TRANSLATIONAL NEUROSCIENCES - REVIEW ARTICLE

Induced neural stem cells (iNSCs) in neurodegenerative diseases

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Abstract Recent advances in somatic cell reprogramming is one of the most important developments in neuroscience in the last decades since it offers for the first time the opportunity to work with disease/patient-specific neurons or other neural cell types. Induced pluripotent stem cells (iPSCs) can be differentiated into all cell types of the body enabling investigations not only on neurons but also on muscle or endothelial cells which are cell types often also of great interest in neurodegenerative diseases. The novel technology of direct lineage conversion of somatic cells into neurons (induced neurons; iNs) or into expandable multipotent neural stem cells (induced neural stem cells; iNSCs) provides interesting alternatives to the iPSC technology. These techniques have the advantage of easier cell culture, but only neurons (iNs) or neuroectodermal cells (iNSCs) can be generated. Although there are several open questions coming along with these new neural cell types, they hold great promises for both cell replacement and cell modelling of neurodegenerative diseases.

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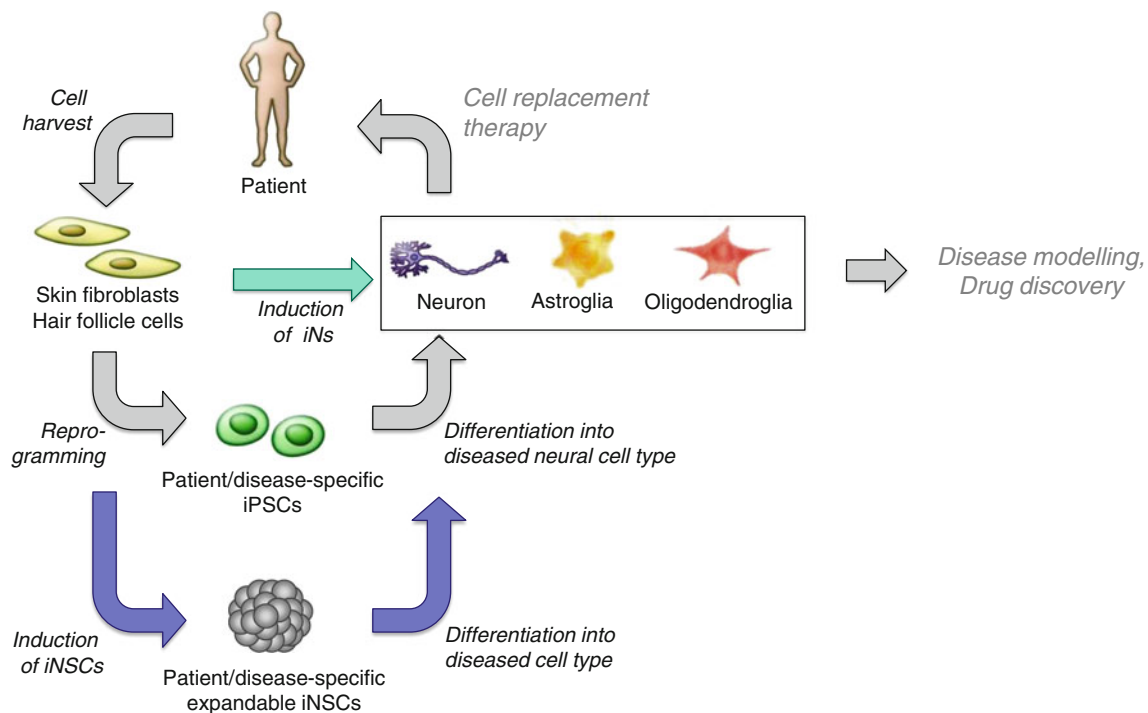University of Technology, 01307 Dresden, Germany

