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SPOTLIGHT

Reprogramming cellular identity *in vivo*

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ABSTRACT

Cellular identity is established through complex layers of genetic regulation, forged over a developmental lifetime. An expanding molecular toolbox is allowing us to manipulate these gene regulatory networks in specific cell types *in vivo*. In principle, if we found the right molecular tricks, we could rewrite cell identity and harness the rich repertoire of possible cellular functions and attributes. Recent work suggests that this rewriting of cell identity is not only possible, but that newly induced cells can mitigate disease phenotypes in animal models of major human diseases. So, is the sky the limit, or do we need to keep our feet on the ground? This Spotlight synthesises key concepts emerging from recent efforts to reprogramme cellular identity *in vivo*. We provide our perspectives on recent controversies in the field of glia-to-neuron reprogramming and identify important gaps in our understanding that present barriers to progress.

KEY WORDS: Adeno-associated virus, Brain repair, Direct reprogramming, Glia-to-neuron conversion, Proneural, Regenerative medicine

Introduction

Many mammalian tissues lack dedicated stem cells yet exhibit remarkable regenerative capacity (Willet et al., 2018). For example, following acute tissue damage, the liver can fully regenerate tissue mass and restore homeostatic functions. Liver regeneration is typically accomplished by differentiated cells (e.g. hepatocytes or cholangiocytes) re-entering the cell cycle to replenish their own cell populations. Interestingly though, recent work suggests that, under certain conditions, both cell types are capable of transdifferentiation into the lineage of the other (Raven et al., 2017; Schaub et al., 2018), providing an example of an intrinsic capacity of some cells to rewrite their identity. However, endogenous regenerative mechanisms are often overwhelmed by severe insults, and some terminally differentiated cell types, such as cardiomyocytes and neurons, show little or no regeneration following injury (Senyo et al., 2013; Zamboni et al., 2020).

Direct lineage reprogramming strives to generate specific cell types by harnessing the inherent cellular plasticity of other cells and engineering conversion of cellular identity (Fig. 1). Reprogramming is usually accomplished through forced expression of transcription factors or regulatory RNAs in a suitable starting cell type, often in conjunction with other protein or small molecule co-factors (Wang et al., 2021a). Recent years have seen an explosion of reports demonstrating high-efficiency conversion of cellular identity *in vivo* across multiple tissues. These studies have restored function in animal models of disabling human diseases (e.g. myocardial infarction, diabetes and Parkinson's disease, etc.), hinting towards transformative translational applications. However, some of the breakthroughs have been met with scepticism, exposing major gaps in our understanding of the biology of reprogramming.

In this Spotlight, we highlight recent studies in reprogramming cellular identity *in vivo*. We draw on progress across multiple tissues; however, we particularly focus on CNS glia-to-neuron conversion. The first section aims to distil some general properties that render cells conducive to lineage conversion *in vivo*. We then consider emerging themes in our currently limited understanding of the journey that reprogramming cells undergo during conversion *in vivo*. Along the way, we discuss controversial technical concerns that have been raised in glia-to-neuron reprogramming and provide perspectives on future directions.

A question of competence

The adult pancreas provides several examples of cellular de-differentiation and transdifferentiation in response to injury (Zhou and Melton, 2018). Many groups have sought to exploit the inherent cellular plasticity of some pancreatic cells to generate new insulin-secreting β-cells, with a view to treating diabetes. A screen of transcription factors important during β-cell development has identified a combination of genes [*Pdx1*, *Neurog3* and *Mafa* (PNM)], the expression of which is sufficient to convert acinar cells of the adult mouse pancreas into insulin-secreting β-like cells (Zhou et al., 2008). Similar transcription factor combinations can convert other pancreatic cell types (e.g. ductal and α-cells) and, surprisingly, even liver hepatocytes into insulin-secreting cells (Ferber et al., 2000; Wang et al., 2018; Xiao et al., 2018). Hepatocytes are ontogenetically more distant from β-cells than from the pancreatic lineages, raising the issue of whether a close developmental relationship between the starting and induced cell type is particularly important in lineage conversion. Indeed, a comprehensive comparison of PNM factors expressed in several lineages *in vivo* has shown that pancreatic duct-derived β-like cells are produced faster and with greater fidelity to endogenous β-cells than those derived from hepatic lineages (Wang et al., 2018). Hepatocyte-derived insulin-secreting cells shut down insulin production over time, whereas pancreatic duct-derived β cells show immature but stable β-cell gene expression, improving long-term glycaemic outcomes in diabetic mice (Wang et al., 2018). Thus, a close ontogenetic distance appears conducive to successful reprogramming outcomes *in vivo* (Fig. 2A).

Even where ontogenetic relations are more distant, lineage conversion is still possible by exploiting injury-induced proliferative states (Fig. 2B). For example, in the heart, cardiomyocytes can proliferate but do so infrequently (Senyo et al., 2013), and at an insufficient rate to regenerate tissue following a significant injury. The heart responds to injury with fibrosis, characterised by 'activation' of cardiac fibroblasts, which proliferate and undergo phenotypic transformation, leading to increased matrix

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deposition that can impair tissue function (Tallquist and Molkentin, 2017). Expression of *Gata4*, *Mef2c* and *Tbx5* converts proliferating cardiac fibroblasts in the infarcted rodent myocardium into cardiomyocyte-like cells, which fire action potentials and improve functional outcomes during recovery (Chang et al., 2019; Isomi et al., 2021; Miyamoto et al., 2018; Qian et al., 2012; Song et al., 2012). These transcription factors engage closed chromatin in fibroblasts and act cooperatively at super-enhancers to promote a cardiomyocyte fate (Stone et al., 2019). Fibroblast-to-cardiomyocyte conversion can also be achieved *in vivo* using regulatory microRNA expression (Jayawardena et al., 2015; Wang et al., 2021d; Yang et al., 2021). Furthermore, regulatory RNAs can stimulate endogenous cardiomyocyte proliferation as an alternative approach for functional cardiac regeneration (Gabisonia et al., 2019) and provide an example of *in vivo* regenerative tissue engineering that does not seek to change cellular identity (Fig. 1).

