Direct neuronal reprogramming: learning from and for development

Giacomo Masserdotti1,2,*, Sergio Gascón1,2,† and Magdalena Götz1,2,3,‡

ABSTRACT

The key signalling pathways and transcriptional programmes that instruct neuronal diversity during development have largely been identified. In this Review, we discuss how this knowledge has been used to successfully reprogramme various cell types into an amazing array of distinct types of functional neurons. We further discuss the extent to which direct neuronal reprogramming recapitulates embryonic development, and examine the particular barriers to reprogramming that may exist given a cell’s unique developmental history. We conclude with a recently proposed model for cell specification called the ‘Cook Islands’ model, and consider whether it is a fitting model for cell specification based on recent results from the direct reprogramming field.

KEY WORDS: Conversion, Direct reprogramming, Neurogenesis, Neuron, Neuronal subtype, Transdifferentiation, Transcription factor

Introduction

During development, specific cell fates are programmed by extrinsic and intrinsic cues. Whereas loss-of-function experiments can test for the requirement of a gene, gain-of-function experiments aim to demonstrate the sufficiency of a gene in instructing developmental fate decisions. For example, MyoD was identified as a crucial regulator of muscle fate for its ability to convert mouse embryonic fibroblasts into muscle cells (Tapscott et al., 1988), and ectopic expression of eyeless, the homologue of Pax6 in Drosophila melanogaster, was sufficient to redirect the fate of antennae cells towards an eye phenotype in vivo (Halder et al., 1995; Ypsilanti and Rubenstein, 2016). Thus, the field of transcription factor-based reprogramming (see Glossary) emerged from testing the potential of key fate determinants that are active during development, and was employed to elucidate when and where such key factors may reach the limit of their function. Indeed, gain-of-function experiments moved from development to postnatal and adult cell types: for example, probing the role of Pax6 in neurogenesis led to the discovery that postnatal glial cells in vitro as well as some glial cells in the adult brain in vivo could be converted into neurons by this single transcription factor (Berninger et al., 2007; Buffo et al., 2008; Heins et al., 2002; Kronenberg et al., 2010; Ninkovic et al., 2013).

Initial attempts at direct reprogramming (see Glossary) were based on the assumption that cells derived from the same lineage or the same germ layer might be easier to convert compared with less closely related cells. This concept derives from and is depicted in the famous Waddington’s landscape model, which describes development as a unidirectional process that over time restricts the potential of a totipotent cell downwards towards a highly specialized cell through the establishment of epigenetic barriers, represented by high mountain peaks (Waddington, 1957). As a consequence of these barriers, conversion between cells derived from different lineages is prevented (Sieweke, 2015). However, over the years this notion has been challenged (Gurdon, 1962; Takahashi and Yamanaka, 2006), and direct reprogramming has proven that cells from various lineages can be directly converted into cells of other lineages, even across germ layers – for example, fibroblasts or hepatic cells into neurons (Marro et al., 2011; Vierbuchen et al., 2010). Indeed, this has been shown to occur naturally in vivo, a process known as transdifferentiation (see Glossary; Zuryn et al., 2014). Thus, lineage boundaries established during cellular specification and differentiation can be overcome, depending on the potency of the factors employed. This raises two fundamental and related questions: (1) to what extent does reprogramming recapitulate a developmental trajectory; and (2) is the developmental origin and, more specifically, the epigenetic history of the starting cell type negligible? The underlying rationale of reprogramming is based on the expression of factors that are essential during development; however, their action during direct reprogramming can be rather different. This is because the factors are active in a very different context, which may or may not provide the proper environment for them to fulfil their potential. Indeed, studying how certain factors function in various reprogramming contexts may bring new insight that can feed back into developmental studies.

In this Review, we provide an overview of the main concepts that have emerged from recent studies in direct neuronal reprogramming, including the lessons this field has learnt from developmental biology, as well as new ideas for future research in neural development. Specifically, we discuss neural specification during development and provide a summary of how direct reprogramming can instruct specific neuronal subtypes, and the remaining challenges for each cell type. We further consider the mechanistic basis for these direct reprogramming approaches, which includes not only transcriptional and epigenetic events, but also regulation of the cell cycle and metabolic state. Sieweke recently proposed a model of cell fate acquisition based on Captain Cook’s voyage through the Pacific islands, hereafter termed the ‘Cook Islands’ model (Sieweke, 2015), which is a more comprehensive and accurate model of direct reprogramming between different cell types, encompassing many variables such as cell cycle and metabolic state. We conclude this Review by highlighting the close interplay between reprogramming and developmental biology, and discuss how the two related areas can inform and advance each other.
The transcription factor Pax6 is expressed in forebrain, sonic hedgehog (Shh) and bone morphogenetic protein (Bmp) signalling instruct the ventral and dorsal telencephalon, respectively (Fig. 1A). The transcription factor Pax6 is expressed in NSCs and NPCs in the dorsal telencephalon, where it antagonizes the transcription factors Gsx1 and Gsx2, which are expressed in the NSCs and NPCs of the ventral telencephalon (Fig. 1A) (Hébert and Fishell, 2008).

