REVIEWS

Leveling Waddington: the emergence of direct programming and the loss of cell fate hierarchies

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Abstract | For decades, Waddington's concept of the 'epigenetic landscape' has served as an educative hierarchical model to illustrate the progressive restriction of cell differentiation potential during normal development. While still being highly valuable in the context of normal development, the Waddington model falls short of accommodating recent breakthroughs in cell programming. The advent of induced pluripotent stem (iPS) cells and advances in direct cell fate conversion (also known as transdifferentiation) suggest that somatic and pluripotent cell fates can be interconverted without transiting through distinct hierarchies. We propose a non-hierarchical 'epigenetic disc' model to explain such cell fate transitions, which provides an alternative landscape for modelling cell programming and reprogramming.

During development, uncommitted stem cells differentiate into various tissue-specific cell types. This differentiation process is established and maintained by complex transcriptional and epigenetic networks[1,2]. Traditionally, lineage commitment and differentiation have been considered to be unidirectional and irreversible. The classic view of cell fate hierarchy is that the undifferentiated, pluripotent state resides 'above' the different committed and differentiated somatic states. Conrad Hal Waddington illustrated this classic concept of lineage specification in an 'epigenetic landscape' model[3]. In his model, a cell moving towards terminal differentiation is represented as a marble rolling down a landscape segregating into different groves on the slope, thereby determining its final fate (FIG. 1a). Thus, Waddington's model intuitively describes the natural restriction of cell differentiation potential during normal development.

Somatic cell nuclear transfer into enucleated oocytes and cell fusion of pluripotent cells with differentiated cells have, however, shown that the somatic epigenome can be reprogrammed to pluripotency[4–7]. More recent studies were able to generate induced pluripotent stem (iPS) cells by transcription factor-based reprogramming[8]. These experiments clearly demonstrated that the route from a pluripotent stem cell to a terminally differentiated somatic fate is not unidirectional but can be reverted using appropriate master transcription factors that impose pluripotency. Considering that iPS cells can be generated by reprogramming different types of somatic cells and that these iPS cells can then be differentiated into virtually any other somatic cell type, the pluripotent state can be regarded as a 'hub' that interconnects different lineage routes at the top of the Waddington landscape model (FIG. 1b). Moreover, such reversibility of somatic differentiation has questioned whether this hub is essential for the transiting from one cell fate or germ layer to another.

Whether cell fates are interconvertible had already been addressed decades ago outside the context of pluripotency. Transdifferentiation induced by the exogenous expression of a transcription factor was shown for the first time in 1987 by Lassar and colleagues, who demonstrated that the transcription factor myoblast determination (MyoD) can induce muscle-specific properties in fibroblasts[9]. Later studies provided additional evidence for successful cell fate conversion between related lineages within the same germ layer (FIG. 1c). However, whether cells of one germ layer can be converted into cell types that belong to another germ layer remained to be investigated. It was not until 2010 that Wernig and colleagues provided proof-of-concept for such 'long-distance' conversions, demonstrating that fibroblasts can be converted into functional neurons with a cocktail of three transcription factors[10] (FIG. 1c). As previously shown for iPS cells, it became clear that transient expression of such programming factors is sufficient to trigger and consolidate a new cell type-specific gene expression programme, which allows cells to retain their newly acquired phenotype even after discontinuation of exogenous transcription factor expression[8,11].

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doi:10.1038/nrm3543
Published online
13 March 2013

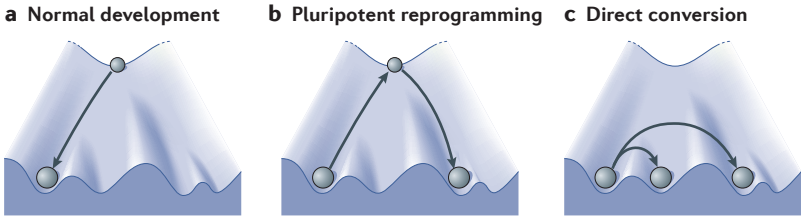


Figure 1 | **Challenging Waddington's epigenetic landscape model.** Schematic illustration of Waddington's epigenetic landscape model. **a** | In normal development, a pluripotent cell (which is represented by a marble at the top of the hill) 'rolls' down a landscape that segregates into different groves on the slope. Depending on in which groove the marble falls, the cell acquires a distinct tissue-specific fate. **b** | A differentiated cell, which is reprogrammed to pluripotency, is symbolized by a marble rolling from the bottom of the hill back to the top. From there, it can be redifferentiated into another somatic cell type. **c** | During direct conversion, a tissue-specific cell directly converts into a related tissue-specific cell (symbolized by a marble 'jumping' over a low hill) or into a cell type of another germ layer (depicted by a marble jumping over a higher hill). The schematics are based on the original model presented in REF. 3.

In this Review, we provide an overview of the current state of the art in somatic cell fate conversion, both within the same germ layer and across different germ layers, and discuss its potential biomedical applications. Because of the unexpected convertibility of pluripotent and differentiated somatic cell fates, we propose a new 'epigenetic disc' model, representing a flat landscape in which the pluripotent stage is merely regarded as 'primus inter pares' among other cell fates. In this model, conversion of cell fates can be achieved by tilting the disc in various directions, with transcription factors serving as 'rails' for precise guiding towards a distinct cell fate.

Intra-germ layer cell fate conversion

The idea that cells from different somatic fates are interconvertible reaches back more than 30 years, when researchers experimentally induced distinct cellular identities from fibroblasts using chromatin remodelling agents. Soon, the role of transcription factors, the DNA-binding proteins responsible for the induction and stabilization of gene expression networks, became evident. By overexpressing such transcription factors, cells from mesodermal, ectodermal and endodermal lineages were successfully converted into distinct somatic cell types of related lineages within the same germ layer. Recently, increasing knowledge of the molecular mechanisms underlying direct lineage conversion (also known as transdifferentiation) has made it possible to perform lineage conversions *in vivo* to directly induce differentiation into biomedically relevant cell types that are affected by common diseases such as diabetes mellitus and myocardial infarction (FIG. 2).

Within the mesoderm: from fibroblasts to myoblasts. Knowledge of experimental conditions that can switch a somatic cell type into another has been accumulating gradually since the 1980s, when it was observed that treatment of immortalized mouse fibroblasts with the demethylating agent 5-azacytidine (5-azaC) induces myogenic, chondrogenic and adipogenic differentiation[12]. This suggested that 5-azaC-treated fibroblasts that turn into muscle cells might express one or more genes capable of inducing myogenic differentiation. Indeed, transfection of genomic DNA from such induced muscle cells into fibroblasts proved to be sufficient to induce muscle formation[13]. Finally, the muscle-inducing helix–loop–helix transcription factor MyoD was identified and cloned the following year[9]. MyoD turned out to be a strong inducer of myogenic genes not only in this particular cell line but also in various other cell types[14,15]. However, whereas most mesodermal starting populations seemed to complete a full phenotypic switch, expression of MyoD in endodermal and ectodermal cells resulted in cellular phenotypes with atypical morphology and residual expression of markers found in the starting population[14,16]. These initial experiments impressively demonstrated how transcription factors can switch the fate of a differentiated somatic cell into another phenotype, although cell fate conversion on the basis of the expression of a single transcription factor remains an exceptional phenomenon.

