



Direct Reprogramming Somatic Cells into Functional Neurons: A New Approach to Engineering Neural Tissue In Vitro and In Vivo

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31.1 Introduction

The concept of reprogramming mature cells into other phenotypes has been around since the 1960s when Gurdon showed that the nuclei taken from the endothelium of tadpoles could be transplanted into tadpole embryos [1]. The properties of the embryo reprogrammed the nucleus, giving rise to a complete tadpole. This seminal work demonstrated the possibility of reprogramming and this process was called nuclear transfer. It also set the stage for more recent effects in this area of reprogramming cells. Dolly, the cloned sheep produced in 1996, also served as an important step in demonstrating the feasibility of reprogramming mammalian cells using the same nuclear transfer process [2]. While using embryos as a method of reprogramming was validated, researchers in 1981 confirmed that such repro-

gramming could be achieved using transcription factors, proteins that regulate which portions of DNA become converted to RNA, altering the protein expression patterns in a cell [3]. Transcription factors specify what types of mature cells will be produced during development [4]. In a 1987 study, the authors expressed the transcription factor MyoD (associated with the development of muscles) in fibroblasts, causing them to become myoblasts, the long tubelike cells that are found in muscle tissue. This work showed that successful cellular reprogramming could be achieved without the use of embryos.

The invention of induced pluripotent stem cells (iPSCs) also demonstrated the power of transcription factors as tools for cellular reprogramming. In 2006, Takahashi and Yamanaka showed that a combination of four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) converted mouse fibroblasts into iPSC lines, which possessed the property of pluripotency [5]. The next year, along with other research groups, they generated human iPSC lines from human fibroblasts, showing that such reprogramming could be accomplished in human cells [6, 7]. This reprogramming process works by virally expressing similar transcription factor patterns compared to those found in embryonic stem cells, which are also pluripotent [8]. These iPSC lines can differentiate into any cell type found in the organism from which the cells were derived. These iPSC lines also avoid the ethical issues associated with

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embryonic stem cells as they can be generated from somatic cells.

In 2010, the Wernig group demonstrated that it was possible to convert fibroblasts into cells they called induced neurons (iN) using a similar transcription factor-mediated reprogramming strategy [9]. The generation of iPSCs and iNs required viral overexpression of the necessary transcription factors to achieve reprogramming. Accordingly, the next section of this book chapter focuses on such viral reprogramming methods. More recent methods for altering transcription factor expression patterns include the use of microRNAs, gene editing, small molecules, and functionalized proteins, which will also be analyzed in this book chapter. Cellular reprogramming can help scientists to understand the different features and progression of neurodegenerative diseases like Parkinson's by reprogramming patient's cells into neurons for further characterization and study [10]. In addition to reprogramming somatic cells into neurons in vitro, reprogramming somatic cells into neurons in vivo using reprogramming also serves as an alternative tissue engineering strategy that could potentially restore function to the damaged nervous system [11, 12]. This book chapter covers the current state of reprogramming for applications in neural tissue using these different methods and closes by examining the barriers that must be addressed before such techniques can be translated for clinical applications.

31.2 Viral Mediated Reprogramming of Somatic Cells into Neural Phenotypes

31.2.1 Direct Reprogramming of Somatic Cells into Neurons Using Lentiviral Vectors

Lentiviral vectors, modified versions of wild-type retrovirus virus, retain only the cis-acting elements of the viral genes that allow RNA encapsidation, reverse transcription, and integration. This modification allows them to deliver a specific nucleic acid sequence that encodes the

target gene to be expressed but removes their ability to replicate within the host cell [4]. Lentiviruses are unique among viruses in that they reverse transcribe their RNA into a triple-DNA strand which is then imported into the nucleus of the host cell without requiring cell division or disruption to the nuclear membrane. Once inside the nucleus, the triple-strand DNA integrates into the host genome through the action of the viral enzyme integrase, where it can then be transcribed into RNA and used to synthesize proteins [13]. Lentiviral expression of transcription factors was the first established method for direct cellular reprogramming. Once inserted into the host genome of a cell, the host cell transcribes the transcription factor DNA into proteins which go on to orchestrate the transcription of other genes, converting the cell from one phenotype to another [13, 14].

Wernig [15], who had previous experience in generating dopaminergic neurons from iPSCs, was the first to raise the question of whether it was possible to reprogram mature cells directly into divergent lineages. They isolated a set of three transcription factors able to successfully reprogram mouse fibroblasts into functioning neurons by screening a set of 19 genes expressed in neural tissues or involved in epigenetic reprogramming [9]. These three factors are Brn2, Ascl1, and Myt1l, often referred to as the BAM factors. Furthermore, they showed that Ascl1 alone was sufficient to activate the reprogramming process, while Brn2 and Myt1l assisted in maturation of functional properties. Conversion was seen to be rapid, taking only 20 days; however efficiency was low, ranging from 1.8 to 7.7%.

The Wernig group next applied the BAM factors to human fibroblasts, and found that although initial conversion into neurons was successful, maturation of functional properties required the help of a fourth transcription factor, NeuroD1 [16]. These induced neurons were mainly an excitatory neuronal subtype, along with a large percentage of peripheral neurons. They could be generated from fetal and adult fibroblasts with similar efficiencies of 2–4%, showing that even adult cells which possess less plasticity than fetal cells can be reprogrammed. Furthermore, viral

BAMN expression induced endogenous expression of BAMN, showing that once the reprogramming process is activated it is self-regulated and does not require further viral mediated expression. The Wernig group next demonstrated the possibility of reprogramming between cells derived from different germ layers. Adult mouse hepatocytes were successfully converted into neurons using the BAM factors with an efficiency of 3%. In this experiment, they silenced the hepatocyte transcriptome, confirming a complete lineage switch as opposed to a hybrid phenotype consisting of traits from both lineages [17]. Figure 31.1 shows that *Ascl1* alone is sufficient to achieve neuronal reprogramming.

Following these experiments, several combinations of transcription factors have been identified for reprogramming specific neuronal subtypes for the treatment of neurodegenerative diseases and spinal cord injury. The rest of this section reviews some of the *in vitro* and *in vivo* reprogramming combinations discovered to date. Parkinson's disease results when the dopaminergic neurons of the midbrain degenerate, making these cells attractive cell therapy targets [18]. A screening process of 11 dopaminergic transcription factors plus the BAM factors identified *Ascl1*, *Nurr1*, and *Lmx1a* as the minimal gene set necessary to generate mature dopaminergic neurons from human fibroblasts [19]. Furthermore, these factors could reprogram fibroblasts taken from both healthy subjects and subjects suffering from Parkinson's disease, with respective efficiencies of $5 \pm 1\%$ and $3 \pm 1\%$.

