Establishment and Characterization of a Human *in vitro* Cell Model for Parkinson's Disease

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1 Abbreviations

AD	Alzheimer's disease
CMV	cytomegalovirus
COR	C-terminal of ROC
GC	gene-corrected
hESCs	human embryonic stem cells
HI	Hertie-Institute
HTS	high-throughput screening
iPSCs	induced pluripotent stem cells
LRRK2	leucine rich repeat kinase 2
MAPT	microtubule associated protein TAU
mDA	midbrain dopaminergic neurons
MPI	Max-Planck-Institute
Mut	mutated
NFTs	neurofibrillary tangles
PD	Parkinson's disease
pTAU	phosphorylated TAU
ROC	ras-of-complex
SEM	standard error of the mean
TALEN	transcription activator-like effector nuclease
TH	tyrosine hydroxylase
UbC	ubiquitin c
WT	wild-type
ZFN	zinc finger nucleases

2 Summary

A major obstacle on the way to understand the molecular pathogenesis of Parkinson's disease (PD) and to develop disease-modifying treatments is the lack of suitable model systems that capture the relevant molecular events leading to human disease and are also accessible to compound screening. This cumulative thesis describes the establishment of two novel cellular model systems based on induced pluripotent stem cells (iPSCs): the generation of an *in vitro* cell model for PD consisting of post-mitotic midbrain dopaminergic neurons, and the derivation and expansion of human neural progenitors for neurodegenerative disease modelling.

For the first model, fibroblasts from two female PD patients with a G2019S mutation in the LRRK2 gene and four age- and sex-matched controls were reprogrammed into iPSCs. This was achieved through infection with the four factors Oct-4, Sox-2, Klf-4 and c-myc published by Shinia Yamanaka and isogenic gene-controls were generated using zinc finger nucleases (ZFNs) that differed from the original iPSCs only in the DNA base responsible for the G2019S mutation. Finally, one gene-corrected wild-type-LRRK2 line was obtained for each G2019S-LRRK2 line. In addition, an isogenic G2019S-LRRK2 line was generated for the wild-type-LRRK2 control C4, also through ZFN technology.

The iPSCs were then differentiated into midbrain dopaminergic (mDA) neurons by an optimized differentiation protocol. Several studies on these mDA neurons showed that they were electrophysiologically active and expressed neuronal as well as dopaminergic neuronal markers.

To clarify whether phenotypic changes can be observed, mDA neuronal cultures were investigated for two already described phenotypes associated with LRRK2: reduced neurite outgrowth and increased sensitivity to oxidative stress. Indeed, a reduced velocity of outgrowing neurons could be observed in G2019S-mutated iPSC-derived neurons. In addition, an increased sensitivity of the mutant neurons against oxidative stress could also be confirmed. As such, these assays suggested that iPSC-derived neurons from PD patients can serve as an *in vitro* PD model.

For further characterization of this PD model, the expression of the proteins α-synuclein and TAU or pTAU was investigated, as these proteins are accumulated or hyperphosphorylated in the brain of PD patients. An accumulation on protein level could be stated for both proteins as well as an elevated RNA level for TAU.

Mechanistic causes of these phenotypes such as the specific dysregulation of certain genes and an increased activation of Erk1/2 by G2019S-LRRK2 are described in the shared publication of Peter Reinhardt and me from 2013 in Cell Stem Cell: „Genetic Correction of a LRRK2 Mutation in Human iPSCs Links Parkinsonian Neurodegeneration to ERK-Dependent Changes in Gene Expression.“

The second cell model was generated to address the question of the suitability of iPSCs for high-throughput screenings. Due to relatively high heterogeneity, iPSC-derived neurons, as described above, are not ideally suited for this purpose. An additional aim was to develop a cell model that is not solely limited to PD but also allows studying other neurodegenerative diseases. Therefore, an iPSC-derived cell model system was established consisting of a robust neural progenitor cell type that is able to give rise to several neuronal subtypes. Results of this project were published in PLoS One in 2013: “Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling.”

3 Zusammenfassung

Die molekularen Pathomechanismen der Parkinson Erkrankung sind bis heute noch immer nicht ausreichend verstanden. Ebenso gibt es keine Behandlungsmöglichkeiten, die effektiv in den Krankheitsverlauf der Parkinson Erkrankung oder auch vieler anderer neurodegenerativer Erkrankungen eingreifen. Der Grund dafür ist, dass keine geeigneten Modelle zur Verfügung stehen, welche relevante, molekulare Merkmale der Krankheiten widerspiegeln oder sich für eine Medikamententestung im Hochdurchsatzverfahren eignen. Im Rahmen dieser kumulativen Dissertation wird die Etablierung zweier neuartiger Zellmodellsysteme beschrieben, die auf induzierten pluripotenten Stammzellen (iPS-Zellen) basieren. Das eine Zellmodell beinhaltet reife und postmitotische, dopaminerge Neuronen als *in vitro* Modell für die Parkinson Erkrankung. Dem anderen Modell liegen proliferierende, neurale Vorläuferzellen zu Grunde, aus welchem ein Modell zur Medikamententestung neurodegenerativer Erkrankungen im Allgemeinen entwickelt wurde.

Für das erste Modell wurden Fibroblasten von zwei Parkinson-Patientinnen mit einer G2019S Mutation im LRRK2-Gen und von vier alters- und geschlechtsangepassten Kontrollen durch Infektion mit den von Shinia Yamanaka publizierten Faktoren Oct-4, Sox-2, Klf-4 und c-myc zu iPS-Zellen reprogrammiert. Mit Hilfe von Zinkfingernukleasen wurden isogene Kontrollen generiert, die sich nur in derjenigen Base von den ursprünglichen iPS-Zellen unterschieden, welche die G2019S Mutation verursacht. So wurde für jede G2019S-LRRK2 Linie eine genkorrigierte Wildtyp-LRRK2 Linie angefertigt. Zusätzlich wurde für die unabhängige Wildtyp-LRRK2 Kontrolle C4, ebenfalls über Zinkfingernuklease-Technologie, eine isogene G2019S-mutierte Linie generiert.

Die iPS-Zellen wurden anschließend nach einem optimierten Differenzierungsprotokoll zu mittelhirnspezifischen dopaminergen Neuronen differenziert. Es konnte gezeigt werden, dass die Neuronen elektrophysiologisch aktiv waren und neuronale und dopaminerge Marker exprimierten.

Um zu klären, ob sich phänotypische Veränderungen in den dopaminergen Neuronen beobachten lassen, wurden die Neuronen auf zwei bereits beschriebene, LRRK2-assoziierte Phänotypen untersucht: Neuritenauswuchs und Sensitivität gegenüber oxidativem Stress. Es konnte gezeigt werden, dass die Neuritenauswuchsgeschwindigkeit bei den mutierten Neuronen verringert war. Zusätzlich wurde eine erhöhte Sensitivität der mutierten Neuronen gegenüber oxidativem Stress gefunden.

Zur weiteren Charakterisierung des Parkinson-Modells aus iPS-Zellen wurde die Expression der Proteine α-synuclein und TAU bzw. pTAU untersucht, welche beim Parkinson-Patienten akkumulieren oder hyperphosphoryliert sind. Für beide Proteine konnte eine Anhäufung auf Protein-Ebene und für TAU auch auf RNA-Ebene nachgewiesen werden.

Mechanistische Ursachen dieser Phänotypen, wie die spezifische Dysregulierung verschiedener Gene und die Hyperphosphorylierung und daher Überaktivierung von Erk1/2 durch G2019S-LRRK2 sind in der gemeinschaftlichen Publikation von Peter Reinhardt und mir im Journal Cell Stem Cell (2013) beschrieben: „Genetic Correction of a LRRK2 Mutation in Human iPSCs Links Parkinsonian Neurodegeneration to ERK-Dependent Changes in Gene Expression.“

Mit dem zweiten Modell wurde die Tauglichkeit der iPS Zellen für Hochdurchsatz Screenings untersucht. Aufgrund ihrer relativ hohen Heterogenität sind Neuronen, die von iPS-Zellen abgeleitet sind, für diese Anwendung nicht ideal. Aus diesem Grund war ein Ziel dieses Projektes, eine robuste, neurale und expandierbare Vorläufer-Linie zu entwickeln. Ein weiteres Ziel war, das Zellmodell so zu gestalten, dass es sich nicht nur auf Screenings für die Parkinson Erkrankung beschränkt sondern sich auch für andere neurodegenerative Erkrankungen eignet. Daher wurde ein Zellmodell auf der Basis eines robusten neuralen Vorläufers etabliert, welcher in mehrere neuronale Subtypen diffrenziert werden kann. Ergebnisse dieser Arbeit wurden 2013 im Journal PLoS One publiziert: “Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling.”

4 List of Publication in the Cumulative Thesis

Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression.

Reinhardt P*, Schmid B*, Burbulla LF, Schöndorf DC, Wagner L, Glatza M, Höing S, Hargus G, Heck SA, Dhingra A, Wu G, Müller S, Brockmann K, Kluba T, Maisel M, Krüger R, Berg D, Tsytsyura Y, Thiel CS, Psathaki OE, Klingauf J, Kuhlmann T, Klewin M, Müller H, Gasser T, Schöler HR, Sterneckert J.

Cell Stem Cell. 2013 Mar 7;12(3):354-67. doi: 10.1016/j.stem.2013.01.008.

***shared first authorship**

Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling.

Reinhardt P, Glatza M, Hemmer K, Tsytsyura Y, Thiel CS, Höing S, Moritz S, Parga JA, Wagner L, Bruder JM, Wu G, **Schmid B**, Röpke A, Klingauf J, Schwamborn JC, Gasser T, Schöler HR, Sterneckert J.

PLoS One. 2013;8(3):e59252. doi: 10.1371/journal.pone.0059252. Epub 2013 Mar 22.

5 Personal Contribution

This study has been a joint project from the groups of Prof. Dr. H. R. Schoeler at the Max-Planck-Institute (MPI) for Molecular Biomedicine in Muenster and of Prof. Dr. T. Gasser at the Hertie-Institute (HI) for Clinical Brain Research in Tuebingen. The lab-work was mainly shared between PhD student Peter Reinhardt (MPI) and me (HI). Direct supervisors of Peter Reinhardt and me were Drs. Jared Sterneckert (MPI) and Martina Maisel (HI). The general idea for the common project was born in a kick-off meeting in Muenster at the beginning of Peter Reinhardt's and my PhD theses in 2008.

The cultivation of fibroblasts of all patients and controls was done by me in Tuebingen. Reprogramming of fibroblasts was shared between Peter Reinhardt (L1 and C1 fibroblast lines) and me together with former master student Ashutosh Dhingra under my supervision (L2, C2, C3, and C4 fibroblast lines). Gene-correction, differentiation and characterization of both, iPSCs and neurons, was shared according to the generation of iPSC lines. An optimized differentiation protocol was established in parallel where each group contributed equally. Electrophysiology experiments were performed by Dr. Ulrike Hedrich from Holger Lerche's group (HI). The generation of the artificially mutated line C4 + G2019S was done by former master-student David Schoendorf under my supervision. Experiments and analyses for neurite outgrowth were performed and established by me and Dr. Lena Burbulla (former PhD student from the lab of Prof. Dr. Rejko Krueger, HI). Experiments and analyses for cytotoxicity was established and performed by Peter Reinhardt. Experiments and analyses for α-synuclein were shared between Peter Reinhardt and me whereas I was working on the protein levels of α-synuclein and Peter Reinhardt on the RNA levels. Experiments and analyses for TAU and pTAU were outsourced to Gunnar Hargus from Tanja Kuhlmann's lab.

Ideas have always been collected in group discussions during conference calls with Peter Reinhardt, Jared Sterneckert, Thomas Gasser and me.