Intoduction

Recent advances in direct lineage conversion of somatic cells into neurons (induced neurons; iNs) or expandable multipotent neural stem cells (induced neural stem cells; iNSCs) provide interesting alternatives to the induced pluripotent stem cell (iPSC) technology to generate neurons or other neural cell populations for both cell replacement and disease modelling for neurodegenerative diseases (see Fig. 1 for schematic presentation of the strategies). Takahashi and Yamanaka (2006) pioneered the field of somatic cell reprogramming (Takahashi et al. 2007). The principle of somatic cell reprogramming was long known by the technique of somatic nuclear transfer, but this technique is highly complicated and always needs an enucleated oocyte as the acceptor cell (for review see Jaenisch and Young 2008). Takahashi and Yamanaka identified four transcription factors which are—when overexpressed in a somatic cell—sufficient to induce a cell harbouring all properties of a pluripotent stem cell (Takahashi et al. 2007; Takahashi and Yamanaka 2006). The so-called induced pluripotent stem cells (iPSCs) are thus able to differentiate into all cell types of the body. This technology is a tremendous breakthrough in medical science since any cell type can now be derived patient-specific with the potential for autologous cell replacement strategies and personalized medicine (Fig. 1). For neuroscientists, a new era begun with the possibility to get access to patient/disease-specific neurons.

Fig. 1 Schematic representation of the three strategies to generate patient/disease-specific neural cell types for cell replacement strategies or disease modelling of neurodegenerative diseases. The first strategy is to reprogram somatic cells into pluripotent stem cells, which are subsequently differentiated into the neural cell type of interest (with or without intermediate generation of NSCs [not shown in scheme, *grey arrows*)]. The second approach is to directly convert somatic cells into neurons (iNs; *green arrow*). The third strategy is to convert somatic cells into expandable neural stem cells (iNSCs), which are subsequently differentiated into the diseased neural cell type (*blue arrows*). *iNs* induced neurons, *iNSCs*, induced neural stem cells, *iPSCs* induced pluripotent stem cells

The induction of iPSCs was subsequently shown from many somatic cell sources, e.g. skin fibroblasts (Takahashi et al. 2007; Takahashi and Yamanaka 2006), hair follicle keratinocytes (Linta et al. 2012), liver cells (Kleger et al. 2012) and blood cells (Okita et al. 2013). It is thus believed that they can be generated from any cell source of the body. It was further on successfully demonstrated that iPSCs perform like embryonic stem cells (ESCs) during cell type differentiation, therefore most protocols already established for ES cell differentiation are—in general—applicable for iPSCs. Exemplarily, dopaminergic differentiation from Parkinson's disease (PD) patient-derived iPSCs was proven in vitro and in vivo by restoration of hemiparkinsonian rats after intrastriatal transplantation (Hargus et al. 2010). This also holds true for sickle cell anemia (Hanna et al. 2007), acute myocardial infarction (Nelson et al. 2009) and diabetes (Alipio et al. 2010). Within this gold rush mood of the recent iPSC years, large parts of the scientific and clinical community neglect some major problems accompanied with the use of pluripotent stem cells such as their tumorigenicity (Miura et al. 2009). Moreover, there are unclear limitations with the use of iPSCs in clinical application such as their genetic and epigenetic instability and heterogeneity (Lister et al. 2011; Gore et al. 2011).

Besides their application in cell replacement strategies, iPSCs are currently believed to be one of the best in vitro cell model systems for various diseases and are hoped to catalyse our understanding in neurodegenerative diseases. However, one major obstacle is whether it will be possible to model neurodegenerative disorders as age-dependent diseases since cellular reprogramming changes the cell to an embryonic stem cell state. Nevertheless, first reports promisingly show neuronal cell degeneration in various iPSC-based disease models, e.g. spinal muscular atrophy (Ebert et al. 2009), PD (Reinhardt et al. 2013), spinocerebellar atrophies (Koch et al. 2011) and Alzheimer's disease (Koch et al. 2012).

