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# Human Pluripotent Stem Cells

## Modeling Neurodegenerative Diseases

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Additional information is available at the end of the chapter

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### 1. Introduction

Modeling of human neurodegenerative diseases in animals has led to important advances in the understanding of pathogenic mechanisms and has opened avenues for curative approaches. However, inherent genetic, developmental and anatomical species differences between humans and animals frequently resulted in imperfect phenotypic correlations between animal models and human diseases. This might account for the observed hampered translation of promising preclinical treatment studies in animal models towards clinics.

Pluripotent stem (PS) cells hold considerable promise as a novel tool for modeling human diseases. Human PS cells include human embryonic stem (hES) cells and induced PS (IPS) cells. IPS cells are generated *via* reprogramming of somatic cells through the forced expression of key transcription factors and share salient characteristics of ES cells, which are derived from the preimplantation blastocyst.

Both types of PS cells show the capacity to self-renew and to differentiate *in vitro* and *in vivo* into the cell types that make up the human body. This includes the various types of mature neurons affected by neurodegenerative diseases. The combination of the key advantages of PS cells allows for the first time to generate large numbers of postmitotic human neurons for preclinical research in cell culture. In particular, the IPS cell technology opens doors for intensified research on human PS-derived neurons because, in comparison to hES cells, ethical concerns can be dispelled. Furthermore, the isolation of patient-derived IPS cell lines from skin biopsies enables the study of pathogenic mechanisms in human cells carrying relevant pathogenic allelic constellations.

During recent years the generation of IPS cell lines from human material has become routine. However, for neurological research a remaining major challenge is to guide *in vitro* differen-

tiation of IPS cells into defined and homogeneous neuronal populations that are required for modeling neurodegenerative diseases. A hallmark of human neurodegenerative diseases is the chronic and progressive loss of specific types of neurons: cerebral cortex glutamatergic and basal forebrain cholinergic neurons in Alzheimer's disease, midbrain dopaminergic neurons in Parkinson's disease, striatal GABAergic neurons in Huntington's disease, motor neurons in amyotrophic lateral sclerosis and spinal muscular atrophy, cerebellar and peripheral sensory neurons in ataxias and others. To fully tap into the potential of the IPS technology and to progress towards a fundamental understanding of the causes of disease selectivity in the loss of neuron subtypes it will be necessary to establish reproducible and tailored protocols for differentiation of IPS cells specifically into these neuronal subtypes *in vitro*.

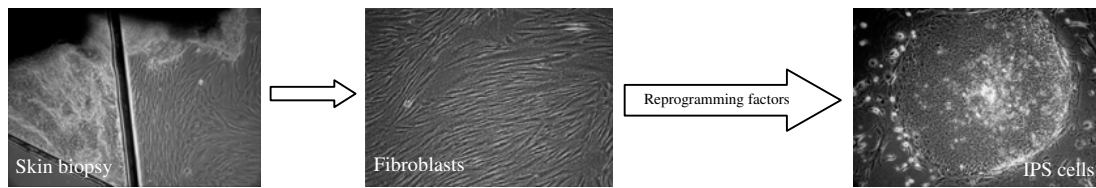
To date, reprogramming of patient somatic cells into IPS cell-based models has been achieved for several neurodegenerative diseases. The results show that IPS cells or their derivatives can display at least some of the cellular and/or molecular characteristics of the respective diseases. These findings provide first proof for etiological validity of these models. Here, we review the existing reports demonstrating the generation of human PS cell-based models for neurodegenerative diseases, including also the studies showing the differentiation of human PS cells, both ES and IPS cells, toward telencephalic neurons (glutamatergic, GABAergic and cholinergic), midbrain dopaminergic neurons, cerebellar neurons, spinal motor neurons and peripheral neurons. We further discuss the perspectives of these cellular models.

## 2. Generation of human IPS cells

It was in 2006 when the first IPS cells were generated by Takahashi and Yamanaka *via* reprogramming of mouse somatic fibroblasts through retroviral transduction with a specific set of factors [1]. A screen of pluripotency-associated genes yielded a successful combination of transcription factors, comprising Oct4, Sox2, Klf4 and c-Myc (OSKM), which are commonly referred to as the 'Yamanaka factors'. Shortly afterwards, the same group [2], concurrently with other groups that used different combinations of transcription factors, for example substituting c-Myc and Klf4 by Lin28 or Nanog [3-5], were able to demonstrate that also fibroblasts obtained from adult human beings can be induced to undergo the transformation into PS cells.

Since these first descriptions of IPS cell derivation significant improvements in efficiency of the protocols, in the quality of the resulting IPS lines and in the depth of their analysis have been achieved. So far, fibroblasts remain the most popular donor cell type, and were used in more than 80% of all published reprogramming experiments. Figure 1 illustrates the steps in generating human IPS cell from skin fibroblasts, as well as cell morphology transition in culture.

However, other cell sources for inducing pluripotency have been used, amongst them keratinocytes [6], cord blood cells [7] and mesenchymal stem cells [8] with sometimes higher efficiency compared to fibroblasts. Furthermore, different combinations of reprogramming



**Figure 1.** Generation of human IPS cells from a skin biopsy.

factors have been developed, ranging in number between two to six [3;4;9]. Each of these reprogramming factors contributes to the kinetics and efficiency of IPS induction.

Genetic material coding for these reprogramming factors has been introduced into cells via a variety of methods, comprising genome integrating as well as non-integrating techniques [10]. The most commonly used method for factor delivery is the transduction using retroviruses, originally with Moloney murine leukemia virus (MMLV), vectors, later on with modified lentiviral vectors. The efficiency of IPS cell generation using sets of four MMLV-derived retroviruses expressing single genes from the OSKM set separately is ~0.01% in human fibroblasts.

Silencing of the permanently integrated transgenes is important because only an IPS cell that has up regulated the endogenous pluripotency gene network but down regulated the expression of the transgenes can be considered fully reprogrammed [11]. Although the use of retroviruses is efficient and yields reproducible results, random insertional mutagenesis, permanent alteration of gene expression as well as reactivation of silenced transgenes during differentiation cannot be excluded. The use of Cre-deletable or dox-inducible lentiviruses has overcome some of these problems and allows factor expression in a more controlled manner [12;13]. Other attempts to generate integration-free IPS cells focused on replication-defective adenoviral vectors, or Sendai viral vectors [14;15] which efficiently deliver foreign genes into a multitude of cell types.

To avoid the use of viral vectors, direct delivery of episomal vectors (plasmids) as well as standard DNA transfections using liposomes or electroporation have also been used, but with low transfection efficiency [16-18]. A polycistronic expression cassette flanked by loxP sites enabled the excision of the reprogramming cassette after expressing Cre recombinase also in the non-viral system [19].