In contrast to cardiomyocytes, terminally differentiated mammalian neurons of the central nervous system (CNS) do not proliferate. A few specialised neural stem cell niches exist in the CNS but, outside of these, there is no neurogenesis during adulthood (Denoth-Lippuner and Jessberger, 2021). Instead, the direct lineage conversion of brain-resident glia to produce new neurons has received much attention (Box 1). Recombinant γ-retroviruses, which rely on mitosis for genome integration, can be used to selectively transduce proliferating glia. It has been shown that, after CNS injury, γ-retrovirus encoding neurogenic transcription factors (e.g. *Neurog2*, *Sox2*, *Ascl1* and *Dlx2*) can steer

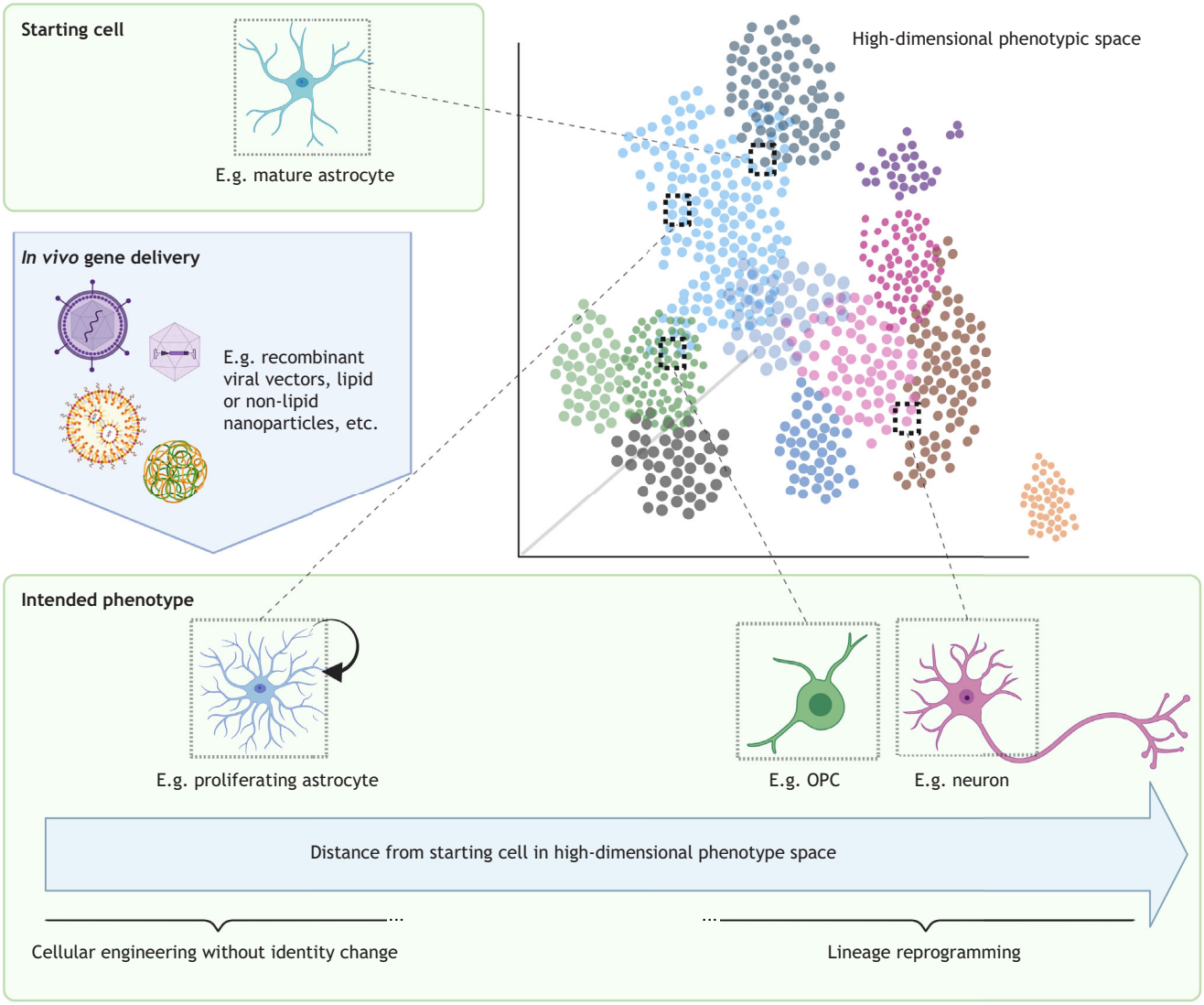

Fig. 1. Cell lineage reprogramming in context. Cellular identity may be viewed as a conceptual grouping of cells that are close neighbours in a high-dimensional space of biological functions and attributes (top right). *In vivo* cellular engineering strategies make defined changes to starting cells (typically by delivering genes or gene products) with a new intended phenotype as the goal. In many cases, these phenotypic changes do not fundamentally change how we might classify their identity (e.g. inducing a proliferative state). How much phenotypic change is necessary to be deemed a new identity is difficult to define. Instead, cellular engineering activities may be viewed to exist on a spectrum of how distant the new phenotype is from the starting cell in high-dimensional phenotypic space (bottom). We loosely define lineage reprogramming as existing towards one end of this spectrum, where the intended phenotype is very distant to the starting cell and, as such, is better described as a new identity (e.g. astrocyte-to-neuron conversion).

