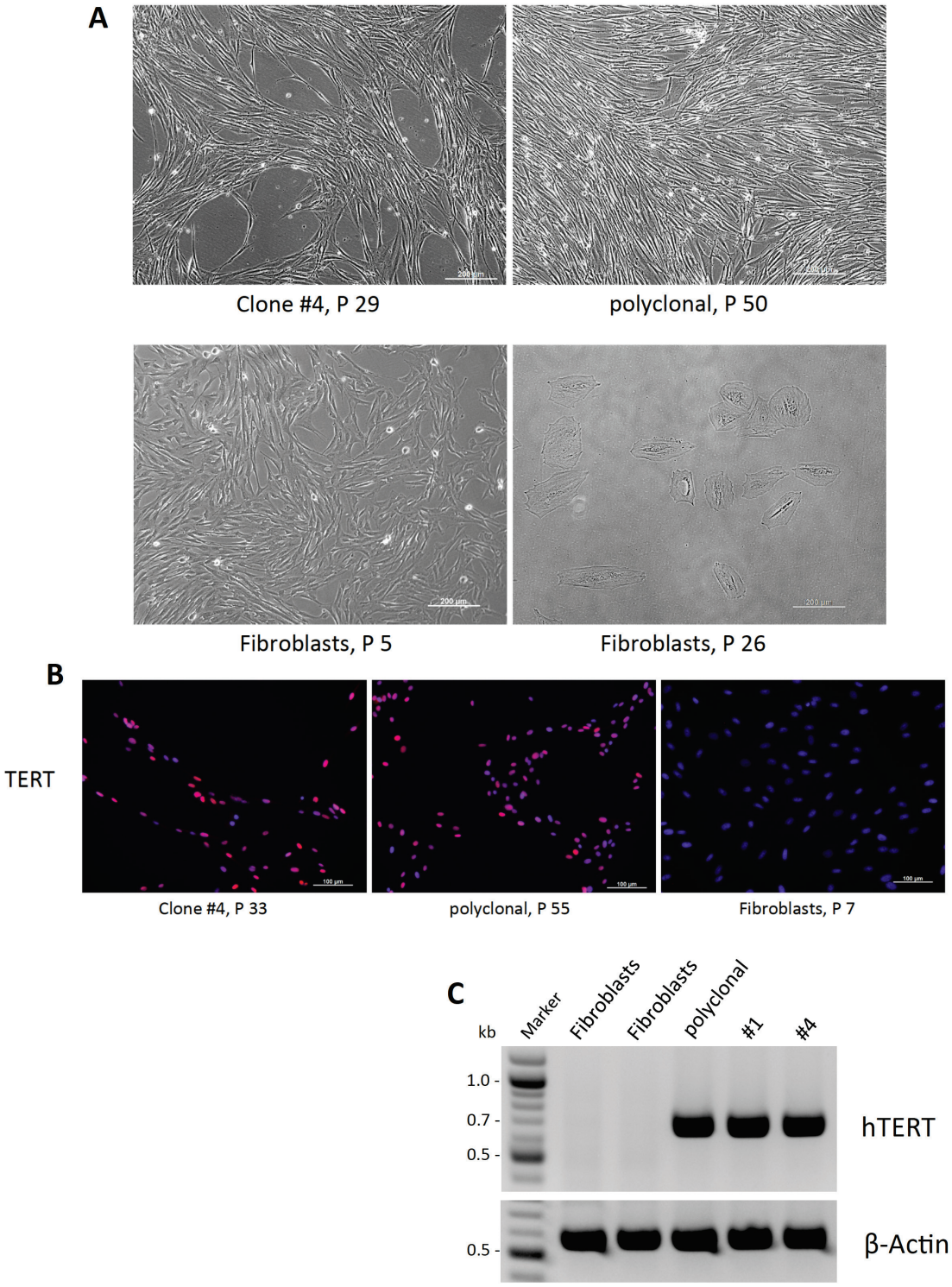

Figure 3.9. A) Phase contrast images of the *hTERT* immortalized cell lines and fibroblast controls. P: passage. Clone #4 and the polyclonal cell line show a thinner more "needle like" morphology when compared to fibroblasts. Fibroblasts in P26 show a flat and broad morphology typical for cells in senescence. Bars = 200µm **B)** *hTERT* immunofluorescence staining of immortalized cell lines. Immortalized cell lines show expected *TERT* expression (red). No expression was detected in fibroblasts. Bars = 200µm. Only images of clone #4 are shown because clone #4 and #1 are indistinguishable from each other **C)** RT-PCR for expression of *hTERT*. Primer pairs specific for the *hTERT* transgene were used. As a loading control, β-Actin was amplified. As template DNA, cDNA of the immortalized cell lines and fibroblasts was used. Fibroblasts of two different common marmosets were used as control. For all templates –RT controls were used.

4. Discussion

The aim of this study was to establish methods for the genomic modification of the common marmoset monkey. This would extend the spectrum of possible applications of the common marmoset as a model organism in biomedical and preclinical research. The different parts of this study were i) the generation of transgenic common marmosets carrying a pluripotency specific marker cassette ii) establishing CRISPR/Cas9 system, a new and promising method for gene targeting, in common marmoset monkey cells, and iii) generation of immortalized common marmoset fibroblast cell lines, and the generation of marmoset iPS cell lines by robust expression of transgenes from the *piggyBac* transposon.

4.1 Generation of EOS-EiP transgenic common marmosets

To achieve the first aim of this thesis, i.e. in vivo transgenesis of marmoset monkeys, it was necessary to learn the required reproductive biology and embryological methods. These methods included the oocyte collection ("oocyte-pick up"; OPU), oocyte maturation ("in vitro maturation"; IVM), oocyte fertilization ("in vitro fertilization"; IVF) and in vitro culture (IVC) of IVF embryos. For all of these methods competence in handling of oocytes and embryos was needed. All the mentioned methods were challenging procedures that needed long-term training of several months. Therefore, most of the time at the CIEA was needed to acquire and perfect the practical skills to effectively generate transgenic common marmosets.