Within the mesoderm: conversions within the blood lineage. Additional insights into the instructive roles of transcriptions factors in lineage specification came from studies in the haematopoietic system, which probably is the best-defined cellular differentiation system in mammals[17,18]. Two transcription factors, GATA1 and PU.1 (also known as SPI1), were shown to modulate early lineage decisions of haematopoietic progenitors towards either an erythrocyte and megakaryocyte or a granulocyte and moncyte fate[19,20] through the direct regulation of basic gene expression programmes in these lineages[21]. Lineage conversion experiments showed that forced expression of GATA1 was sufficient to induce erythroid, eosinophil and megakaryocytic markers and downregulate monocytic markers in monocytic cell lines[22,23]. In freshly isolated granulocyte and macrophage progenitors, GATA1 promoted differentiation into erythroid, eosinophil, basophil and megakaryocytic lineages[24]. Forced expression of PU.1 in megakaryocytic and erythrocyte precursors has also been shown to convert these cells into myeloblasts[25].

Another impressive example of a lineage switch in the haematopoietic system is the conversion of fully committed B cell and T cell progenitors into functional macrophages through the ectopic expression of the basic Leu zipper transcription factor C/EBPα (CCAAT/enhancer binding protein-α), the activity of which is required for the formation of granulocyte and macrophage precursors[26,27]. Insights into the transcriptional mechanisms that facilitate direct conversion of B cells into macrophages were provided by experiments in which a drug-inducible C/EBPα–ER (oestrogen receptor) fusion protein was expressed in a B cell line. This system allowed monitoring of gene expression changes and morphological alterations in living cells. A stable macrophage phenotype could be observed as soon as 48 hours after induction, and most gene expression changes were independent of protein synthesis, which indicates that they were caused directly

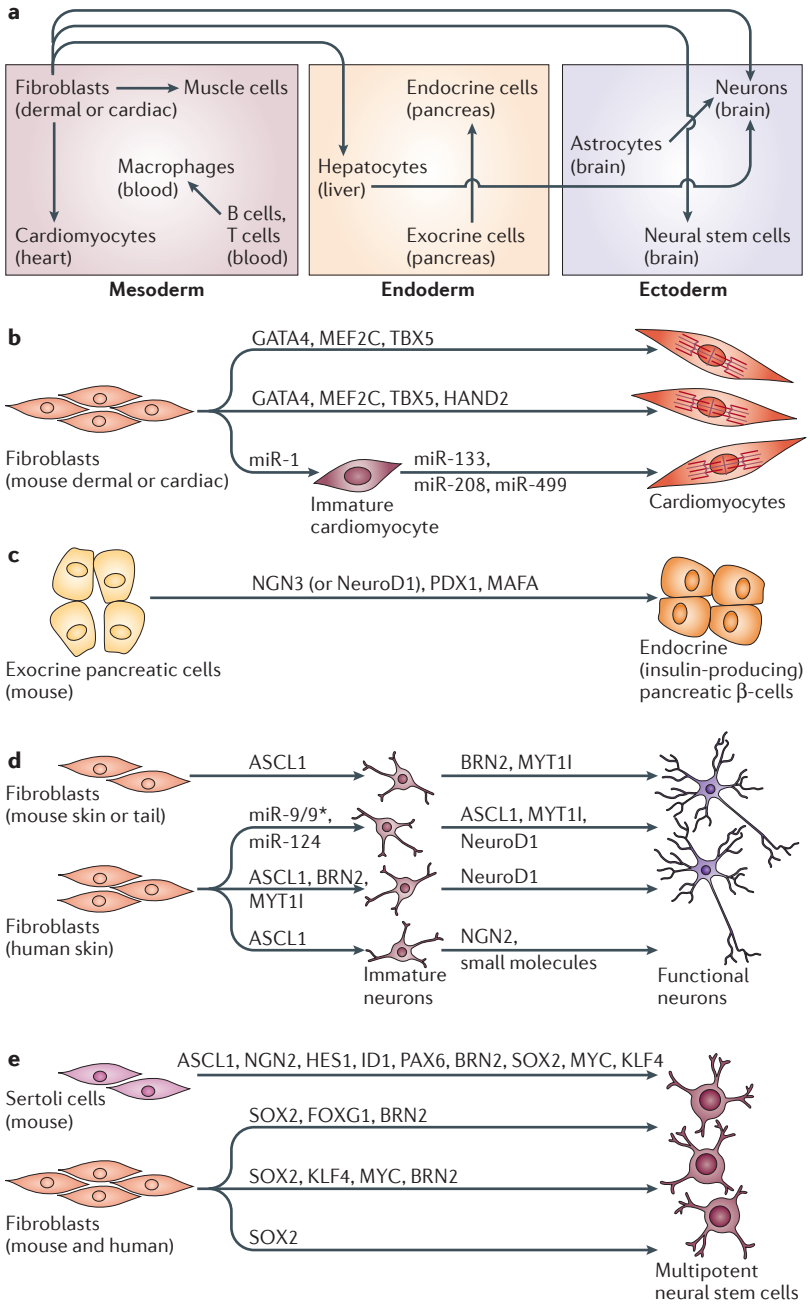


Figure 2 | **Direct conversion of somatic cells.** **a** | The schematic illustrates successful intra- and trans-germ layer conversion approaches. **b** | Transdifferentiation within the mesoderm. Different combinations of transcription factors or microRNAs (miRNAs) have the potential to convert fibroblasts into immature or fully mature cardiomyocytes. **c** | Transdifferentiation within the endoderm. A combination of three transcription factors can convert exocrine pancreatic cells into endocrine insulin-producing pancreatic β-cells. **d** | Transdifferentiation across different germ layers from the mesoderm to the ectoderm. Induced neurons can be generated by different sets of transcription factors or miRNAs. Additional factors increase the maturity of the generated neurons. **e** | Direct induction of proliferating neural progenitors. Different combinations of transcription factors can convert starting mesenchymal cell populations into neural progenitors, which can be further differentiated into neurons and glia. ASCL1, achaete-scute complex homologue 1; BRN2, brain-specific homeobox and POU domain 2; FOXG1, forkhead box G1; HAND2 heart and neural crest derivatives expressed 2; HES1, hairy and enhancer of split 1; ID1, inhibitor of DNA binding 1; KLF4, Krüppel-like factor 4; MEF2C, myocyte-specific enhancer factor 2C; MYT1L, myelin transcription factor 1-like; NeuroD, neurogenic differentiation factor 2; NGN, neurogenin; PAX6, paired box 6; PDX1, pancreas and duodenum homeobox 1; TBX5, T-box 5.

by the activation of C/EBPα[28]. Another study demonstrated that expression of C/EBPα in isolated lymphoid progenitors leads to the generation of granulocytes or macrophages, whereas expression of the transcription factor GATA2 gives rise to mast cells[29]. Interestingly, the order of expression of the individual factors determined the lineage outcome; expression of C/EBPα followed by GATA2 led to eosinophils, whereas the inverse sequential expression resulted in basophils. Further work demonstrated that the combination of C/EBPα and PU.1 is sufficient to induce macrophage-like cells from primary and NIH3T3 fibroblasts[26,30]. Notably, these induced macrophages were dependent on persistent transgene expression. Additional mechanistic studies indicated that PU.1 and C/EBPα bind to macrophage-specific transcriptional enhancer elements in fibroblasts and synergistically facilitate their activation, thereby promoting genome-wide macrophage-specific transcriptional programmes[31]. These studies also demonstrated that the same transcription factor can induce different cell fates depending on the starting population and on the additional transcription factors used for transdifferentiation.