Inhibitory interneuron transplants improve symptoms of epilepsy by correcting an imbalance between excitatory and inhibitory activity in cerebral neuronal networks [20]. Inhibitory interneurons also promote neuronal circuit plasticity and thus are promising therapeutic tools for treating a variety of other neurological disorders including Parkinson's disease and Alzheimer's disease. A screening process identified a set of five factors that can reprogram human fibroblasts into inhibitory interneurons: *Ascl1*, *Dlx5*, *Lhx6*, *Foxg1*, and *Sox2*. The first three are associated with inhibitory neuron development; however the last two, *Foxg1* and *Sox2*, are not and so their

necessity in direct reprogramming was unexpected. It is hypothesized that *Foxg1* is needed to improve the ability of *Ascl1* and *Sox2* to bind to their targets, and thus it may be a "pioneer" factor for inhibitory interneurons, similar to the way that *Ascl1* serves as a "pioneer" transcription factor for excitatory neurons.

In vivo studies began with a proof-of-concept experiment carried out by De La Rossa et al [21] who successfully converted postmitotic neocortical neurons into L5B neurons in mouse embryos using the transcription factor *Fexf2*. Studies have since identified various transcription factors to convert resident cells in damaged areas of the central nervous system into neurons. Spinal cord injury leads to degeneration of neurons and subsequent loss of motor function [22]. Astrocytes, a type of support cell found in abundance in the central nervous system, switch to a reactive state after spinal cord injury and form a scar in order to protect the integrity of the blood-brain barrier. This scar presents a physical barrier which prevents damaged neurons from re-establishing neuronal relays while secreting inhibitory proteins that suppress axonal regeneration around the injury. Reprogramming resident astrocytes into functional neurons near the injury site has been proposed as a strategy to re-establish neural connectivity and make the injury environment more favorable to axonal regeneration [23]. The transcription factor *Sox2* was identified from a pool of 12 factors as the sole factor needed to reprogram resident mouse spinal cord astrocytes into neural precursor cells, with an efficiency of 6–8% as seen in Fig. 31.2 [24]. In this experiment, the cell source of the induced neural precursors was confirmed by genetic lineage tracing to be resident astrocytes. Subsequent treatment with valproic acid, a small molecule that inhibits histone deacetylase enzymes which regulate transcription, induced differentiation of these neural precursors into synapse-forming neurons. In a follow-up study, *Sox2* was applied to mouse brains *in vivo* where it also succeeded in generating neural precursors from resident astrocytes. Promisingly, these cells persisted for months and could also be differentiated into mature neurons using valproic acid, or alternatively by treatment

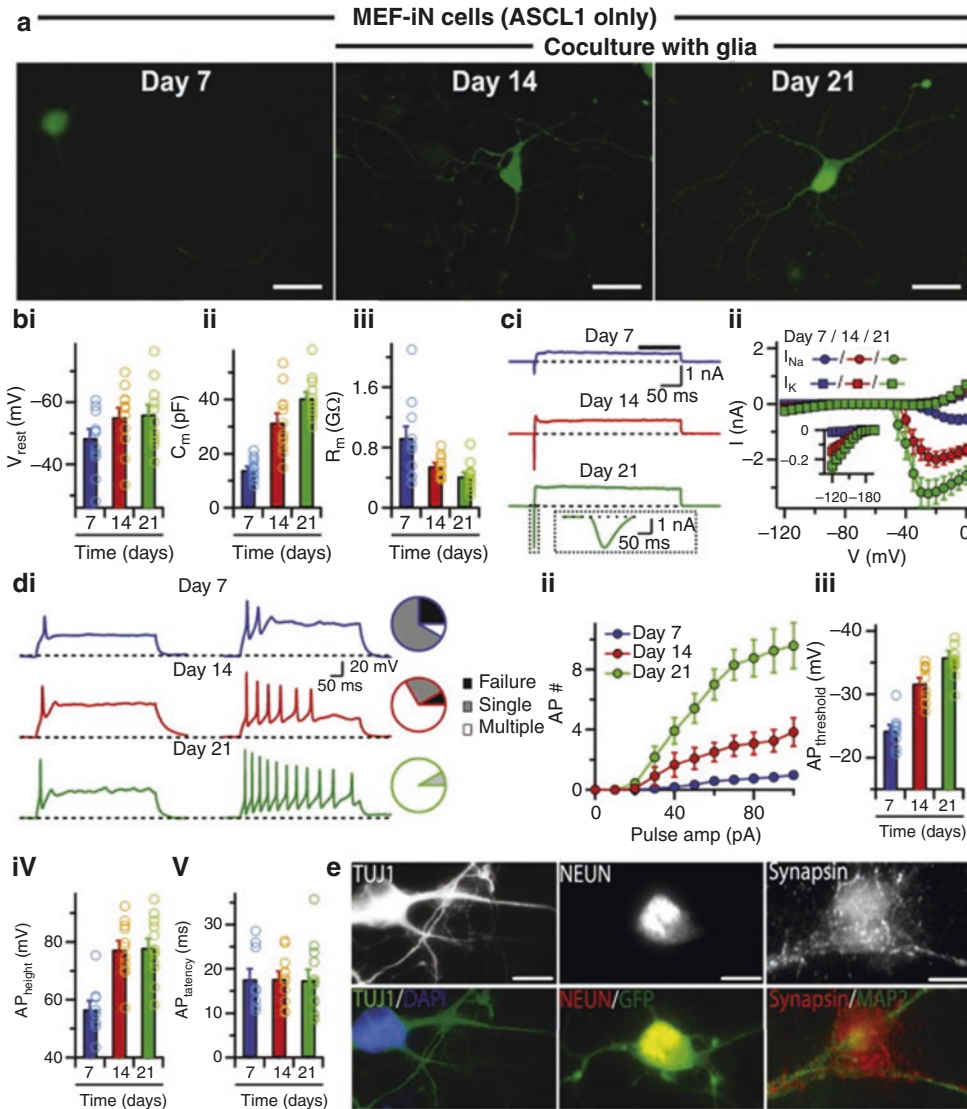


Fig. 3.1 ASCL1 alone is sufficient to generate functional induced neuronal cells from mouse embryonic fibroblasts referred to as MEF-iN cells. **(a)** Gradual development of the morphological complexity of ASCL1-induced single-factor MEF-iN cells at day 7 (left) and after co-culturing with glia until day 14 (middle) or day 21 (right). Scale bars, 10 μ m. **(b)** Average values of resting membrane potential (V_{rest} , *i*), membrane capacitance (C_m , *ii*), and input resistance (R_{in} , *iii*) of ASCL1-induced single-factor MEF-iN cells from day 7 (blue), day 14 (red), and day 21 (green). Bar graphs represent mean values \pm SEM ($n = 12$ for individual averages). Open circles of corresponding colors represent values measured from individual cells. **(c)** Example traces of Na^+/K^+ currents recorded at $V_{hold} = -70$ mV with a step voltage of 50 mV (*i*) and corresponding averages \pm SEM ($n = 12$ for each point), (*ii*) for current–voltage (I – V) relationship (filled circles: Na^+ current and filled squares: K^+ current) recorded from