Alternatively, Warren et al. [20] developed a novel mRNAs-based system and achieved an efficient conversion of different human somatic donor cells into IPS cells using a direct delivery of high dosages of modified mRNAs encoding OSKM and Lin28 packaged in a cationic vehicle. The efficiency reached with this approach was much higher when compared with other non-integrative protocols [20].

Recently, a potential role of specific microRNAs (miRNAs) for pluripotency has been elucidated. The miRNAs from the miR-302 cluster contribute to unique ES cells features such as cell cycle and pluripotency maintenance [21;22]. Based on these findings protocols for highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency were reported [23;24]. The resulting miR-IPS cells are subject to a reduced risk of mutations

and tumorigenesis relative to most other protocols because mature miRNAs function without genomic integration [23;24].

Finally, another promising possibility of inducing pluripotency is to deliver the reprogramming factors directly as proteins. To this end Zhou et al. generated recombinant OSKM proteins fused with a poly-arginine transduction domain [25]. However, this protein-based strategy induced pluripotency with extremely slow kinetics and poor efficiencies [25].

Apart from the delivery methods of reprogramming factors, other parameters, including culture conditions and the application of small pharmacological compounds, exert an influence on reprogramming efficiency. For example, it has been demonstrated that culturing IPS cells under hypoxic conditions mimicking the *in vivo* environment, enhances the efficiency rate [26]. The addition of small molecules, that either modifies epigenetic states like DNA methylation or histone acetylation, or influences specific receptor mediated signaling pathways, enhances the generation of IPS cells [27-31].

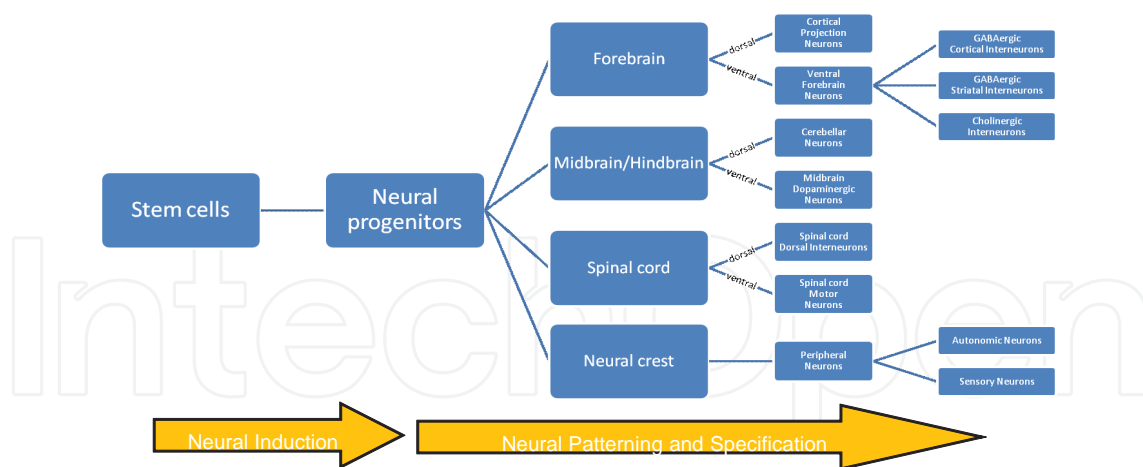
Eventually, the reactivation of endogenous pluripotency genes leads to establishment of cell lines with pluripotent characteristics. However, even though IPS lines share many characteristics with hES cells with regard to morphology and pluripotent gene expression, further research is required to establish more precisely communalities and differences between hES and IPS cells. Differences in epigenetic status and *in vitro* and *in vivo* differentiation potential have been reported [32-34].

### 3. Neuronal differentiation of human PS cells

The *in vitro* production of neurons from PS cells, following similar mechanism as *in vivo* development, involves several sequential steps precisely orchestrated by signaling events (reviewed in [35;36]).

*In vivo*, during embryonic development, the initial step is neural induction, the specification of neuroepithelia from ectoderm cells [37]. When the neuroectodermal fate is determined, the neural plate folds to form the neural tube, from which cells differentiate into various neurons and glia [38;39]. The neural tube is patterned along its anteroposterior (A/P) and dorsoventral (D/V) axes to establish a set of positional cues. The neural plate acquires an anterior character, and is subsequently posteriorized by exposure to Wntless/Int proteins (Wnt), fibroblast growth factors (FGF), bone morphogenic proteins (BMP) and retinoic acid (RA) signals to establish the main subdivisions of the central nervous system (CNS): forebrain, midbrain, hindbrain, and spinal cord, as well as the neural crest from which the peripheral neurons derive [40-42]. Therefore, the precursor cells in each subdivision along the A/P axis are fated to subtypes of neurons and glia depending on its exposure to unique sets of morphogens at specific concentrations (Figure 2).

As reviewed in Petros et al. [35], specific PS cell-bases protocols, following the principles of nervous system development, can generate neuronal types with markers consistent with telencephalic, midbrain, hindbrain spinal cord and peripheral neurons (Table 1).



**Figure 2.** Stem cell fates aligned to nervous system development

Differentiated neural subtype	PS cell type	Key patterning differentiation factors	References
General telencephalic neurons	mES cells	DKK, LeftyA, Wnt3a, Shh	Watanabe et al. (2005)[43], Li et al. (2009)[44]
Cortical pyramidal neurons	mES cells hES cells	Cyclopamine, Fgf2, RA	Eiraku et al. (2008)[45], Gaspard et al. (2008)[46], Gaspard et al. (2009)[47], Ideguchi et al. (2010)[48], Nat et al 2012[36]
Cortical interneurons	mES cells hES cells	Shh, Fgf2, IGF, Activin	Maroof et al. (2010)[49], Danjo et al. (2011)[50], Goulburn et al. (2011, 2012) [51;52], Cambray et al. (2012)[53], Nat et al 2012[36]
Basal forebrain cholinergic neurons	hES cells	RA, bFGF, FGF8, Shh, BMP9	Wicklund et al (2010)[54], Bissonnette et al. (2011)[55]
Striatal medium spiny neurons	mES cells, hES cells	Shh, BDNF, DKK1, cAMP, valproic acid	Aubry et al. (2008)[56], Zhang et al. (2010)[57], Danjo et al. (2011)[50]
Floor plate cells	hES cells	Shh, dual SMAD inhibition	Fasano et al. (2010)[58]
Midbrain dopaminergic neurons	mES cells hES cells, hIPS cells	Shh, AA, FGF8, bFGF	Kawasaki et al. (2000)[59], Lee et al. (2000)[60], Perrier et al. (2004)[61], Yan et al. (2005)[62], Chambers et al. (2009)[63], Sánchez-Danés et al. (2012)[64]