6 Introduction

6.1 Parkinson's Disease (PD)

Parkinson's disease (PD) is the most common neurodegenerative movement disorder and the second most common neurodegenerative disorder in the elderly after Alzheimer's disease (AD). Around 1 out of 1,000 people are affected in Europe but so far only symptomatic treatment is possible.

6.1.1 Clinical Features of PD

The diagnosis of PD is based on clinical criteria as a clear diagnosis through biomarkers is not available. As parkinsonian symptoms can have many reasons like brain tumors or brain injuries, four cardinal features of PD are used for the clinical diagnosis to differentiate PD from parkinsonisms: Beside the most obvious symptom of rest tremor, PD is further characterized by muscular rigidity, bradykinesia even ranging to akinesia, and a loss of postural reflexes.

6.1.2 Cellular Features of PD

On a cellular level, PD is characterized by the specific loss of dopaminergic neurons in the substantia nigra pars compacta. A characteristic of the substantia nigra, which is a part of the midbrain, is its black colour created by dopeminergic neurons expressing the black neuropigment neuromelanine (Kastner *et al.*, 1992). As a consequence of dying dopaminergic neurons of the midbrain (mDA neurons), it can be found that the substantia nigra with its characteristic black colour has disappeared in a post mortem brain of a PD patient compared to a post mortem brain of a non-PD individual (figure 1 A and B).

6.1.3 Subcellular Features of PD

The remaining neurons which haven't died during the disease contain proteinaceous inclusion bodies consisting of misfolded, insoluble proteins. These inclusion bodies are called Lewy Bodies and consist mainly of aggregated and insoluble forms of the protein α-synuclein and ubiquitin. This suggests that α-synuclein may play a central role in PD pathology (Spillantini *et al.*, 1997) possibly in combination with deficiencies

in the proteasomal protein degradation machinery (Wakabayashi *et al.*, 1990). A further observation is the formation of neurofibrillary tangles (NFTs) formed by a hyperphosphorylated form of the protein TAU (Rajput *et al.*, 2006). However, the mechanisms leading to the formation of α-synuclein and TAU aggregation still remain unknown.

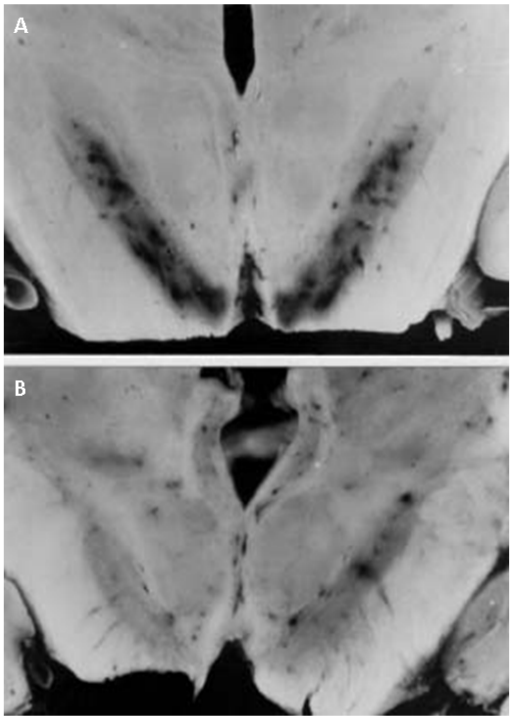

Figure 1: Nigral Degeneration in PD. (A) Post mortem brain of a non-PD individual and a PD patient (B). The brain of the PD patient is lacking the black coloured substantia nigra formed by dopaminergic neurons. Figure by Triarhou *et al.*, Prog Brain Res. 2000.

6.2 Sporadic and Familial Forms of PD

Most frequent cases of PD occur sporadically at an average age of 65 years without signs of heredity within the family. But in around 5 - 10% of the cases, a familial background can be found and the disease seems to be inherited within the family following Mendelian laws (Schiesling *et al.*, 2008). This led to the assumption that there must be genetic causes of PD in these cases. And indeed, several genes have been found and identified in the past which can cause the disease if they are mutated (table1).

Table 1: PD-Associated Loci and Genes. Modified after Corti *et al.* and omim entry 614251 (http://omim.org/entry/614251)

PARK Loci	Gene	Map Position	Inheritance	Disease Onset	Mutations	Susceptibility Variants
PD-associated loci and genes with conclusive evidence						
PARK1/PARK4	*SNCA*	4q21	Dominant; rarely sporadic	Early onset	A30P, E46K, A53T, genomic duplications/triplications	Promoter Rep1, 5' and 3' variants increase risk for PD
PARK8	LRRK2	12q12	Dominant; sporadic	Late onset	>80 Missense variants, <7 of them pathogenic, including the common G2019S	G2385R, R1628P increase risk for PD in Asian populations
PARK2	parkin	6q25-q27	Recessive; sporadic	Juvenile; early onset	Approximately 170 mutations (point mutations, exonic rearrangements)	Promoter polymorphisms increase risk for PD; heterozygous mutations may increase risk for late-onset PD
PARK6	PINK1	1p35-p36	Recessive	Early onset	Approximately 50 point mutations, rare large deletions	Heterozygous mutations may increase risk for late-onset PD
PARK7	DJ-1	1p36	Recessive	Early onset	Approximately 15 point mutations and large deletions	Heterozygous mutations may increase risk for late-onset PD
PARK9	ATP13A2	1p36	Recessive	Juvenile KRS, early-onset PD	>5 Point mutations	Heterozygous variants increase risk for PD
PD-associated loci and genes with unknown relevance						
PARK3	Unknown	2p13	Dominant	Late onset	Not identified	SPR variants may increase risk for PD
PARK5	UCHL1	4p14	Dominant	Late onset	One mutation in a single PD sibling pair	S18Y variant decreases risk for PD
PARK10	Unknwon	1p32	Unclear	Late onset	Not identified	Unknown
PARK11	GIGYF2	2q36-q37	Dominant	Late onset	7 missens variants	None
PARK12	Unknown	Xq21-q25	Unclear	Late onset	Not identified	Unknown
PARK13	Omi/HTRA2	2p13	Unclear	Late onset	2 missense variants	Regulatory variants may contribute to risk for PD
PARK16	Unknown	1q32	Unclear	Unclear	Not identified	Polymorphic SNPs
PARK18	EIF4G1	3q27	Dominant	Late onset	5 missens variants	Unknown
Loci and genes associated with atypical parkinsonism						
PARK14	PLA2G6	22q12-q13	Recessive	Juvenile levodopa-responsive dystonia-parkinsonism	2 missense mutations	Not investigated
PARK15	FBXO7	22q12-q13	Recessive	Early onset parkinsonian-pyramidal syndrome	3 point mutations	Not investigated
PD-associated genes proposed by candidate gene approach						
Not assigned	SCA2	12q24.1	Dominant for SCA2	Unclear	Low-range interrupted CAG expansions in SCA2	Not investigated
Not assigned	GBA	1q21	Recessive for GD	Unclear		Heterozygous GD-associated mutations increase risk for PD

PD patients with a mutation in one of these genes display similar symptoms and nigral degradation as patients with sporadic PD. As such, the function and the mechanism of these genes are of certain interest and have been extensively investigated in order to obtain a better understanding for both, familial and sporadic PD.

6.3 The Role of α-Synuclein and TAU in PD

A common feature of neurodegeneration is the formation of insoluble, accumulating proteins (Ross and Poirier, 2004). Proteins whose aggregation has been found to be implicated in PD are α-synuclein and TAU (Spillantini *et al.*, 1997, Rajput *et al.*, 2006).

6.3.1 α-Synuclein

The gene which was discovered first to be associated with PD was the *SNCA* gene encoding the protein α-synuclein (Polymeropoulos *et al.*, 1996). Mutations within the *SNCA* gene follow an autosomal dominant inheritance pattern (Polymeropoulos *et al.*, 1996). α-synuclein has been described as a synaptic protein and constitutes in its insoluble and multimeric form the major component of Lewy Bodies (Spillantini *et al.*, 1997, Murphy *et al.*, 2000, Cabin *et al.*, 2002). Interestingly, duplications or triplications of the *SNCA* gene also lead to the disease suggesting that sheer overexpression of non-mutated α-synuclein might be sufficient to cause PD (Farrer *et al.*, 2004, Miller *et al.*, 2004).

6.3.2 TAU and phosphorylated TAU (pTAU)

Another protein associated with neurodegeneration and supposedly also with PD is TAU, encoded by the gene *MAPT* (microtubule associated protein TAU) (Rajput *et al.*, 2006, Golub *et al.*, 2009). Stability and function of TAU is linked to its phosphorylation status (Hanger *et al.*, 2009). In PD as well as AD, TAU has been discovered to be hyperphosphorylated (Alonso *et al.*, 2001). Hyperphosphorylation in turn leads to its intracellular accumulation, so called neurofibrillary tangles (NFTs), which is designed as tauopathy (Grundke-Iqbal *et al.*, 1986). Although not consistently present in all PD patients, PD cases with LRRK2 mutations and tauopathy have already been reported (Cookson, 2010).

6.4 LRRK2: The Most Frequent Genetic Cause of Familial PD

The most frequent PD gene identified at present is the LRRK2 gene (leucine rich repeat kinase 2) which was discovered in 2004 (Paisan-Ruiz *et al.*, 2004, Zimprich *et al.*, 2004). Although quite rare in Central European populations, LRRK2 mutations emerge to up to 40% of familial PD in South African cohorts or ashkenazy jewish

populations (Lesage *et al.*, 2006, Healy *et al.*, 2008, Hassin-Baer *et al.*, 2009). LRRK2 is a multidomain protein kinase with several enzyme functions (figure 2).

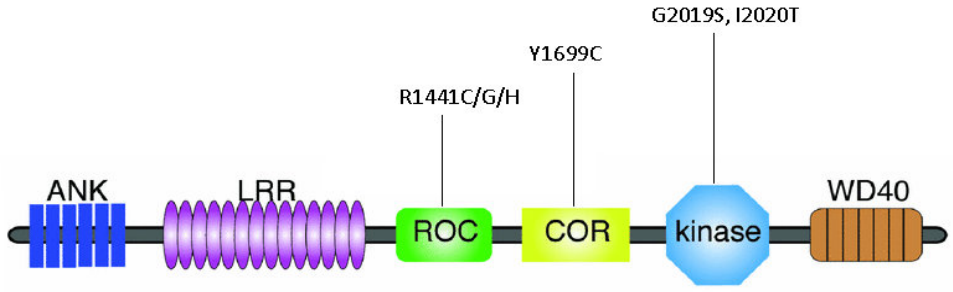

Figure 2: Domains of the LRRK2 Protein. LRRK2 is a multidomain protein containing an Ankyrin domain (ANK), a leucine rich repeat domain (LRR) both for protein-protein interactions, a ras-of-complex domain (ROC) with an intrinsic GTPase, a c-terminal of ROC domain (COR), a kinase domain, and a WD40 domain. Pathogenic mutations are located in the ROC-, COR, and kinase domains. Modified after Lewis *et al.*

It belongs to the superfamily of ROCO-proteins which are defined as enzymes containing a ROC domain (ras-of-complex) and a COR domain (c-terminal of ROC) (Bosgraaf and Van Haastert, 2003). The ROC domain can bind GTP or GDP and functions as an intrinsic GTPase which works, like the ras-protein, as a molecular binary switch between the state “on” or “off” having bound either GTP or GDP, respectively (Bosgraaf and Van Haastert, 2003, Lewis, 2009). Beside the kinase and the GTPase domain, LRRK2 has an Ankyrin domain, a WD40 domain and a leucine rich repeat (LRR) domain for protein-protein interactions (Marin, 2006). Several results point into the direction that pathogenic mutations within the LRRK2 gene lead to a gain-of-function of the kinase domain which in turn leads to a higher basal activity of LRRK2 (Cookson, 2010). Mutations within the LRRK2 gene have already been reported either in the ROC-domain (R1441C/G/H), the COR-domain (Y1699C) or the kinase domain (G2019S, I2020T) (figure 2). The most frequent mutation is the G2019S mutation, where glycine at position 2019 is replaced by a serine. It is assumed, that the mutation generates a novel phosphorylation site within the activation segment which in turn leads to a constitutively active kinase (Luzon-Toro *et al.*, 2007). Phenotypic alterations that are linked to G2019S-LRRK2 comprise neurite shortening and increased sensitivity of dopaminergic neurons (MacLeod *et al.*, 2006, Plowey *et al.*, 2008, Li *et al.*, 2009, Lee *et al.*, 2010, Nguyen *et al.*, 2011, Ramonet *et al.*, 2011, Winner *et al.*, 2011). The pathomechanistic effects of LRRK2 mutations seem to be diverse comprising mitochondrial, autophagic, electrophysiological,

microtubule, and lysosomal deficits (Lewis, 2009, Tong *et al.*, 2009, Parisiadou and Cai, 2010, Gomez-Suaga *et al.*, 2012, Lewis and Manzoni, 2012, Sanna *et al.*, 2012). Also accumulation of α-synuclein and TAU has been reported as LRRK2 associated effects (Rajput *et al.*, 2006, Carballo-Carbajal *et al.*, 2010, Nguyen *et al.*, 2011, Sanna *et al.*, 2012). However, a direct interaction partner of LRRK2 is still unknown.

