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Direct lineage reprogramming to neural cells

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Abstract

Recently we have witnessed an array of studies on direct reprogramming that describe induced inter conversion of mature cell types from higher organisms including human. While these studies reveal an unexpected level of plasticity of differentiated somatic cells, they also provide unprecedented opportunities to develop regenerative therapies for many debilitating disorders and model these ‘diseases-in-a-dish’ for studying their pathophysiology. Here we review the current state of the art in direct lineage reprogramming to neural cells, and discuss the challenges that need to be addressed toward achieving the full potential of this exciting new technology.

Introduction

Regenerative medicine offers new avenues for developing effective therapies for many debilitating injuries and diseases, including neurodegenerations. Success of cell replacement therapy in this field may largely depend on readily available and unlimited supply of desired functional cell types. However, meeting such demands has been a challenge owing to the limited supply of required stem cells (e.g. hematopoietic stem cells, neural stem cells, mesenchymal stem cells, etc.), inefficient technologies to derive, maintain, and manipulate them, or ethical and political issues surrounding their use. Although other exciting technologies like somatic cell nuclear transfer (SCNT) [1,2] and cell fusion [3,4] were successful in experimentally generating pluripotent cells, their current state of the art is far from being useful for human applications. The search for new ways of obtaining stem cells met with considerable excitement when Yamanaka and colleagues showed that pluripotency can be induced by introducing a handful of transcription factors into fully differentiated somatic cells [5,6]. Ever since this groundbreaking discovery, the field of regenerative medicine has been growing in an unprecedentedly rapid pace.

The discovery of induced pluripotent stem cells (iPSCs) has not only offered a promise for realizing personalized cell-based therapy but also provided a platform to change the

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plasticity of differentiated cell types in human body. Notwithstanding the hope and hype surrounding this technology, many practical hurdles still remain before realizing its potential in regenerative medicine. Addressing these problems is a very active area of research in many laboratories including ours.

Meanwhile, the realization that the fate commitment of mature cells is reversible through defined and simple genetic manipulation has led many groups to search for alternative cell reprogramming strategies that are possibly faster, safer, and more efficient than iPSC technology. In this regard, it is logical to test whether one differentiated cell type can be directly converted (i.e. without passing through intermediate or pluripotent state) to another desired cell type. Indeed, the feasibility for experimentally eliciting such conversions in animal cells had long been reported [7]. For instance, Lassar and colleagues were able to show that introduction of a single transcription factor, *MyoD*, can convert fibroblast cells to skeletal myocytes [8,9]. For more such examples of transdifferentiation, see other excellent reviews [10•]. In the past few years, we witnessed an array of reports on forced direct conversion between somatic cells, within or outside their original lineage [11,12]. Direct conversions involve reprogramming of cell fate and this process is also called *lineage reprogramming* in order to distinguish it from *pluripotent reprogramming* that generates iPSCs.

As outlined in Figure 1, the current strategies for lineage reprogramming can be broadly classified into two groups: Somatic cell-specific factor-mediated Direct Reprogramming (SDR) where target somatic cell-specific factors (e.g. transcription factors, microRNAs, etc.) are used, and Pluripotent cell-specific factor-mediated Direct Reprogramming (PDR) that employs iPSC reprogramming factors (Oct4, Sox2, Klf4 and c-Myc; at least some or all of these factors are used). In this review, we will describe these two approaches in the context of neural lineage reprogramming, their applicability in studying and treating neural disorders, and finally we will discuss some of the outstanding challenges that remain in the field.

Lineage reprogramming to neurons

Many cell type-specific transcription factors are shown to be master regulators of cell fate during animal development [13]. This ability of these factors could be taken advantage of in experimentally manipulating cell fate. In fact, iPSCs were generated when twenty four ESC-specific transcription factors were tested to confer pluripotency in fibroblasts [5,6]. Previously, Anderson and colleagues had shown that ectopic expression of *Ngn1* in dermomyotome of chick embryo can induce neuronal morphology and marker gene expression in these cells [14]. Subsequently, Götz and colleagues reported neuronal features in mammalian astroglia overexpressing *PAX6* [15]. When they introduced *Ascl1, Ngn2* and *Dlx2* in neonatal astroglia these cells showed neuronal morphology, generated action potentials and exhibited functional synaptic properties [16]. These and other related studies [17–19] raised the possibility that more easily accessible and abundant cell types in mammals may be amenable for direct conversion to neural cell types by expressing neural specific transcription factors and such phenomenon could be exploited in regenerative medicine.

