

Review

Epigenetic regulation of neural lineage elaboration: Implications for therapeutic reprogramming

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ABSTRACT

The vulnerability of the mammalian brain is mainly due to its limited ability to generate new neurons once fully matured. Direct conversion of non-neuronal cells to neurons opens up a new avenue for therapeutic intervention and has made great strides also for in vivo applications in the injured brain. These great achievements raise the issue of adequate identity and chromatin hallmarks of the induced neurons. This may be particularly important, as aberrant epigenetic settings may reveal their adverse effects only in certain brain activity states. Therefore, we review here the knowledge about epigenetic memory and partially resetting of chromatin hallmarks from other reprogramming fields, before moving to the knowledge in direct neuronal reprogramming, which is still limited. Most importantly, novel tools are available now to manipulate specific epigenetic marks at specific sites of the genome. Applying these will eventually allow erasing aberrant epigenetic memory and paving the way towards new therapeutic approaches for brain repair.

1. Introduction

The central nervous system of mammals, like the pancreas or the heart, has little capacity to replace cells, in particular neurons, lost through damage, stroke or degeneration (Barker et al. 2018; Grade and Gotz 2017). This is notably different from other organs that can regenerate by somatic cells with the ability to de-differentiate (e.g. the liver (Sadri et al. 2016)) or the presence of widespread stem cell niches (e.g. skin (Blanpain and Fuchs 2006)). Conversely, the mammalian brain has few neural stem cell niches left in the adult that are also species-dependent and notably different in human (Obernier and Alvarez-Buylla 2019), leaving large parts of the CNS devoid of any potential to replace lost neurons.

Several strategies have been developed to support brain regeneration during or after degeneration (Barker et al. 2018; Grade and Gotz 2017), amongst which the most appealing one, direct reprogramming, is also the most challenging. The term direct reprogramming or trans-differentiation describes a directed cell identity change usually triggered by the administration of expression constructs of master transcription factor (i.e. reprogramming factor) genes. Cellular

reprogramming might be best known for the conversion of fibroblasts into muscle cells (Weintraub et al. 1989) and the induction of pluripotency (Takahashi and Yamanaka 2006). Direct reprogramming of neurons has been pioneered already in 2002 (Heins et al. 2002), and might be today the most intriguing area of this field, as it has been implemented with great success in vivo. Since the first demonstration of in vivo glia to neuron conversion in the injured brain using the transcription factor Pax6 (Buffo et al. 2005), the use of down-stream transcription factors and modifying viral vector systems has now achieved highly efficient conversion protocols of local glial cells in the injured murine brain using Neurog2 (Gascon et al. 2016; Heinrich et al. 2010; Mattugini et al. 2019), NeuroD1 (Guo et al. 2014; Matsuda et al. 2019; Wu et al. 2020) or others (Vignoles et al. 2019). Recently, it has even been shown that reprogrammed cells in the cortex are developing lamina-specific characteristics and adequate long-distance axonal projections (Mattugini et al. 2019). However, despite the apparent fidelity of neurons in aspects of morphology, axonal projection, marker expression and electrophysiological hallmarks, it is not known how closely they resemble endogenous neurons in regard to their total transcriptome, and to which extent an epigenetic memory of the cell of origin may still be present.

