Neurosci Bull October 1, 2015, 31(5): 589–600. http://www.neurosci.cn
DOI: 10.1007/s12264-015-1527-z

·Review·

Induced neural stem/precursor cells for fundamental studies and potential application in neurodegenerative diseases

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Recent research has shown that defined sets of exogenous factors are sufficient to convert rodent and human somatic cells directly into induced neural stem cells or neural precursor cells (iNSCs/iNPCs). The process of transdifferentiation bypasses the step of a pluripotent state and reduces the risk of tumorigenesis and genetic instability while retaining the self-renewing capacity. This iNSC/iNPC technology has fueled much excitement in regenerative medicine, as these cells can be differentiated into target cells for replacement therapy for neurodegenerative diseases. Patients' somatic cell-derived iNSCs/iNPCs have also been proposed to serve as disease models with potential value in both fundamental studies and clinical applications. This review focuses on the mechanisms, techniques, and applications of iNSCs/iNPCs from a series of related studies, as well as further efforts in designing novel strategies using iNSC/iNPC technology and its potential applications in neurodegenerative diseases.

Keywords: induced neural stem cell; induced neural precursor cell; transdifferentiation; fundamental study; clinical application; neurodegenerative disease

Introduction

Neurodegenerative disease is a condition in which neurons in the brain and spinal cord are gradually and progressively lost, leading to nervous system dysfunction. As the proportion of elderly individuals in the total population is rising, there is an increase in the number of patients afflicted with neurodegenerative diseases. Because of the limited regenerative ability of neurons, neurodegenerative disease may cause permanent damage, implying that cell replacement therapy would be the most effective therapeutic strategy. Although neural stem cells and progenitor cells have self-renewal capacity and differentiation potential, their clinical application is limited due to their insufficient quantities in the body. Patients' somatic cell-derived induced neural stem cells or neural precursor cells (iNSCs/iNPCs) may be a new source for replacement therapy for neurodegenerative disease.

iNSCs/iNPCs are types of pluripotent stem cells that can be generated directly from adult somatic cells by introducing a specific set of "reprogramming factors". iNSCs/iNPCs express NSC/NPC markers, and exhibit cell morphology, gene expression profiles, epigenetic features, differentiation potential, and self-renewing capacity, as well as *in vitro* and *in vivo* functionality similar to those of wild-type NSCs/NPCs.

Recently, a series of experimental studies has shown that somatic cells can be directly converted into iNSCs/iNPCs by different combinations of exogenous factors[1-11]. Compared to induced neurons, iNSCs/iNPCs have the advantage of self-renewal and differentiation. These dividing cells may have considerable clinical applications, being able to generate sufficient amounts of cells.

A patient's somatic cell-derived iNSCs/iNPCs can avoid the ethical issues raised by embryonic stem cells, and have a lower risk of tumorigenesis and genetic instability compared to induced pluripotent stem cells (iPSCs). Besides cell replacement therapy[4], they can

also be used to establish disease models[12] for studying pathogenesis, screening drugs, and monitoring efficacy. But this technology is still in the preliminary stage, and many problems need to be solved before clinical use.

Here we review the techniques, mechanisms, and applications of iNSCs/iNPCs in neurodegenerative diseases, and discuss the limitations and prospects for development.

Mechanisms of Direct Conversion from Somatic Cells into iNSCs/iNPCs

It has been confirmed that adult somatic cells retain broad cellular plasticity, so that they can directly change fate from one lineage to another. Activation of certain key transcription factors in adult somatic cells can realize the change of cell fate. Direct transdifferentiation technology bypasses the pluripotent stage, shortens the induction time, and improves the efficiency of conversion. At present, this technology has been used to successfully obtain iNSCs, iNPCs and induced neurons, but the mechanism of transdifferentiation is still not clear. Nowadays, the process of transdifferentiation is considered to involve the expression of target genes activated by both defined transcription factors and epigenetics. First, transcription factors initiate and control effective gene expression, while the role of epigenetics deals with the challenge of external environment factors, to ensure a constant process of transdifferentiation[13]. Accurate coordination between transcription factors, as well as the epigenetic modifications of target genes, are the key determinants of transdifferentiation. Wernig and colleagues revealed that a precise match between pioneer factors and the chromatin context at key target genes determines transdifferentiation[14].

Basic Function of Transcription Factors

The process of transcription defines the specific phenotypes of differentiated cells during the development of a multicellular organism, implying that transcription factors also play a vital role in regulating the process of transdifferentiation. For adult stem cells (such as iNSCs/iNPCs) with self-renewal capability and differentiation potential, the pluripotent state is regulated by an extremely complicated molecular network. Only if the molecular network is fully activated, and the balance between various factors is precisely regulated to the right level, can the direct conversion of somatic cells to iNSCs/iNPCs successfully proceed.

Kim and colleagues showed that the induction of four reprogramming factors (Oct4, Sox2, Klf4, and c-myc) can efficiently convert fibroblasts into functional iNSCs/iNPCs[1]. Lujan and colleagues infected mouse embryonic fibroblasts derived from Sox2-EGFP mice with a set of 11 transcription factors highly expressed in NPCs, and successfully obtained colonies that expressed NPC-specific genes and differentiated into neurons and astrocytes. Using stepwise elimination, they also found that two different combinations of transcription factors (Sox2/FoxG1; Sox2/FoxG1/Brn2) are capable of generating clonal self-renewing iNPCs[2].