Extrinsic signalling events not only pattern distinct regions of the developing brain, but they also initiate transcriptional cascades that eventually lead to the generation of different neuronal and glial subtypes. In the dorsal telencephalon, Pax6 and Emx1/2+ NSCs upregulate the proneural (see Glossary) basic helix-loop-helix (bHLH) transcription factors Neurog1 (also known as Ngn1) and Neurog2 (also known as Ngn2) in regions where the signalling factor wingless (Wnt) is highly expressed (Fig. 1B) (Ohtaka-Maruyama and Okado, 2015). Neurog1 and Neurog2 instruct the fate of glutamatergic neurons in the dorsal telencephalon through the induction of several downstream targets, such as NeuroD and the T-box brain genes Tbr2 (Eomes) and Tbr1 (Fig. 1B) (Fode et al., 2000; Guillemot, 2007). In this region, glutamatergic projection neurons are generated in successive waves forming distinct horizontal layers of neurons projecting to different targets and receiving distinct inputs (Toma and Hanashima, 2015). Each of these projection neuron subtypes is specified by a set of transcription factors (Lodato and Arlotta, 2015; Molyneaux et al., 2008), some of which have the hallmarks of master regulators or neuronal selector genes (see Glossary) (Hobert, 2011). For instance, the transcription factor Fez2 specifies cortico-spinal glutamatergic projection neurons by directly binding to and regulating genes that govern glutamatergic transmitter identity and axonal pathfinding, while it represses genes of the GABAergic fate (Lodato et al., 2014). Thus, transcription factor cascades regulate both early and late aspects of neuronal identity.

The mechanisms that specify glutamatergic neurons differ along the neuraxis, such that the transcription factors Tlx1 and Tlx3 are postmitotic selectors for glutamatergic fate in the spinal cord (Cheng et al., 2004), whereas in the brain they are not expressed and Tbr1/2 take on their roles (Fig. 1B). Likewise, GABAergic neurons are specified by Dlx genes in the ventral telencephalon (Casarosa et al., 1999; Guillemot et al., 1993; Poitras et al., 2007), by Gata2 and Helt (also known as Megane) in the di- and mesencephalon, and by Prf1a in the spinal cord, cerebellum and retina (Achim et al., 2014). In the dorsal midbrain, the generation of GABAergic neurons requires the expression of Ascl1 and Helt (Guimera et al., 2006; Wende et al., 2015).

Cross-repressive mechanisms ensure the generation of one (glutamatergic) or the other (GABAergic) neuron identity. For instance, deletion of Neurog1 and Neurog2 in the telencephalon causes de-repression of Ascl1 and the aberrant generation of GABAergic neurons in the dorsal telencephalon (Casarosa et al., 1999; Fode et al., 2000; Nieto et al., 2001). Conversely, Neurog2 expression in Ascl1 null embryos rescues ventral neurogenesis but does not induce ectopic expression of glutamatergic markers (Parras et al., 2002), demonstrating the key role of Dlx and Olig2 transcription factors in governing GABAergic neuron fate in this region (Petryniak et al., 2007). Many of the key transcription factors that regulate neuronal identity have been identified (Fig. 1B), and it is clear that they act in a highly region-specific context and as part of specific transcriptional networks.

The knowledge gained from studies on embryonic neurogenesis raised several questions with regard to direct neuronal reprogramming. First, to what extent can one transcription factor induce the correct neuronal subtype if expressed in cells of the same regional specification, for example glial cells of the same region? Second, to what extent does the neuronal reprogramming process recapitulate the programme elicited by a particular
transcription factor in the same region during development, and would the same factor, when expressed in cells with a different regional background, elicit a different neuronal identity, as observed during development? Third, would additional factors be required to convert developmentally more distant cell types, for example fibroblasts into neurons? Finally, given the role of major signalling pathways in establishing regionalization and neuronal specificity during development, to what extent is the signalling information required for specifying neuronal identity during reprogramming? Throughout the remainder of this Review, we will examine each of these questions closely and attempt to answer them based on experimental data.

