

● PERSPECTIVE

Neuroregeneration using *in vivo* cellular reprogramming

Cellular reprogramming is an innovative technology used to artificially convert a mature cell type into a different cell type by molecular manipulation. The general concept of cellular reprogramming is to use master transcription factors to override the endogenous transcriptome profile of a given cell type with the transcriptome profile of the target cell type, thereby altering the cellular function and identity. One of the most well-known examples of cellular reprogramming is the use of four transcription factors, octamer-binding transcription factor 4 (*Oct4*), sex determining region Y-box 2 (*Sox2*), *c-Myc*, and Kruppel-like factor 4 (*Klf4*) to reprogram somatic cells into induced pluripotent stem cells, which led to the award of the Nobel Prize in physiology to Shinya Yamanaka and colleagues in 2012. Beyond induced pluripotency, cellular reprogramming has been used to convert one somatic cell type directly into another somatic lineage, yielding promising results for the *in vitro* conversion of fibroblasts directly into neurons, oligodendrocytes, cardiomyocytes, muscle cells, blood progenitors and hepatocytes.

Also, direct reprogramming has been successfully applied *in vivo* in multiple tissues, such as the heart, liver, retina, the brain and spinal cord. Importantly, *in vivo* reprogramming has the potential to convert endogenous cells into the cell type lost in disease or injury. This regenerative approach bypasses the complications associated with cell transplantation, including issues with immunocompatibility, cell delivery and long term survival/integration of donor cells. *In vivo* reprogramming strategy offers an alternative to transplantation-based therapeutic approaches and has tremendous potential for the advancement of regenerative medicine. Here we will review and summarize the current progress for using *in vivo* reprogramming for tissue regeneration, with particular focus on its use in neuroregeneration.

***In vivo* reprogramming for tissue regeneration:** The feasibility of *in vivo* reprogramming was first demonstrated in the pancreas, using neurogenin 3 (*Ngn3*), pancreatic and duodenal homeobox 1 (*Pdx1*) and v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A (*Mafa*) to reprogram pancreatic exocrine cells to functional insulin-secreting β cells in adult mice (Zhou et al., 2008). Since then, subsequent studies by others have refined the reprogramming techniques, by targeting different endogenous cell types (e.g., liver cells, intestinal cells) to generate different islet cell subtypes. Importantly, Banga et al. (2012) demonstrated that *in vivo* reprogramming to regenerate β cells can attenuate diabetic phenotypes in mice, thus demonstrating the therapeutic potential of this approach.

Similarly in the heart, cardiac fibroblasts are an appealing avenue for *in vivo* reprogramming because of their abundance and their ability to become activated and recruited to damaged sites of the heart. A previous study has demonstrated the feasibility of *in vivo* reprogramming of cardiac fibroblasts into cardiomyocyte-like cells, by viral delivery of the master regulators *Gata4*, myocyte enhancer factor 2c (*Mef2c*) and T-box transcription factor 5 (*Tbx5*) into the heart of mice (Qian et al., 2012). These transcription factors allowed the activation of cardiac gene regulatory networks, yielding successful cardiac reprogramming with a reported efficiency as high as 10–15% (Qian et al., 2012). Notably, *in vivo* reprogramming was reported to attenuate cardiac dysfunction and decrease infarct size in the injured heart, providing a novel strategy to promote cardiac regeneration (Qian et al., 2012).

Neural regeneration in the central nervous system (CNS) using cellular reprogramming: *In vivo* reprogramming in the nervous system has advanced rapidly since 2013. Earlier approaches for neural regeneration mainly focus on targeting neural stem cells to differentiate and generate *de novo* neurons, which recapitulates native processes that takes place during brain development. Direct reprogramming, however, targets terminally differentiated cells that can only convert into a different cell type under a coerced change.

