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Review

Focus: Induced Pluripotency & Cellular Reprogramming

Forward engineering neuronal diversity using direct reprogramming

Rachel K Tsunemoto, Kevin T Eade, Joel W Blanchard & Kristin K Baldwin*

Abstract

The nervous system is comprised of a vast diversity of distinct neural cell types. Differences between neuronal subtypes drive the assembly of neuronal circuits and underlie the subtype specificity of many neurological diseases. Yet, because neurons are irreversibly post-mitotic and not readily available from patients, it has not been feasible to study specific subtypes of human neurons in larger numbers. A powerful means to study neuronal diversity and neurological disease is to establish methods to produce desired neuronal subtypes *in vitro*. Traditionally this has been accomplished by treating pluripotent or neural stem cells with growth factors and morphogens that recapitulate exogenous developmental signals. These approaches often require extended periods of culture, which can limit their utility. However, more recently, it has become possible to produce neurons directly from fibroblasts using transcription factors and/or microRNAs. This technique referred to as direct reprogramming or transdifferentiation has proven to be a rapid, robust, and reproducible method to generate mature neurons of many different subtypes from multiple cell sources. Here, we highlight recent advances in generating neurons of specific subtypes using direct reprogramming and outline various scenarios in which induced neurons may be applied to studies of neuronal function and neurological disease.

Keywords direct reprogramming; neuronal diversity; neurons; transdifferentiation
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Introduction

The human nervous system is comprised of an unknown number of diverse cellular subtypes that collaborate to produce human-specific behaviors and cognitive abilities. Advances in genome sequencing are providing increasing information about human-specific genes and mutations that affect brain function. However, much of our understanding of neuronal diversity and development derives either from animal models or from studies of neurons produced *in vitro* from human stem cells, which may lack critical features of their appropriate counterparts *in vivo*. As such, some of the most intriguing and important problems in human neurobiology have been difficult to address at the cellular or molecular level.

Similarly, many neurological diseases are difficult to study using animal models, perhaps because they selectively impact certain subpopulations of neurons that may differ between species or arise so late in life that short-lived rodent models may not adequately recapitulate disease progression. For example, amyloid lateral sclerosis (ALS) primarily affects certain motor neurons but spares other subtypes, while Parkinson's disease most severely impacts dopaminergic neurons. Because these disorders, along with Alzheimer's and other neurodegenerative diseases, typically emerge in adult humans at ages far greater than the typical 1–2 year lifespan of the laboratory mouse, cellular phenotypes arising in animal models may differ significantly from those underpinning the concordant human disease. Additionally, despite increasing knowledge of their genetic bases, neuropsychiatric and neurodevelopmental disorders such as autism and schizophrenia are difficult to model using animals because their diagnostic symptoms are embedded in behaviors that are largely human specific. Fortunately, recent advances in cellular reprogramming offer a set of powerful methods to approach the problems of human neurobiology and neurological disease at the cellular and molecular level.

One useful method to generate human neurons *in vitro* is to differentiate human embryonic stem cells (ESCs) into neural precursors and then into desired neural subtypes. However, this approach suffers from limited availability of ESC lines with disease-prone or diverse genetic backgrounds. The discovery of human induced pluripotent stem cells (iPSCs) (Takahashi *et al*, 2007; Yu *et al*, 2007) alleviated this concern in that iPSCs may be produced from a broad range of individuals with known disease histories or genetic backgrounds. The power of these models will likely be magnified by applying increasingly powerful genome editing approaches to produce isogenic lines, to engineer disease states or to label desired cell types. Efforts to produce specific neuronal subtypes from pluripotent stem cells typically aim to recapitulate development by applying growth factors or small molecules to cultured cells. However, perhaps because production of specific neuronal subtypes during human brain development unfolds over months and years, *in vitro*

The Scripps Research Institute, La Jolla, CA, USA
*Corresponding author. Tel: +1 858 784 9466; E-mail: kbaldwin@scripps.edu

methods that recapitulate development often require very long incubation periods and result in neurons that more closely resemble embryonic neurons than mature cell types. In contrast, direct reprogramming of fibroblasts or other cell types into neurons using ectopic expression of transcription factors and/or microRNAs offers a different path to neuronal subtype identity that may be able to more rapidly and reproducibly generate neurons that more closely resemble mature neurons or later-born neuronal subtypes. This review will outline progress in this emerging field and speculate about the future prospects of using direct reprogramming to study human neuronal diversity, brain development, and disease.

Direct production of neurons from other cell types and lineages

In 2002, Heins *et al* (2002) showed that expressing *Pax6* in postnatal mouse cortical astrocytes could convert them into neurons. Additional studies showed that expressing either *Ascl1* or *Ngn2* in astrocytes can transdifferentiate them into neurons, demonstrating that neuronal identity can be established via mechanisms not thought to be involved in their specification during normal development (Berninger *et al*, 2007). However, astrocytes, like neurons, are not easily obtained from patients, thereby limiting the utility of these methods for drug screening or translational approaches. In 2006, the Yamanaka laboratory found that they could convert fibroblasts into iPSCs using transient expression of only a few transcription factors. Following this discovery, multiple groups showed that this method was robust and that alternative combinations of factors could also produce iPSCs from human and mouse fibroblasts, blood and a large number of other accessible subtypes (Robinton & Daley, 2012). Together, these studies suggested that it might also be possible to reprogram cells into lineages other than pluripotent stem cells using transient expression of the appropriate combinations of factors.

Testing this hypothesis, using an approach similar to the original Yamanaka paper, the Wernig group discovered that ectopic expression of three transcription factors *Ascl1*, *Brn2*, and *Myt1l* (BAM factors) could produce induced neurons (iN) from mouse embryonic (MEFs) and tail-tip fibroblasts (TTFs) (Vierbuchen *et al*, 2010). They also were able to produce neuronal-like cells using several other combinations of related factors. These iNs expressed neuron-specific proteins, fired action potentials, and formed functional synapses *in vitro*. This method was also shown to be applicable to human cells, although it required the addition of NeuroD1 to produce neurons with mature electrophysiological properties (Pang *et al*, 2011).