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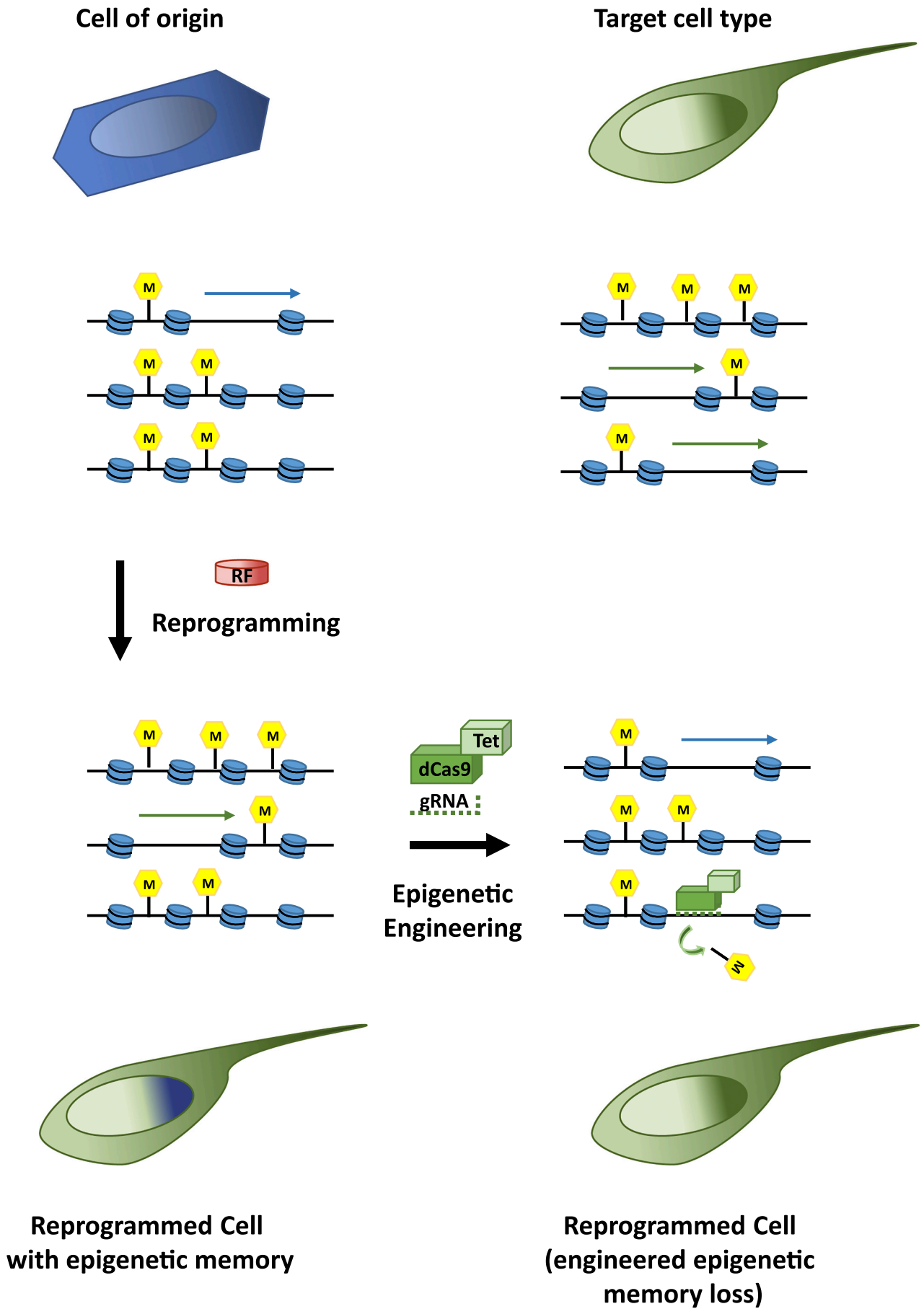
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Fig. 1. Direct reprogramming coincides with epigenetic changes. Cell of origin (blue) is reprogrammed into a target cell type (green) through the expression of a reprogramming factor (RF). During this process, epigenetic marks (e.g. DNA methylation, yellow hexagons) are reorganized and genes are silenced (blue arrow). Other genes get activated (green arrow). Genes and epigenomic marks that do not appropriately reprogram constitute epigenetic memory. Manipulation of chromatin marks by epigenome editing (e.g. dCas9-Tet) enables to remove epigenetic memory. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Indeed, several glial (astrocytes, oligodendrocyte progenitors, microglia) and other (fibroblasts, pericytes, peripheral blood T cells etc.) cell types have been reprogrammed into neurons *in vitro*, but nothing is known to which extent these neurons differ due to incomplete erasure or establishment of adequate chromatin marks. This is however of crucial importance, as it could guide us to know which cell type and which reprogramming factors best to choose to generate the most adequate neurons efficiently.

