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Review Article

Current status of treating neurodegenerative disease with induced pluripotent stem cells

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Degenerative diseases of the brain have proven challenging to treat, let alone cure. One of the treatment options is the use of stem cell therapy, which has been under investigation for several years. However, treatment with stem cells comes with a number of drawbacks, for instance the source of these cells. Currently, a number of options are tested to produce stem cells, although the main issues of quantity and ethics remain for most of them. Over recent years, the potential of induced pluripotent stem cells (iPSCs) has been widely investigated and these cells seem promising for production of numerous different tissues both *in vitro* and *in vivo*. One of the major advantages of iPSCs is that they can be made autologous and can provide a sufficient quantity of cells by culturing, making the use of other stem cell sources unnecessary. As the first descriptions of iPSC production with the transcription factors Sox2, Klf4, Oct4 and C-Myc, called the Yamanaka factors, a variety of methods has been developed to convert somatic cells from all germ layers to pluripotent stem cells. Improvement of these methods is necessary to increase the efficiency of reprogramming, the quality of pluripotency and the safety of these cells before use in human trials. This review focusses on the current accomplishments and remaining challenges in the production and use of iPSCs for treatment of neurodegenerative diseases of the brain such as Alzheimer's disease and Parkinson's disease.

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Introduction to neurodegenerative diseases

The central nervous system is one of the most fragile elements of the body and has a limited capacity to regenerate from both acute injuries, such as stroke and spinal cord injury, or degenerative diseases. Neurodegenerative diseases (NDs) are characterized by progressive loss of neuronal subtypes in the brain and spinal cord and can occur either sporadic or familial. The best known examples of neuron degeneration of function or structure are Parkinson's disease (PD) and Alzheimer's disease (AD). The onset of these types of ND is typically mid- to late life, which means that with an increasing life expectancy of the population the incidence of these diseases will increase. Current therapies are predominantly focused on alleviating symptoms, and there is a need for the development of new therapeutic interventions. Intensive research has increased the knowledge about the pathophysiology of separate diseases and has also indirectly visualized the overlap between them. The most evident parallels between the different NDs are seen in the atypical protein formation and induction of cell death (1). The pathways of synthesis and degradation of these toxic proteins have to be investigated in order to comprehend the complex management

of their steady state levels. For example, it has been shown that the ubiquitin-proteasome and macro-autophagy contribute to degradation of these proteins, implicating that dysfunction of these pathways could be involved in the pathology of NDs. A deeper understanding of these pathways is not only required to develop novel drugs and therapies, but is also required for development of potential cures that reverse the loss of tissue in the brain.

Alzheimer's disease

Of the 24 million individuals that suffer from dementia worldwide, 60% are affected by AD (2). AD is clinically characterized by a slowly progressive decline in learning and memory abilities, caused by gross cerebral atrophy, indicating the loss of neurons. This atrophy is predominantly found in the frontal and temporal lobes, including the hippocampus, and is caused by formation of extracellular plaques of neuritic amyloid-β and intracellular tau protein aggregates, called neurofibrillary tangles (3) (Fig. 1). The etiology of AD remains unclear, although there is strong evidence for involvement of the e4 variant of the gene *APOE*. This variant has been found to increase the risk for AD and lower the age of onset as it is seen in familial AD. ApoE4 is involved in carrying the amyloid-β protein and could therefore be related to the accumulation of amyloid plaques. In addition, the failure of ApoE4 to bind tau protein could be a cause of decreased phosphorylation of the protein, which results in aggregation in neurofibrillary tangles. In contrast, the ApoE2 variant, encoded by the *APOE* e2 allele, has been found to provide slight protection against sporadic AD. Currently, treatments are purely symptomatic as therapeutic interventions are focused on the cognitive impairment as a result of AD. These are targeted to replace or modulate a number of neurotransmitters and enzymes, examples of which are acetylcholinesterase inhibitors (4), cholinesterase inhibitors (5), antioxidants (6), amyloid-β targeting drugs, nerve growth factors, c-secretase inhibitors (7), and vaccines against amyloid-β (8). Unfortunately, these treatments have not been successful in curing AD, nor in attenuating progression of the disease.

Parkinson's disease

An estimated 1% of the entire population is affected by PD. The main characteristic of PD is degeneration of nigrostriatal dopaminergic neurons in the midbrain. In general, at the time, PD shows the first significant symptoms, up to 80% of the dopaminergic neurons in this region have already been damaged permanently. As a result, the dopamine (DA) levels in the brain are reduced, which leads to typical motor symptoms like bradykinesia, rigidity and a resting tremor (9). The neurotransmitter DA is involved in transmission of electrical signals to facilitate physical motion, and thus, dysregulation of DA levels results in the typical abnormal movements. As for AD, the etiology of PD remains elusive, but there is strong evidence for a pathophysiology involving multiple factors in a cascade of deleterious events (Fig. 2). It is, however, known that the protein aggregates in the form of Lewy bodies are included in the cytoplasm of the cells and that pigmented DA-containing neurons are depleted in the substantia nigra. For the last 30 years, the symptoms of PD have been predominantly treated with levodopa, which is a precursor of DA, or DA agonists. These drugs compensate for the loss of DA in the patient, but come with side effects as well, mainly expressing in the development of motor complications and dopaminergic adverse effects, respectively. Moreover, these symptomatic therapies do not slow down the progression rate of the disease, and therefore, new treatments are continuously being investigated to target the cause of disease rather than fighting the symptoms (10).

Treatment of neurological disease with iPS cells

Increasing effort is put in the improvement of stem cell transplantation therapies to revert the damage that is done by diseases such as AD and PD. In other, closely related fields like spinal cord injury, important progress has been made using stem cells to treat patients. Tabakow and Jarmundowicz (11) describe how they collected autologous mucosal olfactory ensheathing cells and olfactory nerve fibroblasts from patients with a complete spinal cord injury and were able to improve their neurological functions without severe adverse effects. Diffusion tensor imaging showed that the white matter tracts in the spinal cord regained continuity, which was in line with the improvement of the transmission and activity of the muscles in the lower extremities. The use of patient-specific stem cells therefore holds the potential for new treatments in the spectrum of neurological diseases.

However, the source of stem cells is a longstanding challenge, together with other drawbacks such as ethical questions about the use of