The required methods are well established at the CIEA, and proved to ensure highly efficient generation of wildtype (Tomioka et al., 2012) and transgenic (Sasaki et al., 2009) common marmosets.

The number of obtained oocytes from animals was dependent on the condition of the animal but also on the experience and skill of the operator performing the OPU. To obtain a maximum number of oocytes at the optimal stage for the IVF, the oocytes were in vitro matured. These procedures guaranteed a maximum output of oocytes for IVF. After IVF the embryos were injected with the used lentivirus and further cultured in vitro to obtain embryos at the optimal stage (8-16 cell stage) (experience at the CIEA) for embryotransfer into surrogate animals.

In this study the EOS-EiP lentiviral vector was chosen to generate EOS-EiP transgenic common marmosets. The EOS-EiP cassette was reported to exhibit pluripotency specific eGFP expression in mice and human iPSCs (Hotta et al., 2009). As in mice and human such a pluripotency specific eGFP marker system would allow to monitor the developmental state of iPS cells. Pluripotency-associated eGFP activity would be a very useful tool study culture conditions of iPS cells and to monitor in vivo, i.e. in the embryo, the effects of the activation of specific signals transduction pathways with regard to pluripotency. EOS-EiP transgenic fibroblasts would also allow the refinement of reprogramming procedures since activation of pluripotency could be easily monitored in living cells. In the common marmoset such a marker system was not yet reported.

We obtained three putatively transgenic marmosets, two of which were proven to carry the full EOS-EiP transgene. The animals are kept in the Central Institute for experimental animals in Kawasaki, Japan. Germline transmission of the transgene was not confirmed yet. The achieved birth rate after embryo transfer (12%) is of an average level for IVF embryos according to experiences at the CIEA (Tomioka et al., 2012).

