REVIEW

Regeneration through Reprogramming Adult Cell Identity *in Vivo*

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The discovery and *in vivo* application of cell fate reprogramming concepts have jumpstarted new technologies aimed at the functional regeneration of damaged tissues. As most adult organ systems retain only a limited potential for self-regeneration after trauma, the production of fate-specific cells by *in vivo* transdifferentiation offers a targeted method for tissue bioengineering. Proof-of-principle studies have demonstrated the induction of neural precursor cells, neurons, cardiomyocytes, and insulin-producing β islet cells. Each of these induced cell types survive, mature, and integrate into the local environment in a functionally meaningful manner. Here, we briefly highlight recent advances in the *in vivo* reprogramming of cell identity and the current challenges that face the clinical relevance of these methods. (*Am J Pathol* 2015, 185: 2619–2628; <http://dx.doi.org/10.1016/j.ajpath.2015.02.025>)

The central focus of regenerative medicine is the development of patient-specific technologies that treat age-, disease-, and trauma-related injuries through the regeneration of damaged tissues. These therapeutic methods are critical to the repair of adult organ systems, which often retain a limited capacity for self-regeneration in adulthood. Currently, state-of-the-art *in vivo* cell fate reprogramming methods are widely regarded as one of the most promising techniques for engineering patient-specific cell therapies.

Adult cell fate conversion was demonstrated by both direct transdifferentiation and the indirect induction of pluripotent stem cells or lineage-specific progenitor states.¹ Induced pluripotent stem cells (iPSCs) have numerous *in vitro* applications as an expandable source of undifferentiated cells. Genome-engineered iPSCs can be differentiated for transplantation or combined with biocompatible materials for three-dimensional tissue printing.^{2–4} In addition, these cells are invaluable for screening novel pharmaceuticals and for investigating the molecular pathways of development and disease.⁵ Although a useful tool *in vitro*, the therapeutic utility of iPSCs *in vivo* is inherently limited by the acquisition of unlimited growth potential and demonstrated propensity for teratoma formation.^{6,7} To overcome this tumorigenicity and to demonstrate clinical feasibility, iPSC-derived neural-specific progenitors were generated *in vitro* and transplanted

to an injured nonhuman primate spinal cord with no observed tumor formation.⁸

Although the transplantation of iPSC-derived cells might be clinically feasible, the *in vivo* transdifferentiation of resident cells has emerged as a more versatile tool in the repair of neural, cardiac, and pancreatic disease and trauma.^{9–19} The ability to bypass pluripotent intermediates during lineage conversion enables fast generation of nontumorigenic, fate-specific cells from an endogenous cell population. In this review, we highlight current methods in adult cell *in vivo* reprogramming and describe the challenges that face the clinical application of technology.

Reprogramming Cell Identity *in Vivo*

The *in vivo* reprogramming of cellular identity was demonstrated in the mouse brain, spinal cord, heart, pancreas, and liver (Table 1).^{10–24} Currently, these state-of-the-art

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Table 1 *In Vivo* Cell Fate Reprogramming Methods

Cell of origin	Reprogramming factor(s)	Induced cell fate
Central nervous system		
Glia		
Astrocyte	ASCL1, BRN2, MYT1L	Neuron ¹⁴
Cortical astrocyte	NEUROD1	Glutamatergic neuron ¹³
Spinal astrocyte	SOX2	Neuroblast ¹¹
Spinal astrocyte	SOX2, VPA	Neuron ¹¹
Striatal astrocyte	SOX2	Neuroblast ¹⁰
Striatal astrocyte	SOX2, VPA*	Neuron ¹⁰
Cortical NG2 glial cell	SOX2 ± ASCL1	Neuron ¹²
Cortical NG2 glial cell	NEUROD1	Glutamatergic and GABAergic neurons ¹³
Neuron		
LGE neural progenitor	FEZF2	Cortical projection neuron ²¹
Spiny neuron	FEZF2	Pyramidal neuron ²²
Callosal projection neuron	FEZF2	Corticofugal projection neuron ²³
Nonneuronal cell		
Nonneuronal cell	NGN2, growth factors	Neuron ²⁴
Cardiovascular system		
Cardiac fibroblast	GATA4, HAND2, MEF2C, TBX5	Cardiomyocyte ¹⁵
Cardiac fibroblast	GATA4, MEF2C, TBX5	Cardiomyocyte ¹⁶
Cardiac fibroblast	miR-1, miR-133, miR-208, miR-499, JAK inhibitor 1	Cardiomyocyte ¹⁷
Pancreas		
Pancreatic acinar cell	NGN3	Somatostatin ⁺ δ cell ¹⁹
Pancreatic acinar cell	NGN3, MAFA	Glucagon ⁺ α cell ¹⁹
Pancreatic acinar cell	NGN3, MAFA, PDX1	Insulin ⁺ β cell ^{18,19}
Liver		
SOX9 ⁺ hepatocyte	NGN3, MAFA, PDX1	Insulin ⁺ β cell ²⁰