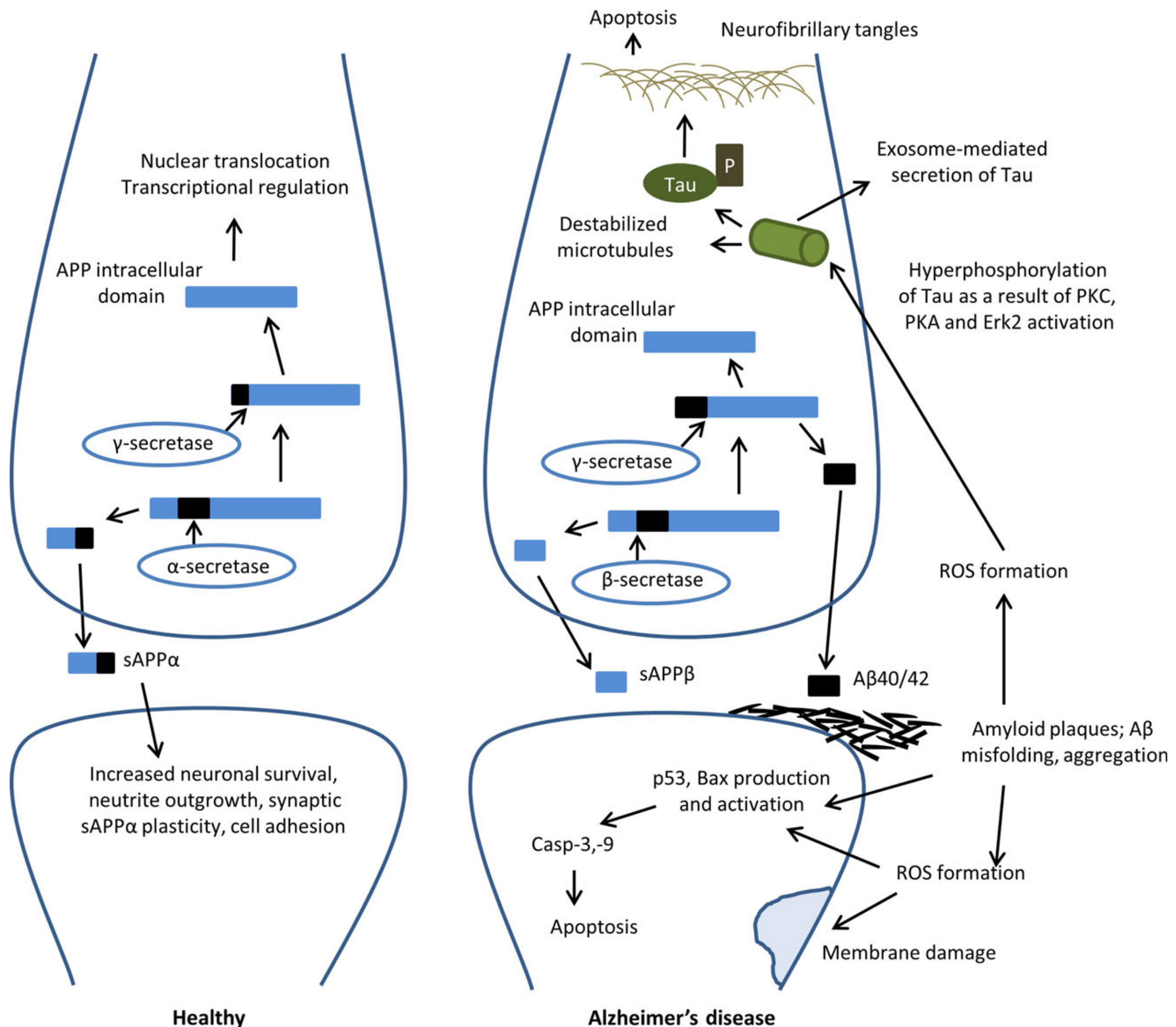

Figure 1. Processing of amyloid-β in healthy neurons and in case of Alzheimer's disease. The APP intracellular domain translocates to the nucleus and is responsible for transcriptional regulation. sAPPα is responsible for increased neural survival, neurite outgrowth, synaptic sAPPα plasticity and cell adhesion. sAPPβ is cleaved to N-APP, which binds DR6 to activate apoptosis. In healthy cells, this results in normal axon pruning, while it causes aberrant neuronal death in diseased cells.

embryonic stem cells (ESCs) and histocompatibility of the patient. Recent developments in stem cell research have shown that it is possible to retrieve the pluripotent state in somatic cells by expressing specific reprogramming factors. These induced pluripotent stem cells (iPSCs) can subsequently be directed to differentiation into neurons *in vitro* and potentially provide an autologous and easily accessible source of neurons for cell transplantation. Currently, the methods used to generate iPSC lines are being improved, although the limited understanding of the underlying process of reprogramming is still in the way of using iPSCs widely in the clinic. This work reviews iPSCs as a potential therapeutic intervention in patients with sporadic NDs. It will cover the current status of methods used to generate iPSCs and their resemblance to ESCs, as well as subsequent differentiation into neurons and how this could benefit patients with AD or PD.

Induced pluripotent stem cells

Pluripotency by defined factors

In 2006, Takahashi and Yamanaka published the first accomplishments in the process of producing ESC-like cells using defined factors (12). Their approach was based on the fact that somatic cells can be reprogrammed either by nuclear transfer into oocytes or by fusion with ESCs. This suggests that oocytes and ES cells contain factors that

