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MINIREVIEWS

Using transcription factors for direct reprogramming of neurons *in vitro*

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Abstract

Cell therapy offers great promises in replacing the neurons lost due to neurodegenerative diseases or injuries. However, a key challenge is the cellular source for transplantation which is often limited by donor availability. Direct reprogramming provides an exciting avenue to generate specialized neuron subtypes *in vitro*, which have the potential to be used for autologous transplantation, as well as generation of patient-specific disease models in the lab for drug discovery and testing gene therapy. Here we present a detailed review on transcription factors that promote direct reprogramming of specific neuronal subtypes with particular focus on glutamatergic, GABAergic, dopaminergic, sensory and retinal neurons. We will discuss the developmental role of master transcriptional regulators and specification factors for neuronal subtypes, and summarize their use in promoting direct reprogramming into different neuronal subtypes. Furthermore, we will discuss up-and-coming technologies that advance the cell reprogramming field, including the use of computational prediction of reprogramming factors, opportunity of cellular reprogramming using small chemicals and microRNA, as well as the exciting potential for applying direct reprogramming *in vivo* as a novel approach to promote neuro-regeneration within the body. Finally, we will highlight the clinical potential of direct reprogramming and discuss the hurdles that need to be overcome for clinical translation.

Key words: Cell reprogramming; Neuronal subtypes; Transcription factors; Direct reprogramming; Glutamatergic neurons; GABAergic neurons; Retinal neurons

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Core tip: Direct reprogramming represents an innovative technology to generate neurons in the lab, which can be used for cell therapy, drug screening and disease modeling for neurodegenerative diseases. In this review we will discuss the current advance in identifying transcription factors to promote direct reprogramming of specialized neuronal subtypes, including glutamatergic, GABAergic, dopaminergic, sensory and retinal neurons. We will also discuss the hurdles that need to be overcome for clinical translation.

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INTRODUCTION

The mammalian nervous system in adults has limited regenerative capacity, thus disease or trauma often cause permanent neuronal damages and have debilitating repercussions[1]. To facilitate regenerative medicine and repair damages in the nervous systems, we must develop robust methods to generate specialized subtypes of neurons efficiently in the laboratory. Cellular reprogramming could be the key to this issue. This is a technique that utilizes transcription factors to convert one cell type into another. This was demonstrated by the early work of Takahashi and Yamanaka who were able to reprogram somatic cells into induced pluripotent stem (iPS) cells, using *Oct-3/4, Sox2, c-Myc*, and *Klf*[2].*This* seminal work established cellular reprogramming as a major game changer in generation of patient-specific cells *in vitro*[3] and enabled subsequent development of direct reprogramming, also known as trans-differentiation. A key characteristic of direct reprogramming is that this method bypasses the pluripotency stage and allows the conversion of one cell lineage directly to another, which represents a potentially faster method to generate cells compared to iPS cell generation and subsequent differentiation[4].

Direct reprogramming allows the rapid generation of patient-derived neurons *in vitro*, providing a cellular source for transplantation, disease modelling, drug screening and gene therapy[5]. Previous studies have identified transcription factors that promote direct reprogramming of multiple starting cell types into specialized neuronal subtypes (Table 1, Figure 1). Here, we will summarize the role of proneural transcription factors in development and highlight their use in direct reprogramming, with particular focus on their use for specification of neuronal subtypes *in vitro*.

MASTER REGULATORS OF THE NEURONAL LINEAGE

Proneural genes were first discovered in Drosophila by knockout studies to determine genes responsible for development of sensory bristles[6]. A lack of bristles in a scute mutant fly led to the discovery of many proneural genes essential to proper neural development of the fly[7]. Likewise in the early developmental stages of humans, proneural factors promote neurogenesis and differentiation of progenitor cells to become specialised neurons[6]. For instances, basic helix loop helix (bHLH) genes are important regulators for the specification of neuronal cell fate and differentiation of neural cells in the central and peripheral nervous system[8]. Due to their importance in neural development, bHLH genes are often utilized in direct reprogramming to direct cells into the neuronal lineages[9]. bHLH genes can be categorized into two subtypes; specification and differentiation, both of which are important in the reprogramming of a neuronal subtype[8].

