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Engineering Cell Fate for Tissue Regeneration by In Vivo Transdifferentiation

I. de Lázaro[1,2] · K. Kostarelos[1,2]

Abstract Changes in cell identity occur in adult mammalian organisms but are rare and often linked to disease. Research in the last few decades has thrown light on how to manipulate cell fate, but the conversion of a particular cell type into another within a living organism (also termed in vivo transdifferentiation) has only been recently achieved in a limited number of tissues. Although the therapeutic promise of this strategy for tissue regeneration and repair is exciting, important efficacy and safety concerns will need to be addressed before it becomes a reality in the clinical practice. Here, we review the most relevant in vivo transdifferentiation studies in adult mammalian animal models, offering a critical assessment of this potentially powerful strategy for regenerative medicine.

Key words Transdifferentiation · Reprogramming · Cell fate · Regeneration · Tissue repair

Introduction

The biological dogma that considered cell differentiation as an irreversible process is now refuted and we are witnessing the increasing development of reprogramming technologies [1]. The conversion of a specific, fully differentiated cell into a different type, has relatively recently been termed *direct reprogramming* or *transdifferentiation* [2]. However, the first reports of the capacity of lineage specific transcription factors to command changes in cell identity date back to the late 1980s, when Davies and Weintraub demonstrated that forced expression of *MyoD* in vitro induced different cell lines to express muscle-specific genes or even converted them into stable myoblasts [3, 4]. Although ectopic expression of *Antp* at different Drosophila larvae stages was soon after found to convert antennae into second legs [5], almost two decades were needed until the possibility to intentionally induce in vivo transdifferentiation in adult mammalian organisms was confirmed [6–8].

Changes in cell fate occur also spontaneously in nature. The outcome of such phenomena can represent the onset of a disease, as it is the case in Barret's metaplasia, whereby *Cdx2* mediated transdifferentiation of stratified squamous into columnar epithelium predisposes to oesophagus carcinoma [9, 10]. Transdifferentiation of different starting cell types into myofibroblasts can result in fibrotic scarring after injury or chronic damage to various tissues, including kidney [11], liver [12] and muscle [13]. In contrast, transdifferentiation is one of the underlying mechanisms in liver [14] and heart [15] regeneration in zebrafish, as well as lens regeneration in axolotls [16]. Endogenous transdifferentiation as a means of tissue repair is however almost restricted to those lower vertebrates with striking regeneration capacities. One of the very few examples in mammals is the replenishment of hair cells after injury in the inner ear via Lgr5^{+} cell transdifferentiation, which is however restricted to neonatal stages and definitely not sufficient to restore significant damage [17]. Based on the paradigm of those organisms with better capacities to regenerate and thanks to the increasing knowledge of the specific

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developmental regulators - mainly transcription factors - that govern each cell type, in vivo transdifferentiation has been proposed as a therapeutic strategy in different mammalian tissues and disease models. The most relevant studies published to date are summarised in Fig. 1, where the targeted tissue and reprogramming factors involved in each study are highlighted. Further details of each particular study are summarised in Table 1 (in vivo transdifferentiation in liver and pancreas), Table 2 (in vivo transdifferentiation in the heart) and Table 3 (in vivo transdifferentiation in the central nervous system). In vivo transdifferentiation has the capacity to become a powerful tool in regenerative medicine. However, the challenges and pitfalls of this technology should be carefully analysed before the excitement surrounding reprogramming and regenerative medicine leads to misleading expectations. We review here the different strategies described so far to induce cell-to-cell fate conversions in vivo and discuss their promises and challenges in view of their therapeutic potential.

In Vivo Transdifferentiation in Liver & Pancreas

Although a few attempts to modify cell identity in vivo via gene transfer had been made before [6, 7, 18–21], the hype triggered by Yamanaka's discovery of transcription factor-induced cell reprogramming to pluripotency in 2006 [22] re-fuelled the attention to reprogramming and transdifferentiation as therapeutically-relevant strategies. Two years later, Zhou et al. attempted to tackle diabetic disease by reprogramming exocrine α-cells directly into endocrine, insulin producing β-cells in mouse pancreas [8]. Taking advantage of the preference of adenovirus to infect exocrine cells but not β-islets, pools of viral vectors carrying different transcription factors involved in the embryonic development and maturation of β-cells were directly administered in the pancreas. The combination of Ngn3, Pdx1 and MafA provided the highest efficiency in the transformation of exocrine cells into induced β-cells. Importantly, such cells could not be distinguished from their endogenous counterparts by size, shape or ultrastructure and, like them, produced VEGF and triggered angiogenesis. While their gene expression profile was almost identical to the endogenous β-cells, the genes characteristic of the exocrine phenotype were silenced, supporting their complete reprogramming. More importantly, induced β-cells not only produced insulin, but were also able to secrete the hormone in response to glucose when the animals were rendered diabetic by streptozotozin administration.

