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·Review·

Direct lineage conversion of astrocytes to induced neural stem cells or neurons

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Since the generation of induced pluripotent stem cells in 2006, cellular reprogramming has attracted increasing attention as a revolutionary strategy for cell replacement therapy. Recent advances have revealed that somatic cells can be directly converted into other mature cell types, which eliminates the risk of neoplasia and the generation of undesired cell types. Astrocytes become reactive and undergo proliferation, which hampers axon regeneration following injury, stroke, and neurodegenerative diseases. An emerging technique to directly reprogram astrocytes into induced neural stem cells (iNSCs) and induced neurons (iNs) by neural fate determinants brings potential hope to cell replacement therapy for the above neurological problems. Here, we discuss the development of direct reprogramming of various cell types into iNs and iNSCs, then detail astrocyte-derived iNSCs and iNs *in vivo* and *in vitro*. Finally, we highlight the unsolved challenges and opportunities for improvement.

Keywords: astrocyte; direct lineage conversion; induced neural stem cells; induced neurons; reprogramming; transcription factor; vector

Introduction

Cerebrovascular diseases, neurodegenerative diseases, and traumatic brain injury are common neurological problems. All lead to different levels of regression of the brain parenchyma and many types of neurons, astrocytes, and oligodendrocytes. Limitations and insufficiencies of current clinical therapies prevent effective tissue repair and functional recovery, which brings a burden of death, disability, and economic loss every year. Therefore, approaches to functional reconstruction of damaged brain tissue are urgently needed. Recently, transplantation of exogenous neural stem cells (NSCs) and stimulation of endogenous neurogenesis have been shown to be promising approaches for treating such damage. However, several issues restrict their clinical applications, such as a lack of reliable resources, ethical issues, immunological rejection after transplantation, integration into neuronal circuits, scarcity of the neuronal repopulation or reduced brain regeneration, and difficulty of new neurons to reaching and repopulating target sites[1].

In 2006, Takahashi and Yamanaka reported the production of induced pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblasts by introducing four transcription factors, and further demonstrated that iPSCs exhibit the morphology and growth properties of embryonic stem cells (ESCs) as well as expressing marker genes of ESCs[2]; this has opened up a new era of regenerative medicine. This cellular reprogramming of fibroblasts, one type of terminally differentiated cells, into iPSCs is a novel technique to produce patient-specific cells for autologous transplantation, establish human disease models, and assist drug screening. Thus since then, research teams worldwide have been enthusiastic about iPSC research. However, Ben-David and Benvenisty reported that the capabilities of self-renewal and multilineage differentiation,

two properties of iPSCs, make them tumorigenic[3]. Also, the transcription factors commonly used for reprogramming are highly expressed in various types of cancers and integrating vectors increase the risk of genetic alterations[3]. So overcoming these limitations is paramount before the widespread therapeutic use of iPSCs. Taking into account these limitations, research workers are now striving to investigate the possibility of direct reprogramming of a committed differentiated cell into targeted cell lines. This emerging technology seems promising for stem cell-based approaches. It has already been reported that human and mouse fibroblasts can be converted into cardiocytes[4], hepatocytes[5] and neurons[6] by transduction with defined factors. Other cell types, such as astrocytes, can be directly reprogrammed into neuronal lineage cells. Astrocytes are the most plentiful cells in the human brain and play a vital role in glial scar formation following neurological insults. Glial scarring, known as reactive astrogliosis, on one hand protects neuronal networks from further damage[7] and on the other hand acts as a primary barrier to functional regeneration[8]. In addition, properties such as cell proliferation and proximity in lineage distance make astrocytes the ideal candidate cell-type to transdifferentiate into neurons[9].

Thus, in this review we mainly focus on recent advances in the direct lineage switching of astrocytes to induced neural stem cells (iNSCs) or induced neurons (iNs), then explain the limitations in this field and finally discuss the possible improvements before its application in the clinical setting.