Glial cells are the most abundant cell type in the adult brain, which represented an attractive therapeutic target for repairing injured or diseased brain. Many studies have demonstrated glial cells can be converted into neurons in the brain and spinal cord. The first proof-of-principle of direct neuronal conversion *in vivo* was demonstrated by Torper et al. (2013), using *Brn2*, *Ascl1*, and *Myt1l*. Using a Cre mouse model, specific overexpression of these reprogramming factors in parenchymal astrocytes allows successful reprogramming to NeuN positive neurons in the striatum.

Further research has simplified the reprogramming cocktail and demonstrated that *Sox2*, a master regulator for neural stem cells, on its own is sufficient to induce neuronal reprogramming *in vivo*. Overexpression of *Sox2* alone can reprogram resident astrocytes into DCX positive neuroblasts in the striatum, which can mature into functional neurons in some cases (Niu et al., 2013). Similarly, Heinrich et al. (2014) showed that *Sox2* alone can induce glia to neuroblast conversion in the adult mouse cerebral cortex post injury. Synaptic connection was also established between the reprogrammed neurons to their neighbouring neurons. However, such reprogramming was not observed in the absence of cortex injury, suggesting striatum and cortical glia possess differences in plasticity for reprogramming.

Overexpression of another single transcription factor, *NeuroD1*, in mouse models of brain injury and Alzheimer's disease can also reprogram cortical astrocytes into glutamatergic and GABAergic neurons (Guo et al., 2014). In particular, this study demonstrated that *in vivo* reprogramming is more efficient in reactive glial cells, such as those found in the setting of injury or diseased states. Thus, *in vivo* reprogramming may be used as a strategy to reduce reactive gliosis, which is widely associated with nerve injury and neurodegenerative disorders.

Although there has been exciting progress in reprogramming CNS neurons *in vivo*, more studies are needed to better characterise the functionality of the reprogrammed neurons and the effect of neuroregeneration in disease and injury. More detailed analysis of integration of the reprogrammed neurons into existing neural circuits would be helpful to understand how newly formed neural circuits can contribute to functional restoration of the CNS.

Reprogramming Müller glia for retinal regeneration: In the eye, Müller glia are retinal glial cells that play a critical role in providing retinal integrity and homeostasis. In response to injury and disease, Müller glia become activated and display altered morphology and physiology, resulting in reactive gliosis. Studies in a number of species indicated that Müller glia represent a cellular source of new neurons (Jadhav et al., 2009). For instances, teleost fish possesses remarkable retinal regeneration capacity. Upon retinal injury, quiescent Müller glia can dedifferentiate into multipotent progenitors and give rise to all retinal neural subtypes, resulting in retinal regeneration and restoration of vision. In postnatal chicks, retinal injury also causes Müller glial proliferation and neural regeneration, albeit with less regenerative capacity compared to that seen in teleost fish.

In contrast, acute injury to the rodent retina can stimulate Müller glia to proliferate and produce a very small number of neurons, but they are not sufficient to contribute to vision restoration *in vivo* (Jadhav et al., 2009). Similarly, primary culture of rodent and human Müller glia *in vitro* have neural stem cell characteristics and can generate retinal neurons, including photoreceptors. This supports the notion that given the appropriate stimuli, human Müller glia may potentially be directed to generate neurons as a regenerative response to repair retinal damage.

Inducing changes in cell fate to restore functional cell types provides a promising therapy for visual loss. In particular, *in vivo* reprogramming can be utilized to convert endogenous mammalian Müller glia into photoreceptors, thereby providing a regenerative therapy for diseases characterized by photoreceptor degeneration such as retinitis pigmentosa. Previous studies have supported the feasibility and therapeutic potential of *in vivo* reprogramming to regenerate photoreceptors. An elegant study by Tom Reh's group have demonstrated that overexpression of *Ascl1* alone is sufficient to reprogram Müller glia *in vivo* to a neuronal fate in the injured retina, producing functional, integrated bipolar cells, amacrine cells and photoreceptors (Ueki et al., 2015). Also, this reprogramming response seemed to be more pronounced in young mice compared to adult mice, suggesting an age-dependent difference in the permis-



siveness for Müller glia reprogramming. Further study to assess the effect of Müller glia reprogramming in restoration of visual function in other retinal disease models would be very interesting. In another study, Sanges et al. (2016) reported that Müller glia can be reprogrammed *in vivo*, by spontaneous cellular fusion with transplanted hematopoietic stem cells, which can further differentiate into photoreceptors. Notably, this reprogramming approach to regenerate photoreceptors rescued the retinal function in a mouse model for retinitis pigmentosa, providing strong support for using this strategy to treat retinal degeneration. Further studies to determine the precise reprogramming factors that allow Müller glia reprogramming would be helpful in simplifying this reprogramming strategy.