Differentiated neural subtype	PS cell type	Key patterning differentiation factors	References
Cerebellar granule cells	mES cells, hES cells	Wnt1, Fgf8, RA, BMP 6/7, GDF7, Shh, JAG1	Salero and Hatten (2007) [65], Erceg et al. (2010)[66]
Cerebellar Purkinje cells	mES cells	BMP4, Fgf8	Su et al. (2006)[67], Tao et al. (2010)[68]
Spinal cord motor neurons	mES cells, hES cells, IPS cells, hMS cells, hADS cells	Shh, RA, SB431542, Olig2, HB9	Wichterle et al. (2002)[69], Li et al. (2005)[70], Soundararajan et al. (2006) [71], Lee et al. (2007)[72], Dimos et al. (2008)[73], Peljto et al. (2010)[74], Patani et al. (2011)[75], Park et al. (2012) [76], Liqing et al. (2011)[77]
Spinal cord interneurons	mES cells	Wnt3A, Shh, RA, BMP2	Murashov et al. (2005)[78]
Neural crest	hES cells, hIPS cells	SB431542, noggin, BDNF, NGF, AA, dbcAMP	Lee et al. (2010)[79], Menendez et al. (2011)[80], Goldstein et al. (2010)[81]

**Table 1.** Neural cell types derived from PS cells to date (modified from Petros et al. 2011[35])

Recognizing that all resulting cell populations, although enriched in specific neurons, remain heterogeneous, there is a need for additional selection methods to further purify neuronal subtype lineages. Whilst a key aim of positionally specifying human neurons is to work towards the generation of cell-based therapies for diseases that target a sub-population of cells, this system will be particularly powerful in attempting to understand disease specificity when applied to patient-derived IPS cells.

#### 4. Neurodegenerative diseases and related models

Neurodegenerative diseases are characterized by the chronic and progressive loss of neuronal functions in selected neurons. Classical neurodegenerative diseases are Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, amyotrophic lateral sclerosis, spinal muscular atrophy and ataxias. Other rare diseases such as familial dysautonomia and Fragile-X syndrome contain neurodegenerative aspects as well.

Here we aim to present the main characteristics of these diseases, focusing on their pathogenesis and its reflection into the disease models, including the recent cellular models derived via IPS cell technology. The most important publications and aspects regarding the IPS cell-related models for neurological diseases are reviewed in Han et al. 2011[82] and updated for the neurodegenerative diseases in Table 2.

Neurodegenerative disease	Types of affected neurons	Histopathology	Gene (Mutation)	Donor cell	Reprogramming method	Reported disease-related phenotype	References
Alzheimer's disease	Basal forebrain cholinergic neurons, cortical neurons	Neurofibrillary tangles, Amyloid plaque, Loss of neurons and synapses	PS1, PS2 mutations; Sporadic and APP duplication	SF	LV: OSKLN RV: OSKM	Increased amyloid $\beta$ 42 secretion, increased $\beta$ (1-40) and phospho- $\tau$ levels	Yagi et al. (2011)[83], Israel et al. (2012)[84]
Parkinson's disease	Midbrain nigro-striatal dopaminergic neurons	Lewy-bodies, loss of dopaminergic neurons	idiopathic	SF	LV: Cre-excisable, DOX-inducible; OSK or OSKM	NA	Soldner et al. (2009)[12], Hargus et al. (2010)[85]
			LRRK2 (G2019S)	SF	RV: OSK	Increased caspase-3 activation and DA neuron death with various cell stress conditions	Nguyen et al. (2011)[86]
			PINK1 (Q456X; V170G)	SF	RV: OSKM	Impaired stress-induced mitochondrial translocation of Parkin in DA neurons	Seibler et al. (2011)[87]
			SCNA triplication	SF	RV: OSKM	Increased neural $\alpha$ -Synuclein protein levels, sensitivity to oxidative stress	Devine et al. (2011)[88], Byers et al. (2011)[89]
Huntington's disease	Striatal GABAergic medium spiny neurons, cortical neurons	Neural inclusion bodies, loss of striatal/cortical neurons	HTT (CAG repeats)	SF	RV: OSKM	Increase in lysosomal activity	Park et al. (2008)[4], Camnasio et al. (2012)[90]
Amyotrophic lateral sclerosis	Upper and lower motor neurons	Ubiquitinated inclusion bodies, loss of motoneurons	SOD1	SF	RV: OSKM, OSK	NA	Dimos et al. (2008)[73], Boulting et al. (2011)[33]
Spinal muscular atrophy, type I	Spinal motor neurons	Loss of anterior horn cells	SMN1 deletion	SF	RV: OSNL Episomal plasmid OSKMNL combinations	Reduced number of motor neurons, decreased soma size, synaptic defects	Ebert et al. (2009)[91] Sareen et al. (2012)[92]
Friedrich Ataxia	Dorsal root ganglia (DRG) peripheral	Reduced size of DRG-neurons, iron	FXN (GAA expansion)	SF	RV: OSKM	GAA repeat instability	Ku et al. (2010)[93], Liu



Neurodegenerative disease	Types of affected neurons	Histopathology	Gene (Mutation)	Donor cell	Reprogramming method	Reported disease-related phenotype	References
	neurons, cerebellar neurons	misdistribution, decreased myelination					et al. (2010) [94]
Spinocerebellar Ataxia Type 3 (Machado-Joseph Disease)	Cerebellar neurons, striatal and cortical neurons	Intranuclear inclusion bodies, neuronal loss	ATAXIN 3(CAG expansion)	SF	RV: OSKM	NA	Koch et al. (2011)[95]
Familial dysautonomia	Sensory and autonomic neurons	Reduced size of DRG neurons, reduced number of non-myelinated small fibers and intermediolateral column neurons	IKBKAP	SF	RV: OSKM	Defects in neurogenesis and migration	Lee et al. (2009)[96]
Fragile-X syndrome	Hippocampal, cerebellar neurons	Dendritic spine abnormalities, neuronal loss	FMR1 (CGG repeat)	SF	RV: OSKM	NA	Urbach et al. (2010)[97]

SF-skin fibroblasts, RV-retroviruses, LV-lentiviruses and NA- not assessed

**Table 2.** Overview of the iPS the cell-related models for neurodegenerative diseases (modified from Han et al. 2011 [82])

4.1. Alzheimer’s disease

Alzheimer’s disease (AD) is the most common neurodegenerative disease, affecting 35 million patients worldwide. Clinically, it is characterized by progressive loss of short-term memory and other cognitive functions toward a state of profound dementia.