According to the iPSC technology and the results of screening for transcription factors with high expression in NSCs/NPCs, the transcription factors noted above play different roles in direct transdifferentiation.

The *Sox2* gene belongs to the *Sry* gene family, and it is widely expressed among cells within the neural tube at early stages of neurodevelopment. Its expression is subsequently localized to the ventricular layer in the cortex, where NSCs/NPCs are present after the mid-fetal period[15]. These findings indicate that the *Sox2* gene may be a key factor with high expression in NSCs and NPCs. Sox2 functions to maintain the pluripotent state and self-renewal ability of iNSCs/iNPCs, and to inhibit the differentiation process[2, 6, 16]. Sox2 collaborates with other transcription factors. In NSCs, Sox2 interacts with the POU (Pit-1, Oct, unc-86) domain transcription factors such as Oct4 and Brn2, to form a specific partnership to regulate the mechanism that maintains undifferentiated pluripotent cells. The target genes of the combination of Sox2 and Oct4 include *Nanog*, *Utf1*, and *FGF4*. Sox2 binds to the regulatory region of the *Nestin* and *Sox2* genes with Brn1 and Brn2 to perform an important function in the regulation of gene expression. Sox2 activates EGFR transcription, and the EGFR signaling in turn activates Sox2 transcription. Similarly, Sox2 activates Shh transcription, and the Shh signaling downstream factor Gli2 in turn activates Sox2 transcription. Sox2 also activates the *Nestin* and *Tlx* genes but represses *NeuroD1* transcription[15]. Therefore, Sox2 functions by acting as a molecular switch in several major signaling pathways.

The *Oct4* gene is a member of the POU transcription

factor family. Oct4 has emerged as a principal regulator of the induction and maintenance of cellular pluripotency, with crucial roles in the early stages of differentiation[17]. Janghwan Kim's team pointed out that Oct4 is the only indispensable reprogramming factor of the four Oct4, Sox2, Klf4, and c-myc to obtain pluripotent stem cells[1]. The functions of Oct4 depend on its ability to recognize and bind to DNA regulatory regions alone or in cooperation with other transcription factors (such as Sox2) and on its capacity to recruit other factors required to regulate the expression of specific sets of genes[17].

Foxg1 is an important member of the *Fox* gene family, known to play a central role in cortical development in that it regulates progenitor proliferation, specification, and telencephalic patterning. It is also expressed dynamically during the post-mitotic multipolar phase to critically regulate the assembly and integration of pyramidal neuron precursors into the cortical network[18]. Being upstream of many genes, *Foxg1* may regulate the proliferation and differentiation of NSCs during the early phase of embryogenesis[19]. Brancaccio and colleagues demonstrated that the main function of *Foxg1* in the cerebral cortex during the embryonic period is to maintain the normal status of the precursor cell bank and ensure the normal process of neuron proliferation, as well as regulating the fate of NPCs by suppressing the genesis of glial cells, while promoting differentiation to neurons[20]. Fasano demonstrated that the cooperation of Bmi-1 and Foxg1 is required to maintain the pluripotency and self-renewal capability of NSCs[21].

C-myc, a member of the proto-oncogene family, can accelerate the rate of cell proliferation and enhance the self-renewal capacity of NSCs[22]. Klf4 is also involved in the regulation of cell proliferation and differentiation, and also participates in maintaining the pluripotent state.

The transcription factors above play different roles through different mechanisms in the process of direct transdifferentiation. A key point to improve the conversion efficiency of iNSCs/iNPCs technology is the appropriate combination of these transcription factors.

Epigenetic Modifications of Target Genes in Direct Transdifferentiation to iNSCs/iNPCs

Epigenetic alterations can modify the activation of certain genes, without changing the DNA sequence. There are three major types of epigenetic mechanisms: DNA methylation, histone modification, and non-coding RNA-mediated regulation[23]. The process of direct conversion from somatic cells to iNSCs/iNPCs also refers to these epigenetic modifications.

DNA methylation DNA methylation is a key element in the hierarchy of control mechanisms that govern gene function and differentiation.

Cortese and colleagues demonstrated significant enrichment of genes involved in neuronal differentiation, such as Jag1 and Tcf4, in a genome-wide screen for differential DNA methylation, providing robust evidence for the relevance of DNA methylation in early neuronal development[24]. Methyl-CpG-binding domain protein 1 (MBD1) facilitates neuronal differentiation by direct binding to the promoter of FGF-2. MBD1-induced methylation of the FGF-2 promoter results in down-regulation of FGF-2 expression to undergo neuronal differentiation[25]. At the same time, *de novo* DNA methylation and hypo-methylation are likely to be important for the process of transdifferentiation and maintenance of the pluripotent state[26].