This has however not been the only attempt to alleviate diabetic disease via in vivo transdifferentiation. Various studies
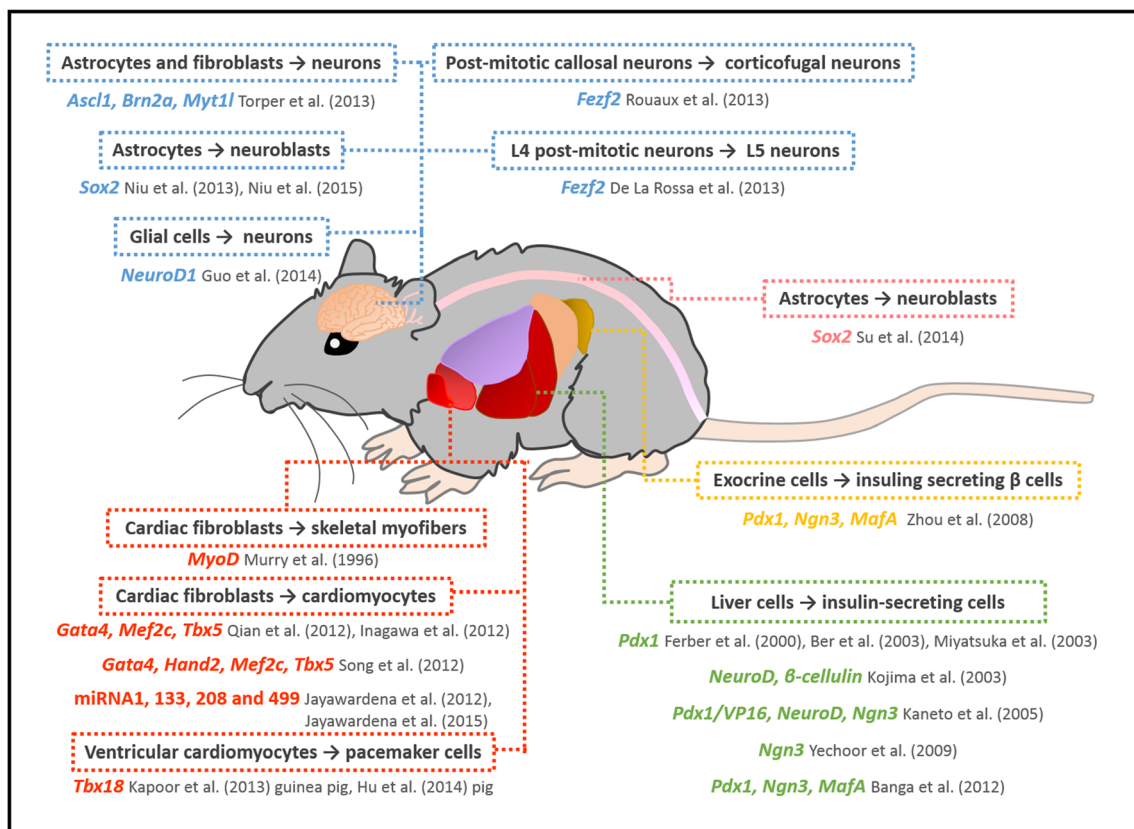

Fig. 1 In vivo transdifferentiation strategies in different tissues

Table 1 In vivo transdifferentiation studies in liver and pancreas. All studies were performed in mice

Tissue	Disease model	Starting cell	Induced cell	Reprogramming factors	Vector	Administration	Outcomes	Ref.
Liver	Diabetes	Liver (cell type not determined)	Mixed phenotype (Insulin and glucagon producing cells)	Pdx1	Adenovirus	Intravenous	Endocrine and exocrine pancreatic markers Insulin production Long term amelioration of hyperglycaemia	[7, 18]
Liver	None	Hepatocyte Cholangiocyte	Mixed phenotype (not complete reprogramming)	Pdx1	N/A (conditional Pdx1 transgenic model)	N/A	Endocrine and exocrine pancreatic markers Liver dysmorphogenesis and jaundice	[19]
Liver	Diabetes	Liver (cell type not determined)	Induced β-islet	NeuroD, β-cellulin	Adenovirus	Intravenous	Appearance of β-islet like structures in liver Long term amelioration of diabetes	[20]
Liver	Diabetes	Liver (cell type not determined)	Insulin-producing cell (hepatocyte phenotype remained)	Pdx1VP16, NeuroD1	Adenovirus	Intravenous	Insulin production in liver Amelioration of hyperglycaemia	[21]
Pancreas	Diabetes	α-cell	Induced β-cell	Pdx1, Ngn3, MafA	Adenovirus	Intrapancreatic	Generation of induced β-cells that secrete insulin in response to glucose. Long term alleviation of diabetes.	[8]
Liver	Diabetes	Hepatic progenitor/oval cells (not confirmed)	Induced (periportal) β-islet	Ngn3, β-cellulin	Adeno-associated viris	Intravenous	Transdetermination of hepatic progenitors No hepatocyte transdifferentiation Amelioration of diabetes	[23]
Liver	Diabetes	Sox9+ cell	Insulin-secreting duct	Pdx1, Ngn3, MafA	Adenovirus	Intravenous	Generation of long-lasting insulin secreting ducts in liver. Long term alleviation of diabetes.	[24]

