Perspective

In vivo direct reprogramming as a therapeutic strategy for brain and retina repair

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Once neurons are lost because of injury or degeneration, they hardly ever regenerate in most mammalian central nervous system (CNS) regions. In adult rodents, some brain regions, such as the subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus, retain neural stem cells (NSCs) and generate new neurons. Although a small population of new neurons derived from NSCs migrate toward lesion sites after brain injury, they are insufficient to completely restore neuronal functions. Cell transplantation using induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) has become an attractive therapeutic strategy for nerve injury or degeneration (Barker et al., 2015; Huang and Zhang, 2019). For Parkinson's disease, transplantation of dopaminergic neurons from human ESCs or iPSCs is emerging as a therapeutic approach (Li and Chen, 2016). However, the risks of immune rejection and tumorigenesis remain substantial drawbacks of this therapeutic approach.

In vivo direct neuronal reprogramming, forcibly converting non-neuronal cells to neuronal cells by artificial manipulation of gene expression patterns, has emerged as a potential therapeutic approach for treating nerve injury and neurological diseases (Niu et al., 2013; Li and Chen, 2016; Chen et al., 2019; Matsuda et al., 2019; Mattugini et al., 2019; Wu et al., 2020; Zhou et al., 2020). Compared to external cell transplantation, direct neuronal reprogramming has several advantages, such as a short induction period, non-immunogenicity, and absence of ethical concerns and risk of tumorigenesis. Astrocytes are the most populous glial cells and tile the entire CNS. In response to focal tissue injury or inflammation, astrocytes proliferate and become reactive to establish scar borders that segregate damaged and inflamed tissue from adjacent viable and functioning neural tissue (Li and Chen, 2016). Therefore, *in vivo* neuronal conversion from astrocytes is a potential strategy to modify gliotic tissues to provide a cellular source for reconstructing disrupted neuronal circuits. The approach of turning astrocytes into induced neuronal (iN) cells was first reported 13 years ago, based on the forced expression of Neurog2 (Berninger et al., 2007). Six years later, Niu et al. (2013) successfully converted astrocytes to iN cells *in vivo* by forcing the expression of SOX2. More recently, astrocytes have been converted into distinct subtypes of iN cells in several brain regions (Chen et al., 2019; Mattugini et al., 2019; Zhou et al., 2020). This strategy has also been investigated as a possible treatment for brain injury (stab wound injury, stroke) and neural degeneration (Parkinson's disease, Alzheimer's disease, Huntington's disease) (Niu et al., 2013; Li and Chen, 2016; Chen et al., 2019; Matsuda et al., 2019; Mattugini et al., 2019; Wu et al., 2020; Zhou et al., 2020). For instance, Chen et al. (2019) revealed that *in vivo* astrocyte-to-iN cell conversion mediated by NeuroD1 not only regenerates new cortical neurons but also protects injured neurons in the cortex of focal stroke induced by the vasoconstrictive peptide endothelin-1 in model mice, and thereby promotes functional recovery. This result implies that astrocyte-to-iN cell conversion may reduce the harmful inflammatory response of astrocytes after injury. Moreover, combinatorial expression of NeuroD1 and Dlx2 has been reported to convert striatal astrocytes into functional GABAergic iN cells that alleviate motor dysfunction in Huntington's disease model mice (Wu et al., 2020).

Microglia are brain-resident immune cells that use their ramified processes to survey the brain parenchyma. Microglia express a set of genes that allow them to sense their surroundings for inflammatory cues, promote neuron survival, and contribute to activity-dependent synaptic remodeling. These cells also gather at sites of injury to engulf damaged cells and debris, becoming one of the major cell types at the glial scar. Furthermore, a recent study indicated that, even after selective elimination of over 99% of microglia in the adult mouse brain, the microglia population can be swiftly reestablished from the remaining 1% (Li et al., 2019). Thus, microglia that have converged at the injured sites may also provide favorable candidates for restoring lost neurons by direct neuronal conversion without exhausting the cell source.

