

● PERSPECTIVE

## Engineering personalized neural tissue using functionalized transcription factors

Diseases and disorders of the central nervous system often require significant interventions to restore lost function due to their complexity. Examples of such disorders include Parkinson's disease, Alzheimer's disease, multiple sclerosis, traumatic brain injury, and spinal cord injury. These diseases and disorders result from healthy cells being destroyed, which in turn causes dysfunction in the central nervous system. The death of these cells can trigger a cascade of events that affect the rest of the body, causing symptoms that become progressively worse over time. Developing strategies for repairing the damage to the central nervous system remains challenging, in part due to its inability to regenerate.

Cell therapy offers great promise for treating diseases and disorders of the central nervous system (Willerth, 2011). The central nervous system contains three major types of neural cells, which act in concert to maintain proper neurological function. Neurons enable the transmission of information throughout the body. These cells are often considered a desirable target for cell transplantation therapies. Neurons come in different varieties, depending on their function and location in the central nervous system. Oligodendrocytes insulate neurons, enabling them to transmit information in an effective manner. Finally, astrocytes play a vital role in providing nutrients and other support to neuronal cells while serving as the main cells found in the blood-brain barrier, which keeps the central nervous system immunoprivileged.

Several questions must be answered when choosing a cell therapy depending on the cellular populations lost. These questions can include the type, number, and source of cells for transplantation, the method for delivering the cells, the need for immunosuppression, and if the transplanted cells could potentially cause harm. One of the major decisions when developing a cell therapy is whether to use endogenous cells present in the patient or transplant exogenous cells into the appropriate site in the nervous system. Each cell source has associated advantages and disadvantages. Endogenous cells often cannot fully repair damage on their own. However, it is possible to deliver appropriate cues that simulate these cells into repairing damage caused to the central nervous system. Using a patient's own endogenous cells eliminates the need for immunosuppression as well as the need for an additional surgery to transplant cells as would be required for exogenous cell transplantation. Exogenous cell transplantation has several advantages, including being able to control the number and type of cells delivered to the appropriate site in the nervous system. The drawbacks include the necessity of matching donor cells to the recipient and the need for transplantation surgery, which can be invasive. Additional considerations arise when working with pluripotent stem cells, such as the possibility of undifferentiated cells causing tumor formation and the need to confirm that the cells differentiate into the desired phenotypes *in vivo*. Cell survival after transplantation often decreases as pluripotent cells become more specialized.

Reprogramming cells into the desired phenotypes can be implemented either using endogenous or exogenous cells. For example, patients' cells could be cultured *in vitro*, reprogrammed into the desired cell type, and transplanted into the appropriate region of the nervous system to treat their disease. Alternatively, their endogenous cells could be treated with factors that would directly reprogram them to become the therapeutically relevant cell phenotypes *in vivo*. Both of these approaches enable the engineering of patient specific neural tissue for clinical applications.

**Transcription factors as a powerful reprogramming tool:** Transcription factors regulate which genes are turned on and off inside of a cell, which in turn determines the levels of proteins being expressed. These proteins act by binding to regions of DNA called

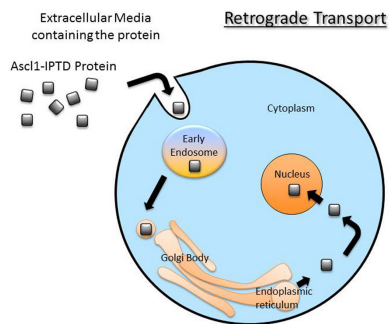
promoters that control transcription to initiate the process, or directly to RNA polymerase, regulating gene expression. This capability makes them a powerful tool for controlling cell behavior, which scientists have been able to harness for applications in cell therapy. Takahashi and Yamanaka screened a large set of transcription factors to determine which ones could be used to reprogram fibroblasts back into a pluripotent state (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The hallmarks of pluripotency or "stemness" include 1) the ability to produce more than one cell type, and 2) the ability to self-replicate. They identified a combination of four transcription factors (Oct3/4, Sox2, Klf4 and c-Myc) necessary for converting an adult cell back into a pluripotent stem cell. These four transcription factors are often referred to as the Yamanaka factors. Both Oct3/4 (POU51), and Sox2 (SRY-box 2) maintain pluripotency. Treatment with leukemia inhibitory factor (LIF) upregulates the expression of both Oct3/4 and Sox2, explaining why it is often used as a supplement when culturing pluripotent stem cells. The other two transcription factors, Klf4 and c-Myc, play important roles in regulating the cell cycle. Klf4 can act as a tumor suppressor and its presence indicates "stemness" in both pluripotent and mesenchymal stem cells while constitutive expression of c-Myc can lead to cancer. In the aforementioned studies, lentiviruses were used to deliver the sequences for these four transcription factors to fibroblasts where, upon infection, these factors began being overexpressed and the result was cellular reprogramming back to a pluripotent state. The resulting cells are referred to as induced pluripotent stem cells (iPSCs).

Recently, other groups used similar approaches to directly reprogram terminally differentiated cells, like fibroblasts, directly into the desired cell types. Generating neurons from terminally differentiated cells holds particular interest as these cells are relevant for therapeutic applications in treating disorders of the central nervous system. The Wernig group screened 19 proneural transcription factors to determine which ones would be sufficient for directly reprogramming mouse embryonic and post natal fibroblasts into neurons (Vierbuchen et al., 2010). They found that Brn2, Ascl1, and Myt1l, collectively referred to as the BAM factors, were the required factors and they termed the resulting cells induced neurons (iNs), as they expressed neuronal markers, generated action potentials, and could form synapses. In terms of function, Brn2 plays an important role in regulating the central nervous system during cortex development, Ascl1 is critical for proper neuronal differentiation, and Myt1l is associated with the differentiation of both neurons and oligodendrocytes.