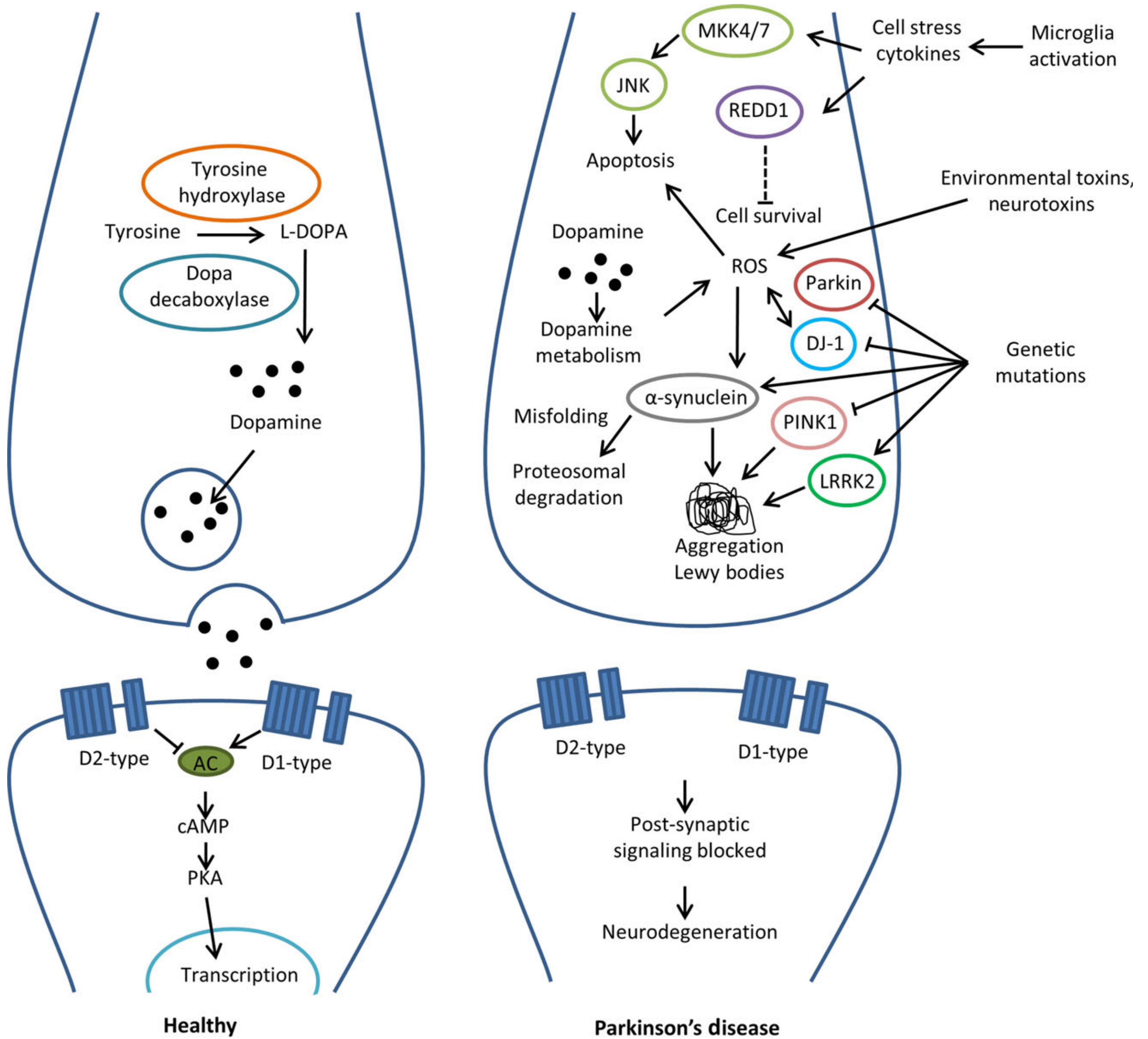

Figure 2. The dopamine pathway in healthy cells and in the case of Parkinson's disease. Normally, L-DOPA is converted into dopamine, which will activate or inhibit downstream effect trough binding of D1- and D2-type receptors. In Parkinson's disease patients, a number of factors can affect dopamine metabolism and apoptosis, leading to a loss of post-synaptic signaling and thus cell death. Misfolding of α-synuclein protein plays a central role in this disease, because of the resulting Lewy Bodies.

induce reprogramming in the somatic cell. By identifying these factors, they hypothesized, it should be possible to reprogram the epigenetic state of a differentiated cell and induce pluripotency without using embryos or oocytes. In case pluripotency is maintained over a longer period, ESCs express specific transcription factors (Oct3/4, Sox2) as well as certain genes that are related to tumorigenesis (Stat3, c-Myc, b-catenin). In their trial, Takahashi and Yamanaka screened different combinations of reprogramming agents from a pool of 24 preselected transcription factors in embryonic fibroblasts from mice (MEFs). Upon successful induction of pluripotency, the cells expressed marker genes for ESCs and early embryos (e.g. *Fbx15* and *Nanog*). The most elementary method to produce ESC-like cells was by retroviral delivery of four genes in fibroblasts and these were called iPSCs (Fig. 3). The induced pluripotency is obtained by overexpression of a specific combination of transcription factors, now known as the Yamanaka factors: Oct4, Sox2, Klf4 and c-Myc. In the process of creating iPSCs, adult somatic cells were collected and infected with a retrovirus containing the reprogramming factors. The culture conditions are similar to that of ESCs and after an average of 2–3 weeks the iPSCs can be obtained by selecting for *Fbx15* expression.

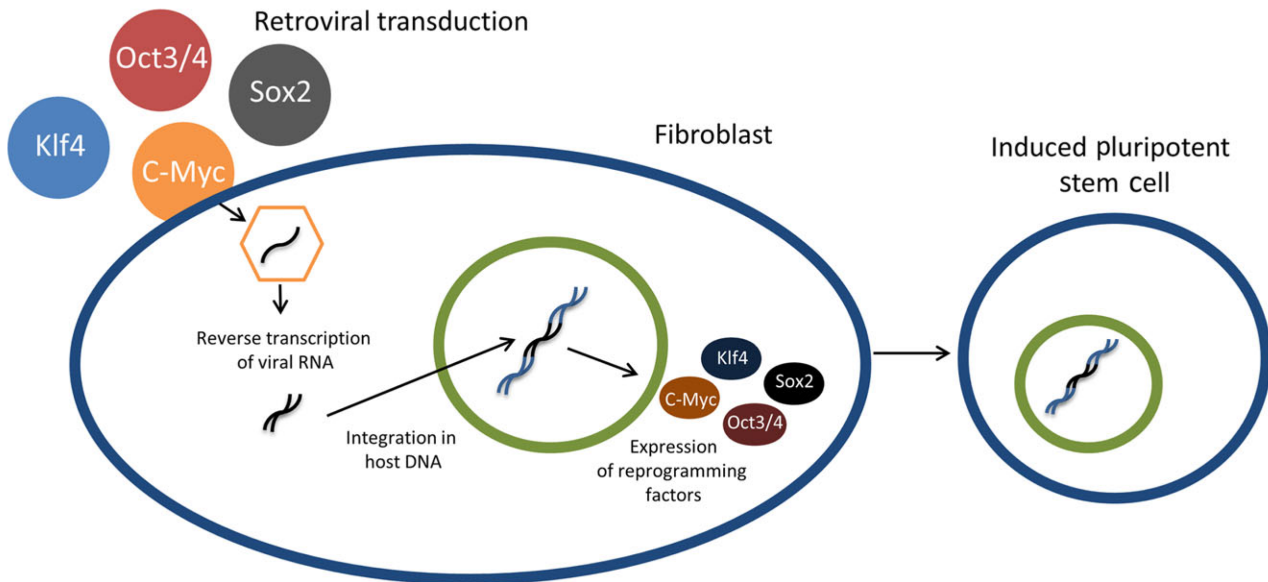

Figure 3. Direct reprogramming by introducing a selection of transcription factors into the somatic cell through viral vector transduction. After integration in the host DNA, the factors will be expressed and the cell reprogrammed to the pluripotent state.

In additional experiments, this specific combination of transcription factors was also found to reprogram cells of different species, including human (13). The studies indicated that adult mouse tail fibroblasts and adult human fibroblasts can be induced to a pluripotent state. iPSCs from mouse fibroblasts could not be distinguished from mouse ESCs in means of morphology, proliferation and teratoma formation. Oct4 and Sox2 are a requisite for direct reprogramming, but combining these two with either Klf4 and c-Myc or Nanog and Lin28 is both possible (14). Other studies have found that pluripotency can be reached using less than four factors, even down to the sole use of Oct4 in case the other factors are endogenously expressed by the converted cells (15).

iPSC vs ESC

The inner cell mass of mammalian blastocysts provides ESCs that are able to grow indefinitely and at the same time maintain their pluripotency. Because of these features, they are potentially useful in the treatment of numerous degenerative diseases, such as AD, diabetes and spinal cord injury. However, the use of human ESCs in the clinic is problematic because of the ethical issues that are involved in the use of human embryos. Additionally, application of foreign cells in patients comes with the risk of tissue rejection after implantation. These issues are circumvented when pluripotent cells are generated directly from the patient's own somatic cells using the Yamanaka factors. With an easily accessible and autonomous source of cells, the options are then open for regenerative medicine, but also for disease modeling and to screen potential new drugs. However, an important question remains how equal the properties of ESCs and iPSCs are. Additionally, it is essential to address a number of safety issues before a clinical trial can be initiated. Here, a number of the epigenomic, transcriptional and genomic states of iPSCs will be compared to those of ESC and their somatic cell parent.