The EOS-EiP virus injected one-cell stage embryos showed different levels of eGFP expression after virus injection. Only embryos that showed a clear strong eGFP signal together with a timely development were used for embryo transfer into recipient females. A timely development of embryos proved to be of great importance to assure proper implantation of embryos and thereby to obtain healthy offspring (Sasaki et al., 2009; Tomioka et al., 2012). By using embryos with a strong eGFP signal, preferably in all blastomeres, the probability of obtaining offspring with a strong transgene expression is the highest. Similar eGFP signal intensity in all blastomeres further suggests transgene integration at an early developmental stage. Earliest integration of the virus is desired since this leads to less extensive genetic mosaicism in the offspring.

Sasaki et al. (2009) showed germline transmission of the eGFP transgene. As a first step to demonstrate germline transmission of the EOS-EiP, sperm samples of the transgenic animal I779tgM should be analyzed. At the time this thesis was written the animal I779tgM was most likely close to sexual maturity, but no analysis was performed yet at the CIEA (E. Sasaki personal communication).

Both living animals (I779tgM, I664tgF) obtained from EOS-EiP injected embryos exhibit normal behavior. Furthermore, they do not show any apparent increased susceptibility to diseases compared to wildtype common marmosets. This was also shown for other marmosets

derived from non-disease inducing lentivirus-injected embryos (Sasaki et al., 2009) (unpublished data). This is important since theoretically already the random integration of lentiviral vectors can cause harmful genetic changes in embryos and later developmental stages. However, if too severe, such mutations prevent proper early development of embryos so that they are either eliminated prior to retransfer, or, if retransferred, result in early stage abortions.

The pluripotency specific activity of the EOS-EiP reporter would mark the pluripotent cell fractions (inner cell mass/embryoblast) in preimplantation and early postimplantation embryos, allowing live imaging of the different developmental stages. This would help to better understand the processes in early embryo development. Furthermore, in cell differentiation for preclinical testing of cell replacement therapies, EOS-EiP based GFP expression would mark residual pluripotent cells, which harbor a high tumorigenic potential when transplanted.

In summary, transgenesis in common marmosets offers new possibilities in biomedical research. Genetically modified marmosets can become valuable disease models for translational research as well as for efficacy and safety testing of new therapeutics.

4.2 iPS cell generation from EOS transgenic common marmoset fibroblasts using the *piggyBac* transposon system

In this study, two iPS cell lines were generated from EOS-EiP transgenic common marmoset I780tgM. Both lines were reprogrammed with the classical four factors *SOX2, OCT4, KLF4, c-MYC* (SOKM) using the *piggyBac* transposon system. The *piggyBac* transposon construct used in this study was designed and constructed by Katharina Debowski (Debowski et al., 2015). All analyses performed including RT-PCR and IF staining as well as the continuous growth and the indistinguishable morphology between the generated iPS cells and the reference ES cells indicate successful and complete reprogramming. However, additional functional assays like the teratoma formation assay and the emryoid body formation assays would be beneficial to prove pluripotency of the iPS cells generated in this study.

The iPS cells presented in this study were generated with only the classical four (SOKM) reprogramming factors. The cells show the same morphology as common marmoset ES cells and are also comparable to published marmoset iPS cells generated with six reprogramming factors (SOKMLN) (Debowski et al., 2015; Tomioka et al., 2010). An important difference between four factor and six factor reprogramming of marmoset cells shown in this study, is