Zhang's group treated NIH/3T3 fibroblasts with a combination of 5-aza-dc, a DNA methylation inhibitor and Trichostatin A, a histone deacetylation inhibitor. By culturing the cells in a neural environment supplemented with retinoic acid (RA), they generated neuron-like cells from fibroblasts, and found that the pluripotent markers Sox2, klf4, c-myc, and Oct4 were expressed in reprogrammed NIH/3T3 fibroblasts and the total DNA methylation level was significantly decreased after treatment, indicating a role of the demethylation process in inducing and maintaining the pluripotent state[27].

Histone modification Histone modification is a major mechanism of regulating the expression of target genes by remodeling chromatin, including histone methylation, acetylation, phosphorylation, and ubiquitination, and plays critical roles in gene activation and inactivation.

Taking histone methylation as an example, Dai and colleagues showed that decreased H3K27me3 accompanied by increased demethylase of H3K27me3 (Jmjd3) at the promoter of Ascl1 enhances the expression of Ascl1 in RA-treated P19 cells, a neuronal differentiation model[28]. Burgold also reported that Jmjd3 controls the expression of key regulators and markers of neurogenesis and is required for commitment to the neural lineage[29]. Zuryn and colleagues reported that Jmjd3.1 and the H3K4

methyltransferase Set1 complex cooperate to ensure invariant transdifferentiation of postmitotic *Caenorhabditis elegans* hindgut cells into motor neurons[13]. H3K27 methyltransferase prevents Wnt-signal-mediated β-catenin action on neuronal genes and results in blockade of neuronal differentiation[23].

Acetylation is another important histone modification, which has impact on transcriptional activation by disrupting the electrostatic interaction between histone and the DNA backbone and acting as a docking site for the recruitment of transcriptional co-activators[23]. Zhu and colleagues formulated a chemical cocktail containing NaB (a histone deacetylase inhibitor, HDACi) that, combined with the ectopic expression of Oct4, converted adult human dermal fibroblasts into human iNSCs[30]. Similarly, another HDACi (Trichostatin A, TSA) combined with 5-aza-2-deoxycytidine (5-aza-dC) dedifferentiated NIH/3T3 fibroblasts into neuron-like cells with RA supplement[27].

Polycomb-group proteins are a family of proteins that remodel chromatin such that epigenetic silencing of *Hox* genes takes place. The Bmi-1 polycomb ring finger protein promotes NSC self-renewal and maintains the pluripotency of NSCs through the cooperation of Bmi-1 and Foxg1[21].

MicroRNAs MicroRNAs (miRNAs) are non-coding RNAs that range in size from 17 to 25 nucleotides and function as important post-transcriptional gene regulators. They also play essential roles in neuronal development and function. MiRNAs interact with gene regulatory motifs to regulate the balance between neural progenitor self-renewal and differentiation[31].

Recently, a set of brain-enriched microRNAs such as miR-9 and miR-124 have been found to promote the conversion of the non-neuronal fate of fibroblasts towards neurons[32]. MiR-9 regulates neural progenitor proliferation and differentiation by targeting Foxg1, Tlx, and Gsh2, among others. The overexpression of miR-9 promotes NSC differentiation by down-regulating Tlx expression, forming a double negative feedback loop with Hes1[33] and suppressing Gsh2 and Foxg1 expression to negatively control progenitor proliferation[34]. MiR-124 is another brain-enriched miRNA. Both loss-of-function and overexpression studies have reported that miR-124 is a promoter of neuronal differentiation and an inhibitor of progenitor self-renewal[33]. Reported main targets of miR-124 to establish neuronal programs include Sox9 and Jag1(a Notch ligand)[33]. The induction of transdifferentiation from fibroblasts to functional neurons can be accomplished *via* the action of miR-124[32].

In general, while miR-124, miR-125b, miR-137, miR-9, and let-7 promote neuronal differentiation, other miRNAs such as miR-134 and miR-184 have been implicated in neural progenitor maintenance and proliferation[31].

The molecular network regulating the proliferation and differentiation of NSCs is complex. The links between transcription factor expression and epigenetic modifiers require further studies. Research on the regulatory pathways of proliferation and differentiation of NSCs may help find effective target points for direct transdifferentiation. Meanwhile, treatment with reprogramming factors on NSCs induces neuronal differentiation to obtain the neuron subtype that is needed. The network of several main signaling molecules[15, 21, 25, 33-37] that may regulate the proliferation and differentiation of NSCs is shown in Figure 1.

Methods of Direct Conversion to iNSCs/iNPCs

Independent groups have demonstrated that directly-induced transdifferentiation technology is capable of converting somatic cells into iNSCs/iNPCs by transient insertion of varied combinations of factors through different methods of transfection, including viral vectors, non-viral plasmids, mRNAs, proteins, and small molecule compounds. Each approach has its avantages and disadvantages.

Viral Vector Transfection

At present, the most commonly used method of transfection is viral, including lentiviral, adenoviral, and retroviral vectors. The ideal viral vector should be packaged into infectious particles and be capable of carrying exogenous genes, mediating transfection, and expressing exogenous genes with high efficiency. Adenoviral vectors infect target cells without insertion into the host genome, thus the duration of transgenic expression is short. Unlike adenoviral vectors, retroviral and lentiviral vectors insert into the host genome, which leads to a long duration and stable expression of exogenous genes, but they can only infect dividing cells. Lentiviral vectors may be a better expression system, which infects a broader range of host cells, including dividing cells and non-dividing cells like neurons.