single-factor MEF-iN cells at day 7 (blue), day 14 (red), and day 21 (green). The black line (upper panel, *i*) indicates time period used for calculating average K^+ currents. The insets depict expanded views of Na^+ current (bottom panel, *i*) and reversal of K^+ current (*ii*). **(d)** Analysis of action potential (AP) firing properties from 1F-iN cells at day 7 (blue), day 14 (red), and day 21 (green). Example traces of single (left) or multiple (right) APs generated by a 90 pA step-current injection, with pie charts representing fraction of iN cells in each condition able to generate single AP (gray), multiple AP (white), or no AP (black) (*i*). Average values presented as means \pm SEM ($n = 12$ for individual averages) for AP number with respect to current-pulse amplitude (*ii*), AP threshold (*iii*), AP height (*iv*), and AP latency (*v*). Open circles represent corresponding values measured from individual cells (*iii*–*v*). **(e)** Immunostaining analysis of 1F-iN cells at day 21 with indicated neuronal markers. Scale bars, 10 μ m [63]

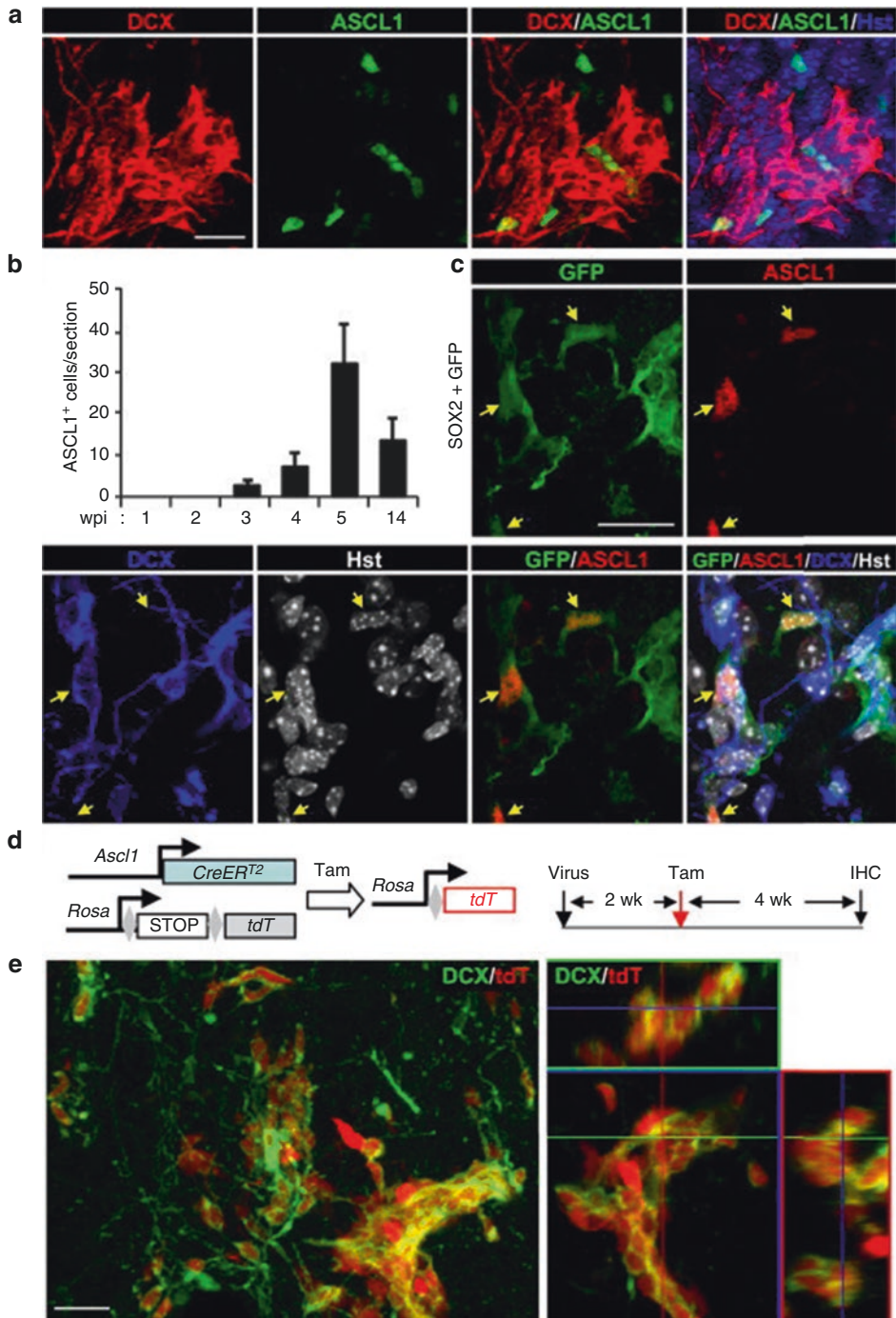


Fig. 31.2 SOX2 induces ASCL1⁺ neural progenitors from astrocytes in vivo. **(a)** The expression of ASCL1⁺ cells in striatal regions with DCX⁺iANBs at 5 wpi. The scale bar represents 20 μm. **(b)** A time course analysis of ASCL1⁺ cells in the reprogramming area (mean ± SD; n = 3 mice at each time point). **(c)** ASCL1 is detected in astrocytes transduced with SOX2 lentivirus. The co-expressed GFP marker is under the control of the human

GFAP promoter. The scale bar represents 20 μm. **(d)** A genetic approach to trace derivatives of ASCL1⁺ progenitors. SOX2-driven reprogramming was induced in adult *Ascl1-CreER²²;Rosa-tdTomato* (tdT) mice. **(e)** SOX2-induced DCX⁺ cells pass through an ASCL1⁺ progenitor stage. Confocal images show genetic labeling of iANBs. An orthogonal view is shown in the right panel. The scale bar represents 20 μm [25]

with growth factor brain-derived neurotrophic factor (BDNF) and the bone morphogenetic protein antagonist Nog [25]. The mechanism of this Sox2 program was seen to follow a distinct sequence involving *Ascl1* and the neural precursor gene *Doublecortin* (*Dcx*) and give rise to mainly inhibitory interneurons [26].

Another potential application for *in vivo* reprogramming is the treatment of retinal degeneration [27]. Pollack et al. [28] discovered that *Ascl1* can reprogram Muller glia, a type of retinal glial cell, into retinal neurons. Nonmammalian vertebrates regenerate injured retinal neurons by dedifferentiating Muller glia via activation of *Ascl1*. However, mammalian Muller glia cannot regenerate; instead they form a glial scar. Delivery of *Ascl1* to mouse Muller glia cultures and intact retinal explants converted 5% of these cells into bipolar retinal neurons. Interestingly, *Insm1* protein levels increased, a protein involved in nonmammalian vertebrate regeneration, suggesting that the conversion mechanism follows a similar process to that first noted in nonmammalian vertebrates. To this end, the study of nonmammalian vertebrates such as fish may hold promise for finding ways to improve the efficiency of retinal regeneration in humans. In another study, Hao et al. [29] tested the ability of the BAM factors to reprogram fibroblast-like cells from human retinas into neurons. While BAM factor expression was unable to reprogram these cells, further testing determined that the combination of *Ascl1* with *Pax6* was sufficient for reprogramming of these cells, generating a mix of inhibitory neurons and dopaminergic neurons, with an efficiency of 8.78%.