Expression of the reprogramming factors as foreign genes using viral transduction systems leading to genomic integration is of limited suitability for clinical use. Therefore, many attempts have been undertaken in order to overcome this issue such as the use of non-integrating adenoviruses (Stadtfeld et al. 2008), oriP/EBNA1-based episomal vectors (Yu et al. 2009), piggyBac transposition systems (Kaji et al. 2009), transient transfection with reprogramming plasmids (Okita et al. 2008), Cre-recombinase excisable viruses (Soldner et al. 2009), recombinant proteins (Zhou et al. 2009; Kim et al. 2009a) and modified

RNA (Warren et al. 2010). In the latter approaches, the need for genomic manipulation and DNA transfection that is associated with viral or other DNA-based reprogramming methods is eliminated. Another attempt has been the reduction of reprogramming factors with or without the combination with small molecules (Kim et al. 2008, 2009b, 2009c). This was accomplished by the use of NSCs as cell origin already expressing three of the four Yamanaka factors. Recent data suggest that there is a dose dependency of the extrinsic factors for optimal reprogramming efficacy potentially explaining the differences between cell types in their suitability for factor reduced reprogramming (Nagamatsu et al. 2012). In addition, somatic cells express their specific set of transcription factors which is important for maintaining the cell identity (Hikichi et al. 2013). Overexpressing these factors was shown to strongly interfere with cell reprogramming and it was shown that these factors differed exemplarily between NSCs and hepatic cells (Hikichi et al. 2013). This means, it is not only important whether the somatic cell already express one of the four reprogramming factors. Additionally, it is also important to overcome the cell type-specific transcription factor network to succeed with iPSCs generation.

Interestingly, even though complete reprogramming has been proven for factor reduced NSC-derived iPSCs they might have a decreased differentiation potential (Lohle et al. 2012). In addition, even though believed to be reprogrammed to a fully immature embryonic stem-like cell, iPSCs seem to retain a distinct epigenetic memory still favouring the subtype differentiation of the respective cell source of origin (Kim et al. 2011b). This is due to incomplete erasure of tissue-specific methylation and aberrant de novo methylation (Kim et al. 2011b).

Directly induced neurons (iNs)

The advances in generating of iPSCs stimulated another approach to generate neurons by direct induction of mature neurons from somatic cells even from other germ layers: Using forced expression of various neuronal key-fate determining factors (mainly transcription factors) was demonstrated to generate fully mature neurons (Vierbuchen et al. 2010; Pfisterer et al. 2011; Pang et al. 2011). The major advantage of this technology is the rapid and rather easy induction of the neuronal phenotype without sophisticated protocols as used in iPSC generation. However, some disadvantages are arising from this technique: The derived cells (postmitotic neurons) are not expandable, thus every experiment starts with a new induction process. Although the induction efficacy is relatively high (~ 5 %) compared to those for iPSCs/iNSCs generation, the population of the cell type of interest is very low thereby limiting their

application. To enhance the efficacy of the conversion process and thus overcome this limitation, Ladewig et al. (2012). recently reported an experimental approach combining two-factor neuronal programming (ASCL1 and NGN2) and small molecule-based inhibition of glycogen synthase kinase-3b and SMAD signalling leading to high yields of functional neurons with high neuronal purity (>80 %) In addition, it seems to be necessary to use different transcription factor combinations for any specific neuronal subtype. This was recently shown for midbrain dopaminergic neurons, where midbrain-specific transcription factors Nurr1 and Lmx1a were used (Caiazzo et al. 2011).

Xue et al. (2013) recently reported the transdifferentiation of fibroblasts to iNs by the use of miRNA-124a. This is in close agreement with our studies in NSC-like cells generated by conversion from bone marrow mesenchymal stem cells (MSCs) without genetic manipulation (Hermann et al. 2004, 2006a): Using genome-wide expression profiling and functional network analysis we detected the miR-124a as an important regulator during the neuroectodermal conversion process (Maisel et al. 2010).