Mash1/Ascl1, is a specification bHLH transcription factor found to be expressed in neural precursors of the developing embryo with a transient expression[8,10]. *Mash1/Ascl1* double knockout mice die within 24 h of birth and show major defects in the development of neuronal progenitors in olfactory epithelium, as well as lack of generation of sympathetic neurons. Hence, *Mash1/Ascl1* plays an important role in the development of neuronal progenitors[11]. In terms of direct reprogramming, *Ascl1* alone was shown to have the ability to reprogram mouse fibroblasts into functional induced neurons (iN). These iN exhibited the expression of mature neuronal markers, including Tuj1, NEUN, MAP2 and synapsin, after 21 d of induction. Notably, the iN

Table 1 Summary of *in vitro* neuronal reprogramming studies discussed in this review with details of transcription factors used and neuronal characteristics

Species	Cell of origin	Target cell	Transcription factor(s)	Neuronal characteristic	Year and ref.
Mouse	Embryonic fibroblast	iN	*Ascl1*	Neuronal morphology Neuronal markers Functional electrophysiology	2014[12]
Human	Fetal fibroblast	iN	*ASCL1*	Neuronal morphology Neuronal markers	2014[12]
Human	Fibroblast	iN	*ASCL1*, *SOX2* and *NGN2*	Neuronal morphology Neuronal markers Neuronal gene expression profile Functional electrophysiology	2015[14]
Mouse	Embryonic fibroblast	iN	*Ascl1*	Simple neuronal morphology Neuronal markers	2010[25]
Mouse	Embryonic fibroblast	iN (mostly GABAergic and glutamatergic neurons)	*Brn2*, *Myt1l*, *Zic1*, *Olig2*, and *Ascl1*	Neuronal morphology Neuronal markers Functional electrophysiology Synaptic maturation	2010[25]
Mouse	Embryonic fibroblast	iN (mostly excitatory neurons)	*Brn2*, *Myt1l*, and *Ascl1*	Neuronal morphology Neuronal markers Functional electrophysiology Synaptic maturation	2010[25]
Mouse	Adult tail tip fibroblast	iN (mostly excitatory neurons)	*Brn2*, *Myt1l*, and *Ascl1*	Neuronal morphology Neuronal markers Functional electrophysiology Synaptic maturation	2010[25]
Human	Fibroblast	iN (mostly dopaminergic neurons)	*BRN2*, *MYT1*, *ASCL1*, *LMX1A* and *FOXA4*	Neuronal markers Functional electrophysiology	2011[26]
Mouse	Embryonic fibroblast	iN (mostly dopaminergic neurons)	*Mash1*, *Nurr1* and *Lmx1a*	Neuronal markers Neuronal gene expression profile Neuronal epigenetic reactivation Functional electrophysiology	2011[33]
Human	Adult fibroblast	iN (mostly dopaminergic neurons)	*MASH1*, *NURR1* and *LMX1A*	Neuronal morphology Neuronal markers Neuronal gene expression profile Functional electrophysiology	2011[33]
Human	Fibroblast	iN (mostly dopaminergic neurons)	*ASCL1*, *NGN2*, *SOX2*, *NURR1* and *PITX3*	Neuronal morphology Neuronal markers Neuronal gene expression profile Functional electrophysiology	2012[35]
Mouse	Embryonic fibroblast	Induced sensory neurons	*Brn3a* and *Ngn1* or *Ngn2*	Neuronal gene expression profile Functional electrophysiology Synaptic maturation	2015[38]
Human	Adult fibroblast	Induced sensory neurons	*BRN3A* and *NGN1* or *NGN2*	Neuronal morphology Neuronal markers Neuronal morphology Neuronal markers Functional electrophysiology	2015[38]

Mouse	Embryonic fibroblast	Induced nociceptors	*Ascl1*, *Myt1l*, *Ngn1*, *Isl2*, and *Klf7*	Neuronal morphology Neuronal markers Neuronal gene expression profile Functional electrophysiology Synaptic maturation	2014[43]
Human	Fibroblast	Induced nociceptors	*ASCL1*, *MYT1L*, *NGN1*, *ISL2* and *KLF7*	Neuronal markers Functional electrophysiology	2014[43]
Human	Iris cells	Photoreceptor- like cells	*Crx*, *Rx* and *Neurod1*	Neuronal morphology Neuronal markers Neuronal gene expression profile Functional light electrophysiology	2012[47]
Human	Dermal fibroblast	Photoreceptor- like cells	*CRX*, *RAX*, *NEUROD1* and *OTX2*	Neuronal markers Neuronal gene expression profile Functional light electrophysiology	2014[48]
Mouse	Müller glia	iN (mostly retinal glia-like neurons)	*Neurog2*	Neuronal morphology Neuronal markers Functional electrophysiology Neuronal gene expression profile	2018[56]
Mouse	Müller glia	iN (mostly retinal neurons)	*Ascl1*	Neuronal morphology Neuronal markers Functional electrophysiology Neuronal gene expression profile	2018[56]
Mouse	Cerebellum astroglia	iN (mostly glutamatergic neurons)	*Neurog2*	Neuronal morphology Neuronal markers Synaptic maturation Functional electrophysiology	2017[57]
Mouse	Cerebellum astroglia	iN (mostly glutamatergic neurons)	*Ascl1*	Neuronal morphology Neuronal markers Synaptic maturation Functional electrophysiology	2017[57]

iN: Induced neurons.