Indeed, in early 2010 Vierbuchen *et al*. elegantly showed that mouse dermal fibroblasts can be directly reprogrammed to functional neurons by forced expression of a set of neural lineage transcription factors [20••] The authors initially tested a pool of 19 transcription factors from which three (*Ascl1, Brn2* and *Myt1l*, abbreviated hereafter as BAM factors) were found sufficient to generate neuronal phenotype from fibroblasts. These induced Neurons (iNs) were capable of firing repeated trains of action potentials, formed functional synapses *in vitro* with a pre-existing network of cortical neurons and exhibited excitatory postsynaptic currents when co-cultured with astrocytes. Majority of these iN cells were excitatory glutamatergic in nature. This is particularly intriguing, given the role of *Ascl1* in specifying inhibitory neurons during brain development. Notwithstanding, this study provided the first demonstration that easily accessible cells like dermal fibroblasts can be robustly converted to functional neurons, and in doing so are capable of crossing the lineage boundaries established during the development. Another interesting feature of this phenomenon is that the conversion seems to be direct, that is, there is neither any involvement of cell divisions nor an occurrence of intermediate stages is apparent during the process. Furthermore, as a solid evidence for a direct conversion, this group was able to recently demonstrate that BAM factors could also convert a pure population of hepatocytes to functional neurons [21].

Within a year after the publication of iN cell generation from mice, seven different groups, including ours, independently reported the generation of human iN cells (hiNs) [22,23•,24•, 25•,26,27•,28,29] (Table 1). One notable aspect in these reports were that except for Pfisterer *et al*. [26], none of the groups were successful in producing functionally mature hiN cells using the same three transcription factors (BAM) that worked in mouse cells. Functional hiNs could be generated when additional factors like NEUROD1/2 [22], ZIC1 [23•], microRNAs [24•,25•] were added to some or all of the BAM factors. Moreover, by adding critical transcription factors involved in neuronal subtype specification, some of these groups were also able to directly generate enriched hiN populations of dopaminergic [26,27•] or motor neurons [28].

Some of the common themes emerging from the above reports are: (i) The conversion seems to be very rapid, that is, within a short duration from the factor introduction. (ii) While neuronal identity is acquired very fast, the subsequent functional maturation seems to take several weeks. (iii) Cell division does not appear to be a requisite. (iv) When embryonic or neonatal cells are used, the efficiency of hiN cells generation (4–10%) seems to be comparable to that of in mouse cells, but adult cells seem to exhibit a much lower efficiency. (v) Similarly, hiNs derived from embryonic or neonatal human cells seems to functionally and physiologically mature much faster than adult cell derived hiNs.

Though majority of the above studies used a cocktail exclusively of transcription factors, we and Crabtree's group sought to explore the utility of microRNA in the conversion experiments [24•,25•]. Analogous to functioning of transcription factors, microRNAs can also simultaneously regulate the activity of multiple targets. Given the expression pattern and the roles of miR-124 in maintaining neuronal identity and function [30–33], this microRNA was in particular an attractive candidate for testing in hiN cell conversion. Thus, by combining miR-124 with *BRN2* and *MYT1L* or with *NEUROD2* and miR-9, we and Yoo

et al. [24•,25•], respectively, were able to generate functionally mature hiNs from adult fibroblasts. These neurons were predominantly excitatory glutamatergic type, but occasional GABAergic and dopaminergic types were also observed. It is noteworthy that so far only in the presence of the microRNAs was it successful to produce adult hiN cells that exhibited functional synapses in the absence of co-culture with helper cells. Moreover, these cocktails also demonstrate that use of *ASCL1*, or bHLH factors are dispensable for conversion.

Lineage reprogramming to neural progenitors A

Major limitation of direct conversion to a terminally differentiated mature cell type is the inability to expand the reprogrammed cells in sufficient quantity for various applications. In addition, many mature cell types are more difficult to be sustained after purification *in vitro* or transplantation *in vivo*. Therefore, direct conversion to expandable neural stem/progenitor cells (NSCs) is desirable in practical applications that demand large amount of cells. Toward this goal, we hypothesized that overexpression of the iPSC-factors might initially activate the cells into an epigenetically unstable and plastic state that allows cells to take alternate fates, other than pluripotency, if provided with appropriate environmental conditions (Figure 1). Proving this hypothesis, we were able to demonstrate that transient expression of the iPSC-factors for as short as three days and subsequently applying NPC-supporting conditions (i.e. FGF2 and EGF), was sufficient to convert mouse fibroblasts to homogenous and $Pax6^{+}$ neural stem cell colonies (iNSCs) that can be isolated, expanded in serial passages, and further differentiated into mature neuronal subtypes and astroglia [34••]. Another advantage of this approach is that the transient expression of transcription factors could principally be more easily replaced by non-integrating approaches or small molecules [35–38]. Along this PDR concept, recently, two other groups could successfully generate tripotent iNSCs from mouse fibroblasts [39,40]. Interestingly, one of these groups replaced *Oct4* with *Brn4* and *E47* in their cocktail [39]. These iNSCs were strikingly similar to wild type NSCs in their gene expression, epigenetic features, self-renewal capacity as well as *in vivo* functionality. Extending the PDR approach to human cell conversion, recently we were able to reprogram postnatal human fibroblasts to stably self-renewing and expandable hiNSCs capable of differentiating into functional neuronal subtypes and astroglia (unpublished observation).