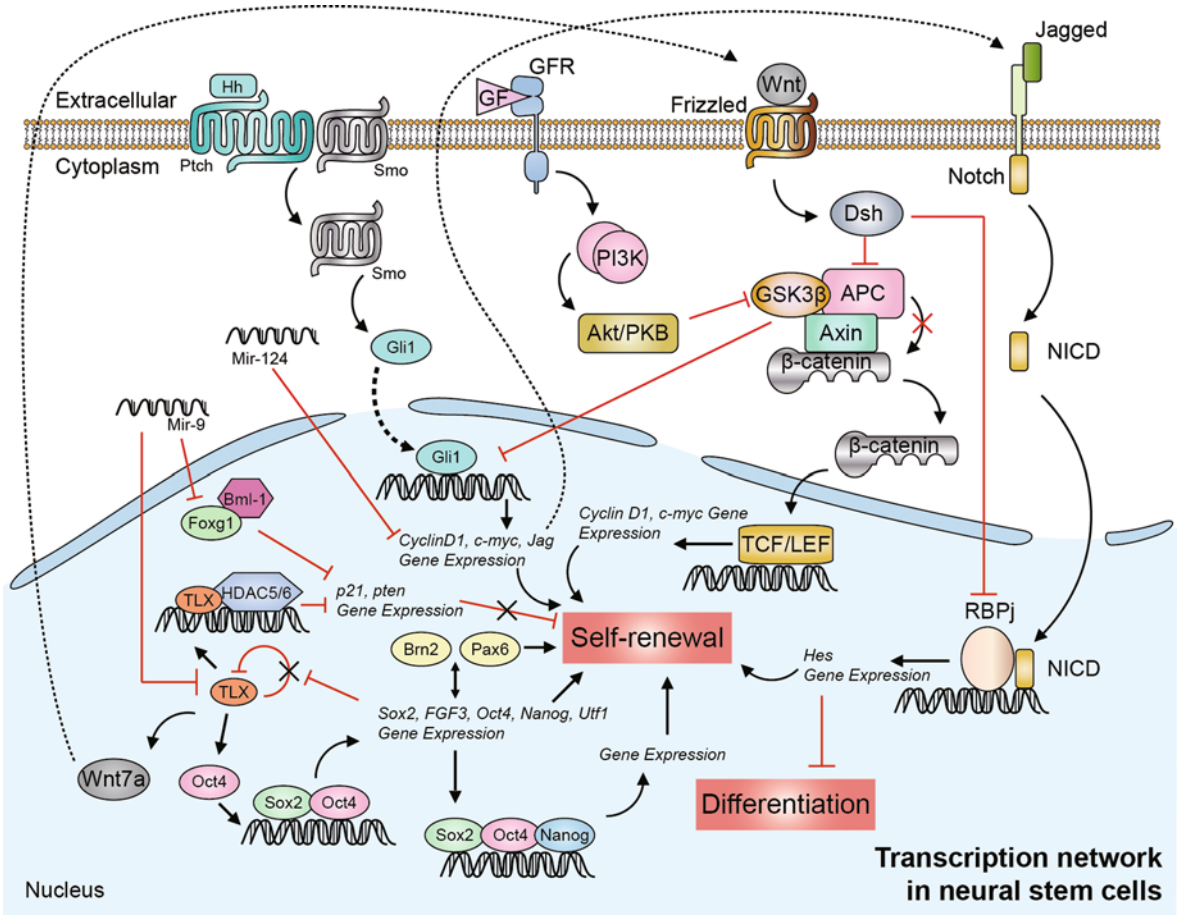

Fig. 1. A possible molecular network regulating the proliferation and differentiation of NSCs. The network includes extracellular signal pathways (such as Wnt, Notch, Shh, and GF) and transcription factors (such as Sox2, Hes, and Numb). The Wnt signaling pathway[25] starts with the combination of the Wnt ligand and receptor (Frizzled) to inactivate a degradation complex comprising Axin, APC, and GSK-3β. In the absence of ongoing β-catenin degradation, stabilized β-catenin enters the nucleus and associates with TCF/LEF transcription factors, resulting in transcription of the *CyclinD1* and *c-myc* genes. In the Notch pathway[25], Notch receptors are activated by ligands (Jagged) resulting in the release of NICD into the cytosol, then this translocates into the nucleus to form the NICD–RBPj complex, which in turn acts as a transcriptional activator and induces the expression of the *Hes* gene and others. Shh signaling[25] acts *via* a receptor complex consisting of Ptch and Smo; after Shh ligand binding to Ptch, released Smo activates the transcription of Gli proteins and other Shh target genes. Various kinds of growth factors function to inhibit the action of GSK-3β by the Akt/PKB pathway. The nuclear orphan receptor Tlx recruits HDACs to repress downstream target genes, including *p21* (cyclin-dependent kinase inhibitor) and *pten* (tumor suppressor gene), which in turn regulate NSC proliferation[35]. Tlx also activates the Wnt signaling pathway, promotes transcription of the *Oct4* gene, and can be inhibited by action of the Sox2 transcription factor. Sox2 and Oct4 coordinate to promote expression of the *Nanog*, *Utf1*, and *Fgf4* genes to regulate the mechanism that maintains the pluripotency of stem cells[15]. MiR-9 regulates neural progenitor proliferation and differentiation by targeting Foxg1, Tlx, and Gsh2, among others[34], while miR-124 targets Sox9 and Jagged[32]. APC, adenomatous polyposis coli; GSK-3β, glycogen-synthetase-kinase-3β; TCF, T-cell factor; LEF, lymphoid enhancer-binding factor; NICD, notch intracellular domain; RBPj, recombination signal binding protein for immunoglobulin κ J region; Hes, hairy and enhancer of split; Shh, sonic hedgehog; Ptch, patched; Smo, smoothened; GF, growth factor; GFR, growth factor receptor; Akt/PKB, protein kinase B; HDACs, histone deacetylases.