were predominantly excitatory as they expressed vesicular glutamatergic transporter 1 but not GABAergic transporter[12]. Furthermore, the use of *Ascl1* in a combinational approach has shown to increase the directionality of target neuronal subtype and complexity of the maturation. This will be discussed further in the review under specific subtypes of neurons.

Another bHLH specification gene is neurogenin 2 (*NGN2*), which is found to have a similar expression pattern to *ASCL1* in undifferentiated neural crest cells during development. In neuronal reprogramming experiments *NGN2* and *ASCL1* can bind and interact when used in combination[13] and increase the neuronal conversion efficiency by up to 13.4%[14]. However, other studies have shown that the expression of *NGN2* returns to basal level after neuronal conversions have occurred, suggesting that it is involved in the initial neuronal specification but doesn't have a long-term neuronal survival effect following reprogramming[15].

Unlike the specification factors, differentiation factors have a role in the later maturation of neurons. The bHLH differentiation factor *NeuroD1* is absent in precursors and is found to increase expression by 50-fold once the neuron has reached terminal differentiation[16]. Its expression is required for both the maturation process as well as the survival of newly generated neurons[17]. *NeuroD1* is found to be upstream to *Ngn2*[18]. These factors show sequential expression which can be attributed to their loci placement. This pattern is commonly seen in transcription factors involved in different stages of the cell cycle. For instance, the specification factors are found upstream to the differentiation factors[19]. In addition, differentiation factors often play a major role in the neuronal reprogramming and maturation processes.