Historical Perspective on Direct Reprogramming from Various Cell Types to Nervous System Cells

Since 2010, many labs have succeeded in directly switching the identity of one cell type to neurons and NSCs by expression of appropriate transcriptional factors with or without the assistance of specific environmental signals (Fig. 1). Vierbuchen *et al.* have converted mouse embryonic and postnatal fibroblasts into functional neurons, which express markers of cortical identity, form functional synapses, and generate action potentials, by the ectopic expression of three transcription factors, Ascl1, Brn2, and Myt1l[10]. The conversion rate was up to 19.5%. However, further exploration is needed, for only a few induced neurons have been identified as GABAergic neurons, without other types of iNs. Recent progress has generated mouse ESC-derived GABAergic neurons[11], glutamatergic neurons[12], motor neurons[13], layer V/VI corticofugal projection neurons derived from layer II/III callosal projection neurons[14], and fibroblast-derived induced dopaminergic (iDA) cells[15-17]. Not only mouse but also human fibroblasts have changed their cell types toward dopaminergic neurons[17]. The direct reprogramming of human somatic cells is an important step toward clinical application and autologous cell replacement therapy, avoiding ethical concerns and potential issues of immune rejection. Pfisterer *et al.* reported that human fibroblasts are directed toward functional neurons by a cocktail of transcription factors such as Ascl1, Brn2, and Myt1l[16]. The functional neurons present a dopaminergic phenotype

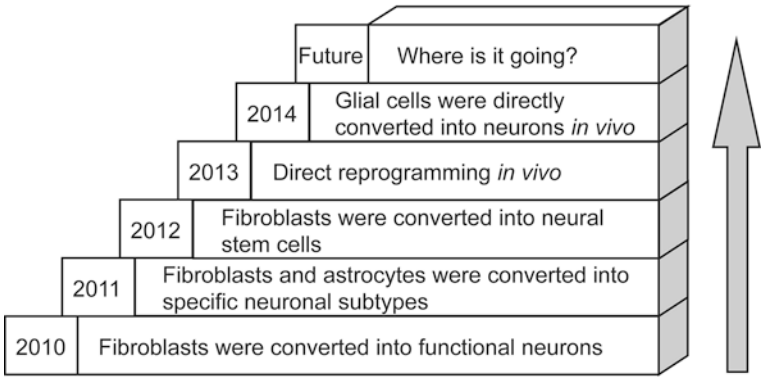

Fig. 1. The history of direct reprogramming of various cell types into iNSCs or iNs.

by expression of the dopamine fate-determining genes *Lmx1a* and *FoxA2*, with a conversion rate of ~10%. But this study did not further explore the properties of iDA cells and lacked *in vivo* functional analysis. However, Caiazzo *et al.* confirmed that iDA cells release dopamine and show spontaneous electrical activity[17]. In addition, Liu *et al.* not only have described the characteristics of iDA cells *in vitro* but also provided evidence of symptomatic relief like stabilization of rotational behavior in a rat model of Parkinson disease after iDA cell transplantation[15].

Although directly reprogrammed neurons open an intriguing possibility for cell-replacement therapy, considerable challenges need to be overcome. For example, some protocols yield a mixture of neuronal cells and other unknown cell types. Meanwhile, it should be noted that iNs are terminally differentiated and cannot proliferate, while iNSCs can self-renew and differentiate. Lee *et al.* reprogrammed fibroblasts into induced neurosphere-like cells using iNSCs line-derived cellular extracts under neurosphere culture conditions[18]. However, the trouble is that the reprogrammed cells undergo incomplete reprogramming, in that they do not possess all the properties of NSCs. For instance, one of the important properties of NSCs is the capacity to expand, whereas neural precursor cells (NPCs) show poor self-renewal, with no more than three passages. Another defect of NPCs is that they cannot differentiate into oligodendrocytes. To overcome these limitations, Lujan *et al.* successfully generated self-renewing tripotent NPCs, which produced neurons, astrocytes, and oligodendrocytes[19]. The limitation of this study is that there was no evidence for generating neurons and astrocytes *in vivo* and mature oligodendrocytes *in vitro* from NPCs. Recently, two groups have obtained iNSCs using different approaches. The iNSCs described by Thier *et al.* are multipotent, with high differentiation of astrocytes and neurons but rare occurrence of oligodendrocytes[20]; the differentiation rates are 100%, 30%, and 3% when iNSCs are cultured in different media. iNSCs can also self-renew and expand for >50 passages. Similarly, although the iNSCs generated by Han *et al.* can differentiate into all neural cell lineages, they also have low efficiency in oligodendrocytes differentiation[21]. Both Han *et al.* and Thier *et al.* used the transcription factor c-Myc, which may lead to unwanted reactivation of c-Myc. c-Myc, an oncogene, probably increases the risk of tumor formation. In the same year, Ring *et al.* converted fibroblasts into iNSCs with only one transcription factor, Sox2[22], reducing the number of activated oncogenes in the reprogramming process.