Using a SDR approach, two other groups recently generated iNSCs from mouse cells (Table 1). While Lujen *et al*. generated tripotent iNSCs from fibroblasts by introducing *Brn2, Sox2*, and *FoxG1* [41•], Sheng *et al*. used *Pax6, Ngn2, Hes1, Id1, Ascl1, Brn2, c-Myc*, and *Klf4* to produce iNSCs from sertoli cells of the testes [42]. Intriguingly, while the former group found the addition of *Sox2* to be beneficial to the process, the latter group found the same to be inhibitory.

Applications of lineage reprogramming

A major attraction of lineage reprogramming is the opportunity to produce patient specific ‘disease-in-a-dish’ models of many neurological disorders where pathologic human tissues are inaccessible for evaluation. As a proof of this concept, hiNs from familial Alzheimer’s disease (FAD) patients recapitulated the pathology of altered processing and aberrant

endosomal localization of amyloid precursor protein (APP) and increased production of Ab peptides [23•]. Similarly, induced DA neurons (iDA) generated from Parkinson's disease (PD) patients shared many properties of midbrain dopaminergic neurons and released monoamine in response to depolarization, although it is not clear whether these iDA neurons recapitulate any disease specific phenotype [27•]. The induced motor neurons (iMNs) from normal mice, produced by Son *et al.*, responded to degenerative stimuli from SOD1G93A mutant glia in non-cell-autonomous fashion, implying their motor neuron identity [28]. Moreover, iMNs from SOD1G93A mouse generated in the same study showed impaired survival compared to control iMN cells when they are cultured on normal glia, showing cell-autonomous disease phenotype [28].

Another important area where this technology may have an impact is in cell replacement therapies. In this regard, iN/iNSC transplantations performed so far has been encouraging in that the cells engrafted survived and produced many neuronal features *in vivo* [23•,27•, 28,39,40,43•]. For example, one study is the iDA transplantation performed by Kim *et al.* These neurons not only shared close similarity in gene expression, pacemaker like electrophysiological activity and many other cardinal features of mesencephalic DA neurons, they also integrated in brain of PD mice resulting in alleviation of some of the disease pathologies [43•].

iN/iNSCs could also serve an excellent source of human cells for studies involving neural development, understanding gene networks, gene–environmental interactions, and in drug screening and toxicity evaluations.

Current limitations and future prospects

As iN cell generation is a rapid process without cell division, its net yield after conversion is limited by the starting cell numbers. Moreover, like in iPSC generation, prolonged *in vitro* culturing of starting cells seems to negatively affect the efficiency of hiN generation. These aspects impose a severe limitation on scalability of these cells for various applications. Expandable iNSCs are better alternative in this regard. But owing to their proliferative nature, a more thorough long term evaluations after transplantations need to be done to fully exclude tumourigenecity. Also post-transplantation follow-ups should evaluate immunogenicity of iN/iNSCs, and ascertain their differences, if any, in survival and functional integration *in vivo*.

Another area for improvement of iN technology is to expand the repertoire of neurons beyond glutamatergic, dopaminergic and cholinergic types that can currently be generated. Moreover, hiNs with specific regional identity will be desirable, for example it would be of great interest in the Parkinson's disease field to produce pure populations of hiNs that are indistinguishable from DA neurons of the substantia nigra pars compacta (SNc), the predominant type of neurons lost in PD.