Table 2 In vivo transdifferentiation studies in the heart

Species	Disease model	Starting cell	Induced cell	Reprogramming factors	Vector	Administration	Outcomes	Ref.
Rat	Freeze-thaw injury	Cardiac fibroblasts	Skeletal myofibers	MyoD	Adenovirus	Intramyocardial	Immature myofibers in tissue	[6]
Mouse	MI	Cardiac fibroblast	Cardiomyocyte	Gata4, Mef2c, Tbx5	Retrovirus	Intramyocardial	Decreased infarct size. Significant attenuation of cardiac dysfunction	[28]
Mouse	MI	Cardiac fibroblast	Cardiomyocyte	Gata4, Mef2c, Tbx5	Retrovirus	Intramyocardial	Cardiomyocyte-like cells in fibrotic area No functional data	[29]
Mouse	MI	Cardiac fibroblast	Cardiomyocyte	Gata4, Hand2, Mef2c, Tbx5	Retrovirus	Intramyocardial	Decreased infarct size Significant attenuation of cardiac dysfunction	[30]
Mouse	MI	Cardiac fibroblast	Cardiomyocyte	microRNAs 1, 133, 208 and 499	Lentivirus	Intramyocardial	Fibroblast to cardiomyocyte conversion in the infarct area Modest attenuation of cardiac dysfunction	[31, 32]
Guinea pig	Complete heart block	Ventricular cardiomyocyte	Pacemaker cell-induced sinoatrial node (iSAN) cell	Tbx18	Adenovirus	Intramyocardial	Establishment of a biological pacemaker Correction of bradycardia	[34]
Pig	Complete heart block	Ventricular cardiomyocyte	Pacemaker cell-induced sinoatrial node (iSAN) cell	Tbx18	Adenovirus	Percutaneous, to heart ventricule	Establishment of a biological pacemaker Correction of bradycardia	[35]

Table 3 In vivo transdifferentiation studies in the central nervous system. All studies were performed in mice

Tissue	Disease model	Starting cell	Induced cell	Reprogramming factors	Vector	Administration	Outcomes	Ref.
Brain	None	Fibroblast Astrocyte	Induced neuron (iN)	Ascl1, Brn2a, Myt1l	Lentivirus doxycycline inducible	Transduced in vitro Doxycycline in drinking water	iNs in tissue	[36]
Brain	None	Astrocyte	Induced adult neuroblast (iANB)	Sox2	Lentivirus	Stereotactic (brain)	iANBs in tissue Mature neurons (+ BNTP/Noggin or VPA)	[37, 38]
Brain	Stab injury Alzheimer's disease	Astrocyte NG2 cell	Induced neuron (iN)	NeuroD1	Retrovirus	Stereotactic (brain)	Mature iN in tissue No functional or behavioural data	[41]
Brain	None	Callosal projection neuron (CPN)	Corticofugal projection neuron (CFuPN)	Fezf2	Plasmid DNA	In utero electroporation	Changes in morphology, gene and protein expression (until P3) Changes in axon connectivity (until E17.5)	[42]
Brain	None	L4 neuron	L5B neuron	Fezf2	Plasmid DNA	In utero Iontoporation	Changes in morphology, gene and protein expression and axon connectivity (until P1)	[43]
Spinal cord	Spinal cord injury	Astrocyte	Induced adult neuroblast (iANB)	Sox2	Lentivirus	Stereotactic (spinal cord)	iANBs in tissue Mature neurons (+VPA) Synapses with resident neurons No functional data	[39]

in the early 2000s explored the possibility to reprogram liver cells into insulin-secreting cells, given the similar developmental origin of both organs [7, 18–21, 23]. However, some of these strategies were accompanied by a considerable degree of liver damage and, more importantly, they generated mixed endocrine/exocrine phenotypes instead of *bona fide* induced β-cells. The identity of the starting cells was also not confirmed, probably due to the limited availability of lineage tracing tools at the time. Notably, Yechoor et al. achieved transdetermination (a fate switch between closely related progenitor cells) of hepatic progenitors but not transdifferentiation of terminally differentiated hepatocytes [23].