A follow-up study a year later confirmed that human fibroblasts could be converted to iNs and that the process was more effective with the addition of the basic helix-loop-helix transcription factor NeuroD (Pang et al., 2011). Other recent work based on these landmark studies showed that additional factors could be expressed to specify neuronal fate. In particular, Caiazzo et al. (2011) showed that using the same methods to express the transcription factors Lmx1a, Ascl1, and Nurr1, referred to as the LAN factors, could generate dopaminergic neurons directly from mouse and human fibroblasts. In 2014, it was confirmed that Ascl1 alone was sufficient to drive the neuronal reprogramming process. This study also concluded that the other transcription factors enhanced the speed of the maturation process. However, the conversion rate for the reprogramming process remains relatively low (~20%).

For these studies, expression of these proteins was achieved using a doxycycline-inducible lentiviral vectors, meaning that target gene expression only occurs after the cells are exposed to doxycycline. These vectors are based on a modified version of human immunodeficiency virus (HIV), which gives them several unique properties (Hu, 2014). They have high transfection efficiency, produce stable gene expression, and can infect non-dividing cells. The drawbacks of lentiviral vectors include a potential lack of control over whether the virus integrates into the host cell's genome, and limited ability to control the levels of gene expression produced after transfection. Additionally, transfection efficiency can vary between cell lines and tissue types with different cells expressing varying levels of protein.

Several research groups, including my own, have acknowledged these limitations and accordingly, we have been evaluating alternative methods for reprogramming human cells into neuronal phenotypes.



**Figure 1** Functionalizing a protein with the intracellular protein delivery technology enables it to be taken up from cell culture media into an early endosome.

It is then transported back through the secretion pathway through the Golgi bodies and endoplasmic reticulum where it is then released into the cytoplasm. From the cytoplasm, Ascl1 protein functionalized with intracellular protein delivery technology (Ascl1-IPTD) can be transported into the nucleus where it regulates gene expression.

For example, a recent paper from Sheng Ding's group screened combinations of small molecules as way of modifying protein expression levels to program cells (Zhang et al., 2016). They demonstrated a cocktail of nine small molecules (M9) could convert mouse fibroblasts into neural stem cells. The use of small molecules potentially provides a low cost alternative method to the use of lentiviral vectors. However, the mechanism of action is fundamentally different from using transcription factors to mediate reprogramming. Small molecules influence cell behavior by modulating the activity of the proteins endogenous to the cell as opposed to directly effecting reprogramming through regulating levels of transcription factor expression.

**Recent progress using intracellular protein delivery technology as an alternative to lentiviral mediated transfection:** Modifying transcription factors to allow efficient uptake by cells serves as an attractive alternative for increasing protein expression levels when compared to the use of lentiviral vectors and small molecules. While transcription factors play important roles in stem cell maintenance and differentiation, they are not traditionally used as media supplements due to their instability. iProgen Biotech has developed a technology called intracellular protein delivery technology (IPTD). In this process, the target protein is modified to contain a signal sequence that enables efficient uptake by human cells and stabilizes the transcription factor. **Figure 1** shows a schematic of the protein delivery technology and how it works. Further details on this technology are contained in their patent (Lee et al., 2014).

The recent Stem Cell Reviews and Reports paper from my research group demonstrates the power of modifying transcription factors using IPTD (Robinson et al., 2016). Treatment of undifferentiated human iPSCs with 15 µg/mL resulted in the rapid generation of NeuN positive neurons compared to both positive and negative controls, demonstrating that IPTD turned Ascl1 into a potent neuronal differentiator of stem cells. This study also confirmed that Ascl1-IPTD construct was stable in the media. We analyzed the average neurite length and amount of neurite branching observed for these cultures and showed that cells treated with Ascl1-IPTD after 12 days had increased neurite length and number of branch points compared to neural progenitors cultured for 24 days. This finding suggests that our protocol rapidly generates mature neurons compared to traditional neuronal differentiation protocols. Overall, our study demonstrates how the Ascl1-IPTD protein can be used to efficiently and inexpensively derive neurons from human iPSCs as an attractive alternative to lentiviral methods.

**Future directions:** The use of IPTD makes it easier to deliver transcription factors in a clinically relevant and controlled fashion to human cells. On-going work is evaluating the different uptake sequences to further improve this technology, resulting in a next generation IPTD technology. It is also possible to use antibodies to target proteins expressed by specific cellular populations to allow the proteins

functionalized with IPTD to only be taken up by these target cells, further enhancing the clinical relevance of this approach. IPTD technology greatly expands the number of potential protein targets for delivery. In terms of neural tissue engineering, the next steps will be to functionalize other desirable transcription factors with this technology, like Brn2, Myt1l, and NeuroD, to determine their effect on iPSC differentiation both alone and in combination. We also want to determine if treatment with Ascl1-IPTD and other functionalized transcription factors can directly reprogram fibroblasts into different types of neurons. Once we have demonstrated this ability, the next challenge will be in vivo delivery as proteins do not cross the blood-brain barrier. Potential delivery methods include direct injection of protein to the site of damage, which is a logical first step to determine appropriate dosing. The use of biomaterial-based microspheres to generate controlled release of these functionalized transcription factors over extended time periods after injection into the nervous system would be a more effective long-term treatment. Overall, the combination of transcription factors and IPTD has the potential to revolutionize cellular reprogramming.

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