Within the mesoderm: conversion into cardiac muscle cells. Cardiac muscle tissue has limited regenerative capacity, although recent evidence suggests that cardiomyocytes in the adult heart can divide and contribute to cell regeneration[32]. The conversion of mouse cardiac and dermal fibroblasts into cardiomyocyte-like cells was first achieved by the forced expression of GATA4, myocyte-specific enhancer factor 2C (MEF2C) and T-box 5 (TBX5)[33] (FIG. 2b). Factors specifically expressed in the developing mouse heart were identified using microarray analysis, and this led to initial experiments in which a pool of 14 factors was screened, and this set was then further refined to the GATA4, MEF2C and TBX5 triplet. Cardiomyocyte-like cells induced with this transcription factor combination exhibited a gene expression profile similar to that of primary cardiomyocytes. Using the same cocktail, it was also possible to directly convert *in vivo* resident cardiac fibroblasts into functional cardiomyocytes[34]. The induced cells exhibited cardiomyocyte-like properties such as action potential generation and electrical coupling and yielded a slight improvement in a model of cardiac dysfunction. In a parallel study, a combination of GATA4, MEF2C, TBX5 and HAND2 (heart and neural crest derivatives expressed 2) was shown to be sufficient to induce cardiomyocyte-like cells *in vivo*[35], and the converted cells contributed to improved cardiac function and enhanced regeneration in a mouse model of cardiac ischemia (FIG. 2b). Interestingly, the chosen factors are not only core transcription factors during early heart development but have also cross- and autoregulatory competence (that is, the transcriptional programme is stabilized by the same initiating factors)[36,37]. Moreover, GATA4 can function as a pioneer transcription factor as it is able to bind to its target sites in the genome even when they are occupied by nucleosomes or packed into heterochromatin[38].

Importantly, other factors in addition to transcription factors have the potential to initiate cell fate conversion. microRNAs (miRNAs) alone can mediate conversion of fibroblasts into cardiomyocytes both *in vitro* and *in vivo*[39]. Six miRNAs known to be involved in cardiac development have been tested, and it was shown that miR-1 alone can convert mouse cardiac fibroblasts into cardiomyocytes. Furthermore, combined overexpression of miR-1, miR-133, miR-208 and miR-499 resulted in more mature cardiomyocytes (FIG. 2b). In contrast to transcription factors, which are often known as transcriptional activators, miRNAs usually exert their function through the direct repression of multiple genes. Thus, it is likely that miRNAs target a specific set of genes that need to be inhibited to achieve or improve lineage conversion. It is also conceivable that miRNAs contribute to cell fate conversion by promoting chromatin remodelling[40].

Cell fate conversion within the endoderm. Several developmental studies in pancreatic lineage specification have indicated that the transcription factors neurogenin 3 (NGN3), neurogenic differentiation factor 1 (NeuroD1), pancreas and duodenum homeobox 1 (PDX1) and MAFA have a role in regulating cell fate determination[41–44]. PDX1, which is one of the first markers indicating pancreatic differentiation[45], was shown to induce the expression of insulin and additional pancreatic genes in various endoderm-derived cells such as adult mouse liver, cultured human liver cells and chick embryonic endoderm[46–49]. Forced expression of the proneural helix–loop–helix transcription factor NGN3 led to endocrine differentiation in early chick endoderm and precocious differentiation of endocrine cells in the early pancreatic anlage[47]. Expression of NGN3 also induced the conversion of hepatic progenitors into islet cells but failed to achieve the same lineage switch in terminally differentiated hepatocytes[50]. NeuroD1 was shown to be involved in normal function of terminally differentiated β-cells[51]. However, NeuroD1 alone does not seem to specify β-cell fate. The basic Leu zipper transcription factor MAFA is known to be specifically expressed in islet β-cells and to directly activate the transcription of insulin-encoding genes by binding to a conserved *cis*-regulatory element in the insulin promoter[52,53]. *In ovo* electroporation of *MafA* into embryonic endoderm and transfection of *MafA* in a pancreatic α-cell line induced insulin expression[54,55].

The knowledge of pancreatic development has been further refined and exploited for the *in vivo* induction of functional insulin-producing β-cells[56]. In this study, 1,100 transcription factors were first screened by *in situ* hybridization in the developing pancreas. Out of these, 20 were identified as being expressed in mature β-cells and their precursors, and from those, nine led to disturbed β-cell development when mutated. Finally, a combination of three transcription factors was sufficient to induce β-cells when expressed in pancreatic exocrine cells. As expected, such factors corresponded to those known from developmental studies, namely NGN3 (or NeuroD1), PDX1 and MAFA (FIG. 2c). Interestingly, these three transcription factors seemed to act in a cell type-specific manner as they were not able to convert fibroblasts into β-cells. Lineage tracing experiments revealed that these three transcription factors converted >20% of the exocrine cells into cells closely resembling β-cells. Remarkably, this *in vivo* cell fate switch was sufficient to alleviate hyperglycaemia caused by insulin deficiency in a mouse model of type I diabetes[56].

Cell fate conversion within the ectoderm. Numerous studies have focused on molecular pathways involved in cellular subspecification in the nervous system (see, for example, REFS 57–60). In the early 1990s, several studies in *Xenopus laevis* revealed the enormous potency of single proneural genes that encode transcription factors of the basic helix–loop–helix (bHLH) class in converting non-neural ectodermal cells into neural cells[61–63]. About 10 years later, it was shown that it is possible to induce transdifferentiation between different neural subtypes. In a series of elegant studies, it was demonstrated that pre- and postnatal astrocytes can be converted into functional neurons using forced expression of the transcription factors paired box 6 (PAX6), achaete-scute complex homologue 1 (ASCL1; also known as MASH1), NGN2 or distal-less homeobox 2 (DLX2), or a combination thereof[64–66]. Cell tracking experiments revealed that cells typically divide before they undergo neuronal conversion[64]. As dividing cells are more amenable to epigenetic changes[67], cell division might be crucial for a lineage switch, which most likely involves epigenetic remodelling. The authors also observed that converted cells undergo morphological changes that are comparable to developing early cortical progenitors[68,69]. They suggested that this is due to the fact that one of the direct transcriptional targets of NGN2 is the small GTP-binding protein RND2, the activity of which has a role in regulating the morphology and migration of early cortical precursors[70]. In addition, they found that induced neurons from astroglia that were forced to express NGN2 predominantly generated cells positive for TBR1 (T-box brain 1; which is a marker for telencephalic glutamatergic neurons), whereas no TBR1-positive neurons could be evoked from ASCL1 expression[64]. It would be interesting to analyse whether NGN2 alone is sufficient to induce a cortical identity or whether the regional identity of the astroglial starting population is involved in determining neuronal fates.