While previous liver-to-pancreas transdifferentiation studies relied on one or two developmental regulators to switch cell identity, Banga et al. later found that the same combination of factors used by Zhou et al. in the pancreas (*Pdx1*, *Ngn3* and *MafA*) was also the most efficient to transdifferentiate Sox9^{+} cells in the liver into insulin-secreting cells. More importantly, such cocktail was the only one generating long-lasting ducts able to produce the hormone [24]. Transdifferentiation was confirmed in this study because the resulting cells expressed various β-cell markers and were able to produce and secrete insulin. However, their phenotype differed significantly from that of endogenous β-cells islets, probably accounting for the different microenvironment (liver vs pancreas), and therefore were regarded as a unique cell type.

Importantly, the therapeutic effects attained against diabetes persisted in both Zhou et al. (analysed for up to 9 weeks) and Banga et al. (analysed for 4 months). These findings agreed with the observations that the phenotype acquired upon transdifferentiation was stable even if achieved via transient expression of the *Pdx1*, *Ngn3* and *MafA* cocktail (in a single administration). Although these two studies represent the most efficient β-cell-like in vivo conversion achieved today, their main hurdle was precisely their limited efficiency. 20 % of the virally transduced cells were successfully converted in the pancreas in Zhou et al., a very satisfactory efficiency in comparison with the various orders of magnitude lower achieved with many in vitro reprogramming methods [25]. However, the resulting numbers of induced β-cells were still significantly lower compared to the endogenous β-cell population in non-diabetic counterparts. In Banga et al., the induced insulin-secreting ducts produced only 23 % of the insulin secreted by endogenous β-cells in healthy animals.

Adenoviral vectors like the ones utilised in these studies elicit strong immune responses that not only constitute a safety risk but also rapidly clear the viral particles and transduced cells from the organism [26]. In fact, Zhou et al. and Banga et al. based their studies on immunodeficient mice, but the latter confirmed a decrease in transdifferentiation efficiency when immunocompetent mice were subjected to the same procedures. It is therefore clear that the results obtained in such models cannot be directly extrapolated to a non-immunosuppressed scenario. Another

limitation in the Banga et al. study was the complete lack of targeting of the adenovirus vectors, which were administered intravenously relying on their preferred sequestration in the liver. However, the mechanism by which viruses were able to target Sox9^{+} cells and not hepatocytes or other cell types in the liver was not provided.

The studies discussed in this section provide encouraging results showing that in vivo transdifferentiated cells not only resembled their endogenous counterparts, but retained functionality within the tissue for extended periods of time. Even though such advances have opened new therapeutic insights in the treatment of diabetes, efficacy and safety concerns need to be addressed to allow clinical translation.

In Vivo Transdifferentiation in the Heart

The heart is perhaps the tissue most intensively investigated for in vivo transdifferentiation, motivated by the high prevalence and mortality rate associated with cardiovascular disease [27]. The first report, published by Murry et al. in 1996, attempted to regenerate freeze-thawed injured rat hearts via *MyoD* mediated reprogramming of cardiac fibroblasts into skeletal muscle fibers [6]. The study was to a certain extent limited by the immune response elicited against the adenoviral vector, which had to be administered at a high dose. In addition, no solid proof of complete transdifferentiation was obtained. The cells transfected with *MyoD* expressed myogenin and embryonic-MHC but there was no clear evidence of cell fusion, nor maturation of such embryonic myofibers into functional ones, let alone electrical coupling with endogenous cardiomyocytes (CMs). A possible explanation for the lack of complete maturation to an adult skeletal muscle phenotype was the presence of these myofiber-like cells in an ectopic (cardiac) microenvironment.

Since then, advances in cardiovascular development research have widened our knowledge on the several transcription factors coordinating the generation and maturation of CMs. As a result, it is now possible to attempt the replenishment of the injured heart with CMs instead of skeletal myofibers. These efforts have so far focused on heart repair after myocardial infarction (MI) and the generation of a biological pacemaker in situ.