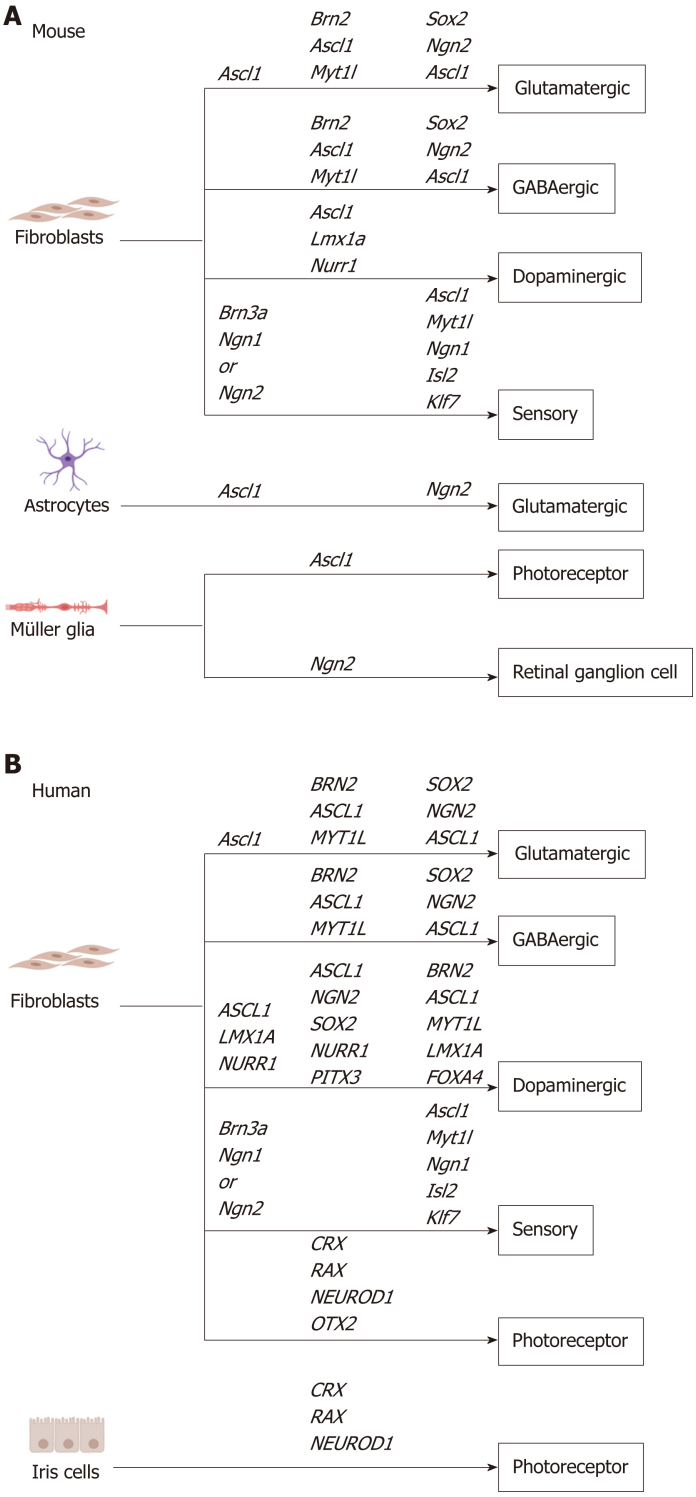

Figure 1 Transcription factor combination used for *in vitro* direct reprogramming to specific neuron subtypes in mouse and human, including sensory neurons, GABAergic neurons, glutamatergic neurons, dopaminergic neurons, photoreceptors and retinal ganglion cells. A: Mouse; B: Human.

TRANSCRIPTION FACTORS IN SPECIFICATION OF NEURONAL SUBTYPES

Glutamatergic and GABAergic neurons

The *in vitro* generation of neuronal subtypes provides an invaluable resource for the study of many neuronal diseases. Figure 1 summarized the transcription factor combinations that were used for *in vitro* direct reprogramming into neuronal subtypes. One such promising effort is in the reprogramming of glutamatergic neurons. Glutamate is the major neurotransmitter of the central nervous system and

an imbalance in its production can lead to major neuronal defects[20,21]. Degradation of glutamatergic neurons is linked to disorders such as schizophrenia[22]. On the other hand, overexpression of glutamate can lead to excitotoxicity and glutamatergic cell death, which are associated with Alzheimer's disease and amyotrophic lateral sclerosis[23,24].

As mentioned previously, *ASCL1* alone is capable of reprogramming fibroblasts into excitatory glutamatergic neurons. However, *ASCL1* is often used in a combinatorial transcription factor approach for direct reprogramming to generate neurons, such as the widely used BAM combination (*ASCL1, BRN2* and *MYT1L)* discovered by Vierbuchen *et al*[25]. In this study, reprogramming using *ASCL1* alone resulted in generation of Tuj1-positive neurons. However, the morphological complexity, maturity and action potentials of the cells could be further improved by the addition of *BRN2* and *MYT1L*. BAM were able to reprogram fibroblasts into predominantly a mix of both glutamatergic and GABAergic neurons; while no other major neuronal subtypes were detected in significant numbers[26]. Unlike glutamate, gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter produced by GABAergic neurons[27]. A further study has discovered that BAM expression is only required in the initial stages of the reprogramming process and removing gene expression 3 days after transduction has no effect on the neuronal reprogramming process[26].

The incorporation of *BRN2* and *MYT1L* into neuronal reprogramming is due to their importance in neural development. *BRN2* has a role in the mouse hypothalamus enhancing neural differentiation during development, as well as promoting activation of other events which play a role in the maturation and survival of paraventricular nuclei, as well as supraoptic nuclei[28,29]. *MYT1L* is a gene expressing a zinc finger protein which can be found in early differentiating neurons but is absent from glial cell populations, suggesting a role in early neuronal differentiation[30]. Although these factors are important for the reprogramming of neurons, neither *BRN2* or *MYT1L* alone is sufficient to induce neurons, as they require *ASCL1* to initiate the specification process[25].

Dopaminergic neurons

Dopaminergic (DA) neurons are the main source of dopamine production in the mammalian nervous system and its degeneration could lead to devastating neurological disorders, such as Parkinson's disease. Restoration of DA neurons in Parkinson's disease could provide a treatment for the disease as demonstrated in rat models[31]. In order to achieve this level of cell type specification, incorporation of fate specification factors during the reprogramming process is necessary.

By incorporating the expression of DA specification factors *LMX1A* and *FOXA4* along with BAM factors, approximately 10% of the human iN reprogrammed from human embryonic fibroblasts were DA neurons. Interestingly, the same study found that *LMX1A* and *FOXA4* drove more human iN to a DA fate but did not increase the overall neuronal conversion rate[26]. The differentiation of DA neurons has been shown to be influenced by a positive feedback system in which *FOXA1* and *FOXA2* promote the expression of *LMX1A* and *LMX1B*, which subsequently leads to the development of mesodiencephalic DA neurons[32].