6.5 Models for PD

The choice of a suited PD model is challenging. Ideally, such a model should display a robust phenotype directly linked to the disease like for example the formation of Lewy Bodies in combination with nirgal neurodegeneration.

6.5.1 Animal Models

Several animal models like mouse-, drosophila- or *C. elegans* models have extensively been used in the past. Usually, the strategy was to insert a mutated PD gene under the assumption the mutation there has a similar pathomechanistic effect as in humans. The obvious advantage of an animal model is the possibility to study PD in a whole organism where other organs are present which may possibly be implicated into the pathomechanism of the disease. As such, animal models have a clear justification and in the past, these models have already suggested valuable insights to the pathomechanism of several PD genes. However, they all failed when it comes to the question of displaying PD related phenotypes like the formation of Lewy Bodies or specific mDA neurodegeneration except some overexpression models, where the respective gene was artificially upregulated and not under the control of the cell itself. A quite obvious disadvantage of these models is their nature as not being human. For that reason, it is not clear whether mechanisms revealed in animal models are also present in human cells.

6.5.2 Human Specimen

Post mortem brains or biospecimens like blood, cerebrospinal fluid (CSF) or fibroblasts constitute an important resource for PD research as they are directly derived from patients and therefore have an immediate link to PD (Ravid, 2009). For example, fibroblasts of PD patients have already been used to study mitochondrial deficits in PD (Mortiboys *et al.*, 2010). Blood- or CSF samples are mainly used for biomarker screenings and specimens derived from post mortem brains have the

potential to provide desired insights e.g. into neurochemical changes (Ravid and Ferrer, 2012). However, the resource is rare and, although samples are derived directly from patients, it is not clear whether alterations found in fibroblasts or blood cells are associated with neurodegeneration. Further, studies based on human material face the problem of individual, unpredictable variability that can only be compensated through a high number of samples.

6.5.3 Conventional *in vitro* Cellular Models

Conventional human cell model systems have the main disadvantage of either not being cell type specific like HEK293 or HeLa cells or being derived from toumor cells like the glioblastoma line SH-SY5Y. These cells are normally used in combination with artificial overexpression of a respective PD gene. Overexpression in turn can lead to cellular stress unrelated to PD and consequently, results of such models remain questionable. Further, mechanisms implicated in PD might not be present in cell types other than neuronal cells.

Another option as an *in vitro* model is the use of human embryonic stem cells (hESCs), which offers the possibility studying PD in dopaminergic neurons with almost unlimited resource. However, the use of hESCs is hampered due to ethical controversies and additionally the association between PD and a hESC line derived from an embryo remains unpredictable.

6.5.4 Patient Specific Induced Pluripotent Stem Cells (iPSCs)

A promising alternative was found in 2007 with the development of techniques to generate iPSCs by the research group of Shinia Yamanaka (Takahashi *et al.*, 2007). The possibility of reprogramming somatic cells like fibroblasts to a pluripotent state enabled the generation of patient specific stem cells which can give rise to any desired cell type. Reprogramming is achieved through delivery of the 4 factors Oct-4, Sox-2, Klf-4 and c-myc into the cells. Possible applications range from basal research over disease models and drug discovery up to cell replacement therapies. For PD research, iPSCs seem to be of special interest as they provide an almost unlimited source for functional human mDA neurons (Chambers *et al.*, 2009, Cooper *et al.*, 2010), and several groups have already described their successful application for PD research (Nguyen *et al.*, 2011, Liu *et al.*, 2012, Sanchez-Danes *et al.*, 2012).

6.6 Genetic modifications in cellular models

In order to study the function of a certain gene, cell culture systems can be genetically modified by various techniques.

6.6.1 Cell Transfection

One possibility for the delivery of a gene into a cell is cell transfection. This method normally requires a plasmid containing the gene of interest in combination with a transfection reagent like Lipofectamine or Fugene followed, dependent on the experiment, by a selection. The plasmid mostly remains transiently expressed which means that the gene doesn't integrate into the genome. In addition, expression is mainly regulated by a strong and permanently active promoter like a CMV- or a UbC promoter and therefore is not under the control of the cell itself. The result is an artificial overexpression possibly inducing several effects unrelated to the mechanistic function of the gene.

6.6.2 Viral Infection

The use of viruses like Lenti- or Retroviruses as a delivery system permits the stable integration of a desired gene into the genome. Beside the advantage of not being transiently expressed, the gene is delivered generally much more efficiently into the cell than through cell transfection. However, the integration site remains a random effect leading to diverse expression levels which are again not under the control of the cell itself. As such, other genes can be destroyed or can get hyper activated through random integration also leading occasionally to chromosomal abnormalities like translocations.

6.6.3 Zinc Finger Nuclease (ZFN) Mediated Gene-Editing

Gene-Editing through homologous recombination is a powerful tool that enables the study of genes at an endogenous level without artificial overexpression. Homologous recombination is a standard technique for the generation of knock-out or knock-in mice. However, this technique has proven difficult in human stem cells and for a long time only few studies included gene-editing in human stem cells due to extremely inefficient recombination events (Zwaka and Thomson, 2003). With the development of gene-specific endonucleases like ZFNs, the efficiency could be optimized to a level that permits the application in the lab (Urnov *et al.*, 2010, Ding *et al.*, 2013).

ZFNs are highly specific endonucleases which can be designed in a way to only recognize one site within the whole genome. Through introduction of double strand breaks at a specific site, ZFNs induce DNA repair through homologous recombination.

7 Objectives

The goal of this study is the generation of iPSCs from PD patients with a G2019S mutation in the LRRK2 gene. These iPSCs shall be genetically modified by ZFN-technology in order to obtain isogenic gene-corrected controls. Then, an efficient differentiation protocol shall be established for the generation of neuronal cultures containing a sufficient amount of functional mDA neurons. The differentiation shall be based on protocols published for the differentiation of human embryonic stem cells. Finally, proof of principle experiments shall be performed comprising neurite outgrowth deficits, neurodegenerative effects in mDA neurons, as well as the accumulation of proteins associated with PD α-synuclein and TAU or pTAU.

Similarly, the use of ZFNs had already been reported in other cell types. However, functionality of ZFNs in human embryonic stem cells or iPSCs hasn't been described in 2008. As an additional part of this thesis, the functionality of ZFNs shall be tested and established in order to generate isogenic gene-corrected iPSC clontrols.

When this study was started in 2008, the technology of iPSC generation had been described for only one year. Although it was theoretically expected that these cells can be differentiated to functional mDA neurons like real human embryonic stem cells, proof of principle studies were required. Further, it was questionable, if such a system - a human stem cell derived *in vitro* system - is suited to study pathomechanistic effects of G2019S-LRRK2. As such, the focus of this study was on foremost basic investigations analyzing the usability of iPSCs as a PD model.

8 Results

8.1 Generation of an iPSC Based *in vitro* Cell Model for PD

8.1.1 Generation of iPSCs from a PD Patient with a G2019S Mutation

Fibroblasts from two female PD patients with a G2019S mutation were used for the generation of iPSCs. One patient was born in 1958 (patient L1) and the other was born in 1931 (patient L2). In addition, four independent healthy control lines were included which were matched regarding sex and age from donors born in 1959, 1931, 1943, and 1932 (C1, C2, C3, and C4, respectievely) (table 2).

Table 2: iPSCs included in this study.

iPSC line	Derived from	Sex / Year born	LRRK2 Genotype	Comment
L1-1Mut	LRRK2 Patient #1	F / 1958	G2019S	
L1-2Mut	LRRK2 Patient #1	F / 1958	G2019S	
L1-1GC1	LRRK2 Patient #1	F / 1958	Wild-type	corrected subclone of L1-1Mut
L1-1GC2	LRRK2 Patient #1	F / 1958	Wild-type	corrected subclone of L1-1Mut
L1-2GC	LRRK2 Patient #1	F / 1958	Wild-type	corrected subclone of L1-2Mut
C1-1	Control #1	F / 1959	Wild-type	
C1-2	Control #1	F / 1959	Wild-type	
L2-1Mut	LRRK2 Patient #2	F / 1931	G2019S	
L2-2Mut	LRRK2 Patient #2	F / 1931	G2019S	
L2-3Mut	LRRK2 Patient #2	F / 1931	G2019S	
L2-1GC	LRRK2 Patient #2	F / 1931	Wild-type	corrected subclone of L2-1Mut
L2-2GC	LRRK2 Patient #2	F / 1931	Wild-type	corrected subclone of L2-2Mut
L2-3GC	LRRK2 Patient #2	F / 1931	Wild-type	corrected subclone of L2-3Mut
C2	Control #2	F / 1931	Wild-type	
C3	Control #3	F / 1943	Wild-type	
C4	Control #4	F / 1932	Wild-type	
C4 + G2019S	Control #4	F / 1932	G2019S	G2019S mutated subclone of C4

iPSCs were obtained by retroviral infection of patient fibroblasts in passage 1 - 4 with Oct4, Sox2, Klf4 and C-myc. The iPSC lines were initially identified by morphology (figure 3A) and further characterized regarding the expression of the stem cell markers Oct4, Nanog, Tra-1-81, and SSEA4 (figure 3B), silencing of the viral factors (figure 3C), pluripotency through either *in vivo* teratoma formation or *in vitro*

spontaneous differentiation (shown in Reinhardt and Schmid *et al.*), promoter demethylation of stem cell markers Nanog, Rex1 and Oct4 (figure 3D), and chromosomal integrity through an Illumina DNA chip analysis (not shown) or karyotyping (figure 3E).