Following on this observation, the Wernig group showed that the BAM factors could also reprogram mouse hepatocytes into iNs, confirming that both endodermal and mesodermal lineages are capable of transdifferentiating into the neural, ectodermal lineage (Marro *et al*, 2011). Single cell and genome-wide expression analyses of iNs derived from fibroblasts with those derived from hepatocytes revealed that direct reprogramming of each lineage involves the coordinated activation of neuronal transcriptional pathways and concomitant silencing of the transcriptional program of the source cell (Marro *et al*, 2011). Importantly, neither protocol involved a dividing precursor or detectable neural stem cell intermediate.

These studies raised the question of whether it would be possible to direct the differentiation of undifferentiated pluripotent cells into neurons using these transcription factor combinations. Indeed, applying the BAM factors to human ESCs resulted in rapid and efficient induction of neurons after only a week of induction (Pang *et al*, 2011). Additionally, *Ascl1* (Chanda *et al*, 2014), *Ngn2* (Thoma *et al*, 2012; Zhang *et al*, 2013), and *NeuroD2* (Sugimoto *et al*, 2009) expressed alone have been show to efficiently and directly generate neurons from both mouse and human ESCs and iPSCs. Therefore, to date, transcription factors have been shown to induce neurons from fibroblasts (Vierbuchen *et al*, 2010; Pang *et al*, 2011), hepatocytes (Marro *et al*, 2011), ESCs/iPSCs (Sugimoto *et al*, 2009; Pang *et al*, 2011; Thoma *et al*, 2012; Zhang *et al*, 2013; Chanda *et al*, 2014), and astrocytes (Torper *et al*, 2013) of both mouse and human origin, underscoring the robustness of this method (Fig 1, Table 1).

How might transient expression of only a few transcription factors in a non-neural cell type irreversibly reprogram cell fate and produce functional neurons, when this does not occur during normal development? An attractive hypothesis is that direct reprogramming taps into conserved feed-forward transcriptional circuits that may be used to actively maintain neuronal identity in mature neurons. Perhaps by "swamping out" the fibroblast or hepatocyte transcriptional circuitry, the reprogramming factors override and down-regulate ongoing transcriptional networks while simultaneously enacting new neuronal feed-forward circuits.

In 2011, the Crabtree laboratory reported that neuronal microRNAs miR-9/9* and miR-124 alone were sufficient to convert fibroblasts into neurons, which might have been thought to be

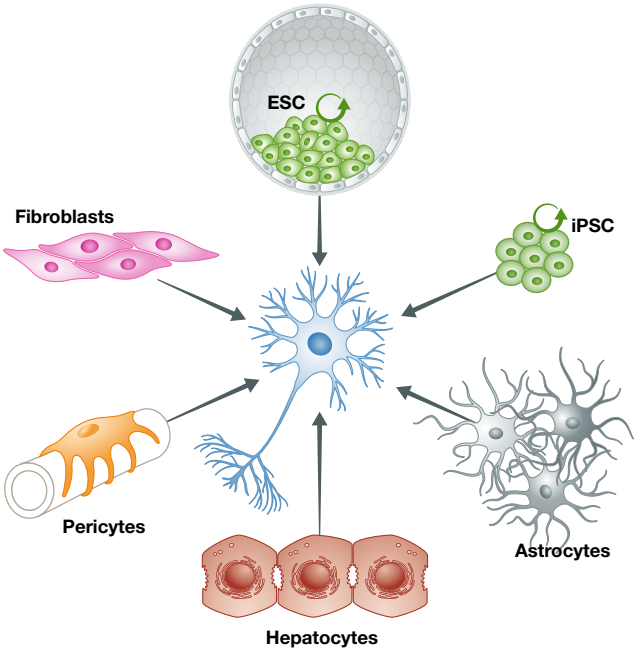

Figure 1. A wide range of cell types can give rise to induced neurons (iNs).
Various somatic cell sources from mouse and human can be reprogrammed into iNs, both *in vitro* and *in vivo*. Embryonic and induced pluripotent stem cells can also be directly converted to iNs using similar transcription factor combinations.

Table 1. Direct reprogramming methods to generate induced neurons.

	Reference	Reprogramming factor(s)	Source tissue	Species	Subtype	Utility (transplanted/ *in vivo*/disease)
Mixed neurons						
1	Heins *et al* (2002)	Pax6	Postnatal cortical astrocytes	Mouse	Mixed lineage (Gabaergic or glutamatergic)	
2	Berninger *et al* (2007)	Ascl1	Postnatal cortical astrocytes	Mouse	Not reported	
		Ngn2	Postnatal cortical astrocytes	Mouse	Not reported	
		Pax6	Postnatal cortical astrocytes	Mouse	Not reported	
3	Sugimoto *et al* (2009)	NeuroD2	Embryonic stem cells	Mouse	Not reported	
4	Heinrich *et al* (2010)	Ngn2	Postnatal astrocytes	Mouse	Glutamatergic	
		Dlx2	Postnatal astrocytes	Mouse	Gabaergic	
		Dlx2, Ascl1				
5	Sugimoto *et al* (2009)	NeuroD2	Embryonic stem cells	Mouse	Not reported	
6	Vierbuchen *et al* (2010)	Ascl1, Brn2, Myt1l	Prenatal fibroblasts, postnatal fibroblasts	Mouse	Mixed lineage (Gabaergic or glutamatergic)	Neuroligin-3 mutant (Chanda *et al*, 2013)
7	Pang *et al* (2011)	Ascl1, Brn2, Myt1l	Embryonic stem cells	Human	Mixed lineage (glutamatergic)	
		Ascl1, Brn2, Myt1l, NeuroD1	Prenatal fibroblasts, postnatal fibroblasts	Human	Mixed lineage (Gabaergic, dopaminergic, or glutamatergic)	
8	Marro *et al* (2011)	Ascl1, Brn2, Myt1l	Hepatocytes	Mouse	Not reported	
9	Karow *et al* (2012)	sox2, ascl1	Pericytes	Human	Excitatory (glutamatergic)	Transplanted into mouse brain
10	Ambasudhan *et al* (2011)	miR-124, Myt1l, Brn2	Postnatal fibroblasts	Human	Mixed (glutamatergic, gabaergic, dopaminergic)	
11	Yoo *et al* (2011)	miR-9*, miR-124, NeuroD2, (Ascl1), (Myt1l)	Postnatal fibroblasts	Human	Mixed (glutamatergic, gabaergic)	
12	Xue *et al* (2013)	Poly-pyrimidine-tract-binding (PTB) protein knockdown	Prenatal fibroblasts	Mouse	Mixed (glutamatergic, gabaergic)	
13	Thoma *et al* (2012)	Ngn2	Embryonic stem cells	Mouse	Glutamatergic	
14	Zhang *et al* (2013)	Ngn2	Embryonic stem cells, iPSCs	Human	Glutamatergic	Knockdown of Munc18-1 mRNA implicated in infantile epileptic encephalopathies, cognitive impairments, and nonsyndromic epilepsy; functional integration into mouse brain
15	Chanda *et al* (2014)	Ascl1	Prenatal fibroblasts, postnatal fibroblasts	Mouse	Mixed (glutamatergic, gabaergic)	
			Prenatal fibroblasts, postnatal fibroblasts	Human	Mixed (glutamatergic, gabaergic)	
			Embryonic stem cells	Mouse	Mixed (glutamatergic, gabaergic)	
			Embryonic stem cells	Human	Mixed (glutamatergic, gabaergic)	