**Direct neuronal reprogramming: learning from development**

The first evidence for direct neuronal reprogramming was obtained when it was shown that Pax6, the deletion of which impairs neurogenesis in the developing forebrain, was sufficient to convert postnatal glial cells isolated from cerebral cortex into neurons (Heins et al., 2002). Neurog2, a downstream target of Pax6, proved to be even more efficient in converting postnatal astrocytes into fully functional glutamatergic pyramidal-like neurons (Fig. 2) (Berninger et al., 2007; Heinrich et al., 2010). Interestingly, forced expression of Ascl1, together with Dlx2, has been shown to elicit the generation of GABAergic neurons in agreement with its role in the developing telencephalon (Casanosa et al., 1999; Heinrich et al., 2010). In essence, a single transcription factor is thus able to reprogramme non-neuronal cells into the respective type of fully functional neuron of the same brain region, thus addressing the first question above. Other factors, however, are not necessarily sufficient on their own to instruct a full neuronal identity, and are therefore not considered to be master regulators (see Glossary). Only factors that are sufficient to achieve direct fate conversion entirely on their own qualify as such, even though they may not necessarily act as pioneer factors (see Glossary) with the capacity to open closed chromatin.

The ability of these proneural factors to impose a specific neuronal phenotype in glial cells paved the way for addressing the second question: namely, to examine the transcriptional events that occur during the conversion process and compare them with those identified during development. The fact that different transcription factors could be used to reprogramme the same cells (astrocytes) from the same region (cerebral cortex) into distinct neuronal subtypes also provided an unprecedented opportunity to examine the transcriptional networks induced by different factors in the same cellular context.
To begin to understand the transcriptional cascade triggered during reprogramming, unbiased transcriptome analysis was performed on astrocytes reprogrammed with either Ascl1 or Neurog2. Many genes showed dynamic changes in expression during the time course analysed, suggesting the rapid acquisition of a neuronal fate (Masserdotti et al., 2015). Surprisingly, Neurog2- and Ascl1-induced transcriptional changes showed little overlap, suggesting that Neurog2 and Ascl1 regulate largely different neurogenic cascades in the same cellular background. However, some, albeit few, genes were regulated by both transcription factors, such as common neuronal components of the cytoskeleton like Dcx and Tubb3 (βIII-tubulin), and also a few common transcriptional regulators such as Insml, Hes6, Neurod4, Proxl, Sox11 and Trnp1 (Bae et al., 2000; Farkas et al., 2008; Inoue et al., 2002; Lavado et al., 2010; Mu et al., 2012; Ninkovic et al., 2013; Stahl et al., 2013). Notably, not only are some of the identified downstream targets required for Neurog2- and Ascl1-induced neuronal conversion, but Neurod4 alone was also sufficient to generate functional neurons, although apparently not sufficient to elicit a neuronal subtype identity. Neurod4 is a close family member of Neurod1, which is also a downstream target of Neurog2 and also appears to be sufficient to reprogramme astrocytes into functional glutamatergic neurons in vitro and in vivo (Fig. 2; Table 1) (Guo et al., 2014). Importantly, in vitro transcriptional analysis revealed that the hierarchy in gene activation during reprogramming was similar to that observed during in vivo development (Masserdotti et al., 2015). For example, Tbr2 and Tbr1, although direct targets of Neurog2 (Schuurmans et al., 2004), were not induced early during reprogramming, but at later time points, suggesting that their activation requires other events, such as chromatin remodelling and/or expression of other factors, to occur earlier (Berninger et al., 2007; Englund et al., 2005; Heinrich et al., 2010; Masserdotti et al., 2015). Despite these commonalities, however, overall there was little overlap between Neurog2-regulated genes during reprogramming and development in cerebral cortex progenitors (Gohlke et al., 2008; Masserdotti et al., 2015), indicating that embryonic neurogenesis is not fully recapitulated in direct neuronal reprogramming, but rather that different transcriptional circuits may be at play.

Reprogramming of astrocytes is particularly useful for understanding the role of regional identity in influencing neuronal subtypes, as astrocytes appear to inherit and maintain their regional identity (Hochstim et al., 2008). For instance, expression of Ascl1 in cerebral cortex-derived astrocytes induces a GABAergic programme (Masserdotti et al., 2015), whereas it leads to the production of both GABAergic and glutamatergic neurons in dorsal midbrain astrocytes in vitro, consistent with its role during embryonic development in that region (Liu et al., 2015; Achim et al., 2014). Interestingly, the efficiency of neuronal conversion is lower when instructing a type of neuron not normally generated from cells of this region. For example, expression of Neurog2 in cortical astrocytes induces neurons with 70-80% efficiency, whereas Ascl1 generates neurons with only about 40% efficiency (Heinrich et al., 2010). These data suggest an important role for regional specification of the starter cell in defining the identity of the induced neurons.