Wernig and colleagues infected mouse fibroblasts with lentiviral vectors containing neuronal lineage-inducing transcription factors and efficiently converted the fibroblasts into iNPCs[2]. Ding and colleagues also used lentiviral

vectors to transfect four transcription factor genes (*Oct4*, *Sox2*, *Klf4*, and *c-myc*) into mouse embryonic fibroblasts, and converted them directly into iNSCs and iNPCs[1]. By first infecting mouse embryonic fibroblasts with lentiviral vectors carrying the *FUW-Oct4* and *M2rtTA* genes and then giving a second transfection with three transcription factor genes (*Sox2*, *Klf4*, and *c-myc*) using retroviral vectors, Their and colleagues induced transdifferentiation of the target cells into neuron-like cells with morphological and molecular characteristics similar to NSCs directly isolated from brain[4].

In fact, multiple independent experiments have demonstrated the feasibility of direct transdifferentiation from somatic cells into iNSCs/iNPCs (Table 1).

Non-viral Plasmid Transfection

It has been demonstrated that insertion of a transgene into target cells is not necessary during the transdifferentiation process. Transient expression of transdifferentiation factors transfected by non-viral vectors is also capable of direct reprogramming. Compared to viral vector transfection, this method has a lower risk of mutagenicity and tumorigenicity.

Xu and colleagues reported the successful generation of iNPCs from fetal pig fibroblasts using non-integrative episomal vectors expressing reprogramming factors (Oct4, Sox2, Klf4, Lin28, and L-myc) without going through a pluripotent state, and showed lower tumorigenicity[38].

RNA or Protein Transfection

Another way to avoid changes in the target cell genome is to induce the reprogramming process through direct transfection of the mRNA or protein of transdifferentiation factors. RNA or protein transfection is safer than viral or plasmid transfection, but transient expression leads to lower transdifferentiation efficiency.

Maucksch and colleagues demonstrated that transient insertion of the transcription factors Sox2 and Pax6 into adult human fibroblasts by protein transduction allows the generation of iNPCs expressing a range of neural stem and pro-neural genes, and can give rise to neurons that exhibit typical neuronal morphologies and express multiple neuronal markers[39]. Yakubov and colleagues presented a method that used transfection of the synthesized RNA of four transcription factors (Oct4, Sox2, Klf4, and c-myc) to

Table 1. Independent experiments showing direct transdifferentiation from somatic cells to iNSCs/iNPCs

Starting Cells	Transgenes	Method	Results	Reference
MEF	*Oct4, Sox2, Klf4, c-myc*	Lentivirus	iNPCs	Ding *et al.*, 2011[1]
MEF	*Sox2, FoxG1, Brn2*	Lentivirus	iNPCs	Wernig *et al.*, 2011[2]
MSC	*Ascl1, Ngn2, Hes1, Id1, Pax6, Brn2, Sox2, c-myc, Klf4*	Retrovirus	iNSCs	Sheng *et al.*, 2011[3]
MEF	*Oct4, Sox2, Klf4, c-myc*	Lentivirus Retrovirus	iNSCs	Their *et al.*, 2012[4]
MF	*5F (Brn4, Sox2, Klf4, c-myc, E47)* *4F (Brn4, Sox2, Klf4, c-myc)*	Pmx Retrovirus	iNSCs	Han *et al.*, 2012[5]
MEF; HFF	*Sox2*	Retrovirus	iNSCs	Ring *et al.*, 2012[6]
HCA	*Oct4, Sox2, Nanog, Lin28*	Lentivirus	iNSCs, N	Corti *et al.*, 2012[7]
HAF	*Oct4*	Lentivirus	iNPCs	Mitchell *et al.*, 2014[8]
HFF	*Sox2, c-myc, Brn2/Brn4*	Lentivirus	NRPs	Zou, 2014[9]
MEF	*Oct4, Sox2, Klf4, c-myc*	Lentivirus	iDPs	Kim *et al.*, 2014[10]
HDF	*Sox2, HMGA2, myc, Lin28*	Retrovirus	iNSCs	Yu *et al.*, 2015[11]

MEF, mouse embryonic fibroblasts; MF, mouse fibroblasts; HFF, human fetal fibroblasts; HAF, human adult fibroblasts; HCA, human cortical astrocytes; MSC, mouse Sertoli cells; HDF, human dermal fibroblasts; N, neurons; iNSCs, induced neural stem cells; iNPCs, induced neural precursor cells; NRP, neuronal restricted progenitors; iDPs, induced dopaminergic neuronal progenitors.

reprogram human fibroblasts into iPSCs[40]. Although there has not been any report on the transfection of somatic cells with modified mRNAs encoding reprogramming factors to generate iNSCs/iNPCs, we still predict that this technique has potential for safer clinical applications.