Basic properties – Inducing the 'ground state' of pluripotency in cells by erasing the epigenetic mark of differentiation is thought to create the optimal starting point for subsequent differentiation, as seen in ESCs. Mouse iPSCs however, appear not to be as successful in developing into live mice as ESCs (16). Chances increase by selection of high quality iPSCs colonies. These will have to be selected, because some highly proliferative colonies will not become pluripotent. Initial selection is therefore based on the morphological similarities to ESCs, such as the small size of the cells and growth in tight colonies (17). Other important basic properties of true iPSCs are proliferation rate, the expression of endogenous genes of pluripotency, as well as the ability of cells to develop into teratomas. When using the retroviral method, an obvious difference with ESCs is the integration of reprogramming genes,

which are randomly distributed in the genome and could possibly disturb other gene functions. On the other hand, one of the characteristics of successful reprogramming is the silencing of these transgenes as the endogenous genes take over. This state is also indicated by the ability of the pluripotent cells to contribute to an embryo when injected into a blastocyst (18).

The ultimate test of pluripotency will be to produce a complete animal through tetraploid complementation, when cells with two different sets of chromosomes are combined to form one embryo (Fig. 4). This technique shows whether the iPSC are truly pluripotent and if its state is nearly identical to ESCs (19). Unfortunately, thorough testing of many cell lines for true pluripotency is problematic. Testing human cells for this feature is ethically impossible and thus quality standards will become lower, while the heterogeneity between different iPSC lines increases. Merely the different culturing conditions between laboratories are enough to introduce variety between the same lines of cells (20). Apart from these relative differences, there is a general problem with human pluripotent cell lines because of their similarities to mouse stem cells from post-implantation embryo epiblasts. This kind of cell is one step further in development and therefore represents a slightly more differentiated stem cell, which also explains their poor ability to generate chimeras and expression of markers that indicate commitment to early lineage (21, 22). Because of these similar features in human iPSC, the question remains whether these features are the result of species differences between human and mice, or that the optimal isolation and culture conditions for human ESCs has not yet been met.

In vitro *differentiation and epigenetics* – Another method to test the human iPSC on their true pluripotency is to induce differentiation *in vitro*. This will allow screening for functionality, which is the essential property for clinical use in the future. Numerous different cell lines have already been created by differentiating iPSC cells, although comparing different iPSC lines on their differentiating potential is problematic due to the large differences in the protocols used in the individual studies. In addition, scoring differentiation can be based on either the efficiency, by measuring differentiation markers or quantity of cells, or the identity of the differentiated cells, in which a large number of cell-specific functions is tested. The comparisons that have been performed between iPSCs and ESCs show either an equal to decreased ability to differentiate of the iPSC, with especially a lowered efficiency in the turnover into differentiated cells. This efficiency difference, however, does not seem to influence the quality of the final cells (23). In these analyses, it also has to be taken into account that *in vitro* culture influences the cell state.

Due to the fluctuation in differentiation, tests have been developed to screen iPSCs on DNA methylation, the transcriptome and spontaneous differentiation potential *in vitro* (24). The scorecards of these analyses indicate the potential of different pluripotent cell lines to differentiate to a certain lineage. Analysis of DNA methylation patterns is a good qualification for iPSC lines because this epigenetic pattern is important for the pluripotency of cells. Genomewide DNA methylation maps are created using high-throughput sequencing, showing large similarity between iPSCs and ESCs in comparison to non-pluripotent cells lines. The majority of epigenetic differ-

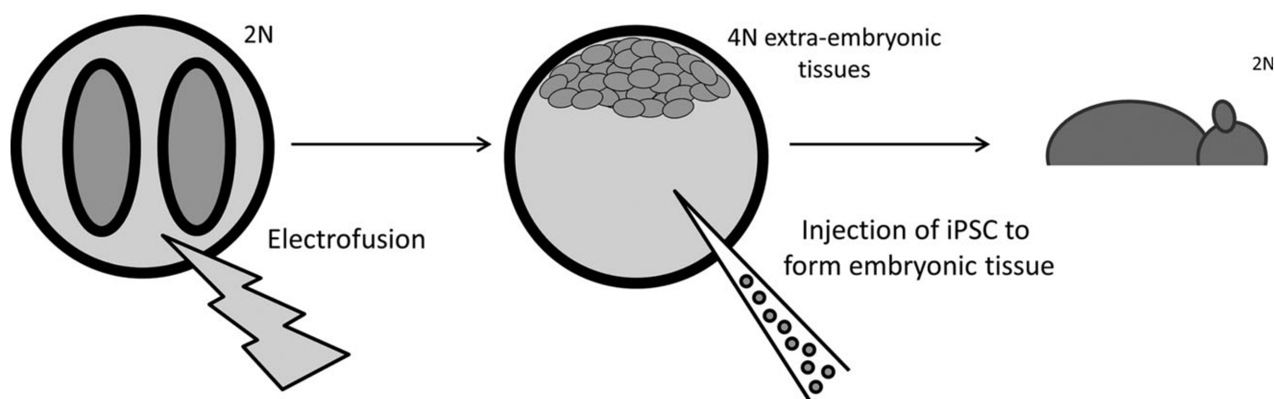

Figure 4. Tetraploid complementation; electrofusion of a two-cell embryo, which becomes 4N, and subsequent injection of the iPS cells, which are 2N. Tetraploid cells will not contribute to the embryo, and thus, the organism will develop completely from IPS cells.

ences showed to be the result of incomplete epigenetic reprogramming of the original epigenome (25). Differential methylation in other regions indicates the acquisition of novel patterns that do not belong to the ESC-like state (26). The ESC-specific non-CpG methylation is found to be regained by iPSCs during reprogramming. Besides DNA methylation, histone modification is an important epigenetic effector on transcription in cells. These markers show a specific, highly similar pattern in both iPSCs and ESCs. As for DNA methylation, the histone modifications showed subtle differences between individual cell lines, for instance in the number of unique regions with the relatively uncommon H3K9Me3 modification in iPSCs which are not seen in ESCs (27).

The next question is whether there is an epigenetic memory of the cell of origin that influences the quality of an iPSC line. Different studies investigated the success of silencing donor-cell genes and activation of genes related to pluripotency, which resulted in the consensus that iPSCs indeed possess a memory from the original cell type (28, 29). Especially iPSC lines with low passage numbers are possibly not entirely reprogrammed. Higher passage numbers appear to lose the donor-cell-specific transcriptome (30).

Genomic mutations – Besides epigenetic changes, reprogramming and *in vitro* expansion events can introduce genomic mutations as well. Mutations providing an advantage during change of cell fate can be carried on in the subsequent proliferation and adaptation. However, this phenomenon is also found in ESCs and many other cell lines. Prolonged culturing of iPSC will introduce similar mutations as seen in tumor development, although the same pattern is seen in ESCs and all immortalized cell lines.

Challenges of Yamanaka's approach

The induction of pluripotency by expression of transcription factors revealed a surprising plasticity of mammalian cells. However, there are also some limitations that need to be addressed. To date, a big challenge has been to increase the throughput the reprogramming process, which generally is very low. Another disadvantage is the retroviral integration that is used in this method, due to the fact that this process comes with the risk of introducing mutations in the genome of the target cells (31). Moreover, the increased risk for tumor development in iPSCs has been described widely and is correlated to increasing efficiency of reprogramming. Apart from the known function of proto-oncogene c-Myc, Oct4 is possibly involved in dedifferentiation toward cancer stem cells (32). Sox2 is suggested to influence the expression of tumor-specific genes in cancer stem cells (33), and there is evidence for oncogene function of Klf4 in certain types of cancer as well (34).