Reprogramming of fibroblasts highlights the relevance of the starting population in influencing the neuronal subtype obtained by expression of the same transcription factor. In terms of their developmental origins, fibroblasts are more distantly related to neurons than are astrocytes, and so an important question is whether and how this affects direct reprogramming. Forced expression of Ascl1 in cultured fibroblasts generates...
<table>
<thead>
<tr>
<th>Reprogramming factors</th>
<th>Source</th>
<th>Species</th>
<th>Subtype</th>
<th>Small molecule/other treatments</th>
<th>Co-culture</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mixed neurons</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Pax6</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td>Heins et al., 2002</td>
<td></td>
</tr>
<tr>
<td>2 Ascl1, Neurog2</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td>Berninger et al., 2007</td>
<td></td>
</tr>
<tr>
<td>Pax6</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Neurog2, Dlx2, Ascl1+Dlx2</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic</td>
<td>No</td>
<td>Heinrich et al., 2010</td>
<td></td>
</tr>
<tr>
<td>4 Ascl1, Brn2, Myt1l</td>
<td>Mouse embryonic fibroblasts, postnatal fibroblasts</td>
<td>Mouse</td>
<td>GABA/Gluta</td>
<td>Glia, cortical neurons</td>
<td>Vierbuchen et al., 2010</td>
<td></td>
</tr>
<tr>
<td>5 Ascl1, Brn2, Myt1l, NeuroD1</td>
<td>Embryonic fibroblasts, postnatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta/Dop</td>
<td>Glia</td>
<td>Pang et al., 2011</td>
<td></td>
</tr>
<tr>
<td>6 Ascl1, Brn2, Myt1l</td>
<td>Hepatocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td>Marro et al., 2011</td>
<td></td>
</tr>
<tr>
<td>7 miR-124, Brn2, Myt1l</td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta</td>
<td>Noggin, FK</td>
<td>Ambasudhan et al., 2011</td>
<td></td>
</tr>
<tr>
<td>8 miR-9*, miR-124, NeuroD2, (Ascl1) (Myt11)</td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta</td>
<td>VPA dbcAMP</td>
<td>Yoo et al., 2011</td>
<td></td>
</tr>
<tr>
<td>9 Neurog2, Ascl1</td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta (mainly Gluta), serotoninergic, Th^+</td>
<td>SB431542, CHIR99021, noggin (or LDN193189)</td>
<td>Ladewig et al., 2012</td>
<td></td>
</tr>
<tr>
<td>10 Ascl1, Sox2</td>
<td>Pericytes</td>
<td>Human</td>
<td>GABAergic</td>
<td>E14.5 cortical neurons</td>
<td>Karow et al., 2012</td>
<td></td>
</tr>
<tr>
<td>11 Ptb knockdown</td>
<td>Prenatal fibroblasts</td>
<td>Mouse</td>
<td>GABA/Gluta</td>
<td>Glia</td>
<td>Xue et al., 2013</td>
<td></td>
</tr>
<tr>
<td>12 Pax6, Brg1, Brn2, Sox11</td>
<td>Postnatal glia</td>
<td>Not reported</td>
<td>Not reported</td>
<td>No</td>
<td>Ninkovic et al., 2013</td>
<td></td>
</tr>
<tr>
<td>13 Ascl1</td>
<td>Prenatal fibroblasts</td>
<td>Mouse</td>
<td>GABA/Gluta</td>
<td>Low oxygen</td>
<td>Glia</td>
<td>Chanda et al., 2014</td>
</tr>
<tr>
<td></td>
<td>Prenatal fibroblasts</td>
<td>Mouse</td>
<td>GABA/Gluta</td>
<td>Low oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prenatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta</td>
<td>Low oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>GABA/Gluta</td>
<td>Low oxygen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 NeuroD1</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic</td>
<td>Guo et al., 2014</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal NG2 cells</td>
<td>Mouse</td>
<td>GABA/Gluta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fetal astrocytes</td>
<td>Human</td>
<td>Glutamatergic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 NeuroD4, Insm1</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic (low %)</td>
<td>No</td>
<td>Masserdotti et al., 2015</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>No</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continued
<table>
<thead>
<tr>
<th>Reprogramming factors</th>
<th>Source</th>
<th>Species</th>
<th>Subtype</th>
<th>Small molecule/other treatments</th>
<th>Co-culture</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NeuroD4, Sox11</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>NeuroD4+Insm1</td>
<td>Mouse embryonic fibroblasts</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>NeuroD4</td>
<td>Fetal astrocytes</td>
<td>Human</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>NeuroD4+Insm1</td>
<td>Fetal astrocytes</td>
<td>Human</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>16 Ascl1, Sox2, FoxG1, Dlx5, Lhx6</td>
<td>Embryonic fibroblasts</td>
<td>Mouse</td>
<td>GABAergic</td>
<td>Glia, hippocampal rat neurons</td>
<td>Colasante et al., 2015</td>
<td></td>
</tr>
<tr>
<td>17 Ascl1, Bcl2</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td>Gascon et al., 2016</td>
</tr>
<tr>
<td>Neurog2, Bcl2</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Glutamatergic</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Ascl1, Bcl2</td>
<td>Embryonic fibroblasts</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2, Bcl2</td>
<td>Embryonic fibroblasts</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Ascl1</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td>FK</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Ascl1, Vdr</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2, Vdr</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Ascl1, Bcl2, Vdr</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2, Bcl2, Vdr</td>
<td>Postnatal cortical astrocytes</td>
<td>Mouse</td>
<td>Not reported</td>
<td></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2, Bcl2</td>
<td>In vivo reprogramming</td>
<td>Mouse</td>
<td>Excitatory (Clp2⁺)</td>
<td>α-T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurog2, Bcl2</td>
<td>In vivo reprogramming</td>
<td>Mouse</td>
<td>Not reported</td>
<td>Calcitriol</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Motor neurons**