The induction of neurons from adult human cortical perivascular cells was successfully achieved by transduction with only two factors, namely MASH1 and SOX2 (Karow et al. 2012). This suggests that similar to iPSCs also for iNs, the choice of the needed transcription factor combination depends on the cell source (via endogenous transcription factor profiles of different somatic cells). This might favour the use of ectodermal or even better neuro-ectodermal somatic cells as a cell source for iN induction.

Concerning possible clinical applications, the major disadvantage of iNs is the lack of expandability and thus the low cell number not only limiting the cells amount per se for applications but also for genetic and microbiology testing prior of use. This disadvantage translates into another limitation: iN cultures contain neurons in various purities and non-neural cells such as the initial cell type (e.g. fibroblasts), but not other neural cell types. On the one hand, the contamination with the initial cell type might provoke side effects and, on the other hand, in some cases the non-neuronal cells might support the neurons in cell replacement studies or provide a more physiological environment in cell culture models. In addition, without a defined intermediate immature cell type transplantation approaches are difficult, because detachment from the surface is harmful for mature neurons with established axo-dendritic arborisation. Finally, the transgenes might remain active in the iNs without being completely silenced.

Together, clinical applications of iNs for cell replacement approaches are limited with the current protocols, but iNs might be a very suitable cell source for applications in which only single neuronal entities on the single cell level are of investigational interest. Consistently, a first study

Table 1 Synopsis of various iNSC strategies

Reference	Cell source	Construct	Factors	Expandability	Transgene silencing	Genome analysis	Promotor analysis	Karyotyping	Multipotent differentiation	Subtype specification	Electrophysiology	In vivo integration
(Kim et al. 2011a)	Fibroblasts	Dox-inducible	Oct4, Klf4, c-Myc, Sox2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.
(Lujan et al. 2012)	Fibroblasts	Dox-inducible	FoxG1, Brn1, Sox2	n.d.	n.d.	n.d.	n.d.	n.d.	+	n.d.	+	(+)
(Han et al. 2012)	Fibroblasts	Retrovirus	Brn4, Klf4, cMyc, Sox2, E47	+	+	+	+	n.d.	+	+	+	+
(Thier et al. 2012)	Fibroblasts	Retrovirus + Oct4	Oct4*, Klf4, cMyc, Sox2	+	+	+	n.d.	n.d.	+		+	+
(Ring et al. 2012)	Fibroblasts	Retrovirus	Sox2, glass cover slides, STO feeders	+	+	+	+	n.d.	+		+	+
(Corti et al. 2012)	Human astrocytes	Lentivirus	Oct4 or Sox2 or Nanog	+	n.d.	+	+	n.d.	+	n.d.	n.d.	+
(Sheng et al. 2012)	Mouse Sertoli cells	Retrovirus	Pax6, Ngn2, Hes1, Id1, Ascl1 Brn2, c-Myc, Klf4	+	+	+	nd	+	nd	+	+	+
(Hermann et al. 2004)	Human MSCs	none	Hypoxia, NSC medium	+	n.a.		n.d.	n.d.	+	n.d.	n.d.	n.d.

n.a not applicable, *n.d* not determined

The study by Hermann et al. (2004) used a non-genetic approach not leading to the novel iNSC type

showed that iNs can be used for disease modelling of Alzheimer's disease (Qiang et al. 2011).