Cell fate conversion across germ layers

The experiments outlined above clearly demonstrated that somatic cellular identities are not entirely fixed but partially interconvertible, at least as far as developmentally related lineages are concerned. In Waddington's epigenetic landscape model, the different 'valleys' are separated by 'hills' of different height (FIG. 1), which implies that interconversion of developmentally related cell types might require less effort than switching from one germ layer to another. Whether such inter-germ layer barriers can be crossed at all, whether cells

Anlage
The initial clustering of embryonic cells from which a part of an organ, or the whole organ, develops.

Exocrine cells
Cells that secrete hormones, factors or other material.

Type I diabetes
A variant of diabetes that is caused by an autoimmune reaction against insulin-producing cells; previously also known as juvenile diabetes.

undergoing such a shift are functional and whether they acquire new and stable epigenetic signatures remained open questions for many years. Inspired by the recent advances in cellular reprogramming and the generation of iPS cells, several teams began to assess combinations of lineage-specific transcription factors for their capacity to directly convert somatic cells into developmentally unrelated phenotypes.

The first successful direct conversion of murine fibroblasts into functional induced neurons was achieved in 2010, and this was followed by a number of studies describing similar switches between developmentally remote cell types. Within 2 years, successful direct conversion of mesoderm into ectoderm and endoderm as well as endoderm into ectoderm was reported for murine and human cells (FIG. 2a). This rapidly evolving field abounds with new questions and aims to exploit these discoveries, for example to induce distinct cellular subtypes and expand progenitor populations, as well as to enhance conversion efficiency and to translate direct conversion into biomedically relevant populations in an *in vivo* setting.

Instructive conversion into differentiated neurons. Direct conversion of fibroblasts into induced neuron-like cells was first shown by Wernig and colleagues[10]. Starting from a pool of 19 candidate genes known to have a role in neural development or epigenetic reprogramming, they found that the proneural gene *Ascl1* alone can convert mouse fibroblasts into cells with neuronal morphology that express neuronal markers. However, expression of additional transcription factors such as brain-specific homeobox and POU domain 2 (BRN2; also known as POUF3F2) and myelin transcription factor 1-like (MYT1L) was required to obtain electrophysiologically functional neurons capable of engaging in synaptic circuitries (FIG. 2d). Remarkably, cells with immature neuronal morphology could be observed as early as 3 days following initiation of transgene expression. These cells did not incorporate 5-bromodeoxyuridine (BrdU), suggesting that conversion from murine fibroblasts into induced neurons is fast and direct. What remained unclear in this first publication was whether the induced neurons would also maintain their neuronal identity after cessation of exogeneous transgene expression and whether fibroblast-specific genes were completely downregulated in induced neuron-like cells.

About 1 year later, this approach was translated to human fibroblasts[11] and endoderm-derived hepatocytes[71] using the same set of transcription factors. The first human fibroblast-derived cells with neuronal morphology were detected 7–10 days following transgene induction, which indicates that longer conversion times are required for human cells. In line with this, human induced neurons remained functionally immature and were not able to generate action potentials at three weeks following transgene induction. Functional maturation was accomplished by combining ASCL1, BRN2 and MYT1L with the additional factor NeuroD1 (REF. 11) (FIG. 2d), which also increased conversion efficiencies threefold. It was also shown that the induced neuron-like cells are transgene-independent for at least 3 weeks and initiate endogenous expression of ASCL1, BRN2, MYT1L and subsequently NeuroD1 — a temporal sequence also observed during normal brain development[62,72]. These results further suggested that the endogenous sequence of transcription factor induction is maintained in induced neurons even in the presence of exogenous NeuroD1.

In an attempt to exploit this approach for studying neurodegenerative diseases in human neurons, the processing of the Alzheimer's disease-associated amyloid precursor protein (APP) and the formation of amyloid-β in induced neuron-like cells generated by overexpression of ASCL1, BRN2 and MYT1L in combination with oligodendrocyte transcription factor 2 (OLIG2) and zinc-finger protein of the cerebellum 1 (ZIC1) were analysed[73]. It was found that induced neuron-like cells from fibroblasts of a patient with Alzheimer's disease carrying a mutation in the presenilin 1 gene showed increased levels of amyloid ß42 compared with amyloid 40, which is characteristic for this condition. This provided a first example of how induced neuron-like cells can be used for studying mechanisms underlying neurodegenerative disorders.

Future studies aiming at disease modelling or cell replacement will most likely include the induction of specific neuronal subtypes affected by the respective disease. Indeed, a number of recent reports suggest that the combination of neuron-inducing factors with region-specific transcription factors can be exploited for the direct induction of region-specific phenotypes such as midbrain dopamine neurons or motoneurons (BOX 1).

Similarly to studies aiming at producing cardiac muscle cells, miRNAs were found to be potent tools for neuronal conversion. Remarkably, expression of miR-9/9* together with miR-124 was shown to convert fibroblasts into neuron-like cells even without overexpression of transcription factors[74]. However, these cells were immature, and conversion efficiencies were low. Thus, the authors combined miRNA expression with overexpression of ASCL1, MYT1L and NeuroD1, which resulted in neurons with mature physiological properties. Interestingly, miR-124 and miR-9 are also known to regulate gene expression by promoting chromatin remodelling[40], suggesting that they might function indirectly via epigenetic mechanisms. The neuron-inducing effect of miR-124 was confirmed in a further study, in which expression of miR-124 together with the transcription factors MYT1L and BRN2 was used to induce functional neurons from human fibroblasts[75]. Recently, repression of a single RNA-binding polypyrimidine tract-binding (PTB) protein was reported to suffice in inducing the conversion of a mesodermal starting population into functional neurons. Interestingly, this phenomenon seems to be due to the relief of a PTB-mediated blockade of miRNA action on several components of the REST (RE1-silencing transcription factor) complex and subsequent derepression of neuronal-specific genes and genes encoding miRNAs[76].

Box 1 | **Combining lineage conversion with sublineage instruction**

Complex tissues are composed of a multitude of several sublineages. In the nervous system, neurons segregate into different subtypes that exhibit region-specific morphology, transcription factor expression, neurotransmitter phenotype and function. Direct conversion with sets of classic neurogenic transcription factors (such as achaete-scute complex homologue 1 (ASCL1), brain-specific homeobox and POU domain 2 (BRN2) and myelin transcription factor 1-like (MYT1L) or variations including neurogenic differentiation factor 1 (NeuroD1), oligodendrocyte transcription factor 2 (OLIG2), zinc-finger protein of the cerebellum 1 (ZIC1) or neurogenin 2 (NGN2)) yields predominantly excitatory neurons with a cortical identity[10,11,77]. This 'default' might be overcome by combining overexpression of generic neurogenic factors with transcription factors that instruct distinct regional subtypes (see the table). Such a strategy was used in several recent studies focusing on the generation of midbrain dopamine neurons and spinal cord motoneurons. Yet, it is important to note that these successful conversion approaches now require thorough follow-up studies addressing the authenticity and functionality of the generated cell types at a level comparable to that used for validating *in vitro* differentiated pluripotent stem cells.