Table 1 (continued)

	Reference	Reprogramming factor(s)	Source tissue	Species	Subtype	Utility (transplanted/ *in vivo*/disease)
Motor neurons						
16	Son *et al* (2011)	Ascl1, Brn2, Myt1l, Ngn2, Lhx3, Isl1, Hb9	Prenatal Fibroblasts	Mouse	Motor neurons	Transplanted into chick, ALS model- SOD1 mutant
		Ascl1, Brn2, Myt1l, Ngn2, Lhx3, Isl1, Hb9, NeuroD1	Fibroblasts (iPSC derived)	Human	Motor neurons	
17	Liu *et al* (2013)	Ngn2, Forskolin, Dorsomorphin	Prenatal lung fibroblasts	Human	Motor neurons	
		Ngn2, Forskolin, Dorsomorphin, Sox11	Postnatal fibroblasts	Human		Converted from ALS patient fibroblasts
18	Mazzoni *et al* (2013)	Ngn2, Isl1, Lhx3	Embryonic stem cells	Mouse	Spinal motor neurons	Transplanted into chick
		Ngn2, Isl1, Phox2a	Embryonic stem cells	Mouse	Cranial motor neurons	Transplanted into chick
Dopaminergic neurons						
19	Pfisterer *et al* (2011)	Ascl1, Brn2, Myt1l, Lmx1a, Foxa2,	Prenatal fibroblasts	Human	Dopaminergic	
20	Caiazzo *et al* (2011)	Ascl1, Nurr1, Lmx1a	Prenatal, postnatal fibroblasts	Mouse	Dopaminergic	Made iNs from Parkinson's patients
21	Addis *et al* (2011)	Ascl1, Nurr1, Lmx1b	Astrocytes	Mouse	Dopaminergic	
22	Kim *et al* (2011a)	Pitx3, Ascl1	Postnatal fibroblasts	Mouse	Dopaminergic	Transplantation, functional integration lesion striatum
		Pitx3, Ascl1, En1, Lmx1a, Foxa2, Nurr1,				
23	Torper *et al* (2013)	Ascl1, Brn2, Myt1l	Embryonic fibroblasts, fetal lung fibroblasts	Human		Transduced with factors *in vitro*, but reprogramming genes not activated until transplanted *in vivo*
		Ascl1, Brn2, Myt1l, Lmx1a, FoxA2, Lmx1b, Otx2	Embryonic fibroblasts, fetal lung fibroblasts	Human	Dopaminergic	Transducted with factors *in vitro* and reprogramming genes were activated when transplanted into the striatum of rats prelesioned with 6-OHDA
		Ascl1, Brn2, Myt1l	*In vivo* astrocytes	Mouse		*In vivo* direct reprogramming
24	Dell'Anno *et al* (2014)	Ascl1/Nurr1/Lmx1a	Prenatal fibroblasts	Mouse	Mid-brain dopaminergic	Transplantation with DREADD
Peripheral sensory neurons						
25	Blanchard *et al* (2015)	Brn3a, Ngn1 or Brn3a, Ngn2	Prenatal Fibroblasts	Mouse	DRG	Respond to pain ligands
		Brn3a, Ngn1 or Brn3a, Ngn2	Postnatal fibroblasts	Mouse	DRG	
		Brn3a, Ngn1 or Brn3a, Ngn2	Fibroblasts (iPSC derived)	Human	DRG	Respond to pain and itch ligands
		Brn3a, Ngn1 or Brn3a, Ngn2	Postnatal fibroblasts	Human	DRG	
26	Wainger *et al* (2015)	Ascl1, Brn2, Myt1l, Ngn1, Isl2, Klf7	Prenatal fibroblast	Mouse	Nociceptive	Respond to pain ligands, pain sensitization
			Postnatal fibroblast	Human	Nociceptive	Made iNs from Familial Dysautonomia patients
Other neurons						
27	Victor *et al* (2014)	mi9*/124 BCL11B/ DLX1 DLX2/MYT1L	Postnatal fibs	Human	Striatal medium spiny neurons	
		Pax6/Ngn2/Ascl1/Dlx2	Astrocytes			

surprising given that microRNAs act primarily to repress genes (Yoo *et al*, 2011). However, the action of these microRNAs during development suggested a potential mechanism. As neurons become specified from their precursors, these microRNAs are known to repress the SWI/SNF-like chromatin remodeling complex BAFa. This repression then induces the cells to assemble the BAFb complex, which facilitates cell cycle exit and helps to establish key features of neuronal identity. Therefore, this study showed that repression of developmentally relevant non-neuronal programs can result in reprogramming that involves the indirect activation of neuron-specific chromatin states. In addition, one effect of expressing miR-124 is to repress the RNA binding polypyrimidine-tract-binding (PTB) protein. Accordingly, in 2013, the Fu group found that down-regulating PTB with specific small hairpin RNA can also generate functional neurons from MEFs (Xue *et al*, 2013). Interestingly, numerous subtypes of neurons were generated through expression of mi9*/124, including both excitatory and inhibitory neurons and cells positive for markers of multiple cortical layers. This shows that not only can mi9*/124 induce neuronal identity, but also suggests that somehow, different cells can also implement distinct downstream programs to further pattern subtype identity.