31.2.2 Limitations of Lentiviral Mediated Reprogramming

Directly reprogrammed cells are converted without an intermediate progenitor state or the need for cell division as would be necessary with directed differentiation from stem cells. Avoiding a pluripotent or mitotic state considerably reduces the risk of tumor formation, making direct reprogramming safer for clinical applications.

However, the underlying mechanisms of reprogramming remain unclear and therefore are difficult to predict and develop into a standardized medical practice. To this end, more work is needed to understand this powerful technology. One possible model put forth to describe the mechanism of reprogramming is that of “pioneer” transcription factors, factors which possess the ability to activate genes that are in a repressed chromatin state, also known as “silenced” genes [30]. In this model it is hypothesized that chromatin states at silenced loci fluctuate between repressed and active configurations, spending more time in a repressed state. “Pioneer” transcription factors bind weakly at these loci, stabilizing active configurations over time and allowing more accessible sites for further transcription factor binding. This process eventually leads to active transcription at these loci.

Another major limitation associated with lentiviral delivery is that they have a pattern of integrating within the core of transcribed genes in the host cell, which can lead to cancer-causing mutations. In other cases, it can lead to inappropriate expression of proteins, harming cell function and resulting in genotoxicity. To circumvent the dangers associated with insertional mutagenesis, research groups have modified lentiviral vectors to contain a defective integrase so that they remain as floating nuclear DNA circles within the nucleus; however, this method is less efficient. Additionally, lentiviral vectors harm the cell by eliciting an immune response if their viral capsids and their associated protein products are detected by the host’s immune system [13]. Another major drawback is that viral mediated reprogramming remains difficult to control. After infection, protein expression levels from cell to cell can vary greatly. These inconsistent protein expression levels can cause incomplete activation of a transcriptional program, and in the case of *Ascl1* they can also lead to activation of a myogenic program resulting in the generation of muscle cells (13, 14). To achieve greater control researchers are engineering switches to activate or deactivate transcription protein expression. Poulou et al. [31] have designed a novel OFF-ON activator of transcription factor expression using

the yeast transcription factor Leu3p- α IPM as seen in Fig. 31.3. Acting as a repressor when bound to its UAS_{Leu} DNA element, it can become an activator of transcription in the presence of α IPM, a lipid-soluble and metabolically stable metabolite involved in leucine biosynthesis. Although it is only found in prokaryotes, fungi, and plants, it is fully functional and nontoxic in

mammalian cells. The group tested the switch in mice using the transcription factor Sox2. After 2 days of Sox2 activation, mouse astrocytes converted into neural progenitor cells with a radial glial phenotype. Radial glial cell proteins nestin and RC2 were upregulated to $85 \pm 13.89\%$ and $93 \pm 6.97\%$, respectively, and the astrocyte protein GFAP was downregulated to $25 \pm 20\%$.

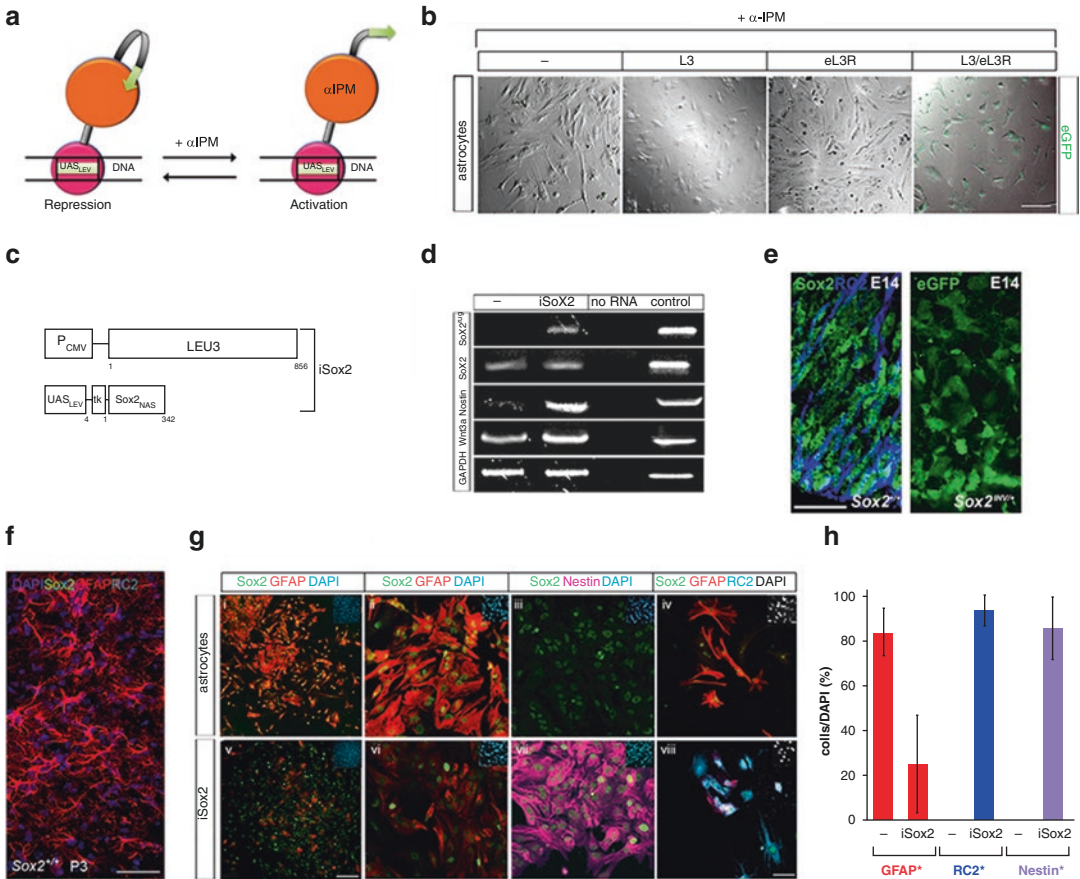


Fig. 31.3 Leu3p- α -IPM inducible fast-track direct reprogramming of astrocytes to neural stem cells. (a) Leu3p- α -IPM mode of action: Transcriptional repressor upon binding to the UAS_{Leu} DNA element and transcriptional activator upon α -IPM ligand binding. (b) Superimposed bright-field and confocal images of transiently transfected primary P3 murine astrocytes (*i–iv*) with either Leu3p protein (L3) (*ii*) or UAS_{Leu}-eGFP reporter (eL3R) (*iii*) or both L3/eL3R (*iv*). eGFP is observed only in cell UAS_{Leu} with both L3/eL3R (*iv*). Scale bar 75 μ m. (c) Generation of iSox2 expression system under the control of Leu3p UAS_{Leu} elements. (d) *iSox2* induces endogenous *Sox2*, *nestin*, and *wnt3a* 48 h post-transfection in P3 primary murine astrocytes. (e) *Sox2*⁺ and RC2⁺ neural progenitors in the proliferating zone of E14

