Direct Lineage Conversions: Unnatural but useful?

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Abstract

Classic experiments such as somatic cell nuclear transfer into oocytes or cell fusion demonstrated that differentiated cells are not irreversibly committed to their fate. More recent work has built on these conclusions and discovered defined factors that directly induce one specific cell type from another, which may be as distantly related as cells from different germ layers. These examples of lineage reprogramming raise the possibility that any cell type may be converted into any other if the correct combinations of reprogramming factors are known.

Introduction

Once programmed to perform a specific function, cells rarely undergo dramatic fate changes *in vivo*. It was long thought that this phenotypic stability was the result of an irreversible loss of developmental potency that accompanied each progressive differentiation step in the embryo. However, the cloning of an animal from the nucleus of a terminally differentiated cell by nuclear transfer unambiguously proved that epigenetic modifications to the genome acquired during development are reversible and that nuclei from even the most functionally specialized cells maintained the potential to generate an adult organism [1–11]. Cell fusion experiments had also indicated that transcriptional reprogramming could occur following exposure to the nuclear and cytoplasmic components of cells from other lineages, such as myotubes and pluripotent stem cells [12–17]. However, it was difficult to measure the mechanism of reprogramming in fused cells due to the continuous presence of the donor cell genome.

Substantial progress was achieved with the discovery that a combination of four transcription factors (*Oct4*, *Sox2*, *Klf4*, and *c-Myc*) was sufficient to reprogram diverse somatic cell types to a pluripotent state *in vitro* [18–26]. These studies raised the question of whether reprogramming to a pluripotent state was fundamentally distinct from other defined lineages (Figure 1). Reprogramming to pluripotency could represent a return to the developmental "ground state", and might simply be the result of the erasure of all developmentally acquired epigenetic information [27]. However, earlier work such as the classic report from Weintraub and colleagues reporting the identification of *MyoD*, a transcription factor capable of converting fibroblast cell lines into myocytes, as well as more recent findings in various cell lineages support the notion that cell fate conversions can be direct without the involvement of de-differentiation even between distantly related somatic cell types (Figure 2) [28–31].

In this review we will first discuss the latest developments of lineage reprogramming in various cell types. For excellent reviews about the history of the field see e.g. [3, 17, 32–35]. Given that multiple examples of direct lineage reprogramming with transcription factors have now been published [18, 28–31, 36–43], we will also discuss emerging themes and important unresolved issues in the field. From the perspective of a developmental biologist, lineage reprogramming offers exciting new experimental tools for interrogating aspects of lineage commitment and terminal differentiation processes observed during normal development. From a translational perspective, it provides a fundamentally new approach for the generation of patient-specific cells for *in vitro* disease modeling or direct therapeutic applications.

MyoD and the myogenic program

Pioneering work by Taylor and Jones demonstrated that treatment of an immortalized mouse embryonic fibroblast cell line with the DNA demethylating agent 5-azacytidine was sufficient to induce myogenic, chondrogenic, and adipogenic clones [44]. The high conversion frequencies suggested that the reactivation of a small number of loci was likely to be responsible for these lineage-conversion events. It is now known that many CpG islands that are typically unmethylated *in vivo* and in primary cells can readily become methylated in immortalized cell lines [45–47]. Therefore it is possible that the effects of 5-azacytidine observed by Taylor and Jones were due to reactivation of CpG island promoters to their *in vivo* state.

Subsequent heterokaryon experiments by Blau and colleagues, in which two somatic cell types were stably fused but maintained separate nuclei (and thus did not divide), demonstrated that myogenic factors present in myocytes could elicit expression of myocyte-specific genes from amniocytes and other cell types [13]. Heterokaryon studies also indicated that DNA methylation was important for silencing lineage-inappropriate gene expression. For example, 5-azacytidine treatment prior to myotube-fusion was required for the induction of muscle-specific gene expression from HeLa cell nuclei [48].

These observations led Weintraub and colleagues to screen cDNA libraries from 5-azacytidine-induced myogenic clones for factors that could explain the myogenic effects of 5-azacytidine treatments. This led to the discovery of the gene *MyoD*, a basic helix-loop-helix (bHLH) transcription factor that could induce expression of myogenic markers in various fibroblast and adipose cell lines [28, 49]. Importantly, *MyoD* could convert primary mouse dermal fibroblasts into myocytes, whereas 5-azacytidine treatments could only induce myogenic differentiation in immortalized cell lines [49, 50]. These findings provided the first evidence that the intrinsic activity of a single gene was sufficient to drive lineage-specific differentiation programs in a lineage-independent manner [28, 49, 51, 52].

Further studies confirmed that forced expression of *MyoD* induced expression of skeletal muscle specific genes (i.e. desmin and myosin heavy chain) in a variety of cell types *in vitro*, including melanoma, neuroblastoma, liver, retinal pigmented epithelia, and adipose-derived cell lines [49, 50]. However, only mesodermal cell types appeared to complete a full phenotypic switch, whereas endodermal and ectodermal cells generally failed to

downregulate expression of their pre-existing tissue-specific genes [49, 53]. Similarly, work utilizing heterokaryons had indicated that trans-acting factors present in muscle cells could induce muscle-specific genes in cells derived from all three germ layers, although ectodermal and endodermal cells exhibited slower kinetics of muscle gene activation [14, 54]. In general, cell fusion appeared to be more powerful at inducing myogenic programs than transcription factors alone. For example, forced expression of *MyoD* in the MRC5 human fetal lung fibroblast line was not sufficient to activate myosin heavy chain expression, however, when stable heterokaryons were produced between MRC5 cells and muscle cells, myosin heavy chain expression was readily detectable [53, 55]. These data suggested that additional trans-acting factors were important for regulating transcription of muscle-specific genes from non-muscle cells [56–58].

Later work identified three additional myogenic bHLH factors (*Myf5*, *MRF4*, and *myogenin*) that regulate partially overlapping but distinct processes during the development of skeletal muscle [59]. *MyoD* and *Myf5* are both sufficient but not essential for myogenesis, whereas *MRF4* [60] and myogenin seem to act downstream of these factors to regulate terminal differentiation processes [61–65]. *MyoD* and *Myf5* share a histidine- and cysteine-rich domain (H/C domain) and a C-terminal amphipathic α-helix (helix III domain) that is not present in the other myogenic bHLH genes. These two domains are not required for transcriptional activation and instead appear to be important for the recruitment of chromatin remodeling proteins to a specific subset of *MyoD* target genes (including the *myogenin* promoter) that are bound by *Pbx* proteins prior to *MyoD* recruitment [66]. These unique features could facilitate the activation of genes in heterochromatin by recruiting SWI/SNF chromatin remodeling complexes or histone acetyltransferases to a specific subset of *MyoD* target genes or tissue-specific enhancer elements [67], which is likely important for imparting their specification functions during development [68].

The discovery that a single transcription factor can be sufficient to activate lineage-specific genes outside of its normal cellular context raised a variety of important questions about the basic biology of cellular differentiation, some of which have been studied intensively over the last 25 years and some of which are only beginning to be addressed. For example, how does *MyoD* find its physiologically relevant binding sites in heterochomatic regions of the genome? Similarly, how does the expression of a single gene recapitulate the temporal patterns of gene expression found during normal muscle differentiation [64]? Genome-scale studies of gene expression and transcription factor occupancy during muscle differentiation have begun to provide insight into these issues. Chromatin immunoprecipitation of *MyoD* followed by high throughput sequencing (ChIP-seq) in muscle cells and fibroblasts expressing *MyoD* indicated that *MyoD* binds to the promoters of many genes that regulate muscle differentiation at early and late stages and its binding is correlated with acetylated histones genome-wide [69]. Paradoxically, *MyoD* was also shown to bind constitutively to thousands of other genomic regions, independent of differentiation status. These results suggest that additional factors likely impart gene-specific regulation at *MyoD* bound promoters and enhancers. Along these lines, a key question will be to elucidate the mechanisms that regulate the temporal activation of genes controlled by *MyoD* (as well as the other myogenic bHLH factors), which is likely to involve *MyoD*-associated cofactors

(e.g. *Mef2* family proteins, *Pbx/Meis* family proteins, *p38 MAPK*, *PCAF*, and *p300*, among others) [66, 70, 71], chromatin remodeling complexes [72, 73], and the cis-regulatory organization of the promoters of *MyoD*-regulated genes, including the pre-existing, developmentally-regulated chromatin marks at these promoters [56, 64, 67]. These studies provide an important paradigm for the reprogramming field, and continue to be particularly relevant given the demonstrated importance of other bHLH family transcription factors in lineage-specification, terminal differentiation, and lineage-reprogramming (see below).