This method avoids gene insertion and may be developed to replace the use of DNA vectors in the formation of iNSCs/iNPCs.

Epigenetic Modifications Using Small Molecule Compounds

Epigenetic modification is a key determinant to complete the process of transdifferentiation using epigenetic small molecules instead of transcription factors. These epigenetic molecules regulate the reprogramming process through DNA methylation and histone modification, which has been demonstrated to promote transformation efficiency combined with specific transcription factors in a series of transdifferentiation studies.

A recent study showed that infection of postnatal and adult human and monkey fibroblasts with Sendai virus containing the Yamanaka factors (Oct3/4, Sox2, Klf4, and c-Myc), cultured in a chemically-defined medium containing leukemia inhibitory factor, the transforming growth factor-β (TGF-β) inhibitor SB431542, and the glycogen synthase kinase-3β (GSK-3β) inhibitor CHIR99021, caused the generation of iNPCs[41]. Zhu and colleagues identified a small-molecule combination of A83-01 (a TGF-β inhibitor) and CHIR99021 that enabled reprogramming of Oct4/Sox2-transduced human neonatal fibroblasts into colonies expressing the human NSC marker Pax6[30]. They also found that a combination of lysophosphatidic acid (a phospholipid derivative), rolipram (a phosphodiesterase-4 inhibitor), and SP600125 (a c-Jun N-terminal kinase inhibitor) facilitated the reprogramming of adult human dermal fibroblasts transduced with Oct4 alone[30]. Cheng and colleagues reported that iNPCs can be generated from mouse embryonic fibroblasts using a chemical cocktail, VCR (VPA, an inhibitor of HDACs; CHIR99021; and RepSox, an inhibitor of TGF-β pathways), under physiologically hypoxic conditions. Further experiments showed that another combination of inhibitors of histone deacetylation, glycogen synthase kinase, and TGF-β pathway molecules had similar efficacy for induction. Thus their studies demonstrated that lineage-specific conversion of somatic cells to NPCs can be achieved using chemical cocktails without introducing exogenous factors[42].

Biochemical Agents

Some biochemical agents can also be used to induce direct transdifferentiation or enhance this process when combined with transcription factors, such as chemicals and cytokines.

The main categories of chemical inducers of transdifferentiation into iNSCs/iNPCs are antioxidants and calcium channel blockers. Cai and colleagues examined the most representative antioxidant, β-mercaptoethanol, and found that it induces adipose-derived stromal cells to rapidly and efficiently differentiate into neurons *in vitro*[43]. At the same time, calcium channel blockers play a role in transdifferentiation. Besides, numerous traditional Chinese medicines also appear to be inducers of the transdifferentiation process, such as *Lycium barbarum* polysaccharide[44], *Salvia miltiorrhiza*[45], and *Rehmannia glutinosa* polysaccharide[46] with antioxidation similar to β-mercaptoethanol, and *Panax notoginseng* saponins[47], ligustrazine[48], and salidrosides[49], which belong to the calcium channel blockers. However, because of the toxicity and short survival time of the differentiated cells, the clinical applications of chemical inducers are limited.

The most commonly used cytokines in iNSC/iNPC technology are basic fibroblast growth factor, neurotrophic factor, brain-derived neurotrophic factor, nerve growth factor, and RA. These cytokine inducers often play a supplementary role during the transdifferentiation process when used in combination with transcription factors to improve the efficiency. Zhang and colleagues elicited the generation of neuron-like cells by exposure of reprogrammed cells to RA-containing medium[27].

Applications of Somatic Cell-Derived iNSCs/iNPCs in Neurodegenerative Diseases

Current therapies for neurodegenerative diseases are restricted to controlling symptoms, and their long-term use is limited due to the inevitable side-effects. At present, there is no effective treatment to prevent or delay the clinical progression of these diseases.

The iNSCs/iNPCs derived from patients have potential value in both fundamental studies and clinical applications. Application of iNSCs/iNPCs would be very useful in various

fields, such as obtaining target cells for transplantation therapy, establishing disease models, and drug screening, as well as for monitoring curative effects.