| 18 Ascl1, Bm2, Myt11, Neurog2, Lhx3, Isl1, Hb9 | Prenatal fibroblasts | Mouse | Motor neurons | Myoblasts from E10 epaxial muscles (chick embryo) | Son et al., 2011 |
| Ascl1, Bm2, Myt11, Neurog2, Lhx3, Isl1, Hb9 | Tail fibroblasts     | Mouse | Motor neurons |                                                |            |
| ASCL1, BRN2, MYT1L, NEUROG2, LHX3, ISL1, HB9, NEUROD1 | Fibroblasts (derived from iPSCs) | Human | Motor neurons |                                                |            |
| 19 NEUROG2 | Prenatal lung fibroblasts | Human | Cholinergic neurons | FK, DM                  | C2C12-derived myotubes | Liu et al., 2013 |
| NEUROG2, SOX11 | Postnatal fibroblasts | Human | Cholinergic neurons | FK, DM                  |                                                |            |
| 20 NEUROG2, SOX11, ISL1, LHX3 | Adult fibroblasts | Human | Motor neurons | FK, DM                  | Glia, skeletal myotubes | Liu et al., 2015 |
| NEUROG2, SOX11, ISL1, LHX3 | Adult fibroblasts from ALS patients | Human | Motor neurons | FK, DM                  |                                                |            |

**Dopaminergic neurons**

| 21 Ascl1, Bm2, Myt11, Lmx1a, Foxa2 | Prenatal fibroblasts | Human | Dopaminergic | No | Pfisterer et al., 2011 |
| 22 Ascl1, Nurr1, Lmx1a | Prenatal fibroblasts | Mouse | Dopaminergic | No | Caliazzo et al., 2011 |
| Ascl1, Nurr1, Lmx1a | Postnatal fibroblasts | Mouse | Dopaminergic | No |            |
| 23 Ascl1, Nurr1, Lmx1b | Astrocytes | Mouse | Dopaminergic | No | Addis et al., 2011 |

Continued
<table>
<thead>
<tr>
<th>Reprogramming factors</th>
<th>Source</th>
<th>Species</th>
<th>Subtype</th>
<th>Small molecule/other treatments</th>
<th>Co-culture</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 Ascl1, Pitx3</td>
<td>Postnatal fibroblasts</td>
<td>Mouse</td>
<td>Dopaminergic</td>
<td>No</td>
<td>Kim et al., 2011</td>
<td></td>
</tr>
<tr>
<td>25 Ascl1, Brn2, Myt1l</td>
<td>Embryonic fibroblasts, postnatal fibroblasts</td>
<td>Human</td>
<td>Not reported</td>
<td>No</td>
<td>Torper et al., 2013</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 Ascl1, Nur1, Lmx1a</td>
<td>Prenatal fibroblasts</td>
<td>Mouse</td>
<td>Dopaminergic</td>
<td>No</td>
<td>Dell’Anno et al., 2014</td>
<td></td>
</tr>
</tbody>
</table>