We have recently shown that the expression of a single transcription factor, NeuroD1, induces direct neuronal conversion of microglia, both *in vitro* and in the mouse brain (Matsuda et al., 2019). We also revealed that NeuroD1 accesses closed chromatin associated with bivalent histone modifications, including active (trimethylation of histone H3 at lysine 4 [H3K4me3]) and repressive (H3K27me3) marks, and induces the expression of neuronal genes. These chromatin regions are subsequently converted to a monovalent active state, altering the epigenome state by, at least in part, direct induction of genes involved in epigenetic modifications and chromatin remodeling. NeuroD1 also induces transcriptional repressors that silence microglia-specific genes, while reprogramming the microglial epigenetic landscape around promoter and enhancer regions to suppress microglial identity. We have also reported that NeuroD1 could convert microglia to striatal projection neuron-like cells in the adult mouse striatum, and these iN cells were functionally integrated into brain circuits through synaptic connections with other neurons. Although further investigation is required to reveal whether these iN cells generated from microglia contribute to functional recovery after brain disease and injury, control of neurogenesis from brain-resident non-neuronal cells by *in vivo* direct reprogramming holds promise as a potential therapeutic strategy to treat brain diseases.

The mammalian retina, which is anatomically and developmentally known as an extension of the CNS, has almost no potential to regenerate new neurons, and the loss of photoreceptor cells or retinal ganglion cells can lead to irreversible visual impairment or blindness. Müller glia, the major glial cell type in the retina, serve to provide structural support and maintain homeostasis of retinal neurons. Müller glia proliferate and then produce new neurons as retinal stem cells in cold-blooded vertebrates such as zebrafish, but not in mammals, possibly due to the inability to proliferate in the physiological condition. In the injured retina, Müller glia are known to become reactive and release inflammatory factors. Yao et al. (2016) revealed that N-methyl-D-aspartate (NMDA)-induced retinal injury initiated the proliferation of Müller glia through Wnt/β-catenin signaling. They also found that forced expression of β-catenin in Müller glia induced their transient proliferation, resulting in the generation of a small number of new neurons, even under physiological conditions, although most cell-cycle-reactivated Müller glia underwent cell death (Yao et al., 2016). Recently, Yao et al. (2018) further reported that sequential gene transfer of β-catenin and transcription factors Otx2, Crx and Nrl can convert Müller glia to rod photoreceptors in mice. Müller glia-derived photoreceptors restored visual responses in Gnat1rd17Gnat2cpfl3 double-mutant mice, which lack photoreceptor-mediated light responses, throughout the visual pathway from the retina to the primary visual cortex (Yao et al., 2018). In addition, Zhou et al. (2020) reported that downregulation of a single RNA binding protein, Ptbp1, can convert Müller glia into retinal ganglion cells, leading to the alleviation of disease symptoms associated with retinal ganglion cell loss in an NMDA-induced retinal injury mouse model (Zhou et al., 2020). However, since Müller glia play a crucial role in retinal homeostasis, long-term evaluation is necessary to reveal how partial reduction of the number of Müller glia due to neuronal conversion affects the survival and function of retinal neurons.

In the intact retina, microglia reside in both inner and outer plexiform layers, where they exhibit elaborate ramified processes responsible for immune surveillance of the retina. Retinal insults such as oxidative stress, hypoxia, or inherited mutations trigger microglia reactivity, as manifested by amoeboid morphology, increased proliferation, and migration to sites of injury. Like microglia in the brain, microglia in the retina of adult mice can be rapidly repopulated after selective elimination of most microglia (> 99 %) (Li et al., 2019). Therefore, retinal microglia should also be useful for restoring lost retinal neurons after injury or degeneration without exhausting the retinal cell sources, although direct conversion of microglia into retinal neurons has not yet been achieved.

Generation of the appropriate neuronal subtypes corresponding to particular regions