Many studies have focused on direct neural reprogramming *in vitro*. Later, it was reported that beta cell and cardiomyocyte conversion can occur *in vivo*[23,24] . So can postmitotic neurons change their phenotype from one subtype into another within the central nervous system? Rouaux *et al.* and Rossa *et al.* acquired corticofugal neurons from embryonic or early postnatal neurons using the transcription factor Fezf2 *in vivo*[14,25]. The fact that neurological disorders are more prevalent in the aging population raises a problem of whether the adult brain must overcome more obstacles when switching cell identity. It has been reported that astrocytes from adult brain can undergo fate changes *in vivo*[26-28]. Neuron-to-neuron and astrocyte-to-neuron are two major foci in the field of *in vivo* reprogramming. Actually, the literature has reported direct reprogramming of astrocytes into neurons *in situ*.

Directing Reprogramming of Astrocytes *in vitro*

As astrocytes are terminally differentiated, why have they been selected as a starting cell? The reasons are manifold[29]. First, they are spatially ubiquitous throughout the nervous system. Compared with the NSCs in the subventricular zone (SVZ) of lateral ventricles and the dentate gyrus of the hippocampus, the conscription of astrocytes is not confined by limited resources and sophisticated migration. Second, astrocytes share many characteristics with radial glia, the NSCs[30] capable of generating neurons, astrocytes, and oligodendrocytes. Third, injury and a favorable environment induce astrocytes into tripotent differentiating and self-renewing cells[31]. Accordingly, astrocytes are likely to be ideal starting cells for neuronal conversion in neuronal injury or neurodegenerative diseases.

There are two states of astroglia: quiescent and reactive. The difference between them is that quiescent astrocytes cannot divide, but resume proliferation and differentiation after being activated by injury[31] or other pathological conditions like stroke[32] and neurodegenerative disease[33]. The astrocytes activated under pathological conditions are reactive. The postnatal stage astrocyte,

which can retain potential for neurogenesis, is intermediate between radial glia and quiescent astroglia[29]. Indeed, an earlier study revealed that early postnatal astrocytes give rise to multilineage precursors and NSCs[34]. Several reports[35-38] have shown that reactive astroglia in the injured brain have increased plasticity and acquire the potential of NSCs. Activated astrocytes can self-renew and are able to differentiate into neurons, astrocytes, and oligodendrocytes *in vitro*[35,37,38]. However, Buffo *et al.* and Shimada *et al.* failed to verify that reactive astrocytes can generate neurons *in vivo*[31,37]. Nevertheless, reactive astrocytes still seem to be a promising cell type for further exploration of regenerative medicine.

Astrocytes are able to give rise to neurons or NSCs when provided with proper transcription cues *in vitro*[39-42]. Astrocytes from early postnatal cerebral cortex infected by Pax6-encoding virus transform into neurons[39]. However, there is no evidence of whether these neurons are functional and which types they belong to. Therefore, to further answer these questions, Berninger *et al.* started to study the physiological properties of astroglia-derived neurons[40]. They used the other proneural genes neurogenin-2 and Mash1 which are also able to induce astrocytes from early postnatal cerebral cortex toward a neuronal identity. The neurons derived from reprogrammed postnatal astrocytes *in vitro* did display the functional properties of neurons. They adopted the morphological and immunocytochemical characteristics of neurons and fired action potentials, but failed to establish spontaneous synaptic input within the culture period, indicating no synaptic activity. However, these neurons received functional synaptic input when co-cultured with cortical neurons, which suggests that spontaneous or evoked synaptic activity is regulated by some other molecular mechanisms. Given that iNs cannot generate presynaptic output, they fail to integrate into neuronal networks and thus cannot function in brain repair. This raised the question of whether astrocyte-derived neurons could be transformed into specific neuronal subtypes and finally restore a damaged neuronal network. Besides, Heinrich *et al.* generated neurons capable of establishing functional synapses following the expression of neurogenic fate determinants[41]. As presented in the report, Dlx2 directed postnatal cortical astroglia towards a transition to synapse-forming GABAergic neurons, while Neurog2 directed towards glutamatergic neurons. Inspiringly, the approach of expansion is not restricted to postnatal astroglia, and likewise could be applied to astrocytes from the injured cerebral cortex. By transduction with Neurog2 or Dlx2 *in vitro*, reactive astrocytes are driven towards fully-functional neurons which are able to establish functional connection. This might have vital implications for regeneration of the central nervous system using endogenous astroglia after brain injury or pathological conditions, and hence this may be closer to clinical application.