Cell Transplantation Therapy

At present, therapies for neurodegenerative diseases mainly rely on drug treatment, but it is difficult for drugs to pass through the blood-brain barrier and target the location of neuronal loss, so the curative effects are limited. As a novel approach to neurodegenerative diseases, cell transplantation therapy has proven effective in animal disease models[4, 50]. The ideal cell resource should have the following characteristics: the ability to self-renew, proliferation capacity *in vitro*, differentiation into the target cell type, and having a low tumorigenic risk. To realize this therapeutic strategy, iNSCs/iNPCs may be safer and more therapeutically effective. Compared with iPSCs, iNSCs/iNPCs have a lower risk of tumorigenesis, while maintaining the capacity of self-renewal *in vitro*, the ability to give rise to multiple neuronal subtypes, and higher survival after transplantation than iNs.

For example, Thier and colleagues have addressed the question of whether iNSCs are suitable for cell replacement. They transplanted iNSCs derived from mouse fibroblasts through overexpression of transcription factors (Sox2, Klf4, and c-myc) into the left and right hemispheres of neonatal myelin-deficient rat brain, and the results clearly demonstrated that grafted iNSCs survived and gave rise to differentiated neurons in *vivo*[4].

Parkinson's disease Parkinson's disease (PD) results from greatly reduced activity of dopamine-secreting cells caused by cell death in the substantia nigra. At present, the main treatment is replacement therapy of with levodopa, which is effective in the early stage. With disease progression, the effect is reduced and a series of movement complications occurs. Because of the specific injury site, cell transplantation therapy is currently the most promising treatment for PD.

Aleksandra and colleagues transfected mesenchymal stem cells isolated from bone marrow with the *Notch1* gene (NICD), resulting in human bone marrow-derived neural progenitors, which have the potential to elicit the recovery of damaged dopaminergic and serotoninergic neurons in a partial lesion rat model of PD[51].

Multiple sclerosis Multiple sclerosis is an inflammatory disease in which the insulating covers of nerve cells in the brain and spinal cord are damaged. The symptoms may be ameliorated by stem cell therapy. Luca and colleagues converted mouse somatic skin fibroblasts into iNSCs, which displayed significantly high intrinsic migratory features and anti-inflammatory capacity when co-cultured with lipopolysaccharide-activated macrophages. After the injection of iNSCs, chronic experimental allergic encephalomyelitis in mice is ameliorated[50].

In addition, stem cell transplantation therapy has also made certain progress in animal models of other neurodegenerative diseases such as Huntington's disease[12] and amyotrophic lateral sclerosis (ALS)[52]. iNSCs/iNPCs possess features that could solve some of the main problems in stem cell therapy. But there is still a lack of experimental evidence demonstrating the effectiveness of iNSC/iNPC transplantation therapy for the above diseases. The key to the success of iNSCs/iNPCs transplantation in the treatment of neurodegenerative diseases is how to promote the differentiation of iNSCs/iNPCs into the target neuronal subtypes needed. In the future, more research will be needed to confirm its advantages.

Neurodegenerative Disease Models

In vitro neurodegenerative disease models have potential applications for observing disease initiation and progression, studying the pathophysiological mechanisms, and screening for new drugs. Patient-derived iNSCs/iNPCs that carry the disease genotype can be a very powerful and convenient tool to establish neurodegenerative disease models *in vitro*, including cell-based and molecular-based models. Differentiation of patient-specific stem cells carrying disease-specific genes has enabled the establishment of neurodegenerative disease models caused by certain multiple gene mutations. Compared with the traditional methods, these patient-specific models are much closer to the real situation, allowing investigation of the relevant neuronal phenotypes, and serving as a platform for new attempts to benefit human neural development, tissue repair and regenerative medicine, and disease modeling, in addition to a powerful tool for personalized drug tests[53].

Studies of the pathophysiology mechanism of neurodegenerative disease First of all, disease-associated specific phenotypes can be investigated by comparing the induced neural cells in patients and unaffected individuals.

Research on *in vitro* disease models established with patient-derived induced neural cell lineages has indicated a series of pathogenic gene mutations. Overexpression or inhibition of certain genes by transgenic technology can demonstrate the roles of these genes in pathological processes. Then knock-in or knock-out of a certain gene in induced neural cells by gene targeting technology excludes the influence of patients' genetic background. Therefore, the combined application of gene targeting and induced transdifferentiation technology is an important approach to studying the relationship between a single mutation and neurodysfunction.

iNSC/iNPC technology can be used to model neurodegenerative diseases such as ALS. In a recent study, fibroblasts from ALS patients and age-matched healthy controls were converted to iNPCs by transfection with four reprogramming factors (Oct4, Sox2, Klf4, and c-myc), and they subsequently had the potential to generate motor neurons (iMNs) and astrocytes (i-astrocytes). In addition, astrocytes carrying the *C9orf72* mutation displayed toxicity toward iMNs, thereby corroborating a crucial role of this cell type in ALS pathogenesis. Furthermore, these findings demonstrated that the toxicity is an intrinsic property of ALS patient-derived astrocytes that is independent of the neuro-inflammatory environment of the end-stage ALS spinal cord. Co-culture of i-astrocytes and iMNs now provides a tool for testing pathogenic hypotheses and opens the door to personalized modeling of toxicity in ALS[52].

We predict that, in future, it may be feasible to evaluate the pathophysiological mechanisms of neurodegenerative diseases using iNSC/iNPC models, while animal disease models cannot perfectly mimic the development and progression of certain diseases.