The fact that Taylor and Jones also observed adipogenic differentiation following 5-azacytidine treatment of immortalized fibroblasts has received less attention. Building upon this work, Spiegelman and colleagues recently made the surprising discovery that *Myf5*-expressing muscle precursors can give rise to brown fat tissue *in vivo* [74]. The transcription factor *PRDM16* plays a critical role in this developmental decision by co-regulating transcriptional programs of adipogenesis via its interaction with *PPAR-γ*, another important adipogenic determinant [75]. When expressed in primary mouse myoblasts, PRDM16 induces brown fat differentiation with nearly 100% efficiency, and when *PRDM16* expression is reduced, primary brown fat cells inappropriately express skeletal muscle lineage genes such as *MyoD* and *myogenin* and exhibit a myotube-like morphology [74]. However, unlike the myogenic bHLH factors, forced expression of *PRDM16* is not sufficient to induce brown fat differentiation in non-myogenic fibroblast cell lines. Using an unbiased proteomics approach to search for potential *PRDM16* interaction partners, the authors identified *C/EBP-β* as a *PRDM16* binding partner and found that it also exhibits highly specific expression in brown versus white adipose tissue. Strikingly, forced co-expression of *PRDM16* and *C/EBP-β* was sufficient to convert mouse and human dermal fibroblasts into functional brown fat-like cells [76]. These reprogrammed brown fat-like cells could form fat pads and function in glucose uptake following transplantation of transduced fibroblasts into adult mice. These elegant studies highlight the power of proteomic approaches for discovering novel, biologically relevant interactions between transcription factors, an approach that should prove similarly informative for lineage reprogramming studies in other tissues.

Induction of Cardiac Muscle Phenotypes

Extensive developmental studies have failed to identify a single "master regulator" of cardiac muscle fate analogous to the myogenic bHLH genes. Instead, cardiac development appears to be controlled by a highly conserved core module of transcription factors (*Gata4*, *Tbx5*, and *Mef2c*, along with *Nk2* and *Hand* family transcription factors) that regulate cardiac specification, morphogenesis, and terminal differentiation [77, 78]. Transient transfection of small groups of these core regulators into cultured early stage mouse embryos (E6.5–E8.75) provided the first evidence in mammals that reprogramming of non-cardiogenic mesoderm toward a cardiac fate was possible [41]. Strikingly, the combination of *Gata4*, *Tbx5*, and the chromatin remodeling protein *Baf60c* (also known as *Smarcd3*) was sufficient to precociously induce ectopic beating cardiomyocytes in the posterior mesoderm and the extra-embryonic amniotic tissue, whereas during normal development cardiomyocytes are generated in the lateral mesoderm [77, 78]. However, these factors were not sufficient to induce cardiomyocyte differentiation during later stages of development or from cultured embryonic fibroblasts.

The combination of *Baf60c* and *Gata4* was sufficient to induce the expression of some early cardiac markers, as well as other crucial regulators of cardiac development (*Nkx2–5*), however, ectopic *Tbx5* expression was essential for the generation of beating heart tissue. Interestingly, *Baf60c* expression was necessary for *Gata4* to bind at least a subset of its target genes, suggesting that tissue-specific chromatin remodeling events might be required for the activity of these cardiogenic transcription factors when they are expressed outside of their normal embryonic milieu. Accordingly, *Baf60c* is selectively expressed in the pre-cardiac mesoderm during heart development, and loss-of-function studies in mouse indicate that it is essential for the proper heart morphogenesis and the execution of terminal differentiation of cardiac tissues [79]. Furthermore, *in vitro* assays indicated that *Baf60c* promotes a physical interaction between *Tbx5*, *Gata4*, *Nkx2–5* and *Brg1* [79], suggesting that SWI/SNF chromatin remodeling complexes containing *Brg1* and *Baf60c* might play a crucial role in regulating cardiac development. It is also interesting to note that there is evidence that *Gata4* can function as a "pioneer" transcription factor, meaning that it can bind to its target sites in the genome even when they are in tightly packed nucleosomes in heterochromatin [80, 81]. During liver development, *Gata4* is one of the first genes to bind to the albumin enhancer, where it might play a role in creating a chromatin environment that allows other enhancer-binding transcription factors to access their target sites [80].

Recently, Srivastava and colleagues demonstrated direct conversion of mouse cardiac and dermal fibroblasts into cardiomyocyte-like cells (termed induced cardiomyocytes, or iCMs) by forced expression of three transcription factors (*Gata4*, *Mef2c*, and *Tbx5*) [42]. To identify potential reprogramming factors they chose to focus on genes that exhibited clear developmental defects in cardiac tissue when mutated in mice and also exhibited high levels of expression in cardiomyocytes compared to cardiac fibroblasts [33, 36]. They utilized an iterative process of elimination to define a minimal pool of three genes (*Gata4*, *Mef2c*, and *Tbx5*) that were sufficient to induce rapid and efficient activation of early cardiomyocyte markers, with kinetics and efficiencies similar to previous examples of lineage reprogramming in other tissues [29, 36]. When compared with parental fibroblasts, iCMs exhibited genome-wide expression patterns that were statistically closer, but not identical to, neonatal cardiomyocytes, indicating that the cardiac fibroblasts underwent global transcriptional reprogramming. The authors also noted evidence of concomitant epigenetic reprogramming by comparing active and repressive histone methylation marks and DNA methylation at a small set of cardiac specific genes. Interestingly, they noted a slight increase in trimethylated-H3K27 at these promoters in tail tip-derived iCMs compared to cardiac fibroblast-derived iCMs, suggesting that the tail tip fibroblasts were more refractory to epigenetic reprogramming. It will be interesting to compare these active and repressive epigenetic marks genome-wide, both in iCMs from tail tip and cardiac fibroblasts to gain a more comprehensive understanding of the epigenetic reprogramming process. Importantly, electrophysiological assays in individual iCMs provided evidence for the adoption of functional properties similar to ventricular cardiomyocytes, and a low percentage of iCMs contracted spontaneously. It will be important to explore whether protocols can be developed to more efficiently generate functional iCMs and whether other types of cardiomyocytes such as cardiomyocytes of the atrium or sinus node can be generated directly from fibroblasts.

The three identified transcription factors *Gata4*, *Tbx5*, and *Mef2c* are well known players in cardiac development that act as a core regulatory module. This transcriptional module is stably maintained by extensive cross-regulation and auto-regulation between the component transcription factors [77]. Interestingly, genetic lineage tracing methods provided compelling evidence that the fibroblast-iCM conversion occurred directly, without passing through a cardiac progenitor-like state, as critical markers of cardiac progenitors (*Islet1* and *Mesp1*), were not activated during reprogramming [42]. This suggests that these transcription factors drive a transcriptional program of cardiomyocyte terminal differentiation in fibroblasts [82], rather than initiating de-differentiation of fibroblasts to a cardiac precursor state and recapitulating the entire program of heart development. This would be consistent with a feed-forward model of cardiac differentiation, in which the transcription factors that are critical for early cardiogenesis are also important for the activation and regulation of genes that are critical for terminal differentiation of cardiomyocytes, although genome-wide occupancy studies of these genes during the reprogramming process will be necessary to confirm that their binding patterns during reprogramming are similar to that found during development. Furthermore, given that reprogramming of mesodermal tissue to a cardiac fate has now been accomplished in two different contexts, it will be interesting to investigate why the genes that are sufficient for cardiac reprogramming in embryonic mesoderm cannot reprogram postnatal fibroblasts, and, conversely, what effect the three gene cocktail identified by Srivastava and colleagues would have when transfected into embryonic mesodermal tissue. An important area of future study will be to determine whether any combination of these factors can induce iCMs from more distantly related cell types such as ectodermal or endodermal cells.

Reprogramming Blood Cells

The hematopoietic system is arguably the most thoroughly characterized cellular differentiation system in mammals [83, 84]. All blood cells are derived from long term hematopoietic stem cells (LT-HSCs), functionally defined as cells that can reconstitute a mouse's blood system and can be serially passaged into other recipients. Given its analytical power, it is not surprising that one of the first clear demonstrations of direct lineage conversion was in the blood system (see [34] for an excellent review).

Although "lineage infidelities" such as immunoglobulin or T cell receptor gene rearrangements had been observed previously in some cases of myeloid leukemia, it was unclear whether this phenomenon was unique to transformed cells [85–87]. Overexpression and genetic loss-of-function studies aiming to decipher the mechanisms regulating normal hematopoietic differentiation identified various transcription factors and cytokine signaling pathways as instructive lineage-determining factors [34]. For example, forced expression of the zinc-finger transcription factor *GATA-1*, which is required for erythroid development [88–91] was sufficient to induce erythroid and megakaryocytic markers in monocytic cell lines [92, 93]. Conversely, forced expression of the ETS-domain transcription factor *sfpi1* (also known as *PU.1*), a critical regulator of myeloid and B-cell development, was shown to repress *GATA-1* expression and upregulate monocytic markers in an erythroid-megakaryocytic cell line [94]. Much of this early work utilized established cell lines with restricted differentiation potential and thus the significance of these findings was

initially unclear. However, ensuing studies demonstrated that forced expression of *GATA-1* in freshly isolated granulocyte-macrophage progenitors was sufficient to induce erythroid cell types [95].