Although cell fate reprogramming largely attributes to the ability of specific transcription factors to bind target sequences across the genome and form complexes with other transcription factors (including co-activators, corepressors, and enzymes, etc. to remodel the

epigenome), it would be fascinating to understand precisely how a small number of 'factors' could rapidly 'rewire' the molecular circuitry that governs the identity of a mature cell and confer upon it a totally new identity and functionality. This is even more puzzling as there is no involvement of cell division, during which cells are considered to be more amenable to transcriptional and epigenetic remodeling. Moreover, global gene expression and chromatin analysis of iN/iNSCs revealed that their molecular identity is closer to neural cells than the starting cell type [21,22,23•,27•,28,39,40,43•]. However, it needs to be determined whether their extent of 'closeness' to the *bona fide* neural cells is sufficient to ensure long term maintenance of a stable phenotype and whether the remaining 'memory' of starting cells would have any detrimental effect in this regard. Similarly, single cell/clonal studies could be undertaken to understand whether the conversion process involves stochastic events or whether it can be controlled to yield more homogenous products. In all these regards, it will be fascinating to understand the molecular mechanisms that underlie direct lineage conversions. Insights could also be drawn from studies on naturally occurring direct reprogramming events as in the conversion of rectal cells to motor neurons in *C. elegans* [44]. These mechanistic studies could reveal critical signaling and molecular pathways, perturbation of which using small molecules or microRNAs could result in more efficient and clinically safer direct reprogramming methods. Such safe factors can also be exploited for direct *in vivo* reprogramming in therapeutic paradigms.

In summary, direct lineage reprogramming from readily available cells offer an exciting new option in regenerative medicine for faster and efficient generation of therapeutically relevant neural cells. Though there are some hurdles to overcome before clinical translation of this technology, its current state of the art is highly promising toward achieving such goals.

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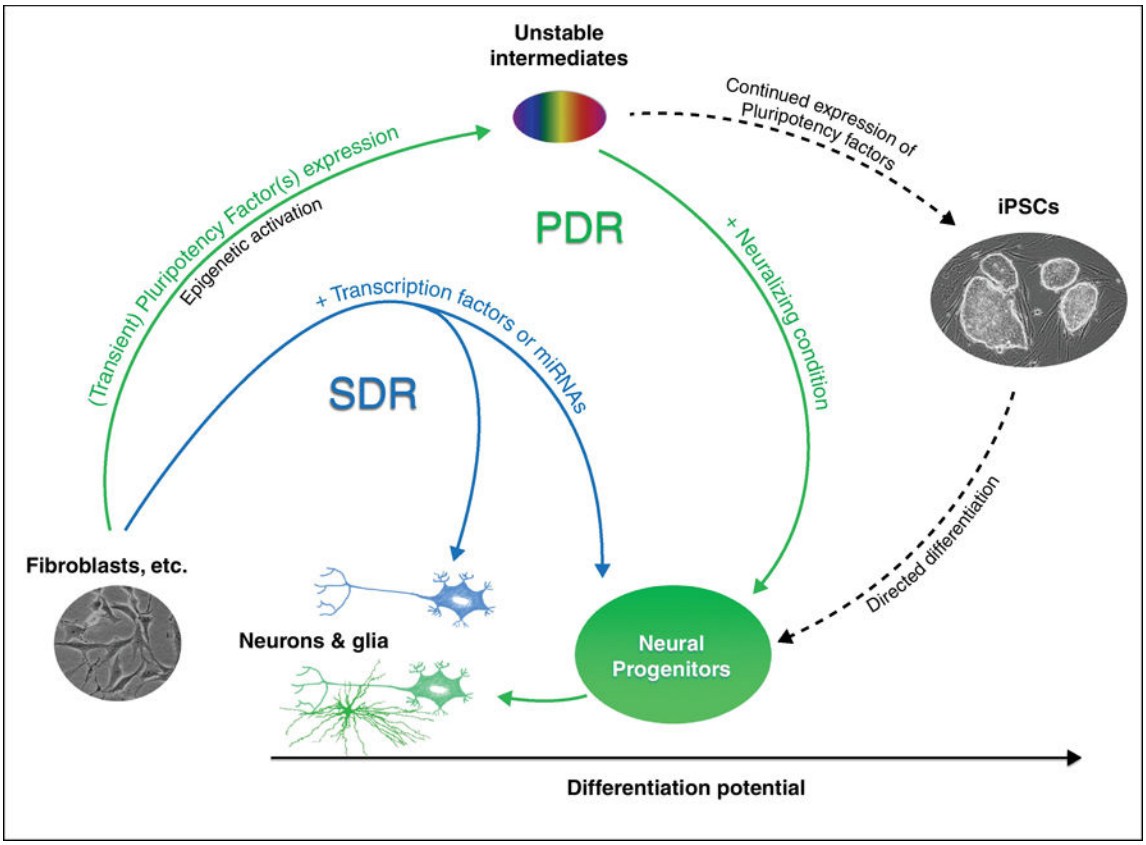

Figure 1.