the time necessary for colony formation. The first colonies of four factor reprogrammed cells were picked after 91 days. In contrast, six factor reprogrammed cells only needed 28 days until first colonies were picked. However, other reports of common marmoset iPS cells generation with only the classical four factors (Wiedemann et al., 2012; Wu et al., 2010) showed different morphologies as observed in this study. The iPS cells published by Wu et al. 2010 did not exhibit the typical ES/iPS cell characteristics e.g. epitheloid cells forming tightly packed cell colonies with a clear colony boundary. Wiedemann et al. 2012 reported iPS cells that displayed rather dome-like colony morphology, characteristic for mouse ES cells. The differences in methodology between the four factor iPS cells in this study and from Wu and Wiedemann are the origin of the reprogramming factors the used method for delivery of reprogramming factors and the time in culture before colony picking/passaging. The reprogramming factors used in this study are derived from common marmoset cDNA whereas the other groups used human derived reprogramming factors. Wu et al. used retroviral transduction and cultured the cells for 21 days until colonies were transferred further. Wiedemann et al. used a lentiviral vector system for reprogramming factor delivery and cultured the cells for 12-22 days until colonies were further passaged. Although it is difficult to compare different ways of cell reprogramming, the mentioned differences in methodology between the four factor reprogrammed cells in this study and from Wu et al. and Wiedemann et al. might explain the observed differences in iPS cell morphology. Moreover, due to the very limited reprogramming period in the Wu and in the Wiedemann studies, it is likely that the cells cultured and characterized in these studies were only partly reprogrammed. In our lab mature iPS cell lines were never isolated after less than four weeks, even if six factors were used for reprogramming.

The EOS-EiP cassette inserted into the common marmoset genome via lentiviral integration is supposed to exhibit pluripotency associated eGFP activity. Hence, it was expected that the generated iPS cells show an eGFP signal. However, the cells showed no eGFP activity visible in fluorescence microscopy, and flow cytometric analysis was also negative for eGFP expression. However, RT-PCR results indicate eGFP mRNA expression. Real time quantitative PCR, however, detected only very low levels of eGFP mRNA that were only slightly above the negative control levels. Therefore, we conclude that eGFP expression is not sufficient for eGFP detection through fluorescence microscopy or flow cytometry, but that very low levels of eGFP mRNA are produced that are sufficient for RT-PCR detection.

Inactivity of eGFP reporter cassette in reprogrammed iPS cells could be due to viral rearrangement of the EOS-EiP integration cassette leading to changes in the promoter or eGFP sequence. However, sequencing revealed intact sequences for the EOS promoter (early transposon promoter + *Sox2/Oct4* enhancer elements) and for the eGFP ORF.

Hotta and colleagues showed EOS-EiP based eGFP expression in human and mouse iPSCs and reported that no viral vector silencing was detected. However, lentiviral vector silencing through epigenetic effects (DNA methylation, histone modifications) is a known issue (Antoniou et al., 2013; Ellis, 2005) and it is likely that this is the reason for the inactivity of the EOS-EiP cassette in common marmoset iPS cells. However, this has to proven by appropriate methods like DNA methylation analysis.

Hotta and colleagues used an early transposon promoter that was shown to be highly active in mouse and human pluripotent cells but inactive in differentiated cells. (Maksakova and Mager, 2005). This promoter, as well as the *Sox2* and *Oct-4* enhancers in the EOS-EiP vector, were all derived from mouse DNA. Even though the EOS-EiP reporter worked in human cells, the mouse origin could also influence the activity of the construct in marmoset cells.

If common marmoset iPS cells with EOS-EiP activity can be obtained, they would be a valuable system for investigating cell reprogramming and cell differentiation. In reprogramming experiments developing pluripotent colonies would be marked by GFP fluorescence before a selection through colony morphology would be possible. This would allow investigating the earliest stages of reprogramming helping to better understand the underlying mechanisms of cell reprogramming. Furthermore, EOS-EiP would be beneficial for preclinical testing of cell replacement therapies. Since differentiation of iPS cells would cause GFP silencing, GFP expression would mark residual pluripotent cells. This would assure that only properly differentiated cells are used for cell transplantation but at the same time also that residual pluripotent cells, which harbor a high tumorigenic potential when transplanted, could be eliminated.

The *piggyBac* based reprogramming system used in this study is reversible, i.e. the integrated reprogramming cassette can be excised (Kim and Pyykko, 2011). Therefore, using the *piggyBac* system, it is possible to obtain transgene free iPS cells. This reduces the risk of tumorigenicity because no exogenous genes remain randomly integrated in the cell genome. Furthermore, it is especially desirable to obtain *c-MYC* transgene free iPS cells because *c-MYC* is a proto-oncogene and its overexpression can result in tumor formation. Other advantages of the *piggyBac* system include that no silencing has been reported yet and that larger transgenes can be transferred then for instance by lentiviruses.