Transdetermination of primary cells had also been observed following alterations in cytokine signaling pathways. In attempts to test whether interleukin 2 (IL2) signaling regulates natural killer (NK) cell differentiation, Weissman and colleagues observed that primary common lymphoid progenitors (CLPs) isolated from human IL2 receptor β-chain transgenic mice and stimulated with human IL2 *in vitro* gave rise to granulocytic and monocytic lineages (GM) [96]. The same effects were seen with pro-T cell progenitors, but not with pro-B cell progenitors. However, when the cells were cultured prior to transduction (and thus more differentiated) transdetermination did not occur. These results were surprising as the primary role of cytokines in hematopoiesis was thought to be the regulation of survival and growth of specific blood cell lineages as opposed to instructive functions. It is interesting to note that ectopic activation of the erythropoietin or IL7 receptors, both of which have downstream signaling effects very similar to human IL2R, cannot induce GM lineages in primary CLPs [96]. However, the receptor for granulocyte/monocyte colony stimulating factor (GM-CSF) mimics the effects of human IL2R signaling, suggesting that upregulation of GM-CSF receptors during HSC differentiation is one of the key factors regulating common lymphoid versus myeloid progenitor cell determination. Thus, it is possible that in unique cellular differentiation contexts (such as the differentiating HSC) certain signaling pathways can instruct specific lineages, but the effects of their ectopic activation may be limited to the induction of lineages sharing the same immediate progenitor cell.

Transdetermination has also been induced in different lineages of blood progenitor cells by deletion of transcription factors. For example, deletion of the B cell determination factor *Pax5* in pro-B cells can promote robust differentiation into the T cell lineage [97, 98]. T cell reconstitution was also observed *in vivo* when *Pax5* was deleted in mature B cells [99]. To determine the reconstitution potential of these reprogrammed T cells, *Pax5*-deficient B cells (CD19+, IgM+, IgD+) were isolated and transplanted into lymphocyte-deficient RAG2 mice. The appearance of donor pro-B cells and the gradual development of donor-derived T cells indicated that loss of *Pax5* led to dedifferentiation of mature B cells rather than a direct conversion of B into T cells. Further evidence for this conclusion is the observation that donor pro-B cells isolated from these mice could give rise to macrophages when cultured in appropriate conditions. The dedifferentiation efficiency seemed very low, however, as converted T cells were oligoclonal, and the process was highly dependent on the continued expression of *Bcl2*, indicating that apoptotic signals are also induced following loss of *Pax5* [99].

Work by Graf and colleagues has provided clear evidence that direct conversion can occur between two mature cell types in the hematopoietic system. They demonstrated that forced expression of the basic leucine zipper (bZIP) transcription factors CCAAT/enhancer binding protein *C/EBPα* or *β* efficiently induced the macrophage marker Mac1 in primary bone marrow or spleen B cells within 4 days [38]. The B cell-derived, Mac1+ cells downregulated 7 of the 8 B cell genes analyzed, upregulated 5 macrophage markers, and failed to express granulocytic markers. The reprogrammed Mac1+ cells also exhibited concomitant

morphological changes as well as the capacity to phagocytose fluorescent beads, indicating that they had acquired functional properties normally attributed to macrophages. Interestingly, the downregulation of B cell markers was shown to be *Pax5*-dependent, perhaps resulting from a direct interaction with CEBPα at the CD19 promoter [38]. Conversely, the activation of macrophage markers was dependent on *PU.1* but not on the downregulation of B cell markers, and the conversion efficiencies were increased when *C/EBPα* and *PU.1* were combined. Later work demonstrated that the combination of those two genes was sufficient to induce macrophage-like cells from primary fibroblasts and from an immortalized fibroblast line (NIH3T3) [37, 38]. However, these induced macrophages were dependent upon continued expression of the exogenous factors, indicating that they were not sufficient to induce a stable phenotypic conversion.

Genome-scale studies of *PU.1* occupancy in macrophages and fibroblasts ectopically expressing *PU.1* have provided an initial insight into the potential mechanisms underlying fibroblast-to-macrophage reprogramming[100–102]. The distributions of *PU.1*, *p300*, and H3K4me1 occupancy suggest that *PU.1* is associated with active enhancer elements in macrophages, and that *PU.1* binding at these gene-distal regulatory elements appears to be important for tissue-specific inflammatory responses following LPS-stimulation. When expressed in fibroblasts, preliminary evidence suggests that *PU.1*-binding can activate these macrophage enhancer elements (measured by H3K4me1 enrichment) [101]. Furthermore, *PU.1* occupancy at enhancers tends to be associated with motifs of other lineage-determining transcription factors, both in macrophages (*C/EBP*, *AP-1*) and in B-cells (*E2A*, *EBF*, *Oct*, and *NF-κB*) [100]. This is consistent with a model of direct conversion in which *PU.1* and *C/EBPα* bind to macrophage-enhancer elements in fibroblasts and synergistically facilitate their activation (most likely through direct or indirect recruitment of *p300*). Once activated, these enhancers would then promote macrophage-specific transcriptional programs genome-wide, potentially by mediating cell-type specific enhancer-promoter interactions [102]. Given that enhancer activity appears to be highly cell type specific [103, 104], transcription factors that bind active enhancers in a tissue-specific fashion would be prime candidates for lineage reprogramming. The recent identification of increasingly detailed chromatin signatures of active and "poised" enhancers should permit more detailed studies of their roles during lineage reprogramming [105, 106].

Graf and colleagues have further examined the mechanistic aspects of the B cell to macrophage conversion by utilizing a drug-inducible estrogen receptor *C/EBPα*–ER fusion protein in a permissive B cell line [107]. This system allows for temporally regulated direct conversion of B cells into macrophages with an efficiency of nearly 100% over a 2–3 day period. As early as 3 hours after *C/EBPα* induction bidirectional expression changes of ~ 2,400 genes were observed. Time-lapse microscopy indicated that the first morphological changes occurred within 10 hours. Forced expression of *C/EBPα* for 48 hours was sufficient to induce a stable macrophage phenotype, and most expression changes appeared to be independent of protein synthesis, suggesting that they are caused directly by the activity of *C/EBPα*[107]. This defined system should provide considerable insights into the transcriptional mechanisms that facilitate direct lineage reprogramming, especially at the earliest stages following activation of reprogramming factors.

A recent study reported the induction of several traits of multipotent blood progenitors in human dermal fibroblasts [43]. The resulting cells were capable of generating cells expressing markers of granulocytic, monocytic, megakaryocytic and erythroid lineages. The induction of hematopoietic lineage markers was achieved by expression of the pluripotency and tumor-promoting gene *Oct4* [108] in combination with a permissive culture environment. This result is unexpected because *Oct4* is an important transcription factor regulating pluripotency but is not expressed in the hematopoietic system [109]. Future studies may resolve this conundrum by addressing whether Oct4 is mimicking other Pou-domain containing transcription factors such as *Oct1* or *Oct2* that are expressed in hematopoietic cells [110–112] or whether *Oct4* may induce some partially reprogrammed iPS cells that can subsequently be differentiated into blood lineages with the assistance of growth factor and cytokine signaling provided by the culture media.

Reprogramming Endodermal lineages

Given the clinical interest in insulin-producing β-cells, numerous studies have focused on characterizing the gene regulatory networks controlling the development of the early endoderm and endocrine pancreatic tissues [113–115116, 117]. Interestingly, gene knockout, over-expression and lineage tracing studies have provided evidence of plasticity during the development of the hepatic and pancreatic lineages [114, 118–123]. These data have fueled numerous studies attempting to recapitulate this lineage plasticity by forced expression of lineage-determining transcription factors. In the following we will discuss the role of these reprogramming factors during development and speculate on the specific properties that might differentiate these genes from other important developmental regulators.

C/EBPβ was one of the first transcription factors involved in lineage conversion of endodermal tissue [124]. This basic region/leucine zipper (bZIP) transcription factor is expressed during differentiation of adipose tissue and liver and, as described above, has also been involved in the ectopic induction of brown fat and macrophage fates [34, 76]. Slack and colleagues observed that *C/EBPβ* was sufficient to mimic the effects of the synthetic glucocorticoid dexamethasone and induce hepatic properties in pancreatic exocrine cells, both in immortalized cell lines and cultured primary pancreatic buds from E11.5 mouse embryos [124]. This conversion could occur in the absence of cell division and was blocked by a dominant negative form of *C/EBPβ.*

Ectopic expression of the homeobox-containing transcription factor *Pdx1* can induce expression of insulin and other pancreatic genes in a variety of endoderm-derived cells, including adult mouse liver [125–127], cultured human liver cells [128, 129], and chick embryonic endoderm [130]. *Pdx1* has been proposed to function as a selector gene for pancreatic development, as its expression is the first marker of pancreatic specification from the foregut endoderm [131]. In the absence of *Pdx1* expression, the ventral pancreas fails to form, and the dorsal pancreas has a severe growth defect [132]. In addition, *Pdx1* appears to have a later function in glucose homeostasis in mature β-cells [133], perhaps by transactivating genes that are important for glucose sensing and metabolism, such as *insulin* and *Glut2* [134, 135]. *Pdx1* is expressed in the earliest pancreatic progenitors as well as in mature endocrine cells

suggesting that the later role of *Pdx1* might be more relevant for its ability to induce insulin expression and other β-cell specific features.