Alternative and improved reprogramming

Alternative transcription factors – After the first descriptions of iPSCs, several other groups investigated the use of alternative reprogramming factors. Yu and Vodyanik (14) successfully replaced Klf4 and c-Myc with the transcription factor Nanog and the mRNA binding factor Lin28 to induce a pluripotent state in human somatic cells. In other studies, the transcription factor Glis family zinc finger 1 (Glis1) has been used as a replacement of c-Myc. Glis1 is a factor found in embryos during the one cell stage. This factor is able to increase the efficiency of reprogramming even more than c-Myc, but with a lower risk of tumorigenesis in the iPSCs (35). On the other hand, Glis1 expression has to be controlled very carefully during reprogramming due to its inhibition of proliferation after pluripotency is reached, leading to death of the iPSC colonies (36). It has also been shown that arresting the primary cells by serum starvation and subsequent release to continue through the G2/M phase synchronously, improves the efficiency of retroviral integration. The success of retrovirus mediated programming can thus be improved by starving cells from serum and thereby aligning the cell cycle rhythms (37).

Alternative vectors – The risk that is involved with insertion of transcription factors into the host genome by retroviral transduction can be avoided by the use of alternative vectors, such as adenoviruses or plasmids. The first adenovirus iPSC lines were produced by Stadtfeld and Nagaya (38) and showed that fibroblasts and liver cells from mice could be reprogrammed using non-integrating adenoviruses to express the four Yamanaka factors. Alternatively, plasmids can be used to express the transcription factors, as was shown by Okita and Nakagawa (39). These cells were less likely to obtain harmful genomic mutations as a result of vector insertion, although the continued use of proto-oncogene c-Myc was of influence on the development of tumors as well. However, when the pluripotency genes are not inserted in the target cell genome, the throughput

of pluripotent cells is significantly lower. Recently, vectors based on oriP/EBNA-1 have been used to generate pluripotent cells from fibroblasts and B-Lymphocytes. The advantage is that these vectors do not integrate, but provide enough stability to the expression of the Yamanaka factors to allow the endogenous factors to be upregulated without vector integration. This leaves the host genome free from integrating events (40, 41).

Drug-like chemicals, RNA and small compounds – A number of different drug-like chemicals have shown to significantly increase the rate of iPSC formation when using the standard retroviral induction method. For example, the TGF-beta receptor type-1 (ALK5) inhibitor SB341412 and mitogen-activated protein kinase (MEK) inhibitor PD0325901 (42). Additionally, RNA molecules have shown potential to aid in reprogramming of cells but are not able to fully complete reprogramming. The use of miRNA, such as miR-291, miR-294 and miR-295 can enhance the throughput of iPSCs because of their effect downstream of c-Myc (43, 44). Another method to solve the problems encountered with viral integration of DNA or the low efficiency in plasmid transfection is to induce pluripotency with small-molecule compounds. These molecules are able to exert an effect through mimicking the function of transcription factors and there is no need for genomic integration. In 2008, the first studies indicated that the effect of c-Myc could be adopted by valproic acid, a histone deacetylase inhibitor (45). In addition, Shi and Desponts (46) showed that use of BIX-01294, to inhibit histone methyl transferase and simultaneously activating calcium channels located in the plasma membrane, increased the efficiency of iPSC production. More recently, it was shown that a combination of seven different small molecules, one of which was DZNep that is known for the catalyzing effect on late reprogramming stages, was able to reprogram mouse somatic cells with an efficiency that was comparable to the standard methods of inducing pluripotency (47). These chemically induced pluripotent stem cells (CiPSCs) shared all characteristics with other iPSC lines and were able to be transferred via the germline. The production of human CiPSCs with this technique is expected to be a matter of fine-tuning.

Use of iPSCs in treatment of ND

The number of stem cells in the adult brain is restricted and reserved for specific areas. Additionally, the contribution of these cells to functional recovery is very low. In recent years, the use of stem cells to regenerate brain function has received increasing interest for treatment of a wide variety of diseases and injuries (Fig. 5). The principle is already proven in animals, where stem cells of different sources were able to replace neural cells that were targeted (48, 49). This has been shown to regenerate the function that was lost upon spinal cord injury or PD. However, trials that have been performed with transplantation of neural stem cells in humans have shown to be a

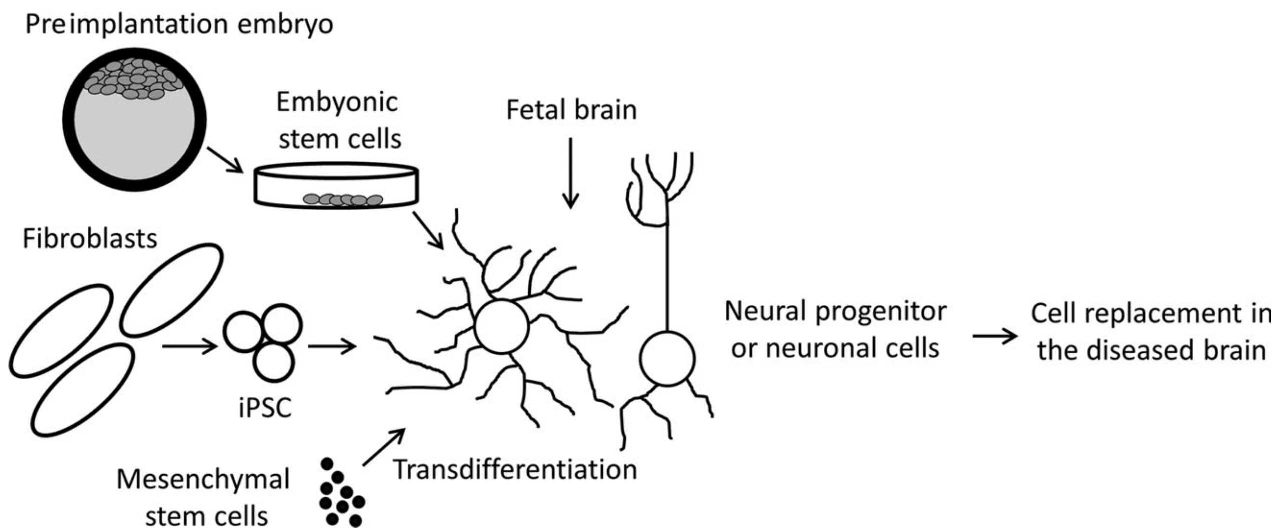

Figure 5. The origin of different cell types, which have been of interest for cell therapy in neurodegenerative diseases. It is possible to obtain fetal brain cells from aborted embryos, hESC can be obtained from the inner cell mass of the blastocyst, iPSC are often derived from fibroblasts and mesenchymal stem cells are obtained from cord blood or bone marrow. After the cells have (trans)differentiated into neural progenitors or neuronal cells they can be transplanted into the diseased brain.

greater challenge and yield strongly varying results. In some cases a significant improvement was reported, while other experiments showed a further decrease of cell function and different severe side effects (50). Moreover, neural stem cells are difficult to obtain and upon transplantation, problems can arise with histocompatibility between the donor and the patient. To circumvent these issues, it is now investigated whether iPSC lines could be derived from the patient's own cells and provide in the need for autologous neurons to treat ND.