Starting population	Desired neuronal subtype	Generic neurogenic factors	Factors instructing regional subtypes	Readout	Ref
Human fibroblasts	Midbrain dopamine neurons	ASCL1, BRN2, MYT1L	LMX1A, FOXA2	Expression of Tyr hydroxylase, AADC, NURR1	111
Human fibroblasts	Midbrain dopamine neurons	ASCL1	NURR1, LMX1A	Expression of Tyr hydroxylase, genome-wide transcriptional profiling (indicating substantial differences between the Tyr hydroxylase-positive induced neurons and brain-derived dopamine neurons)	112
Human fibroblasts	Motoneurons	ASCL1, BRN2, MYT1L, NeuroD1	LHX3, HB9, ISL1, NGN2	Expression of HB9 and vesicular acetylcholine transporter	113
Mouse fibroblasts	Midbrain dopamine neurons	ASCL1	NURR1, LMX1A	Expression of Tyr hydroxylase, VMAT2, DAT, ALDH1A1, calbindin, release of dopamine as determined by amperometry and HPLC	112
Mouse PITX3–EGFP knock-in fibroblast reporter cell line	Midbrain dopamine neurons	ASCL1	PITX3, NURR1, LMX1A, FOXA2, EN1 and the morphogens SHH and FGF8*	Detection of EGFP-positive cells, expression of Tyr hydroxylase, DAT, AADC and VMAT2, expression of midbrain-specific genes, dopamine release, partial functional restoration of the dopamine system in a mouse model of Parkinson's disease	114
Fibroblasts from HB9–GFP mice	Motoneurons	ASCL1, BRN2, MYT1L	LHX3, HB9, ISL1, NGN2	HB9 reporter expression, endogenous expression of motoneuron-associated transcription factors, functional neuromuscular junctions, gene expression analyses	113

AADC, aromatic L-amino acid decarboxylase; ALDH1A1, aldehyde dehydrogenase 1 family, member A1; DAT, dopamine amino transporter; EGFP, enhanced GFP; EN1, engrailed 1; FGF8, fibroblast growth factor 8; FOXA2, forkhead box A2; HB9, homeobox HB9; HPLC, high performance liquid chromatography; ISL1, ISL LIM homeobox 1; LHX3, LIM homeobox protein 3; LMX1A, LIM homeobox transcription factor 1α; NURR1, NUR-related factor 1; PITX3, pituitary homeobox 3; SHH, sonic hedgehog; VMAT2, vesicular monoamine transporter 2. *PITX3 is essential and the other factors increase efficiency.

Neuronal conversion efficiency. Considering that the direct conversion approaches vary in the starting cell type and the conversion factors used, a common set of quality control parameters is needed to enable comparison of transdifferentiation success (BOX 2). One of these parameters is the conversion efficiency, which is crucial as direct conversion into neurons yields post-mitotic cells not amenable to further expansion. In the initial studies, the conversion efficiencies varied considerably depending on the mode of quantification. We recently proposed that two parameters are necessary to provide a precise assessment of conversion efficiency[77]. One parameter is the 'yield', which is the number of converted cells related to the number of initially plated cells, as also suggested in the initial publications describing induced neuron-like cells[10,11]. In order to also account for non-converted or aberrantly converted cells, 'purity' (that is, the percentage of neurons in the final preparation) is required as a second parameter. It is important though that yield and purity are considered in the context of the quality of the converted cells, which requires thorough assessment of cell identity, stability, functionality and safety (BOX 2).

Box 2 | **Quality control in direct conversion**

Efficiency
Quantification of conversion efficiency is important to determine the best set of conversion factors in combination with the most suitable media conditions. The quantification method should take into account that the starting population might continue to proliferate and that fate conversion might be associated with significant cell death. For example, a conversion protocol yielding a final population that contains 80% neurons might still be inefficient if only 100 of initially plated 100,000 cells survive the process. Therefore, a combination of two parameters is needed to comprehensively assess overall conversion efficiency: the 'yield' describes the percentage of converted cells or colonies relative to the number of initially plated cells; 'purity' denotes the percentage of converted cells in the final population (see also, REF. 77).

Cell identity
Comprehensive comparative analyses are required to assess whether and to what extent the converted cells represent or resemble their native counterparts. This should include morphological and immunofluorescence data, transcriptomic, proteomic and metabolomic data sets as well as epigenetic analyses. Although population studies can provide information about the comparability to the native population, single cell studies are required to assess the heterogeneity within the converted population.

Stability
A major criterion is the stability of the converted cell type in the absence of the conversion factors. This would include the excision of the transgenes that have been used for conversion or the use of non-integrative strategies. Common transgene delivery methods for direct cell fate conversion include retroviral and (inducible) lentiviral systems or non-integrating microRNAs (miRNAs). An interesting non-integrative system for the future might be the use of synthetic mRNAs. Ideally, stability of the induced cell type should not only be assessed *in vitro* but also following transplantation.

Functionality
Functionality might be the most relevant parameter for biomedical applications of induced cells. Following detailed functional testing *in vitro*, the most important question is how the cells perform in an *in vivo* context and whether they can restore function in diseased tissues and organs.

Safety
Safety of converted cells is closely associated with their proliferative and tumorigenic potential. Safety validation of directly converted non-proliferative cells might be limited by the fact that stochastic events resulting in genomic alterations and other aberrations in individual cells might not be recognized. Direct conversion into proliferative progenitor cells offers the advantage that individual clones can be validated and pre-selected before further expansion and subsequent downstream applications.

We became interested in how to increase neuronal conversion efficiency and at the same time reduce the number of required transcription factors. We found that inhibition of glycogen synthase kinase 3β (GSK3β) and bone morphogenetic protein (BMP)–SMAD signalling with small molecules in combination with forced expression of ASCL1 and NGN2 significantly enhances conversion of human fibroblasts into functional neurons[77]. Inhibition of GSK3β and BMP–SMAD signalling have previously been used for highly efficient neural differentiation of human embryonic stem (ES) cells and iPS cells[78,79]. Activation of SMAD signalling via transforming growth factor-β (TGFβ) has been reported to promote epithelial-to-mesenchymal transition (EMT), whereas TGFβ inhibition in return promotes mesenchymal-to-epithelial transition (MET), a phenomenon that is implicated in the initial stages of iPS cell generation[80–82]. It is thus tempting to speculate that MET might also have a role in the direct neuronal conversion of human fibroblasts, although further studies are required to address this hypothesis. Interestingly, NGN2 and ASCL1 have consensus sequences that correspond to GSK3 phosphorylation sites, and it has been suggested that their function can be inhibited by GSK3β, and that this may provide a regulatory mechanism[83]. Using this combination of small molecule inhibitors and two transcription factors, we achieved conversion yields of up to 200% (two neurons per initially plated fibroblast) and purities of up to 80% (REF. 77). It would be interesting to re-evaluate the conversion efficiencies of previous studies on the basis of these two parameters and use the results to define the optimal combination of transcription factors, miRNAs and small molecules required for neuronal conversion.