Proneural bHLH transcription factors are also known to play important roles in cell fate decisions during pancreatic development. For example, forced expression of *Ngn3* can initiate endocrine differentiation in early chick endoderm, however, it generates glucagon and somatostatin expressing cells and is insufficient to induce insulin expression in the early gut epithelium [130]. Notably, *Pdx1* expression was also insufficient to induce insulin transcription in this assay [130]. On the other hand, forced expression of *Ngn3* in the early pancreatic anlagen causes precocious differentiation of endocrine cells [136]. In the adult liver it can instruct hepatic progenitor cells to differentiate into pancreatic islet tissue [137]. In embryonic endoderm, *Ngn3* specifically regulates development of the endocrine lineages of the pancreas [131] in combination with components of the notch-signaling pathway [136], whereas its expression is used as a putative marker of islet precursor cells in the mature organ [138]. In the absence of *Ngn3*, there is a complete loss of the endocrine lineages of the pancreas and intestine, with a corresponding loss of expression of crucial genes downstream of *Ngn3* that regulate β-cell development, such as *NeuroD1*, *Isl1*, and *Pax4* [139]. In mice there is some evidence that *Ngn3* expression is maintained in adult β-cells, and that conditional loss of *Ngn3* can slightly impair β-cell function in the adult animal [140], although further studies are needed to determine the exact function of *Ngn3* in the adult pancreas [141, 142].

Similarly, recent data indicate that *NeuroD1* might also be important for the proper function of terminally differentiated β-cells, indicating that it might have a distinct function in later β-cell development [143]. In adult mouse liver, expression of *NeuroD1* in combination with *betacellulin* can induce formation of islet-like structures that contain cells resembling all of the major endocrine cells of the islet [144], indicating that *NeuroD1* alone does not specify β-cell fate, and is instead a general regulator of endocrine lineages in the pancreas. It is also interesting to note that both *NeuroD1* and *Ngn3* are also expressed during central nervous system development, suggesting that they are likely to interact with unique tissue specific co-factors during the development of these disparate lineages [145].

MafA is a basic-leucine zipper transcription factor that is specifically expressed in islet β-cells and directly activates insulin transcription by binding to a conserved cis-regulatory element in its promoter [146–148]. In contrast to *Ngn3* and *Pdx1*, *in ovo* electroporation of *MafA* into embryonic endoderm is sufficient for induction of insulin expression [130, 149]. Induction of insulin expression in a pancreatic α-cell line was also observed following transfection of *MafA* [150]. However, when *MafA* was prematurely expressed under the control of the *Pdx1* promoter in early pancreatic epithelium, it caused severe growth defects, possibly due to an increase in cell cycle exit in progenitors, and did not lead to a relative increase in β-cell specification [151]. Loss of *MafA* leads to a diabetic phenotype due to impaired glucose-stimulated insulin secretion, but does not impair the initial specification of β-cells, indicating that it is only essential for later stages of β-cell function [150]. Unlike *Pdx1* and *Ngn3*, its initial expression coincides with the terminal differentiation of β-cells at E13.5 [152, 153].

Major breakthroughs in attempts to ectopically induce insulin-producing cells were achieved when these master regulators of pancreatic development were combined. Of note, the combination of *MafA*, *Pdx1* and *NeuroD1* synergistically increased insulin expression in the liver *in vivo* and ameliorated glucose tolerance in a streptozotocin-induced mouse model of Diabetes mellitus [154]. Melton and colleagues utilized an unbiased, systematic approach to identify transcription factor combinations that could convert pancreatic exocrine cells (acinar cells) into insulin-producing β cells *in vivo* [36]. Of the 20 transcription factors that are expressed in pancreatic endocrine progenitors or mature β-cells [113], they focused on the 9 genes that exhibited defects in β-cell specification or differentiation when deleted in mice. As a proof of principle, all 9 factors were directly introduced into the adult pancreas with adenoviral vectors. One month after viral injection, insulin-expressing cells were observed at ectopic sites throughout the pancreas. After eliminating the genes that were not required for this effect, they found that the combination of *Pdx1*, *Ngn3 (or NeuroD1)*, and *MafA* was sufficient to convert exocrine cells into functional β-like cells with high efficiency (>20% of co-infected cells). The conversion process was rapid, with the first signs of insulin expression appearing after only 3 days; it could occur in the absence of cell division; and it appeared to be stable over a period of months in the absence of transgene expression. Importantly, the induced β-cells secreted insulin, recruited vasculature, and rescued streptozotocin-induced hyperglycemia in adult animals, demonstrating that the reprogrammed cells had attained functional properties. Lineage tracing analysis utilizing a mouse strain expressing CreERT2 under the control of a mature acinar cell specific promoter (*Cpa1*) confirmed that the induced β-cells were derived from terminally differentiated exocrine cells. Interestingly, attempts by the authors to reprogram skeletal muscle (*in vivo*) and mouse embryonic fibroblasts (*in vitro*) using these genes were unsuccessful.

It is noteworthy that many of these reprogramming factors function not only during the development (i.e. specification stages) of endocrine lineages but also in the continued maintenance of proper function in mature β-cells. Similar to the situation in the heart and hematopoietic system, this suggests that these genes act in a feed-forward manner to control the development of β-cells. This raises the question of the relative contribution of "specification" versus "terminal differentiation" functions of reprogramming factors. Furthermore, it will be interesting to determine whether factors that are normally involved in tissue specification (i.e. selector genes) are critical for establishing an appropriate chromatin context for reprogramming factors to be fully functional. This could be addressed if the specification and differentiation functions of *Pdx1* could be functionally separated, similar to the functional differences that underlie specification-competence in *MyoD/Myf5* compared to *myogenin* and *MRF4*. In this regard, it might be equally informative to pay careful attention to the genes that are known to be critical regulators of organ development but are not required to induce lineage conversion [36, 116]. How do these genes differ from those that are competent to induce lineage reprogramming? Genome-wide location studies may begin to shed light on this question by determining the degree of co-regulation of various sets of target genes throughout different stages of endoderm development. This would also allow for detailed studies of the cis-regulatory logic of individual sets of promoters and enhancers that are co-regulated by these genes (e.g. the insulin promoter).

Recently, two groups have demonstrated the conversion of mouse fibroblasts to hepatocyte-like cells (iHep cells) by forced expression of *Gata4*, *Hnf1α*, and *Foxa3* and inactivation of p19[Arf][31] or *Hnf4α* and *Foxa1*, *Foxa2* or *Foxa3* [155]. These iHep cells exhibited global transcriptional reprogramming, expressed mature hepatocyte-specific markers, engrafted into the adult liver and increased survival in a genetic model of liver failure. However, iHep cells exhibited important differences in gene expression compared to primary hepatocytes and could only partially rescue liver function in the transplantation model. It is also interesting to note that *Gata4* is one of the three factors used to convert fibroblasts to cardiomyocytes, and both *Gata4* and *Foxa3* are thought to be capable of acting as "pioneer" factors during liver development [80].

Inducing Neurons

Numerous studies have characterized the critical molecular pathways underlying the diversification of cell types in the developing nervous system (reviewed in [156–162]). The discovery and characterization of the proneural bHLH genes ignited significant interest in their roles during neural development, given their obvious similarity to the "master regulator" *MyoD* [145, 163, 164]. Comparisons to *MyoD* seemed justified, given reports that under certain circumstances, proneural bHLH genes could induce expression of neuronal markers in non-neural cells *in vivo* [165–169] and *in vitro* [170]. Of particular interest, ectopic expression of *Ngn1* in the dermomyotome *in vivo*, as well as in cultured chick embryonic fibroblasts, induced the expression of a variety of pan-neural markers and immature neuronal morphologies in these mesodermal cells [168]. However, there was no further validation that these cells had acquired any of the functional properties of neurons.

Ascl1 is a master regulator of neural development that controls neuronal versus glial specification from multipotential stem cells of the central and peripheral nervous systems [171, 172], balances progenitor self-renewal and neuronal differentiation by promoting intercellular notch signaling [173], and influences neuronal subtype specification processes in a context dependent manner [174–176]. In the mammalian forebrain, *Ascl1* is expressed primarily in the proliferative progenitors in the ventricular zone of the ventral telencephalon, where it promotes differentiation of GABAergic interneurons [174], whereas in the peripheral nervous system it is involved in the generation of autonomic neurons [177]. These data strongly suggest that proneural genes depend on specific cofactors to generate different types of neural cells in different contexts [178, 179]. Consistent with this idea, *Ascl1* knockout mice show dramatic defects in neural differentiation in the olfactory epithelium and the sympathetic, parasympathetic, and enteric ganglia [180]. *Ascl1* also appears to be important for the proper development of the oligodendrocyte lineage [181–184]. Forced expression of *Ascl1* in adult hippocampal progenitors in the dentate gyrus can promote oligodendrocyte differentiation at the expense of neuronal differentiation [185]. However, if these cells were first cultured *in vitro*, *Ascl1* expression resulted primarily in neuronal differentiation [185], again highlighting the context-specific function of this gene.