cortex of wild-type mouse embryos. eGFP expression detected in the proliferating zone of E14 cortex after *Sox2* ablation in radial glia cells. Scale bar 75 μ m. (f) *Sox2* is not expressed in GFAP⁺ cells in the proliferating zone of P3 mouse cortex. Scale bar 50 μ m. (g) *iSox2* (*v–viii*) reduces the astrocytic marker GFAP (*i*, *ii*, *iv–vi*) in P3 primary murine astrocytes and induces a Nestin⁺ (*vii*) radial glia (RC2⁺) (*viii*) NSC phenotype 48 h post-transfection. DAPI staining of nuclei is present in the upper right corner in all panels. Scale bars for panels *i* and *v* represent 250 μ m and for panels *ii–iv* and *vi–viii* represent 75 μ m. (h) Graphs depicting the GFAP⁺ and either Nestin⁺ or RC2⁺ cells in untransfected astrocytes and in reprogrammed cultures. Reprinted with permission [31]

These cells also began to express endogenous Sox2. Overall, lentiviral mediated reprogramming provides a valuable tool for directly reprogramming cells into neural phenotypes. However, it has major limitations that must be addressed before such viral methods can be used for reprogramming in clinical settings with regard to engineering neural tissue from endogenous cells.

31.3 Small-Molecule-Mediated Reprogramming of Somatic Cells into Neural Phenotypes

31.3.1 Direct Reprogramming of Somatic Cells into Neurons Using Small-Molecule Cocktails

An increasingly successful approach for inducing neuronal transdifferentiation of somatic cells uses small molecules to alter the gene expression patterns [32]. Such cocktails induce epigenetic modifications, which are regulations of gene expression caused by alterations in DNA accessibility, changing it from one somatic type into neural lineages. As cells uptake the molecules directly, it avoids several of the complications associated with viral transfection mentioned earlier. For example, He et al. [33] produced an optimal medium known as 5C comprised of N2 supplement, bFGF, leukemia inhibitory factor, vitamin C, and β -mercaptoethanol. This media successfully converted mouse embryonic fibroblasts into Tuj1-positive neuronal-like cells that remained viable after implantation into a mouse brain, and rat astrocytes into electrophysiologically mature neuronal-like cells in vitro, which also facilitated recovery of brain injury when transplanted into mouse brain. Further, such 5C medium could induce neuronal characteristics when administered to human somatic cell types in vitro.

In another example, Pfisterer et al. [34] utilized human induced neurons to screen 307 compounds (kinase inhibitors, epigenetic modulators, Wnt pathway, nuclear receptors, and phosphatase inhibitors) and found a combination of 6

(Gsk3beta inhibitor kenpaullone, cAMP/PKA modulator prostaglandin E2 (PGE2), adenylyl cyclase activator forskolin, HDAC inhibitor BML210, SIRT1 activator amino resveratrol sulfate, and Src kinase inhibitor PP2) that successfully converted human fibroblasts to MAP2+ human induced neurons with neuronal morphology at concentrations very different from their toxic dose—a highly attractive feature for basal medium development in vitro. These identified conversion-optimizing compounds are not the only that are successful, as many others such as CHIR99021, valproic acid, and ROCK inhibitor Y-27632 work at a subthreshold level as defined by this research group, but show promise in small-molecule reprogramming nonetheless.

Researchers Han et al. [35] used small-molecule reprogramming to convert mouse embryonic fibroblasts into small-molecule induced neuronal stem cells (SMINS-MEF-7). Administering seven small molecules (valproic acid, Bix01294, RG108, PD0325901, CHIR9901, vitamin C, A83-01) in a six-cycle protocol generated SMINS that stained positive for alkaline phosphatase (a marker for pluripotent embryonic stem, and related cells that exists in tissue specific isoforms), and neural stem cell (NSC) markers Sox2 and Nestin. These cells remained multipotent and morphologically indistinguishable from native NSCs for 2 years of expansion post-experiment. They also found a subset of three (Bix01294, RG108, PD0325901) factors sufficient for conversion of tail-tip fibroblasts (TTF) to SMINS-TTF-3 cells, which share common significant findings with their seven-molecule counterparts. The SMINS-TTF-3 cells express NSC marker genes Sox2, GFAP, Olig2, and Gli2 (which are not expressed by TTFs) and did not express pluripotent genes Oct4 and Nanog. Like most small-molecule-mediated reprogramming studies, the reprogramming mechanism remains unknown. Perhaps the most striking results of this study are the achieved multipotency of both SMINS-MEF-7/-TTF-3 cells with additional small-molecule reprogramming in vitro (both SMINS cell lines were differentiated to astrocytes (GFAP+), neurons (MAP2+), and oligodendrocytes (O4+)).

Li et al. [36] identified a small-molecule cocktail consisting of forskolin, cyclic AMP agonist ISX9, CHIR99021 a GSK3 inhibitor, and SB431542 out of 5000 candidate molecules for endogenous reprogramming of mouse fibroblasts into neurons. Next, Li screened 1500 more small molecules associated with neurite outgrowth and morphology and found that the BET family bromodomain inhibitor protein (I-BET151), which disrupts fibroblast-specific programs, improved reprogramming dramatically. The cocktail was then revised to contain forskolin, ISX9, CHIR99021, and I-BET151. The new combination had a conversion efficiency of >90% TUJ1+ cells with neurite outgrowth after 16 days of induction. Co-culturing the induced neuron-like cells with primary astrocytes for 2–3 weeks allowed further maturation marked by extended neurite outgrowth, a gene expression profile indicative of excitatory glutamatergic neurons, and membranes capable of forming functional synaptic connections with each other. Astonishingly, Li concluded that his chemically induced neurons bypassed an intermediary pluripotent state by witnessing a lack of 5-bromodeoxyuridine incorporation by the TUJ1+ cells throughout the reprogramming process—a finding that coincides with the speculative data of Han et al. [35]. The small molecules replace reported lineage reprogramming genes, activating desired cell-type-specific and silencing initial cell-type-specific gene expression, without the need for exogenous transgenes or cell-fate-specific factors (for example, microRNAs) [36]. This conclusion is therapeutically relevant and applicable to human somatic cell conversion, as demonstrated by Hu et al. [37] who identified a cocktail of seven small molecules (valproic acid, CHIR99021, Repsox, forskolin, SP600125, G06983, Y-27632) that convert human fibroblasts to neuronal cells.