The remarkable success of cellular reprogramming raises hopes to provide soon therapeutic options for any disease that manifests itself by the loss of a particular cell type that the human body cannot regenerate. Apart from type 1 diabetes (Xiao et al. 2018), in which beta cells vanish, and the loss of cardiomyocytes after heart attack (Qian et al. 2012), these are primarily diseases caused by neuron loss. This could be focal, as e.g. after TBI, stroke or in specific neurodegenerative diseases like Parkinson's or Huntington's disease, or more wide-spread as in Alzheimer's or other neurodegenerative disorders. This is highly relevant to the method of neuronal replacement – transplantation of new neurons is largely restricted to diseases accompanied by focal loss of neurons, while reprogramming can be applied in more wide-spread manner e.g. by systemic injection of viral vectors (ref AAV9), and do not require immune suppression. Importantly, however, the specific neuronal subtypes lost in the respective disease have to be specifically and reliably induced (Gan et al. 2018; Torper and Gotz 2017). Consequently, three aspects have to be optimized, before *in vivo* reprogramming can become a therapeutic option: (1) Reprogramming factors delivery has to be efficient, safe and cell-type specific. Recent developments, in particular the option of AAV serotypes in the brain has been reviewed in (Haery et al. 2019). (2) Reprogrammed neurons have to gain specific sub-type identities to replace the lost cells (see (Lodato and Arlotta 2015) and (Maserdotti et al. 2016)). (3) The impeccability of the reprogrammed neurons, showing no memory of the former cell identity, which we will address in the following.

2. Chromatin hallmarks implicated in epigenetic memory during reprogramming into induced pluripotent stem cells

Much has still to be learned about epigenomic changes during direct neuronal reprogramming and how epigenetic memory affects the efficiency and accuracy of the cell conversion. In iPSC reprogramming, incomplete and inefficient reprogramming coincide with a residual epigenetic memory of the cell of origin (Kim et al. 2010). Indeed, low passage iPSCs have significant functional differences to ESCs and tend to differentiate more easily to lineages from which they have been derived from (Kim et al. 2010). This epigenetic memory can also be seen on the transcriptional level, containing mRNA traces of the cell of origin (Ohi et al. 2011). The exact molecular basis for this epigenetic memory has not yet always been functionally verified at precise sites (see outlook), but examining chromatin hallmarks has much improved our understanding of hurdles and requirements in direct fate conversion (for review see e.g. (Brumbaugh et al. 2019)).

Generally, epigenetic memory consists of left-over signatures of the cell of origin, which can be derived from the starter cell's identity, its metabolism or age (Fig. 1: Khoo et al. 2020). It manifests itself by a failure to repress active genes of the cell of origin fully (On-memory genes) or by an incomplete activation of gene expression of the induced fate (Off-memory genes; (Hormanseder et al. 2017)). We start by discussing first the former and then the later. Genes that are particularly difficult to be activated are those that have been inactivated for long-

Table 1

Examples of chromatin marks, writers and erasers implicated in epigenetic memory mentioned in the text.

Chromatin mark	Writer (e.g.)	Eraser (e.g.)	Epigenetic memory
DNA methylation	Dnmt3a, Dnmt3b, Dnmt1	Tet1, Tet2, Tet3	Off memory
H3K9 methylation	Ehmt1, G9a, Suv39h1, Suv39h2, Setdb1	Jmjd2c	Off memory
H3K27 methylation	Prc2 complex, Ezh2	Jmjd3	Off memory
H3k4 methylation	Trx complex, Mll proteins,	Lsd1	On memory
H2A ubiquitination	Prc1 complex, Ring1b		Off memory
H3K79 methylation	Dot1l		On memory