Directly induced neural stem cells (iNSCs)

Having some of the limitations of iNs in mind, an alternative approach is to derive induced neural stem cells (iNSCs) from somatic cells as an expandable cell type which can be differentiated into all neuroectodermal lineages. Our group and others established protocols to convert bone marrow-derived MSCs into NSC-like cells without genetic modifications, but by culturing the cells in NSC-promoting conditions (Hermann et al. 2004, 2006a; Lee et al. 2003). Although the generated NSC-like cells express some major properties of expandable NSCs, their functional neuronal differentiation capacity is limited and comparative transcriptome analyses revealed significant differences between these NSC-like cells and brain-derived primary NSCs (Maisel et al. 2010). It is thus now believed that these protocols only lead to partial (and probably transient) neuroectodermal conversion of mesenchymal stem cells. The novel approach using heterologous expression of various key fate-determining transcription factor combinations successfully generated expandable iNSCs from somatic cells expressing all major properties of primary NSCs (Han et al. 2012; Lujan et al. 2012; Ring et al. 2012; Thier et al. 2012) (Table 1). These iNSCs easily differentiated into astrocytes, oligodendrocytes and functional neurons including neuronal subtypes (Han et al. 2012). The first report by Kim et al. (2009a) showed induction of iNSCs from fibroblasts by using the classical Yamanaka-factors as used in iPSC generation. The difference was a shorter time of expression of the foreign genes and the change to a neural medium which seems to convert these intermediate cells into a NSC phenotype. Trilineage differentiation capacity of iNSCs was first described by Lujan et al. (2012) using *Brn2, FoxG1* and *Sox2* as foreign genes. In both reports, no data were shown concerning expandability, silencing of foreign genes, neuronal subtype specification or whole genome analysis (Kim et al. 2009a; Lujan et al. 2012). Our report together with the studies by Thier and colleagues showed subsequently the generation of stably expandable iNSCs exhibiting a NSC-like transcriptome (Han et al. 2012; Thier et al. 2012). Consistently, both protocols led to silenced transgenes within the iNSCs additionally confirming complete and stable conversion (Han et al. 2012; Thier et al. 2012). Glutamatergic, GABAergic, cholinergic and dopaminergic neuronal subtype specification was only with our protocol (Han et al. 2012). The article by Ring et al. (2012) is of additional note since it reports iNSC induction by the single factor *Sox2*. However, the use of a very

specific feeder cell type for iNSC induction might be the crucial point delivering a yet unknown set of reprogramming factors. Corti and co-workers converted human astrocytes using a single genetic factor (*OCT4* or *KLF4* or *NANOG*) into iNSCs with neuronal differentiation capacity (Corti et al. 2012). Sertoli cells were recently shown to be a possible cell source for iNSC generation, but a combination of eight transcription factors were needed for iNSC induction (Sheng et al. 2012).

Another big issue in generating iNSCs is that the definition of the NSC state is by far not that strict as for ESCs and iPSCs. NSCs are defined by expression of some key transcription factors (such as Sox2, Pax6) and their differentiation potential into the main neuroectodermal lineages, namely neurons, astrocytes and oligodendrocytes (Hermann et al. 2006b; Storch and Schwarz 2002). What we learned from both embryonic brain development and ESC/iPSC differentiation is that a regionalization takes place already very early in the developmental/differentiation process to region-specific NSCs (Reinhardt et al. 2013; Pankratz et al. 2007) and it remains enigmatic whether a pan-neural NSC state exists (Pankratz et al. 2007). Thus, neuronal subtype specification might depend on the generation methods of iNSCs.

Conclusions

The novel technology for generating iNSCs from easily accessible cell sources such as skin fibroblasts opens up a new field in personalised medicine for neurodegenerative diseases. Although the available studies convincingly demonstrate the similarities of iNSCs with primary NSCs, there are several open questions such as (1) are the retroviral vectors completely silenced in iNSCs?, (2) does the iNSC conversion process leads to genomic alterations?, and (3) is the conversion into the iNSC state stable and thus leading to a stable change of the epigenome to a NSC-like status? In addition, in contrast to the iPSC technology with readily available differentiation protocols, protocols for neuronal (subtype) differentiation are not available for iNSCs yet. However, expandable iNSCs seem to provide some advantages over iNs and possibly also over iPSCs towards both cell replacement and cell modelling of neurodegenerative diseases.

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