Hu's method also demonstrated a progenitor bypass when the four-molecule cocktail was introduced at optimal concentrations and time course of administration. Hu also replaced the induction media with maturation media containing CHIR99021, forskolin, and dorsomorphin, and extra neurotrophic factors: BDNF, GDNF,

and NT3. The resulting induced neurons expressed the mature neuronal markers Tau, NeuN, and synapsin. The induction protocol was successfully applied to eight different human cell lines. All induced neuronal cells showed electrotonic potential, membrane current, functional glutamate and GABA receptors, and spontaneous calcium transients comparative to those of hiPSC-derived neurons, indicating physiological validity and therefore experimental reliability. Microarray analysis revealed that the induced neuronal cells expressed genes related to neuron differentiation, synapse, and synaptic transmission and downregulated expression of genes related to the extracellular regions, extracellular matrix, and motility. They concluded that CHIR99021 and SP600125 are critical to neuronal gene regulatory network upregulation since only fibroblast gene expression was obtained by removal of these compounds [37].

Zheng et al. [36] used a combination of four small molecules (A-83-01, thiazovivin, purmorphamine, and valproic acid) to reprogram mouse embryonic fibroblasts (MEF) to chemically induced neuron stem cells (iNSCs). TGF β inhibition by A-83-01 improves efficiency of reprogramming of somatic cells to pluripotent stem cells and neural induction of said cells thereafter [38].

A recent study provides insight into how small-molecule reprogramming can bypass the need for a pluripotent state. While chemically reprogramming mouse fibroblasts to iPSCs, Deng et al. [39] defined an expandable extraembryonic endoderm (XEN)-like state, which could explain small-molecule-mediated reprogramming. Deng's group established a three-step chemical induction process to establish pluripotency from mouse somatic cells. They induced a XEN-like state by administering the following seven compound cocktails, VPA, TD114-2, 616452, tranlycypromine, forskolin, AM580, and EPZ004777, which activate the genes (Sall4, Sox17, Gata4, and Gata6). The XEN-like cells can be identified by the presence of hallmark downregulated fibroblast genes and captured in culture, a feature that makes this method attractive to researchers as they can exploit the plastic-

ity of the newly defined intermediate XEN-like state during small-molecule reprogramming. Deng's group was the first to attempt this by recapitulating and applying chemical compounds that facilitate neural lineage differentiation and reprogramming for neural fate specification to the XEN-state cells with a >50% conversion efficiency after 12 days of induction.

31.3.2 Limitations of Using Small-Molecule-Mediated Reprogramming

While Sect. 3.1 discusses successful applications of small-molecule-mediated reprogramming, these methods still have limitations. Researchers are left with the challenge of finding the proper molecules, concentrations, combinations, methodologies (orchestrated timing of administration and validation protocols), and a basal medium that acts as an effective solvent and life support for the cells. Off-target effects caused by these small molecules also remain a concern.

31.4 MicroRNAs and CRISPR/Cas9 for Reprogramming Somatic Cells into Neural Phenotypes

Cellular reprogramming serves as a critical and promising new avenue of research as well as a key milestone for regenerative medicine. It has the potential to generate neural phenotypes for therapeutic uses as well as provide materials for systematic investigation [40–42]. In this section, we will discuss two methods of cellular reprogramming: miRNA (microRNA)-mediated reprogramming and CRISPR/Cas9-mediated genetic modification of protein expression. MicroRNAs are small, endogenous noncoding RNA molecules, typically 20–30 nucleotides in length, and are integral to the development and growth of plants and animals [41–43]. They regulate gene expression at a posttranscriptional level and affect many key cellular processes including metabolism, differentiation, and cell proliferation [2]. Their role in differentiation makes them an

attractive method for altering transcription inside of cells (Fig. 31.4).

MicroRNAs function as a fine tuner of gene expression. They can silence the translation of mRNA by associating with the 3' UTR (untranslated region) of mRNA (messenger RNA) and marking it for cleavage and/or translational repression by associating with the ribonucleoprotein RISC (RNA-induced silencing complex). Once the miRNA has associated with the RISC, it proceeds to find perfect, or near-perfect matches to the RNA guide (miRNA) and will cleave the mRNA on-site, regulating the overall expression in the cell [44]. Certain miRNAs enhance translation, such as miR-10a which interacts with the 5' UTR of mRNAs encoding for ribosomal proteins [45].

An important aspect of cellular reprogramming is epigenetic modification, specifically DNA methylation. miRNA can target epigenetic regulators which in turn affect methylation patterns allowing transcriptional machinery to access these genes and promote reprogramming into neural phenotypes [41]. We can use this method of regulation to induce pluripotency of somatic cells and thereby influence and reprogram cell lines without affecting the nuclear DNA. MiRNAs promoting pluripotency generally occur in clustered regions of the DNA, specifically regions miRNA-302-367 [44, 46, 47]. Overexpressing this region in conjunction with optimal growing conditions can lead to the reprogramming of human fibroblasts into functional neurons (39). These regions are highly expressed in ESCs and their mode of action often involves transcriptional silencing/activation and/or chromatin remodeling to promote reprogramming [37].

Recent approaches include direct conversion or transdifferentiation of patient's somatic cells to neurons as well as iPSCs followed by differentiation into neurons [48]. The miRNAs associated with reprogramming fibroblasts to neurons are miRNA-9/9 and miRNA-124 in addition to the cluster mentioned previously (miRNA-302/367) [37, 39]. Reprogramming takes less time and is significantly more efficient than inducing pluripotency and then differentiating [39]. Using transcription factors, small molecules and fusion proteins can enhance the conversion