The potential for plasticity in neural cells *in vitro* became apparent following careful studies that elucidated the normal developmental potential of some of the numerous neural stem/precursor cells *in vivo* [186]. Kondo and Raff demonstrated that the well-characterized

oligodendrocyte/type 2 astrocyte (O2A) precursor cells could dedifferentiate to a multipotential neural stem cell state following sequential exposure to serum (or BMPs) and bFGF [187]. Building upon the discovery that radial glial cells in the developing rodent brain give rise to neurons [188, 189], Götz and colleagues attempted to determine whether more differentiated glial cells could also be instructed to differentiate into neurons. Indeed, in a candidate gene approach they found that *Pax6* could induce neuronal markers in postnatal astrocytes [40]. Follow-up studies indicated that *Ascl1, Ngn2, and Dlx2* could robustly convert neonatal astrocytes into neuronal cells that were capable of generating action potentials and forming functional synapses, although *Ascl1* alone was not capable of producing fully functional neurons [39, 190]. Time-lapse imaging studies showed that cell division occurred in some cases but was not a requirement for this neuronal conversion, providing strong evidence that the cells underwent a direct fate switch. Furthermore, genetic lineage tracing demonstrated that these induced neurons were derived from *GLAST* (L-glutamate/L-aspartate transporter) expressing astroglia [186]. However, given that radial glia and adult neural stem cells share essentially all known markers with astrocytes including *GLAST* [191] it is currently impossible to formally prove that terminally differentiated astrocytes were the source of induced neurons.

Consistent with their roles during development, these astrocyte-derived induced neuronal cells exhibited different neurotransmitter phenotypes depending on which genes were used for reprogramming. *Ngn2*-induced neurons from mouse and rat astrocytes exhibited markers and functional properties consistent with their acquisition of an excitatory, glutamatergic fate, whereas *Dlx2*-induced cells (with or without *Ascl1* expression) exhibited functional and molecular properties of GABAergic neurons [39, 192]. These results are consistent with the well-described role of *Dlx2* as an important determinant of neuronal fate in the ventral telencephalon downstream of *Ascl1*, with loss of *Dlx1/2* causing *Ascl1*-expressing interneuron/oligodendrocyte progenitors to develop exclusively as oligodendrocytes [184]. Thus, the reprogramming process seems to recapitulate some of the same general principles of normal neuronal development [145, 160, 161]. The fact that cell division is not required for this epigenetic reprogramming to occur suggests the possibility that transcription factors might also be capable of reprogramming one neuronal subtype into another [193]. In fact, there is accumulating evidence suggesting that this is possible *in vivo*, although it has yet to be shown with neurons at later stages of development [194–196].

In order to determine whether lineage-reprogramming might be possible between more distantly related lineages, we set out to determine whether forced expression of critical neural lineage-determining transcription factors would be sufficient to directly convert fibroblasts into neurons. Starting from a pool of 19 genes, we observed that transduction of *Ascl1* was sufficient to induce some neuronal traits in fibroblasts, such as expression of pan-neuronal proteins and immature active membrane properties [29]. We then added each of the remaining 18 genes one at a time to screen for additional factors that could induce expression of neuronal markers in combination with *Ascl1*. This ultimately led to the identification of a pool of three genes, *Ascl1*, *Brn2*, and *Myt1l,* that were sufficient to directly reprogram embryonic and postnatal fibroblasts into functional neurons that we termed induced neuronal (iN) cells. Importantly, these iN cells were capable of firing

repeated action potentials and forming functional synapses with mouse cortical neurons and with each other *in vitro*. Electrophysiological recordings from iN cells co-cultured with primary astrocytes showed that only excitatory postsynaptic potentials could be recorded, providing functional proof that a large majority, if not all, iN cells exhibited a glutamatergic phenotype. At earlier time points, rare cells expressing some markers of GABAergic differentiation, such as GABA and GAD67 were detected, which suggests that it might be possible to generate both functional glutamatergic and GABAergic neurons, and that our culture conditions are not optimized to promote the survival of GABAergic neurons.

Future studies will be necessary to more conclusively determine whether the glutamatergic phenotype represents a default state generated by *Ascl1*, *Brn2*, and *Myt1l* during the reprogramming process or instructed by the reprogramming factors. In light of these data, it is worth noting that *Brn2* is expressed during cortical development in progenitors that give rise to glutamatergic neurons in layers II/III and V [160, 197]. Loss of *Brn1* and *Brn2* disrupts migration of upper layer neurons in the cortex and disrupts proliferation of cortical progenitors in the VZ and SVZ after E14.5 [198, 199]. Given that *Ascl1* and *Myt1l* are both expressed in neurons with a variety of different neurotransmitter phenotypes, the putative role of *Brn2* in regulating differentiation of sub-populations of glutamatergic cortical neurons could explain the glutamatergic phenotype of iN cells generated by *Brn2*, *Ascl1*, and *Myt1l*. Further studies in the *Brn1/Brn2* knockout mice will be necessary to determine the importance of these genes for the specification of a glutamatergic neurotransmitter phenotype during cortical development [160]. In this regard, it will also be important to determine the expression patterns of additional cortical layer-specific subtype markers in iN cells. These studies should provide insight into future attempts to generate other specific types of neurons directly from fibroblasts. Surprisingly, the generation of iN cells was rapid, with the first neuronal markers detected as early as three days following the induction of the viral transgenes, and reached efficiencies of nearly 20% after two weeks. The reprogramming process could also occur in the absence of cell division, similar to lineage reprogramming in other cell types [36, 39] and is not dependent on continued expression of the exogenous reprogramming factors [30, 200]. While this work provides an important proof of concept that neurons can be derived from readily available dermal fibroblasts, the exact cell of origin has not been determined. Even though RT-PCR analysis detected expression of one neural crest marker in the tail tip cultures several observations argue against the possibility that the majority of iN cells are derived from infected neural crest cells: First, neural crest stem cells were not present in the cultures because even in permissive conditions no neurons were detected. Second, iN cells had a central nervous system phenotype. If neural crest cells were infected with the reprogramming factors one would expect the generation of peripheral neurons. Finally, it is highly unlikely that 20% of the cells in the cultures are neural crest-derived. To ultimately clarify this important question, future lineage-tracing studies will be critical to demonstrate that iN cells can be derived from mesodermal fibroblasts or from other defined somatic cell types. Gene expression patterns and chromatin modifications on a genome-wide scale will then determine whether the transcriptional program of the donor cell type is silenced. These data would allow for a more thorough evaluation of the fidelity of reprogramming.

More recently, multiple labs have found that iN cells can also be generated from human fibroblasts using these same three genes [30, 200]. The conversion of human fibroblasts to fully functional iN cells appears to be greatly aided by the addition of the bHLH transcription factor *NeuroD1*. The *NeuroD* family of transcription factors are bHLH proneural genes thought to function downstream of neurogenins and *Ascl1* during neuronal differentiation, somewhat analogous to *MyoD1* and *myogenin* in skeletal muscle [145, 165]. Interestingly, forced expression of *NeuroD1* and *Brn2* in human fibroblasts can efficiently generate *Tuj1*-postive neuronal cells, although their functional development appears delayed compared BAM+ *NeuroD1* iN cells. Given that *NeuroD1* can partially replace *Ngn3* during *in vivo* exocrine-to-endocrine reprogramming in the pancreas (see above), it will be interesting to perform more thorough structure-function studies of these factors to elucidate their shared and unique functions during reprogramming in the context of neuronal and pancreatic tissues. Two recent studies reported that the addition of transcription factors that are involved in dopaminergic neuronal development can generate iN cells from mouse and human fibroblasts that express tyrosine hydroxylase (TH), however, it is not clear whether these cells are mature enough to form synapses [200, 201]. Interestingly, each study used a distinct group of transcription factors for conversion (BAM plus *Lmx1a* and *Foxa2* vs. *Ascl1*, *Nurr1*, and *Lmx1a*).

In general, human iN cells appear to be less functionally mature compared to mouse, are generated at 3–5 fold lower efficiency and require longer periods of time to develop active membrane properties and to receive synapses from mouse cortical neurons. Further optimization of the transcription factor combinations used for reprogramming and the neuronal culture conditions may improve the generation and functional maturation of human iN cells.

Another critical area of future investigation will be to determine how *Brn2* and *Myt1l* collaborate with *Ascl1* to induce neuronal differentiation from fibroblasts. Interestingly, functional synergy between proneural bHLH proteins and *Myt* family proteins has been described during *Xenopus* neuronal differentiation and during endocrine cell differentiation in the mouse pancreas [167, 202]. *Myt1l* is a CCHC-domain containing zinc finger transcription factor that is expressed throughout the central and peripheral nervous systems in differentiating neurons as they become post-mitotic [203, 204], whereas the closely related *Myt1* is expressed in proliferating neural progenitors, oligodendrocyte progenitor cells, and differentiating neurons [205]. In Xenopus, forced expression of the proneural bHLH transcription factor *X-Ngnr1* is sufficient to induce ectopic neurogenesis throughout the ectoderm, even in the absence of neural induction [167]. This ectopic neurogenesis often occurred without cell division, thus providing strong evidence that these cells are generated as a result of a direct conversion of prospective epidermal and neural crest cells into neurons, analogous to neural lineage reprogramming studies in mammalian cells *in vitro*. *Xenopus Myt1* was required for ectopic neurogenesis induced by *X-Ngnr1,* and it appears to be a downstream target of *X-Ngnr1*. Whether this synergy is conserved during neuronal differentiation in mammals is currently unclear.