*Striatal astrocytes can be reprogrammed to functional neurons after treatment with either SOX2 + VPA or SOX2 + noggin + brain-derived neurotrophic factor.

LGE, lateral ganglionic eminence; VPA, valproic acid.

reprogramming strategies use direct transdifferentiation and the induction of lineage-specific progenitors to target injury- and disease-responsive cell types resident to adult organ systems. These pioneering studies have shed new light on the nuanced mechanisms that govern cell fate commitment, maintenance, and plasticity in postmitotic and lineage-committed cells.

Reprogramming Central Nervous System Glia

The central nervous system is a complex network of functionally diverse cell types that cooperatively process and transmit information throughout an organism. In early development, neurogenic signaling cascades modulate transcription to specify distinct neuronal lineages from neural stem cell populations. As the architectural and functional complexity of the adult nervous system increases, neurogenesis slows and nonneurogenic cell populations predominate the brain and spinal cord. With only limited capacity for the generation of adult-born neurons, these structures are particularly vulnerable to traumatic injury and neurodegenerative disease. Therefore, therapeutic reprogramming strategies have used the mechanisms of neurogenesis and neural stem cell multipotency for targeted regeneration after neural damage (Figure 1).

Induced Neuroblasts

In one such technique, the *in vivo* conversion of adult striatal astrocytes to proliferative neuroblasts was achieved with lentiviral-mediated sex determining region Y-box 2 (SOX2) expression.¹⁰ This proof-of-principle study specifically targeted astrocytes by using the *GFAP* promoter to drive SOX2 expression, which diminished in a time-dependent manner during the acquisition of neuroblast identity. Importantly, constitutive SOX2 expression resulted in a 37-fold reduction in the induced neuroblast population, indicating that exogenous SOX2 down-regulation is critical to the maintenance of this induced fate.

Cell-of-origin tracing studies eliminated migrating neuroblasts from the lateral ventricle, a neurogenic region of the adult brain which maintains neural stem cell populations, as a source for striatal DCX⁺ neuroblasts. Further, lineage-specific tracing validated astrocytes as the cell of origin by excluding neurons, oligodendrocytes, microglia, and NG2 glia. In addition, bromodeoxyuridine tracing revealed that induced neuroblasts pass through a proliferative state with peak induction 7 weeks after lentivirus injection. Remarkably, SOX2-induced neuroblasts survive at least 14 weeks after reprogramming and can be generated in the brains of 24-month-old mice.

The clinical relevance of this discovery relies on the generation of neurons that integrate into local neuronal circuitry

Figure 1 A selection of *in vivo* reprogramming methods demonstrate the generation of neural progenitors and neurons from nonneuronal cells. The induction of proliferative neuroblasts from astrocytes was achieved by lentiviral expression of SOX2 in the adult mouse striatum and spinal cord.^{10,11} These neuroblasts differentiated to a mature neuronal fate on treatment with VPA. The direct transdifferentiation of NG2 glia and astrocytes to neurons by NEUROD1 in the mouse cortex, as well as, NG2 glia converted to neurons by SOX2 ± ASCL1 demonstrate a dominant role for transcription factors in cell fate determination.^{12,13} The induction of neurons in the mouse striatum from endogenous nonneuronal cells and transplanted human astrocytes further demonstrates the plasticity of cells within the neural microenvironment.^{14,24} GF, growth factor; VPA, valproic acid.

