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Understanding direct neuronal reprogramming — from pioneer factors to 3D chromatin

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Cell replacement therapies aim at reestablishment of neuronal circuits after brain injury, stroke or neurodegeneration. Recently, direct reprogramming of resident glial cells into the affected neuronal subtypes has become a feasible and promising option for central nervous system regeneration. Direct reprogramming relies on the implementation of a new transcriptional program defining the desired neuronal identity in fully differentiated glial cells implying the more or less complete down-regulation of the program for the former identity of the glial cell. Despite the enormous progress achieved in this regard with highly efficient *in vivo* reprogramming after injury, a number of hurdles still need to be resolved. One way to further improve direct neuronal reprogramming is to understand the molecular hurdles which we discuss with the focus on chromatin states of the starting versus the reprogrammed cells.

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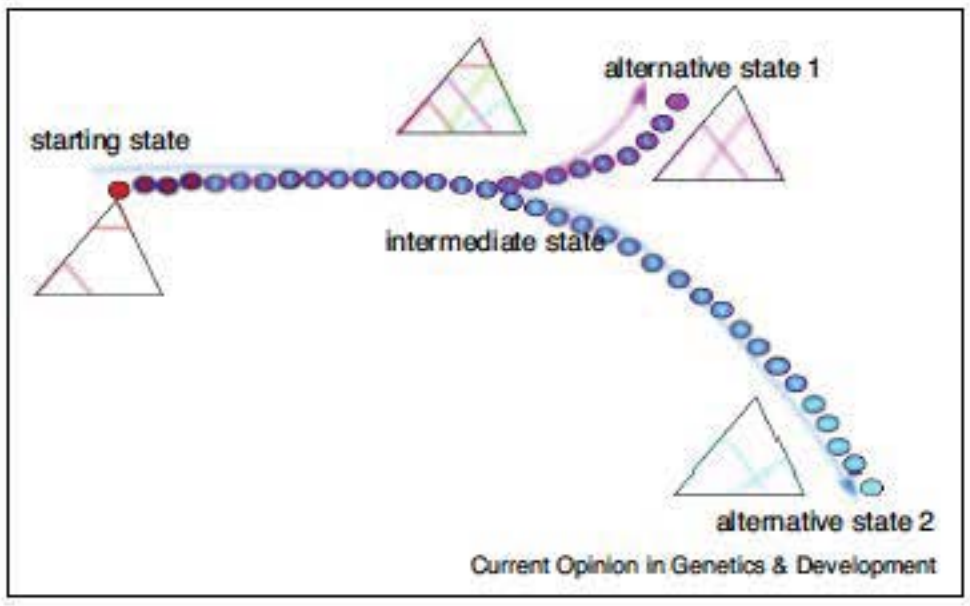
Brain injury induces an orchestrated reaction of resident glial cells and infiltrating monocytes [1–6], leading to changes in the extracellular matrix (ECM) and formation of non-functional glial scar tissue [1,3,7]. Direct reprogramming of reactive scar forming glial cells is a novel approach to reduce scar formation and simultaneously replace the degenerated neurons at the injury site [8,9•]. Glial cells are converted to a neuronal fate bypassing the progenitor stage (direct reprograming) by expression of neurogenic fate determinants *in vitro* or *in vivo* [10,11,12••,13,14]. Recently even in the inflamed environment after invasive stab wound injury amazing conversion rates of over 90% have been achieved including behavioural recovery [9•,11, 13,15–18], but there is still some room for improvement. For example, long term survival of the induced neurons, generation of exact neuronal subtype identity and the appropriate long-range connectivity are still unresolved challenges (for recent review see Grade and Götz [19]; Barker *et al.*, in press). Towards this aim, it is essential to understand the conversion process at the molecular level, and particularly identify the molecular hurdles (for recent review see Gascon *et al.* [17]). Here we discuss the power and limitations of pioneer TFs (TFs) and their effects on 3D chromatin architecture, a so far unexplored field in direct neuronal reprogramming (Figure 1).