During pancreatic endocrine cell differentiation in mice, *Myt1* and *Ngn3* appear to form a feed-forward transcriptional circuit that helps to promote endocrine fate [202]. *Myt1* is

expressed in both the endocrine progenitors and terminally differentiated endocrine cells and appears to function primarily as a transcriptional activator in this context [206]. Thus, *Myt* family proteins appear to be critical and conserved regulators of proneural transcription factor activity during neuronal differentiation in vertebrates, however, further studies will be necessary to determine the exact functions of *Myt* proteins during mammalian neural development.

In mice, *Brn2* is broadly coexpressed with *Ascl1* in the ventricular and subventricular zones of the spinal cord and ventral telencephalon, where it appears to synergistically regulate transcription of the *Delta1* gene, as well as other critical regulators of notch signaling (e.g. *Dll1*, *Jag2*, *Lfng*), indicating that it might cooperate with *Ascl1* to regulate neuronal differentiation [207]. Similarly, in the chick neural tube, electroporation of *Brn2* causes neural progenitors to exit the cell cycle and differentiate into neurons [207]. Studies in *Xenopus* have also shown that *XIPOU2* (homologous to mammalian *Brn4)* is a downstream target of *noggin* during neural induction and is also sufficient to drive neuronal differentiation in uncommitted ectodermal tissue [208]. These studies should inform future efforts to understand the mechanism of action of these factors during the reprogramming process.

Common features of different somatic lineage conversions

When comparing the direct lineage conversion experiments published to date common themes emerge despite the differences in cell types involved. First, in almost all cases of lineage reprogramming, transcription factors were identified as reprogramming factors. It is conceivable that other classes of factors such as components of chromatin modifying complexes, microRNAs and other RNA species, signaling pathway modulators or small molecules may also be sufficient to induce lineage conversion (and some examples have indeed been demonstrated, see [41, 96, 209–213]. The predominance of transcription factors in reprogramming experiments should not be surprising, given that they are the primary effectors of lineage decisions during normal development.

Modulation of signaling pathways would be an attractive alternative to transcription factors because lineage conversion could be achieved without genetic modification. Given the limited number of signaling pathways compared to the vast number of somatic cell types it seems unlikely that the possible combinations of existing signaling pathways are sufficient to induce specific cell fates outside of the context of the developing embryo. Similarly, cellular responses to growth factors and other extracellular signaling molecules are limited by the repertoire of receptors present on the starting cell type. Furthermore, receptor exchange experiments suggested limitations of receptor specificities. For instance, lymphoid cell lines have been engineered to express the muscarinic acid receptor in place of the T cell receptor (TCR) [214]. Remarkably, the stimulation of the muscarinic acid receptor induced immune gene transcriptional responses. Similarly stimulation of PDGF and FGF receptors induces almost identical transcriptional responses in fibroblasts [215]. Thus, it is possible that modulation of signaling pathways is able to induce transdetermination (as described for the blood lineage [96]) but would be insufficient to induce conversion between more distantly related lineages. Specific signaling pathways ultimately act by altering the transcription of downstream genes, but these outcomes are highly context specific, as illustrated by the

repeated utilization of the same signaling pathways for diverse outcomes during different stages of development. Forced expression of lineage-specific transcription factors would thus be more likely to be sufficient to activate the necessary regulatory networks for generating distinct cellular fates *de novo* [216].

Another striking theme from these studies is that the lineage conversion often appears to take place without the generation of an intermediate progenitor cell type [29, 39, 42]. This conclusion is compatible with the notion that transcription factor-mediated lineage reprogramming is a direct phenotypic conversion between two distinct cell types and not a dedifferentiation process followed by differentiation to an alternative fate. This suggests that some lineage-determining transcription factors might be capable of modulating expression of their physiological targets independent of the pre-existing chromatin context, thus acting as "pioneer" factors during the reprogramming process [81].

Equally surprising is the fact that many direct cell fate conversion examples do not require cell division [29, 39, 107]. In dividing cells unmodified histones are incorporated into DNA during S-phase, perhaps making them more prone to epigenetic flexibility than postmitotic cells [217]. In line with this notion is the finding that cell proliferation is a critical component of iPS cell formation [218]. Modulation of cell cycle regulators such as *p53*, *p16* and *p19* also dramatically increases the efficiency of iPS cell reprogramming [219–223]. Nonetheless, mice have been cloned from the nuclei of post-mitotic olfactory neurons and heterokaryons between mouse ES cells and human fibroblasts exhibit rapid activation of pluripotency markers without undergoing cell division [8, 9, 224]. Finally, compared to iPS cell reprogramming somatic lineage conversions appear to be very rapid, with the first marker genes of the target lineage being expressed hours to days after gene induction [29, 42, 107]. This is consistent with the fact that no cell division is required and that the conversion efficiencies are at least an order of magnitude higher than iPS cell reprogramming [29, 107, 225, 226]. How the genome-wide chromatin remodeling that is presumably associated with somatic lineage conversion can be accomplished without cell division is completely obscure, and will likely be a critical area of future investigation.

Possible mechanisms and open questions

Perhaps one of the most fascinating questions arising from direct lineage conversion studies is how a handful of transcription factors can induce such dramatic genome-wide transcriptional and epigenetic changes (Fig. 3). It is clear that the transcriptional activity of a gene is dependent on both transcription factor binding and the chromatin configuration at the regulatory regions for that locus. Whereas the concept of gene regulation by transcription factors is well studied [227, 228], research over the last fifteen years has uncovered the importance of various chromatin modifications and remodeling factors [229, 230]. Some of these chromatin modifications, e.g. DNA methylation, are considered stable and heritable [231]. However, it appears that even DNA methylation can be more dynamic and flexible than previously appreciated [224, 232]. Some general rules appear to apply for lineage conversion: (i) the process is initiated with high levels of ectopic transcription factors, (ii) the activation of early lineage reporters is fast, (iii) it can occur without cell division, and (iv) the activation of markers of mature cells and acquisition of functional properties are

generally delayed. Thus, the most appealing model is that the ectopic transcription factors are upstream of the subsequent chromatin remodeling and act as "pioneer" transcription factors, i.e. factors that can activate target genes in a repressed chromatin state [81, 233] (Fig. 3a). We speculate that chromatin modifications are dynamic, fluctuating between the repressive and partially active configuration[229]. At silenced loci the majority of marks are repressive at any given time (Fig. 3b). When the reprogramming transcription factors (Fig. 3c) are introduced they may weakly bind to the subset of randomly accessible sites in a promoter (green in Fig. 3c). This initially weak interaction may then interfere with the random fluctuation and stabilize active configurations. With accumulating accessible sites more and more transcription factors could be recruited which would eventually lead to active transcription of that locus. Loci coding for transcription factors could further enhance activation of target genes by positive feed-back and feed-forward loops (Fig. 3d).

In some cases different combinations of transcription factors can drive the same cell fate conversion process (e.g. reference [29]). This suggests a model in which many (if not all) of the key upstream transcription factors regulate one another. Thus, rather than one "master transcription factor" there seems to be a group of "master" transcription factors that can be induced by various subsets of the component genes. Once activated, they would regulate each other in a self-organizing network and execute the downstream lineage differentiation program, resulting in the concerted induction of the various structural proteins required for the function of the particular differentiated cell type [77, 216, 234, 235]. The closer the reprogramming factor combination is to this master regulatory network, the more efficient and accurate the reprogramming would be, although smaller subsets are often sufficient to initiate reprogramming [42]. Once the endogenous self-maintaining network is activated the exogenous transcription factors should become dispensable, which would explain why the reprogrammed state tends to be stable in the absence of the exogenous reprogramming factors.

Somewhat puzzling is the observation that many genes characteristic of the donor cell type are rapidly downregulated, even before the target program is activated [107, 236, 237]. Similarly, the fact that the same transcription factors can induce one cell type from many different donor cell types and thus downregulate many different host cell transcriptional programs seems counterintuitive [37, 226, 238, 239]. A potential explanation for this phenomenon is that the high levels of exogenous transcription factors simply saturate the transcriptional and translational machinery of the cell and thus actively outcompete the original transcriptional program. A similar mechanism has been proposed to regulate the switch between neurogenesis and gliogenesis from multipotent neural stems, where the bHLH transcription factor *neurogenin1* interacts with *p300* and sequesters it away from the promoters of genes important for gliogenesis [240]. Alternatively, there may be a general binary switch once a novel cell fate is induced that causes the pre-existing transcriptional program to collapse.

Surprisingly, temporally regulated expression of the reprogramming factors does not need to precisely mimic the temporal expression patterns of the corresponding endogenous factors. This suggests that these transcriptional networks are highly robust, resulting in the same outcome despite different levels and timing of expression of their component genes [241].

Furthermore, constitutive expression does not appear to significantly affect the induction and maintenance of the endogenous transcriptional networks, suggesting that post-transcriptional regulation, such as microRNA-mediated degradation or post-translational modifications of the transcription factors within the network is involved.

Finally, it remains to be seen whether in direct somatic lineage conversions the reprogramming is truly complete, or whether any epigenetic memory of the previous cell fate remains, as has been seen with nuclear transfer [242, 243] and induced pluripotent stem cells [244, 245]. It will also be interesting to compare induced cell types originating from different donor cell types and determine their impact on reprogramming efficiency and fidelity (see Box 1 for a summary of important unresolved issues in the field).