Because transcription factor activation and microRNA-mediated repression can both lead to neuronal fate conversion, it is of interest to know whether these approaches would act synergistically. Neuronal reprogramming with miR-9/9* and miR-124 can be enhanced by including *NeuroD2,* and/or *Ascl1* and *Myt1l* (Yoo *et al*, 2011). And, in a separate study, human fibroblasts were converted into function factor neurons by co-expressing miR-124 with *Brn2* and *Myt1l* (Ambasudhan *et al*, 2011). These results show that the transcription factor and microRNA pathways are compatible and suggest that it may be useful to identify additional neuronal subtype-specific microRNAs and transcription factors in order to improve the quality or subtype identity of induced neurons.

The brain contains hundreds if not many thousands of distinct neuronal subtypes. Therefore, it is of interest to know whether the BAM factors produce any recognizable subtypes, and if so, which ones and how many types. While this is a difficult question to answer due to currently imprecise definitions of neuronal subtype identity, characterization of mouse iNs generated by the BAM factors alone and in combination with microRNAs showed that they contain neurons with mixed characteristics. The largest group resembled excitatory glutamatergic neurons based on their expression of markers such as VGluT1, VGluT2, and Tbr1. However, some iNs produced using the same factors expressed markers of inhibitory GABAergic neurons such as GAD, GABA, GAD65, and GAD67 (Vierbuchen *et al*, 2010; Ambasudhan *et al*, 2011; Pang *et al*, 2011; Yoo *et al*, 2011). In human studies, where induced neurons were generated by adding *NeuroD1* to the BAM factors, 9% of cells expressed the dopaminergic neuronal marker tyrosine hydroxylase (TH), and 21% expressed peripherin, a marker of peripheral neurons (Pang *et al*, 2011). Induced neurons produced from hepatocytes also displayed diversity; approximately 4% of neurons expressed markers of cholinergic neurons (choline acetyltransferase, ChAT), and a similar proportion expressed peripherin, a marker of peripheral neurons that can be cholinergic (Marro *et al*, 2011). These results are consistent with two models. In one, the same set of factors can produce different neuronal subtypes when added to different cells, either due to differences in the donor cell epigenome or due to intrinsic stochastic mechanisms. Alternatively, because these studies used combinations of factors delivered in independent viruses, subtype diversity could arise based on differences in the ratio or identity of factors expressed in distinct donor cells. One way to address this question would be to produce cell lines carrying inducible multi-cistronic reprogramming cassettes integrated at defined locations. Similar approaches have been highly informative in delineating the roles of stochastic versus determined mechanisms in reprogramming to pluripotency (Hanna *et al*, 2009; Carey *et al*, 2011).

Biasing direct reprogramming to produce specific neuronal subtypes

The high demand for certain human neuronal subtypes has led to rapid progress in refining direct reprogramming methods to enrich for neurons with desired characteristics (Fig 2, Table 1). Several studies aimed to produce dopaminergic neurons because of their importance for Parkinson's disease (PD). PD is marked by the specific loss of midbrain dopaminergic neurons and subsequent loss of dopamine in the target striatum. If it were possible to produce autologous dopaminergic neurons for transplantation into patients, symptoms of the disease might be alleviated. As such, production of dopaminergic neurons from pluripotent stem cells has long been a focus of stem cell research (Wakeman *et al*, 2011). Encouragingly, combining the BAM factors with two genes involved in midbrain and dopamine neuron specification (*Lmx1a* and *FoxA2)* produced human iNs that expressed TH (Pfisterer *et al*, 2011).

Additional studies showed that TH-expressing dopaminergic neurons could also be generated with only three factors, *Ascl1*, *Lmx1a*, and *Nurr1* (Caiazzo *et al*, 2011). In these studies, 85% of the iNs generated from MEFs were TH positive, suggesting that it might be possible to generate highly enriched or even pure populations of desired neuronal subtypes with direct reprogramming. Dopaminergic neurons, however, comprise a very diverse set of subtypes. To try to generate iDAs that more closely resemble endogenous midbrain DA neurons and are functional when transplanted into the brain, Kim *et al* (2011a) screened factors and assayed for Pitx3 expression, a specific reporter for midbrain DA neurons. These studies identified a cocktail of six transcription factors, *Ascl1*, *Pitx3*, *Lmx1a*, *Nurr1*, *Foxa2*, and *EN1*, and two exogenous factors, Shh and FGF8, that could generate Pitx3-expressing iNs. Remarkably, when transplanted into the midbrain striatum after lesioning, which is a mouse model of PD, these induced midbrain TH-positive neurons could increase dopamine levels and also alleviate motor impairments in the treated mice. Finally, microRNAs miR-181a/a* and miR-125b can also be used to produce dopaminergic neurons from human long-term self-renewing neuroepithelial-like stem cells (Stappert *et al*, 2013). These results support the idea that it might be possible to directly reprogram neurons into a wide diversity of desired cell fates and provide an important proof of principle regarding the suitability of induced neurons for cell replacement therapy.