in a functionally meaningful manner. However, the neuroblasts induced by SOX2 overexpression rarely matured into NEUN⁺ neurons. Therefore, these cells were coaxed to adopt neuronal fate by treatment with valproic acid or a combination of brain-derived neurotrophic factor and noggin. The induced calretinin⁺ neurons fired action potentials and exhibited spontaneous synaptic currents that resulted from functional integration with endogenous striatal neurons. An intensive electrophysiologic study of these neurons revealed that SOX2 induces a functionally heterogeneous pool of calretinin⁺ neurons that can be classified into four predominant subtypes.²⁵

In parallel with this finding, astrocytes of the injured adult spinal cord were transformed into neuroblasts and mature interneuron-like cells in response to SOX2 overexpression and treatment with valproic acid.¹¹ DCX⁺ neuroblasts were detectable in both normal and injured spinal cords of young and aged mice. Proliferative astrocytes were confirmed as the origin of induced neuroblasts by lineage tracing, bromodeoxyuridine, Ki-67 tracing, and the transplantation of fluorescence-sorted *in vitro* SOX2-reprogrammed mouse spinal astrocytes. NEUN⁺ MAP2⁺ mature neurons were detectable 8 weeks after treatment and survived at least 30 weeks after induction. This population of mature neuroblast-derived neurons was heterogeneous, containing both inhibitory GABAergic and excitatory VGLUT1⁺ glutamatergic neurons. In addition, synapsin-1 colabeling revealed synaptic connections with endogenous cholinergic neurons. This remarkable transformation of cellular identity by a single

transcription factor highlights a surprising and therapeutically valuable plasticity in glial cells of the adult nervous system.

Induced Neurons

By highlighting the multifunctional roles for individual transcription factors in cell fate reprogramming, SOX2 was recently demonstrated to directly induce functional neurons from cortical NG2 glia in models of stab wound injury.¹² The retroviral delivery of *Sox2* 3 days after traumatic injury induced DCX⁺ cells with immature neuronal characteristics. The co-expression of *Sox2* and *Ascl1* further increased the efficiency of reprogramming to 30% of total infected cells. Genetic tracing in *Sox10-iCreER^{T2}*/green fluorescent protein transgenic mice revealed NG2 glia as the predominant source for reprogrammed cells. Functional analyses revealed voltage- and time-dependent conductances and synaptic inputs from local interneurons, indicating that SOX2-induced neurons functionally integrate into cortical circuitry.

Reminiscent of the striking role for SOX2 in neuroblast induction and induced neurogenesis, the retroviral expression of neuronal differentiation 1 (NEUROD1) in the mouse somatosensory cortex after traumatic brain injury was sufficient to promote the transdifferentiation of reactive astrocytes and NG2 glia to neurons.¹³ NEUROD1, a proneural transcription factor, rapidly transformed glial identity. Bipolar DCX⁺ cells were induced within 3 days, and NEUN⁺ neurons appeared 1 week after infection with extensive

neurite outgrowth and the formation of functional synapses within 3 weeks. The long-term survival of these cells in the brain microenvironment was confirmed 2 months after induction. Interestingly, reactive astrocytes directly adopted an excitatory glutamatergic identity, whereas NG2 glia reprogrammed into heterogeneous glutamatergic and GABAergic neurons. Further, neurons that originated from both astrocytes and NG2 glia expressed the deep layer cortical markers chicken ovalbumin upstream promoter transcription factor-interacting protein 1 (CTIP2), orthodenticle homeobox 1 (OTX1), and T-box brain protein 1 (TBR1).

In addition to reprogramming stab-wound-activated glia, NEUROD1 induces resident cortical astrocytes in a transgenic mouse model of Alzheimer disease to transdifferentiate into neurons with robust synaptic activity within 14 to 16 days. Unexpectedly, the relative efficiency of neuronal conversion was higher in aged mice than in young transgenic and wild-type mice.