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proliferating glia towards a neuronal identity (Gascón et al., 2016; Guo et al., 2014; Heinrich et al., 2014; Lentini et al., 2021; Yamashita et al., 2019). In the first weeks of reprogramming, transduced glia acquire immature neuronal morphology and express immature neuronal markers, such as neuronal migration protein doublecortin (DCX) (Gascón et al., 2016; Guo et al., 2014; Heinrich et al., 2014; Lentini et al., 2021; Yamashita et al., 2019). During the following weeks, some induced neuroblasts die; however, others express proteins that are found in mature neurons (e.g. NeuN and MAP2) develop more complex morphology and fire action potentials (Fig. 3A) (Gascón et al., 2016; Guo et al., 2014; Heinrich et al., 2014; Lentini et al., 2021). By using lentivirus (which can also transduce non-mitotic cells), it has been shown that an injury is a pre-requisite for *Sox2*-mediated glia-to-neuron conversion in the adult neocortex (Heinrich et al., 2014). Therefore, injury induces reactive and proliferative glial states (Box 1) that appear conducive to lineage reprogramming (Fig. 2B,C).

In the striatum, astrocytes can activate a neurogenic programme after injury alone (Magnusson et al., 2014; 2020; Nato et al., 2015), contrasting with the neocortex, where exogenous reprogramming factors are required for glia to undergo neurogenesis after injury (Sirko et al., 2013; Zamboni et al., 2020). Lentiviral expression of *Sox2* induces proliferation of striatal astrocytes and activates a neurogenic programme even in the absence of injury (Niu et al., 2013; 2015; Wang et al., 2016). In the retina, injury appears important to facilitate the lineage conversion of Müller glia (MG) to neurons (Jorstad et al., 2017; 2020; Sanges et al., 2016; Todd et al., 2021; Ueki et al., 2015; VandenBosch et al., 2020). However, recent work suggests that MG-to-neuron conversion is possible in the absence of injury – if *Ascl1* is co-expressed with another transcription factor, *Atoh1* (Todd et al., 2021). These data from the retina seem consistent with findings in the cochlea, where forced expression of *Atoh1* alone can convert supporting glia-like cells (SCs) of the organ of Corti into sensory hair cells (HCs) in the absence of injury (Liu et al., 2012; Walters et al., 2017). SCs are usually non-proliferative but proliferate during *Atoh1*-mediated conversion to HCs (Lee et al., 2020; Sun et al., 2021; Walters et al., 2017; Yamashita et al., 2018). Our interpretation of these data is that lineage reprogramming heavily exploits heightened cellular plasticity that exists during proliferation (Fig. 2). Injury is also conducive to reprogramming; however, it is less clear whether this is through stimulating proliferation or independent cellular processes that promote other progenitor-like properties.

Another intuitive but important property of starting cells that make them amenable to lineage conversion is developmental age (Fig. 2D). In the mouse retina, MG-to-neuron conversion with *Ascl1* is possible only up to around the third postnatal week, after which additional molecular interventions are necessary for reprogramming (Jorstad et al., 2017; 2020). Similarly, SC-to-HC conversion can be achieved by forced expression of *Atoh1* in neonatal and juvenile mice, but not in adults, where additional factors are required (Lee et al., 2020; Sun et al., 2021; Walters et al., 2017).

Taken together, direct lineage conversion *in vivo* exploits states of heightened cellular plasticity that exist across many organs and cell types following injury, during proliferation and in youth. The process is assisted by close ontogenetic relationships between converted cells, presumably because they have favourable epigenetic makeup. Understanding how reprogramming factors cooperate with cellular processes that occur following cell injury will be crucial to inform targeted approaches to improve cell conversion *in vivo*.

AAVs for glia-to-neuron conversion: a cautionary tale

Recombinant adeno-associated viruses (AAVs) are highly efficient gene transfer tools with an excellent clinical safety profile and extremely broad tropism (Wang et al., 2019). AAV-mediated