Box 1

Broad questions in direct lineage conversion experiments

- How can transcription factors find their relevant binding sites in a cell type that has a different pattern of chromatin modifications than it is used to encountering?

- How do transcription factors downregulate the transcriptional program of the starting cell?

- To what extent does lineage reprogramming mimic normal terminal differentiation processes and create a novel cell type that faithfully replicates a normal counterpart? Can transcription factors mimic the various patterning cues that govern development?

- Do different types of induced somatic cells maintain epigenetic memory from their previous state? What effect does the starting cell type have on the phenotype of the reprogrammed cell? How similar are directly converted cells to ES or iPS-derived somatic cells *in vitro*?

- Why is direct reprogramming more difficult with human cells compared to mouse cells? What are the molecular mechanisms that underlie these differences?

- Are lineage-reprogramming approaches applicable to all cell types? Will it be possible to generate progenitor cells from a variety of tissue types by direct conversion?

- How can reprogramming occur without cell division? Is this a reflection of some special property of the reprogramming factors? Or is it evidence of previously underappreciated mechanisms of chromatin remodeling?

- Can lineage reprogramming be used to study developmental processes in non-traditional model organisms for which other tools (such as ES cells) are not available?

Potential applications for basic research and medicine

The examples of direct reprogramming described above may very well also become important tools for both basic biology and regenerative medicine. These applications fall into 3 principal categories: (i) Utilization of induced cell types to study basic mechanisms of transcription factor action, chromatin remodeling processes, and lineage determination; (ii) Efficient access to human tissue not otherwise accessible for drug testing and disease modeling; (iii) Use of induced cell types for therapeutic cell transplantation. In principle these applications are very similar to iPS cell-based approaches which have been reviewed extensively before (see e.g. [246–248]) but certain advantages and disadvantages compared to direct cell type induction do exist that are often specific to the respective cell type (see Table 1 for a general comparison). In the following we will discuss various aspects within those three classes of applications and compare direct lineage conversion to iPS cell-based approaches.

In contrast to ES/iPS cell differentiation driven by signaling molecules and growth factors, direct lineage conversion provides a new arena to study the activity of transcription factors. The classic approach to study gene function is by introduction of mutations and gain-, or loss-of-function experiments. Typically these perturbations are analyzed in a physiological context such as the phenotypic analysis of a knockout mouse strain or the ectopic expression of gene products in related compartments (e.g. in various regions of the developing neural tube [249–251]). Transcription factor-mediated direct lineage conversion allows the study of a transcription factor outside its normal and physiological context. For example, based on the current literature it was somewhat unexpected that iN cells generated by transcription factor combinations containing *Ascl1* generated exclusively excitatory neuronal subtypes [29]. *Ascl1* is highly associated with the generation of inhibitory neurons and can cause expression of ventral markers when expressed in the dorsal telencephalon [176]. Moreover, a large number of transcription factors can be tested in different cellular contexts, which may facilitate the characterization of common transcription factor networks or the identification of novel mechanisms for restricting the activity of a transcription factor to a specific context [252, 253].

This approach has already been elegantly applied to investigate the context specific activity of ectopically expressed transcription factors in *C. elegans* [210]. By generating worms that express the sensory neuron specific transcription factor *CHE-1* in every tissue, the authors were able to perform an RNAi screen of all known chromatin-associated proteins to find genes that are important for limiting the activity of *CHE-1* to sensory neurons. They found that *lin-53* knockdown allowed *CHE-1* (and other neuronal subtype-specific transcription factors) to convert mitotic germ cells into neurons *in vivo. lin-53 (Rbap46/48* in humans) is a histone chaperone that plays a role in numerous chromatin remodeling and silencing complexes. This effect could be phenocopied by treating the worms with the histone deacetylase inhibitors valproic acid and trichostatin. Furthermore, the authors provided preliminary evidence that the effect was specific to the neuronal transcription factors tested, as *lin-53* loss did not promote conversion of mitotic germ cells to myocytes following forced expression of the *C. elegans MyoD* homologue *hlh-1*. The use of model organisms to perform *in vivo* shRNA screens should continue to provide significant insight into the

chromatin regulatory processes that permit and prevent transcription factors from being active in diverse cellular contexts.

Along with differentiation of specific cell types from pluripotent stem cells, direct lineage conversion provides a simplified tool for studying developmental processes *in vitro* [254–256]. These accessible culture systems can be used to search for novel cell fate determinants by candidate gene approaches and with unbiased genomic screens. While ES/iPS cell differentiation is preferable for studying early developmental processes (such as neural induction), the strength of direct lineage reprogramming approach may lie in studying terminal differentiation and maturation, as well as the acquisition of functional properties, processes which are relatively poorly understood, especially in the nervous system. The combination of these two approaches provides a powerful toolkit for studying the development of a variety of cell types *in vitro*. The use of human cells should also allow the study of human developmental processes that may exhibit important differences to rodents [30, 256, 257]. One potential limitation of this approach is that it is unclear how similar these reprogramming processes are to the normal development of the a particular cell type of interest in vitro, thus it will be critical to evaluate specific genes of interest in both contexts to determine their physiological relevance.

Perhaps the most exciting future application of these novel "tools" is the possibility of increased experimental accessibility to human cell types in culture. However, only a few examples of direct reprogramming of human somatic cells have been reported, although many are likely forthcoming in the near future [30, 43, 200]. For example, the major reason for our limited understanding of many human brain disorders is arguably the lack of human neurons suitable for experimentation. The development of iPS cell technologies has provided a first method to generate patient-derived cells, but certain limitations exist that may be circumvented by utilizing lineage reprogramming approaches (summarized in Table 1). While iPS cells have the clear advantage of unlimited growth, thus making them amenable for use in high-throughput assays, it is technically very challenging to produce iPS cell lines from numerous individuals. Unless a proliferative intermediate can be induced, direct lineage converted cells would also need to be scaled up as fibroblasts (or other cells types) before conversion. However, since reprogramming is fast and efficient the screening of dozens or even hundreds of individuals may become feasible as methods for lineage reprogramming improve.

Despite major recent breakthroughs the differentiation of human ES/iPS cells is highly variable, ES/iPS cell-line dependent, and generates immature (or in some cases fetal-specific) cells that do not mimic those found in mature organs *in vivo* [258]. Regarding the neural lineage, current protocols often do not produce homogenous neuronal subtypes and differentiated neurons tend to be functionally immature [259–262]. Lineage reprogramming may offer the possibility to produce more homogenous cell populations at least with regard to reproducibility and subtype generation [29], although a direct comparison of ES/iPS-derived cells and cells generated by direct conversion has not yet been performed.

Despite these challenges several recent studies reported the successful recapitulation of disease phenotypes in differentiated iPS cells from patients with monogenetic diseases.

Studer and colleagues generated iPS cells from patients with familial dysautonomia, a rare disorder most frequently caused by a point mutation in the IKBKAP gene that results in peripheral and autonomic neuronal degeneration [263]. After differentiation into neural crest progenitor cells, patient-derived cells exhibited decreased levels of *IKBKAP* transcripts as well as migration defects, providing a possible explanation for the pathogenesis of the disease and a platform for the identification of disease-modifying agents. Recently, Gage and colleagues generated iPS cells from patients with Rett Syndrome and schizophrenia [264, 265]. Rett Syndrome is caused by mutations in the X-linked *MECP2* gene and is a neuro-developmental disorder affecting female children. In both cases patient iPS cell-derived neurons exhibited several differences in synaptic function compared to neurons derived from wild type iPS cells. The schizophrenia study is particularly exciting given it is a complex genetic disorder. Furthermore, iPS cell-based models of human neurodegenerative disorders such as genetic Parkinson's disease are also being developed [266].

Cardiovascular disorders have also been modeled *in vitro* using patient-derived iPS cells. LEOPARD Syndrome is caused by dominant mutations in genes involved in *ras* signaling and is characterized by various malformations including hypertrophic cardiomyopathy [267]. LEOPARD patient-derived cardiomyocytes derived from iPS cells exhibited various signaling abnormalities and were larger when compared to control cells from an unaffected sibling. Another study generated iPS cells from patients with mutations in L-type Ca^{2+} channels causing Long QT Syndrome [268]. iPS cell-derived ventricular cardiomyocytes showed several electrophysiological abnormalities that could explain the pathological cardiac conductive system.

Finally, induced somatic cells could be used for autologous therapeutic cell transplantation. Again, iPS cells offer the advantage of scalability but are known for their ability to form teratomas when not properly differentiated. Directly induced somatic cells on the other hand would bypass the pluripotent state and thus would presumably be less tumorigenic, provided integration-free gene delivery methods are applied [269, 270]. Another interesting potential application of direct lineage reprogramming would be the use of the reprogramming factors directly *in vivo* e.g. [33]. Although limited by the well-known complications associated with *in vivo* gene delivery, this approach would eliminate the lengthy process of culturing explanted cells for lineage conversion. Such approaches may be interesting to explore for myocardial infarction, Diabetes and neurodegenerative disorders such as Parkinson's disease.