Striatal astrocytes can be similarly transdifferentiated to neuronal fate in transgenic *Gfap-Cre* mice with doxycycline-regulated *Ascl1*, *Brn2* (official name *Pou3f2*), and *Myt1l* (ABM).¹⁴ The functional integration of SOX2-, NEUROD1-, ASCL1-, and ABM-induced neurons into local brain circuitry suggests that *in vivo* reprogramming of reactive glia might offer a mechanism for the repair of synapses destroyed by trauma and neurodegenerative disease.

Switching Neuronal Subtype

In parallel with the development of injury-responsive glial cell reprogramming strategies, numerous studies have altered the expression of lineage-specific transcription factors to directly modify neuron subtype. The *in vivo* transdifferentiation of neuron fate by Fez family zinc finger protein 2 (FEZF2) highlights the magnitude of transcriptional regulation in the specification of region- and subtype-specific neurons.^{21–23} This transcription factor has played an integral role in dissecting the developmental mechanisms of fate specification in the mouse cortex. These molecular insights are crucial to therapeutic strategies that target brain regions with distinct neuron populations.

Striatal progenitors of the lateral ganglionic eminence normally differentiate into GABAergic medium spiny neurons and interneurons in the developing mouse embryo. However, the *in utero* electroporation of these cells with *Fezf2* is sufficient to catalyze differentiation to a glutamatergic corticofugal projection neuron-like fate.²¹ In addition to the absence of striatal markers and acquisition of pyramidal structural characteristics, *Fezf2*-transduced neurons formed functional synapses with the targets of endogenous corticofugal projection neurons instead of neighboring neurons in the local striatal niche.

The striking role for FEZF2 in lineage commitment was further demonstrated by the *in vivo* transdifferentiation of postmitotic neocortical layer IV interneurons and early postnatal layer II/III callosal projection neurons to a corticofugal fate.^{22,23} In addition to the molecular, structural, and

functional similarities, FEZF2-induced neurons self-directed the rearrangement of axonal connections to mimic the architecture of endogenous layer V/VI circuitry. Importantly, Cre-inducible *Fezf2* expression revealed that this neuronal reprogramming strategy is an age-dependent phenomenon restricted to the embryonic and early postnatal stages.²³ These developmental reprogramming studies demonstrate the feasibility of engineering subtype-specific neurons from postmitotic cells in the nervous system.

Reprogramming Cardiac Fibroblasts

Rhythmic function of the mammalian heart drives the delivery of oxygen and nutrients to tissues through an extensive network of blood vessels in the cardiovascular system. Any disruption in global blood flow due to cardiac trauma or disease has systemic consequences and results in irreversible damage to adult cardiac tissues. Therefore, therapeutic reprogramming approaches have targeted cardiac fibroblast populations that neighbor regions of functional loss for the induction of beating cardiomyocytes. Grounded in the molecular pathways of early cardiomyocyte specification, these methods have used transcription factor- and miRNA-based overexpression strategies to minimize fibrosis and promote functional tissue remodeling (Figure 2).

Transcription Factor–Mediated Cardiomyocyte Induction

Proof-of-principle *in vitro* reprogramming of adult cardiac fibroblasts to troponin T⁺ α -MHC⁺ cardiac-like myocytes was achieved with the three-transcription factor cocktail GATA binding protein 4, myocyte-specific enhancer factor (MEF)2C, and T-box (TBX)5 (GMT).²⁶ The addition of a fourth transcription factor, heart and neural crest derivatives expressed 2 (GHMT), enhanced cardiomyocyte identity and *in vitro* reprogramming efficiency.¹⁵ Spontaneous contractions were observed within 5 weeks, and the beating cardiomyocytes exhibited calcium transients and action potentials.¹⁵ Importantly, only transient expression of these factors is required for the induction and maintenance of a cardiomyocyte fate.

These findings were translated *in vivo* by using coronary artery ligation to model cardiac injury and induce cardiac fibroblast proliferation.^{15,16} The retroviral expression of GMT or GHMT induced local proliferating fibroblasts to adopt cardiomyocyte fate.^{15,16} In addition to immunohistochemical analysis of green fluorescent protein-control retrovirus-infected cells, multiple cell-of-origin tracing studies were used to pinpoint starting cell identity. The promoters of fibroblast-specific protein 1 and the fibroblast-enriched gene periostin were used to drive Cre recombinase expression in *LacZ* reporter mice.^{15,16} Specific β -galactosidase expression was detected only in cardiac fibroblasts and induced cardiomyocytes of fibroblast origin. Further, pulse labeling eliminated cell–cell fusion as a mechanism for cardiomyocyte induction.