term by silencing, such as those embedded in heterochromatin. Notably, these are also enriched in specific locations within the nucleus, namely in distinct compartments close to the nuclear lamina (Stadhouders et al. 2019). Heterochromatin is associated with specific chemical modifications implicated in gene regulation (Stricker et al. 2017), in particular with methylation (m5C) of cytosines in the DNA and tri-methylation of Lysine 9 of histone H3 (H3K9me3, Table 1) (Nicetto and Zaret 2019). Indeed, H3K9 methylation is known already as a key regulator of cell fate establishment during development (see e.g. (Nicetto and Zaret 2019)) and during reprogramming often prevents the binding of the reprogramming factors; interfering with acquisition of new fates (Becker et al. 2017; Brumbaugh et al. 2019; Nicetto and Zaret 2019). Consistent with this, overexpression of the H3K9 de-methylases Jmjd2c and/or depletion of the H3K9 methyltransferases Ehmt1, G9a, Suv39h1, Suv39h2 and/or Setdb1 can increase rates of iPSC generation (Chen et al. 2013; Onder et al. 2012; Sridharan et al. 2013) or direct reprogramming e.g. into hepatocytes (Becker et al. 2017). Moreover, the re-activated sites have been observed to move from nuclear lamina position to other nuclear compartments, e.g. in B-cells to macrophage reprogramming (Stadhouders et al. 2019). However, some heterochromatin regions are particularly resistant to activation in reprogramming (Becker et al. 2016). Besides, since inducing a new cell identity also requires formation of new heterochromatin, a permanent repression of enzymes responsible for depositing these marks is not a good option. Consistent with this, Suv39h1 has not only been shown to suppress (Chen et al. 2013), but also to facilitate induced pluripotency (Onder et al. 2012).

A particularly detailed and comprehensive knowledge about chromatin changes occurring during reprogramming has been obtained during induction of pluripotency (for a detailed review see (Nashun et al. 2015)). Even a temporal order of epigenetic events, designating different phases of the process, has been described in this model (Apostolou and Hochedlinger 2013). A typical example is the reorganization of H3K4 methylation (Table 1), which is amongst the earliest changes during iPSC reprogramming. While loss of H3K4 di- and trimethylation on somatic genes is accompanied by concurrent transcriptional downregulation, gains of H3K4 methylation only correlate to transcriptional upregulation of stem cell genes much later (Apostolou and Hochedlinger 2013; Soufi et al. 2012). The fact, that iPSC generation can be enhanced through knockdown of the H3K4 de-methylase Lsd1 seems contradictory to these findings (Wang et al. 2011), although it corroborates the general relevance of this mark. Likewise, it

is not well understood, why the effect of Wdr5, a critical cofactor of the Trithorax complex mediating H3K4 methylation is greater the earlier it is removed during reprogramming (Ang et al. 2011). This already highlights the urgent need to understand if specific chromatin marks are required particularly at specific sites, and if so, which, or if rather global rewiring of the chromatin is required.

New H3K27 methylation marks are deposited during all phases of iPSC reprogramming, first on somatic, later on some bivalently marked genes in pluripotent cells (Table 1). Interestingly however, loss of the H3K27 methyltransferase Ezh2, is not sufficient to decrease rates of pluripotency induction despite a global reduction of H3K27me3 (Fragola et al. 2013). Only upon further knockdown of EED (another core PRC2 component) a reduction of reprogramming rates have been reported (Fragola et al. 2013). Further complicating is the fact that the H3K27 demethylase Jmjd3 has been found to be an inhibitor of reprogramming (Zhao et al. 2013). Members of the PRC1 complex, mediating H2A ubiquitination, typically act as facilitators of reprogramming, as knock-down of Bmi1 or Ring1b strongly reduce iPSC generation (Onder et al. 2012) indicating these factors may be required to stabilize newly induced fates.