Selecting the somatic cell

After the description of iPSCs derived from fibroblast cells, many other somatic cell populations have been converted into pluripotent cells using the same technique. Among these are keratinocytes (51), pancreatic b cells (52), neural cells (53), mature B and T cells (54), melanocytes (55), hepatocytes (56), amniotic cells (57), dental pulp stem cells (58), hair follicles (59) and cells derived from adipose tissue (60). Although the use of different target cells generally provides iPSC lines with highly similar characteristics, it has also been found that the source of the induced cells leaves a certain epigenetic memory which provides an advantage to differentiate into cell lineages more related to that of the initial cell type. Small differences like these might form a disadvantage for differentiation to other tissues by decreasing the efficiency or timing. The subsequent differentiation might also be influenced by these markers, implicating that using target cells of the same germ layer could be favorable over those of other layers. In the case of neurons, an ectodermal cell line might therefore be favorable over others. Moreover, the choice of somatic cells is found to influence the degree of tumor formation of the different iPSC lines. Tail-tip fibroblasts from an adult mouse generated a higher amount of teratomas than embryonic fibroblasts or stomach tissues (61). Additionally, Aasen and Raya (51) showed that using the classical reprogramming method in human keratinocyte cells was approximately a 100-fold more efficient and twice as fast as conversion of fibroblasts, while they possessed the same main characteristics of iPSCs. The increased efficiency could be due to the higher similarity between keratinocytes and human ESCs, seen from the levels of stem cell-related genes and higher endogenous expression of Klf4 and c-Myc. Still, the majority of iPSC lines are derived from fibroblasts from the adult mouse or human. These cells have proven to work in many different independent studies, and they are easy to obtain and culture compared to many other cell types. For these reasons, also the development of neural cells is generally performed on fibroblast iPSC lines.

Inducing pluripotency and differentiation to neurons

A number of different methods have been used to induce pluripotency in somatic cells with the intention of generating neurons. Over recent years, the focus has been predominantly put on improving the reprogramming techniques and thereby also improving the safety and quality of iPSCs. One of the promising developments has been successful generation of iPSC lines using the PiggyBac (PB) transposon method in mice, which were differentiated into neuronal stem cells (NSCs). Salewski and Buttigieg (62) describe the use of the PB transposon to generate iPSCs, culturing these to neurospheres and subsequently differentiating them to neural precursors. This method showed efficient induction of the pluripotent state and ability to excise the transgenes when the iPSCs were stable. Subsequent differentiation into a restricted neural precursor carries the potential to provide a safe source of cells for regenerative medicine, in case this technique can be transferred to human cells as well. The iPSC lines obtained from patients could additionally be genetically corrected for mutations which cause NDs. This was used, for example, to create a disease model of TAUopathy, in which a mutation in the *TAU* gene was shown to cause proteolysis of TAU protein and axonal degeneration, predominantly affecting the dopaminergic neurons.

After successful reprogramming, the next challenge is to generate a population of a specific cell type that is functional and highly pure. This process is quite challenging, because differentiation of iPSCs generally yields a heterogeneous population that will interfere in estimating the quantitative and qualitative outcome of the experiments. One of the methods that is used, depends on differential expression of cell surface markers of populations such as NSCs, glia, and neurons (63). Neural differentiation has already been refined in ESCs, and starts with the 'neutralization', where either the embryonic bodies from ESCs are cultured with retinoic acid (64), ESCs are cultured with stromal cells (65) or a monolayer of ESCs is cultured free of serum or feeder cells without the inhibitory signal from bone morphogenetic protein (66). Subsequently, a specific neuron subtype is generated in either neurospheres or monolayers of NSCs by addition or

inhibition of particular morphogens (Fig. 6). To date, most of these methods are well-developed in mice, and are based on the knowledge of *in vivo* development of neuronal cells. Examples are the use of retinoic acid and sonic hedgehog (SHH) to differentiate the cell into a spinal cord motor neuron (67), and inhibition of Wnt and Nodal to specify telencephalic neurons (68). The overexpression of Lmx1a and Msx1 transcription factors resulted in generation of dopaminergic neurons from ESCs (69). The insight that is created in the development of mouse neurons can be partly transferred to human cells. For example, directed differentiation to dopaminergic neurons from ESCs was accomplished when using Heparin, N2, cAMP, ascorbic acid, BDNF, GDNF, SHH, and FGF8 (70). It was shown by Kriks and Shim (71) that SHH and WNT activation with small molecules resulted in the differentiation of human ESCs to midbrain floor-plate precursors which were able to differentiate to dopaminergic neurons and could be transplanted into an animal model of PD. Differentiation of human iPSCs into midbrain A9 dopaminergic (mDA) neurons has also been accomplished by generating embryoid bodies and culturing them in the presence of Rock inhibitor (Y276323), which was shown to increase the size of cell colonies of neural progenitor derived from ESCs by preventing cell death related to detachment cAMP (72). The subsequent neural patterning was performed by a dual SMAD inhibition strategy after which SHH and FGF8a were used to direct the cells to become ventral mDA neurons. The neurons matured in a dopaminergic differentiation medium containing ascorbic acid, BDNF, GDNF and cAMP (72). These cells were characterized and expressed markers of midbrain dopaminergic neurons, one of which was the marker for substantia nigra pars compacta A9 GIRK2 expressed by more than half of the population.

In the case of AD, the focus has mainly been on basal forebrain cholinergic neurons, which have been successfully produced for human AD disease models (73). These cells are generally the first to show dysfunction in AD, which is seen from the aberrant cortical projections. Differentiation of the iPSCs was performed similarly to mDA neurons, but the cells were positively selected for expression of CD24 and negatively for CD184 and CD44 expression. Subsequently, a number of tests were performed to determine the neuronal subtypes, such as qPCR, which indicated glutamatergic, GABAergic, and cholinergic subtypes. It is, however, commonly believed that these basal forebrain cholinergic neurons are lost during the early stages of the disease. In