The developmental maturity and functional integration of induced cardiomyocytes was confirmed by immunohistochemical staining for the cell surface adhesion molecule

Figure 2 A selection of cardiac *in vivo* reprogramming methods that demonstrate the conversion of cardiac fibroblasts into functional cardiomyocyte-like cells. Overexpression of the cardiogenic transcription factors GATA4, MEF2C, TBX5, and HAND2 induces endogenous cardiac fibroblasts to adopt a cardiomyocyte fate.^{15,16} Similarly, the expression of four microRNAs combined with JAK inhibitor 1 treatment induces functional cardiomyocytes *in vivo*.¹⁷ GATA4, GATA binding protein 4; HAND2, heart and neural crest derivatives expressed 2; JAK, Janus kinase; MEF2C, myocyte-specific enhancer factor 2C; TBX5, T-box 5.

N-cadherin and the gap junction protein CX43. GMT- and GHMT-reprogrammed cells coupled to neighboring cardiomyocytes detected by the intercellular flow of calcein transfer dye through newly formed gap junctions. Similarly, these cells mimicked the contractility patterns and calcium transients of native myocytes, suggesting a functionally mature phenotype. Underscoring the important therapeutic potential of GHMT-induced cardiomyocytes to promote functional recovery after trauma, long-term functional improvements in fractional shortening, ejection volume, and stroke fraction were detected 6 and 12 weeks after injury.¹⁵

miRNA-Mediated Cardiomyocyte Induction

Similarly, the transformative effects of miRNA overexpression on cardiac fibroblast identity were demonstrated in a combinatorial screen of six cardiogenic miRNAs.¹⁷ Purified cardiac fibroblasts transduced by lentivirus were evaluated for cardiomyocyte-specific markers, including α -MHC and troponin I type 3. A pool of miR-1, miR-133, miR-208, and miR-499 induced cardiomyocyte-like cells from fibroblasts with low efficiency. The addition of a Janus kinase signal transduction inhibitor, Janus kinase inhibitor 1, promoted an 8- to 10-fold increase in reprogramming efficiency and functional hallmarks such as spontaneous contraction and rhythmic calcium oscillations suggest developmental maturity. The absence of the pluripotency markers *Oct4* and *Nanog* and immediate up-regulation of early cardiomyocyte markers support a direct mechanism of reprogramming. *In vivo* intramyocardial injections of miRNA-expressing lentivirus immediately after coronary artery ligation resulted in the induction of α -MHC⁺ myocytes. Lineage tracing in an *Fsp1-Cre/tdTomato* mouse model revealed that approximately 1% of cardiac fibroblasts colabeled with the cardiomyocyte marker α -MHC⁺, and within 4 weeks these cells were incorporated into the surrounding cardiac syncytium.

These novel *in vivo* reprogramming studies have provided a proof-of-concept model for the therapeutic utility of cell fate engineering and its potential applications in the regeneration of cardiac function after injury.

Reprogramming Pancreatic Acinar Cells and Hepatocytes

The metabolic regulation of blood glucose homeostasis is governed by the release of insulin from pancreatic β cells. The autoimmune-mediated destruction of β islet cells characteristic of type 1 diabetes mellitus results in persistent hyperglycemia due to deficits in insulin secretion. The reprogramming of pancreatic exocrine cell identity to insulin-secreting β cells has emerged as an elegant strategy for rescuing insulin synthesis and the self-regulation of blood glucose concentrations *in vivo* (Figure 3).¹⁸

Induced Insulin⁺ β cells

Adult pancreatic exocrine cells were induced to adopt β cell-like identity upon adenoviral expression of the transcription factors neurogenin 3 (NGN3 or NEUROG3), pancreatic and duodenal homeobox 1 (PDX1), and v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MAFA).¹⁸ The reprogramming of 2-month-old adult mouse exocrine cells to insulin⁺ cells was achieved at >20% efficiency *in vivo*, and within 10 days the quantity of insulin produced by these cells was indistinguishable from endogenous β cells. Interestingly, the transcription factor NEUROD1, which was shown to induce astrocyte–neuron transdifferentiation,¹³ is sufficient to replace NGN3 in pancreatic β cell reprogramming, although a marked reduction in efficiency is observed.¹⁸ Similarly, the expression of these factors in other cell types such as skeletal muscle cells or skin fibroblasts is not sufficient to induce a pancreatic fate, suggesting that