Acknowledgments

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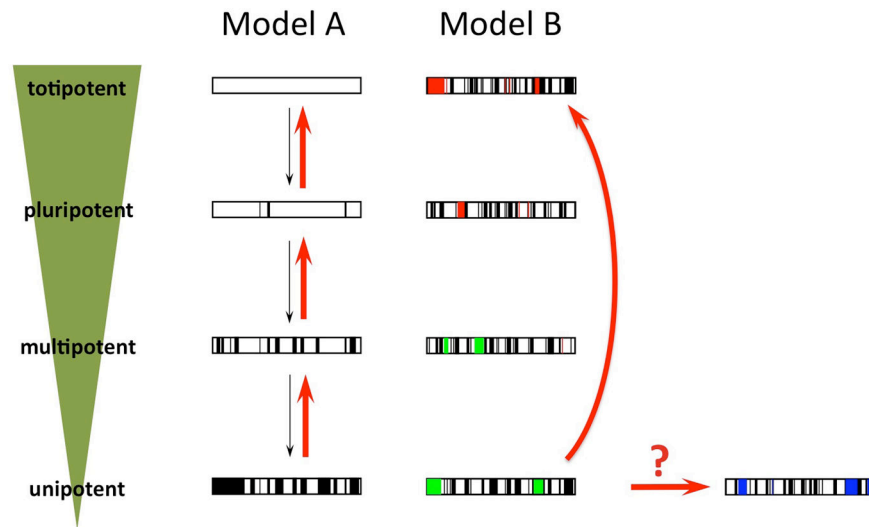
Figure 1. Epigenetic models of development and reprogramming
During development, cells are gradually restricted in their developmental potential (left). It is believed that this irreversible restriction is accompanied and caused by the progressive acquisition of epigenetic modifications (symbolized as accumulating black stripes in bars) that help to stabilize cell fate decisions and to restrict the adoption of inappropriate fates. The pluripotent state is characterized by a highly "open" chromatin configuration, which is thought to permit differentiation to a variety of cell types. In one model (Model A) reprogramming to a pluripotent state (by nuclear transfer, cell fusion, or by defined transcription factors) could occur by the stepwise erasure of epigenetic marks associated with differentiation (red arrows), which would allow cells to regain the open chromatin state and, by default, pluripotency. Alternatively, (Model B) the pluripotent state can be thought of as a defined and actively regulated epigenetic state rather than an epigenetically erased space. This model would suggest that the reprogramming factors actively establish the pluripotent chromatin state (red arrow), and that reprogramming represents an acquisition of pluripotent characteristics (red bars) rather than a loss of epigenetic lineage-restriction (green bars). This model suggests that inducing pluripotency should not be considered fundamentally different than inducing other defined cell types, and that it should be possible to convert one differentiated cell type into another (blue bars) with the right combination of factors.
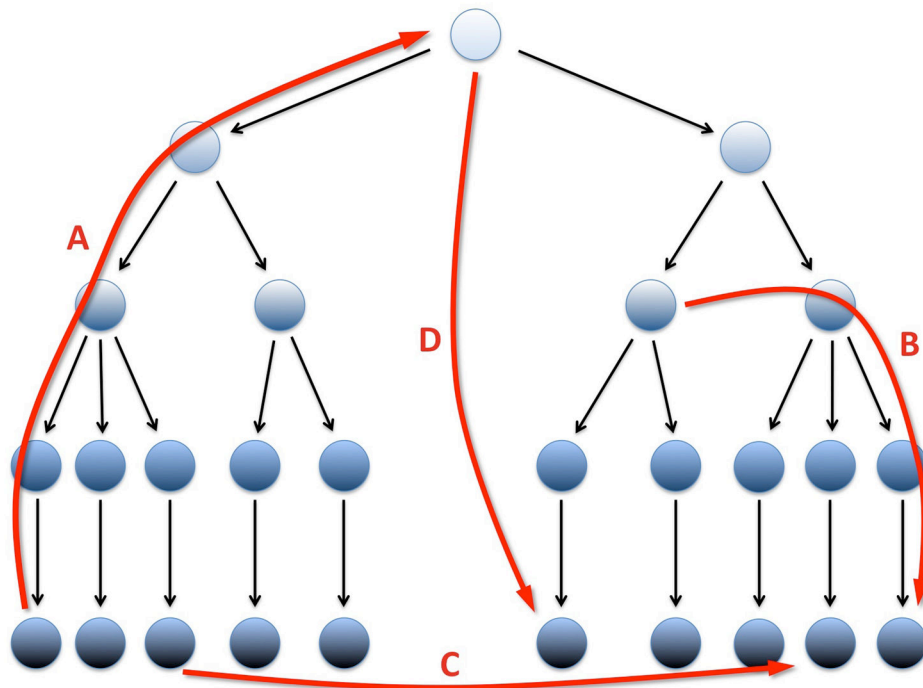
Figure 2. Various modes of induced cell fate changes

a) Dedifferentiation: reversion to a less differentiated state. Typical examples include iPS cell reprogramming and loss of *Pax5* in mature B cells

b) Transdetermination: conversion between two closely related progenitor cells that share a direct common progenitor. Also, most gain-of-function experiments interrogating lineage-determining factors during embryonic development fall into this category.

c) Transdifferentiation: direct fate switch between two distinct cell types. Examples include lineage conversion of mature hematopoietic cell types, exocrine to endocrine pancreatic cells, or the conversion of fibroblasts into cardiac cells, skeletal myocytes, neurons, or hepatocytes.

d) Directly induced differentiation: direct conversion studies suggest that it might be possible to directly induce a more differentiated cell type without passing through the corresponding intermediate progenitor state. For example, *MyoD1* expression in human ES cells rapidly generates multinucleated myotubes [270]. Similarly, forced expression of iN cell reprogramming factors in pluripotent human cells rapidly generates neurons [30, 170]
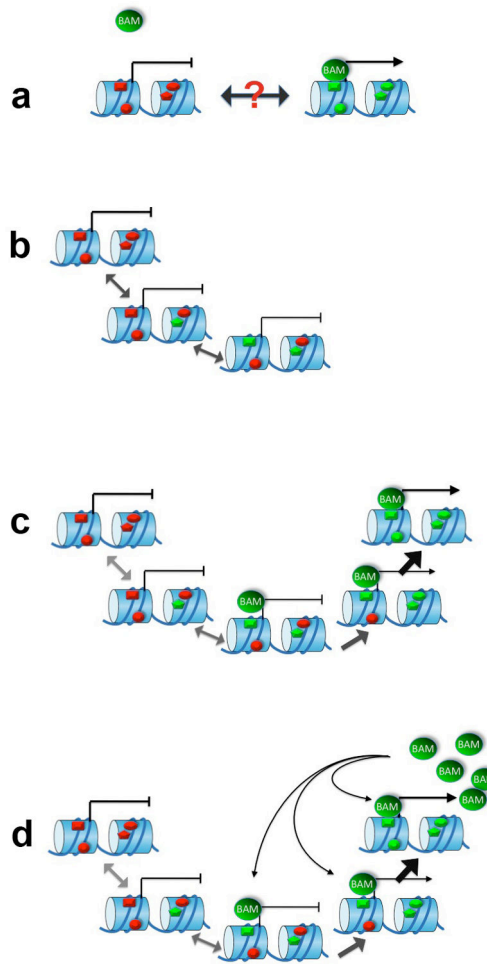
Figure 3. Potential mechanism of the stepwise activation of silent genes by reprogramming factors

a) How can a transcription factor or a combination of transcription factors modify gene expression at epigenetically silenced loci? The various repressive marks are symbolized with red objects of different shapes (left) on DNA (blue line) or nucleosomes (blue cylinders). Green objects represent active chromatin marks (right). Dark green circles represent lineage reprogramming factors (TFs) that promote gene transcription in a permissive chromatin state.

b) The repressed chromatin state is likely to be more dynamic than classically assumed. Epigenetic marks may stochastically fluctuate between active and repressive states with the majority of marks being repressed at any given time point. During cell division there is also a potential window for epigenetic plasticity as unmodified histones are incorporated into duplicated strands of DNA.

c) The stochastic loss of repressive modifications or the sliding/displacement of nucleosomes may allow transcription factors to weakly bind DNA and access their cognate binding sites. This interaction may interfere with the stochastic fluctuation, and can potentially recruit additional coactivators and/or histone modifying enzymes to this

regulatory region, thus stabilizing transcription factor binding and eventually leading to transcriptional activation.

d) The newly activated genes may code for endogenous reprogramming factors or other transcription factors that could promote activation of the novel transcriptional program via a positive feedback loop. This would activate a self-maintaining transcriptional program and the exogenous reprogramming factors would no longer required to maintain the new cell lineage identity.

Table 1

iPS cell reprogramming versus direct conversion

	iPS reprogramming	lineage conversion
cell division	required	(when tested) not required
reprogramming dynamics	slow	fast
reprogramming efficiency	low	high
potential tumor risk	high	low
target cell generation	2 steps[*]	1 step
cell scaling	feasible	limited
Screening numerous individuals	laborious	feasible

[*] reprogramming followed by targeted differentiation

[**] very much dependent of respective differentiation protocol and lineage.