Induced PS cells have great potential in the search for new treatments for cell and tissue degenerative diseases, e.g. Parkinson's disease (PD). Induced PS cell-derived cells and tissues could be used for tissue or cell replacement therapies. For the development of such therapies it is crucial to perform efficacy and safety testings of the highest standard possible. Non-human primate (NHP) iPS cells and NHP themselves are of immense value for such testings because of their genetical, developmental, behavioral and physiological comparability to humans (t Hart et al., 2015). Furthermore, due to their long life span, NHP allow safety testings over longer periods which might be especially valuable in progressive diseases like PD. Therefore efficacy and safety testing in NHP are of very high translational value (Belmonte et al., 2015; t Hart et al., 2015).

4.3 Targeting the *LRRK2* gene of the common marmoset using the CRISPR/Cas9 system

In this part of the study I applied the CRISPR/Cas9 system to modify common marmoset monkey cells. Generation of gene-targeted common marmoset cells and animals may have a big impact on future biomedical research allowing generation of transgene free genetic disease models.

The CRISPR/Cas9 experiments performed in this study caused NHEJ based gene knockouts in marmoset cells. Preliminary work for this study also showed activity of CRISPR/Cas9 in marmoset fibroblasts for other genes (data not shown). The generation of fibroblasts carrying the LRRK2 Gly2019Ser pointmutation by CRISPR/Cas9 induced HDR was not successful although overall 576 clones from four different experiments were checked. Other experiments with CRISPR/Cas9 in common marmoset cells, performed by scientists at the CIEA, were also unsuccessful in obtaining cells with HDR mediated gene modification (unpublished data). This additionally indicates the difficulty of CRISPR/Cas9 mediated HDR based targeted gene modification in the common marmoset.

Even though some groups reported successful CRISPR/Cas9 mediated HDR in other mammalian species (Wang et al., 2013; Zhu et al., 2015), others report only partial, or no integration of a homologous template (Whitworth et al., 2014). However, successful HDR only show very low efficiencies.

Several approaches to increase CRISPR/Cas9 based HDR are known. Reports showed that modified Cas9 nuclease, active as nickase with paired gRNAs, can lead to higher HDR effi-

ciency (Mali et al., 2013a; Rong et al., 2014). In this altered CRISPR/Cas9 system the Cas9 nuclease only nicks a single strand of the DNA. Therefore, two gRNAs, one for the leading strand and one for the lagging strand, are necessary to achieve a doublestrand break. This further reduces the possibility of off-target effects because proximity of both gRNAs is necessary to induce a double strand break. In *Drosophila melanogaster*, it was recently shown that knockout or knockdown of the DNA Ligase4 (Lig4) gene highly increases the occurrence of CRISPR/Cas9 based HDR events (Böttcher et al., 2014; Gratz et al., 2014). The DNA Ligase4 joins double-strand breaks during NHEJ. Knockout or knockdown of Lig4 subsequently results in a repression of the NHEJ pathway and in an increase in HDR events. In human cells, a recent report (Ho et al., 2015) showed successful knockdown of LIG4 via RNAi with subsequent increase of HDR events. An alternative to LIG4 as target to increase HDR is the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). Pharmacological inhibitors of DNA-PKcs were reported to repress the NHEJ pathway, thereby increasing the occurrence of HDR events (Robert et al., 2015) Both, Lig4 and DNA-PKcs based NHEJ repression might allow successful HDR also in other species. In future experiments the application of these refined strategies should be tested in marmoset cells.

Possible off-target effects of the CRISPR/Cas9 system are well documented (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). However, in this study the main aim was to achieve modification of the target site in the common marmoset. Therefore, possible off-targets were not analyzed. Though, the used gRNAs were selected using the CRISPR design tool *(http://crispr.mit.edu/)*, operated by the Zhang lab. This online tool analyses the gRNAs for possible off-target effects through cross-referencing with the target genome, thereby minimalizing the likelihood of off-target activity. However, the used gRNAs were only cross-referenced with the human genome, since the common marmoset genome is not available in the CRISPR design tool yet. For future applications of the CRISPR/Cas9 system, analyzing possible off-target loci is of great importance to rule out off-target mutations.