Instructive conversion into differentiated hepatocytes. Further recent examples for trans-germ layer conversion include the generation of hepatocyte-like cells from mouse fibroblasts by forced expression of the transcription factors GATA4, hepatocyte nuclear factor 1α (HNF1α) and forkhead box O3 (FOXO3) in combination with the inactivation of the tumour suppressor gene *p19*^{Arf} (REF. 84) or by forced expression of HNF4α in combination with either FOXA1, FOXA2 or FOXA3 (REF. 85). Both groups extracted their candidate factors from a pool of transcription factors known to be involved in liver development. Compared with the three-factor combination with GATA4, the conversion efficiency of the two-factor protocol was lower and the latency until conversion was considerably longer. The generated hepatocyte-like cells generated by the two-factor protocol were still expandable. When using GATA4, HNF1α and FOXA3, only limited expandability was observed, and this could be overcome by downregulation of the cell cycle-dependent kinase inhibitor p19^{ARF}, which is normally involved in controlling cellular senescence. Hepatocyte-like cells generated with both methods expressed hepatocyte-associated markers such as albumin and α-fetoprotein, exhibited comprehensive transcriptional reprogramming, could be engrafted into the adult liver and were able to slightly increase survival times in a genetic model of liver failure. Compared with primary hepatocytes, the induced hepatocyte-like cells, however, still showed significant differences in gene expression.

Instructive conversion into somatic progenitor cells. A major drawback of induced neurons is that these cells are by nature post-mitotic and thus not amenable to further expansion. Direct induction of a proliferative stem or progenitor cell would bypass this limitation. Recently, induced neural precursor cells were generated from a mesoderm-derived starting population by using lineage-specific transcription factors (FIG. 2e). In one approach, forced expression of a combination of ASCL1 and NGN2 together with HES1 (hairy and enhancer of split 1), ID1 (inhibitor of DNA binding 1), PAX6, BR2, SOX2, MYC and KLF4 (Krüppel-like factor 4), which are all transcription factors that are highly expressed in neural stem cells, was shown to convert mesoderm-derived Sertoli cells into an induced neural precursor

cell population that is competent to differentiate into electrophysiologically functional neurons, astrocytes and oligodendrocytes[86]. Interestingly, from these nine factors only SOX2 was dispensable in the generation of induced neural precursor cells. Furthermore, residual exogenous transgene expression was detected in these lines, indicating a potential transgene dependency of the induced neural cells.

In another study, SOX2 and FOXG1, or FOXG1 and BRN2, or a combination of all three transcription factors was sufficient to convert mouse fibroblasts into expandable induced neural precursor cells. Induced neural precursor cells generated with SOX2 and FOXG1 showed bipotential differentiation into neurons and astrocytes only and failed to downregulate fibroblast-specific genes. By contrast, induced neural precursor cells generated from combinations that include BRN2 were tripotent and showed reduced expression of fibroblast-associated markers[87]. However, all of these induced neural precursor cell populations were dependent on continuous transgene expression. A surprising aspect of this study was the apparent role of FOXG1 as a programming factor. As FOXG1 is expressed in post-mitotic neurons of the telencephalon[88], it would be interesting to explore whether these particular induced neural precursor cells give rise to telencephalic neurons only. Soon after this study, expandable induced neural precursor cells were generated from mouse fibroblasts by a direct conversion approach using the transcription factors SOX2, KLF4, MYC and BRN4 (REF. 89). Interestingly, SOX2, KLF4 and MYC are all expressed in pluripotent stem cells and neural stem cells, and the three factors together with OCT4 (also known as POUF51) contribute to reprogramming to pluripotency[8]. By replacing only one factor, OCT4, with neural-specific BRN4 the same starting population could be guided towards the formation of committed neural precursors rather than pluripotent iPS cells. The emerging induced neural precursor cells were tripotent, and the retroviral transgenes were largely silenced in these cells. Very recently, direct conversion of mouse and human fibroblast into induced neural precursor cells was reported by exogenous expression of only SOX2 (REF. 90). The data show that the SOX2-induced induced neural precursor cells can be expanded, are multipotent, can give rise to functional neurons and survive and differentiate *in vivo*, although the work in human cells awaits some confirmatory studies such as clonal analysis to provide multipotency at a single cell level.

Direct conversion versus destabilization. Transient expression of reprogramming factors (such as OCT4, SOX2, KLF4 and MYC) has been used as an alternative approach to interconvert somatic cell types. This strategy differs from classic direct conversion and is based on the notion that the reprogramming factors push the cells back to a dedifferentiated or destabilized intermediate state, from which they can be guided with appropriate media conditions towards the desired phenotype. The first report using this method described the conversion of fibroblasts into multilineage blood progenitors by expression of *Oct4* in combination with permissive culture conditions[91]. An activation of haematopoietic transcription factors and cells expressing the pan-leukocyte marker CD45 was observed in this study. These cells were further shown to give rise to granulocytic, monocytic, megakaryocytic and erythroid lineages and were engraftable *in vivo*. Interestingly, and in contrast to iPS cell-derived blood cells, activation of adult and not embryonic haematopoietic programmes was reported. However, it remained unclear whether OCT4 had a dedifferentiating effect or whether it was mimicking other POU domain-containing transcription factors such as OCT1 or OCT2, which are expressed in the haematopoietic system[92,93].

In another study, overexpression of the three reprogramming factors OCT4, SOX2 and KLF4 in mouse embryonic fibroblasts was combined with sequential treatment of cells with small molecule inhibitors of the JAK–STAT (Janus kinase–signal transducer and activator of transcription) pathway and BMP4 under media conditions that promote differentiation of cardiomyocytes. Cells were observed that exhibited spontaneous contractions and atrial-like electrophysiologycal properties[94]. Furthermore, it was found that overexpression of all four reprogramming factors with subsequent culture in neural media containing fibroblast growth factor 2 (FGF2), FGF4 and EGF induces mouse neural progenitors[95]. The generated cells expressed typical neural and rosette cell markers such as zonula occludens 1 (ZO1) and promyelocytic leukaemia zinc-finger protein (PLZF)[96]; however, their self-renewal and tripotential differentiation capacity was not fully investigated. In an independent approach, combined retroviral-mediated expression of SOX2, KLF4 and MYC with a 5-day application of OCT4 fused to the TAT protein derived from the human immunodeficiency virus (OCT4–TAT) was shown to give rise to tripotential mouse neural precursors that are capable of long-term proliferation[97]. In another study, neural precursors directly generated by overexpression of OCT4, SOX2, KLF4 and MYC were reported to exhibit a more pronounced glial differentiation propensity[98]. In addition, these cells showed temporal GFP expression from the Nanog–GFP reporter construct during the conversion process, which indicates that cells subjected to such destabilization approaches might pass through the stage of pluripotency. Clearly, this question deserves more detailed studies involving reporter constructs that reliably and permanently indicate potential transits through pluripotency.