Figure 3 A selection of pancreatic and liver *in vivo* reprogramming methods. Endogenous mouse pancreatic acinar cells transdifferentiate into insulin-producing β cells on overexpression of NGN3, MAFA, and PDX1.^{18,19} Subsets of this transcription factor cocktail, NGN3 + MAFA, and NGN3 alone, successfully induce related pancreatic cell subtypes such as glucagon⁺ α cells and somatostatin⁺ δ cells.¹⁹ Outside of the pancreas, expression of the pro- β -cell transcription factors NGN3, MAFA, and PDX3 generates insulin-producing β -like cells from liver hepatocytes.²⁰ MAFA, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A; NGN3, neurogenin 3; PDX1, pancreatic and duodenal homeobox 1.

lineage-specific transdifferentiation depends on both cell-of-origin and reprogramming factors.

Of the five major cell types in the adult pancreas, genetic tracing identified mature amylase⁺ exocrine cells as the source of reprogrammed insulin⁺ β cells. The ultrastructure, characteristic structure, presence of dense secretory granules, and expression of specific β cell markers collectively suggest that these cells adopt mature β cell identity and silence the cell-of-origin genetic program.¹⁸ Importantly, *Ngn3*, *Pdx1*, and *Mafa* transgene expression are only required transiently during the acquisition of β cell identity, which is stably maintained in the absence of these exogenous factors.

The therapeutic relevance of this discovery hinges on the ability of induced β cells to dynamically regulate blood glucose concentrations in hyperglycemia models. The synthesis of vascular endothelial growth factor indicates that these cells can promote angiogenesis and local blood vessel remodeling to enable insulin secretion into the bloodstream. To evaluate the functional effects of insulin release, adenoviral-mediated reprogramming was performed in mice rendered diabetic by administration of streptozotocin, which specifically ablates β islet cells. Remarkably, the release of insulin from reprogrammed β cells substantially improved glucose tolerance and homeostatic blood glucose concentrations.

Induced Pancreatic α , β , and δ Cells

A comprehensive analysis of the molecular mechanism underlying NGN3-, MAFA-, and PDX1-mediated transdifferentiation revealed an additive transcriptional program that instructs the differentiation of multiple pancreatic lineages.¹⁹ The misexpression of *Ngn3*, *Ngn3* + *Mafa* or *Ngn3* + *Mafa* + *Pdx1* reprograms pancreatic acinar cells to somatostatin⁺ δ cells, glucagon⁺ α cells or insulin⁺ β cells,

respectively. Ultrastructural analysis of secretory granules, hormone secretion, cell-specific marker expression, and modifications to patterned DNA methylation indicate both phenotypic and functional maturity after cell identity conversion. Because fate respecification occurs in the absence of cell proliferation, the mechanisms of reprogramming are direct without passing through an intermediate proliferative state. NGN3 acts as a pan-endocrine activator and promotes general endocrine fate by suppressing acinar factors that act as a molecular barrier to fate conversion. The expression of PDX1 and MAFA modulate transcription to specify α or β cell fate and repress somatostatin⁺ δ cell identity.

Hepatocyte-Derived Insulin⁺ β Cells

Engineering insulin-producing cells in easily targeted non-pancreatic tissues is a novel approach to the normalization of blood glucose concentrations in models of hyperglycemia. SOX9⁺ hepatocytes transduced with *Ngn3*, *Mafa*, and *Pdx1* transform into insulin-secreting β -like cells sufficient to ameliorate the symptoms of diabetes in streptozotocin-treated nonobese diabetic severe combined immunodeficient mice.²⁰ Although these cells are capable of forming ectopic duct-like structures and glucose-responsive insulin secretion, they lack the characteristic structure and phenotype of endogenous pancreatic β cells.