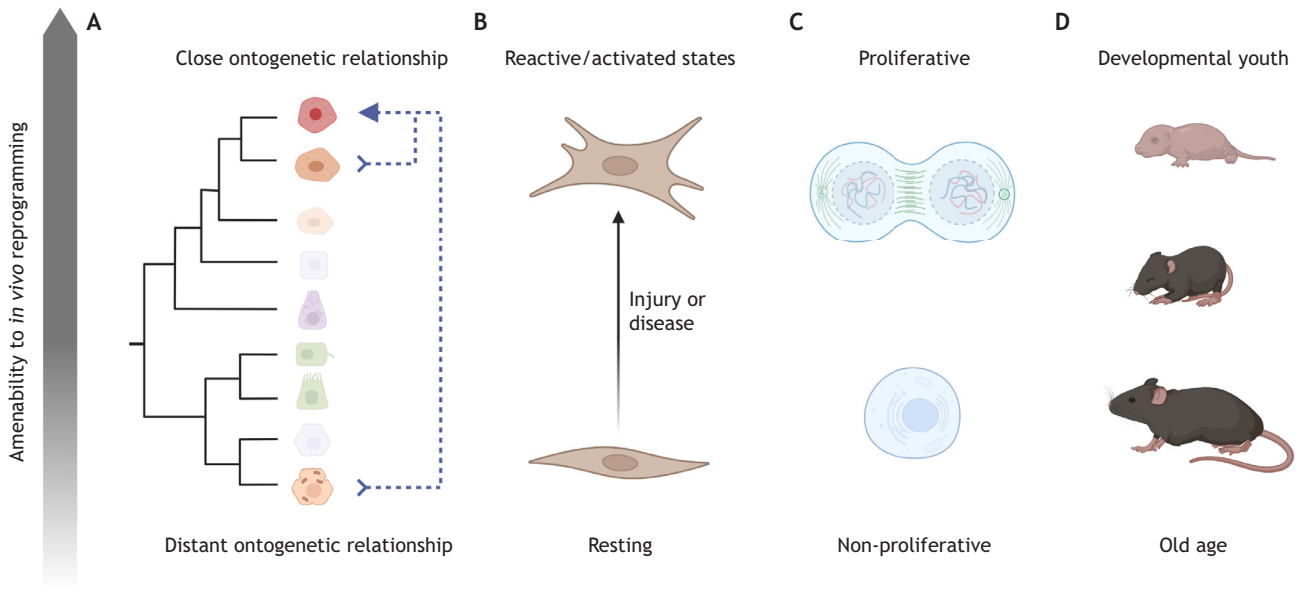

Fig. 2. General properties of starting cells that are conducive to *in vivo* lineage reprogramming. Lineage reprogramming *in vivo* exploits certain properties of endogenously residing cells that are permissive to manipulation of cellular identity. A close ontogenetic relationship with the intended identity (A), post-injury response state (B), proliferation (C) and developmental age (D) are general features of starting cells that researchers commonly exploit to engineer conversion of cellular identity across all tissues. Understanding how these states interact with reprogramming factors may help us to understand how cellular identity is safeguarded and to identify molecular candidates for improved engineering strategies.

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Box 1. Glia: facultative neural stem cells of the CNS?

Astrocytes and oligodendrocyte progenitor cells (OPCs) are abundant in the CNS with diverse homeostatic roles and participate in various aspects of neuronal function (Zuchero and Barres, 2015). Astrocytes, OPCs and neurons are all derived from radial glial cells during neurodevelopment (Zuchero and Barres, 2015).

OPCs in the white matter differentiate into myelinating oligodendrocytes. Despite their name, OPCs have distinct functions in CNS grey matter that are still being investigated (Bedner et al., 2020). These cells are proliferative throughout life, although they are usually slow cycling. As a response to injury, OPCs can proliferate rapidly, acquire progenitor-like features and may even be able to transdifferentiate into other lineages, such as astrocytes (Bedner et al., 2020; Kirdajova et al., 2021).

Most astrocytes are post-mitotic during adult life (Ge et al., 2012). However, emerging evidence suggests that diencephalic astrocytes show transcriptional similarity with neural stem cells and can continue proliferating into adulthood, giving rise to new astrocytes (Ohlig et al., 2021). Following injury, astrocytes adopt reactive states whereby they acquire progenitor-like features (Buffo et al., 2008), as well as mediate important aspects of inflammation and neuroprotection (Sofroniew, 2020). Some reactive astrocytes can proliferate, although this tends to be limited (Bardehle et al., 2013). Astrocytes can activate a neural stem cell-like programme after injury and manipulation of Notch or Sonic hedgehog signalling (Sirko et al., 2013; Zamboni et al., 2020). In the injured striatum, injury alone can lead to neurogenesis, although this is limited (Magnusson et al., 2014; 2020; Nato et al., 2015).

Thus, astrocytes and OPCs show stem cell-like properties that can be stimulated in certain contexts, echoing evolutionarily conserved mechanisms of brain regeneration in other vertebrate species (Alunni and Bally-Cuif, 2016). Thus, both cell types make attractive starting cell populations for neuronal reprogramming *in vivo*.

conversion of activated myofibroblasts to hepatocytes have been used to mitigate chronic liver fibrosis (Rezvani et al., 2016). AAVs have also been used to treat diabetes in mice by generating induced insulin-secreting β-like cells in the pancreas (Xiao et al., 2018).

In recent years, numerous publications have reported highly efficient glia-to-neuron conversion in the CNS using AAVs (Ge et al., 2020; Mattugini et al., 2019; Pereira et al., 2017; Qian et al., 2020; Tang et al., 2021; Wu et al., 2020; Zhou et al., 2020). These putative induced neurons display remarkable fidelity to mature endogenous counterparts and improved functional outcomes in animal models of neurological disease (Qian et al., 2020; Tang et al., 2021; Wu et al., 2020; Zhou et al., 2020). Naturally, this has attracted enormous excitement within – and beyond – the field.

However, some lessons from direct *in vivo* reprogramming discussed in the previous section (Fig. 2) are quite different from the picture presented in reports using AAVs in the CNS, even where similar reprogramming factors have been used. First, AAV-mediated glia-to-neuron reprogramming is reported to be highly efficient throughout the adult CNS, even without acute injury (Liu et al., 2015; Wu et al., 2020; Zhou et al., 2020). Second, putative reprogrammed neurons are derived from mature glia that do not pass through proliferate stages during conversion (Liu et al., 2015; Mattugini et al., 2019; Weinberg et al., 2017). Finally, there is a curious absence of compelling evidence for immature phenotypes or intermediate states (Ge et al., 2020; Liu et al., 2015; Mattugini et al., 2019; Pereira et al., 2017; Qian et al., 2020; Tang et al., 2021; Wu et al., 2020) (Fig. 3B). Therefore, these studies suggest that adult, non-proliferative glia may exhibit more plasticity and neurogenic potential than younger, proliferating glia.

This paradox has raised much scepticism about the interpretation of these results, culminating in a landmark paper suggesting that the genetic tools used to restrict AAV transgene expression to glia have not been sufficiently specific (Wang et al., 2021c). Careful lineage-tracing experiments (including retrograde labelling of endogenous neurons in the motor cortex via spinal cord injection before reprogramming) have shown that AAVs gradually express reporter transgenes in pre-labelled endogenous neurons (Fig. 3B). It remains unclear why these tools lose glial specificity over time. It may be due to the specific genetic elements used to regulate viral transgene expression in these studies. In this case, using different regulatory elements may solve the technical problem. However, if this phenomenon results from molecular communication between glia and neurons, or other more nuanced features of AAV biology, then these issues may be less tractable. As AAVs are also widespread tools for the experimental study of CNS cell types outside of the reprogramming field, urgent work is needed to resolve this.