AD is histopathologically characterized by neuronal and synapse loss and the appearance of extracellular amyloid plaques (AP) and intracellular neurofibrillary tangles (NFTs) in affected brain regions, especially cerebral cortex, hippocampus and basal forebrain [98;99]. The AP and NFTs form by aggregation of two proteins, beta amyloid (Aβ) and hyperphosphorylated tau (pTau), respectively [100]. Aβ is formed from the cleavage of the amyloid precursor protein (APP) into soluble monomers that then aggregate into fibrils and are eventually deposited in the extracellular space [101]. Tau is a microtubule-associated protein that undergoes hyperphosphorylation and accumulates as intraneuronal inclusions or tangles in the brains of individuals with AD [100;102].

Degeneration of basal forebrain cholinergic neurons is a principal feature of AD and the reduction in the level of acetylcholine and choline acetyltransferase activity in the hippocampus and cerebral cortex has been reported in the brains of AD patients [103;104].

The majority of AD cases are sporadic; in these cases the major genetic risk factor disease is the *APOE* gene. ApoE is synthesized in astrocytes and acts as a ligand for the receptor-mediated endocytosis of cholesterol-containing lipoprotein particles. Whether ApoE affects A $\beta$  clearance or operates through its function in lipid metabolism is not yet fully established [105].

Few familial AD (FAD) cases are an early-onset autosomal dominant disorder. Three genes have been identified that account for FAD: the first mutations causing Mendelian AD were identified in the *APP* gene [106], although mutations in two other genes, *presenilin 1* and 2 (*PSEN1* and *PSEN2*), that form the  $\gamma$ -secretase complex components, are more commonly found. The mutations cause different clinical phenotypes, but for all the aberrant processing of A $\beta$  led to its aggregation [107].

By classical transgene and knockout approaches, there were established mouse models that reflect different aspects of AD [108]. Representative models are APP mutant strains (such as PDAPP, J20, APP23 or Tg2576) with a robust APP/A $\beta$  pathology and tau mutant strains with NFT formation such as (JNPL3 or pR5). The histopathology in these strains is associated with behavioral impairment [109].

The modeling of AD via IPS cell technology was recently reported [83;84]. The first study used AD patient fibroblasts carrying mutations in PS1 and PS2. The IPS cells kept the mutations and differentiated into neural cells, showing increased amyloid  $\beta$ 42 secretion as compared to the healthy controls [83].

In the second study, IPS cells were generated from both patients with sporadic AD or carrying APP duplication. Interestingly, increased level of both A $\beta$  (1-40) and pTau were detected in neural cells cultures after neural progenitor expansion of about five weeks, followed by differentiation of about four weeks [84].

#### 4.2. Parkinson's disease

Parkinson's Disease (PD) is the second most common neurodegenerative disorder, afflicting over 6 million people worldwide. Clinically, there are progressive motor dysfunctions comprising bradykinesia, rigidity and tremor, as well as non-motor features.

Pathologically, PD is identified by intracellular inclusions known as Lewy bodies and dopaminergic neuronal loss that initiates in the substantia nigra.

PD is largely a late onset sporadic neurodegenerative condition. However, 5–10% cases are familial, transmitted in either an autosomal-dominant or autosomal recessive fashion [110]. A number of genes have been linked to our understanding of pathogenesis. The gene  $\alpha$ -synuclein (*SNCA*) product is the major component of the Lewy body in sporadic and in some cases of autosomal dominant types and therefore appears to be central to PD pathophysiology [111;112]. The most common mutation related to autosomal-dominant PD occurs in the gene encoding leucine-rich repeat kinase-2 (*LRRK2*) [113]. One missense mutation, the G2019S mutation, occurs in 5% of familial cases and 1–2% of sporadic cases of PD. Mutations in *PARK2*, *PINK1* and *PARK7* (also known as *DJ1*) cause autosomal-

recessive, early onset PD [114-116]. These genetic discoveries have highlighted the importance of the ubiquitin proteasome system, mitochondrial dysfunction and oxidative stress in PD pathogenesis.

The most common genetic risk factor for PD appears to be heterozygous mutations in the *glucocerebrosidase* gene (*GBA*) [117]. The frequency of heterozygous mutations in *GBA* reaches ~4% in sporadic PD populations.

Because PD results from the loss of dopaminergic neurons, the prospect of utilizing cell replacement therapies has attracted substantial interest. Several methods are able to improve the effectiveness of midbrain dopamine neuron generation and/or retrieval from fetal tissue and stem cells.

The ability of deriving large quantities of correctly differentiated dopamine neurons makes stem cells promising cell sources for transplantation in PD; having the transplantation as a main goal, many studies improved the directed differentiation of PS cells toward dopaminergic neurons, opening the doors to IPS cell-derived models.

Soldner *et al.* induced pluripotency in fibroblasts derived from idiopathic PD patients and controls and subsequently differentiated both into dopaminergic neurons. As they did not find significant differences between the expression of *SNCA* or *LRKK2* between patients and controls, they went on to suggest that it might still be necessary to further accelerate PD-pathology related phenotypes *in vitro* with neurotoxins such as MPTP, or the overexpression of PD-related genes such as *SNCA* or *LRKK2* in order to obtain a valid PD model [12].

Hargus *et al.* [85] used a similar protocol of inducing PS cells for idiopathic PD patients and controls, and further differentiated them into dopaminergic neurons. Additionally, they performed intrastriatal transplantation studies into 6-OHDA lesioned rats, demonstrating improvements in motor symptoms.

Regarding familiar PD, Nguyen *et al.* [86] used a classical protocol for IPS cells generation and differentiation and found that IPS cell-derived dopaminergic neurons from patients carrying a *LRRK2* mutation had increased expression of oxidative stress response genes and  $\alpha$ -synuclein protein. The mutant neurons were also more sensitive to caspase-3 activation and cell death caused by exposure to hydrogen peroxide, MG-132 (a proteasome inhibitor), and 6-hydroxydopamine than control neurons. The finding of increased susceptibility to stress in patient-derived neurons provides insights into the pathogenesis of PD and a potential basis for a cellular screen.

Seibler *et al.* [87] generated IPS cells from PD patients carrying mutation in *PINK1* gene (Q456X; V170G). They compared the mitochondrial translocation of Parkin in DA neurons under mitochondrial stress conditions and found a difference between patients and controls, making a step forward into PD pathogenesis *in vitro*.