Liang et al. (2015) reported CRISPR/Cas9 based gene modification in human tripronuclear embryos. Even though the embryos were not viable (because of triploidy) the study was highly controversial. Even though the experiments are highly controversial and ethically discussible, the theoretical potential of the CRISPR/Cas9 technology for the treatment of human diseases is indisputable. However, a major obstacle for the application of CRISPR/Cas9 in human embryo found in that study was the occurrence of mutations at several off-target locations. A NHP model like the common marmoset would be helpful for further testing and “fi-

ne-tuning" of CRISPR/Cas9 before it should be further used in human embryos or cells designated for future cell replacement therapy.

I was able to show that CRISPR/Cas9 allows efficient gene knockout in common marmoset cells. This will allow generation of in vitro and in vivo common marmoset diseases models in the future. For the introduction of point mutations or additional DNA sequences, the system needs to be further refined.

4.4 *piggyBac*-mediated expression of *hTERT* resulted in immortalization of common marmoset fibroblasts

In this study three immortalized fibroblast cell lines were generated by *piggyBac* mediated expression of *hTERT*. Two of the generated cell lines were derived from a single cell and are therefore defined as monoclonal cell lines.

The high proliferation potential of the generated cell lines in comparison to control cells indicates successful immortalization. However, the slight changes in morphology also suggest a change in gene expression that results from stable *hTERT* expression.

Several studies report similar changes in cell morphology (Mondello et al., 2003; Toouli et al., 2002; Wang et al., 2000; Zongaro et al., 2005) while others report a wildtype phenotype and no further changes in *hTERT* immortalized cells (Bodnar et al., 1998; Morales et al., 1999; Simonsen et al., 2002). If the observed morphological changes result from *hTERT* expression or the random *piggyBac* transposon induced integration cannot be determined. The changed cell morphology of the immortalized cells in this study compared to primary fibroblasts could indicate cancer associated changes in gene expression (Belgiovine et al., 2008; Zongaro et al., 2005). Further analysis of the cells is necessary to determine possible cancer associated changes. Therefore, a transcriptome analysis of the immortalized cells and control cells will be performed to check for differences in gene expression of cancer associated genes like tumor protein p53 (*p53*) or retinoblastoma (*RB*). However, the obtained immortalized cell lines allow long-term studies with at least fibroblast like cells under a constant cellular phenotype. This offers new possibilities for research with these cells that are not possible with primary cells with a limited lifetime. An advantage of monoclonal cell lines is that they offer the possibility to perform analysis multiple times under genetically and phenotypically identical conditions. Moreover, stable marmoset cell lines could be useful for the development of biologics, e.g. cytokines, monoclonal antibodies.

An alternative to immortalization with *hTERT* is the use of the viral oncogene simian virus 40 large T antigen (SV40T). It was shown that SV40T is sufficient to immortalize rodent cells (Allen et al., 2000), but in human cells it is only sufficient to overcome senescence but not crisis. Furthermore, SV40T treated cells generally exhibit more severe changes in morphology and gene expression than *hTERT* treated cells when compared to wildtype cells. However, some studies showed that *hTERT* expression is not always sufficient to achieve cell immortalization (Darimont et al., 2002; Kiyono et al., 1998; Yudoh et al., 2001). Especially in immortalization-refractory cell types, e.g. adult hepatocytes or respiratory tract epithelial cells, *hTERT* alone is not always sufficient to stably immortalize cells (Lundberg et al., 2002; Ramboer et al., 2014). However, the combined expression of *hTERT* and other factors like SV40 Tag was shown to successfully immortalize such cell types (Nguyen et al., 2005)(Kemp et al., 2008). Human *TERT* based immortalization of differentiated cell types could be used to generate cellular in vitro models. Examples of possible candidate cell types of the respiratory epithelium and hepatocytes. As mentioned above, such cells would most likely require a combined expression of *hTERT* and SV40T. However, marmoset or other NHP in vitro models of e.g. the respiratory epithelium or the liver could be used to investigate infectious diseases and to test new drugs for such diseases. The advantage of such immortalized cells in comparison to primary cells or tissue culture would be the reproducibility of such experiments under genetically and phenotypically defined conditions. Furthermore, such immortalized in vitro models would reduce the need for primary cells and tissues, thereby reducing the number of animals needed for cell and tissue collection.