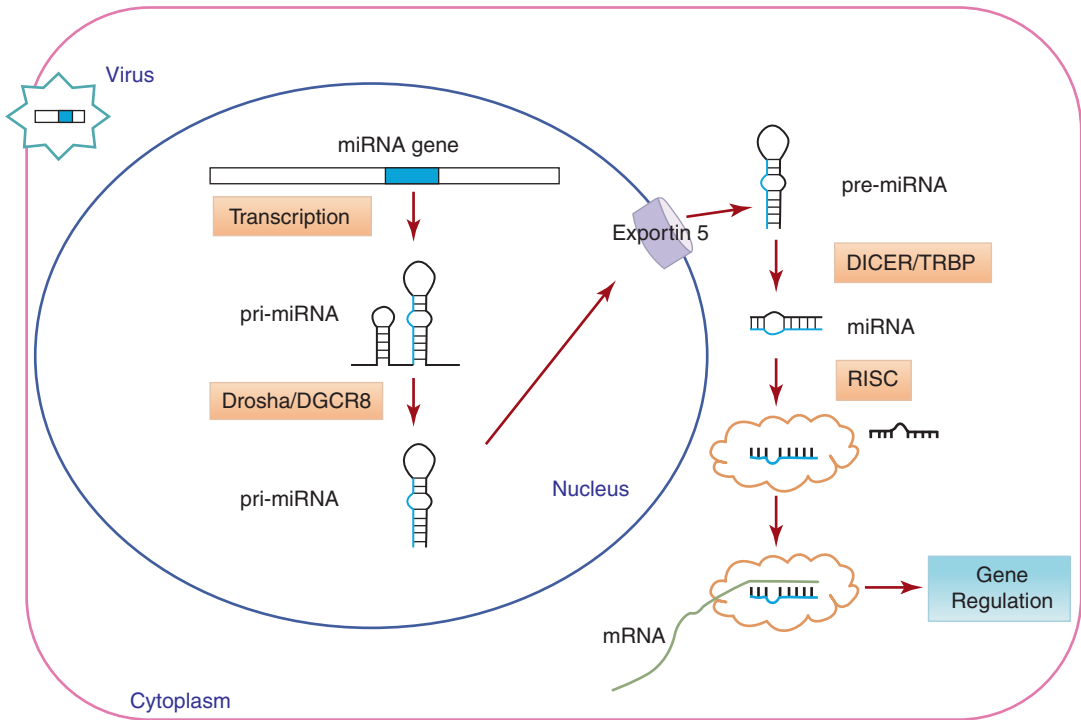


Fig. 31.4 The mechanism for microRNA-mediated gene expression regulation. Reprinted with permission

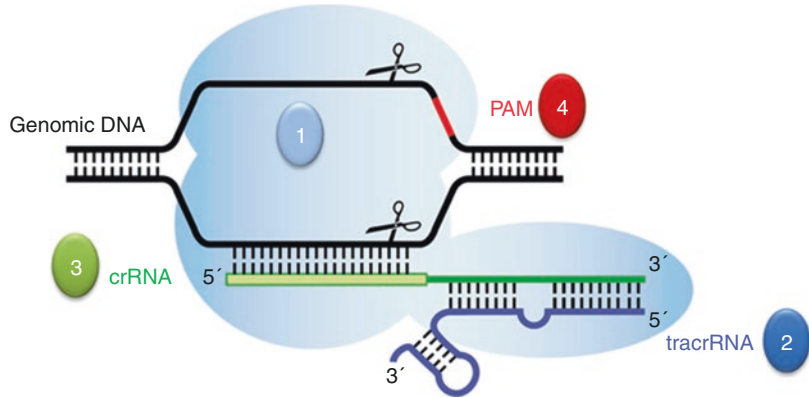
efficacy [2, 49]. Using transcription factors and miRNAs together, Richner et al. [49] have been able to directly convert human fibroblasts to striatal medium spiny neurons (MSNs).

Expressing the miR302/367 cluster increases the efficiency of reprogramming fibroblasts to iPSCs without the use of exogenous transcription factors. This method is more efficient by two orders of magnitude when compared to the standard Oct4/Sox2/Klf4/Myc-mediated methods.

Disadvantages of the miRNA method include specificity issues due to the multiple potential off targets in the transcriptome [44, 50]. Additionally, the uncontrollable expression after reprogramming induction can increase these off-targeting effects [44, 50]. The primary importance of miRNA is that they directly alter the adult transcriptome and proteome while leaving inheritable DNA unaltered. MiRNA leads to the increased efficiency of cellular reprogramming and decreases the necessity for the use of potentially harmful chemicals which can influence the cell's differentiation in a manner foreign to what it would experience *in vivo*.

CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9) technology radically changed the field of genetic engineering. Until its discovery and implementation, humans relied on clumsy, blunt, and work-intensive methods to alter genomes using gene editing [51]. CRISPR-Cas9's programmable, RNA-guided, endonuclease activity allows precise and efficient genome editing in plants and animals. Its ease of use and customization have allowed many new possibilities for genetic studies in neurological diseases as seen in Fig. 31.5 [52]. CRISPR-Cas9 is a microbial adaptive immune system. The Cas enzyme has the endonuclease activity, and the CRISPR is the region of DNA which codes for the many RNA guides which are used to recognize infiltrating viral DNA/RNA [53]. There are three types of CRISPR systems, but we will focus on type II because of its ease of use and practical applications in the lab. The type II system requires a protospacer adjacent motif (PAM sequence), two RNAs, and a Cas9 enzyme. The two RNAs are a target-specific CRISPR RNA (crRNA) and a

Fig. 31.5 CRISPR-Cas9. This illustrates the Cas9 protein (labeled 1) orchestrating a double-stranded break in a genomic strand of DNA. This double-stranded break can follow one of the two repair pathways, nonhomologous end joining (NHEJ), or homology-directed repair (HDR). Used with permission



trans-activating crRNA (tracrRNA); together these two make up the guide RNA (gRNA) which is named so because it guides the Cas9 enzyme to its specific genomic locus. The PAM sequence is essential to the enzymatic activity of the Cas9. The PAM sequence is a 2–6-nucleotide sequence immediately downstream of the 3' target region [54]. Without the PAM sequence, the enzyme will not cut the DNA. Cas9 creates double-stranded breaks, cleaving the two phosphodiester bonds (the backbone) of the DNA [55]. Some CRISPR/Cas9 variants, which contain inactivating point mutations at the catalytic site, demonstrate the ability to function as a “nickase” [53]. When incubated with a native DNA plasmid, the mutant Cas9 enzyme performs single-stranded breaks yielding nicked open circular plasmids [53]. It is also possible to inactivate its enzymatic activity so it may be used as a gene location device if fused with a fluorescent protein, or can be used to recruit molecular machinery if fused with a transcription factor [56].

Genetic modification using CRISPR/Cas9 can use homology-directed repair (HDR) and nonhomologous end joining (NHEJ). HDR allows the insertion of DNA into the cut site, enabling the knock-in of genes, and auxotrophic/selection markers. NHEJ relies on the error-prone DNA repair mechanism of the cell. After the Cas9 performs a double-stranded break, the cell enacts its DNA repair which often causes a frameshift mutation resulting in an unreadable and discarded mRNA. NHEJ is a quick way to perform gene knockouts. The CRISPR/Cas9 system has been

used to induce endogenous gene expression, allowing the direct conversion of fibroblasts to neuronal cells [57]. This effect is achieved through the overexpression of Brn2, Ascl1, and Myt1 which rapidly remodel the epigenetic state of specific regions of chromatin/DNA [16, 57]. This shows how transcriptional activation and epigenetic remodeling of native master transcription factors can convert between cell types [57]. Human neurons can be differentiated/reprogrammed in vitro from human fibroblasts, hPSCs, and hNPCs [52]. The two genes inactivated by Rubio et al. [52] using CRISPR-Cas9 were TSC2 and KCNQ2 with an 85% efficiency on gene targeting in differentiated cells. However, the new CRISPR/Cas9 system was employed by Rubio et al. [52] in combination with neurogenic factors to generate functional human neurons enriched for the gene modification of interest within 5 weeks [52]. This is a faster, more thorough method of reprogramming.