There are also other studies that utilized alternative combination of factors with similar results. Caiazzo *et al*[33] were able to induce DA neuron reprogramming using fibroblasts with a combination of *Ascl1, Nurr1* and *Lmx1a*. This 3-factor cocktail was able to induce approximately 85% iN that are positive for the DA marker, TH. In this regard, *Nurr1* is a DA specific receptor, essential for the formation and survival of DA neurons[34]. It is not activated by ligands but rather forms a heterodimer complex with retinoid X receptor (RXR), and together this complex is able to bind RXR ligands that produce signalling essential for the survival of DA neurons[34].

A subsequent study identified another set of 5 transcription factors which successfully converted human fibroblasts into human-induced DA neurons: *ASCL1, NGN2, SOX2, NURR1* and *PITX3*. Importantly, *ASCL1, NGN2* and *SOX2* are required for this reprogramming process. On the other hand, exclusion of *NURR1* and *PITX3* did not have an effect in the early reprogramming process, rather these factors increased the dendrite network and facilitated maturation of DA neurons[35].

Sensory neurons

Diverse subtypes of sensory neurons are responsible for pain and itch perception. Mutations in sensory neuron-specific proteins result in development of a wide range of sensory disorders, like Friedreich's ataxia[36]. Due to limited availability of human sensory neurons, research in this field is largely dependent on animal models, especially rodents. Therefore, many mechanisms involved in human pain and itch perception remain uncharacterized. Studies from human primary dorsal root ganglion

(DRG) neurons have identified differences between human and mouse nociceptors in function of individual channels, receptors and their response to chemical stimuli[37]. Hence, the development of protocols for the generation of bona-fide human sensory neurons in sufficient numbers is vital for accurate modeling of processes like pain and itch.

A previous study has demonstrated the feasibility of reprogramming fibroblasts into sensory neurons using a combination of *Brn3a* either with *Ngn1* or *Ngn2*[38]. The cells displayed pseudounipolar morphology, and selectively responded to chemical mimics of pain, temperature, and itch. Both combinations with *Ngn1* or *Ngn2* equally induced the differentiation into three functional classes of sensory neurons, expressing one of the tropomyosin receptor kinase TrkA, TrkB, or TrkC[38]. *Ngn1* and *Ngn2* are alternative neurogenic bHLH factors that are expressed within progenitors of the developing DRG[39]. Both factors promote cell cycle exit through the induction of *NeuroD1* and *NeuroD4*, and regulate two waves of neurogenesis[40-42]. *Ngn1* and *Ngn2* are co-expressed during much part of the early phase of DRG neurogenesis, and are both required for the generation of TrkB+ and TrkC+ sensory neurons. On the other hand, in the later phase *Ngn1* is exclusively expressed in neural precursors and is largely responsible for the development of TrkA+ class sensory neurons[39]. At the time of cell cycle exit, the sensory neurons express the "pan-sensory" factors *Brn3a* and *Islet1*, which terminate the expression of the bHLH neurogenic factors and initiate the expression of definitive sensory markers to complete neurogenesis[42].

Similarly, functional nociceptor neurons can be generated through the transgenic expression of *Ascl1*, *Myt1l*, *Ngn1*, *Isl2*, and *Klf7* in mouse and human fibroblasts[43]. The resultant iN expressed functional receptors for the noxious compounds menthol (TrpM8), mustard oil (TrpA1) and capsaicin (TrpV1). They also displayed the nociceptor-specific TTX-resistant Nav1.8 sodium channel. Notably, these iN were able to replicate inflammatory and chemotherapy-induced sensitization, which form the basis of pathological pain. Furthermore, this reprogramming method was successful in the generation of patient-derived neurons from familial dysautonomia, thus representing a promising approach for disease modeling[43]. For the five factors employed, *Ascl1* and *Myt1l* are members of the three-factor combination for neuronal lineage reprogramming BAM[25]; *Klf7* is a factor involved in TrkA expression maintenance[44]; and *Isl2* was selected based on its expression profile in DRG[45]. The extent of *Isl2* contribution to the lineage reprogramming remained unknown and further studies are needed to clarify this.