Regeneration of Infarcted Hearts

In 2012, four independent publications aimed to regenerate the mouse myocadium after MI using similar strategies that converged in the attempt to transdifferentiate cardiac fibroblasts into induced CMs (iCMs) in vivo. Qian et al. and Inagawa et al. exploited the capacity of retroviruses to infect only dividing cells, so that the transcription factors *Gata4*,

Mef2c and *Tbx5* (GMT) would be delivered to the target cells (cardiac fibroblasts) exclusively and not to post-mitotic CMs [28, 29]. MI is an ideal disease platform to evaluate this therapeutic strategy since fibroblasts migrate to the injury site and proliferate actively, providing more cells available for retroviral transfection. Qian et al. confirmed the fibroblast origin of iCMs and their close resemblance to their endogenous counterparts in terms of morphology, sarcomeric organisation, gene expression, cell-to-cell junctions and electrical behaviour. Similar to ventricular CMs, iCMs contracted upon electrical stimulation. Importantly, cardiac function was significantly improved in the GMT transduced group from week 8 after MI. This effect was sustained for the duration of the study (12 weeks) and was accompanied by a significant reduction in the scar area and an improvement in the levels of relevant MI markers. While the safety profile was satisfactory - no arrhythmias or sudden deaths were reported - efficiency seemed to be the main limitation in this work. 4 % of the fibroblasts in the boundaries of the infarcted zone were transduced and of those only 10–15 % were successfully transdifferentiated. Although transdifferentiation efficiency did not change with the co-administration of Thymosin-β4, known to stimulate fibroblast proliferation, the increased numbers of total and subsequently transduced fibroblasts eventually led to a significant improvement in cardiac function compared to the group that received GMT alone.

Lack of efficiency was an even more detrimental obstacle in Inagawa et al. Although retroviral vectors were similarly used to deliver GMT factors, the efficiency of cardiac fibroblast transdifferentiation was 10-fold lower compared to Qian et al., and the resulting iCMs were not as mature. Due to these disappointing results, the electrical properties of iCMs and the recovery of cardiac function after infarction were not investigated further. However, this study provided valuable observations. First, the fast clearance (2 weeks) of GMT transduced cells observed in immunocompetent mice was not found when the study was repeated in an immunodeficient strain. Immune clearance of the transduced cells might therefore have been one of the causes for the low efficiency encountered by Inagawa et al. However, other factors may also play a role, since the efficiency achieved in immunodeficient mice still failed to reach the levels obtained by Qian et al. On the other hand, the latter did not suffer from immune clearance limitations even though no immunosuppression was involved. In fact, transduced cells were detected within the tissue even 4 weeks after viral administration in Qian et al. Overall, the discrepancies discussed here manifest that further efforts are needed to determine the host responses to different vectors before such transdifferentiation strategies can be extrapolated to the clinical scenario.

Many of the studies published today have found that transdifferentiation is more complete when performed in vivo than in the culture dish, most probably due to the effects of the native microenvironment in cell survival and maturation and the interaction with neighbouring cells [8, 28]. Inagawa et al. observed that in vitro generated iCMs shared great similarity with neonatal CMs, whilst in vivo iCMs were closer to endogenous adult CMs. This finding further highlighted the importance of the native microenvironment to achieve fully mature and functional cells that could be able to contribute to the functional recovery of injured tissue.

Song et al. followed a similar strategy, but added an extra transcription factor, *Hand2*. The rationale behind this was the improvement in cell transdifferentiation efficiency observed when *Hand2* was co-administered with the GMT cocktail in vitro [30]. In vivo, the addition of *Hand2* to this combination of reprogramming factors boosted the efficiency of iCM generation from 1.4 to 6.8 %. In addition, the recovery of cardiac function after MI was more significant when *Hand2* was included and this improvement, which was sustained for the duration of the study (12 weeks), was accompanied by a reduction of fibrosis and increase in muscle tissue compared to controls.

Jayawardena et al. attempted the same cell fate conversion after MI, but based on the lentiviral administration of a cocktail of four micro-RNAs (miRNA 1, 133, 208 and 499) involved in CM development [31]. An initial study only reported morphological and molecular characterisation of in vivo iCMs. Although the potential advantages of using micro-RNAs instead of transcription factors were highlighted, these were not confirmed by the endpoints of the study. First, the smaller size of miRNA allowed higher nucleic acid doses loaded on a single vector and therefore the delivery of more copies of reprogramming elements per virion, yet substantial improvements in reprogramming efficiency were not evidenced. It was also argued that miRNA could be relatively easily delivered without the need of viral vectors, however this study still relied on lentivirus for the administration of the reprogramming cocktail. A second report by the same group [32] confirmed that the electrical properties of a subset of iCMs were similar to those of endogenous CMs and that the generation of such cells in situ resulted in a modest improvement in cardiac function compared to controls. However, a substantial subset of reprogrammed cells remained as small-sized, immature iCMs, indicating that further optimisation was required to ensure the generation of sufficient numbers of fully functional cells to replenish the infarcted heart tissue.