**Sensory neurons**

<table>
<thead>
<tr>
<th>27 Neurog2 (or Neurog1), Brn3a</th>
<th>Prenatal fibroblasts</th>
<th>Mouse</th>
<th>Sensory</th>
<th>No</th>
<th>Blanchard et al., 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurog2 (or Neurog1), Brn3a</td>
<td>Postnatal fibroblasts</td>
<td>Mouse</td>
<td>Sensory</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2 (or Neurog1), Brn3a</td>
<td>Fibroblasts (derived from iPSCs)</td>
<td>Human</td>
<td>Sensory</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Neurog2 (or Neurog1), Brn3a</td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>Sensory</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>28 Ascl1, Brn2, Myt1l, Neurog1, Is1, Klf7</td>
<td>Prenatal fibroblasts</td>
<td>Mouse</td>
<td>Sensory</td>
<td>Glia</td>
<td>Wainger et al., 2015</td>
</tr>
<tr>
<td>Ascl1, Brn2, Myt1l, Neurog1, Is1, Klf7</td>
<td>Postnatal fibroblasts</td>
<td>Human</td>
<td>Sensory</td>
<td>Glia</td>
<td></td>
</tr>
</tbody>
</table>

**Striatal neurons**

| 29 miR-9*, miR-124, Bcl11B, Dlx1, Dlx2, Myt11 | Postnatal fibroblasts | Human | Striatal medium spiny neurons | Glia                            | Victor et al., 2014    |

**Photoreceptors**

| 30 NeuroD1, Crx, Rax, Obx2 | Dermal fibroblasts | Human | Photoreceptor cells | Seko et al., 2014 |

**Small molecule-converted neurons**

<table>
<thead>
<tr>
<th>31 Fibroblasts</th>
<th>Mouse</th>
<th>GABA/Gluta</th>
<th>FK, ISX9, CHIR99021, I-BET151</th>
<th>Li et al., 2015</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 Fibroblasts</td>
<td>Human</td>
<td>Glutamatergic (80%)</td>
<td>VPA, CHIR99021, Repsox, FK, SP600625, GO6983, Y-27632</td>
<td>Hu et al., 2015</td>
</tr>
<tr>
<td>Fibroblasts from FAD patients</td>
<td>Human</td>
<td>Not reported</td>
<td>VPA, FK, CHIR99021, Repsox, SP600625, GO6983, Y-27632</td>
<td></td>
</tr>
<tr>
<td>33 Astrocytes</td>
<td>Human</td>
<td>Glutamatergic (90%), GABA (8%)</td>
<td>LDN193189, SB431242, TTNPB, thiazovivin, CHIR99021, DAPT, SAG, Purmo</td>
<td>Zhang et al., 2015</td>
</tr>
</tbody>
</table>

**Notes:**
- DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
- DM, dorsomorphin; dbcAMP, dibutyryl-cyclic AMP
- E, embryonic day
- FAD, familial Alzheimer’s disease
- FK, forskolin
- GABA/Gluta, mixed population of GABAergic and glutamatergic neurons
- GABA/Gluta/Dop, mixed population of GABAergic, glutamatergic and dopaminergic neurons
- iPSCs, induced pluripotent stem cells
- ISX9, isoxazole 9
- Ptb, polypyrimidine tract binding protein (Ptbp1)
- Purmo, purmorphamine
- SAG, smoothened agonist
- Th, tyrosine hydroxylase
- TTNPB, artonoid acid
- VPA, valproic acid

**References:**
- Kim et al., 2011
- Torper et al., 2013
- Dell’Anno et al., 2014
- Blanchard et al., 2015
- Wainger et al., 2015
- Victor et al., 2014
- Seko et al., 2014
- Li et al., 2015
- Hu et al., 2015
- Zhang et al., 2015
glutamatergic neurons (Chanda et al., 2014), whereas it induces a mix of glutamatergic and GABAergic neurons from midbrain astrocytes (Achim et al., 2014; Liu et al., 2015). Likewise, fibroblasts transduced with Neurog2 and treated with small molecules (see next section) do not turn into glutamatergic neurons but rather acquire a cholinergic/motor neuron identity (Liu et al., 2013). Thus, it is clear that the starting cell identity influences the neuronal subtype obtained (Table 1); however, the extent to which this is linked to the developmental memory rather than the specific transcriptome or proteome of the starting cell remains to be understood. Initial attempts to reprogram human fibroblasts into neurons relied on four factors, a finding that many believed was owing to their relative developmental distance from neurons (Fig. 2) (Pang et al., 2011). However, as we will discuss below, manipulation of key signalling pathways can lead to the efficient conversion of human cells with relatively few transcription factors, and thus careful determination of the correct reprogramming environment seems to be equally important as, if not more important than, using multiple factors for reprogramming.

