In vivo reprogramming for tissue repair

Christophe Heinrich, Francesca M. Spagnoli and Benedikt Berninger

Vital organs such as the pancreas and the brain lack the capacity for effective regeneration. To overcome this limitation, an emerging strategy consists of converting resident tissue-specific cells into the cell types that are lost due to disease by a process called in vivo lineage reprogramming. Here we discuss recent breakthroughs in regenerating pancreatic β-cells and neurons from various cell types, and highlight fundamental challenges that need to be overcome for the translation of in vivo lineage reprogramming into therapy.

Regenerative medicine aims at restoring lost organ function by replacing the cells that have been damaged by disease or injury. Classically, cell-replacement approaches have involved the transplantation of cells, either expanded ex vivo or generated in vitro, into the dysfunctional tissue. Although bone marrow transplantation has been remarkably successful in regenerating the haematopoietic system, in most other organs there are still major obstacles to overcome before the potential of transplantation can be employed fully, and so alternative strategies for cell replacement are being explored. An emerging strategy aims at promoting a tissue’s ability for self-repair either by inducing the proliferation of residual cells or fate conversion of other resident cells into the desired cell type. This conversion is also referred to as in vivo lineage reprogramming or transdifferentiation.

The strategy behind successful lineage reprogramming has its fundamentals in developmental biology and is based on the knowledge of transcriptional networks underlying the establishment and maintenance of cellular identity. Re-wiring the transcriptional circuitry by a handful of developmentally relevant transcription factors can impose a new molecular program, and thereby a new cellular identity, onto terminally differentiated cells. Although the past decade has seen enormous progress in regenerating the haematopoietic system, in most other organs there are still major obstacles to overcome before the potential of transplantation can be employed fully, and so alternative strategies for cell replacement are being explored. An emerging strategy aims at promoting a tissue’s ability for self-repair either by inducing the proliferation of residual cells or fate conversion of other resident cells into the desired cell type. This conversion is also referred to as in vivo lineage reprogramming or transdifferentiation.

The heart is another organ endowed with poor intrinsic regenerative capacity, for which in vivo lineage reprogramming strategies are currently being explored with remarkable success and have been reviewed elsewhere.

Defining milestones for successful in vivo lineage reprogramming

When considering the best-suited design of an in vivo reprogramming strategy, it is useful to set milestones with which the factual outcomes can be compared (Box 1). The first milestone would be the identification of the source cell type that is most suited to undergoing conversion into the desired cell type, and is available in the right place. The selection of the source cell type imposes a specific molecular context, defined by its gene expression profile and epigenetic signature, in which reprogramming factors have to operate. An embryological origin that is common to both the source and desired cell types might facilitate reprogramming. Moreover, the cell cycle state could exert an influence on the permissiveness to reprogramming. Also, reprogramming could potentially target a cell population that undergoes constant homeostatic replenishment of its pool, thus avoiding loss of source cell function after cell conversion.

The second milestone consists of identifying the optimal reprogramming route. This entails the identification of a minimum set of reprogramming factors required to superimpose the program of the desired cell type. Identification of signalling molecules or small compounds that mimic transcription-factor-mediated reprogramming will bring the process closer to clinical translation. Likewise, efficient and safe delivery methods of the reprogramming factors in vivo are yet to be developed. The route of lineage conversion might involve an intermediate fate-restricted stage allowing for amplification of the desired cell type, or occur by direct switching of source into desired cell type. Each strategy has advantages and disadvantages that are dependent on the type of tissue and disease in question.

The third milestone involves generating cells of the same molecular identity and phenotype as the desired cell type while erasing their original cellular identity. Lineage reprogramming into cells that only partially exhibit the desired phenotype may fall short of correcting the functional deficits in the organ, and even may exert adverse side effects.

Christophe Heinrich is at INSERM U836, F-38000 Grenoble, France and Univ. Grenoble Alpes, Grenoble Institut des Neurosciences, GIN, F-38000 Grenoble, France. Francesca M. Spagnoli is at the Max Delbrück Center for Molecular Medicine, D-13125 Berlin, Germany. Benedikt Berninger is at the Institute of Physiological Chemistry and the Focus Program Translational Neuroscience, University Medical Center of the Johannes Gutenberg University Mainz, D-55128 Mainz, Germany, and Physiological Genomics, Institute of Physiology, Ludwig Maximilians University Munich, D-80336 Munich, Germany.