The induction of glucose-responsive β -like cells sufficient to normalize blood glucose concentrations in diabetic mice is a promising first step in the development of therapeutic reprogramming strategies that target hyperglycemia.

Fate-Specific *in Vitro* Reprogramming Methods

High-throughput *in vitro* transdifferentiation studies facilitate the identification of novel conditions that enable cell fate reprogramming. Further, these systems are ideal for

teasing apart the mechanistic events that underlie lineage-specific conversion. Often it is molecular insights from these studies that inform the basis and refinement of *in vivo* reprogramming strategies. An inability to direct subtype-specific cell differentiation *in vivo* stifles the clinical utility of most current reprogramming technologies; however, this deficit might be overcome through the use of *in vitro* screens that optimize the induction of defined and homogeneous cell populations.

The plethora of neuron and glial cell types in the central nervous system poses a particular challenge to regenerative therapeutics. Early *in vitro* studies described methods for the induction of heterogeneous pools of excitatory glutamatergic and inhibitory GABAergic neurons by the overexpression of three proneural transcription factors.²⁷ Expanding this core set of transcription factors, cholinergic spinal motor neurons relevant to amyotrophic lateral sclerosis and spinal muscular atrophy were specified upon inclusion of *Ngn2* (official name *Neurog2*), *Lhx3*, *Isl1*, and *Hb9* (official name *Mnx1*).²⁸ In addition to cholinergic neurons, dopaminergic neurons with potential applications in models of Parkinson disease were generated.^{29,30}

In an attempt to derive fate-specific neurons and model reprogramming in neural cell types relevant to *in vivo* reprogramming, cultured astrocytes were functionally reprogrammed into both glutamatergic and GABAergic neurons.³¹ Pericytes, present in brain and spinal cord injury sites, were also successfully targeted for reprogramming into GABAergic interneuron-like cells.³² The *in vitro* induction of multipotent neural stem cell populations, borne out *in vivo*,^{10,11} and NG2 oligodendrocyte precursor cells exemplify the important therapeutic potential of cell fate reprogramming, particularly when applied endogenously.^{33–35}

The transplantation, survival, and integration of thymic epithelial cells, hepatocyte-like cells, and hematopoietic cells generated by direct reprogramming *in vitro* suggests vast potential for *in vivo* therapies that target these organ systems.^{36–40} For instance, transplanted *Foxn1*-induced thymic epithelial cells self-organize into a functional thymus capable of T-cell generation, and fibroblast-induced hepatocytes repopulate damaged tissues to rescue liver function.^{36–38} Moreover, instructive cues of the cellular microenvironment have integral roles in the induction of hematopoietic stem cells, indicating that *in vivo* strategies might be inherently better suited for the induction of functional fate-specific cell types.^{39,40}

Challenges

Delivery of Reprogramming Factors

The therapeutic utility of cellular reprogramming depends on the safe, efficient, and reproducible delivery of reprogramming factors to target cells. Currently, viral transduction is the predominant method for *in vivo* gene delivery.^{10–20} In pursuit of a virus-free induction strategy, recent studies have

demonstrated pluripotent stem cell generation *in vitro* with minicircle DNA constructs in human adipose stromal cells and *in vivo* via hydrodynamic tail-vein injection of DNA constructs in the adult mouse liver.^{41,42}

With a specific focus on neuronal transdifferentiation, a bio-reducible linear poly (amido amine) was used as a vehicle to transport ABM gene constructs into cultured mouse fibroblasts.⁴³ In an attempt to minimize the number of transgenes required for the induction of functional neuron-like cells, a single proneural gene *NEUROG2* was combined with the small molecules forskolin and dorso-morphin to generate cholinergic neurons.⁴⁴ Taking this one step further, iPSCs were generated from somatic cells *in vitro* by using a seven-compound cocktail without any transgene overexpression.⁴⁵

Recent advances in genome editing technologies such as the clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided endonuclease Cas9 system have enabled the high-fidelity targeting of specific DNA sequences in the human genome. This breakthrough offers a powerful mechanism for *in situ* editing of disease-related genes and, when paired with cell fate reprogramming strategies, offers a new mechanism for the regeneration or replacement of diseased tissues.^{46,47}