Several reports using AAVs for transgene expression have demonstrated significant improvement in animal models of Parkinson's disease, Huntington's disease and stroke (Ge et al., 2020; Qian et al., 2020; Tang et al., 2021; Wu et al., 2020). However, these could have explanations other than glia-to-neuron conversion, including rejuvenation of endogenous neurons. For example, it has been suggested that epigenetic rejuvenation of endogenous retinal neurons by AAV-mediated overexpression of *Oct4*, *Sox2* and *Klf4 in vivo* can restore vision in a mouse model of glaucoma (Lu et al., 2020). Alternative explanations should now also be considered.

These specificity issues may not be unique to AAVs. It has recently been reported that microglia are amenable to neuronal reprogramming *in vivo* using lentiviral delivery of the transcription factor *Neurod1* (Matsuda et al., 2019). If confirmed, this would be a fascinating discovery because microglia are derived from a distinct (myeloid) lineage to neurons. However, a conflicting report suggests that *Neurod1* is unable to reprogramme microglia *in vivo* and instead causes microglial cell death with non-specific labelling of endogenous neurons (Rao et al., 2021).

These controversies are an important cautionary tale for the entire field of *in vivo* lineage reprogramming and an opportunity to develop consensus on best practices for lineage tracing (Box 2). This applies to well-established viral vectors, as well as exciting new *in vivo* gene-delivery tools that are increasingly entering reprogramming paradigms, such as the Sendai virus, non-viral nanocarriers and microbubbles (Chang et al., 2019; Isomi et al., 2021; Miyamoto et al., 2018; Wang et al., 2021d; Yang et al., 2020). Although these issues temporarily setback what was believed to be possible in lineage conversion, they have opened interesting new avenues of investigation and, ultimately, will lead to improved tools for studying glial biology, as well as performing *in vivo* tissue engineering.

Reprogramming trajectories

The success of *in vivo* lineage reprogramming strategies is often measured by the resemblance of induced cells to the desired phenotype. However, as we have discussed (Fig. 3; Box 2), lack of scrutiny of the intermediate stages of reprogramming can be misleading and obscures our knowledge of how reprogramming occurs *in vivo*. Understanding the journey that cells undertake to rewrite their identity is a top priority for reprogramming research because it will help to deliver translatable strategies for lineage conversion *in vivo*. Transcriptomic and epigenetic data, along with computational resources to interrogate them, are deriving crucial

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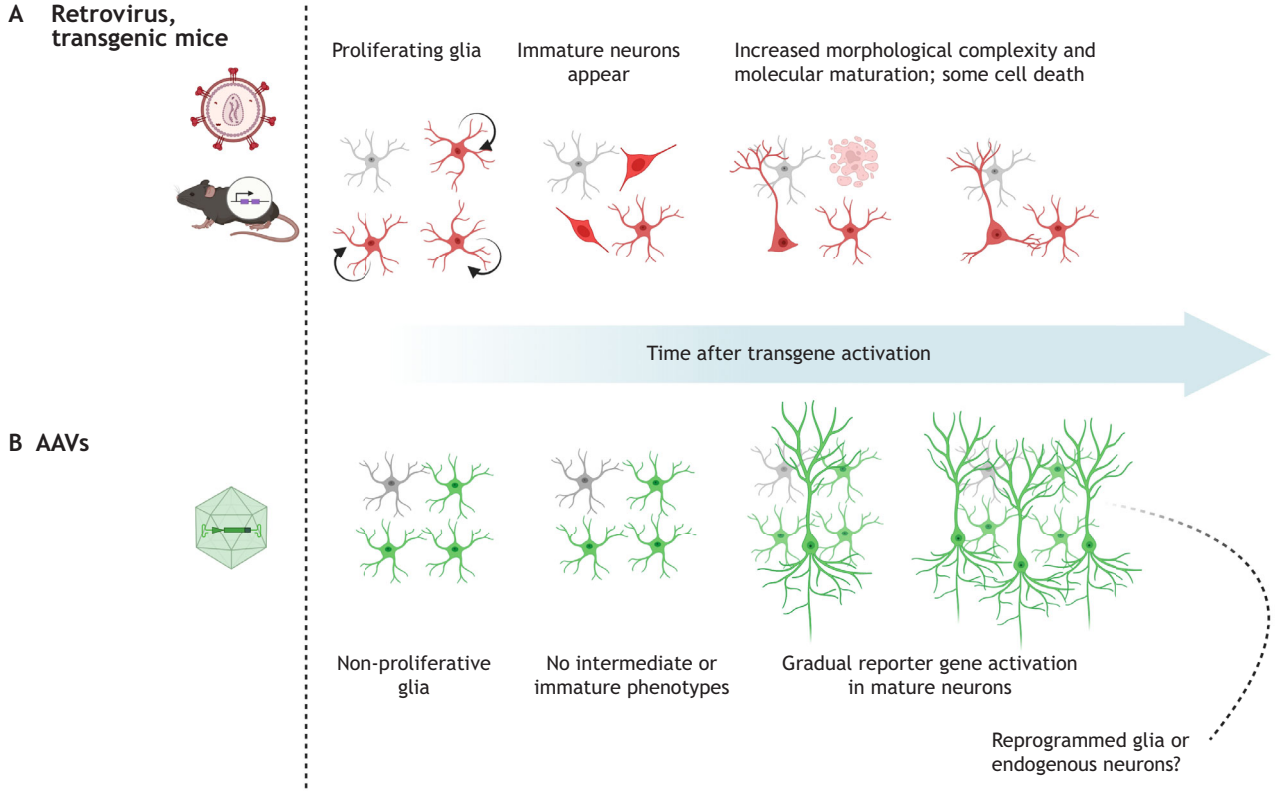