Current strategies for direct lineage reprogramming to neural cells. In Somatic cell-specific factor-mediated Direct Reprogramming (SDR), neural lineage-specific factors such as transcription factors or microRNAs are introduced to readily available cells. The factors introduced starting cells then rapidly undergo direct conversion to desired neural cell type without undergoing any cell division. Pluripotent cell-specific factor-mediated Direct Reprogramming (PDR) employs iPSC reprogramming factors (Oct4, Sox2, Klf4 and c-Myc; at least some or all of these factors are used) and neuralizing conditions (that include growth factors, small molecules, etc.) to facilitate conversion. In this approach, pluripotency factors mediate a short ‘epigenetic activation’ of starting cells and the resulting unstable intermediate cells are coaxed to take a neural fate with the help of neuralizing conditions.

Table 1

Summary of recent direct lineage reprogramming to neural cells

Species	**Mouse**										**Human**							
Starting cells (Developmental stage)	**Fib. embryonic & adult**	**Hep. postnatal**	**Fib. embryonic**	**Fib. adult**	**Fib. embryonic & adult**	**Fib. embryonic**	**Fib. embryonic & adult**	**Fib. embryonic**	**Fib. embryonic**	**Sertoli cell postnatal**	**Fib. postnatal & adult**	**Fib. postnatal**	**Fib. fetal & postnatal**	**Fib. adult**	**Fib. postnatal & adult**	**Fib. fetal & adult**	**Fib. embryonic & fetal**	**Fib. embryonic**
Target cells	**iN**		**iDA**		**iMN**	**iNSC**					**hiN**					**hiDA**		**hiMN**
Reprogramming factors	**Ascl1 Brn2 Myt1L**	**Ascl1 Brn2 Myt1L**	**Ascl1 Lmx1a Nurr1**	**Ascl1 Lmx1a Nurr1 Foxa2 EN1 Pitx3**	**Ascl1 Brn2 Myt1L Lhx3 Hb9 Isl1 Ngn2**	**Brn2 FoxG1 Sox2**	**c-Myc Klf4 Sox2 Oct4**	**c-Myc Klf4 Sox2 Oct4**	**c-Myc Klf4 Sox2 E47 Brn4**	**c-Myc Klf4 Ascl1 Brn2 Pax6 Ngn2 Hes1 Id1**	**BRN2 MYT1L miR-124**	**Ascl1 Brn2 Myt1L**	**Ascl1 Brn2 Myt1L NeuroD1**	**Ascl1 Brn2 Myt1L Zic1 (Olig2)**	**ASCL1 MYT1L NEUROD2 miR-9/9* miR-124**	**Ascl1 Lmx1a Nurr1**	**Ascl1 Brn2 Myt1L Lmx1a FoxA2**	**Ascl1 Brn2 Myt1L NEUROD1 Lhx3 Hb9 Isl1 Ngn2**
Number of factors	**3**	**3**	**3**	**6**	**7**	**3**	**4**	**4**	**5**	**8**	**3**	**3**	**4**	**5**	**5**	**3**	**5**	**8**
Efficiency (%)[a]	19	6	18	9[b]	10	–	0.7	0.008	0.004	0.002	4–11	4	4	9	10	3–6	5–10	0.05
Time (weeks)	1–2	2	2	2–3	1–2	3–4	13	3	4–5	3–4	2–3	2	2–5	3	3	2–3	3–4	4–5
In vivo test	–	–	mouse	mouse	chick	mouse	–	rat	mouse	mouse	–	–	–	mouse	–	–	–	–
Disease model	–	–	–	PD	ALS	–	–	–	–	–	–	–	–	FAD	–	PD	–	–
Reference	[20••]	[21]	[27•]	[43•]	[28]	[41•]	[34••][c]	[40][c]	[39][c]	[42]	[25•]	[26]	[22]	[23•]	[24•]	[27•]	[26]	[28]

Abbreviations: Fib., fibroblasts; Hep, hepatocytes; iN/hiN, induced neurons; iDA/hiDA, induced dopaminergic neurons; iMN/hiMN, induced motor neurons; PD, Parkinson's disease; ALS, amyotrophic laternal sclerosis; FAD, familial Alzheimer's disease.

[a] Approximate percentage.

[b] Percentage of total cells.

[c] Studies used Pluripoent cell-specific factor-mediated Direct Reprogramming (PDR) approach.