*e-mail: francesca.spagnoli@mdc-berlin.de; berningerb@uni-mainz.de

© 2015 Macmillan Publishers Limited. All rights reserved
As host environments differ in their permissiveness to accommodate new cells and can therefore sometimes create barriers against their functional integration, the fourth milestone involves identifying potential environmental constraints. Such constraints may be responsible for the limited self-regenerative potential of these tissues in the first place, and may cause the abortive integration of the reprogrammed cells. Knowing these restrictions is the first step in developing strategies to overcome them.

The final milestone is accomplished with proper functional integration of reprogrammed cells, thereby restoring lost functions in the organ. To promote integration, reprogrammed cells may require local signals that allow overcoming the bottleneck steps towards full differentiation. Tissues where successful integration of new cells takes place physiologically, such as the adult neurogenic niches, serve as useful models.

**In situ reprogramming in the pancreas**

Lineage reprogramming potentially offers a cure to diabetes, a complex metabolic disorder characterized by loss or dysfunction of pancreatic β-cells. Recent studies have unveiled an unsuspected degree of cellular plasticity in the adult pancreas, and have pointed to pancreas-resident cells as potential sources for new β-cells. In vivo lineage reprogramming holds distinct advantages over stem-cell-based replacement strategies, such as the new β-cells being autologous in origin and residing within their native tissue, and with a lower risk of tumorigenesis. Moreover, in contrast to other *in situ* approaches, lineage reprogramming does not require a significant remnant β-cell mass.

A natural place to search for cellular sources of new β-cells is the pancreatic islet itself, given the common developmental lineage and close endocrine functions of α-, β- and δ-cells. Herrera and colleagues have reported that fate interconversion between endocrine cell types can occur spontaneously, following extreme toxin-induced β-cell damage in adult mice. Restoration of endogenous insulin-producing cells from non-β-cells occurs throughout life through distinct age-dependent reprogramming events, originating from somatostatin-producing δ-cells before puberty and glucagon-producing α-cells thereafter. Importantly, ageing does not alter the plasticity of α-cells, even long after β-cell loss. α-to-β-cell conversion might be advantageous, given that only a few α-cells are needed to ensure blood glucose homeostasis in the mouse. Furthermore, given that elevated glucagon levels contribute to the pathophysiology of diabetes, reducing glucagon by reducing the number of α-cells might be beneficial to diabetic patients.

Studies on pancreatic embryonic development revealed that endocrine cell fate allocation is dependent on the interplay between the transcription factors Pax4 and Arx. Consistently, forcing Pax4 or inactivating Arx expression in α-cells results in α-to-β-cell conversion even after birth. By testing different combinations of transcription factors associated with β-cell development, Melton and colleagues found that ectopic expression of Pdx1, Neurog3 and MafA induces direct *in vivo* reprogramming of acinar cells into β-like cells. Although they did not cluster into islets, indicative of constrained tissue remoulding, these β-like cells ameliorated blood glucose levels in diabetic mice. Recently, it was shown that all three major islet endocrine subtypes can be generated by transcription-factor-mediated acinar cell reprogramming as well as in injury models. By testing different combinations of transcription factors associated with β-cell development, Melton and colleagues found that ectopic expression of Pdx1, Neurog3 and MafA induces direct *in vivo* reprogramming of acinar cells into β-like cells. In this experimental model, the reprogramming route involves the re-activation of the endocrine progenitor transcription factor Neurog3 (ref. 35). Acinar cells constitute about 80% of pancreatic mass, so conversion of only a small proportion would not affect the digestive functions of the organ. However, because of the production of a variety of digestive enzymes, *in situ* manipulation of acinar cells carries a risk of pancreatitis.

As an alternative to pancreatic exocrine or endocrine cells, the duct cell compartment, which is the second most abundant cell population...
enteroendocrine progenitors that continually arise from intestinal stem cells and express Neurog3 (ref. 46). Conditional ablation of the transcription factor Foxo1 in these cells results in ectopic insulin expression in the gut, suggesting that Foxo1 is required in vivo to prevent the acquisition of β-cell features by intestinal enteroendocrine cells45. Nonetheless, this is not true reprogramming of gut epithelium into a pancreatic fate, because the induced insulin-positive cells retain intestinal properties45.