Fig. 3. Controversies in glia-to-neuron conversion in the CNS. (A) Time course of CNS glia-to-neuron reprogramming using retroviral vectors and inducible transgenic mouse lines to express reprogramming factors and reporter transgenes. Reporter transgene expression is indicated in red. Proliferating glia pass through immature intermediate states (e.g. indicated by DCX expression or immature morphology) before gradual and incomplete maturation. (B) Time course for recently published work using adeno-associated viruses (AAVs) for transgene delivery. Mature neurons appear in high numbers without clear transitional or immature phenotypes. Labelled neurons are not labelled by thymidine analogues, which suggests that they do not proliferate during reprogramming. Originally interpreted as reprogrammed glia, recent work suggests reporter transgene expression (green) in endogenous neurons (Wang et al., 2021c). This controversy reflects the challenges of studying reprogramming *in vivo* and a consensus is urgently needed to ensure lineage-tracing strategies are robust (Box 2).

molecular insights into cell trajectories during direct reprogramming in cell culture (Cates et al., 2021; Horisawa et al., 2020; Karow et al., 2018; Kempf et al., 2021; Kim et al., 2021; Stone et al., 2019; Treutlein et al., 2016; Wang et al., 2021b; Zhou et al., 2019).

Complex layers of epigenetic organisation control transcriptional access to nuclear DNA, which in turn establishes cell identity. 'Pioneer' transcription factors, which are capable of engaging condensed chromatin and regulating downstream fate effectors, continue to be at the heart of direct reprogramming strategies (e.g. *Ascl1*, *Gata4*, *Foxa1-Foxa3*, etc.) (Donaghey et al., 2018; Horisawa et al., 2020; Karow et al., 2018; Stone et al., 2019). Various co-factors that assist the erasure of epigenetic barriers to reprogramming (e.g. repressive DNA methylation and histone modifications) can improve transcription factor-mediated reprogramming (Elhanani et al., 2020; Garry et al., 2021; Jorstad et al., 2017; VandenBosch et al., 2020; Wang et al., 2021b). Now, increasing attention is turning towards exploiting pathways through which microRNAs post-transcriptionally regulate cell identity for lineage reprogramming (Cates et al., 2021; Jayawardena et al., 2015; Wang et al., 2021d; Yang et al., 2021). For example, miR-124 and miR-9/9* are capable of orchestrating epigenetic erasure of fibroblast identity *in vitro* through the upregulation of KLF-family transcription factors and the subsequent establishment of chromatin states permissive to neuronal identity (Cates et al., 2021). miR-124 is involved in an intricate loop of cross-repression with the RNA-binding protein PTBP1 and the RE-1 silencing transcription factor (REST) complex (Cates et al., 2021; Xue et al., 2013). REST is a transcriptional repressor that acts as a barrier to the establishment of both induced neuronal and pancreatic β-cell fates during direct reprogramming (Elhanani et al., 2020; Masserdotti et al., 2015; Qian et al., 2020). Forced expression of miR-124 or knockdown of PTBP1 inhibits REST, resulting in the neuronal conversion of fibroblasts and astrocytes *in vitro* (Cates et al., 2021; Qian et al., 2020; Xue et al., 2013).

Lineage conversion involves the erasure of starting cell epigenetic landscape and reconfiguration towards the new cellular identity, often passing through intermediate, stem cell-like states (Cates et al., 2021; Karow et al., 2018; Treutlein et al., 2016). It is unclear whether molecular trajectories are highly stereotyped or whether there are many routes to the same outcome. It is clear, however, that cells have an epigenetic memory, which is not completely erased by direct lineage programming (Hörmanseder, 2021). Presumably, this epigenetic memory is why cells that are close ontogenetic relatives exhibit more complete phenotypic conversions than more distant cousins (Wang et al., 2018) (Fig. 2A). This phenomenon is also reflected in the phenotypes of glia-derived reprogrammed neurons, which, as well as by the specific reprogramming factors used, are strongly determined by the regional identity of the starting glia (Chouchane et al., 2017;

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Box 2. Demonstrating direct lineage reprogramming *in vivo*

Gradual increase in non-specific reporter transgene expression in endogenous cells can mislead interpretation, as appears to be the case in glia-to-neuron reprogramming using AAVs (Wang et al., 2021c) (Fig. 3). Considering this, it will be important to develop a consensus on what constitutes robust evidence of *in vivo* reprogramming. Different model systems, tissues and experimental contexts will require different standards; however, as a starting point, we propose that meeting the following conditions would be useful in future work:

(1) the endogenous starting cell population should be labelled with *high specificity* before reprogramming, followed by identification of the label in induced cell types (e.g. using transgenic reporter lines) (Wang et al., 2021c);

(2) the endogenous counterparts of intended cell type (e.g. endogenous neurons in glia-to-neuron reprogramming) should be labelled with high sensitivity before reprogramming, followed by a demonstration that putative induced cells lack this label (Mattugini et al., 2019; Wang et al., 2021c); and

(3) the existence intermediate cell states during reprogramming should be demonstrated to provide evidence of the transition (e.g. through live *in vivo* imaging, characterisation of immature phenotypes, etc.).

Demonstrating intermediate states is not, by itself, sufficient to confirm reprogramming and in no way obviates the need for detailed lineage tracing. For example, in glia-to-neuron reprogramming, immature neuronal phenotypes could derive from rejuvenated mature neurons rather than reprogramming glia. In addition, demonstrating intermediate phenotypic states at one-time point is not necessarily evidence that these are the precursors of mature neurons found at later timepoints. These challenges mean that care will always be needed to interpret evidence of intermediate cell states from *in vivo* reprogramming experiments. However, we take the view that the inability to detect any intermediary cell phenotypes could be a warning sign of technical artefacts and so efforts to characterise them are crucial.