Generating spinal cord motor neurons

Spinal cord motor neurons are a neuronal subtype with relevance to motor neuron diseases such as amyotrophic lateral sclerosis (ALS)

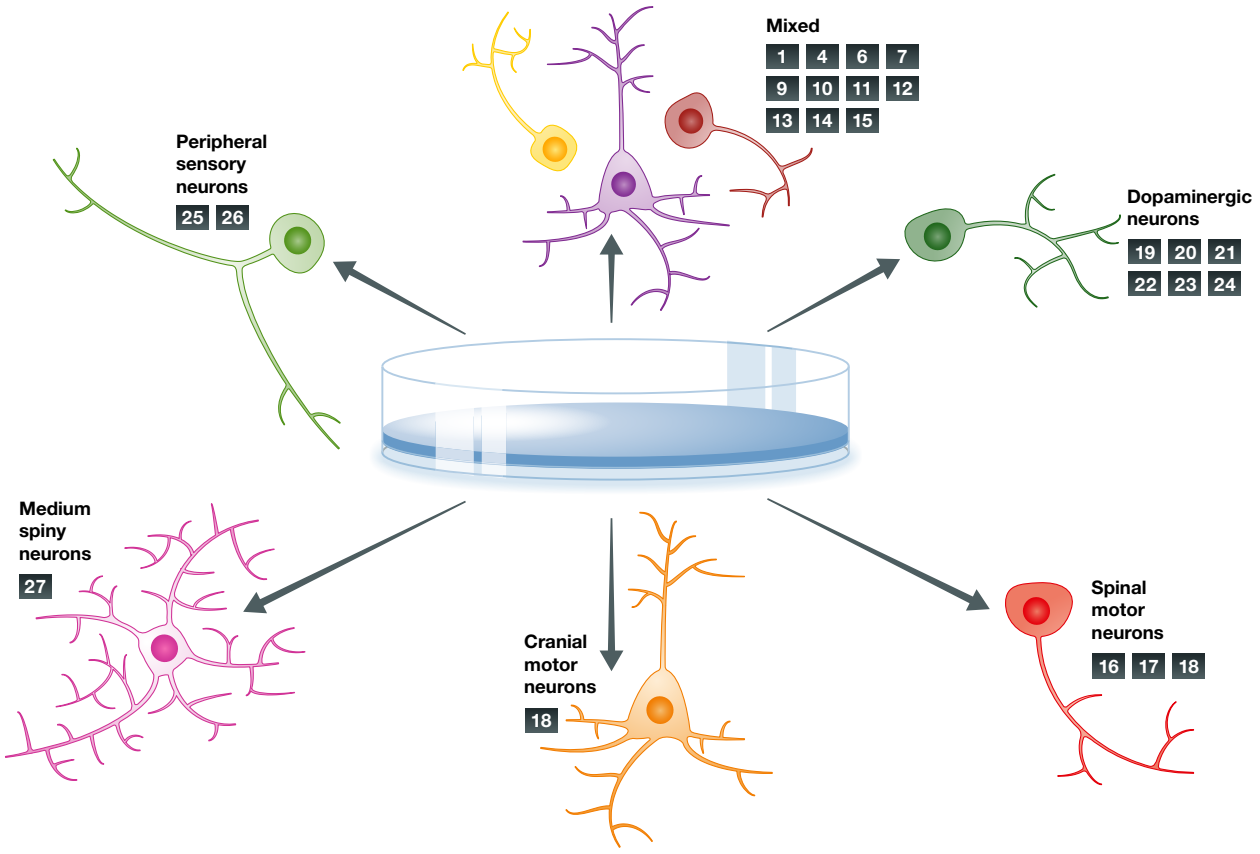

Figure 2. iNs resembling diverse endogenous neuronal subtypes can be produced by using multiple transcription factor combinations.
By altering or adding transcription factors to the original Wernig BAM combination, multiple groups have demonstrated that different source cell types can generate iNs that express markers of diverse neuronal subtypes. Interestingly, many different combinations of factors can produce iNs that resemble the same general subtype. The boxed numbers correspond to the studies listed in Table 1.

and also of interest for patients with spinal cord injuries. By combining the BAM factors and *NeuroD1* with four transcription factors highly expressed in motor neurons, *Lhx3*, *Hb9*, *Isl1*, and *Ngn2*, Son *et al* (2011) were able to generate neurons that expressed the key motor neuron marker Hb9 from mouse and human fibroblasts. These induced motor neurons (iMNs) expressed motor neuron-specific markers, were capable of forming cholinergic synapses with muscle, and migrated appropriately to the ventral horn and projected out to the ventral root after transplantation.

More recently, Liu *et al* (2013) were able to convert human fetal lung fibroblasts into cholinergic neurons of greater than 90% purity. Similar to previously published iMNs, these cholinergic neurons exhibited motor neuron markers and formed functional neuromuscular junctions. In this study, transdifferentiation to a motor neuron identity was accomplished by simply overexpressing one factor, *Ngn2*, in combination with exposure to two small molecules, forskolin and dorsomorphin. However, these factors were not sufficient to reprogram postnatal and adult skin fibroblasts, which required *Sox11*. These results suggest that specific aspects of the epigenetic or transcriptional state of the donor cell can be critical to the successful application of direct reprogramming methods.

Reprogramming may also be influenced by relative levels of transcription factor expression, as has been reported in studies of iPSCs (Carey *et al*, 2011). To investigate this, Mazzoni *et al* (2013) generated mouse ESC lines that harbored the transcription factors *Ngn2*, *Isl1* and *Lhx3* driven by an inducible promoter. Upon induction, they observed highly efficient conversion; more than 99% of the cells expressed the spinal motor neuron marker Hb9. Furthermore, they were able to shift the subtype identity by replacing *Lhx3* with *Phox2a*. In these experiments, 99% of the ESCs converted into induced neurons that expressed the cranial motor neuron marker *Phox2b*. It is intriguing that so many different factor combinations and methods are capable of generating neurons with shared patterns of gene expression such as expression of TH or Hb9. Future RNA-seq and mechanistic studies will be required to determine whether the downstream mechanisms of these distinct reprogramming methods converge on a limited number of feed-forward transcriptional circuits or rather, whether each set of induced neurons is quite different from the other.