in the brain and retina is crucial for neuronal repair and functional recovery. A recent study showed that combined expression of Neurog2 and Nurr1 in upper- or lower-layer astrocytes in the cortex induces reprogramming into different subtypes of iN cells, namely, Cux1-positive upper-layer or Ctip2-positive lower-layer neurons (Mattugini et al., 2019), respectively. Furthermore, Ptbp1 converts two different types of brain cells, striatal astrocytes and Müller glia, into glutamatergic neuron-like cells and retinal ganglion-like cells, respectively (Zhou et al., 2020) (**Figure 1**). These facts suggest that extrinsic signals from the surrounding environment determine the effects of neuronal reprogramming factors and subsequent specification of neuronal subtypes. Neuronal reprogramming has also been reported to be affected by epigenetic signatures of the original cells. For instance, in the conversion of mouse embryonic fibroblasts (MEFs) to neurons, there is a trivalent chromatin state, composed of two marks associated with an active state (H3K4me1 and acetylation of histone H3 at lysine 27 [H3K27ac]) and a repressive mark (H3K9me3), on many Ascl1-bound loci (Wapinski et al., 2013). NeuroD1 can efficiently reprogram microglia into neurons, but Ascl1 cannot (Matsuda et al., 2019), probably because Ascl1 target sites lack such a trivalent state in microglia. Furthermore, non-reactive astrocytes cannot be reprogrammed by NeuroD1, whereas oligodendrocytes can (Matsuda et al., 2019). This is due to the fact that oligodendrocytes, but not non-reactive astrocytes, have a bivalent signature (H3K4me3 and H3K27me3) in NeuroD1-bound loci around neuronal genes (Matsuda et al., 2019). Recent studies have shown that astrocytes in the corpus callosum cannot be reprogrammed into neurons by expression of either NeuroD1 or the combination of Neurog2 and Nurr1, whereas astrocytes in the cortex can (as described above), implying that different epigenetic signatures of astrocytes in distinct brain regions affect reprogramming efficiency. All of these facts taken together indicate that the epigenetic profiles in the original cells affect reprogramming efficiency, but once the original cells are committed to the neuronal lineage by reprogramming factors, they may become suitable neuronal subtypes in response to the surrounding environment. Further investigation is therefore warranted to examine whether pan-neuronal transcription factors such as NeuroD1 or Neurog2 can convert microglia into appropriate neuronal subtypes in each region in the brain and retina according to the external milieu (**Figure 1**).

The recent attempts to repair neuronal circuits using direct reprogramming systems have considerably increased our knowledge of the molecular mechanisms involved in this regenerative process. Deciphering the molecular and cellular mechanisms by which NeuroD1 controls the cell fate of microglia is an exciting starting point for designing future strategies for human brain and retina repair using microglia-to-neuron conversion. We believe that further advances in direct reprogramming technology will greatly improve the chance of moving this strategy towards successful clinical implementation.

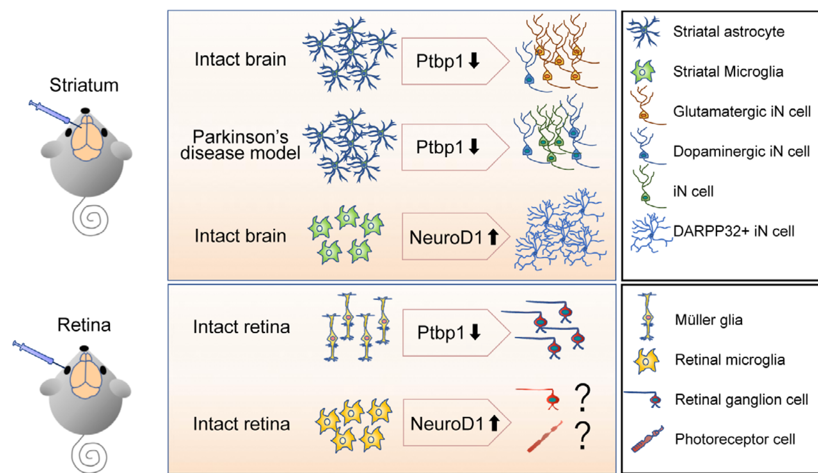

Figure 1 | Direct neural reprogramming in different locations by manipulation of the same genes' expression.
Knockdown of Ptbp1 expression converts striatal astrocytes mainly into glutamatergic iN cells in the intact brain, whereas the conversion into dopaminergic induced neuronal (iN) cells is more permissive in a Parkinson's disease model that induces degeneration of dopamine neurons (Zhou et al., 2020). The same approach also induces production of retinal ganglion cells from Müller glia (Zhou et al., 2020). In contrast, expression of NeuroD1 in striatal microglia efficiently converts them into DARPP32-positive striatal neuron-like cells (Matsuda et al., 2019).

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