Herrero-Navarro et al., 2021; Kempf et al., 2021; Tsunemoto et al., 2018). Retention of differentiated epigenetic marks can be a blessing or a curse. If the goal is to replace local region-specific neurons in the CNS, this aspect of direct lineage reprogramming may be advantageous. It may also limit uncontrolled proliferation, de-differentiation and subsequent tumorigenesis (Gao et al., 2016). Speculatively, however, the epigenetic conflict between starting and induced cell identity could prevent appropriate maturation (Fig. 4). In support of this, it has recently been shown *in vitro* that epigenetic memory can lead to metastable cell types that retain the potential to revert towards their original cell identity or further differentiate towards the new lineage (Kim et al., 2021).

It is increasingly recognised that direct reprogramming imposes significant cellular stress (Babos et al., 2019; Russo et al., 2021; Zhou et al., 2019). Neurons have very different metabolic needs from astrocytes. Death can result from failure of metabolic switching and accumulation of damaging metabolic by-products (Gascón et al., 2016). Assisting cells with the metabolic transition by preventing ferroptosis or upregulating mitochondrial antioxidant proteins, enhances glia-to-neuron conversion (Gascón et al., 2016; Russo et al., 2021). Additionally, it has been recently suggested that conflict between DNA replication and transcription causes significant genomic stress that impedes reprogramming. Resolving these biophysical conflicts using a topoisomerase-dependent mechanism, improves the reprogramming efficiency of fibroblasts to both neurons and cochlear hair-like cells *in vitro* (Babos et al., 2019).

Pulling together the strands of knowledge into a clearer model that can be used for precision tissue engineering is a key challenge. Computational approaches are increasingly available to tease out broader principles from transcriptomic and other big data, which are already helping to identify strategies to improve direct lineage conversion and understand cellular decision making (Kamaraj et al., 2020; Kamimoto et al., 2020 preprint; Merlevede et al., 2021; Rackham et al., 2016; Sáez et al., 2021). Leveraging these approaches, with phenotypic data from cells undergoing lineage conversion *in vivo*, will undoubtedly fuel progress in tissue engineering in years to come.

Functional integration

To accomplish intended functions, lineage-converted cells must develop mature functional properties and interact appropriately with local tissue in their given physiological context. Engineering precise functional integration is a particularly daunting prospect when generating new neurons in the CNS. The addition of new neurons to existing adult neuronal circuits does occur naturally in some mammals at specialised neurogenic niches, such as in the hippocampal dentate gyrus and olfactory bulb. Even here, there are waves of cell death of newborn neurons, with their successful integration involving a stereotyped sequence of innervation by host neurons and communication with surrounding glia (Denoth-Lippuner and Jessberger, 2021). It remains unknown whether the rules that apply in these stem-cell niches will be broadly similar during glia-to-neuron lineage reprogramming.

However, there is strong evidence to support the idea that functional integration of induced neurons is at least possible. Recent discoveries using rabies-mediated monosynaptic tracing have shown that endogenous neurons make synaptic connections with glia-derived neurons in the spinal cord and hippocampus (Lentini et al., 2021; Tai et al., 2021). Reactive glia in a mouse model of mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE-HS), where most endogenous interneurons neurons are lost, can be converted into predominantly GABAergic inhibitory neuronal subtypes by retrovirally encoded *Ascl1* and *Dlx2* (Lentini et al, 2021). Electrophysiological recordings in acute brain slices can detect inhibitory postsynaptic potentials in endogenous granule neurons when nearby induced interneurons are activated using optogenetics (Lentini et al., 2021), suggesting that induced neurons can form synaptic connections that functionally output onto endogenous neurons. Remarkably, these *Ascl1*/*Dlx2*-induced neurons reduce chronic epileptic activity in the hippocampus of MTLE-HS mice.

Direct *in vivo* reprogramming has also provided beneficial functional outcomes in animal models of diabetes (Wang et al., 2018; Xiao et al., 2018), liver fibrosis (Rezvani et al., 2016; Song et al., 2016), cardiac disease (Hu et al., 2014; Isomi et al., 2021; Jayawardena et al., 2015; Miyamoto et al., 2018), spinal cord injury (Tai et al., 2021) and Parkinson's disease (Rivetti Di Val Cervo et al., 2017). These results indicate that *in vivo* lineage reprogramming can generate cells capable of functional tissue integration. However, in addition to amelioration of disease phenotypes, future work must expand our knowledge of the details and context-specific mechanisms of tissue integration.

Perspectives

Fuelled by the toolbox of modern biology, we are constantly discovering that many mammalian cells, once considered immovably differentiated, are far more dynamic and malleable than we thought. Still, from the perspective of the cell, rewriting its identity is a Herculean task. Cells are complex biophysical entities packed full of intricate machinery and cytoskeletal infrastructure, with intimate relationships with their neighbouring cells. Lineage