The power and limits of TFs

Direct lineage conversion relies on the implementation of the transcriptional program of the desired cell type, neurons in this case, and the downregulation of the transcriptional program defining the identity of the starting cell, glia in this case [9•,20–26]. Implementing the new neuronal fate is mostly achieved in direct reprogramming by utilizing genes specifying neurons in development [27]. The choice is mostly for TFs that are very potent during development, such as the master regulator Pax6 or the proneural bHLH TFs. However, it is important to notice that direct reprogramming starts in a very different cellular and transcriptional context, namely in a differentiated cell of a different identity, rather than in a neural stem or progenitor cell from which neurons normally differentiate. Thus, it would be plausible to expect that the developmental factors do not entirely copy the canonical programs and progenitor states we learned about from the development, but also employ non-canonical molecular programs and pass though unusual intermediate (progenitor) states. Indeed, single cell RNA-sequencing after Ascl1 mediated induction of neuronal fate in fibroblasts revealed intermediate states, including the lineage bi-furcation leading to either neuronal or myogenic fate [28•]. The neuronal fate can be stabilized by the addition of the transcriptional repressor Myt1L [29•]. Myt1L represses genes specific for other lineages and thereby stabilizes the neuronal fate [29•]. This function resembles the concept of terminal selectors, a single or group of TFs specifying neuronal subtypes during development and maintaining this identity by repressing others [30–32]. Importantly, the terminal selector concept applies to closely related alternative fates within neural tissue. In contrast, the alternative fates in reprogramming are developmentally distant from the new

Figure 1



Changes in the 3D chromatin organization (triangles) are associated with the reprogramming trajectories (blue and magenta) from starting state to the final alternative fate. Note that at the node of reprogramming trajectories has both chromatin organizations representing at least a part of alternative identity states and one chromatin organization needs to be over-written in order to achieve successful reprogramming.

fate, but seemingly closely related to the starter cell fate (fibroblasts and muscles are both mesoderm-derivatives) [28•]. One explanation for this may be that terminal selectors are not sufficient to repress fates more distant from their normal function in the nervous system. These considerations prompt the question to which extent developmental history (i.e. of fates closely related in their normal lineage) applies in direct reprogramming (for review see Masserdotti *et al.* [33•]).

One additional outcome of clashing cell identity programs is cell death. For example, excessive ROS levels that arise during direct neuronal reprogramming causing most of the cell death [9•] may be due to dysregulation during the metabolic conversion of glycolytic astrocytes into neurons relying predominantly on oxidative phosphorylation. Indeed, the neurogenic TFs seem to induce transcriptional short-cuts in direct reprogramming, such as the fast activation of relatively mature neuronal hallmarks, for example, of ion channels as fast as 4 hours after Neurog1 transduction [25]. In regard to the metabolic conversion this may imply changing the metabolism towards oxidative phosphorylation prior to implementing the protective machinery.

How can we then identify reprogramming short-cuts and the barriers that these short-cuts need to overcome? One wide-spread, pragmatic approach is using a cocktail of developmentally active TFs and then subtracting TFs that are not needed to overcome the lineage barriers [17,27,34,35]. Interestingly, this approach almost invariantly ends with pioneer TFs either on their own or together with other TFs [12••,13–15,20,21,23,24,36,37]. Pioneer TFs are defined by their capacity to bind compacted chromatin, increase the TF target site accessibility and foster the binding of other, lineage specific TFs to instruct the fate specification of progenitors during development [38–40]. Therefore, it is not surprising that a number of neuronal reprogramming cocktails contain the pioneer TFs Ascl1, Neurog2, NeuroD or Sox2 [10,12••, 17,18,20,21,25,28•,29•,33•,37,41•,42,43,44•,45••]. These pioneering factors are then combined with a number of cooperating, lineage specific factors that define different neuronal subtype lineages. Following the developmental logic of pioneering TF function [40], it is plausible to speculate that binding of the pioneer TF precedes the binding of the lineage specific, cooperating TFs and is necessary to establish the competence of the target cell to implement the developmental programs introduced by the cooperating factors. However, this concept has to be revised in reprogramming as — at least in some cases — binding of the pioneering TF is dependent on the cooperating TFs [46]. Finally, according to the developmental concept of pioneer TFs they would establish mainly competence for a fate, rather than implementing the new identity. Again this seems to be different in direct reprogramming as Ascl1, a bona-fide pioneering factor, is capable to instruct astrocytes to generate GABA-ergic neurons that fire action potentials without any further cooperating TFs [10,23,25,36]. However, in this case some of the cooperating lineage-specific TFs may already be present in astrocytes and hence not needed to be added exogenously. Taken together, direct reprogramming is implemented by the coordinated action of pioneer and cooperating lineage specific TFs with especially the former opening closed chromatin sites. But is this action of the pioneering TF sufficient to overcome all epigenetic barriers?