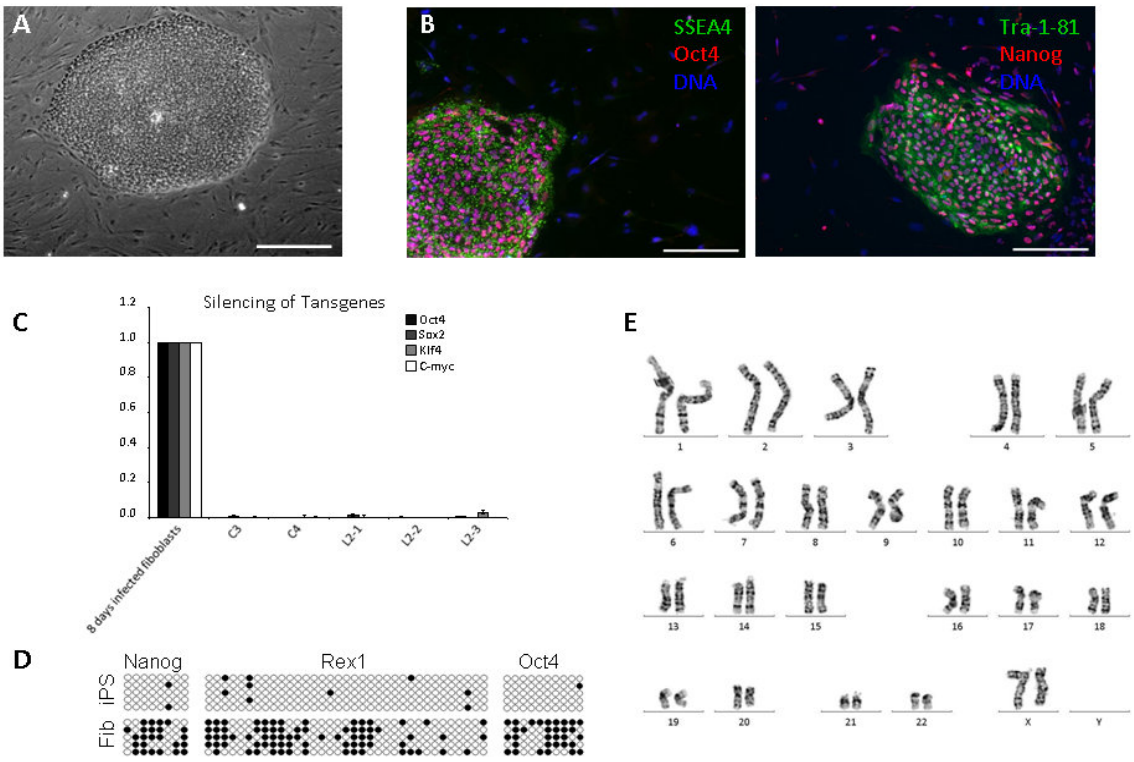

Figure 3 Characterization of iPSCs. (A) Light microscopy picture of a representative iPSC colony (by Peter Reinhardt) (B) iPSCs expressed the stem cell markers SSEA4, Oct4, Tra-1-81, and Nanog. (C) Expression of viral transgenes in the indicated lines compared to fibroblasts 8 days post infection. Expression was standardized on HMBS expression. Error bars give variations from two experiments. (D) Bisulphite sequencing result of promoter regions of indicated genes in fibroblasts (Fib) and iPSCs (iPS). Open circles stand for demethylated CpG islands; closed circles stand for methylated CpG islands. (E) Representative karyotype of iPSC line C4 + G2019S. All scale bars represent 100 µm.

After characterization, 2 clones of patient L1 (L1-1Mut and L1-2Mut) and 3 clones of patient L2 (L2-1Mut, L2-2Mut, and L2-3Mut) were obtained (table 2). For independent age- and gender-matched controls, 1 clone per control was included into this study (tabe 2).

8.1.2 Generation of Isogenic Gene-Corrected Controls

The ZFNs for gene-correction of G2019S-LRRK2 were obtained from Sigma and were designed to target Exon 41 of the LRRK2 gene. The pEasy Flox vector was used as backbone for the homologous construct (addgene plasmid 11725). In the completed homologous construct, the neomycin resistance cassette of the pEasy Flox vector was flanked by homologous arms targeting exon 41 and the adjacent introns (figure 4A).

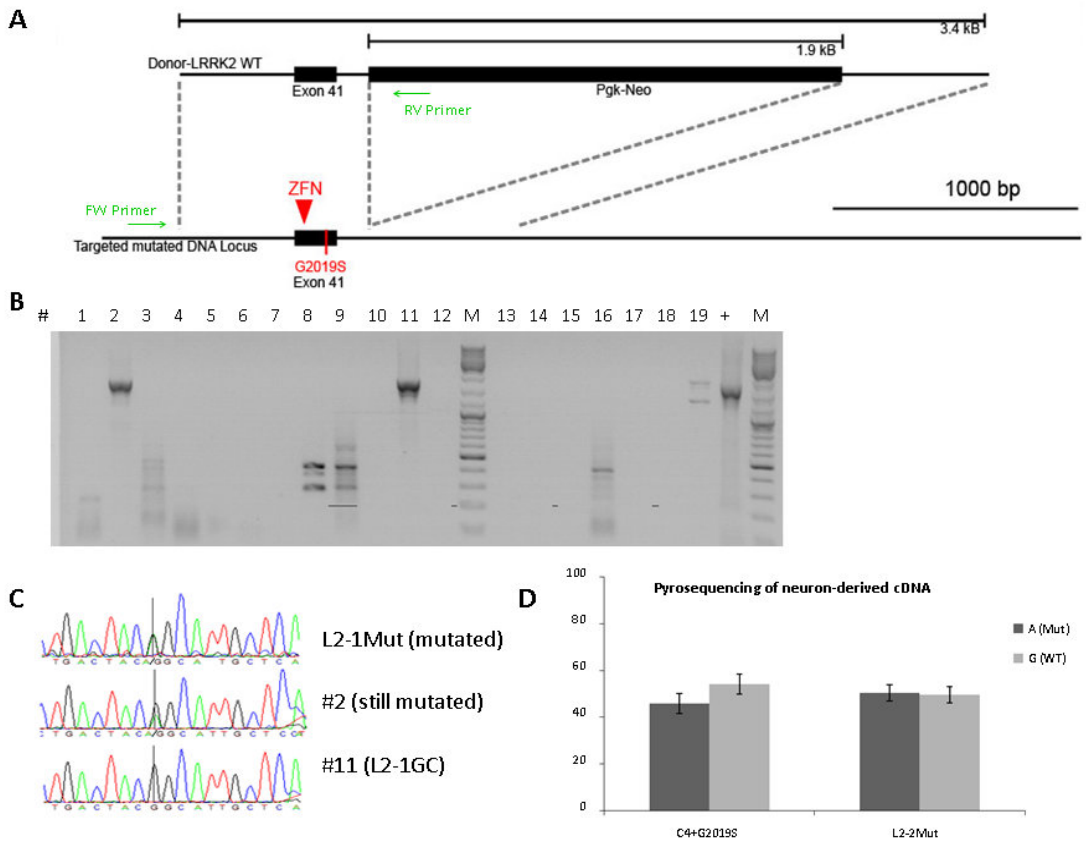

Figure 4: ZFN Mediated Gene-Editing. (A) Schematic draft of the homologous template (Donor-LRRK2 wild-type) with a neomycin resistance casette flanked by homologous arms. ZFN cutting site in Exon 41 is shown in red, primers for integration PCR in green. (B) Representative result of an integration PCR analysis with two successfully targeted clones (#2 and #11). (C) Sequencing analysis result of the initial iPSC line with a G2019S mutation (upper lane) and its subclones #2 (middle lane, still mutated) and #11 (bottom lane, gene-corrected). (D) Quantification of the expression of the inserted base causing the G2019S mutation in the line C4 + G2019S compared to L2-2Mut by pyrosequencing. Error bars represent variations from two experiments.

Homologous construct as well as plasmids encoding the ZFNs were introduced into the cells by nucleofection. In case of successful recombination, the neomycin resistance marker will be inserted into intron 41. Targeted clones were identified through a specific integration PCR test (figure 4B) followed by DNA sequencing. As

expected, only 50% of all clones were gene-corrected after successful targeting as only 1 allele harboured the G2019S mutation (figure 4C). Finally, at least one isogenic gene-corrected line was obtained for each of the G2019S-mutated iPSC lines, designated as L1-1GC, L1-2GC, L2-1GC, L2-2GC, and L2-3GC (table 2). As an additional set, the LRRK2 locus of iPSC line C4 was targeted by ZFNs and artificially mutated designated as C4 + G2019S using a homologous construct containing a G2019S mutation (table 2). Pyrosequencing analysis from exon 40 to exon 42 using cDNA of clone C4 + G2019S revealed that expression and splicing of the targeted allele was not affected by the resistance marker inserted into intron 41 (figure 4D). This indicated that insertion of a resistance cassette into the adjacent intron at this specific site in the LRRK2-gene does not affect splicing. Therefore, it was not necessary to cut off the resistance marker by Cre-lox technology as initially planned.

8.1.3 Generation of Functional mDA Neurons

iPSCs were differentiated to mDA neurons combining two published protocols (Chambers *et al.*, 2009, Cooper *et al.*, 2010). The resulting protocol involved the use of several small molecules and patterning factors over a period of at least 30 days. After 30 days, the differentiated iPSCs were characterized in triplicates for expression of the neuronal marker Map2 as well as the dopaminergic markers tyrosine hydroxylase (TH) and FoxA2 indicating midbrain specificity when coexpressed. Further, presence of LRRK2 in dopaminergic neurons was confirmed (figure 5B). Quantification revealed that around 20% of the cells were positive for both, the neuronal marker β-III-tubuline and TH (figure 5B and 5C). Importantly, this result was quite consistently achieved in all the lines indicating that differentiation efficiencies were comparable. Further quantification of TH and FoxA2 showed that all TH positive neurons expressed FoxA2 indicating midbrain specificity of all TH positive neurons. In order to prove functionality of neurons, patch-clamping analyses were conducted. Experiments revealed that neurons had a plausible negative resting potential and further were able to generate action potentials in a repetitive manner upon stimulation through depolarization. Taken together, these data indicated successful generation of functional human mDA neurons (further characterization of dopaminergic neurons are shown in Reinhardt and Schmid *et al.*).

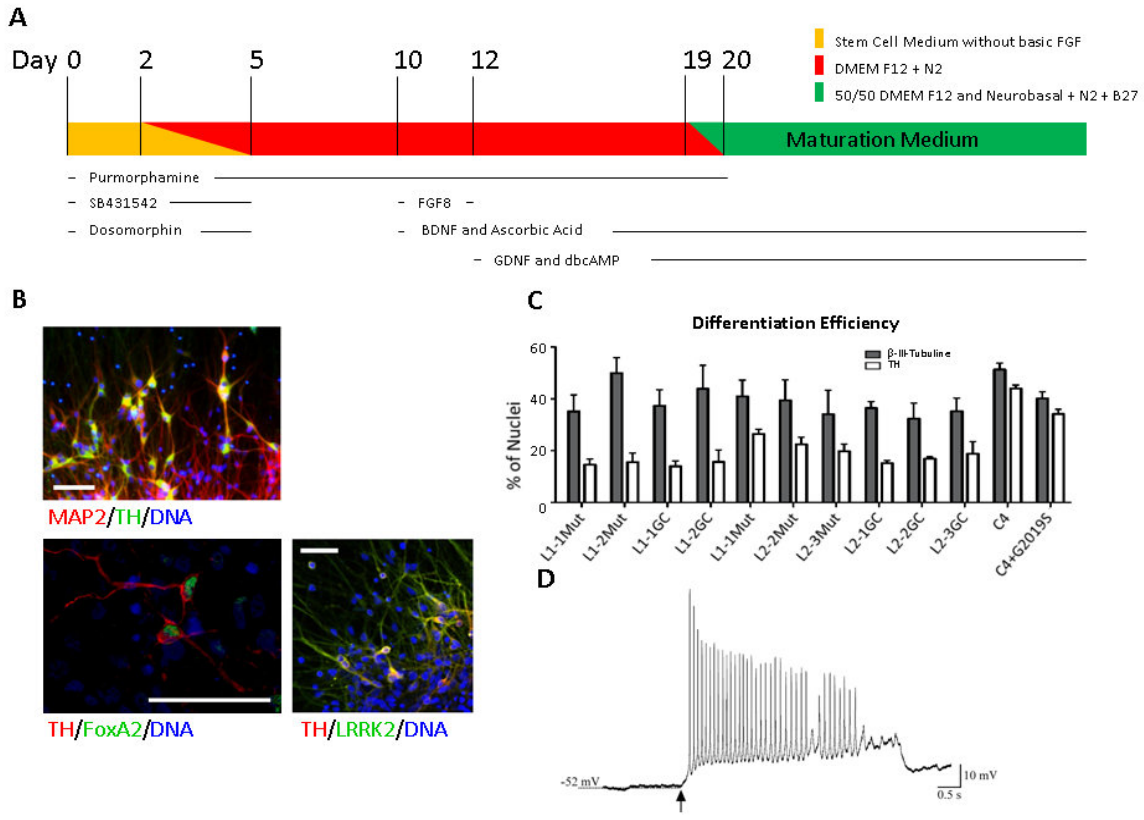

Figure 5: mDA Differentiation. (A) Flow chart for the differentiation of iPSCs to dopaminergic neurons. (B) After 30 days of differentiation, iPSC-derived TH positive neurons expressed MAP2, FoxA2 and LRRK2. (C) Differentiation efficiency was comparable between all lines quantified through immunocytochemistry. n = 3 – 5 for each line, error bars indicate SEM. (D) iPSCs showed repetitive action potentials after injection (arrow) of currents by patch clamp technique. All scale bars represent 100 µm.