4.5 Summary

I was able to successfully perform transgenesis and gene modification in the common marmoset on the cellular and organismic level.

I generated two transgenic common marmoset monkeys carrying the EOS-EiP transgene using lentiviral transduction. Furthermore, I was able to generate two iPS cell lines from primary fibroblasts of the EOS-EiP transgenic marmosets using the virus-free *piggyBac* transposon system. Notably, I succeeded in reprogramming the fibroblasts making use of only the four classical reprogramming factors SOX2, OCT4, KLF4 and c-MYC, omitting LIN28, which was reported to be essential for common marmoset monkey cell reprogramming. However, refinement is necessary to achieve the initial goal of obtaining cells with an active pluripotency reporter. I also successfully applied the CRISPR/Cas9 system to modify the Parkinson's

disease associated gene *LRRK2* in common marmoset primary fibroblasts. However, to achieve CRISPR/Cas9 induced homology directed repair (HDR) further research is necessary. Additionally, I generated immortalized common marmoset monkey fibroblast cell lines by stably introducing the *hTERT* transgene, using the *piggyBac* transposon system.

Overall, the achievements in this study hopefully contribute to an expanded application of the common marmoset in biomedical and translational research.

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6. Appendix

6.1 Oligonucleotide sequences

Sequences of all used oligonucleotides in this study.

Oligonucleotides used for RT-PCR

Target	Primer name (orientation), Primer sequence (5'➔3')	PCR-Product size (bp)
SOX2	G0086 (fwd): TCTTCCTCGCACTCCAGGGC G0030 (rev): CCGCTCGAGAATGCCTCCCCCGTCCAGTTCG	228
OCT4	G0022 (fwd): GATCGGATCCTTGGGGCGCCTTCCTTC G0035 (rev): CAGGGTGATCCTCTTCTGCTTC	510
KLF4	G0091 (fwd): GGAAGACGATCTTGGCCCCG G0021 (rev): GTACTCTAGACAGTGTGGGTCATATCCACTG	323
c-MYC	G0079 (fwd): ATAAGAATGCGGCCGCACTGGATTTTTTTCGGGCAGTGG G0142 (rev): CCTGGATGATGATGTTTTTGATG	456
LIN28	G0305 (fwd): GACGAGCTGTACAAGGGGAGTGAGAGGCGGCCAAAGGGG G0025 (rev): GACTCTCGAGATAGCCAAAGAATAGCCCC	334
NANOG	G0018 (fwd): GATCAAGCTTCCTTTTCCCCAATAATAACATG G0075 (rev): TTATAGAAGGGACTGCTCCAGG	754
S-O (SOX2-OCT4)	G0086 (fwd): TCTTCCTCGCACTCCAGGGC G0035 (rev): CAGGGTGATCCTCTTCTGCTTC	735
L-N (LIN28-NANOG)	G0302 (fwd): AGCCATATGGTAGCCTCATG G0070 (rev): GGTTGCTCCAGGTTGAATTGC	811
β-Actin	G0336 (fwd): GACGACATGGAGAAGATCTGG G0337 (rev): GGAAAGAAGGCTGGAAGAGTG	562
eGFP	eGFP 5-4 (fwd): CAAGGACGACGGCAACTACAAGACC eGFP3-3es (rev): GCTCGTCCATGCCGAGAGTGA	407
hTERT	G0957 (fwd): CTGGACGATATCCACAGGGC G0958 (rev): AAGTTCACCACGCAGCCATA	653