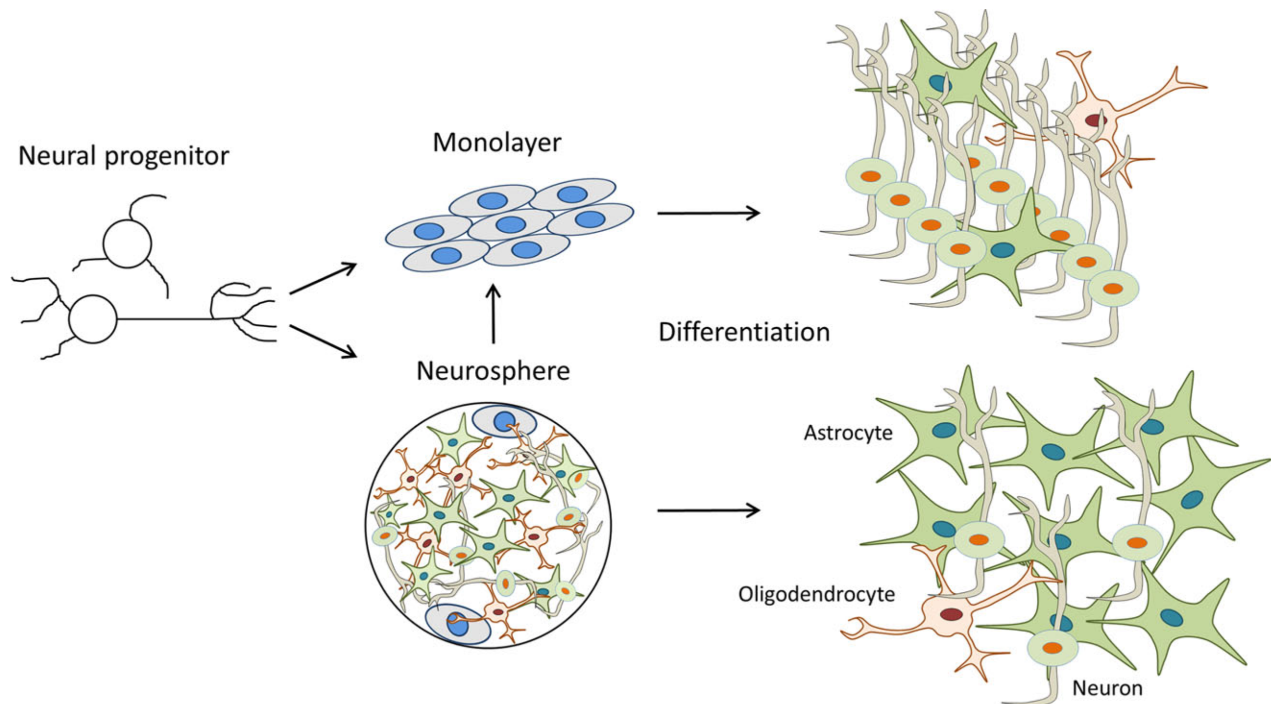

Figure 6. The different sources of cells seen in Fig. 5 can be cultured in monolayers or in neurospheres. Cells in a neurosphere or monolayer are then called tripotent, because they are able to produce neurons, astrocytes and oligodendrocytes. Cellular compositions are mixed for the neurosphere and homogeneous for the NSC monolayer, resulting in a neurogenic potential that is low and high, respectively.

advanced stages, there is an additional loss of neuronal subtypes, which complicates a treatment strategy with iPSCs significantly (74). Much effort is also put in determining whether ESCs and iPSCs have similar differentiating potentials. Initial studies concluded that human ESCs were able to differentiate approximately 90% of the cells to neurons, while human iPSC lines were successful in only 10–50% of the cases (23). Thus, neuronal differentiation appears to be more difficult from iPSC than from ESC, but this is not influenced by the source of fibroblasts, age, choice of reprogramming vectors, residual transgene expression. It may, however, be attributed to the fluctuating response to neural inducers and possibly the incomplete repression of fibroblast gene expression (75). This implicates that the actual pluripotent level of iPSCs is lower than that of ESCs. However, further investigations showed a larger variation in differentiation efficacy for human ESCs as well (76), and it was shown that cortical specification was similarly efficient in differentiated ESCs and iPSCs (77). In addition, those studies have shown that there does not appear to be a difference in gene expression, synapse functionality and electrophysiological properties between the neurons that were derived from iPSCs or ESCs. These results seem promising for the intended transplantation of iPSC derived neural precursors to treat ND.

Transplantation

Diseases like AD and PD can potentially be treated with either neuronal precursor cells or cells that have been differentiated *in vitro* to a subtype that will aid the recovery of the damaged brain. Although transplantation of patient iPSCs has not been allowed in humans yet, there are studies that show the successful production of human neural precursor cells through retroviral transduction and their potential to survive, migrate and differentiate into different lineages upon transplantation in the developing brain of animals, for example a rat model with PD (49). It was shown that transplanting midbrain DA neurons derived from iPSCs were able to recover brain function in these rats. A recent meta-analysis of stem cell transplantation into a large number of different rat and mice models of AD indicated that this procedure has a positive effect on memory and learning recovery in the animals (78). It is, however, indicated that these results are limited by the methodological quality that is currently at hand. Despite these successes in animals, the safety issues of iPSCs are still in the way of clinical trials. Not only the aforementioned risk for tumorigenesis is involved, there is also a risk for post-differentiation loss of cell phenotype. Obtaining cells with high fidelity is required for a long-lasting effect of the transplantation and will have to be guaranteed before using reprogrammed cells in patients.

Besides the safety issue regarding iPSCs, the transplantation of these cells in humans will meet the same challenges as other sources of stem cells. Some of these challenges have already been met in both open and randomized trails. In 2001, Freed et al. (79) published a randomized study on fetal stem cell transplantation in PD. Although positive effect was shown, it was only significant in the younger patients. Furthermore, several of the patients developed side effects in form of dystonia and regidity. This raised questions on both the most optimal anatomical target within the putamen and whether duration of the disease will have strong impact on the efficacy of the treatment. Although integration of the transplanted stem cells into the putamen was shown, the fact that the duration of the disease may be an important issue and suggest that other cell types are lost during the course of disease. This will again challenge the usefulness of animal models in small animals as such decay may be difficult to model. On the other hand, more detailed knowledge of the tissue degeneration in the patients that do not respond to the fetal stem cell therapy may pave the way for a more complex therapy using iPSCs that have been differentiated into all the cellular components needed to restore normal tissue function.

The challenges in using iPSCs as an external source in AD will be more extensive due to the wide spread degeneration that causes the dementia. Therefore an obvious target region cannot as yet be identified. However, transplantation of human neurogenic progenitors into aged rats has been shown improve cognitive function (80). The target zone was the lateral ventricle that is known to host stem cells in the adult brain and the transplanted human stem cells were shown to integrate into the host brain by both proliferation and migration. Whether this approach will work in humans is yet to be proven. Other routes of administration of stem cells have been suggested and the first human phases II trail using intravenously injected mesenchymal stem cells has received approval from the FDA and should initiate late in 2015 (81). It has been debated whether to treat patients early or late in the cause of disease and this should be seen in parallel to the lack of effect when treating patients with PD

late in the cause of disease where tissue integrity may be too compromised to welcome exogenously administered stem cells. However, once the problem regarding the optimal route has been solved, the iPSC technology has the advantage of being able to delivering one or more types of progenitor cells and in the optimal ratio.

Somatic intermediates and transdifferentiation

As mentioned previously, one of the problems arising from reprogramming with defined transcription factors is that many of the generated iPSCs are reprogrammed incompletely compared to ESCs. The intermediates of reprogramming are likely to express several markers specific to a certain lineage, although this is not enough to distinguish them as a different cell type (17). This is due to the extreme differences that have to be made during transition to iPSC compared to transition into another subtype of the same lineage. On the other hand, several different groups found that capturing the intermediate states can be used to lower the threshold for differentiation into other, non-iPSC lines, by directing them toward a desired cell type with the appropriate conditions. Time-limiting exposure of mouse fibroblasts to the Yamanaka factors resulted in spontaneous development into neural precursor colonies, called iNPCs, which were able to differentiate into neurons and astroglia cells, but showed restrained expansion (82). The epigenetic alterations are thought to create an unstable cell state that allows conversions to different lineages. The potential of this method was confirmed by Thier and Worsdorfer (83) who used transient expression of Oct4 to induce an iNPC state in fibroblast cells. These cells were tripotent, able to expand up to 50 passages and expressed genes that belong to neural stem cells.