Other subtypes including DRG

Many broad classes of neurons including midbrain dopaminergic and motor neurons are comprised of an array of functionally distinct subtypes that differ in their connectivity, expression profiles, and electrophysiological properties. Because iNs are generated *ex vivo*, in the absence of their endogenous circuitry, it is difficult to characterize

the precise subtypes of neurons generated within each iN population. In a recent study, Blanchard *et al* (2015) showed that two different transcription factor combinations (*Ngn1* with *Brn3a*, or *Ngn2* with *Brn3a*) could produce induced neurons with many characteristics of the diverse set of peripheral sensory neurons found in the dorsal root ganglion (DRG) and/or trigeminal nerve. These sensory neurons comprise three subclasses of neurons that include nociceptors that detect temperature, pain, and itch, mechanoreceptors that detect pressure or touch, and proprioceptors that innervate muscles and detect limb position. *In vivo*, these subsets of neurons are distinguished by their selective expression of one of three members of the Trk receptor family, TrkA, TrkB, and TrkC, which are found in different proportions in different regions of the peripheral nervous system. Remarkably, in direct reprogramming experiments in both human and mouse, Blanchard *et al* found that the induced neurons generated with either pair of transcription factors diversified into three populations of neurons expressing TrkA, TrkB, or TrkC in roughly equal proportions and showed that more than 90% of iNs expressed one of the three markers, indicating that conversion using this method was highly selective. These iSNs also expressed appropriate neurofilaments, neurotransmitters, and neuropeptides that are enriched in the endogenous DRG. Functionally, the iSNs fired action potentials and a subpopulation exhibited TTX resistance, a hallmark of nociceptive neurons. In addition, using calcium imaging, they were able to show that smaller subsets of iSNs exhibited selective responses to compounds that mimic pain and temperature including capsaicin, menthol, and mustard oil, as well as itch-inducing ligands histamine, chloroquine, BAM 8-22, and SLIGRL.

Concurrently, Wainger *et al* (2015) set out to produce nociceptor neurons that expressed TrpV1, using a genetically engineered reporter for this channel. They identified an alternative path to produce these neurons using the BAM factors plus Isl2, Ngn1, and Klf7. These induced TrpV1-positive neurons exhibited appropriate electrophysiological properties and responded to capsaicin, a known ligand of TrpV1. To establish the utility of these induced nociceptive neurons for drug screening, the authors showed that prostaglandin E2, a known TrpV1 sensitizer, and the common chemotherapy drug oxaliplatin that can cause chemotherapy-induced neuropathy can sensitize the TrpV1 response to capsaicin. They also derived induced nociceptive neurons from human fibroblasts from patients with familial dysautonomia (FD). They show that although fibroblasts from these patients express a mixture of normally spliced and abnormally spliced isoforms of the IκB kinase complex-associated protein gene, induced nociceptive neurons derived from those fibroblasts exclusively expressed the disease-associated splice-form. Together, these two studies show that quite different direct reprogramming approaches can produce functional induced nociceptive neurons and provide further evidence that direct reprogramming techniques may be useful for pharmacologic studies and modeling of human genetic neurological diseases.

Modeling neurological disease with iNs

One of the most promising therapeutic applications of generating human iNs is using them to directly model human neurological diseases *in vitro*. Neurons generated from iPSCs have been used to model loss of function monogenic diseases (Ebert *et al*, 2009; Lee *et al*, 2009; Marchetto *et al*, 2010), as well as, more complex disorders such as schizophrenia (Brennand *et al*, 2011). However, some studies using iPSC-derived neurons have noted high variability in differentiation of neurons between lines and also can require quite extended periods of culture to produce neurons. Furthermore, the electrophysiological properties of these neurons tend to resemble those of immature or embryonic neurons. The studies outlined in this review raise the possibility that direct reprogramming may offer a more rapid method to produce desired neuronal subtypes and suggest that direct reprogramming methods may produce more mature neurons than differentiation of iPSCs. To ultimately determine which methods are best for various applications, it will be useful to directly compare neurons of the same subtype derived from traditional differentiation protocols with those produced by direct reprogramming of fibroblasts or via direct conversion of iPSCs.

Another advantage of using direct reprogramming to study disease is that one can mix neurons carrying disease-associated genetic variants with other cell types that do not carry the changes and vice versa. This approach allows one to pinpoint the cell type in which a disease gene acts, which will be increasingly important as human genetic studies uncover additional gene variants linked to human neurological diseases. To test the utility of iNs in modeling neuropathies mediated by uncertain cell types, Son *et al* (2011) generated iMNs from SOD1G93A mutant mice and observed a significant decrease in survival of mutant iMNs compared to wild-type neurons. They next co-cultured wild-type iMNs on SOD1G93A mutant glia and observe the same cell type-specific, non-cell-autonomous toxicity that has been observed in ESC-derived motor neurons (Di Giorgio *et al*, 2007; Nagai *et al*, 2007).

To determine whether iNs can also be used to study variation in synaptic function, which may be important for understanding autism spectrum disorders (ASD) and neuropsychiatric disorders such as schizophrenia, Chanda *et al* (2013) generated neurons using the BAM factors from mice carrying an ASD-associated point mutation in the postsynaptic cell-adhesion molecule *neuroligin*-3. They found that iNs carrying the mutation exhibited a selective decrease in AMPA-type glutamate receptor-mediated responses at excitatory synapses as seen in endogenous neurons. These findings show that transdifferentiated iNs may be useful not only for examining cell survival or responses to exogenous ligands, but may also offer insight into some aspects of neuronal connectivity, such as synaptic function.

Two therapeutic strategies using iNs: transplantation and *in vivo* conversion

Ultimately, for many diseases, it would be useful to transplant functional neurons into the brain, or alternatively to generate desired subtypes *in vivo* through induced transdifferentiation. For example, in the case of PD, iDAs could be used to restore dopamine to physiological levels in the affected striatum (Wakeman *et al*, 2011). As previously mentioned, transplanting midbrain-specific iDAs discovered by Kim *et al* (2011a) into lesioned striatums of a PD mouse model replenished dopamine levels and alleviated motor impairments (Kim *et al*, 2011a). Dell'Anno *et al* (2014) further analyzed the properties of *Ascl1*-, *Nurr1*-, *Lmx1a*-derived TH

neurons after transplantation and found that their iDAs survived transplantation over long periods of time, integrated into the host circuitry, and improved motor function in PD mice. Furthermore, Dell'Anno *et al* (2014) were able to pharmacologically manipulate activity of their engrafted iDAs *in vivo* using DREADD technology, which enhanced the recovery of motor impairments in PD mice.