The studies discussed in this section were able to confirm via different lineage tracing or co-transduction strategies, the fibroblastic origin of iCMs. As discussed previously, targeting the abundant fibroblast population is a strategic approach to generate replacement cells in infarcted heart tissue, but some questions arise from the design of these studies. First, fibroblast targeting was either not explained, as in both works by Jawayardena et al., or relied on the incapacity of retroviral vectors to infect non-dividing CMs. More clinically-friendly

strategies should be designed to ensure fibroblast targeting. Secondly, reprogramming factors were in all cases administered immediately after MI induction by coronary artery ligation. While prompt treatment is indeed desirable after MI, it might not always be readily available, thus it would be very interesting to investigate how increasing time after MI affects the efficiency and outcomes of in vivo transdifferentiation. Transdifferentiation might be more efficient if induced at later time points after the injury, since more fibroblasts are predicted to migrate towards the infarct site. This was also the rationale behind Murry et al. reporting the 1 week post-injury administration of *MyoD* to convert fibroblasts into skeletal muscle. One week after injury the majority of the post-infarction inflammatory response had been resolved and fibroblasts had proliferated, however there was still no dense scar tissue that could hinder the delivery of the nucleic acids [6].

Generation of an Endogenous Pacemaker

The generation of a biological pacemaker to replace electronic devices – the only currently available option in the clinic – has long been pursued with little success [33]. In vivo transdifferentiation has however been utilised to offer an alternative. *Tbx18* plays an important role in the embryonic development of the sinoatrial node (SAN), a small group of approximately 10,000 cells responsible for initiating the heartbeat, but this transcription factor is no longer expressed after birth. Kapoor et al. found that forced expression of *Tbx18* reprogrammed rat neonatal ventricular CMs into induced SAN (iSAN) cells in vitro. Converted cells resembled their endogenous neonatal counterparts, not only in terms of morphology, gene expression and epigenetic signature but also in their electrophysiological properties and capacity to beat spontaneously [34]. More importantly, the same cell fate conversion was induced in situ via direct injection of adenoviral vectors expressing *Tbx18* in the apex of adult guinea pig hearts. In agreement with the in vitro studies, in vivo iSAN cells closely resembled the morphology and electrophysiology features of endogenous adult pacemaker cells, however no studies were performed to investigate their gene expression profile or epigenetic status. This information would be of considerable interest since the native microenvironment is predicted to have an effect on the maturation of the cells and, while in vitro studies were conducted on neonatal specimens, adult cells would be a more relevant model. Alleviation of bradycardia after complete heart block using the guinea pig model encouraged the same group to translate their findings in a large animal model (pig) [35]. In this subsequent work, an electronic pacemaker was implanted as a backup prior to surgically-induced complete heart block. Immediately after, the adenoviral vectors expressing *Tbx18* were administered via a percutaneous catheter. The median and maximum heart

rates were higher in *Tbx18* - transduced animals compared to controls as early as 2 days after viral administration and for the duration of the study. In agreement, *Tbx18*-transduced animals relied less in the electronic backup pacemaker than the control group. In line with the observations in other in vivo transdifferentiation studies, the efficiency of reprogramming was not high in the guinea pig (9.2 %) nor in the porcine model (24 %). However, this was not regarded as a limitation given that the cell numbers forming the endogenous SAN are indeed low. Even if this region consists of a small cell population, the role of the SAN node is crucial in generating the heartbeat, hence close monitoring for potential disruptions in the heart rate was mandatory. Generation of an ectopic pacemaker via iSAN transdifferentiation did not increase arrhythmic risk, therefore positioning this work as the first study to provide significant heart rate support with a biological pacemaker, induced via a minimally invasive surgery and without major cardiovascular safety concerns.

The switch in cell fate was also stable, and SAN phenotype was sustained irrespectively of the levels of *Tbx18* expression. However, the functional improvements reported in the *Tbx18* transduced group in the porcine model tended to decrease over time when monitored for 14 days after heart block and adenovirus administration. In spite of this decay, the heart rates remained significantly improved compared to controls. Such trend was however not observed in the guinea pig model, where animals were treated with cyclosporine A for the duration of the study, and might be caused by immune clearance of adenovirus transduced cells. Longer-term studies should be designed to investigate whether functional improvement is eventually lost. Together with the suspected immune clearance of iSAN cells, the lack of vector targeting also merits attention. Although only 2.1 % of virus was sequestered in the spleen and 0.3 % in the lungs, potential off-target effects in these organs should be carefully addressed. In addition, there was no conclusive evidence that adenovirus did not infect other cell types in the heart, apart from CMs.