Directing Reprogramming of Astrocytes *in vivo*

In addition to studies demonstrating that reactive astrocytes or postnatal mouse astrocytes can be directly reprogrammed into neurons or stem-like cells by overexpression of transcription factors in culture, astrocyte-derived iNSCs and iNs have also been induced *in vivo* (Fig. 2). For the first time, Niu *et al.* demonstrated a feasible method for directly converting astrocytes into neuroblasts within mammalian tissues[27]. Different from the previous studies, most induced neuroblasts were derived from quiescent astrocytes, indicating that quiescent astrocytes also exhibit surprising plasticity *in vivo*. Niu *et al.* reprogrammed quiescent astrocytes into induced adult neuroblasts (iANBs) with the single transcription factor SOX2. However, few iANBs can differentiate into mature neurons unless they are supplied with brain-derived neurotrophic factor and noggin or the histone deacetylase inhibitor valproic acid, suggesting that a permissive microenvironment is critical for cell reprogramming. After facilitation, the differentiated neurons have electrophysiological functionality, so that they integrate into local neuronal circuitry. It is worth mentioning that the differentiation efficiency of iANBs is no more than 0.3%, so they are less valuable for clinical application compared to iNs. In another study, SOX2 was also capable of inducing resident astrocytes in the injured adult spinal cord into neuroblasts. The histone deacetylase inhibitor likewise promoted neuronal maturation, leading neuroblasts to form synapse-forming neurons *in vivo*[26].

Direct neuronal conversion has also been performed *in vivo*[28,43]. Torper *et al.* provided the first evidence of conversion of endogenous astrocytes to iNs following infection with Ascl1, Brn2a, and Myt11 Cre-inducible