**The role of region-specific signalling pathways in direct reprogramming: links to development**

Given the importance of signalling pathways in specifying and patterning neural tissue during development, one may anticipate that small molecule agonists or antagonists influencing these pathways could also contribute to reprogramming cells into neurons. Strikingly, manipulation of these and other pathways is indeed sufficient to convert fibroblasts (mouse and human) and astrocytes (human) into neurons (Hu et al., 2015; Li et al., 2015; Zhang et al., 2015).

Table 2: Summary of the small molecules employed in direct neuronal reprogramming, and their action on the known target pathways

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pathway</th>
<th>Effect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td>cAMP pathway</td>
<td>Activation</td>
<td>Liu et al., 2013, 2015; Li et al., 2015</td>
</tr>
<tr>
<td>Dorsomorphin</td>
<td>TGFβ, BMP</td>
<td>Inhibition</td>
<td>Liu et al., 2013, 2015</td>
</tr>
<tr>
<td>Noggin</td>
<td>BMP</td>
<td>Inhibition</td>
<td>Ladewig et al., 2012</td>
</tr>
<tr>
<td>LDN193189</td>
<td>TGFβ, BMP</td>
<td>Inhibition</td>
<td>Hu et al., 2015</td>
</tr>
<tr>
<td>RepSox</td>
<td>TGFβ, BMP</td>
<td>Inhibition</td>
<td>Hu et al., 2015</td>
</tr>
<tr>
<td>SB431542</td>
<td>SMAD</td>
<td>Inhibition</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>Y27632</td>
<td>ROCK</td>
<td>Inhibition</td>
<td>Hu et al., 2015</td>
</tr>
<tr>
<td>Thiazovivin</td>
<td>ROCK</td>
<td>Inhibition</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>SAG</td>
<td>Shh pathway</td>
<td>Activation</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>Purmorphasmine</td>
<td>Shh pathway</td>
<td>Activation</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>DAPT</td>
<td>Notch signalling</td>
<td>Inhibition</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>GSK-3</td>
<td>Inhibition</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>TTNPB</td>
<td>Retinoic acid</td>
<td>Activation</td>
<td>Zhang et al., 2015</td>
</tr>
<tr>
<td>Valproic acid</td>
<td>Chromatin modification</td>
<td>HDAC</td>
<td>Hu et al., 2015</td>
</tr>
<tr>
<td>ISX9</td>
<td>Ca²⁺ signalling</td>
<td>Activation</td>
<td>Li et al., 2015</td>
</tr>
<tr>
<td>I-BET151</td>
<td>BET family proteins</td>
<td>Inhibition</td>
<td>Li et al., 2015</td>
</tr>
</tbody>
</table>

Neuronal conversion of human astrocytes and fibroblasts mediated by small molecules seems to require sequential phases of reprogramming, or a first phase of neuronal fate induction followed by a second phase of neuronal maturation (Table 1; Hu et al., 2015; Zhang et al., 2015). This strict order suggests some degree of organization in the molecular cascades involved in reprogramming that may recapitulate a developmental programme. Remarkably, most of these small molecules influence key developmental pathways. For example, each chemical-mediated reprogramming paradigm contains at least one compound that interferes with the activation of the transforming growth factor β (TGFβ) family of proteins (Table 2; Ladewig et al., 2012; Liu et al., 2013; Zhang et al., 2015; Hu et al., 2015; Li et al., 2015), or their immediate downstream effectors, the SMAD family of transcription factors (Ladewig et al., 2012; Li et al., 2015). As these pathways abolish neural induction during embryogenesis (Chang and Harland, 2007; Yaguchi et al., 2007) and regulate epithelial-to-mesenchymal transition (EMT) (Dunn et al., 2004; Yang and Weinberg, 2008), their inhibition may contribute to the repression of alternative cell fates, in particular in cells of mesenchymal origin such as fibroblasts. Interestingly, inhibition of the TGFβ pathway has also been found to enhance neuronal conversion of astrocytes (Zhang et al., 2015), which is consistent with the role of TGFβ in restricting neuronal differentiation of NSCs and promoting their progression towards the astrocytic lineage (Stipursky and Gomes, 2007).