The very last phase of reprogramming is characterized by global changes in DNA methylation (Apostolou and Hochedlinger 2013). DNA methylation is also the chromatin mark most commonly associated with epigenetic memory (Table 1). During iPSC reprogramming, DNA methylation patterns reminiscent of the cell of origin can be found in early passage cells (Kim et al. 2010). As a consequence, DNA methylation differences between ESCs and iPSCs can be surprisingly extensive; they have been reported to span megabases at regions proximal to telomers and centromeres, and to coincide as well with altered histone marks (Lister et al. 2011). Interestingly however, the de novo methyltransferases Dnmt3a and Dnmt3b have been shown to be dispensable for mouse iPSC generation (Pawlak and Jaenisch 2011). Conversely, the Tet enzymes catalyzing the hm5C mark are critically required for reprogramming to drive enhancer demethylation necessary to active gene expression to induce the new fate by the reprogramming transcription factor (Sardina et al. 2018). Importantly, this critical Tet activity is occurring very early in reprogramming, preceding opening of the chromatin and binding of the reprogramming factor at several sites. However, causal relations by inducing hydroxymethylation at specific sites using epigenetic engineering (see below) have not yet been established directly.

Another enzyme largely associated with marks activating gene expression is the only known histone methyltransferase without a SET domain, Dot1L, which mediates H3K79di- and tri-methylation (Table 1) (Bovio et al. 2019; Farooq et al. 2016). Interestingly, this enzyme is a hurdle to reprogramming (Onder et al. 2012) and apparently responsible mostly for continued gene expression of the original cell fate (Brumbaugh et al. 2019). This highlights the importance of examining these marks and enzymes in the context of reprogramming, which may differ from development or during stable identity in differentiated cells.

To activate gene expression enhancer and promoter regions need to be brought into close contact. During normal differentiation 80% of such contacts change and this also needs to be achieved during reprogramming (Stadhouders et al. 2019). There are different levels of local and more distant chromatin organization important for regulation of gene expression and some or many of these topological rearrangements may be regulated by liquid-liquid phase transitions in the nucleus (for review see (Stadhouders et al. 2019)). Relevant for our discussion here is that the failure to change some of the pre-existing cell-type-specific long-range interactions may result in partially aberrant gene expression, as has been shown e.g. in generating iPSCs from NSCs (Beagan et al. 2016).

In principle, three strategies have been experimentally used to improve reprogramming into iPSCs and erase epigenetic memory of the starter cells. It has been shown for example that transcriptional, epigenetic and metabolic differences between iPSCs and ESCs diminish in higher passage numbers (Panopoulos et al. 2012; Polo et al. 2010), a

strategy that is not available for reprogrammed neurons that are post-mitotic. Second, the use of chemical inhibitors of chromatin modifying enzymes, as a number of studies suggest that the blockage of canonical chromatin processes increases the plasticity of cells and consequently induced pluripotency. Examples include the use of 5-Aza-cytidine, a DNA methyltransferase inhibitor, (Mikkelsen et al. 2008; Theunissen et al. 2011), the HDAC inhibitors Valporic acid and TSA (Huangfu et al. 2008), and the inhibition of Dot1l with EPZ004777 (Onder et al. 2012). And finally, the use of organic molecules stimulating chromatin modifying enzymes, such as Vitamin A and C increasing Tet activity and DNA de-methylation during the reprogramming process (Blaschke et al. 2013; Hore et al. 2016). However, these are all global treatments and hence may lead to reduced or increased modifications at sites with beneficial effects as well as sites with adverse effects on the conversion progress.

3. Epigenetic changes during direct neuronal reprogramming

The observations made in other reprogramming context of course also apply to neuronal reprogramming, but with additional layers of complexity due to the high diversity of neuronal subtypes and the potential subtle but highly relevant effects that slight aberrations may exert. In vitro converted neurons show residual expression of some fibroblast genes (Flitsch and Brustle 2019; Tsunemoto et al. 2018), however it is however still unclear, to which degree this epigenetic memory interferes with neuronal function. Indeed, the gold standard of electrophysiological parameters of neurons seem not to be affected by remnant astrocyte gene expression (Masserdoti & Götz, unpublished observations). This is a particularly important, but also particularly challenging issue in neuronal reprogramming, as differentiation may not complete in vitro, and only be completed when neurons in vivo connect and function within a neuronal network. Yet isolating reprogrammed neurons from in vivo is notoriously difficult and does not yield large amounts for molecular analysis. New developments in single cell RNA and DNA sequencing, improvement of in vitro conditions as well as screening for subtype and maturation regulating transcription factors might help to overcome these issues.