Drug screening The *in vitro* cell-based models of neurodegenerative diseases are advantageous over primary neuronal cultures or transformed cell lines, holding promise for high-throughput screening of candidate drugs on patient-derived neurons carrying specific phenotypes, which come from the differentiation of iNSCs/iNPCs. The aim is to screen for a series of drugs that can improve or restore normal neuronal phenotype and function, especially among the drugs have already obtained approval in clinical trials, and can be directly used in clinical treatment once found to be effective.

Phenotypic changes caused by a certain disease mainly appears in mature cells (such as dopaminergic neurons and motor neurons), resulting in the need for a time-consuming process to test target drugs that are not conducive to high-throughput screening. Proliferative iNSCs/iNPCs can make up for these shortcomings. Recently, *in vitro* cell-based models such as embryonic stem cells[54] and iPSCs[55] have been used for the rapid screening of drug candidates for potential therapeutic effects and toxicity. So far, *in vitro* models derived from iNSCs/iNPCs of neurodegenerative diseases have been established and gradually improved, but have not yet been reported in the field of drug screening. In future studies, drug screening experiments should be designed based on neural cells at an early stage of differentiation, i.e. NSCs or NPCs.

Challenges in the Application of iNSC/iNPC Technology

The direct conversion of somatic cells into iNSCs/iNPCs provides a more convenient, efficient, and safer cell source for the clinical treatment of neurodegenerative diseases. It is also a new tool to study the pathological mechanisms of neurodegenerative diseases. However, research of this method is still in the preliminary stage, and many problems need to be solved before its clinical application.

Reducing the Risk of Tumorigenesis

The oncogenes *c-myc* and *Klf4* play important roles in controlling the stemness of NSCs, while their sustained expression might lead to a tumorigenic tendency in target cells. Several recent experiments have shown that c-myc and Klf4 are dispensable for the production of iNSCs/iNPCs[2, 6-8], although the reprogramming process is significantly delayed and less efficient in the absence of these oncogenes. The process of direct conversion of somatic cells into iNSCs by a single factor, Sox2, does not generate tumors[6].

Risks of Viral Vectors

The viral vector is the most commonly used tool in transfection technology. But its clinical application is limited due to the potential risk of DNA damage caused by integration of exogenous transgenes into the host genome and the potential tumorigenesis associated with such DNA damage.

In order to improve the safety of transdifferentiation technology, the viral vectors can be replaced with inducible expression vectors[56] to regulate transgene expression, non-integrating plasmid vectors[38], small molecule compounds[27, 30, 41, 42], mRNA[40] or protein[39], to make the transfection system safer and more efficient for clinical trials.

Impurity of the Transdifferentiation Product

The transdifferentiation product is not pure, but contains a mixture of pluripotent cells, cells with different degrees of differentiation[57], and even untransformed cells. Those untransformed cell colonies may result from incomplete reprogramming or the unstable status of iNSCs/iNPCs which may return to the initial state. Future research should attempt to optimize the conditions for induction and cell culture, promote the process of complete cell transdifferentiation, and maintain the long-term status of iNSCs/iNPCs.

Difficulty in Controlling the Differentiation Direction of iNSCs/iNPCs

iNSCs/iNPCs are capable of differentiating into three main neural lineages, neurons, astrocytes, and oligodendrocytes[1, 3, 4, 6]. However, it is difficult to regulate the differentiation process in the direction needed.

Neuronal restricted progenitors (NRPs) are a type of transitional intermediate cells that lie between multipotent neural progenitors and terminally-differentiated neurons during neurogenesis[9]; these may be an ideal source for transplantation, as they only differentiate into neurons, rather than glial cells and other cell types. Lai and colleagues provided evidence of the direct conversion of primary human fibroblasts into NRPs by three defined factors, Sox2, c-myc, and Brn2/Brn4. When injected into the subventricular zone, the human induced NRPs migrated widely and integrated into different encephalic regions, differentiated into various neuronal subtypes but not glial cells, and contributed to the repair of the brain[9]. Their research provides a new source of cells for cellular replacement therapy of neurodegenerative diseases.

Conclusions

The generation of iNSCs/iNPCs provides a unique platform for the fundamental study and clinical treatment of neurodegenerative diseases. Further research should focus on better protocols for direct transdifferentiation, clinical applications of iNSCs/iNPCs, timing and efficiency of transdifferentiation, and sufficient amounts of target cells for transplantation therapy.

A series of studies has shown the process of direct transdifferentiation from somatic cells to iNSCs/iNPCs by different combinations of exogenous factors, involving defined transcription factors and epigenetic modifications that activate the expression of target genes. A key point to promote the development of iNSC/iNPC technology is combining these defined factors with high conversion efficiency. The applications of iNSCs/iNPCs vary among fields, such as obtaining target cells for transplantation therapy, establishing disease models, drug screening, and monitoring curative effects. However, many questions remain to be answered.

ACKNOWLEDGEMENTS

This review was supported by the National Natural Science Foundation of China (81271248 and 81400933).

Received date: 2015-01-26; Accepted date: 2015-04-01

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