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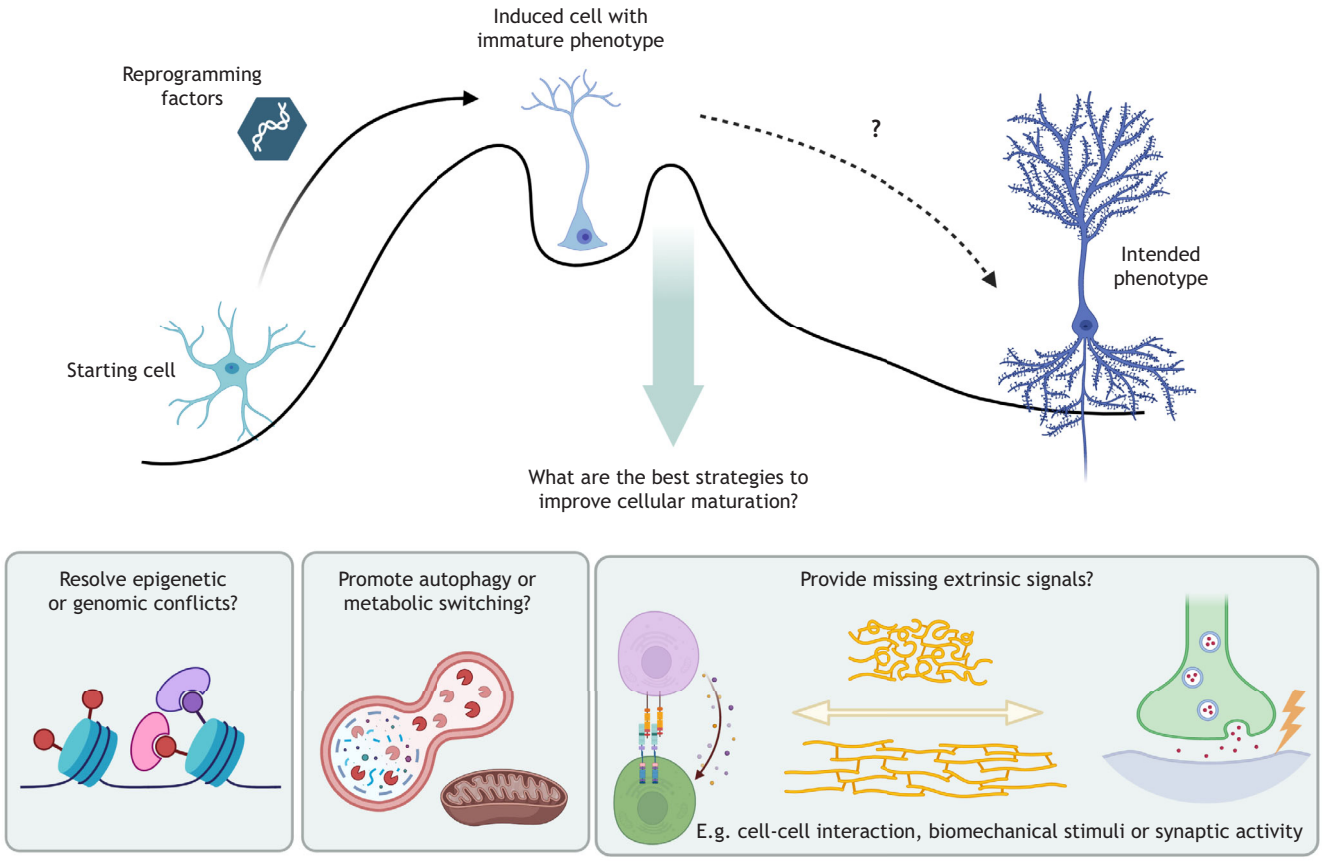

Fig. 4. Removing barriers to reprogrammed cell maturation. The immaturity of induced cells remains a significant problem in lineage reprogramming across many tissues *in vivo*. Overcoming barriers to maturation are a top priority for *in vivo* reprogramming research. Strategies to further erase the old cell identity (e.g. by autophagy/recycling of old cellular infrastructure and removal of repressive epigenetic marks at targeted genomic loci) may release the breaks and permit full maturity. Alternatively, cells may be primed to mature but lack necessary extrinsic maturation signals, usually provided during development (e.g. cell-cell signalling, biomechanical forces or stimulated electrical activity). These examples are not necessarily mutually exclusive and may well conspire to prevent maturation.

reprogramming asks that all of this be overhauled. Reprogramming cells must then struggle through reorganisation of genomic architecture, probably harbouring incompletely repurposed cell machinery and incompatible aspects of metabolism. During this process, they find themselves in a microenvironment that is not accustomed to their unexpected transition. This may well lead to terse or unsatisfactory negotiations with neighbours and uncompromising local immune cells.

The barriers to lineage conversion probably scale with increasing distance from the phenotype of the starting cell (Fig. 2A). However, it is clear from the limited single-cell transcriptomic data collected *in vivo* (Sun et al., 2021; Todd et al., 2021; Yamashita et al., 2018) that, across multiple tissues and organs, induced cells are immature (Fig. 4). It will be interesting to see whether there are common mechanisms that limit the maturation of reprogramming cells across all tissues or whether these are highly contextual. Characterising immature and intermediate phenotypes during reprogramming is essential to overcome challenges to cellular maturity and for moving forward on a scientifically firm footing (Box 2).

It is exciting to see rapid advances in our ability to genetically access and manipulate specific cell types *in vivo*, using molecular know-how and improved viral and non-viral tools. It is also now widely possible to manipulate the regulation of endogenous genes using regulatory RNAs and CRISPR activation, and these tools are being adopted in lineage reprogramming (Jayawardena et al., 2015; Qian et al., 2020; Russo et al., 2021; Wang et al., 2021d; Yang et al., 2021). Advances in all these technologies will enable precision tissue engineering of the future for both discovery and translational science.

The observation that rewriting cell identity *in vivo* can restore tissue function in various models of disease illustrates the power of *in vivo* tissue engineering. The plethora of lineage conversions being performed *in vitro* outnumbers those attempted *in vivo*. These include musculoskeletal, cancer, immune, gastrointestinal and other cell types, which will – no doubt – make their way towards *in vivo* model systems soon. Indeed, early phase clinical trials using *Atoh1* for direct SC-to-HC conversion in hearing loss are already under way (NCT02132130). It is crucial to emphasise, however, that the field is still in its infancy and an enormous amount of work remains. Nonetheless, progress in rewriting cellular identity *in vivo* seems inevitable and may well herald a coming medical revolution.

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