Pioneering factors and epigenetic landscape

Appropriate changes in the chromatin of the somatic cell and acquisition of the adequate metabolic state have been identified as a major hurdle in direct lineage reprogramming [9•,17,25,26,44•]. The chromatin is highly structured in the differentiated cell to ensure existence of the coherent transcriptional programs defining the cellular identity [17,25,26] and a number of epigenetic mechanisms including chromatin remodelling factors, REST complex and DNA methylation have been implicated in direct reprogramming. Based on the classical Waddington epigenetic landscape model, the major difference between the fate specification during development and direct reprogramming could be the higher order chromatin organization due to the sequence of events leading to the establishment of the lineage barriers. According to the Waddington model, lineage barriers are established as the progenitor roles downhill in the epigenetic landscape and the mountains between the valleys act as lineage barriers stabilizing the specific fate. The fate stabilization also includes the desired chromatin organization that favours binding of the developmental TFs in the active chromatin as well as the

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establishment of the repressed chromatin domains decreasing or inhibiting the binding of the alternative lineage specific TFs. The reprogramming would require the rewiring of the epigenetic landscape to allow cells to cross the developmentally established hills of the Waddington model. However, as discussed above, reprogramming does not necessarily follows the logic of developmental lineages. Therefore, the James-Cook Island model may be better suited to visualize the ease of fate conversion with some still submerged hurdles, such as corals [33•]. In either model or picture, the epigenetic hurdles need to be overcome. While pioneer TFs can achieve opening of some important closed sites, alternative or aberrant fates observed in direct reprogramming may be due to incomplete resolution of higher order chromatin. This prompts the question why only some alternative lineages emerge and according to which logic they emerge. Answering this question will be crucial to predict the alternative fates and improve the conversion to the appropriate fully differentiated cellular identity.

Understanding higher order chromatin changes during reprogramming — pioneering efficient full lineage re-specification.

As for transcription factor function, our knowledge about 3D chromatin looping changes during cell fate acquisition comes largely from development [47,48•,49] with few studies examining these changes in direct reprogramming — so far only in the context of induction of pluripotency from somatic cells [50•]. Parts of the single chromosomes self-interact and form topologically associating domains (TADs) with the help of architectural proteins such as CTCF or cohesin [51,52]. During neural differentiation (most often from ES cells) many if not most newly appearing TADs are associated with active transcription, but are not formed by CTCF, that is involved in most chromatin loops in ES cells. Indeed, the association of different TADs is highly dynamic during differentiation across different lineages [48•,50•,53]. The neural lineage-specific non-CTCF loops are instead formed by the TF YY1 and mostly involved in smaller loops within larger TADs [50•]. Most interestingly for direct reprogramming, also lineage-specific TFs, such as Pax6, NeuroD2 and Tbr1 [48•] suggesting that these functions may explain why these TFs are also powerful in reprogramming. However, to which extent these or other TFs can truly instruct new TADs and if so whether this works only within larger TADs and in an appropriate manner remains to be determined. In this regard, it is interesting to consider that high mobility group proteins, such as Sox2, are involved in many direct reprogramming protocols — even for different lineages, such as neurons or pluripotent stem cells [14,54]. Sox2 has the capacity to alter the chromatin structure [55] and promote reprogramming *in vitro* [24] and *in vivo* together with the pioneering factor Ascl1 [18]. As the pioneer factors bind repressed chromatin, they could act as drivers in the chromatin re-compartmentalization during direct reprogramming. However, they may need architectural and TAD forming proteins to implement this re-compartmentalization in the appropriate manner for a given cell type, for example, neurons. Indeed, direct reprogramming from neural towards pluripotent stem cells is accompanied by retaining some NSC-specific TADs and missing some ESC-specific TADs which is accompanied by respective changes in transcription of the genes affected [50•]. In this case, these aberrant loops can be fixed by growing the cells in 2i/LIF conditions to convert them to fully reprogrammed iPSCs. Such mis-wiring of the chromatin may also explain why direct reprogramming can also result in aberrant fates, such as muscle cells in neuronal reprogramming. Imprecise TADs or inappropriate formation of new TADs within the wrong larger TAD area that belongs to the previous lineage might be leading to the establishment of aberrant fates. Therefore, understanding the higher order chromatin structures and changes during reprogramming is essential to avoid incomplete re-wiring and aberrant transcription. This is not only essential to achieve a fully functional new cell identity, but also for utilizing these cells for repair purpose.

Conflict of interest statement

Nothing declared.

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