In Vivo Transdifferentiation in the Central Nervous System

Intense efforts are also made to change cell identity in situ in the brain and central nervous system (CNS) due to the prevalence of neurodegenerative diseases. However, the literature is more scattered in terms of starting and induced cell types, reprogramming factors used, disease models investigated and outcomes achieved.

Torper et al. offered the first proof-of -principle of in vivo transdifferentiation in adult mouse brain, achieved via viral delivery of *Ascl1*, *Brn2a* and *Myt1l* and using both transplanted (fibroblasts and astrocytes) and resident cells (astrocytes) as starting cells. The feasibility to induce specific

neuronal populations (dopaminergic, DA neurons) was also confirmed [36]. The sophisticated transplantation and transgenic approaches used in this study make it more relevant to basic research than to clinical translation. However, this work already pointed at a specific glial cell type, the astrocytes, as convenient target cell population to be reprogrammed into neurons due to their abundance and ubiquitous distribution in the brain.

That study is not unique in attempting astrocyte to neuron transdifferentiation. Niu and colleagues published a study in which administration of a lentiviral vector with the astrocyte-specific GFAP promoter, converted these target cells into neuroblasts in adult mouse brain. Importantly, a single factor (*Sox2*) was sufficient to command the change in cell identity [37]. In contrast to Torper et al., where induced cells expressed neuronal markers and did not divide, this strategy yielded proliferative precursors similar to endogenous neuroblasts, thus termed 'induced adult neuroblasts' (iANBs). Co-administration of neurogenic factors (BNTP and Noggin) or an epigenetic modulator (valproic acid, VPA) was required to promote further maturation of iANBs into electrophysiologically functional, mature neurons that integrated into local circuits in vivo. It has been recently reported that such induced mature neurons belong to different subtypes but predominantly express calretinin [38].

The same group has recently reproduced the astrocyte to neuroblast conversion following spinal cord injury, again mediated by *Sox2* [39]. GFAP-lentiviral vectors were also used to benefit from increased astrocyte proliferation in the spinal cord after the insult, set to form the glial scar. According to this approach, what is normally seen as a detrimental event, since glial scarring ultimately blocks axon regeneration [40], can be used to obtain a larger pool of reprogrammable cells. In vivo transdifferentiation in adult brain and spinal cord has however proven as inefficient as in other organs. Only 3–6 % of the spinal cord transduced astrocytes were effectively reprogrammed into iANBs and, as in the brain, VPA co-administration was indispensable to generate mature neurons. iANB-derived GABAergic interneurons formed synapses with resident neurons, however the study did not explore whether such synapses supported exchange of information or contributed to functional rehabilitation. In addition, the administration of the virus at the time of injury may act in detriment of the efficiency of cell conversion, since higher numbers of astrocytes are expected to be available for reprogramming at later time points.

Retroviral expression of a different transcription factor, *NeuroD1*, similarly converted reactive glia (including astrocytes and NG2 cells) into mature and functional neurons which integrated into local neural circuits and survived for at least 2 months in the mouse brain [41]. *NeuroD1* was sufficient to drive the generation of mature neurons without co-administration of other factors or epigenetic modulators. Such

cell fate conversion was achieved in two injured scenarios, stab-injured brain tissue (as a result of the stereotactic injection) and degenerated brain tissue (using the 5xFAD mouse transgenic model of Alzheimer's disease). Higher numbers of induced neurons were generated in diseased vs. wild-type mice, and even more were obtained in diseased aged animals, which correlated with the increased glial cell population present in such conditions as a result of the disease or age-related degeneration.

Such findings confirmed that cell reprogramming can be induced even when a disease phenotype has been established for a prolonged period of time and that age does not seem to pose an obstacle in the conversion to a different cell type. The fact that *NeuroD1* has also been reported to transdifferentiate human astrocytes into glutamatergic neurons in vitro is in addition very encouraging. However, these conclusions should be perceived with caution, since they have been based on observations obtained from a single model and evidence of behavioural rehabilitation remains unconfirmed.

Induction of fate changes between different types of neurons is thought to be more challenging due to their post-mitotic status. Rouaux and Arlotta relied on in utero electroporation to attempt transdifferentiation between excitatory neurons in the mouse brain cortex [42]. *Fefz2*, a key factor in corticofugal projection neurons (CFuPNs), was specifically expressed in callosal projection neurons (CPNs) at embryonic day 14.5 (E14.5), or using a conditional expression cassette at later embryonic (E17.5) and post-natal (P3, P21) stages. When *Fezf2* was expressed at E14.5, E17.5 or P3, CPNs acquired gene and protein expression profiles similar to those of CFuPNs. A significant proportion of converted cells sustained those changes for up to 4 weeks, although it was not clear whether the CPN phenotype was completely abolished. At P21, *Fefz2* expression was unable to trigger any changes in the molecular signature of CPNs. Changes in axon projection and connectivity were also found when the conversion was induced at E14.5 or E17.5. There is no confirmation that such effect can be achieved at later stages, since after E17.5 axonal extension towards the different anatomical regions of the brain is completed in normal development.