Two recent studies focused on the IPS cell-derived models of PD carrying a triplication in *SNCA* genes. Devine *et al.* showed that the levels of  $\alpha$ -Synuclein protein were increased in the dopaminergic population derived from patients, compared to the healthy controls [88], while

Byers *et al.* focused on the differences in sensitivity to oxidative stress in correlation with this mutation [89].

#### 4.3. Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder resulting from an expanded CAG triplet repeat in the Huntingtin gene (*HTT*) on chromosome 4 [118]. This expansion accounts for an attachment of a polyglutamine strand of variable length at the N-terminus of the protein leading to a toxic gain of function [119]. HD together with eight other CAG triplet repeat expansion disorders forms the group of PolyQ diseases which share some specific pathophysiological features [120].

Although the protein huntingtin is ubiquitously expressed in mammalian cells, mainly striatal GABAergic medium spiny neurons with a dopamine- and cyclic AMP-regulated phosphoprotein (DARPP-32)-positive phenotype are the most susceptible to neurodegeneration in HD [121]. As a consequence a prominent cell loss and atrophy in the caudate nucleus and putamen can be observed. Other brain regions and neuronal subtypes involved in HD comprise the substantia nigra, hippocampus, cerebellar Purkinje cells and thalamic nuclei [119;122].

One of the histopathological hallmarks of Huntington's disease, as in other PolyQ disorders too, is the appearance of nuclear and cytoplasmic inclusion bodies containing the mutant huntingtin and polyglutamine [123;124]. Much debate regarding the meaning and function of these inclusions is going on, and although indicative of pathological mutant protein processing they do not correlate with cellular dysfunction and might even confer a protective role [125;126].

Numerous studies indicated that wild-type huntingtin might be involved in a variety of intracellular functions such as in protein trafficking, vesicle and axonal transport, mitochondrial function, postsynaptic signaling; transcriptional regulation, as well as in anti-apoptotic pathways [127;128]. Therefore a disruption and detrimental impairment of these various intracellular pathways is supposed to be the consequence of accumulation of mutant huntingtin, finally leading to neuronal death.

Over the years, several different HD models had been introduced, ranging from invertebrate models like *Drosophila* and *C. elegans* to various rodent models [129;130]. Genetically modified animals (especially mouse) models such as transgenic, knock-in and conditional ones recapitulated some features of HD like neuronal polyglutamine inclusions [131].

The intrastriatal injection of excitotoxic glutamic acid analogues like kainic acid, quinolinic acid and 3-nitropropionic acid into animals resulted in neuronal cell death similar to the pathology observed in HD patients [132-134]. They proved to be useful in studying pathogenic processes involved in the progressive disease course although some limitations regarding the selective neuronal cell loss as well as aggregate formation properties and variable phenotypes have to be kept in mind.

Transplantation studies in animal HD models aimed at providing neuroprotective support or intended to replace damaged and lost neuronal subtypes. Successful application of stem cell-

based therapy in animal models of HD with functional recovery has been reported [135;136]. Different cell types ranging from neural stem/progenitor cells from mouse and rat or human fetal brain tissue to bone marrow and mesenchymal stem cells have been transplanted into excitotoxic animal HD models[137].

In order to facilitate research in the HD field with human material, Bradley *et al.* [138] derived four hES cell lines containing more than 40 CAG repeats from donated embryos obtained through an informed consent. Those hES cells were able to differentiate in to neuronal cells expressing the mutant huntingtin protein.

The first HD-IPS cell lines were successfully generated by Park *et al.* from patient with a 72 CAG repeat tract using the classical lentiviral vectors [4]. In a subsequent study Zhang *et al.* used these patient-specific IPS cells in order to generate HD specific neural stem cells that were then differentiated into striatal neurons. Besides a stable CAG repeat expansion in all patient-derived cells, an enhanced caspase 3/7 activity was found.

A second group successfully generated HD-specific IPS cells via lentiviral transduction of transcription factors and was able to demonstrate a stable CAG triple repeat length in all IPS cell clones as well as in IPS cell-derived neurons. Interestingly, they observed an enhanced lysosomal activity in IPS cells and their derived neuronal populations [139].

#### 4.4. Motor neuron diseases

Motor neurons (MNs) are essential effector cells for the control of motor function. Degenerative MN diseases, such Spinal Muscular Atrophy (SMA) and Amyotrophic Lateral Sclerosis (ALS) are devastating disorders due to a selective loss of MNs, which in turn leads to progressive muscle atrophy and weakness.

SMA is the most common form of degenerative motor neuron disease in children and young adults, characterized by the selective degeneration of lower MNs in the brainstem and spinal cord [140]. SMA is a classical autosomal recessive disorder with the vast majority of SMA cases caused by homozygous mutations in the gene named Survival of Motor Neuron-1 (SMN1) [141;142].

Interestingly, in humans the SMN exists in a telomeric copy, SMN1, and several centromeric highly homologous copies, SMN2, with both genes being transcribed [143]. Due to the fact that the vast majority of SMN2 transcripts lack an exon due to a splicing defect, it is only partially and poorly able to compensate for reduced SMN1 levels [144;145].

SMN is a ubiquitously expressed gene involved in the biogenesis of small nuclear ribonucleoproteins important for pre-mRNA splicing, but might also have a specific role in RNA transport in neurons [146]. However, it remains to be elucidated how a deficiency in SMN is responsible for the selective degeneration of lower motor neurons [147].

Several experimental models have been used to study the putative cellular and molecular processes involved in SMA. Mouse models have become the most often used, albeit lacking the duplication of the SMN gene in humans. As a consequence, homologous recombination technology of the *Smn* locus in mice leads to complete depletion of the SMN protein, causing



early embryonic lethality [148], which has necessitated generating transgenic mice that harbor human SMN2 [149;150] on a SMN<sup>-/-</sup> background. Although this model provided invaluable protein and disease information, reflecting a gene dosage-dependent phenotype similar to severe forms of SMA, these mice normally die shortly after birth.

It was in 2009 when patient-derived IPS cells were used for the first time to model SMA [91]. Therefore, skin fibroblasts from a three-year old child with SMA as well as from the unaffected mother were successfully reprogrammed via transduction with lentiviral vectors comprising OCT4, SOX2, NANOG, and LIN28. Characterization of the obtained IPS cells demonstrated lack of SMN1 expression and reduced levels of the full-length protein compensated by SMN2. Patient and control IPS cells were further differentiated into neurons. Within these neural cultures, significant differences regarding the number of motoneurons as well as their soma size and synapse formation ability could be observed between patient-specific and control cells, therefore reflecting disease-specific phenotypes. Furthermore, valproic acid and tobramycin, two drugs known to increase full-length SMN mRNA levels from the SMN2 locus, were tested on this human cellular SMA model. A 2-3-fold increase in SMN protein expression in SMA-IPS cells and an increased nuclear punctuate localization of SMN protein were found.