8.1.4 G2019S-LRRK2 Causes Neurite Shortening

Several studies of G2019S-mutated neurons including neurons of transgenic G2019S-LRRK2 mice showed neurite shortening (MacLeod *et al.*, 2006, Plowey *et al.*, 2008, Winner *et al.*, 2011). As an initial proof of principle for the iPSC-derived cell model system with mutated and gene-corrected neurons, this phenotype was repeated by measuring the velocity of outgrowing neurons via live cell imaging. All neurons were differentiated in triplicates. After 30 days of differentiation, neuronal clusters were transferred onto a chamber slide for live cell analysis. The next day, the neurite outgrowth was analyzed over a total time of 30 minutes. The experiment revealed that the velocity for outgrowing neurons from cultures with a G2019S mutation was significantly reduced compared to that of gene-corrected neurons and healthy controls. With other words: gene-correction of G2019S-LRRK2 raised the

velocity of outgrowing neurons to a level comparable to that from independent healthy controls. This effect was consistent for all the lines from both patients as well as for the artificially mutated line C4 + G2019S. When all the lines were taken together, a highly significant result could be found (figure 6A and 6B; results from single lines are shown in Reinhardt and Schmid *et al.*).

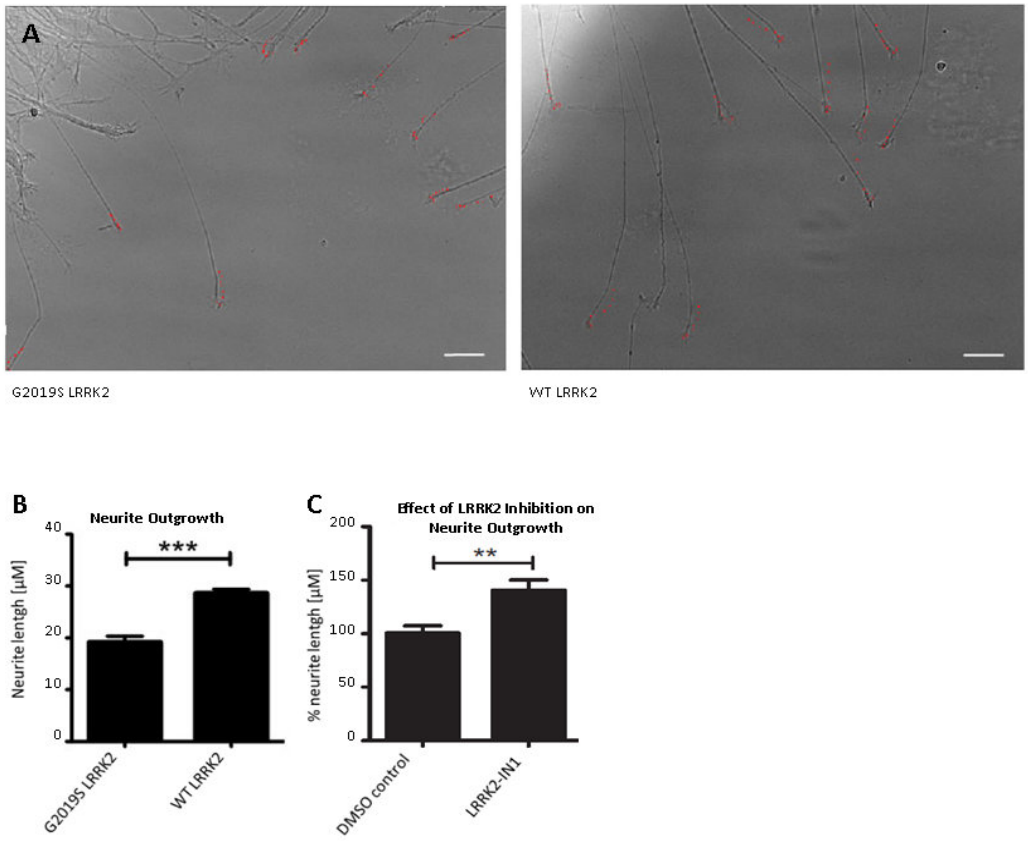

Figure 6: Neurite Outgrowth. (A) Sample picture of a neurite outgrowth experiment. Distance between two adjacent red dots represents 5 minutes. Pictures were taken over a total time of 30 minutes. (B) Quantification of neurite outgrowth per 30 minutes. All G2019S-mutated lines were compared to all wild-type lines including gene-corrected lines as well as healthy independent controls. (C) Quantification of neurite outgrowth per 30 minutes of G2019S-mutated lines treated either with the LRRK2 inhibitor IN1 (1.5 µM) or DMSO as a control. Error bars represent the standard error of the mean. $^{**}p < 0.01$, $^{***}p < 0.001$ according to t test.

To address the question, whether impaired neurite outgrowth was dependent on kinase activity, G2019S-mutated neurons were treated in triplicates with the LRRK2 inhibitor IN1. After 6 days of incubation with IN1 at a concentration of 1.5 µM, an increased neurite outgrowth could be observed compared to a DMSO treated control (figure 6C). The next question was, whether altered neurite outgrowth was specific to dopaminergic neurons. Therefore, outgrowing neurites were analyzed by

immunofluorescence microscopy. Representive areas from neuronal clusters were stained against TH and beta-III-tubuline and subsequently quantified. A subset of 20% from outgrowing neurons could be identified as TH positive suggesting that the reduction of neurite outgrowth was a general neuronal effect and not specific for dopaminergic neurons (shown in Reinhardt and Schmid *et al.*). Taken together the data show that G2019S-LRRK2 either directly or indirectly reduces neurite outgrowth velocity in iPSC-derived neuronal cultures. This effect is reversible upon inhibition of LRRK2 kinase activity through IN1.

8.1.5 G2019S-LRRK2 Leads to Increased Sensitivity to Oxidative Stress

A previous study showed that dopaminergic neurons with G2019S-LRRK2 derived from human iPSCs were more sensitive to oxidative stress than neurons from healthy unrelated controls (Nguyen *et al.*, 2011). As such, the next question was whether this finding was true also for the model system described here of mutated and gene-corrected controls. Oxidative stress was induced through addition of rotenone that inhibits complex I of the respiratory chain and causes PD related phenotypes in mice like nigral degeneration and accumulation of α-synuclein. Sensitivity was determined via immunocytochemistry by counting apoptotic dopaminergic neurons using activated caspase3 and TH as a respective marker (figure 7A). The effect of rotenone was assessed in triplicates at two different concentrations of either 50 nM or 100 nM. Immunocytochemical quantification revealed that dopaminergic neurons with G2019S mutation were significantly more sensitive to oxidative stress (figure 7B, effect of both concentrations are taken together, single results are shown in Reinhardt and Schmid *et al.*). As a further result of this experiment it could be observed that higher concentrations of rotenone induced slightly more apoptotic cells in the G2019S-mutated lines compared to their gene-corrected counterpart. This effect could be again consistently detected in all the lines of both patients as well as in the artificially mutated line C4 + G2019S (shown in Reinhardt and Schmid *et al.*). In a subsequent experiment, the effect of LRRK2 inhibition through IN1 was assessed in the G2019S-mutated lines. The Inhibitor was applied at a concentration of 1.5 µM over 6 days in triplicates. The analyses revealed that inhibition of LRRK2 decreased the number of apoptotic cells to a level comparable to that from wild-type LRRK2 lines. Taken together, the results indicate that G2019S-LRRK2 leads to a

higher sensitivity of mDA neurons to oxidative stress. This effect increases with higher dosage and can be rescued through inhibition of LRRK2 kinase activity.

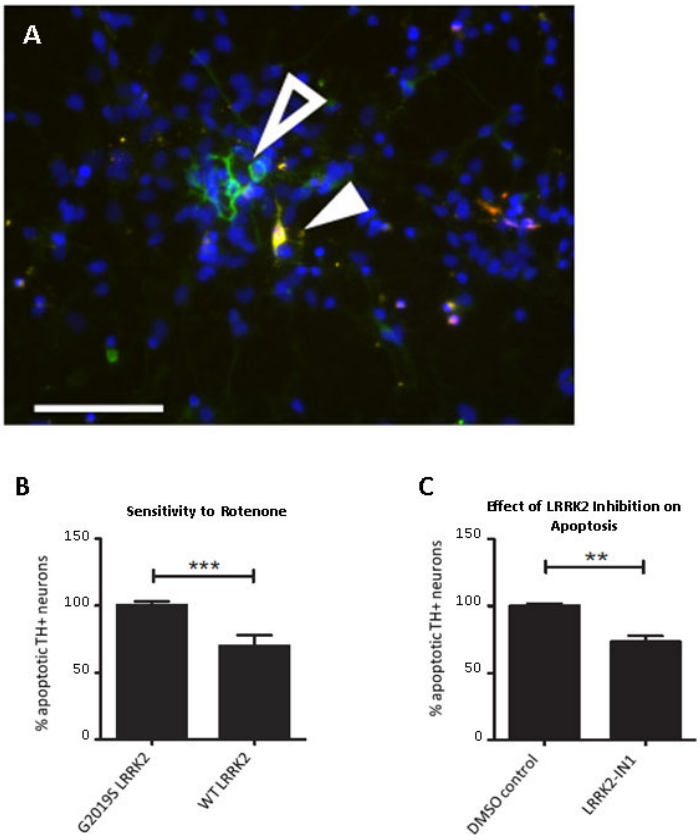

Figure 7: Sensitivity against Oxidative Stress. (A) Sample picture of the sensitivity assay. The open arrow head points to a TH positive and caspase3 negative neuron. The closed arrowhead points to an apoptotic neuron which is positive for both, TH and caspase3. Scale bar represents 100 µm. (B) G2019S-mutated lines show an increased sensitivity to oxidative stress. Effects of two different concentrations, 50 nM and 100 nM, were taken together. (C) Inhibition of LRRK2 by IN1 in G2019S-mutated lines had a beneficial effect and reduced the sensitivity of dopaminergic neurons against rotenone compared to a DMSO treated control. Error bars indicate SEM. **p < 0.01 and ***p < 0.001 according to t test.