Functional Maturation of Reprogrammed Cells

Cell fate reprogramming as a therapeutic tool relies on the induction, survival, maturation, and functional integration of reprogrammed cells. To evaluate the feasibility of reprogramming human cells *in vivo*, human astrocytes and fibroblasts were transplanted into hippocampal and striatal regions of the adult rat brain then successfully transdifferentiated into neurons by doxycycline-regulated transgene expression.¹⁴ The brain microenvironment might therefore be conducive to the survival and maturation of induced neurons. Further, the innervation of muscle by cholinergic neurons transplanted in the chick spinal cord and the rescue of Parkinson disease-related symptoms by transplanted dopaminergic neurons demonstrate a remarkable level of regenerative plasticity in the neural microenvironment.^{28,30,48} Notably, extracellular cues of the cortical and striatal microenvironments and the focal injury microenvironment uniquely instruct nonneuronal cells to adopt distinct neuronal subtypes of varying functional maturity when transduced with *NGN2*.²⁴ These regional cues might have further roles in patterning the formation and rearrangement of synaptic connections by corticofugal projection neurons induced by FEZF2.^{21–23}

The therapeutic significance of functionally mature reprogrammed cells relies on the high-efficiency induction of the desired cell type. Although this remains a noteworthy hurdle *in vivo*, recent *in vitro* experiments have demonstrated conversion efficiencies >90%.⁴⁴ Likewise, these methods must generate a sizable population of cells sufficient to mediate functional recovery. The induction of an expandable

progenitor such as the neuroblast might offer an elegant solution to this population issue in the nervous system without depleting large numbers of endogenous glia.^{10,11} Finally, immune response to cells undergoing transdifferentiation *in vivo* remains an important unresolved question that faces the long-term survival of engineered human cells.⁴⁹

Molecular and Epigenetic Roadblocks

The forced overexpression of pioneering transcription factors is often sufficient to stably transform cellular phenotype. These factors modify the transcriptome by engaging a relatively small number of lineage-specific genomic targets that trigger secondary mechanisms of epigenetic remodeling. However, this limited scope of targets often results in a hybrid epigenetic landscape containing residual elements of the origin cell and functional regions of the adopted identity.⁵⁰ A detailed model of DNA methylation, histone tail marks, three-dimensional chromatin structure, and other mechanisms of epigenetic regulation in transdifferentiating cells will be essential to the development and refinement of clinically meaningful *in vivo* reprogramming technologies.^{51,52}

Mechanistic studies of induced pluripotency revealed dynamic stages of transcriptional and epigenetic regulation in fibroblast-derived iPSCs.⁵³ Exogenous transcription factors induce waves of transcription with corresponding biphasic changes in miRNA expression and histone marks followed by later-stage changes in DNA methylation patterning.⁵³ Studies of cytosine demethylation in early iPSCs, however, indicate that the early generation of methyl-oxidation products promote DNA accessibility and enhanced transcription factor binding.^{54–56} These studies provided early insight into the mechanisms of reprogramming and laid the groundwork for lineage-specific explorations.

The DNA occupancy profiles for multiple pro-neural transcription factors during neuronal transdifferentiation are dictated by repressive and activating histone marks.⁵⁷ Moreover, dramatic alterations to histone methylation signatures in gene enhancer regions precede transcriptional activation and short-hairpin RNA knockdown of histone methyltransferases substantially affect reprogramming efficiency.^{58,59} Therefore, high-efficiency *in vivo* reprogramming strategies should target cells with chromatin states poised for on-target binding of selected transcription factors. Taken together, these studies highlight the epigenetic landscape as a central regulator of transcription factor-mediated reprogramming and demonstrate how the pre-existing chromatin landscape should inform the design of reprogramming strategies.

Conclusion

The discovery of cell fate reprogramming and its subsequent application to disease- and trauma-damaged organ systems represents a new frontier in regenerative therapeutics. As a rapidly evolving technology, *in vivo* reprogramming faces numerous technical limitations that currently challenge its

clinical utility. As these limitations are overcome through innovation and refinement, a diverse array of novel reprogramming technologies will emerge in a new generation of patient-specific regenerative medicine.

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