An independent study reported very similar findings almost simultaneously. *Fefz2* was also administered in the mouse cortex, however iontoporation and CreERT2 technology were utilised to trigger the expression of the transcription factor at different developmental stages [43]. In addition, the use of specific promoters allowed restriction of expression to specific neuronal populations. L4 spiny neurons were successfully transdifferentiated into L5B neurons in terms of morphology, molecular signature, axon disposition and input/output connectivity. Very importantly, and in agreement with the findings by Rouaux and Arlotta, such conversion was only possible during the first post-natal week.

Even if the contribution of these two studies seems far from therapeutic applications, they have provided sound evidence that the identity of post-mitotic neurons can be reprogrammed. More importantly, they have for the first time pointed at the existence of a time window (from embryonic to early post-natal stages) when a specific cell-to-cell conversion is possible. This will critically determine the therapeutic applications – if any – envisioned for such cell identity switch. Neurodegenerative and age-related diseases will most probably be the first to be crossed out from the list.

Future Perspectives

Promises an Challenges of in vivo Transdifferentiation

The interest in reprogramming technologies – including transdifferentiation – is growing as new applications are unveiled and is reflected in the increasing number of studies exploring such strategies in the last few years. This review article attempted to highlight the efforts to transdifferentiate cells in vivo with therapeutic purposes. The reports published to date, even if still scarce and preliminary, have already generated valuable knowledge regarding the plasticity of various differentiated cell types within their native microenvironment. In addition, some of the studies discussed here have generated encouraging results in utilising cell transdifferentiation within diseased or injured tissue, which highlights its therapeutic potential even though still in early days.

Common evidence drawn from these reports is the crucial role of the native microenvironment in the survival and maturation of the induced cells. Attempts to generate cells in an ectopic microenvironment, such as in Murry et al. [6], provided inconclusive results. In addition, many of these studies relied on in vitro experimentation to screen different developmental regulators that would trigger cell-to-cell changes, before moving to the in vivo setup once the optimal single transcription factor or combination of factors was identified. Direct comparison of in vitro and in vivo reprogrammed cells suggested that those switched to a different phenotype within living tissue, better resembled their endogenous counterparts in terms of morphology, function, degree of differentiation and interaction with neighbouring cells.

This observation can place in vivo transdifferentiation in an advantageous position over traditional cell therapy. Indeed it will be extremely difficult to recapitulate all the combinations of biochemical and mechanical cues offered by the native microenvironment of the tissue to induce a particular phenotype in the culture dish. In addition, in vivo transdifferentiation can circumvent other limitations associated to cell transplantation strategies such as culture-derived genomic aberrations and the challenges of cell delivery.

Despite such advantages, there are various other aspects of the in vivo transdifferentiation approach that need to be resolved prior to clinical translation. The efficiency of cell conversion acts as a limiting factor in most of the studies. Co-administration of substances to mobilise and increase the pool of cells available for reprogramming can modestly alleviate the problem [28]. Other strategies that include an intermediate proliferative stage have not yet reported significantly higher efficacy compared to those involving direct reprogramming to a non-dividing phenotype [37, 39]. Reprogramming the target differentiated cells to a more plastic, pluripotent state and relying on the native microenvironment to drive re-differentiation may also provide an alternative approach in some disease settings, especially now that fears of teratoma generation have been overcome and there is growing evidence of successful re-integration of the reprogrammed cells within the tissue [44–46].

In vivo transdifferentiation has so far relied heavily on the use of viral vectors for the delivery and expression of reprogramming factors, which may also complicate to a certain extent the development towards the clinic. Various studies have used retrovirus in order to restrict the infection exclusively to dividing cells. Engineering safer vectors that avoid the use of viruses and offer more sophisticated targeting of specific cell types is desirable. Finally, in those approaches with therapeutic endpoints, it is essential to investigate the effects and outcomes of in vivo transdifferentiation by administration of the transcription factors at different time points *after* the onset of injury. All the studies published so far, except that from Murry et al. [6], induced cell fate conversion at the time of injury. This not only diminishes clinical relevance, but may have also underestimated the therapeutic potential of those strategies that rely on the injury-triggered proliferation of particular cell types to be available for transdifferentiation.

Overall, in vivo transdifferentiation has the potential to become a very powerful tool in tissue repair and regeneration, however careful and systematic work still needs to be performed before overenthusiastic and unmeasured expectations of the therapeutic promise of the approach are communicated.

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