Oligonucleotides used for EOS-EiP analysis

Genotyping Primer

Target	Primer name (orientation), Primer sequence (5'➔3')	PCR-Product size (bp)
EOS-EiP cassette	G1108_EOS_fwd_02 (fwd): CTTGGCATTCCGGTACTGTT G1109_EOS_rev_02 (rev): AAGTCGTGCTGCTTCATGTG	283

Sequencing Primer

Target	Primer name (orientation), Primer sequence (5'➔3')	PCR-Product size (bp)
EOS-EiP cassette	G1106_EOS_fwd_01 (fwd): CTTGCTCAGGGGTGGAGCTT eGFP3-3es (rev): GCTCGTCCATGCCGAGAGTGA	1048
EOS-EiP cassette	G1267_EOS_Prom_fwd (fwd): CTGGATGGAGTGGGACAGAG G1107_EOS_rev_01 (rev): GATCGCAGATCTTGTTGCGG	1105
EOS-EiP cassette	G1108_EOS_fwd_02 (fwd): CTTGGCATTCCGGTACTGTT G1268_EOS_GFP_rev (rev): AGACCCCTAGGAATGCTCGT	954

Oligonucleotides used for real-time qPCR

Target	Primer name (orientation), Primer sequence (5'➔3')	PCR-Product size (bp)
eGFP	G1261_GFP_fwd1 (fwd): AGTCCGCCCTGAGCAAAGA G1262_GFP_rev1 (rev): TCCAGCAGGACCATGTGATC	56
18s rRNA	G0871 (fwd): ATTAAGGGTGTGGGCCGAAG G0872 (rev): GAGTTCTCCTGCCCTCTTGG	81

Oligonucleotides used for CRISPR/Cas9

gRNA sequences

gRNA name	Oligo name (orientation), Oligo sequence (5'➔3')
LRRK2 gRNA po86	gRNA_LRRK2_Exon41_po86_fwd (fwd):CACCGATTGCAAAGATTGCTGACTA gRNA_LRRK2_Exon41_po86_rev (rev): AAACTAGTCAGCAATCTTTGCAATC
LRRK2 gRNA po113	gRNA_LRRK2_Exon41_po113_fwd (fwd): CACCGGCTCAGTACTGCTGTAGAAT gRNA_LRRK2_Exon41_po113_rev (rev): AAACATTCTACAGCAGTACTGAGCC

Primer used for T7EN1 assay and cloning into pcDNA3.1(-)

Target	Primer name (orientation), Primer sequence (5'➔3'), resriction sites are shown in lower case	PCR-Product size (bp)
LRRK2 Exon41	G1033_LRRK2_Exon41_fwd (fwd): GAGgctagcAAGGGTCAAAGTGAGCACAGA G1034_LRRK2_Exon41_rev (rev): GAGctcgagGTTTGCTTAGGTTTTGCCACC	742

ssODNs

Name	ssODN sequence (5'➔3')
ssODN 162NT	GACTTGAAGCCCCACAATGTGCTGCTTTTCACCCTGTATCCCAATGCTGCCATCATTGCAAAGATTGCTGACTACAGCAT TGCTCAATACTGCTGTAGAATGGGGATAAAAACGTCAGAGGGCACACCAGGTAGGTAATCAGGTTTGTCTCATAATTCTG TC
ssODN 92NT	GTATCCCAATGCTGCCATCATTGCAAAGATTGCTGACTACAGCATTGCTCAATACTGCTGTAGAATGGGGATAAAAACGT CAGAGGGCACAC

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel “Modifying the common marmoset monkey (*Callithrix jacchus*) genome: transgenesis and targeted gene modification in vivo and in vitro“ selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Diese Arbeit ist bislang keiner anderen Prüfungsbehörde vorgelegt worden und auch nicht veröffentlicht worden.

Ich bin mir bewusst, dass eine falsche Erklärung rechtliche Folgen haben wird.

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Ort, Datum, Unterschrift