A novel epigenetic landscape model

Taken together, the recent advances in cell programming have dramatically changed our view of epigenetic stability. Not only does temporary overexpression of a few transcription factors suffice to revert fully differentiated somatic cells into a pluripotent state; with comparable experimental approaches somatic cells can even be transdifferentiated into derivatives of other germ layers. Transdifferentiation across germ layers questions the long-held traditional concept that the pluripotent state occupies a superior position within the cell fate hierarchy — a notion implied by the Waddington scheme.

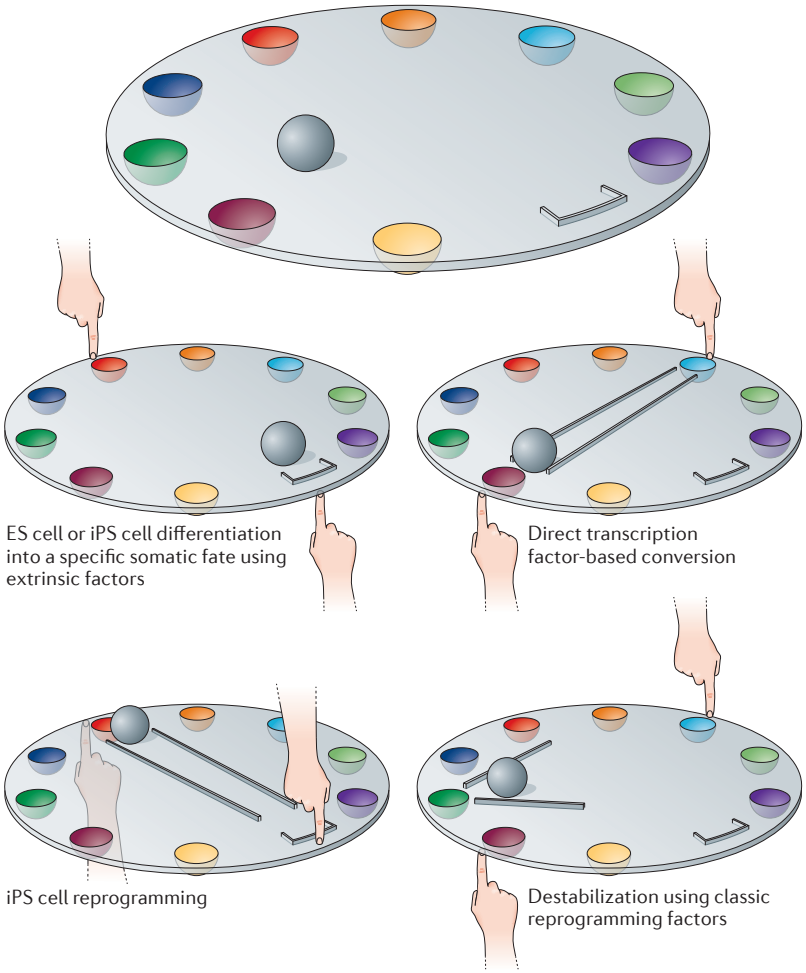


Figure 3 | **The epigenetic disc model.** Whereas Waddington's concept of the epigenetic landscape provides an exquisite model for cell fate specification during normal development, it is becoming increasingly difficult to fit this model with the tremendous plasticity of cell fate that is being uncovered in recent reprogramming and direct conversion studies. It has become clear that it is possible to interconvert various cell types. Moreover, it seems to be as easy to directly induce a neuronal fate from a fibroblast as it is to convert a fibroblast into an induced pluripotent stem (iPS) cell. The disc model lacks a slope and thus hierarchy between different cell types. In this model, the pluripotent state is just one of many possible states, which is not any higher in hierarchy than other cell states and, therefore, can be bypassed in the process of cell fate conversion. Conversion of one cell type into another can be achieved by extrinsic factors that 'tilt' the disc and/or transcription factors that function as 'guide rails' between distinct fates. As the pluripotent state is metastable, extrinsic factors are sufficient to induce differentiation into somatic fates (left). Transcription factors serve as a guide rail to direct cell fate conversion (right). In the case of iPS cell reprogramming, transcription factors initiate the pluripotent state, which is further stabilized by culture conditions (left). A similar approach is used when somatic cells are destabilized by reprogramming factors. Depending on the extrinsic factors, different somatic cell fates can be triggered.

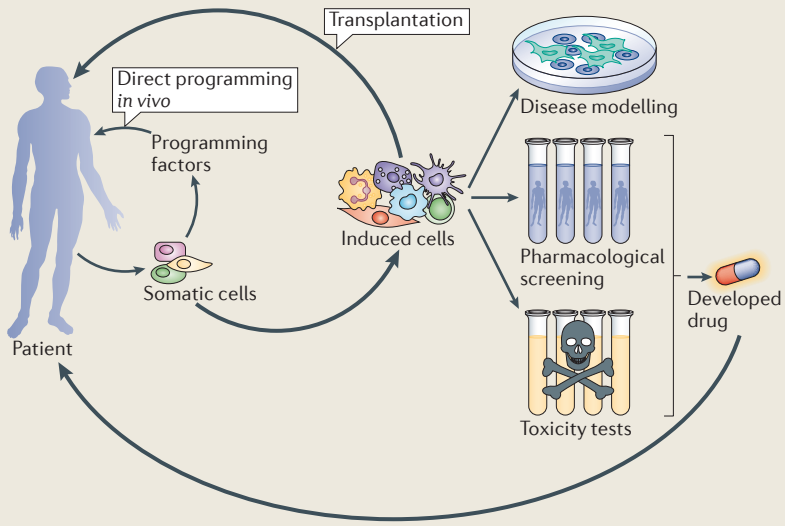
In Waddington's epigenetic landscape model, the pluripotent state resides at the top of a downward slope that represents the differentiation process. Such a valley landscape implies that the process of cell differentiation is, in principle, irreversible, and that 'direct' somatic transdifferentiation routes (that is, across hills and valleys) require much more energy than 'indirect' (up and down the valley) routes via the pluripotent state. Both notions are hard to reconcile with the results of recent cell fate conversion studies, in which one or two factors can convert fibroblasts into either pluripotent cells or differentiated neurons. These remarkable observations raise questions as to whether the differentiation potential of different cell types can still be allocated to a hierarchic model in which the pluripotent state resides at the top of this hierarchy rather than a flat system in which pluripotency merely represents one of many equally attainable states. In such a flat system, the sole difference between pluripotency and other states is that pluripotency is a metastable state, which can only be maintained under very specific culture conditions. *In vivo*, pluripotency is by nature transient and unstable, serving as transit between totipotency and germ layer differentiation. In this context of developmental instability, Waddington's concept will indeed continue to serve as an attractive model for embryonic tissue formation. However, it falls short of accommodating somatic cell fate transitions that have been demonstrated in recent direct conversion studies.