In a continuative experiment, Sareen et al [92] generated SMA-specific IPS cells using a virus-free plasmid-based approach with subsequent differentiation of IPS cells into NSCs and further MN differentiation. Besides the already described SMA-specific phenotypes, increased apoptosis was detected in SMA-specific cells, which might be another potential target for therapeutic intervention.

ALS, also known as Lou Gehrig's disease is the most common form of MN disease; it is a rapidly progressing, fatal disorder, usually as a result of respiratory failure. In contrast to SMA, it is characterized by a progressive loss of both upper and lower motor neurons in the cerebral cortex, brainstem and spinal cord. Two forms of ALS can be distinguished, the more frequent sporadic form accounts for about 90% of cases and the less common familial form (FALS) for the remaining 10% [151].

Mutations in the Cu/Zn superoxide dismutase 1 (SOD1) gene are responsible for about 20% of the familial cases [152]. Recently, several other gene mutations were identified as important causing typical FALS, such as the gene encoding the TAR DNA-binding protein 43 (TDP-43) [153]. The role of TDP-43 was first suspected when it was identified as one of the major constituents of the intra-neuronal inclusions characteristically observed in ALS and in frontotemporal lobar degeneration-ubiquitin (FTLD-U); [154]. Subsequently, mutations in the TARDBP gene encoding TDP-43 were identified in some FALS [153].

On macroscopic and microscopic examination of the nervous system in ALS variable neuronal inclusion bodies in lower motor neurons of the spinal cord and brain stem can be detected [155]. Morphologically these inclusions are reliably demonstrated only by their immunoreactivity to ubiquitin, and have been reported in both sporadic and familial cases and are present in transgenic models of ALS. It is now well established that ALS is typically characterized by the presence of these inclusion bodies.



Furthermore, it has been reported that an ALS genotype in glial cells (astrocytes) has an effect on the survival of motor neurons and contributes a crucial role in motor neuron degeneration [156].

ALS research has focused mainly on models of the familial SOD1-mediated form, although all forms of ALS share striking similarities in pathology and clinical symptoms. A toxic gain of function of this enzyme with the exact mechanism still unclear is thought to be responsible which subsequently results in mitochondrial dysfunction, oxidative damage, glutamate excitotoxicity, protein aggregation, proteasome dysfunction, cytoskeletal and axonal transport defects and inflammation [151;157].

Transgenic mice or rats overexpressing mutant SOD1 develop MN degeneration with progressive muscle weakness, muscle wasting and reduced life span [158]. Furthermore, mutant SOD1 as well as TDP-43 models have been generated in zebrafish and *C. elegans*, mimicking at least some of the pathological hallmarks (e.g. selective vulnerability of MN and MN dysfunction) and therefore making them suitable for genetic and small compound screening [157;159].

Transgenic ALS models have also already been utilized for stem cell therapies by transplanting different types of cells comprising human as well as rodent fetal neural stem and progenitor cells, umbilical cord blood stem cells, mesenchymal stem cells and bone marrow. In some of the studies, a moderate improvement of motor function and a delayed disease progression could be observed [160]. However, the translation of stem cell transplantation therapies into clinical trials did not show any therapeutic benefit in ALS patients.

In order to get more insight into human pathophysiology, Dimos *et al.* [161] were the first to generate ALS-patient specific IPS cells using retroviral transduction of the classical Yamanaka factors OSKM. They successfully obtained IPS cells from an 82-year old sibling suffering from a familial form of ALS with a mutation in the SOD1 gene. Subsequently, patient-specific IPS cells were forced to differentiate into MN and glia. Due to the fact that more than 90% of ALS cases are sporadic, patient-specific IPS cell models from sporadic ALS might overcome this drawback through the integration of the genetic as well as environmental individual background.

#### 4.5. Ataxias

The degenerative ataxias are a group of hereditary or idiopathic diseases that are clinically characterized by progressive ataxia resulting from degeneration of cerebellar-brainstem structures and spinal pathways [162].

Autosomal recessive cerebellar ataxias are heterogeneous, complex, disabling inherited neurodegenerative diseases that become manifest usually during childhood and adolescence.

Friedreich Ataxia (FRDA), an autosomal-recessive ataxia, is the most common inherited ataxic disorder in the white Caucasian population with a prevalence of 2-4/100,000 and with an age of onset in the teenage years. Clinical characteristics include progressive ataxia of gait and limbs, dysarthria, muscle weakness, spasticity in the legs, scoliosis, bladder dysfunction, and

loss of position and vibration sense [163;164]. Cardiomyopathy and diabetes mellitus are systemic complications in some patients [165].

FRDA is caused in 96% of individuals by a GAA triplet expansion in the first intron of the Frataxin (*FXN*) gene on chromosome 9q13 [166]. The mutation leads to transcriptional silencing as a result of heterochromatin formation, adoption of an abnormal DNA-RNA hybrid structure, or triplex DNA formation [167] with reduced Frataxin protein expression. About 4% of the individuals affected with FRDA are compound heterozygous. Disease-causing expanded alleles present with 66 to 1700 GAA repeats with the majority ranging from 600 up to 1200 GAA repeats [166;168]. Major neuropathologic findings comprise a degeneration of dorsal root ganglia (DRG), with loss of large sensory neurons, followed by degeneration of posterior columns, corticospinal tracts and spinocerebellar tracts, and the deep nuclei in the cerebellum [165;169].

The gene product Frataxin is a ubiquitously expressed and evolutionary conserved mitochondrial protein that has been proposed to exhibit roles in mitochondrial iron metabolism and the production of iron-sulfur (Fe-S) clusters.

Several FRDA disease models, from yeast, *C. elegans* and *Drosophila* to mice have been used to get more insight into the disease [170;171]. Viable transgenic mouse models were generated through conditional gene targeting [172] which have been crucial in the development as models for FRDA, although some with ambiguous results. The complete knock-out of Frataxin resulted in embryonic lethality [173], whereas conditional mouse models under the control of different promoters were capable to recapitulate some of the disease phenotypes [174]. In order to circumvent the non-physiologic complete loss of Frataxin at a specific time point in conditional models, GAA based mouse models were introduced [175;176], shedding more light on tissue-dependent GAA dynamics and putative pathophysiologic pathways.

Despite a general genotype-phenotype correlation it is not possible to predict the specific clinical outcome in any individual based on GAA repeat length. The inherent variability in FRDA may be caused by genetic background, somatic heterogeneity of the GAA expansion [177;178], and yet other unidentified factors.