Given the conceptual similarity of transcription factor induced reprogramming systems, it seems likely that residual chromatin features influence the accuracy and efficiency of direct cell fate conversion too (Flitsch and Brustle 2019). This may be particularly important, as aberrant epigenetic settings may reveal their adverse effects only in certain brain activity states. Indeed, the primary risk represented by aberrant epigenetic memory in this context could result in only slightly aberrant connectivity (only 1 of 20 brain regions) that would become functionally relevant only if this one brain region becomes particularly activated, e.g. in states of stress, drugs, infection, hormonal bias etc. Likewise, minimal alterations in transmission of electric signals would only be revealed in particular brain states. These issues can only be investigated in in vivo settings and call for examining them in various brain states.

Moreover, there are already clear indications that epigenetic memory might be even more relevant in direct neuronal reprogramming than during induced pluripotency. It has for example been suggested, that the S-phase of the cell cycle is a particularly permissive window for reprogramming (Nashun et al. 2015), yet glial cells converting to neurons proliferate little with only 10–20% of the converted neurons derived from a proliferating cell in vitro (Gascon et al. 2016) and in vivo (Mattugini et al. 2019). Moreover, the molecular correlate of cellular age, a DNA methylation signature (Horvath 2013), is readily reset in iPSCs (Bell et al. 2019), but not in induced neurons (Huh et al. 2016; Mertens et al. 2015), demonstrating the limitations in resetting epigenomic marks in direct somatic reprogramming. Moreover, the differences are not limited to DNA methylation signatures, but extend to metabolic, mitochondrial and nuclear defects (Kim et al. 2018; Tang et al. 2017). This could be a major drawback for the therapeutic use of direct reprogramming in neurodegenerative diseases, as not only

genetic, but also environmental and epigenetic risk factors (e.g. age) might remain in the newly generated neurons.

On the other hand, however, little is known about the epigenetic processes regulating direct reprogramming, and what is, is often dissimilar to what has been found in iPSCs. Results from neural conversion of microglia cells using the reprogramming factor NeuroD1 for example indicate that (in contrast to iPSC reprogramming) loss of H3K27 methylation and upregulation of direct target genes constitute the first epigenetic changes in this process, while microglial genes are down-regulated in a secondary event, implemented by the repressor proteins *Scrt1* and *Meis2* (Matsuda et al. 2019). Directed neuronal reprogramming of fibroblasts using the BAM factors *Ascl1*, *Brn2* and *Myt1l* causes global alterations in DNA methylation that result in methylomes reminiscent of cortical neurons (Luo et al. 2019). These changes include the emergence of a particular feature of neurons, methylation at rather atypical sites, so called CpH sites and not at the usual DNA methylation targets, the CpG sites (Lister et al. 2013). In contrast to induced pluripotency, efficiencies of direct reprogramming of fibroblasts to neurons are reduced when de novo methylation is abolished, although these effects are minor (Luo et al. 2019). Analysis of *Ascl1* binding sites indicate that a highly unusual chromatin state, consisting of two active marks, H3K27ac and H3K4me1, and the repressive mark H3K9me3, is highly receptive for *Ascl1* binding in fibroblasts (Wapinski et al. 2013). Indeed, this triple mark at *Ascl1* target sites predicts if cells can be efficiently reprogrammed (like fibroblasts) or not (like osteoblasts). Moreover, overexpression of the H3K9 de-methylase *Jmjd2d* decreased reprogramming rates in this system, indicating a necessity for this mark for binding of the reprogramming factor *Ascl1* to its targets and eliciting fate conversion of fibroblasts (Wapinski et al. 2013). Interestingly, this is in sharp contrast to iPSC reprogramming, where overexpression of the H3K9 de-methylases *Jmjd2c* has been reported to increase reprogramming rates and H3K9 methylation has been not identified as a prerequisite, but rather as the main reprogramming barrier (Becker et al. 2016). Thus, requirements for reprogramming are clearly lineage-specific, and while important insights have been made about the key molecular steps in direct neuronal reprogramming (see also (Mall et al. 2017; Masserdotti et al. 2015) very little is still known about epigenetic memory in induced neurons and how they may differ from endogenous neurons.