In order to illustrate or model transdifferentiation in the context of active cell programming, it might thus be more appropriate to use a *primus inter pares* model, in which all cell fates are situated as dents on the circumference of a flat disc, with the unstable pluripotent state symbolized as a open hutch (FIG. 3). In such a model, cells can be moved towards other fates by exogenous factors that 'tilt' the disc in a specific direction, and targeted cell fate conversions can be further refined by using transcription factors as 'guide rails'. The disc model might also be more suitable for illustrating approaches using (incomplete) transcription factor-based reprogramming with subsequent enrichment of distinct somatic cell types with extrinsic factors as shown in the various destabilization approaches. Yet, it is obvious that such a reductionist model can only represent an approximation. It neglects, for example, differences in cell fate stability and thus resistance to transdifferentiation observed among different somatic cell types.

Conclusions

Although still at early stages, direct lineage conversion (also known as transdifferentiation) holds great promise for biomedical applications such as regenerative medicine and cell-based disease modelling (BOX 3). However, several crucial issues need to be addressed to achieve these goals. A key question, which had already emerged in the context of classic reprogramming is whether and to what extent converted cells retain epigenetic marks of the original donor cell and how much such an 'epigenetic memory' may impair cell functionality. In our classic way of thinking, we consider differentiation-associated fate determination to be accompanied and caused by progressive acquisition of epigenetic changes that stabilize the cell fate decision[99]. However, what happens if a fully committed somatic cell directly converts into another without passing through intermediate fates? In the case of reprogrammed pluripotent cells, an epigenetic memory of the cells was proposed and used to explain differences in differentiation propensity between iPS cell lines of different origins[100,101].

Box 3 | **Biomedical application of direct cell fate conversion**



Somatic cells (for example, skin fibroblasts) can be directly converted into other tissue-specific cell types. The converted cells can be used in disease modelling (to analyse a disease-specific phenotype in a disease-associated somatic cell type *in vitro*), pharmacological screening (to identify drugs interfering with the disease phenotype) and drug toxicity testing (to identify side effects of potential drugs in different somatic cell types) (see the figure). The generation of cells by direct conversion is particularly attractive for poorly accessible, non-regenerating tissues such as the nervous system, the heart and pancreatic β-cells. As direct conversion is considerably faster than any transit through the pluripotent state, samples from large cohorts of patients might be handled in parallel, thereby opening new dimensions for disease modelling in the context of genome-wide association studies. Moreover, the generation of expandable intermediate cell populations, as shown for neural progenitors[86,87,89,90,95,97,98], might bypass the limitations related to total yield and heterogeneity of the induced population. This is of particular importance when it comes to the validation of the induced cells as clone-derived populations share transgene integration events and karyotypic abnormalities, whereas bulk-converted populations contain cells with different genetic status and are thus less amenable to quality control. From a clinical point of view, the induction of individual cell types directly *in vivo* might be the most promising aspect. In animal models, pancreatic β-cells and functional cardiomyocytes have been successfully induced *in situ*[33,34,56], which points to potential of this approach for the treatment of acute and chronic disease such as cardiac infarction or diabetes. The combination of neuronal induction and regional instruction might be exploited for generating specific neuronal subtypes that are affected by diseases such as Parkinson's disease, Huntington's disease or amyotrophic lateral sclerosis, either *in vitro* or by direct conversion of resident glial or non-neural cells. Provided that *in situ* delivery of programming factors becomes efficient and controllable and that the induced cell types are stable and functional, *in vivo* direct cell fate conversion might provide a highly attractive alternative to classic cell transplantation.

Ground state
A physics and chemistry term that denotes the state of having the least energy of all the possible states. This term was recently adopted in cell biology to denote the most primordial or authentic state, or differentiation stage (such as ground state pluripotency), of a cell.

It was also suggested that this memory vanishes during extended proliferation[102], which is consistent with the observation that dividing cells are more open to epigenetic changes[67]. Such considerations are of particular importance in scenarios in which converted cells divide rarely or not at all[10,33,56]. Whether or not cell division is at all necessary for genome-wide remodelling associated with direct cell conversion remains currently enigmatic.

Interestingly, most transcription factors shown to be efficacious in direct lineage conversion are pioneer transcription factors that act in positive feedback or feed-forward loops. They can activate target genes even in a repressed chromatin state, are able to recruit additional transcription factors, enhance the activation of target genes and establish transcriptional networks[103–105]. Interestingly, the same set of transcription factors can induce a specific cell fate from different species and starting populations[10,11,71], which suggests that conserved regulatory networks are initiated. Moreover, the fact that conversion efficiencies correlate with viral titres and levels of transgene expression implies that supra-physiological transgene levels might override the original transcriptional network of the starting cell population. This might lead to the downregulation of characteristic genes expressed in starting cells even before the new cell type-specific programme becomes activated, as shown for the generation of iPS cells[106,107]. For a more comprehensive understanding of the steps involved in direct programming it will be important to study the exact function of each individual programming factor including any downstream effects on target genes. The temporal sequence of transgene induction might not only affect conversion efficiencies but also influence the phenotype of the resulting cell types, as shown for the direct conversion of lymphoid progenitors to eosinophils or basophils[29]. Remarkably, miRNAs can replace transcription factors during cell programming and reprogramming as observed for the induction of iPS cells[108], neurons[74,75] or cardiomyocytes[39]. As miRNAs do not directly induce transcription but modify the expression of genes at the post-transcriptional level, miRNA-dependent initiation of new transcriptional programmes may be supported by directly targeting the chromatin remodelling machinery[40]. We have recently shown that small molecule inhibitors of developmentally relevant signalling pathways can be used to dramatically increase the efficiency of converting human fibroblasts into neurons[77]. It will be interesting to explore whether certain small molecules are also competent to replace pioneer transcription factors in direct cell conversion, as recently shown for iPS cell generation[109].

Manipulating the 'ground state' of the starting population by small molecules might also help to overcome a major and so far completely unsolved obstacle in transdifferentiation — the question of why cells from young donors can be converted with much higher efficiency than those from adult or aged donors. Although obvious differences such as shorter telomeres or increased mitochondrial age might contribute to this observation, it seems unlikely that they are the sole reason. An alternative explanation might be that the amenability of cells to epigenetic remodelling decreases with age (for example, histone H4 Lys12 acetylation in the context of age-dependent learning and memory decline)[110]. A related important question is whether direct conversion resets developmental timing or whether the age of the starting population (for example, mitochondrial age, proliferation capacity and telomere status) will have an impact on the age of the target cell. Despite these open questions, direct fate induction opens exciting new avenues for cell-based disease modelling and regenerative medicine (BOX 3).

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Acknowledgements
Work in the laboratory of O. B. is supported by the European Union (FP7-HEALTH-2007-B-22943-NeuroStemCell and FP7-HEALTH-2010-266753-SCR&Tox, COLIPA; to O.B.), the Federal Ministry of Education and Research (BMBF; grants 01GS0860, 01GN1008C, 01GN1009B, 0315799; to O.B.; ERA-Net for Research Programmes on Rare Diseases 01GM1309A; to P.K.), BIO.NRW (project StemCellFactory; to O.B. and P.K.), the Hertie Foundation (to O.B.), the Ministry of Innovation, Science and Research of the State of North Rhine-Westphalia (to J.L. and P.K.) and BONFOR (to J.L.).

Competing interests statement
The authors declare no competing financial interests.

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