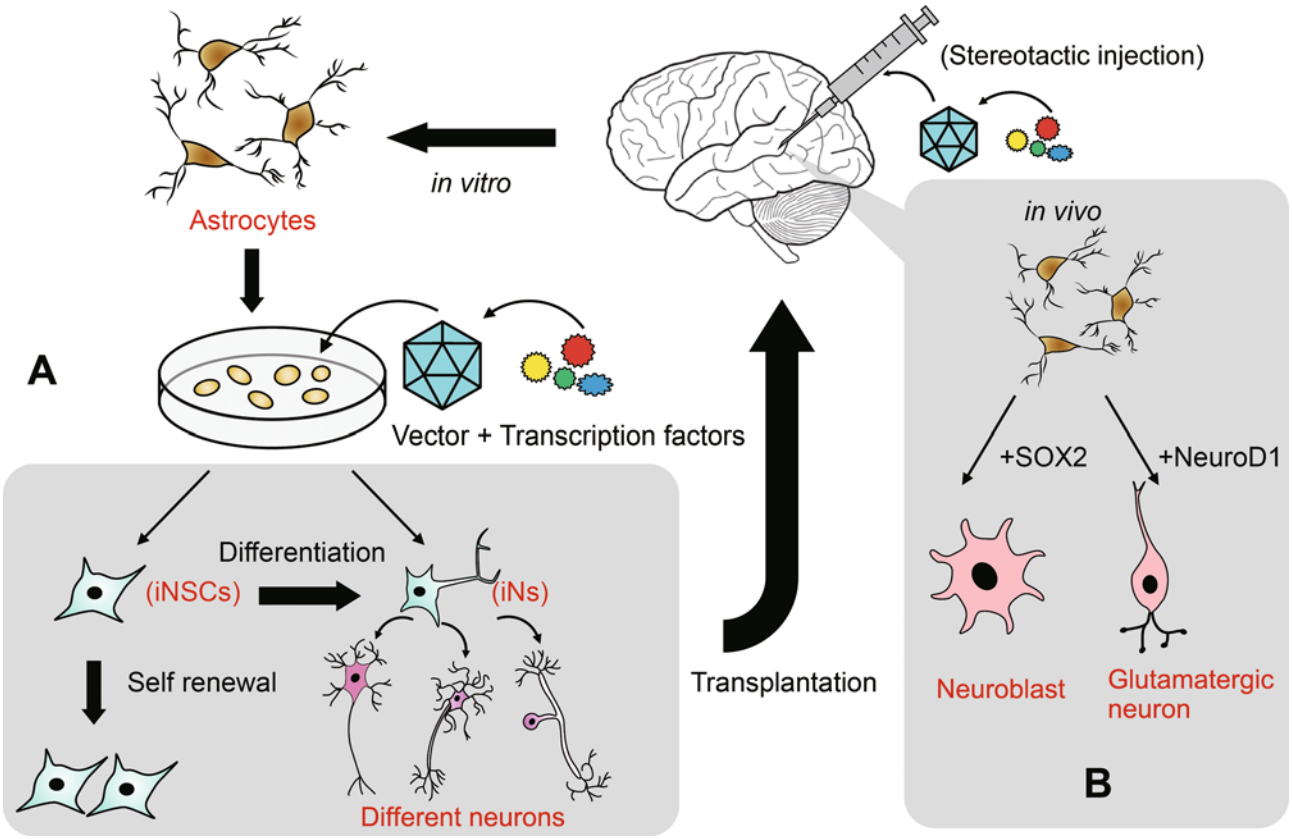

Fig. 2. Direct reprogramming of astrocytes into iNSCs or iNs. (A) Specific transcription factor-encoding vectors reprogram astrocytes *in vitro*. Then the targeted cells are transplanted into the central nervous system. (B) Examples of *in vivo* direct reprogramming of astrocytes into induced glutamatergic neurons by overexpression of NeuroD1 factor and induced neural stem-like cells by overexpression of SOX2.

lentiviral vectors[43]. The reprogrammed cells finally showed neuronal morphology and expressed NeuN. The restriction of this study is that the phenotypes and functions of the iNs were not explored. Recently, Guo *et al.* have made great strides in direct neuronal cell fate conversion[28]. Most importantly, in contrast to Torper *et al.*, Guo *et al.* used reactive astroglia cells in the cortex of stab-injured or an Alzheimer's disease (AD) mouse model as starting cells instead of resident astrocytes. Then functional neurons were generated by overexpression of just a single neuronal fate determinant, NeuroD1, which might reduce the risk of multiple transcription factors integrating into the host genes. Finally, Guo *et al.* applied retroviral vectors instead of lentiviral vectors to deliver NeuroD1. According to the report by Zhao *et al.*, retrovirus only infects proliferating cells but does not target quiescent cells such as normal astrocytes, neurons, and other normally-functioning cells in the brain[44]. The authors showed that NeuroD1 can reprogram reactive astrocytes in both injury and AD disease models into glutamatergic neurons. The NeuroD1-induced neurons display repetitive action potentials and spontaneous synaptic responses, suggesting that the converted neurons establish functional connections with surrounding neurons. Therefore, not only active but also chronic and progressive gliosis can be induced to undergo fate-change by extrinsic and intrinsic cues. Significantly, the more reactive astrocytes, the more reprogrammed cells, because of the greater increase of NeuroD1-induced neurons in the older AD model. Besides, the approach likewise enables human astrocytes to give rise to functional glutamatergic neurons in culture. Further, Lu *et al.* addressed a number of major issues that remain to be solved[45]. Besides the known

mechanisms and safety problems, two other problems are worthy of attention. One is whether the converted neurons can improve functional recovery in animal disease models or even in the human brain. The other is whether reactive astrocytes can be directed into other disease-specific neuronal subtypes such as motor and dopaminergic neurons.

iNSCs or iNs, Which Will Be a Therapeutic Option in Future?

We rate the current achievements in direct reprogramming highly. The most significant achievement that astrocytes are capable of direct conversion into iNSCs or iNs *in vivo* without cell transplantation is desirable for clinical application. Now, we consider the question of whether iNSCs or iNs are more appropriate for cell replacement therapy.