So far, in vivo reprogramming of liver cells to β-like cells has been reported in the mouse following systemic adeno-virus-mediated expression of various pancreatic transcription factors, resulting in partial correction of hyperglycaemia in diabetic mice47–50. However, delivery of these factors through adeno-associated viruses (AAVs), which have low immunogenicity but high transduction efficiency, or following transgenic expression, did not exert the same effects51,52. These observations suggest that adenoviruses may alter cellular reprogramming efficiency by inducing a general inflammatory response51. Hence, it seems that the appropriate route for reprogramming hepatocytes to pancreatic fate has yet to be defined. The identification of developmental factors controlling the fate decision between the two lineages53 might help to define new candidate factors and provide the proper niche for hepatocyte-to-β-cell reprogramming.

**In situ reprogramming in the CNS**

Neurodegenerative diseases and acute CNS trauma are leading causes of patient disability and death. The standard strategy for experimental cell replacement in the brain has been cell transplantation54. Yet transplantation is a highly invasive method and accompanied by considerable cell death among the grafted cells55. An alternative line of research pursues the possibility of mobilizing endogenous neural progenitors towards diseased brain tissue56,57. Although promising, this approach is likely to be limited to the immediate vicinity where progenitors naturally reside. For more remote brain areas, reprogramming of abundant resident cells may be a more viable strategy.

A decade of in vitro studies has shown that brain-resident cells, such as astroglia, NG2 glia and pericytes, can be converted into functional neurons58–63. Single transcription factors were sufficient to direct astroglia towards a glutamatergic (Neurog2 or NeuroD1) or GABAergic (Ascl1 or Dlx2) neuron fate64,65. Similar studies in fibroblasts have broadened the spectrum of transcription factors and microRNAs (for example, miR-9/9* and miR-124) that have reprogramming capacity64,65, resulting in the generation of other clinically relevant neuron subtypes, such as dopamine neurons, motor neurons or medium spiny neurons66–69. Even if fibroblast-derived induced neurons are of no direct use for in situ reprogramming, they are a valuable heuristic tool in the quest for new reprogramming factors, for understanding the molecular mechanisms underlying reprogramming, and for identifying epigenetic barriers that prevent it70. They can also serve as a source of cells for human disease modelling71,72 as well as transplantation68,73.

Encouraged by these in vitro studies and early work in vivo74, several groups have embarked on assessing the possibility of reprogramming astrocytes directly in situ (Table 1). Targeted expression of the transcription factors Ascl1, Brn2 and Myt11 in parenchymal astrocytes in the adult striatum in vivo was able to convert them into induced NeuN+ neurons, providing proof-of-principle evidence for the viability of this approach75 (Fig. 2e). Zhang and colleagues reported that the
transcription factor Sox2 can also convert striatal astrocytes into doublecortin (DCX)-positive cells\(^76\) (Fig. 2d). This finding was unexpected, as Sox2 generally regulates self-renewal of neural stem cells rather than neuronal differentiation. Intriguingly, induced DCX\(^+-\) cells rarely developed into mature neurons but instead continued to proliferate, forming DCX\(^+-\) cell clusters. Maturation into electrophysiologically functional neurons required treatment with differentiation-promoting factors such as BDNF (brain-derived neurotrophic factor) and the BMP (bone morphogenetic protein) inhibitor noggin, or blocking histone deacetylase activity\(^76\). This work differs conceptually from Torper et al.\(^75\) in that reprogramming into neurons occurred through an intermediate proliferative neuroblast state and hence may imply an additional yield of induced neurons due to local amplification (Fig. 2d).