Current challenges and future directions: Compared to cell transplantation therapy, a key advantage of *in vivo* reprogramming is that this strategy generates new neurons from endogenous cells which are compatible with the host and already integrated with the tissue, obviating issues such as immunorejection and transplant mechanisms. However, many challenges remain for translation of *in vivo* reprogramming to the clinic. In many cases, the reprogramming efficiency remains low. In this regard, overcoming the epigenetic barrier during cellular reprogramming is a key challenge for the field. Also, the oxidative stress caused by the change in redox state during cell fate conversion can potentially trigger cell apoptosis. Even though cellular reprogramming has been demonstrated in many tissues using combinations of lineage-restricted regulatory factors, in-depth knowledge of cell fate-determining gene networks are needed to improve reprogramming efficiency.

The difficulty of delivering multiple reprogramming factors remains a major challenge with *in vivo* reprogramming strategies, which may be required for certain cell types. A promising approach to address this issue is the use of the CRISPR/Cas9 system that allow multiplex activation of endogenous genes. This was demonstrated in a recent study by Black et al. (2016), where the authors utilised dCas9d-based transactivators to activate endogenous *Brn2*, *Ascl1*, and *Myt1l* and directly reprogrammed mouse embryonic fibroblasts to neuronal cells *in vitro*. Further development to apply the CRISPR activation system *in vivo* would facilitate the translation for using cellular reprogramming as regenerative therapies.

Many studies have relied on viral methods to deliver the reprogramming factors *in vivo*. However, lentiviruses or retroviruses can randomly integrate into the genome and thus development of safer gene delivery method, such as adeno-associated viruses, to target specific cell types within the tissue would be desirable. An interesting direction would be the use of small molecules to promote cell reprogramming. Zhang et al. (2015) has demonstrated reprogramming of glial cells by using a cocktail of small molecules to convert cultured human astrocytes into neurons, including LDN193189, SB431542, TTNPB, Tzv, CHIR99021, DAPT, VPA, SAG and Purmo. The chemical reprogramming of astrocytes is achieved through both transcriptional and epigenetic regulation. Small molecules promote conversion into functional neurons by activating transcription factors such as *ASCL1*, *NGN1/2*, and *NEUROD1*. Developing small molecules that can be easily synthesized and administered to patients would be a pragmatic approach to *in vivo* reprogramming. However, the screening process to discover small molecules that target specific transcription factors can be time-consuming and expensive. Other issues related to drug toxicity and delivery should also be taken into consideration for development of *in vivo* reprogramming using small molecules.

Conclusion: *In vivo* reprogramming is an emerging field that attracts enormous interest for its therapeutic potential. This technology can be used to convert endogenous cells into the target cell types, thus providing an alternative approach for regenerative medicine that bypasses many of the major obstacles posed by transplantation. In particular, a panel of transcription factors have been demonstrated to promote glial-to-neurons reprogramming, providing a promising approach for neuroregeneration. Nevertheless, several hurdles for *in vivo* reprogramming remained, such as delivery and improvement of reprogramming efficiency. Further improvements with direct reprogramming approaches using small molecules

and CRISPR/Cas9 technologies would advance development of this technology as a novel regenerative therapy for tissue repair.

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Comments to author: The author did a great job in delivering his perspective on current state of *in vivo* neuroregeneration using *in vivo* reprogramming. It is simplified and easy to read. The author has divided the topic into cellular, tissue and organ regeneration.

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