Retinal neurons

Retinal neurons are another promising target for therapeutic *in situ* reprogramming strategies. The retina is a highly organized structure bearing several major types of neurons, including rod and cone photoreceptors, bipolar, amacrine, horizontal and retinal ganglion cells (RGC)[46]. Rod and cone photoreceptors are responsible for detecting light stimuli and converting it to electrical signals that are later sent to the brain for visual perception. The loss of photoreceptors is a key hallmark of many blinding diseases and currently there are no effective treatments to cure blindness once photoreceptors are lost. Therefore, cellular reprogramming is a powerful technique for developing novel approaches for photoreceptor regeneration.

Akihiro Umezawa's group used a promising combination of factors (*CRX, RAX* and *NEUROD1*) to induce the conversion of both iris cells[47] and fibroblasts into photoreceptor-like cells[48]. These factors induced the expression of photoreceptor-related genes and the reprogrammed cells became positive for the rod marker rhodopsin and the cone marker blue-opsin. Notably, some of the reprogrammed cells were photoresponsive. Interestingly, the authors showed that although *OTX2* enhanced the upregulation of retinal genes, it was not essential for the reprogramming[48]. The same combination of factors was used to reprogram peripheral blood mononuclear cells[49], which facilitate clinical applications of this reprogramming strategy as blood samples are easier to collect from patients than fibroblasts. The generated photoreceptor-like cells expressed blue and red/green opsin, and some of the cells were able to respond to light stimuli. However, the reprogrammed cells expressed low levels of rod marker rhodopsin and some photoreceptor-related genes were not detected, which indicated that additional factors might be required to produce mature and functional photoreceptors[49].

In the developing retina, *OTX2* is expressed in progenitors and early precursors that become committed to a photoreceptor fate[50,51]. *NEUROD* and *CRX* are factors expressed in developing photoreceptors and their expression is maintained in photoreceptors of mature retina, therefore they are proposed to participate in cell fate specification, as well as in maturation and survival processes[41,51]. Additionally, *NEUROD* is known to promote neuron specification in retinal progenitors and regulates interneuron differentiation to direct the amacrine cells fate[41]. On the other

hand, *CRX* is downstream of *OTX2* activity, it enhances the expression of photoreceptor-specific genes and is important for terminal differentiation into rod and cone photoreceptors[51,52]. *RAX* is a factor expressed in retinal and hypothalamic progenitor cells and is a key transcription factor for eye development in vertebrates. In the developing retina, it is involved in retinal progenitor proliferation and photoreceptor fate specification[53-55].

RGC are another interesting therapeutic target within the retina. These specialised retinal neurons convey the visual cues to the brain and form the optic nerve[46,51]. Degeneration of RGC is a major hallmark in glaucoma, a major blinding disease affecting the aging population[56]. Thus, *in vitro* generation of RGC offers an exciting avenue to develop regenerative therapy for this disease.

A study by Guimarães *et al*[56] demonstrated that RGC can be reprogrammed from postnatal Müller glia through the overexpression of *Ngn2*. The forced expression of *Ngn2* produced a pool of iN which express genes associated with photoreceptors, amacrine cells and RGCs. However, this was only possible with Müller glia from young mice, as P(21) Müller glia cells failed to reprogram into iN. They also showed that the presence of mitogenic factors like EGF or FGF2 during the expansion of Müller glia enhanced the efficiency of the reprogramming. This finding supports the theory that the starting cell types for reprogramming have a strong influence on the neuronal subtypes in the resultant iN. For instance, *ASCL1* or *NGN2* alone can reprogram astroglia from the cerebellum and neocortex into neuron subtypes in the brain[57]. On the other hand, these factors can drive reprogramming of Müller glia into iN subtypes with retinal neuronal identities[56,58]. Thus, careful consideration should be taken in choosing the starting cell type in reprogramming to generate specialized neuronal subtypes.

NEW TECHNOLOGICAL ADVANCES IN CELLULAR REPROGRAMMING

Recent studies have demonstrated several exciting approaches to further develop the direct reprogramming processes. Here we will highlight and discuss the use of new technological advances in overcoming the hurdles of direct reprogramming as well as the potential of *in vivo* application of direct reprogramming to promote regeneration.