Most neurological conditions associated with neuronal loss are accompanied by reactive gliosis\(^77\). The acquisition of hallmarks of neural stem cells by reactive astrocytes\(^78\) makes them appealing targets for direct reprogramming into induced neurons\(^77\). Furthermore, following stroke, astrocytes in the adult mouse striatum can give rise spontaneously to Ascl1-expressing transit-amplifying cells that in turn generate clusters of DCX\(^+-\) cells and some NeuN\(^+\) neurons\(^79\). This surge of neurogenesis was found to be elicited by a reduction of endogenous Notch signalling in striatal astrocytes\(^79\). Genetically abrogating Notch signalling triggered a similar latent neurogenic program in striatal astrocytes, even in the absence of injury\(^79\). Intriguingly, this genetic manipulation is capable of evoking a neurogenic program in a few brain areas, such as the striatum and superficial medial cortex, hinting at differences in the molecular mechanisms controlling maintenance of astrocyte fate. The similarities in the response of striatal astrocytes to Ascl1\(^+\) versus Ascl1\(^-\) astrocytes suggest that the genetic manipulation may mimic certain aspects of stroke, including reactive gliosis and neurogenesis, in the adult brain.

To overcome the restrictions presented by most cortical astrocytes, Chen and colleagues combined a mild injury paradigm with forced NeuroD1 expression. These manipulations resulted in the highly efficient conversion of reactive astrocytes into NeuN\(^+\) and Tbr1\(^+\) neurons...
in vivo, and the induced neurons were able to fire action potentials and received synaptic input (Fig. 2a). Surprisingly, astrocytes could be retrovirally targeted at an early stage of reactive gliosis, when astrocytes typically do not yet proliferate. Also, the high conversion rate contrasts with the low rate of induced neurogenesis in a similar experimental paradigm following expression of Neurog2 (ref. 81), a transcription factor upstream of NeuroD1. These distinct reprogramming potencies may hint at differences in the epigenetic status of loci targeted by Neurog2 and NeuroD1, underscoring the importance of the epigenetic context when choosing reprogramming factors. Moving towards disease models, NeuroD1-induced conversion of reactive astroglia into NeuN+ neurons was also observed in a mouse model of Alzheimer’s disease.

Reprogramming of non-neuronal cells into neurons in the context of severe CNS injury represents a major challenge. In parallel to the work of the Zhang and Chen labs, we have assessed whether Sox2 and Ascl1 could induce reprogramming of proliferating glia during ongoing reactive gliosis in the cerebral cortex, following an acute stab wound lesion. We found that expression of Sox2 and Ascl1, but strikingly also Sox2 alone, caused the emergence of DCX+ cells that received synaptic inputs from endogenous GABAergic neurons (Fig. 2b). Instead of converting reactive astrocytes, genetic fate mapping revealed an NG2+Sox2/(Ascl1)Quiescent (?) astrocyte +Ascl1/Brn2/Myt1l

Figure 2 Examples of in vivo lineage reprogramming in the brain. A schematic of the brain is shown, indicating the regions where attempts aiming at in situ lineage reprogramming have been conducted successfully. (a–c) The cerebral cortex. (a) Forced expression of NeuroD1 directly converts reactive astrocytes and NG2 glia into induced neuronal cells in the adult mouse cortex63. In this study, the virus-injection needle itself was considered as a lesion paradigm. (b) Following an acute invasive injury inflicted to the adult cerebral cortex (a stab wound lesion), forced expression of Sox2, alone or in combination with Ascl1, induces reactive NG2 glia to adopt a neuronal identity during ongoing reactive gliosis82. Some induced DCX+ neuronal cells spontaneously mature into NeuN+ neurons (dashed arrow). (c) Embryonic and early postnatal callosal projection neurons of layer II/III can be post-mitotically reprogrammed by Fezf2 into layer V/VI corticofugal projection neurons87.

(d, e) The striatum. (d) Parenchymal astrocytes residing in the adult striatum can be reprogrammed by Sox2 into proliferative neuroblasts76. Maturation of these induced neuroblasts into functional neurons requires additional differentiation-promoting factors such as BDNF and the BMP inhibitor noggin, or blockade of histone deacetylase activity with VPA (valproic acid)76. (e) Parenchymal astrocytes residing in the adult striatum are converted into neuronal cells by the combination of Ascl1, Brn2 and Myt1l (ref. 75). The question marks in d and e indicate that it remains unknown whether the astroglia were quiescent at the time of lentiviral transduction. Question marks in b and e indicate that the functional properties of NeuN+ neurons (action-potential-firing) were not determined in these studies.
glial origin of the DCX+ cells. Targeting NG2 glia for reprogramming may have the added benefit that converted cells are replaced by homeostatic control of proliferation, preventing exhaustion of the NG2 glia pool. In contrast, by specifically targeting astrocytes in the hemi-sected spinal cord, Zhang and colleagues showed that Sox2 can reprogram these cells into neuroblasts that can give rise to mature neurons with features of GABAergic interneurons. Thus, the CNS harbours distinct glial cell populations of different reprogramming permissiveness depending on the region and health status. Future studies will reveal whether non-neural cell populations that contribute to scar formation in response to CNS injury, such as pericytes, are also amenable to reprogramming not only in vitro, but also in vivo.