8.1.6 G2019S-LRRK2 Induces Higher Expression Levels of α-Synuclein, TAU and Phospho-TAU

A hallmark of PD is the accumulation of proteins such as α-synuclein and TAU or pTAU encoded by the genes *SNCA* and *MAPT*, respectievely. A previous study already suggested that human iPSC-derived dopaminergic neurons with a G2019S mutation tend to overexpress or accumulate monomeric α-synuclein compared to healthy controls (Nguyen *et al.*, 2011). However, it was not clear, if this effect was specific for G2019S-LRRK2 since unrelated healthy controls were used in this study and the expression level of α-synuclein can vary a lot between different individuals

depending on their genetic background. Consequently, the next question of this study was whether the model of mutated and gene-corrected neurons is able to display dysregulation of α-synuclein and TAU or pTAU. iPSCs of all lines were differentiated in triplicates. After 30 and 60 days of differentiation, RNA and protein were harvested. qRT-PCR analyses revealed that after 30 days of differentiation, the gene-corrected lines had around 40% less *MAPT* RNA compared to the G2019S-mutated lines (figure 8A).

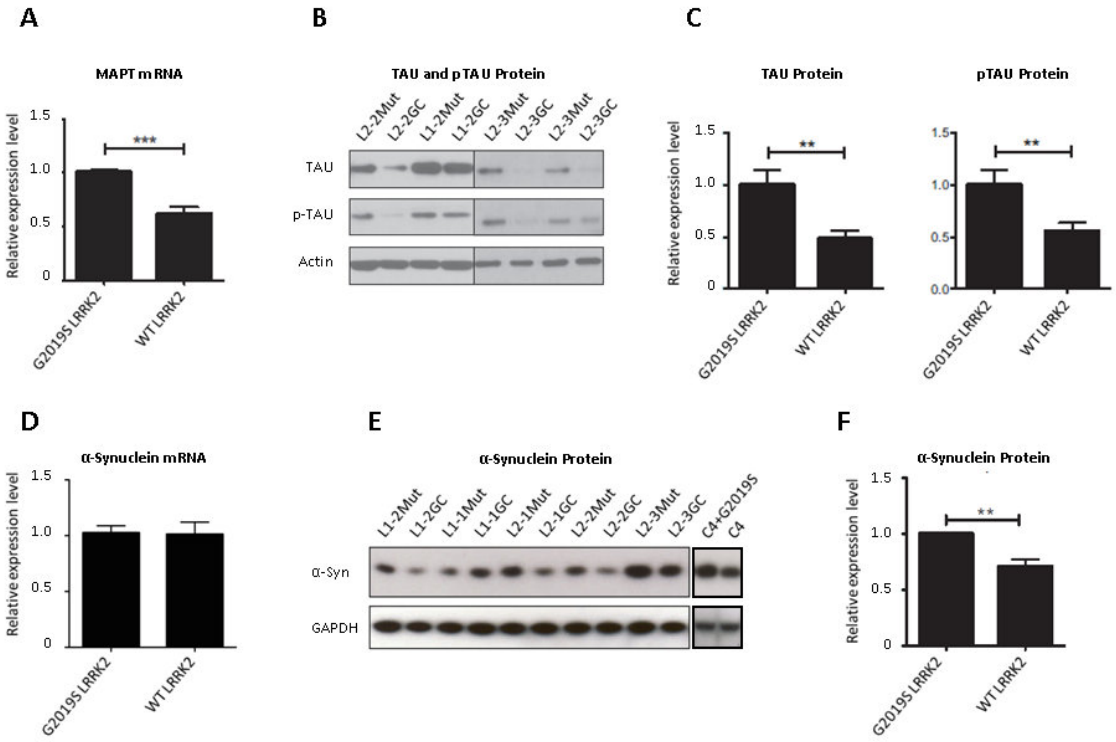

Figure 8: Expression Levels of TAU, pTAU and α-synuclein. (A) RNA expression levels of *MAPT* by qRT-PCR analysis. (B) Sample picture for the expression of TAU and pTAU by Western blot analysis. (C) Quantification of TAU and pTAU protein levels from all mutated and gene-corrected lines. (D) Expression levels of α-synuclein by qRT-PCR analysis. (E) Sample picture for the expression of α-synuclein by Western blot analysis. (F) Quantification of α-synuclein protein level from all mutated and gene-corrected lines. Error bars indicate SEM in all panels. **p < 0.01, ***p < 0.001 according to t test.

This result was confirmed by Western blot analysis showing a reduced level of TAU and pTAU in the gene-corrected lines (figure 8B and 8C). Dysregulation of *SNCA* expression was analyzed after 30 and 60 days of differentiation. qRT-PCR analyses revealed, that there was no change in α-synuclein expression on RNA level between mutated and gene-corrected lines after 30 days and even after extended cultivation up to 60 days (figure 8D). However, Western blotting revealed that significantly more

monomeric α-synuclein of around 30% could be stated after 30 days of differentiation in cultures with G2019S-LRRK2 compared to their isogenic gene-corrected counterpart. Interestingly, when absolute amount of α-synuclein in all wild-type lines including the healthy unrelated controls was compared to all mutated lines, no difference in α-synuclein level could be found. Taken together, the data show that G2019S-LRRK2 leads to an accumulation of monomeric α-synuclein and TAU or pTAU on protein level as well to an accumulation of TAU on RNA level in iPSC-derived neuronal cultures.

8.1.7 Continuation of this Study (Summary)

Further analyses based on this study are described in detail in Reinhardt and Schmid *et al.*, Cell Stem Cell 2013. Briefly, the global gene expression profile of G2019S-mutated neuronal cultures was compared with isogenic gene-corrected controls. This study revealed that 5 genes were consistently dysregulated by G2019S-LRRK2 in all lines. Dysregulation of these genes was confirmed through independent qRT-PCR analysis and Western blotting. Further experiments showed that the dysregulation of these genes contributes to deficiencies in neurite outgrowth and increased sensitivity to oxidative stress. Finally, increased phosphorylation and activation of ERK mediated by G2019S-LRRK2 could be identified as cause for the observed gene-dysregulation.

8.2 Derivation of Human Neural Progenitors for Neurodegenerative Disease Modeling

To exploit the full potential of iPSCs, they must be optimized for use in high-throughput screening (HTS). However, several problems reduce the accessibility of HTS for iPSC-derived models of neurodegenerative diseases. Large amounts of neurons are required for HTS campaigns, necessitating the use of high quantities of expensive patterning factors for differentiation. This limits the quantities of patterning factors that can be used, which constrains the size of the initial iPSC culture and eventually results in mature, post-mitotic neurons that are incapable of further expansion. Furthermore, beginning the long process of differentiation with iPSCs can result in heterogeneous neural cultures, which introduce unwanted variables and negatively influence the results of HTS campaigns. One possibility to reduce this heterogeneity is to establish a robust neural progenitor whose differentiation phase

stably resides between pluripotent stem cells and post-mitotic neurons. Neural progenitors also divide and expand without limitation; post-mitotic neurons do not.

Therefore, in an additional study, a neural progenitor line was established on the basis of inexpensive small molecules, including purmorphamine, dorsomorphin, CHIR99021, and SB43152, which induce neuronal differentiation through inhibition of BMP and TGFβ signalling and the activation of Wnt and SHH signalling. These neural progenitors formed both neural tube and neural crest cell lineages and expanded for more than 150 passages without a reduction in cell doubling time, the formation of chromosomal aberrations, or any adverse impact on differentiation. As such, motor neurons, midbrain dopaminergic neurons, peripheral neurons, and mesenchymal cells were obtained. After differentiation to midbrain dopaminergic neurons, lines derived from PD patients with a LRRK2 mutation and from isogenic gene-corrected controls successfully displayed PD-associated phenotypes, indicating their suitability for HTS. Results of this study are published in Reinhardt *et al.*, PLoS One 2013.

9 Discussion

The generation of iPSCs has been a breakthrough in stem cell research as it allows studying pathomechanisms of diseases in a specific cell type that otherwise would not be available. Potential applications include basic research, drug screening or even cell replacement therapies. This work describes the generation of two cellular model systems based on iPSCs.

The goal of the first model was the establishment of a neuronal cellular system that is able to recapitulate relevant features of PD in order to study underlying pathomechanisms. The goal of the second model was the establishment of an expandable and inexpensive neural progenitor line that is able to give rise to several neuronal subtypes and would therefore be suitable for high-throughput screening (HTS) for targets in neurodegenerative diseases.

9.1 Reprogramming of Skin Fibroblasts to iPSCs

iPSCs were obtained after retroviral infection with the four factors Oct4, Sox2, Klf4 and C-myc (Takahashi *et al.*, 2007). However, retroviral integration remains a random event and can lead to chromosomal disruption (Baum *et al.*, 2003). In this study, chromosomal aberrations were excluded as far as possible through karyopying; smaller modifications such as gene disruptions or duplication were excluded by Illumina DNA chip analyses. To date, several reprogramming techniques are available based on viral as well as non-viral methods. Viral approaches include the use of retroviruses, lentiviruses, adenoviruses, and sendaiviruses. The first report of iPSC generation involved the use of retroviruses (Takahashi *et al.*, 2007). iPSCs were obtained after 11 - 25 days post infection with an efficiency ranging between 0.01 – 0.001%. As such, efficiency and duration of reprogramming seem to be improvable but to date no remarkably optimized approaches were published. Subsequently, mainly lentiviral approaches were developed, as these viruses, in contrast to retroviruses, are able to introduce the factor into non-dividing cells. Lentiviral strategies were further refined, and polycystronic, inducible and excisable constructs were generated (Soldner *et al.*, 2009, Sommer *et al.*, 2009). However, both retroviral as well as lentiviral approaches are facing the problem of random

genome integration – a problem that was solved through the use of replication-deficient adenoviruses and sendaiviruses (Stadtfeld *et al.*, 2008, Fusaki *et al.*, 2009). Because adenoviruses don't possess mechanisms to accumulate in a cell, this approach is limited by extremely low efficiency. In contrast, RNA-based sendaiviruses can replicate over several passages within dividing cells and therefore lead to long term expression of reprogramming factors (Fusaki *et al.*, 2009). iPSCs can be obtained after 21 – 28 days and the efficiency is even higher compared to that of retroviruses. Non-viral approaches that involve the use of plasmids (Okita *et al.*, 2008), proteins (Kim *et al.*, 2009, Zhou *et al.*, 2009) or RNA (Yakubov *et al.*, 2010) are restricted by low efficiencies. As such, sendaiviruses may constitute to date the most advanced technique of reprogramming with the best ratio of cost/benefit.

9.2 Gene-correction through ZFN-induced homologous recombination

A few works have already reported the use of iPSCs as a PD model, for example by studying the effect of the G2019S-LRRK2 mutation in comparison with healthy independent controls. Effects have been described for α-synuclein expression, sensitivity to oxidative stress and impaired autophagy (Nguyen *et al.*, 2011, Sanchez-Danes *et al.*, 2012). However, the use of independent controls always faces the problem of unpredictable variability due to individual gene expression and it was not clear whether the effects found were specifically associated with the G2019S-mutation. The most important requirement to an ideal patient derived cellular model therefore is that variability of genomic background should be reduced to a minimum. To this end, we took advantage of ZFN technology to derive isogenic gene-corrected controls that differ only in one base. As such, phenotypic changes can therefore be linked directly to that single base. As an example, in our experiments alterations of α-synuclein expression levels were not detected when neuronal cultures from mutated lines and unrelated wild-type controls were compared. However, the use of isogenic gene-correction revealed a significant reduction of a-synucein protein in the gene-corrected lines. A similar result was obtained when global gene expression patterns of mutated lines, isogenic gene-corrected controls and independent controls were compared (Reinhardt *et al.*, 2013). Mutated lines clustered most closely with their isogenic gene-corrected counterpart. In contrast, healthy controls were significantly different from all other lines although differentiation efficiencies were comparable.