As we now know, CRISPR/Cas9 system uses DNA targets. However, scientists have recently discovered a single-component, programmable, RNA-guided, RNA-targeting CRISPR effector known as C2c2 [58]. It can be programmed to cleave ssRNA targets carrying the appropriate protospacers, yielding a programmable and easy-to-use system for effecting the expression of cells without altering genomic DNA. The identification of new Cas9 orthologs as well as the engineering of variant strains are leading to new specialized functions, flexibility, and targeting range due to size, PAM recognition, and catalytic variation [52, 59, 60].

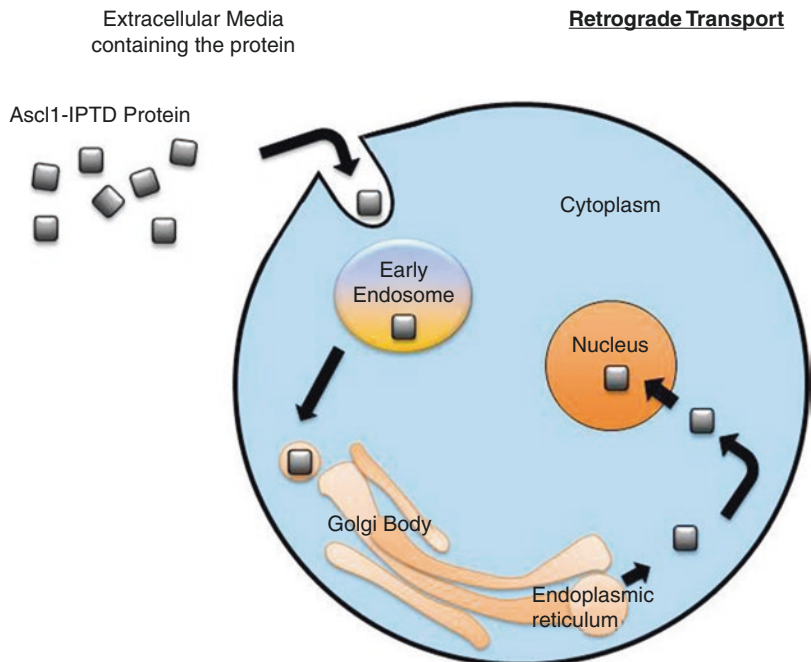
31.4.1 Limitations of miRNA- and CRISPR-Cas9-Mediated Reprogramming of Somatic Cells into Neural Tissue

When working with eukaryotic organisms generally off targets increase with the complexity of the genome. When examining partially mismatched sites, four out of six CRISPR RGENs (RNA-guided endonucleases) displayed off-target alterations [61]. Yangfang et al. [61] demonstrated that RGENs, even with up to five base pair DNA-RNA mismatches with the genome, are still highly active in human cells. Doudna et al. [51] have evidence to refute this claim. Using immunoprecipitation assays and high-throughput sequencing, they showed that catalytically inactive RGENs will associate with many regions of the genome, but active RGENs rarely cleave at off-target sites. This implicates a decoupled binding and cleaving event. CRISPR-Cas9 has a limited ability to access regions of heterochromatin. Thus, it is important to note the location and the spatial arrangement of the DNA and how it will affect the binding efficiency.

31.5 Functionalized Transcription Factors as a Way to Directly Reprogram Somatic Cells into Neural Phenotypes

While transcription factors play important roles in cell maintenance and differentiation, they are typically unstable due to their transitory nature. Also, they are not efficiently taken up by cells if introduced exogenously. Modifying factors to allow efficient uptake and improve their stability could serve as a promising alternative to the use of viral based methods and small molecules for applications in reprogramming. iProgen Biotech has created a modification which allows for efficient uptake of transcription factors in human cells and has shown to effect rapid reprogramming using a novel intracellular protein delivery technology called IPTD shown in Fig. 31.6. This modification fuses a target protein to a secretion signal peptide, indicating that the protein should be retained by the cell. The secretion signal of a peptide is usually cleaved during maturation in the cell and so is protected by the addition of a cleavage inhibition sequence. While improving the stability of the

Fig. 31.6 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-IPTD can be transported into the nucleus where it regulates gene expression [64]



protein, this combination appears to allow for efficient entry into the cell via receptors on the cell surface, which is unique from conventional mechanisms that involve direct interaction with the phospholipid bilayer. The use of IPTD to deliver transcription factors means protein expression levels can be more precisely controlled and thus easier to translate to a clinical setting. A functionalized version of the transcription factor Ascl1, Ascl1-IPTD, was shown to effect rapid induction of mature neurons from human induced pluripotent stem cells in 12 days. These neurons were NeuN positive and exhibited morphologically mature features like neurite length and branching than those cultured for over 4 weeks using a standard neural differentiation protocol.

Ongoing work is being done to functionalize more transcription to enhance reprogramming efficiencies and generate specific neuronal subtypes. The use of antibodies to target surface cellular markers of specific cell populations is being investigated to allow reprogramming of a subset of a heterogeneous population of cell types for in vivo tissue engineering. Overall, the use of novel functionalized transcription factors serves as a promising, more clinically translatable approach for in vivo reprogramming for neural tissue engineering.

31.6 Future Directions and Conclusions

This chapter has covered a number of studies evaluating methods of directly reprogramming somatic cells into neural tissue along with their limitations and potential. The use of adeno-associated virus provides a potential promising alternative for generating transcription factor expression that avoids some of the concerns about lentiviral transfection [62]. It does not integrate into the genome, eliminating the concerns associated with improper integration. While small-molecule cocktails have shown promise for reprogramming applications, the mechanism behind these cocktails needs to be elucidated. Also, such combinations should be subjected to extensive preclinical testing to ensure that no harmful off-target effects are observed. Such dos-

ing of these cocktails may be hard to translate for clinical applications. Off-target effects also remain a concern with gene editing approaches as well, but this technology is evolving at a rapid pace. In terms of functionalized transcription factors, major concerns involve ensuring that the purity of the protein is sufficient for clinical applications along with dosing similar to the use of small molecules. In terms of future work, direct reprogramming using plasmid-based methods successfully converted adult human fibroblasts into induced neural stem cells. Such DNA-based methods of reprogramming will require more work to determine how to translate delivery and dosing to achieve in vivo reprogramming.

While these studies show the possibility of direct reprogramming, the question remains over whether or not reprogramming endogenous cells will result in enough functional cells to promote recovery after neurological disorders. Even if it does not, these studies provide insight into how transcription factors alter cell behavior and such insights can be used to engineer exogenous cell therapies for transplantation. In summary, direct reprogramming of somatic cells into neural phenotypes serves as an exciting strategy for engineering tissues both in vitro and in vivo.

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