As an alternative to glia-to-neuron reprogramming, conversion of neuronal progenitors or post-mitotic neurons of one subclass into neurons of another has been pioneered by the Arlotta lab, which has shown that the transcription factor Fezf2 can convert striatal progenitor cells into pyramidal-like corticofugal neurons during development. Furthermore, Fezf2 could also re-direct early post-mitotic callosal projection neurons towards a corticofugal neuron fate, including corticospinal motor neurons (Fig. 2c). Likewise, Fezf2 expression in early post-mitotic layer-IV stellate neurons converted these into neurons with layer-Vb physiological properties and synaptic connectivity. Reprogramming by Fezf2 seems to be restricted to early stages of the post-mitotic life of a neuron, suggesting that maturation closes a window of nuclear plasticity required for reprogramming.

Milestones reached and future challenges

The field of in vivo reprogramming is very young and much of the initial pioneering work needs confirmation. Nonetheless, we here recapitulate how close we are to accomplishing the milestones described above (Table 1).

Various suitable source cell types for in vivo reprogramming have been identified in the adult mouse, but this search has certainly not been exhaustive (milestone 1). More knowledge is needed of the molecular context of the source cell, which would require studying their transcriptomes, epigenomes and proteomes, ideally following direct isolation from the organism. To demonstrate the relevance of in vivo reprogramming for therapy, we need to validate the feasibility of fate conversion in other species, including non-human primates. Combining induced pluripotent stem cell and 3D-organoid technologies may allow for developing human tissue models that could address issues of cell inter-convertibility in an organ-like context. Likewise, human xenografts or humanized organs could serve as models to study in vivo lineage reprogramming of human cells before a viable reprogramming strategy can be tested in patients.

Various potential routes of reprogramming are emerging and deserve closer investigation (milestone 2). Forced expression of development-inspired transcription factors to induce fate conversion has proved to be a successful approach common to both the pancreas and CNS. One important concern is that virus-mediated transcription factor expression is often maintained beyond the reprogramming stage with unknown consequences. It may turn out to be important to restrict transcription factor expression to only the time window of reprogramming and switch it off thereafter. Several approaches towards β-cell restoration have relied on the ablation of specific molecules, whereas reprogramming through genetic loss-of-function of the Notch pathway could elicit neurogenesis in the adult striatum. This indicates that in both the pancreas and the CNS, release from cell fate-maintaining factors or signalling pathways can trigger some degree of reprogramming. In both cases, release from fate control often results in the reawakening of a progenitor- or stem-cell-like state that allows for subsequent differentiation into β-like cells or induced neurons.

A more complete knowledge of the molecular mechanisms underlying gene regulation, including the role of microRNAs and other non-coding RNA species, may lead to new tools for overcoming molecular barriers, largely of epigenetic nature, that impede the action of fate determinants. Identifying the epigenetic state in which target cells are caught during the conversion process may enable the remodelling of their epigenome to render them more permissive to reprogramming. Can we extract general rules for the identification of reprogramming-competent cells? Work on Ascl1-mediated reprogramming in fibroblasts and other cell types into induced neurons has provided first insights by correlating the success rate of reprogramming with the state of histone modifications at Ascl1 target genes. Although Ascl1 seems to act as a pioneer factor opening chromatin, it can only achieve this when its target gene loci exhibit a particular trivalent histone modification signature. If this model holds true, it will be of great interest to assess the epigenetic status of key target genes in different resident cells in the healthy and injured CNS. Along similar lines, evidence from human pancreatic islets has suggested a correlation between histone modifications and reprogramming potential. With respect to the question of how to render cells more responsive to reprogramming factors, work in model organisms can be helpful. For instance, seminal work in Caenorhabditis elegans showed that interfering with epigenetic modifiers such as the Polycomb repressive complex-2 can make otherwise non-permissive cells susceptible to transcription-factor-mediated neuronal reprogramming. Natural fate conversion of hindgut cells into motor neurons constitutes another intriguing example of epigenetic plasticity in worms where sequential histone-modifying activities determine the robustness of the conversion process.