Therefore, this study demonstrates that it is possible to detect subtle effects in iPSC-derived models only through gene-correction. To date, only two studies have reported the use of isogenic gene-corrected iPSC lines for PD. One study describes gene-correction of iPSCs derived from a PD patient with an A53T mutation in the *SNCA* gene (Soldner *et al.*, 2011). This study was the first publication dealing with a gene-correction of a pathogenic PD mutation but it didn't show any phenotypic alterations. The strength of this publication was that no resistance marker was used for selection, which potentially might influence gene-expression of the targeted allele. However, we showed that gene-expression of the targeted allele remained unchanged after the insertion of a neomycin resistance cassette into the adjacent intron. The other study described viral-based approach to obtain isogenic gene-corrected lines from G2019S-mutated iPSCs (Liu *et al.*, 2012). The authors described phenotypic changes in pre-neuronal precursors corresponding to an early developmental stage. These changes comprise a dramatically reduced capability of cell division after a certain number of passages. Mechanistically, this effect was associated with an impairment of the nuclear envelope in the neural progenitors. As such, the data lack a direct link to PD but suggest that rather regenerative mechanisms for mDA neurons are disturbed. Indeed, there has been speculation that loss of mDA neurons in PD might be caused by disrupted neuroregeneration. Notably, one study claimed that there is evidence for neurogenesis in the substatia nigra of adult mammalian brains (Zhao *et al.*, 2003). However, this study was subsequently followed by a report from another group claiming exactly the opposite (Frielingsdorf *et al.*, 2004).

9.3 Off-target effects of ZFNs

A major criticism addressing the use of ZFNs is the risk of off-target effects, which means that ZFNs have the potential of cutting other genes with a related DNA sequence in an unspecific way. Such effects have already been described in previous reports (Gabriel *et al.*, 2011, Pattanayak *et al.*, 2011). A major problem is that ZF motifs interact in unpredictable ways, which creates the need for rigorous testing. Sigma, the company which has commercialized the Zink finger technology, incorporates this testing as part of their quality control. However, the studies mentioned above were based on self-made ZFNs with a limited test for off-target effects. As an example, another study reported the use of several ZFNs generated by

Sigma (Hockemeyer *et al.*, 2009). Interestingly, none of the ZFNs used in their study showed off target activity which was confirmed through sequencing of related DNA sequences. The risk of off-target effects of ZFNs generated by Sigma was therefore regarded as negligible. Nevertheless, the costs of ZFNs from Sigma are high and range to date between 10,000 and 20,000 € what causes the urge for alternatives. Previously, such an alternative tool for gene-editing has been published called "transcription activator-like effector nucleases" (TALENs) (Cermak *et al.*, 2011). TALE domains are entirely different compared to ZF motifs. Structurally, TALEN domains are completely separate from each other and only two amino acids interact with the DNA within each domain. As such, they are entirely predictable. This allows generating self-made TALENs with costs ranging between 30 and 50 € per TALEN.

9.4 Phenotypic Changes

Reduced neurite outgrowth or neurite shortening caused by G2019S-LRRK2 is an observation without mechanistic basis, which has already been reported before (MacLeod *et al.*, 2006, Plowey *et al.*, 2008, Li *et al.*, 2009, Winner *et al.*, 2011). Independent reports describe an effect of LRRK2 mutations on the cytoskeleton (Gloeckner *et al.*, 2006, Gandhi *et al.*, 2008, Parisiadou *et al.*, 2009, Parisiadou and Cai, 2010, Meixner *et al.*, 2011). This of course leads to the speculation that an impairment of the cytoskeleton is preceding a reduced neurite outgrowth. Further, it was shown that it is not specific to dopaminergic neurons but a rather general effect (Reinhardt *et al.*, 2013). As such, there is no obvious link to PD in contrast to the accumulation α-synuclein and TAU or pTAU. Interestingly, increased sensitivity to oxidative stress was exclusively observed in mDA neurons as sensory neurons didn't manifest such an effect (Reinhardt *et al.*, 2013). Even though both analyses lack a mechanistic basis, they seem suited as an easy and reliable readout potentially suited for HTS campaigns. Accumulation and hyperphosphorylation of TAU and the formation of NFTs have been reported for PD as well as for AD (Rajput *et al.*, 2006, Golub *et al.*, 2009, Cookson, 2010). In this study, RNA and protein levels have been found to be upregulated in G2019S-mutated neuronal cultures. This result is in accordance with a previous study indicating that G2019S-LRRK2 is associated with higher levels of TAU and pTAU in PD patients (Rajput *et al.*, 2006). Although NFTs have not been investigated in this work, accumulation and hyperphosphorylation of TAU constitutes already an interesting finding, which might possibly be the initiation

of NFT formation. So far, hyperphosphorylation of TAU has only been found in PD patients but never in an animal or cellular model for PD suggesting that iPSC-derived models are able to recapitulate aspects of PD where other models fail to do so. Mutations in the *MAPT* gene have already been described to be involved in the disruption of microtubule dynamics, and phosphorylation of TAU at Thr181 causes neurite retraction (Maldonado *et al.*, 2008). As such, hyperphosphorylation of TAU in the G2019S-LRRK2 lines could contribute to the effect in neurite outgrowth. Aggregation of α-synuclein constitutes a hallmark in PD pathogenesis. Its insoluble and aggregated form is the major compound in Lewy Bodies. In this study, the monomeric and soluble form has been investigated as accumulation is thought to precede aggregation. Aggregated forms couldn't be detected in this system possibly due to an insufficient incubation time. The monomeric form of α-synuclein was found to be slightly but significantly upregulated in the G2019S-mutated lines compared to the gene-corrected controls. Interestingly, hyperphosphorylation of TAU has already been shown to enhance α-synuclein accumulation and aggregation in cellular models (Badiola *et al.*, 2011). In contrast to TAU, the RNA levels of α-synuclein remained unchanged. This finding points into the direction that the cause of α-synuclein accumulation is at a post-translational stage like impaired autophagy. Interestingly, LRRK2 has indeed already been linked to impaired autophagy in several publications (Mizushima *et al.*, 2008, Plowey *et al.*, 2008, Alegre-Abarrategui *et al.*, 2009, Gomez-Suaga *et al.*, 2012, Sanchez-Danes *et al.*, 2012), and accumulated α-synuclein could possibly be a result of that.

9.5 iPSCs as PD Model

A potential risk of animal or human cellular models has always been that pathomechanisms underlying PD might not be present as these models are either not human, not cell-type-specific or derived from tumour cell lines. The use of iPSCs implicates several advantages such as an almost unlimited resource of functional human dopaminergic neurons. The possibility to combine iPSC technology with gene-editing techniques such as ZFN or TALEN mediated homologues recombination allows conducting studies without artificial overexpression. An obvious limitation of iPSC-derived neuronal cultures is that only neurons, astrocytes and glial cells are accessible. However, initiation and progression of PD pathology may require the presence of cell-types other than neural cells or even a whole organism.

As such, it seems somehow questionable whether an iPSC-derived system is capable of displaying phenotypes that are indeed relevant for the disease. There is evidence that PD might be a multi-system disorder and for example some groups have suggested a link between PD and an impairment of the immune system (Kim *et al.*, 2012, Moehle *et al.*, 2012). These reports seem somehow corroborated by the fact that LRRK2 expression is highly expressed and inducible in macrophages and monocytes (Thevenet *et al.*, 2011). Further it has been shown that transgenic G2019S-LRRK2 mice harbour abnormally coloured and enlarged kidneys (Tong *et al.*, 2012) indicating that other organs are involved either as a primary causative effect or a secondary reaction. However, the model system of iPSCs does show several phenotypic changes of which a few seem to be directly linked to PD pathology like the accumulation of α-synuclein and TAU or pTAU and the increased sensitivity to oxidative stress that is specific to mDA neurons. These findings therefore suggest that iPSC-derived neuronal cultures represent a model that fulfills at least the most essential frame conditions to reliably study pathomechanistic effects of PD. Of course, this doesn't exclude that other organs are also affected but it points rather into the direction that effects in cell types other than neural cells might be secondary. Another point which needs to be discussed here is the incubation time. Symptoms in PD normally occur in the elderly with a typical age at onset of 65 years. However, phenotypic changes in iPSCs could already be detected in less than two months. Several studies, including this work, have reported a neurodegenerative effect in an iPSC or hESC based model system after a few weeks (Di Giorgio *et al.*, 2008, Nguyen *et al.*, 2011, Kondo *et al.*, 2013). Reasons for that may be compensatory mechanisms *in vivo* which are not present *in vitro* and/or the exposure of an *in vitro* model to a more stressful and less protected environment that potentially enforces pathology.

The still young technology of iPSCs has already shown its first promising successes indicating that it may possess the potential to fulfill the high expectations of scientists to eventually pave the way towards an urgently needed molecular understanding of PD and other neurodegenerative diseases.

10 Literature

1. Alegre-Abarrategui J, Christian H, Lufino MM, Mutihac R, Venda LL, Ansorge O, et al. LRRK2 regulates autophagic activity and localizes to specific membrane microdomains in a novel human genomic reporter cellular model. Human molecular genetics. 2009;18(21):4022-34.

2. Alonso A, Zaidi T, Novak M, Grundke-Iqbal I, Iqbal K. Hyperphosphorylation induces self-assembly of tau into tangles of paired helical filaments/straight filaments. Proceedings of the National Academy of Sciences of the United States of America. 2001;98(12):6923-8.

3. Badiola N, de Oliveira RM, Herrera F, Guardia-Laguarta C, Goncalves SA, Pera M, et al. Tau enhances alpha-synuclein aggregation and toxicity in cellular models of α-synucleinopathy. PloS one. 2011;6(10):e26609.

4. Baum C, Dullmann J, Li Z, Fehse B, Meyer J, Williams DA, et al. Side effects of retroviral gene transfer into hematopoietic stem cells. Blood. 2003;101(6):2099-114.

5. Bosgraaf L, Van Haastert PJ. Roc, a Ras/GTPase domain in complex proteins. Biochimica et biophysica acta. 2003;1643(1-3):5-10.

6. Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, et al. Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2002;22(20):8797-807.

7. Carballo-Carbajal I, Weber-Endress S, Rovelli G, Chan D, Wolozin B, Klein CL, et al. Leucine-rich repeat kinase 2 induces alpha-synuclein expression via the extracellular signal-regulated kinase pathway. Cellular signalling. 2010;22(5):821-7.

8. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic acids research. 2011;39(12):e82.

9. Chambers SM, Fasano CA, Papapetrou EP, Tomishima M, Sadelain M, Studer L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nature biotechnology. 2009;27(3):275-80.

10. Cookson MR. The role of leucine-rich repeat kinase 2 (LRRK2) in Parkinson's disease. Nature reviews Neuroscience. 2010;11(12):791-7.

11. Cooper O, Hargus G, Deleidi M, Blak A, Osborn T, Marlow E, et al. Differentiation of human ES and Parkinson's disease iPS cells into ventral midbrain dopaminergic neurons requires a high activity form of SHH, FGF8a and specific regionalization by retinoic acid. Molecular and cellular neurosciences. 2010;45(3):258-66.

12. Corti O, Lesage S, Brice A. What genetics tells us about the causes and mechanisms of Parkinson's disease. Physiological reviews. 2011;91(4):1161-218.

13. Di Giorgio FP, Boulting GL, Bobrowicz S, Eggan KC. Human embryonic stem cell-derived motor neurons are sensitive to the toxic effect of glial cells carrying an ALS-causing mutation. Cell stem cell. 2008;3(6):637-48.

14. Ding Q, Lee YK, Schaefer EA, Peters DT, Veres A, Kim K, et al. A TALEN genome-editing system for generating human stem cell-based disease models. Cell stem cell. 2013;12(2):238-51.

15. Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS, Hulihan M, et al. Comparison of kindreds with parkinsonism and alpha-synuclein genomic multiplications. Annals of neurology. 2004;55(2):174-9.