Computational predictions of transcription factors

Most of the reprogramming studies mentioned above have taken an elimination approach to identify the optimal set of transcription factor(s) required for direct reprogramming, which is a tedious and laborious screening process. Recent advances in computational biology provide an alternative approach to predict the required transcription factor for cellular reprogramming in a faster and more efficient manner. One early example is CellNet, a computational algorithm that analyze, categorize and predict the function of transcription factors[59]. Subsequently, several other programs have been described to predict the transcription factors for cellular reprogramming, including Mogrify[60], BART[61], MAGICACT[62] and CellRouter[63]. Most of these programs work by comparing the quantitative amount of transcription factor in one cell type to another cell type to create a specificity score, which is used to create a ranking in which transcription factor are sorted and categorized depending on the cell type they are most prominent in[59,64]. Another exciting approach to predict transcription factors for cellular reprogramming is by creating computer models of cells. These programs, such as DeepNEU, can be used to create a simulation model of the cell using deep learning and provides a simulation of the events after the introduction of selected transcription factors[65]. Although these advanced algorithmic models are still in their infancy, further development will improve our understanding of the fundamental properties of cells and the molecular interplay of transcription factors that promote cellular reprogramming.

In vivo application of direct reprogramming

The potential to apply direct reprogramming *in vivo* represents an exciting direction for regenerative medicine. Therapeutic approaches using *in vivo* reprogramming have the potential to re-purpose local cells into the cells lost following injury or disease, thus providing an alternative regenerative approach to transplantation[66]. This has already been demonstrated for many neuronal subtypes, including glutamatergic and GABAergic neurons[67]. It was shown that overexpression of *NeuroD1* is sufficient to convert astrocytes to glutamatergic neurons in rodents *in vivo*, as characterized by vGlut1 expression and glutamate-mediated synapses. Compared to astrocytes, interestingly the same study found that the NG2 glial cells have a larger repro-

gramming capacity into multiple neuronal subtypes, as the presence of both glutamatergic and GABAergic neurons was detected after *NeuroD1* overexpression[67].

There has also been an extensive effort to re-purpose local cells for retinal regeneration. Many studies target the Müller glia within the retina for reprogramming, which are cells responsible for maintaining the integrity and homeostasis of the retina[68]. Interestingly, the Müller glia exhibit some progenitor properties. In the teleost fish and chicken, upon retinal injury the Müller glia can dedifferentiate into multipotent progenitors and give rise to all retinal neural subtypes[69]. This remarkable trait makes Müller glia an excellent candidate for reprogramming studies.

In several elegant studies by Tom Reh's group, the author has demonstrated successful reprogramming of mouse Müller glia into a range of retinal neurons both *ex vivo* and *in vivo*, by the forced expression of the pro-neural factor *Ascl1*[58,70]. When *Ascl1* was overexpressed in retinal explants and in Müller glia dissociated cultures, the cells re-entered the mitotic cell cycle and expressed neural progenitor genes[58]. This reprogramming was accompanied with chromatin remodeling, acquisition of neural morphology and the ability to respond to neurotransmitters[58]. In addition to its role as a pioneer proneural factor for direct reprogramming and retinal development, *Ascl1* induction in response to injury is required for retinal regeneration in the fish[71-73]. Conversely, organisms with limited regenerative capacity in the retina do not upregulate *Ascl1* following retinal damage[58,74]. Subsequently, in a landmark study the same group extended the application of *Ascl1* to reprogram Müller glia into retinal neurons *in vivo* following retinal injury[70]. Marker analysis showed that the reprogrammed retinal neurons were able to functionally integrate with the existing retinal circuit. Interestingly, this reprogramming approach was less effective in adult mice compared to young mice[75]. The restrictive regenerative capacity of adult mice Müller glia is thought to be caused by reduced epigenetic accessibility of progenitor genes in the cell, as the addition of histone deacetylase tricostatin A is able to overcome this epigenetic hurdle in adult Müller glia[70]. In support of this, epigenetic profiling using ATAC-seq demonstrated that the treatment with trichostatin A favored the accessibility of genes associated with neural development and differentiation, such as *Otx2*[70]. In this case, *Otx2* is known to regulate genes associated with bipolar and amacrine cells. However, it should be noted that most of the reprogrammed retinal neurons are bipolar cells, suggesting that additional factors are required to induce photoreceptors *in vivo*.

A subsequent study from Bo Chen's group demonstrated that adult mice Müller glia can be reprogrammed to rod photoreceptors without the necessity of retinal injury[76]. In this study, the authors first stimulated Müller glia proliferation by forced expression of β-catenin, followed by overexpression of *Otx2, Crx* and *Nrl* after two weeks. This approach allowed successful generation of rod photoreceptors *in vivo* that functionally integrate into the retinal and visual cortex circuits. In a remarkable experiment, the authors were able to use this *in vivo* reprogramming approach to restore light response in a mouse model of photoreceptor degeneration. Collectively, these studies demonstrate the potential of *in vivo* reprogramming as a novel approach for neural regeneration.