Both iNSCs and iNs have the potential to replace lost and damaged cells, but NSCs display several capabilities that help to promote neuronal repair. First, NSCs are anti-inflammatory[46-48]. For instance, Lee *et al.* demonstrated that NSC transplantation reduces cerebral inflammatory infiltration and attenuates the activation of inflammatory factors[46]. Second, Koraria *et al.* and De Feo *et al.* noted that NSCs have beneficial effects such as immune modulation and neuronal trophic support[49,50]. Third, NSC treatment enhances dendritic plasticity, increases axonal rewiring, and facilitates axonal transport, all of which are critical for axonal function and may improve functional recovery[51]. Fourth, anti-apoptosis is also involved. Xia *et al.* found that NSCs down-regulate the expression of apoptosis genes, coinciding with a decrease of apoptotic cells in lesions[52]. Finally, iNSC therapy protects the injured brain in mice with stroke partly through enhanced neurogenesis and angiogenesis[53]. In conclusion, the profiles of NSCs, such as anti-inflammatory function, modulation of the immune response, trophic effects, enhanced structural plasticity, anti-apoptosis potential, neurogenesis, and angiogenesis as well as self-renewal and differentiation, have attracted attention. However, the major challenge of directing the fate of iNSCs to specific neuronal subtypes to replace neurons lost to disease remains unsolved. Besides, the low differentiation rate[27] and tumorigenicity are stumbling blocks. Although the studies by Niu *et al.*, Corti *et al.*, and Ring *et al.* showed that iNSCs do not form tumors *in vivo*[22,27,42], iNSCs can lead to tumor formation if their growth is unlimited or growth control is impaired. Consequently, further studies are warranted to fully evaluate the tumorigenic potential of such therapy.

iNs are terminally-differentiated mature cells, thus circumventing the pluripotent stage, shortening the experimental procedures and avoiding re-differentiation processes[54]. So far, astrocyte-derived neurons include iDA, GABAergic, motor, and glutamatergic neurons. Parkinson disease, characterized by the loss of iDA neurons in the substantia nigra of the midbrain, shows symptomatic relief after treatment with DA neurons[15]. In a sense, iNs might hold promise as a replacement for iNSCs in certain diseases that are defined by the death and degeneration of specific subtypes of neurons.

Previous reports have demonstrated that iNSCs and iNs have potential applications in neurology, but problems still remain. First of all, are the reprogrammed cells identical to their endogenous counterparts? Do the converted cells maintain a memory of their original identity? If they do, how does that influence their functionality[9]? In conclusion, it is difficult to reach agreement as to which is better, since iNSCs and iNs each have their advantages and disadvantages.

Challenges for Direct Conversion of Astrocytes in the Central Nervous System

Although both iNSCs and iNs can be generated from astrocytes *in vitro* and *in vivo*, limitations such as viral infection and possible undesirable reactivation of transgenes prevent their clinical application. So this new and advanced technology is urgently in need of optimization mainly in three aspects: the choice of integrating vectors; the screening of reprogramming factors; and the mechanisms of astrocyte conversion into iNSCs or iNs.

Vectors

Given that viral vectors are comparatively efficient, researchers have principally used lentiviral or retroviral vectors for gene delivery in pre-clinical studies. Notwithstanding their efficiency, viral vectors also pose a series of risks including insertional mutagenesis, transgene integration, cell senescence, strong immunogenicity[55]