While the most efficient strategy to overcome epigenetic memory in iPSCs, passaging, is not applicable in non-dividing neurons, the use of small molecules has however been adopted to neuronal conversion. Several strategies for the chemical in vitro conversion of fibroblasts and astrocytes have been recently reported, many of them are based on complex cocktails that contain the HDAC inhibitor VPA (Cheng et al. 2015; Xu et al. 2019; Zhang et al. 2015). It is however unclear which role this inhibitor plays during fate conversion and whether the effects are due to global epigenetic memory removal or rather by the activation of endogenous reprogramming factor genes, e.g. *NeuroD1*. Moreover, another HDAC inhibitor, TSA, has been shown recently to restore together with the reprogramming factor *Ascl1*, the ability of Müller glia to give rise to neurons (Jorstad et al. 2017; Jorstad et al. 2020). Although it is unclear to which degree this represents a reprogramming or a programming effect, it does suggest histone de-acetylation contributing as a barrier.

4. Epigenetic engineering to improve fidelity of reprogrammed neurons

Although much has still to be figured out, it is likely that epigenetic processes are determining the efficacy and pertinence of reprogrammed neurons. Above all, two open questions remain, which are more closely linked than it seems on the first glance: (A) Are there chromatin barriers common to all cell fate transitions, or do the critical epigenetic mechanisms differ depending on the starting cells, the reprogramming factors and the converted fate? (B) Are barriers constituted by the sum (or at

least many) of the occurrences of a specific chromatin feature differing between cell of origin and target fate, or are there only few (or even single) critical marks that make the epigenome unpassable. For direct reprogramming, published data indicates currently the latter. Reprogramming by *Ascl1*, for example, depends on an unusual chromatin state, consisting of H3K27ac, H3K4me1 and H3K9me3, it can, however, be replaced by one single *Ascl1* target, the zinc finger, *Zfp238* (Wapinski et al. 2013).

To definitely answer these questions conclusively, more functional experiments are needed. In particular those that have the potential to distinguish global effects from those of local chromatin changes. Experimental strategies to engineer or eradicate specific epigenetic marks are on the rise (Köferle et al. 2015). Most of these are a side product of recently developed gene targeting systems, such as CRISPR (see Fig. 1). Fusion of enzymatically dead versions of the Cas9 nuclease to enzymatically active domains of chromatin modifying enzymes has been proven to be a powerful tool to manipulate individual chromatin marks on defined genomic regions (Braun et al. 2017; Hilton et al. 2015; Liu et al. 2016). This approach has been highly versatile and often successful; constructs for the manipulation of all canonical chromatin marks have been published and new options are getting available on almost a weekly basis (Breunig et al., 2021). These have been successfully targeted to specific sites, but multiplexing gRNAs (see e.g. (Breunig et al. 2018; Cong et al. 2013)) also allows targeting many sites to determine their role in fate conversion. Interestingly, DNA methylation has been identified recently as a hurdle in transcriptional engineering of the *Sox1* locus to re-activate neurogenic stem cell fate, and could at least be partially overcome by targeting Tet to these specific sites (Baumann et al. 2019). This allowed successful fate conversion, highlighting the success of combining transcriptional and epigenetic engineering techniques. Combining these epigenome editing tools with libraries of gRNAs, defining their genomic targeting sites, will soon allow distinguishing critical chromatin marks from merely bystanders in epigenomic screens. Importantly, however, we need to identify the epigenetic settings in the induced neurons, ideally in vivo, as this is the setting to achieve repair. This knowledge, combined with epigenetic engineering to remove aberrant marks, will help to pave the way to improve fidelity of reprogrammed neurons towards therapeutic reprogramming.

Declarations of interest

None.

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