Any potential clinical application will require safe and efficient delivery methods for reprogramming factors. These may include non-invasive systemic delivery of modified RNAs, extracellular microvesicles or safer viral systems. In particular, AAVs may allow for selective in vivo targeting of a specific source cell type, as it has been already achieved for the mouse retina. Such target selectivity is of greatest importance for the translation to patients. Small molecules acting on fate-maintaining signalling pathways or harnessing epigenetic plasticity are likely to complement the arsenal of reprogramming tools.

A general limitation of in vivo reprogramming studies is the lack of extensive characterization of the reprogrammed cells (milestone 3), hampering a global assessment of cell maturation. Partially reprogrammed cells may be prone to malignant transformations or to revert to their original cellular identity. In the pancreas, reprogrammed β-like cells are often tested for their ability to counteract hyperglycaemia in diabetic mice, a key characteristic of bona fide β-cells, but besides that only a few markers are usually checked. Similarly, limited information is available regarding the identity of induced neurons. The only evidence for subtype-specific reprogramming has been provided by direct conversion of one neuronal subtype into another using Fezf2, which emerges as a selector gene. It will be of
great interest to discover the effect of Fez2 in the context of a more generic reprogramming as induced by NeuroD1. To fully achieve this milestone, strategies are required to isolate the reprogrammed cells and perform transcriptional, epigenetic and proteomic profiling, and functional analysis, ideally at the single-cell level.

Regarding the role of the host environment in which cell fate conversions take place (milestone 4), little is known. One conspicuous similarity between the pancreas and CNS is the reprogramming-facilitating effect exerted by prior tissue damage, which induces resident cell types to become more plastic and thereby renders the cellular context more amenable to fate switch.26,28,32,33. Also, there are marked regional differences as illustrated by different permissiveness for glia-to-neuron conversion of striatal and cortical astrocytes.28,32 It remains unclear whether distinct degrees of permissiveness are due to cell intrinsic differences and/or result from environmental influences that promote or constrain cell fate conversions. Moreover, the immune cells may add a layer of complexity by activating signalling pathways that interfere with the transcriptional cascades underlying reprogramming.

With respect to the last milestone, reprogrammed cells that undergo full identity switch and maturation should be able to integrate into the existing tissue, reconstituting its 3D-architecture. In the pancreas, acinar-derived β-like cells did not cluster into islets, most likely because their maturation was not completed. Tissue integration and connection to the bloodstream is crucial for proper functioning of the new β-cells, but the islet niche might also actively contribute to preserve β-cell function. Due to the complexities of CNS organization, the consequences of reprogrammed cells on neural circuit function have not yet been evaluated. This milestone probably represents the most difficult hurdle. Aberrant neurogenesis or connectivity patterns may promote circuit dysfunction such as epilepsy or dyskinesias as described following transplantation of neuronal progenitor cells in Parkinson's patients.38 Restoring relatively simple relay functions by regenerating relay neurons within a circuit or replacing neurons of a single cell type is more likely to work at this early stage rather than regenerating an entire neuronal tissue.

In conclusion, there is still a long way to go before in vivo lineage reprogramming will become a clinical reality. Much fundamental research needs to be conducted in appropriate models to fully weigh the potential of this approach against alternative strategies, such as cell transplantation. Considering the broad spectrum of potential medical applications, each of these approaches is likely to have its own particular merits, which only future studies will disclose.

ACKNOWLEDGEMENTS
Our apologies go to all authors whose important work could not be mentioned due to space limitations. We are grateful to Célia Lentini for her help with the design of the figures and Marisa Karow for critically commenting on the manuscript. C.H. is supported by CNRS and Citizens United for Research in Epilepsy (CURE), F.M.S. is supported by the Helmholz Association, ERC-St.-Grant HEPATOPANCREATIC, and FP7 IRG-ENDOPANC. B.B. is supported by the DFG, BMWF, and the Bavarian research network Induced Pluripotent Stem Cells (ForIPS).

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.