and viral infection. Now investigators are seeking an ideal vector for gene delivery to alleviate these potential risks. Most importantly, an ideal vector[56] would avoid vector-related side-effects and efficiently transduce the starting cells, tissues, or organs while minimally transducing unrelated targets. Then the ideal vector would express the transcription factors at appropriate levels and for a sufficient length of time without multiple transfections. Although the ideal vector has not been developed, these properties provide hints for currently-available vectors to further facilitate transgene expression and even reprogramming technologies. As the potential risks caused by transgene integration are the major concern, a series of studies have focused on polycistrons[57], the Cre-LoxP system[58], plasmids[59], episomal vectors[60], RNA, PiggyBac transposition[61], proteins[62,63], and adeno-associated virus (AAV) to eliminate or avoid gene integration and achieve safe delivery. Here, we cite several instances, beginning with more details about RNA. microRNAs not only play a crucial role in the transcription process in regulating gene expression after transcription, but also participate in the progress of reprogramming. For instance, it has been demonstrated that microRNA and neurogenic transcription factors induce the cell-fate change toward functional neurons[64]. In the same year, Ambasudhan *et al*. acquired human fibroblasts-derived neurons with a combination of miR-124 and two reprogramming factors, MYT1L and BRN2[65]. This indicates that microRNAs play an essential role in neuron formation, so it is thought that microRNAs can help develop a vector-free strategy. Co-transfection with miR-375 and miR-186 differentiates iPSCs to insulin-like cell clusters[66]. This provides hints for the direct conversion of astrocytes. Besides microRNAs, mRNA encoding reprogramming factors are transfected into human cells to induce pluripotency with high efficiency, but this needs repetitive transfections[67]. This mRNA-based technology shows no genome integration. The Cre-Loxp system is also under investigation. The transgenes are first integrated, then are excised from the reprogrammed genome by the Cre-Loxp system, reducing the potential for transcription factor integration[58]. Finally, we should mention that AAV is a promising vector for gene delivery. It exhibits no pathogenicity and high efficiency, so it has been widely used in animal experiments[68] and clinical trials[69]. However, AAV also faces challenges, such as controlling transgene expression and increasing cellular targeting specificity[70]. In fact, since viral and non-viral vectors have their pros and cons and an ideal vector seems far from reality, can differentiated cells change their fate using external stimuli only without the introduction of multiple transcription factors? A study has reported that chemical stimulation does work: using inhibitors of TGF-B pathways, glycogen synthase kinase, and histone deacetylation, workers have converted somatic cells to NPCs under physiological hypoxia without introducing exogenous factors[71].

Transcription Factors

As for transcription factor screening, optimized factors should be safe and have high efficiency. Reprogramming factors can result in uncontrolled reactivation and residual expression, which may finally lead to tumor formation and gene mutation. In terms of safety, most transcription factors, known as oncogenes, are associated with cancer. The stem-cell marker genes Sox2, Oct4, and Nanog have been detected in samples of bladder carcinomas, colon cancer, and prostate cancer, as well as other cancers[72]. Taking Sox2 as an example, it is overexpressed in squamous cell carcinomas in several tissues such as the lung[73], hypopharynx, larynx, and sinonasal area[74]. Besides safety, the combination of transgenes is also related to efficiency. In a study by Vierbuchen *et al*., the conversion rate in the Ascl1, Brn2, and Myt1l pool was 2–3-fold higher than that in the Ascl1, Brn2, Myt1l, Zic1, and Olig2 pool[10]. Taken together, transgene-free reprogrammed cells need further investigation, and may become an emerging strategy in regenerative medicine.

Mechanisms

In the years to come, other than developing ideal vectors and vector-free or transgene-free strategies, better understanding of the exact underlying mechanisms is needed. If all the mechanisms of the reprogramming process are clearly elucidated, the currently perplexing technological hurdles can be overcome and probably only simple signaling molecules can be used to regulate genetic manipulation.

Conclusion

The generation of iNs and iNSCs by direct reprogramming is a promising field for cell-replacement therapy in the central nervous system. Investigators can directly

reprogram astrocytes into neural lineage cells *in vitro* and even *in vivo* without passing through an undifferentiated pluripotent state, which is time-consuming and technically demanding. Importantly, increasing numbers of studies have focused on the direct reprogramming of astrocytes *in vivo* since 2013. We hypothesize that *in vivo* reprogramming is an alternative to *ex vivo* reprogramming. Compared with *in vitro* methods, *in vivo* reprogramming saves the trouble of cell culture and transplantation, benefits from the natural environment that can provide all the necessary molecular and spatial factors, and leads to the accurate reconstruction of endogenous cells, tissues, and even organs[75]. Therefore, direct reprogramming from astrocytes into iNSCs or iNs may be advantageous for treatment of the central nervous system. Despite the fact that several diverse cell types have been produced by astrocyte conversion, many more neuronal subtypes exist in the central nervous system. Therefore, it is necessary to facilitate the protocols to generate many other neuronal cell subtypes or induce iNSCs to differentiate into different functional neurons. Finally, improvement concentrated on safety and efficiency requires the development of a standard protocol that mainly involves a desired vector system and transcription factors. In the future, it may be possible to target cells by intravenous delivery of safe vector and transcription factors instead of by stereotactic injection. Even more, cells can be targeted by chemical stimuli[71]. Notwithstanding the many unsolved problems, direct reprogramming of astrocytes has profound implications in the field of neurological disorders.

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