Another option is to use transdifferentiation, where fibroblast cells from patients are converted directly to neurons when they are exposed to transcription factors of the neural lineage. Induced neurons were produced from mouse fibroblasts by expressing *Ascl1*, *Brn2,* and *Myt1l* with transgenes and showed expression of neuron-specific proteins, were able to fire action potentials and formed functional synapses (84). Interestingly, the efficiency of this conversion reached up to 20%, although it produced a mixed population of cells with both glutamatergic and GABAergic neurons. Fine-tuning of the culture conditions might decrease this heterogeneity. The conversion to induced dopaminergic neurons (iDAs) has also been described by adding Lmx1 and Foxa2 to the group of compounds, as well as the combinations of Ascl1, Nurr1 and Lmx1a, and Ascl1, Pitx3, Lmx1a, Foxa1, and EN1 have induced the dopaminergic neuron state in mouse fibroblasts (85, 86). These iDAs share the most important properties of dopaminergic cells, such as release of DA and regular spikes of spontaneous electrical activity. These findings are especially interesting in the light of finding a cell source to treat PD. However, direct conversion to the final, fully differentiated neuron does not allow further expansion of the necessary product, as a result of mitotic arrest in these cell types. In more recent studies, the induction of neural precursor from fibroblasts was performed by expressing Brn2, Sox2, and FoxG1, generating iNPCs with a significantly increased efficiency compared to the intermediates of standard reprogramming (87). However, there have not been thorough investigations of the safety of these cells, which will be required before they can be tested in humans. More thorough investigations on transdifferentiation will also have to answer other questions, such as why keratinocytes are less likely to transform into neurons than cells of another germ layer, like fibroblasts.

In vivo reprogramming

Currently, the majority of studies focus on reprogramming cells *in vitro* to expand patient-specific pluripotent cells and transplant these into the damaged brain areas. Transplantation in this extremely delicate area will be challenging by means of surgery, graft survival and functional integration. Therefore, there is increasing interest in reprogramming of cells *in situ*, which will convert cells at the diseased site using endogenous cells that are already present. In case these cells can be converted efficiently, the transplantation of cells becomes unnecessary altogether. Moreover, the risk of introducing mutations in cells by culturing them is decreased significantly. Proof of principle was already shown, when Zhou and Brown (88) transformed exocrine cells to endocrine cells in the pancreas. Other groups now try to determine which neurons could be converted to revert the damage done by ND. It has been shown that certain adult CNS cells (pericytes) can be reprogrammed and converted to neurons by expressing Sox2 and Mash1 with the retroviral method (89). Choosing a target cell that already possesses epigenetic marks of the neural lineage can possibly increase the efficiency of reprogramming *in situ*. These cells are more likely to respond to the factors used for the conversion

and could aid the formation of specific neurons by expressing the genes required for CNS cells. An example of candidate cells for reprogramming are astrocytes, which are close to neurons in development (90). These cells have already been shown to convert to GABAergic neurons using *Ascl1* and *Dlx2*, glutamatergic neurons after *Ngn2* expression and dopaminergic neurons by expressing *Ascl1, Lmx1* and *Nurr1* (91, 92). One of the advantages of this method is that the risk for mutations is decreased when cells are not expanded in culture, although the efficiency will therefore also be questionable. Further studies will have to clarify whether the damaged brains of patients with AD or PD are capable of regenerating by stimulating other cell types to differentiate into those that have been affected, without posing a risk for the other brain functions.

iPSCs disease models

The use of pluripotent stem cells is not restricted to therapeutic intervention, but can also aid to the understanding of pathological events in several diseases. Due to the large number of different pathological conditions, there is a need for specific models in which the pathways of each disease can be investigated, and possible therapies and drugs can be tested. Tissue samples from the central nervous system are extremely difficult to obtain during life, which complicates the study of neurological diseases in its early stages. Additional animal models are used widely to create transgenic or knockout disease systems, which are of great importance to understand monogenic diseases. However, there is a large group of complex diseases that cannot be covered by these models and the difference in species is a considerable problem as well. There is a strong need for advanced human models, which can be generated from patients with ND by inducing pluripotency in somatic cells and redifferentiating these to the desired cell type to study the development of disease.

Neurodevelopmental disorders are generally caused by aberrant gene expression and can be modeled relatively easily in iPSC lines because of the early onset (93, 94). Late-onset NDs are generally caused by a complex mechanism that involves both abnormal gene expression and the influence of environmental factors. This complicates the production of iPSC models from these diseases, because of slow phenotype development. Therefore, specific gene mutations have to be used to stimulate the symptom development in iPSCs (95, 96). AD and PD disease models from iPSCs have already been developed and can be used for drug screening (97) or investigating disease mechanisms (98). Unfortunately, stem cells do not make up for a whole organism, which complicates the investigations of complex pathological pathways. However, the improvement of both reprogramming and differentiation techniques show the potential to ultimately lead to patient-specific disease models to test drugs prior to administration (99).

Concluding remarks

With the increasing life expectancy, our society will be burdened with an increasing number of patients with NDs in the future. Therefore, it is necessary to develop advanced treatments that target the cause of neurodegeneration effectively and interfere in its progression and possibly even improve the patient's condition. Transplantation of neural stem cells derived from ESCs into the brain has been shown to improve its function in animals and to a varying extend in humans. However, these trials are troublesome because of the restriction in cell sources due to ethical issues and histocompatibility, among other factors. Recently, the findings that pluripotency can be induced in terminally differentiated cells have opened doors to a solution to some of these challenges. The iPSC technique has the potential to produce a sufficient number of autologous cells to treat these patients by regenerating part of the brain tissue that has been lost during the course of disease. However, these cells come with a number of issues as well, mostly on the account of safety. Those issues will have to be addressed prior to the use of iPSC derived transplants for NDs.

Ongoing studies of different research groups are pursuing the answers to some fundamental questions about iPSCs. One of the most interesting, and simultaneously most challenging, will be to uncover the molecular mechanisms underlying the reprogramming events when producing iPSCs. A better understanding of the pathways involved in the process will most likely allow us to improve the methods that are used, as well as the selection procedures for high quality pluripotent cells. To date, the majority of research has focused on the Yamanaka factors and fibroblast cells, although it will be very valuable to continue to investigate the use of other factors, methods and cell lines. The different kinds of molecules that have been found to affect the reprogramming process indicate that it can be influenced in several different ways. This emphasizes the plasticity of cells even more, which implicates that it could be possible to follow different reprogram-

ming routes as well. It is therefore very plausible that there is a variety of techniques that needs to be tested in the future, which in turn will also increase the understanding of the mechanisms involved. Additionally, these improvements will be essential for enhancing the throughput and increasing the quality, and thus safety, of iPSCs. A number of these safety issues have already been addressed since the first descriptions of these cells, for instance reducing the risk for tumorigenesis. However, there is still a long way to go before these cells can be used in humans with high confidence of safety. It is also presumable that the production of high quality iPSCs for transplantation into humans will not be as simple as the initial method described by Takahashi and Tanabe (13). A combination of different strategies, such as those discussed earlier in this work, might yield optimal results in terms of efficiency and quality of pluripotency.

To date, only few studies have attempted to transplant adult stem cells in patients with NDs, and these have yielded varying results. Because of the complexity of cells, it can be challenging to use them as a treatment; they can exert many different functions, which cannot be controlled, making them unpredictable. This is much less complicated when using, for example, chemical compounds as a treatment. However, complex diseases such as AD and PD require these advanced treatments, and continued intensive research will have to prove whether iPSCs are going to fulfill the potential they are believed to have in treating them.

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