16. Frielingsdorf H, Schwarz K, Brundin P, Mohapel P. No evidence for new dopaminergic neurons in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(27):10177-82.

17. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M. Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proceedings of the Japan Academy Series B, Physical and biological sciences. 2009;85(8):348-62.

18. Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppel C, et al. An unbiased genome-wide analysis of zinc-finger nuclease specificity. Nature biotechnology. 2011;29(9):816-23.

19. Gandhi PN, Wang X, Zhu X, Chen SG, Wilson-Delfosse AL. The Roc domain of leucine-rich repeat kinase 2 is sufficient for interaction with microtubules. Journal of neuroscience research. 2008;86(8):1711-20.

20. Gloeckner CJ, Kinkl N, Schumacher A, Braun RJ, O'Neill E, Meitinger T, et al. The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. Human molecular genetics. 2006;15(2):223-32.

21. Golub Y, Berg D, Calne DB, Pfeiffer RF, Uitti RJ, Stoessl AJ, et al. Genetic factors influencing age at onset in LRRK2-linked Parkinson disease. Parkinsonism & related disorders. 2009;15(7):539-41.

22. Gomez-Suaga P, Fdez E, Blanca Ramirez M, Hilfiker S. A Link between Autophagy and the Pathophysiology of LRRK2 in Parkinson's Disease. Parkinson's disease. 2012;2012:324521.

23. Grundke-Iqbal I, Iqbal K, Quinlan M, Tung YC, Zaidi MS, Wisniewski HM. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. The Journal of biological chemistry. 1986;261(13):6084-9.

24. Hanger DP, Anderton BH, Noble W. Tau phosphorylation: the therapeutic challenge for neurodegenerative disease. Trends in molecular medicine. 2009;15(3):112-9.

25. Hassin-Baer S, Laitman Y, Azizi E, Molchadski I, Galore-Haskel G, Barak F, et al. The leucine rich repeat kinase 2 (LRRK2) G2019S substitution mutation. Association with Parkinson disease, malignant melanoma and prevalence in ethnic groups in Israel. Journal of neurology. 2009;256(3):483-7.

26. Healy DG, Falchi M, O'Sullivan SS, Bonifati V, Durr A, Bressman S, et al. Phenotype, genotype, and worldwide genetic penetrance of LRRK2-associated Parkinson's disease: a case-control study. Lancet Neurol. 2008;7(7):583-90.

27. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nature biotechnology. 2009;27(9):851-7.

28. Kastner A, Hirsch EC, Lejeune O, Javoy-Agid F, Rascol O, Agid Y. Is the vulnerability of neurons in the substantia nigra of patients with Parkinson's disease related to their neuromelanin content? Journal of neurochemistry. 1992;59(3):1080-9.

29. Kim B, Yang MS, Choi D, Kim JH, Kim HS, Seol W, et al. Impaired inflammatory responses in murine Lrrk2-knockdown brain microglia. PloS one. 2012;7(4):e34693.

30. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, Han BS, et al. Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell stem cell. 2009;4(6):472-6.

31. Kondo T, Asai M, Tsukita K, Kutoku Y, Ohsawa Y, Sunada Y, et al. Modeling Alzheimer's disease with iPSCs reveals stress phenotypes associated with intracellular Abeta and differential drug responsiveness. Cell stem cell. 2013;12(4):487-96.

32. Lee BD, Shin JH, VanKampen J, Petrucelli L, West AB, Ko HS, et al. Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. Nature medicine. 2010;16(9):998-1000.

33. Lesage S, Durr A, Tazir M, Lohmann E, Leutenegger AL, Janin S, et al. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. The New England journal of medicine. 2006;354(4):422-3.

34. Lewis PA. The function of ROCO proteins in health and disease. Biology of the cell / under the auspices of the European Cell Biology Organization. 2009;101(3):183-91.

35. Lewis PA, Manzoni C. LRRK2 and human disease: a complicated question or a question of complexes? Science signaling. 2012;5(207):pe2.

36. Li Y, Liu W, Oo TF, Wang L, Tang Y, Jackson-Lewis V, et al. Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. Nature neuroscience. 2009;12(7):826-8.

37. Liu GH, Qu J, Suzuki K, Nivet E, Li M, Montserrat N, et al. Progressive degeneration of human neural stem cells caused by pathogenic LRRK2. Nature. 2012;491(7425):603-7.

38. Luzon-Toro B, Rubio de la Torre E, Delgado A, Perez-Tur J, Hilfiker S. Mechanistic insight into the dominant mode of the Parkinson's disease-associated G2019S LRRK2 mutation. Human molecular genetics. 2007;16(17):2031-9.

39. MacLeod D, Dowman J, Hammond R, Leete T, Inoue K, Abeliovich A. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. Neuron. 2006;52(4):587-93.

40. Maldonado H, Ortiz-Riano E, Krause B, Barriga A, Medina F, Pando ME, et al. Microtubule proteins and their post-translational forms in the cerebrospinal fluid of patients with paraparesis associated with HTLV-I infection and in SH-SY5Y cells: an in vitro model of HTLV-I-induced disease. Biological research. 2008;41(3):239-59.

41. Marin I. The Parkinson disease gene LRRK2: evolutionary and structural insights. Molecular biology and evolution. 2006;23(12):2423-33.

42. Meixner A, Boldt K, Van Troys M, Askenazi M, Gloeckner CJ, Bauer M, et al. A QUICK screen for Lrrk2 interaction partners--leucine-rich repeat kinase 2 is involved in actin cytoskeleton dynamics. Molecular & cellular proteomics : MCP. 2011;10(1):M110 001172.

43. Miller DW, Hague SM, Clarimon J, Baptista M, Gwinn-Hardy K, Cookson MR, et al. Alpha-synuclein in blood and brain from familial Parkinson disease with SNCA locus triplication. Neurology. 2004;62(10):1835-8.

44. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069-75.

45. Moehle MS, Webber PJ, Tse T, Sukar N, Standaert DG, DeSilva TM, et al. LRRK2 inhibition attenuates microglial inflammatory responses. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2012;32(5):1602-11.

46. Mortiboys H, Johansen KK, Aasly JO, Bandmann O. Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. Neurology. 2010;75(22):2017-20.

47. Murphy DD, Rueter SM, Trojanowski JQ, Lee VM. α-synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2000;20(9):3214-20.

48. Nguyen HN, Byers B, Cord B, Shcheglovitov A, Byrne J, Gujar P, et al. LRRK2 mutant iPSC-derived DA neurons demonstrate increased susceptibility to oxidative stress. Cell stem cell. 2011;8(3):267-80.

49. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. Science. 2008;322(5903):949-53.

50. Paisan-Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron. 2004;44(4):595-600.

51. Parisiadou L, Cai H. LRRK2 function on actin and microtubule dynamics in Parkinson disease. Communicative & integrative biology. 2010;3(5):396-400.

52. Parisiadou L, Xie C, Cho HJ, Lin X, Gu XL, Long CX, et al. Phosphorylation of ezrin/radixin/moesin proteins by LRRK2 promotes the rearrangement of actin cytoskeleton in neuronal morphogenesis. The Journal of neuroscience : the official journal of the Society for Neuroscience. 2009;29(44):13971-80.

53. Pattanayak V, Ramirez CL, Joung JK, Liu DR. Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. Nature methods. 2011;8(9):765-70.

54. Plowey ED, Cherra SJ, 3rd, Liu YJ, Chu CT. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. Journal of neurochemistry. 2008;105(3):1048-56.

55. Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, et al. Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science. 1996;274(5290):1197-9.

56. Rajput A, Dickson DW, Robinson CA, Ross OA, Dachsel JC, Lincoln SJ, et al. Parkinsonism, Lrrk2 G2019S, and tau neuropathology. Neurology. 2006;67(8):1506-8.

57. Ramonet D, Daher JP, Lin BM, Stafa K, Kim J, Banerjee R, et al. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. PloS one. 2011;6(4):e18568.

58. Ravid R. Biobanks for biomarkers in neurological disorders: the Da Vinci bridge for optimal clinico-pathological connection. Journal of the neurological sciences. 2009;283(1-2):119-26.

59. Ravid R, Ferrer I. Brain banks as key part of biochemical and molecular studies on cerebral cortex involvement in Parkinson's disease. The FEBS journal. 2012;279(7):1167-76.

60. Reinhardt P, Schmid B, Burbulla LF, Schondorf DC, Wagner L, Glatza M, et al. Genetic correction of a LRRK2 mutation in human iPSCs links parkinsonian neurodegeneration to ERK-dependent changes in gene expression. Cell stem cell. 2013;12(3):354-67.

61. Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nature medicine. 2004;10 Suppl:S10-7.

62. Sanchez-Danes A, Richaud-Patin Y, Carballo-Carbajal I, Jimenez-Delgado S, Caig C, Mora S, et al. Disease-specific phenotypes in dopamine neurons from human iPS-based models of genetic and sporadic Parkinson's disease. EMBO molecular medicine. 2012;4(5):380-95.

63. Sanna G, Del Giudice MG, Crosio C, Iaccarino C. LRRK2 and vesicle trafficking. Biochemical Society transactions. 2012;40(5):1117-22.

64. Schiesling C, Kieper N, Seidel K, Kruger R. Review: Familial Parkinson's disease--genetics, clinical phenotype and neuropathology in relation to the common sporadic form of the disease. Neuropathology and applied neurobiology. 2008;34(3):255-71.

65. Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW, Cook EG, et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. Cell. 2009;136(5):964-77.

66. Soldner F, Laganiere J, Cheng AW, Hockemeyer D, Gao Q, Alagappan R, et al. Generation of isogenic pluripotent stem cells differing exclusively at two early onset Parkinson point mutations. Cell. 2011;146(2):318-31.

67. Sommer CA, Stadtfeld M, Murphy GJ, Hochedlinger K, Kotton DN, Mostoslavsky G. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. Stem cells. 2009;27(3):543-9.

68. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M. Alpha-synuclein in Lewy bodies. Nature. 1997;388(6645):839-40.

69. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K. Induced pluripotent stem cells generated without viral integration. Science. 2008;322(5903):945-9.

70. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131(5):861-72.

71. Thevenet J, Pescini Gobert R, Hooft van Huijsduijnen R, Wiessner C, Sagot YJ. Regulation of LRRK2 expression points to a functional role in human monocyte maturation. PloS one. 2011;6(6):e21519.

72. Tong Y, Giaime E, Yamaguchi H, Ichimura T, Liu Y, Si H, et al. Loss of leucine-rich repeat kinase 2 causes age-dependent bi-phasic alterations of the autophagy pathway. Molecular neurodegeneration. 2012;7:2.

73. Tong Y, Pisani A, Martella G, Karouani M, Yamaguchi H, Pothos EN, et al. R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice. Proceedings of the National Academy of Sciences of the United States of America. 2009;106(34):14622-7.

74. Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. Nature reviews Genetics. 2010;11(9):636-46.

75. Wakabayashi K, Takahashi H, Ohama E, Ikuta F. Parkinson's disease: an immunohistochemical study of Lewy body-containing neurons in the enteric nervous system. Acta neuropathologica. 1990;79(6):581-3.

76. Winner B, Melrose HL, Zhao C, Hinkle KM, Yue M, Kent C, et al. Adult neurogenesis and neurite outgrowth are impaired in LRRK2 G2019S mice. Neurobiology of disease. 2011;41(3):706-16.

77. Yakubov E, Rechavi G, Rozenblatt S, Givol D. Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochemical and biophysical research communications. 2010;394(1):189-93.

78. Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB, et al. Evidence for neurogenesis in the adult mammalian substantia nigra. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(13):7925-30.

79. Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, et al. Generation of induced pluripotent stem cells using recombinant proteins. Cell stem cell. 2009;4(5):381-4.

80. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 2004;44(4):601-7.

81. Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. Nature biotechnology. 2003;21(3):319-21.