Huntington's disease (HD), another neurodegenerative disease, is characterized by the specific degeneration of medium spiny neurons (MSNs) of the caudate and putamen. Previous studies have examined the capacity of fetal striatal neurons and MSNs derived from hESCs for transplantation and potential rescue of disease symptoms (Benraiss & Goldman, 2011). Victor *et al* (2014) used microRNAs miR-9/9* and miR-124, in combination with transcription factors *BCL11B, DLX1, DLX2, MYT1L* to produce induced neurons that resemble medium spiny neurons. When transplanted into the mouse striatum, these iMSNs survived more than 6 months *in vivo*, exhibited dense dendritic spines, displayed electrophysiological membrane properties similar to endogenous MSNs, and projected their axons to the appropriate targets. Together, these studies suggest that production of autologous cell types by direct reprogramming and transplantation may be a viable therapeutic avenue, providing that the transplanted cells do not pose a risk for tumorigenesis. Encouragingly, because direct reprogramming does not involve a dividing or pluripotent stem cell intermediate, this method may prove to be less likely to pose a risk of tumor formation than using cells derived from iPSCs or ESCs. Additional long-term engraftment studies comparing neurons derived by both methods will be required to determine which cell source provides the best benefit to risk profile.

Because transplantation and engraftment of exogenous neurons can potentially harm the brain, an attractive alternative to transplantation is to reprogram cells already found in the surrounding regions of the affected area into the desired neuronal subtype. It has been previously shown that neurons can be derived from mouse astroglia *in vitro* by overexpressing single transcription factors: *Pax6*, *Ngn2*, *Ascl1*, and *Dlx2* (Heins *et al*, 2002; Berninger *et al*, 2007; Heinrich *et al*, 2010, 2012). More recently, Addis *et al* (2011) demonstrated that iNs expressing TH could be generated from mouse postnatal astrocytes *in vitro* using a single polycistronic lentiviral vector encoding for three transcription factors: *Ascl1*, *Lmx1b*, and *Nurr1*. Taking this a step further, Torper *et al* (2013) transplanted human embryonic fibroblasts, fetal lung fibroblasts, and astrocytes that had been transduced with the BAM factors, then induced them to convert into neurons after transplantation. Similarly, *in vivo* conversion could be accomplished when using dopaminergic neuron reprogramming factors: *Ascl1*, *Brn2*, *Myt1l*, *Lmx1a*, *Foxa2*, *Lmx1b*, and *Otx2*. However, the conversion efficiency into TH-positive cells remained low, potentially due to the fact that the seven reprogramming factors were delivered in individual viral vectors.

As these studies showed, astrocytes can be converted into iNs after engraftment, but can transdifferentiation be accomplished solely *in vivo*? Transdifferentiation *in vivo* has the advantage of generating the desired cell type directly in its environmental niche and has previously been accomplished using transcription factors in other organs (Fu *et al*, 2014). In the nervous system, Torper *et al* (2013) demonstrated that endogenous astrocytes could be reprogrammed *in vivo* by specifically expressing the BAM factors in GFAP-expressing astrocytes of the mouse striatal parenchyma. Astrocytes were also converted into proliferative neuroblasts by expressing a single transcription factor, *Sox2,* in the adult mouse brain (Niu *et al*, 2013). When supplied with specific small molecules, these neuroblasts can further develop into functionally mature induced neurons.

For technical and safety reasons, direct reprogramming of human cells in humans has not been tested. However, human iNs can be produced from pericytes cultured from human brain specimens provided during surgical procedures. In addition, Karow *et al* (2012) discovered that overexpressing *Ascl1* and *Sox2* in pericytes produced iNs that were predominately GABAergic, indicating that subtype specificity can be imparted to neurons derived from a potentially useful cell type found in the brain. Many additional studies in model organisms will be required before such approaches are feasible to test in patients.

Mechanism and efficiency

In addition to its potential impact on translational medicine, drug screening, and disease modeling, direct reprogramming may also provide insight into unknown mechanisms governing neuronal differentiation. Cell identity is determined by the patterns and expression levels of a particular subset of our roughly 25,000 genes and non-coding RNAs. How can ectopic expression of as few as one, two, or three genes erase the identity of the cell of origin while simultaneously activating new, highly specific coordinated patterns of gene expression that allow iNs to maintain key features of neuronal identity such as electrical excitability, synapse formation and neuronal morphology? Some insight into this question may be gleaned from the fact that nearly all reprogramming factors reported to date are direct regulators of transcription, either transcription factors or miRNA. In one of the most well-characterized examples of reprogramming, the conversion of fibroblasts into iPSCs, individual reprogramming factors were replaced by their immediate downstream effectors demonstrating that a hierarchical transcriptional cascades form a cornerstone of iPSC reprogramming, which may also be the case in direct reprogramming into neuronal cells (Buganim *et al*, 2012). Although fewer mechanistic studies of direct reprogramming to neurons have been reported, a recent ChIP-seq study of reprogramming with the BAM factors identified *Zfp238* as key target gene of *Ascl1*. Accordingly, expression of *Zfp238* could partially replace *Ascl1* during reprogramming (Wapinski *et al*, 2013). Additional studies of this sort may be able to establish some of the mechanisms governing direct reprogramming and offer paths to more precise programming of desired subtypes of neurons or perhaps help to produce induced neurons with more mature properties.

Direct reprogramming to iPSCs and to neurons does not occur in 100% of transduced cells, suggesting that cells may have intrinsic barriers to direct reprogramming. The *Ink4a* locus has recently been identified as a barrier to iPSC reprogramming (Li *et al*, 2009; Utikal *et al*, 2009). The *Ink4a/Arf* locus encodes $p16^{Ink4a}$ in addition to $p21^{Cip1}$, and Trp53. Upregulation of $p16^{Ink4a}$ is thought to be the main inducer of cellular senescence during culture *in vitro*. Reduced proliferative potential has been thought to impair iPSC reprogramming because iPSCs are a highly proliferative cell type. In contrast, during neuronal reprogramming, fibroblasts exit the cell cycle as early as 3 days post-induction and conversion to neurons does not

appear to require a dividing neural stem cell intermediate (Vierbuchen *et al*, 2010; Blanchard *et al*, 2015). Yet, modulation of Ink4a can also impact direct neuronal reprogramming (Price *et al*, 2014). Further, treating fibroblasts with CHIR99021, a potent GSK3β inhibitor that induces proliferation, in conjunction with Tgfβ inhibition by SB431452, was demonstrated to increase reprogramming during BAM reprogramming from > 5 to ~80% (Ladewig *et al*, 2012). These results suggest that blocking cellular senescence pathways, or perhaps inducing fibroblast proliferation prior to the later stages of direct reprogramming may enhance cell fate conversion.