Therefore, FRDA-IPS cell lines have already been established by Ku et al. [93] and Liu et al. [94]. Data showed that, although a specific disease-related phenotype was not reported, these FRDA IPS cells were able to recapitulate some of the molecular genetic aspects of FRDA, including the phenomenon of repeat-length instability, epigenetic silencing of the *FXN* locus and low levels of Frataxin expression [93].

With regard to GAA repeat instability, IPS cells showed repeat expansions whereas parental fibroblasts did not [93]. Instability was specific to the abnormally expanded *FXN* as GAA expansions in normal *FXN* alleles or at two unrelated loci with short GAA repeats remained unchanged. To understand the mechanism of instability in this IPS cell system, analysis of differences in mRNA expression showed that MSH2, a critical component of the DNA mismatch repair (MMR) machinery and important for mediating repeat-length instability, was highly expressed in FRDA-IPS cells relative to donor fibroblasts. ShRNA-mediated silencing

of MSH2 resulted in shorter repeat lengths suggesting that FRDA IPS cells could be a useful system to evaluate the mechanisms of repeat expansions and contractions in disease.

GAA repeat mutations are unstable and progressive and postnatal instability occurs in various tissues throughout life. For example, large GAA repeat expansions are especially prominent in the dorsal root ganglia of FRDA patients, which harbor cell bodies of sensory neurons, a neuronal subtype especially affected in FRDA [179].

Given FRDA-IPS cells can be directed to differentiate into sensory neurons, as well as cardiomyocytes [94], the presence and mechanisms of tissue-specific expansion should be testable. The major focus of FRDA IPS cell differentiation research is currently focused on generating appropriate disease-relevant cell types. For example, sensory neurons of the DRG are crucially affected in individuals with FRDA.

The autosomal dominant Spinocerebellar Ataxias (SCAs) comprise a genetically and clinically heterogeneous group of inherited neurodegenerative progressive disorders affecting various parts of the CNS. The number of known SCAs continues to grow and comprises meantime over 30 entities.

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is the most frequent entity among the autosomal dominantly inherited cerebellar ataxias in Europe, Japan, and the United States [180].

Genetically, SCA3 belongs to the group of CAG-triple repeat disorders, also known as PolyQ-disorders due to abnormally long polyglutamine tracts within the corresponding protein. The majority of patients suffering from SCA3 carry one allele of the ataxin3 (*ATXN3*) gene with 60–82 CAG repeats and a second allele containing the normal number of repeats, which is usually between 13 and 41 [181].

As in most of these polyglutamine diseases, patients with a repeat expansion above a critical threshold form neuronal intranuclear inclusion bodies, one important hallmark of polyQ diseases [182]. Further neuropathological features include a depigmentation of the substantia nigra as well as a pronounced atrophy of the cerebellum, pons and medulla oblongata, altogether culminating in an overall reduced brain weight compared to healthy individuals [183].

As most of the PolyQ disease proteins are ubiquitously expressed it still remains unclear why only specific neuronal cell populations are prone to neurodegeneration. Many animal models, like rodents, *C.elegans* and *Drosophila*, overexpressing specific forms of *ATXN3* are available to study the molecular and phenotypic aspects of MJD involving aggregation, proteolysis and toxicity of expanded *ATXN3*, as well as the apparent neuroprotective role of wild-type *ATXN3* [184].

Kakizuka's group was the first to demonstrate neurodegeneration and a neurological phenotype in mice transgenic for the CAG repeat expansion [185]. Mouse models further provided evidence for the subcellular site of pathogenesis, the processing and trafficking of the mutant protein in order to cause cellular dysfunction and neuronal cell loss.

While some of the transgenic mouse models expressing the full-length ATXN3 under control of various exogenous promoters were able to mimic some aspects of the disease, they all overexpress only a single isoform of ATXN3. Taken this into account, a YAC MJD transgenic model was established which more closely recapitulates the human disease as all elements, including regulatory regions of the gene, are present [186]. Research in animal models of SCA has now begun to focus on therapeutic strategies to prevent protein misfolding and aggregation in polyglutamine diseases by overexpressing chaperones.

Koch *et al* [95], investigated the formation of early aggregates and their behavior in time by making use of patient-specific IPS cell-derived neurons. They demonstrated that MJD-IPS cell derived neurons constitute an appropriate cellular model in the study of aberrant human protein processing. Moreover, they concluded that neurons are able to cope, at least in the beginning, with the aggregated mutant material and cytotoxicity evolved over time. Besides, a key role for the protease calpain in ATXN3-aggregation formation was found which could further display a putative benefit of calpain inhibitors.

#### 4.6. Familial dysautonomia and fragile X syndrome

Familial Dysautonomia (FD), also known as Riley-Day Syndrome or Hereditary Sensory Autonomic Neuropathy (HSAN) Type III, is a rare autosomal recessive disease mostly occurring in persons of Ashkenazi Jewish descent [187]. The disease is characterized by degeneration of sensory and autonomic neurons, leading to severe and often lethal central and peripheral autonomic perturbations, as well as small-fiber sensory dysfunction. The underlying mutation induces a splicing defect in the I $\kappa$ B kinase complex-associated protein (*IKBKAP*) gene, which results in tissue-specific loss of function or reduced levels of the IKAP protein [188]. Individuals affected with FD suffer from incomplete neuronal development as well as progressive neuronal degeneration with the sensory and autonomic neurons mainly affected [189].

Although the exact function of the IKAP protein is not clearly understood, researchers have identified IKAP as the scaffold protein required for the assembly of a holo-elongator complex [190]. As a consequence, an impaired transcriptional elongation of genes responsible for cell motility is thought to be the cause for the observed cell migration deficiency in FD neurons [191]. Besides, the IKAP protein is also thought to be involved in other cellular processes, including tRNA and epigenetic modifications and exocytosis [192].

To better understand the function of IKAP, Dietrich *et al.* [193] created a mouse model with two distinct alleles that result in either loss of *Ikbkap* expression, or expression of the mutated truncated protein. Besides, a humanized IKBKAP transgenic mouse model for FD had been created that recapitulated the tissue-specific splicing defect, i.e. skipping of exon 20, in nervous tissues [194].

In order to untangle the tissue-specific pattern of IKBKAP mRNA splicing in FD, Boone *et al.* [195] created a human olfactory ecto-mesenchymal stem cell (hOEMSC) model of FD. It has been shown that these multipotent hOE-MSCs exhibit the potential to differentiate *in vitro* into neurons, astrocytes, and oligodendrocytes as well as other cell types [196]. Classical features



of the FD phenotype, like the expression of the mutant IKBKAP transcript, notably lower IKBKAP levels as well as an impaired migration, were observed. Besides, drug testing experiments with kinetin, which had been shown effective in previous studies [197], had the potential to correct the splicing in a dose-dependent manner in FD hOE-MSCs.