Alternatives to transcription factor-mediated reprogramming

Beside transcription factors, alternative direct reprogramming strategies using small chemicals and microRNAs are also two exciting research directions to improve the usability of the technology. For instance, chemical reprogramming using small chemicals has been shown to induce functional neurons without the need for transcription factor. A cocktail of 7 small molecules (Valproic acid, CHIR99021, Repsox, Forskolin, SP600125, GO6983 and Y-27632) was successful at reprogramming human fibroblasts into neurons with functional electrophysiology representative of glutamatergic and GABAergic cells[77]. Furthermore, the use of a combinational approach of small molecules together with transcription factors can improve direct reprogramming. In a study by Liu *et al*[15], *NGN2* along with small molecules, forskolin and dorsomorphin, directly reprogrammed human fetal lung fibroblasts to cholinergic neurons with functional electrophysiology. In this regard, forskolin is a cAMP activator in the PKA signalling pathway and dorsomorphin acts as a BMP inhibitor in the BMP signalling pathway, both of which are signaling pathways involved in neurogenesis.

Similarly, microRNAs have also been used in combinational approaches with transcription factors to induce neuronal reprogramming. microRNAs are known to play important roles in post transcriptional regulation, neural differentiation, morphological and phenotypic development[78-82]. In a study by Yoo *et al*[83], miR-9/9* and miR-124 were used in combination with *NEUROD1* to convert human fibroblasts into neurons, however these cells would not always demonstrate repetitive action potential, signifying immature neurons. In order to tackle this issue the same group

also introduced two other factors, *ASCL1* and *MYT1L*, and in turn were able to induce neurons with higher maturity which demonstrated repetitive action potentials and even the ability to convert adult human fibroblasts into functional neurons. These studies highlighted alternative approaches to transcription factors that promote neuronal reprogramming.

CLINICAL PERSPECTIVES OF *IN VITRO* NEURONAL REPROGRAMMING AND CHALLENGES FOR CLINICAL APPLICATION

Allogeneic transplantation represents a promising cell therapy approach to replace neurons lost by injury or neurodegenerative disease, such as Parkinson's disease[84]. However, there are two major challenges related to transplantation: (1) The shortage of donor tissue for transplantation; and (2) Immuno-rejection issues of the grafted tissue. Development of stem cell and cell reprogramming technology to generate patient-specific cells *in vitro* would be critical to overcome these hurdles. Cell therapies strategy using pluripotent stem cells have been extensively highlighted previously[85-87]. In comparison, notably direct reprogramming bypasses the pluripotent stem cell state, thus this is potentially a faster and more cost-effective approach to generate neurons *in vitro*, with less tumorigenic risks compared to pluripotent stem cell strategy. Moreover, there is also the exciting opportunity to combine with gene therapy to correct disease-causing mutation(s) in the cells *in vitro*, prior to transplantation to patients to treat hereditary neurodegenerative diseases.

To facilitate clinical translation of direct reprogramming technology, it is critical to develop robust reprogramming protocol to generate target cells with high purity and efficiency. Optimization of transcription factors for direct reprogramming, as well as improved method for gene delivery would be key to improving the reprogramming efficiency. Flow cytometry or magnetic-activated cell sorting can be used to enrich the purity of the target cell type prior to transplantation. For clinical applications, cells derived by direct reprogramming should be produced under good manufacturing practices conditions. To ensure the quality of the reprogrammed cells, it is important that the derived cells are extensively characterized for marker expression and functional studies, and screened to ensure the derived cells have a normal karyotype. In the latter case, the use of non-integrative methods for direct reprogramming is desirable, such as Sendai viruses or episomal vectors.

CONCLUSION

In summary, direct reprogramming allows the conversion of one somatic cell type directly to another cell type. The *in vitro* reprogramming of neurons provides an exciting avenue to generate patient-specific neurons for disease modelling, drug testing and cell therapy for neurodegenerative diseases. Transcription factors responsible for the specialization and differentiation of neuronal cells during development are commonly for direct reprogramming to generate multiple neuronal subtypes. Future studies that optimize the precise combinations of transcription factors for neuronal reprogramming would improve the reprogramming efficiency, expand the neuronal subtypes that can be generated and facilitate the translation of cellular reprogramming to the clinics. Emerging computational algorithms and alternative reprogramming approaches will further improve the technique of direct reprogramming, and future application of direct reprogramming *in vivo* would provide a novel approach to promote regeneration in the nervous system.

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