Perspectives

In this review, we have highlighted some of a larger group of recent studies that have generated induced neurons that resemble endogenous neurons of various subtypes based on their morphology, patterns of gene and protein expression, and functional and electrophysiological properties. However, a key unsolved issue with iNs is the question of how well they truly recapitulate the features of their endogenous counterparts. For some applications, such as production of dopamine or measures of responses to stimulating ligands such as capsaicin, it may be sufficient simply to screen for these desired neuronal features. Alternatively, to model more complex neurological diseases with more subtle impacts on neuronal specification, morphology, or synaptic connectivity, induced neurons may need to more fully recapitulate the transcriptional programs that characterize bona fide neuronal subtypes in the CNS or PNS.

However, at present, for most types of iNs, only a limited number of cellular features have been examined. Larger scale single cell and bulk population RNA-Seq experiments on various types of induced neurons and their presumed endogenous counterparts represent one approach to begin to identify neuronal features which are properly reset after reprogramming. Similarly, these studies could identify neuronal features that might require additional factors or be induced with exogenous cues that can mimic their niche *in vivo*. For example, the position of a neuronal cell body results in differential expression of Hox family genes in otherwise similar cells (Rinn *et al*, 2006). *In vivo*, retinoic acid gradients can drive differential expression of Hox genes in motor neurons. Mazzoni *et al* (2013) found that Hox transcription factors that control the further diversification of motor neurons into subpopulations were not upregulated in their induced spinal motor neurons. However, when retinoic acid was added during reprogramming, these iMNs increased expression of specific Hox genes, and appeared to acquire a cervical MN identity. It is likely that other aspects of neuronal identity that are induced by cues provided by local glial cells or appropriate potential synaptic partners will fail to emerge properly in iNs. Intriguingly, however, Blanchard *et al* showed that specific aspects of sensory neuron morphology, such as a pseudounipolar neurite structure, could be generated by reprogramming with only two transcription factors in the absence of any known exogenous cues. With additional studies, it may be possible to exploit direct reprogramming to distinguish between cell-intrinsic features of neurons and those that require exposure to a particular local environment.

An additional related question is whether it will be possible to produce all neuronal subtypes in the nervous system, or rather, whether some types of neurons might be difficult or impossible to produce using direct reprogramming approaches. Since so few neuronal subtypes have been generated, it is unclear whether these represent a privileged group of neuronal subtypes for which direct differentiation is possible. Future directed and perhaps also unbiased attempts to produce neurons with different transcription factors may help to answer this question. Similarly, it is not yet clear whether the generation of iNs is limited to producing defined neuronal subtypes that truly exist in the nervous system. An intriguing alternative possibility is that transcriptional circuits that underlie discrete attributes of different cellular subtypes are interchangeable allowing for the generation of completely new hybrid subtypes. Again, more thorough characterizations of the transcriptional profiles of different induced neuronal subtypes alongside parallel characterization of endogenous neurons will help to distinguish between these possibilities.

If it turns out that many desired neuronal subtypes cannot be produced using direct reprogramming, several alternative strategies may be useful to consider. One can potentially augment direct reprogramming with small molecules or chromatin modifiers. Indeed, small molecules can drastically increase neural reprogramming efficiency (Ladewig *et al*, 2012) and also reduce the number of reprogramming factors required (Liu *et al*, 2013). This suggests that additional chemical approaches that increase neural reprogramming diversity, efficiency, and utility will likely be identified. Along the same lines, addition of trophic factors during reprogramming and maturation may prove critical to generating particular subtypes and phenotypes.

Another attractive strategy to better recapitulate endogenous neuronal subtypes is to first generate the neural progenitor of the desired subtype and then induce differentiation of this precursor. It has been previously demonstrated that proliferative cells of the nervous system can also be generated with direct reprogramming. From fibroblasts, this includes bipolar neural precursors (Kim *et al*, 2011b), self-renewing, tripotent neural precursors (Lujan *et al*, 2012), and oligodendrocyte precursor cells (Yang *et al*, 2013). Tripotent neural precursor cells were also directly generated from human cortical astrocytes (Corti *et al*, 2012).

Finally, once the field has matured, it is likely that some of these iNs may be seen as suitable for testing in human transplantation-based therapies, as has recently been reported for retinal pigment epithelial cells derived from human iPSCs (Eiraku *et al*, 2011). Before such trials, it is essential to further develop this technique to ensure the safety of the patients. One hurdle is to produce iNs lacking genomic insertions which could lead to degeneration, dysfunction, or tumorigenesis. Recently, Adler and colleagues demonstrated that nonviral-mediated neuronal direct reprogramming can also be accomplished by delivering plasmids encoding the BAM factors with a bioreducible polymer (Adler *et al*, 2012). Additionally, mRNA approaches that have proved useful for iPSC generation (Warren *et al*, 2010) may be applied to neural reprogramming. It will also be important to understand the extent and role of somatic mutation in the donor cell lineages as well as the potential mutagenicity of the direct reprogramming methods. Similar to iPSCs, iNs will carry with them any somatic mutations that arose in the original donor cell. While these mutations might be benign in the context of a non-neural cell, they could be deleterious in the context of the brain. Fortunately, both neurons and iNs are post-mitotic, suggesting that barriers to potential tumorigenesis may be higher

using iNs than when using iPSCs or tissues derived from them. The remarkable progress made in the field of direct reprogramming to neurons in < 5 years suggests that this set of technologies will lead to many more discoveries relevant to understanding the human brain and the many currently untreatable disorders of the nervous system.

Conflict of interest

The authors declare that they have no conflict of interest.

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