Furthermore, IPS cells were generated from a patient with FD using the classical Yamanaka factors and subsequently differentiated into neural crest derivatives [96]. This was one of the earliest reports of a phenotype for a neurological disease to be modeled with IPS cells. FD-IPS cell derived neural precursors showed particularly low levels of IKBKAP, mis-splicing of IKBKAP, and defects in neurogenic differentiation and migration behavior. Again, the plant hormone kinetin was tested as a candidate and showed a reduction of mutant IKBKAP splice forms, an improvement in neuronal differentiation, but not in cell migration.

Fragile-X (FX) syndrome belongs to the autism spectrum disorders, and is the most common cause of inherited mental retardation with a prevalence of 1/3600 [198]. In the vast majority of cases, the disease is caused by a silencing of the *FMR1* gene due to a CGG repeat expansion (>200 repeats) in the 5'-UTR of the *FMR1* gene [199]. The *FMR1* gene codes for the cytoplasmic protein FMRP, which has RNA-binding properties and is thought to play a role in synaptic plasticity and dendrite maturation. This could be demonstrated in histopathological studies of FX where dendritic spine abnormalities were found [200].

Several animal models revealed important insights into the role of the FMR protein. A *Drosophila* model showed a role of FRMP in the regulation of the microtubule network [201].

The first *fmr1* KO mouse model was generated shortly after the discovery of the disease-causing gene and showed classical clinical features of FXS like macroorchidism, learning deficits, and hyperactivity [202].

Although current mouse models for FX syndrome are useful for studying the clinical phenotype, they do not recapitulate the hallmark, i.e. silencing of the *FMR1* gene due to the triplet repeat expansion [203]. Loss of function studies using morpholino antisense oligonucleotides in zebrafish revealed a function of FMRP in terms of normal axonal branching.

Primary and transformed cell cultures obtained with an unmethylated full mutation in the *FMR1* showed that the CGG expansion per se does not block transcription [204]. In undifferentiated human FX embryonic stem cells (FX-ES cells) derived from affected blastocyst-stage embryos, *FMR1* is expressed and gene silencing occurs only upon differentiation [205] indicating a developmentally dependent process.

Recently, Urbach et al. [97] generated FX-IPS cell lines from three patients. In contrast to FX-ES cells, FX-IPS cells presented with a transcriptionally silent *FMR1* gene, both in the pluripotent and differentiated states. This was reflected by corresponding epigenetic heterochromatin modifications in the gene promoter. IPS cells were further differentiated into neural derivatives and different potential epigenetic modifiers were tested. Amongst those, 5-azacytidin showed an upregulation of *FMR1* transcripts both in pluripotent as well as neuronal FX-cells.

## 5. Conclusions and perspectives

In this chapter we have described the first successful attempts to harness the IPS technology for the generation of models for neurodegenerative diseases of the human nervous system. The key advantage of IPS based models over animal models is that they offer researchers for the first time a realistic chance to work in cell culture with large numbers of primary human cells that closely resemble the postmitotic neurons affected by neurodegeneration.

The first studies in which patient-derived disease-susceptible cellular phenotypes were compared with those of cells derived from healthy individuals, provide strong indications that such cellular models reflect key pathological molecular and cellular aspects of the neurological diseases. Therefore a future concept for patient-derived cellular models will be to correct neuronal malfunctions diseases by *in vitro* treatment of affected cells. A first such attempts aspect has been in the SMA models [91].

These *in vitro* treatments will include hypothesis driven approaches based on knowledge about pathophysiological mechanisms. Equally important patient derived lines will be used as *in vitro* assays for the screening of compound libraries. Drug safety screens with IPS cell-derived neurons will help to reduce the animal dependency of the current drug development pipeline. Finally, IPS cell technology will be an important driver of personalized medicine. Prior to patient treatment drug types and doses can be tested on patient-derived IPS cells or differentiated progenies in order to tailor a personalized curative approach according to the individual genetic and cellular profile.

There is even hope that the novel approach bypasses the laborious, time-consuming and expensive IPS cell generation by direct reprogramming of mouse and human somatic cells into functional neurons, called induced neurons (INs) [206;207], will come to fruition. Several groups have already generated dopaminergic INs [208;209] and motor INs [210]. Patient-specific INs could be generated to enhance the study of developmental disorders and other neurological diseases [211]. The significant decrease in time and resources to derive neurons directly from somatic cells justifies further investigation into this strategy.

But despite the enormous potential of IPS cell derived neurons for studies involving cell biological, physiological and pharmacological methods important question remain to be solved. One major drawback is that we still know very little about the specific cell biology of IPS cells and even less of their neuronal derivatives. This includes for example changes in chromatin structure and epigenetic signatures that accompany the reprogramming process. And there is exceedingly little information about membrane physiology of the IPS cell-derived neurons. Electrophysiological recordings and parallel studies of synaptic proteins and ion specific channel composition should be a focus of future research.

We have already pointed out the difficulties to design specific differentiation protocols for specific neuronal populations from IPS cells. The underlying hypothesis for all existing protocols is that cells should be guided through a shortcut version of embryonic development. A hindrance for progress in this regard is the lack of specific information of human embryonic development since most of our knowledge about vertebrate brain development derives from



work with rodents. Recent reports about surprising differences between rodent and human developmental processes emphasize the demand for further comparative studies of human and rodent brain development [36;44].

The biggest limitation of IPS cell models is that they do not offer straightforward possibilities to study functions of neurons *in vivo*, as parts of the brain circuitries that regulate higher brain functions and organismic behavior. Obviously, cellular models alone will never be able to produce clinically important read-outs, such as memory dysfunction and behavioral changes in AD, tremor, bradykinesia, and rigidity in PD, or reduced forced vital capacity, swallowing dysfunction, dysarthria, or limb motor impairment in ALS. Therefore, in the foreseeable future research on neurodegenerative diseases will combine *in vitro* and *in vivo* approaches. *In vivo* transplantation of stem cell derivatives in relevant animal models could bring additional information regarding the potential of hIPS cells for *in vivo* differentiation and their survival in a pathological brain environment. This is first exemplified in a study of directed differentiation of IPS cells to midbrain neurons and their transplantation into a rat model of PD, which led to functional recovery [64].

This result and many others that we summarized in this chapter raise hopes that IPS cells derived from affected and healthy human individuals will provide a unique opportunity to gain insights into the human pathophysiology and pharmacologic responses in yet incurable neurodegenerative diseases.

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