Another approach for small molecule-based neuronal reprogramming has been the use of inhibitors to block the activity of Rho-associated protein kinase, otherwise known as ROCK inhibitors (Hu et al., 2015; Zhang et al., 2015). During neural development, Rho family molecules regulate neuronal differentiation, migration (Cappello, 2013; Cappello et al., 2012) and neurite development (Govork et al., 2005). In vitro, RhoA inhibits nerve growth factor (NGF)-induced formation of the actin-rich filaments that initiate neurite growth in neurons and prevents neurite outgrowth promoted by neurotrophin 3 (NT-3; Nf3) and brain-derived neurotrophic factor (BDNF) (Da Silva et al., 2003; Schwamborn and Püschel, 2004; Yamaguchi et al., 2001). Accordingly, ROCK inhibitors promote differentiation of embryonic stem cells into neurons and induce neurite outgrowth of reprogrammed neurons (Hu et al., 2015). In agreement with the role of Rho kinase pathways in cell death and survival during embryogenesis, ROCK inhibition also improved survival in direct neuronal reprogramming (Shi and Wei, 2007; Zhang et al., 2015).

Shh specifies neuronal subtypes in a concentration-dependent manner in vivo (Briscoe and Small, 2015), and agonists of Shh have also been used in reprogramming of astrocytes in vitro (Fig. 1; Table 2; Zhang et al., 2015). It would be interesting to test whether exposure to different concentrations of Shh could convert astrocytes into different neuronal subtypes, either by re-specifying dorsoventral patterning or by co-regulating neuron-specific genes (Fig. 1). This could also contribute to uncovering the molecular mechanisms underlying the specification of neuronal identities during development. Another fundamental cascade involved in the maintenance of progenitor cells and inhibition of neuronal differentiation during embryogenesis is Notch signalling (Loui and Artavanis-Tsakonas, 2006), which also promotes astrogliogenesis (Namihiro et al., 2009) besides many other roles in neural development. Treatment with DAPT, a γ-secretase inhibitor that blocks the proteolytic cleavage of the activated Notch receptor and promotes neuronal differentiation in competent cells (Pierfelice et al., 2011), has been shown to facilitate the conversion of human astrocytes towards induced neurons (Zhang et al., 2015).
et al., 2015). In summary, these observations clearly demonstrate some similarities between the processes that regulate normal neuronal differentiation during development and direct neuronal reprogramming mediated by small molecules.

Despite the examples discussed above, the link between well-known signalling pathways active in development and reprogramming is not always so clear. For instance, all reprogramming paradigms include GSK-3 (Gsk3b) inhibitors, which seem to activate neuronal genes during changes in cell fate, such as Ncam1, Dcx, Map2 and several neurogenic fate determinants, such as Ascl1 or Neurog2 (Hu et al., 2015). However, during development, GSK-3 activity enhances neuronal differentiation of radial glia and reduces progenitor expansion (Kim et al., 2009). In agreement with these observations, GSK-3 inhibitors significantly improve the reprogramming efficiency of fibroblasts towards pluripotency (Kirby et al., 2012; Lyssiotis et al., 2009) and facilitate self-renewal of mouse embryonic stem cells (Knockaert et al., 2002), probably through activation of Wnt signalling (Marson et al., 2008). These observations suggest that the downregulation of the GSK-3 pathway opposes neuronal differentiation and promotes a progenitor-like stage. Perhaps a reversion to a more undifferentiated state may have some beneficial effect in direct reprogramming, although progenitor genes such as Sox2, Pax6 or Olig2 are induced very little, if at all (Hu et al., 2015; Zhang et al., 2015).

Overall, these recent approaches relying entirely on small molecules uncover the possibility of instructing a neuronal fate without any genetic manipulation, and demonstrate the power of these versatile pathways to specify cell fate. However, more work is needed to understand how the interplay among such pathways mediates fate conversion and to what extent this mimics their activity during development.

**Neuronal subtype specification: from dopaminergic neurons to photoreceptors**

Rather than recapitulating the exact developmental signalling environment required for the activation of specific transcription factor networks (Achim et al., 2014; Briscoe and Small, 2015), a more direct approach to neuronal subtype specification could be to overexpress the respective transcriptional regulators of a core specification programme active during development. Indeed, this approach has been used for inducing many other neuronal subtypes lost in traumatic injury or neurodegenerative diseases, and generation of which *in vitro* could be potentially useful for cell-based therapies as well as for disease modelling (Blanchard et al., 2015; Caiazzo et al., 2011; Gascón et al., 2016; Rouaux and Arlotta, 2013; Son et al., 2011; Victor et al., 2014; Wainger et al., 2015). Especially for the latter, direct reprogramming has the advantage of maintaining the age of the starter cell (Mertens et al., 2015), in contrast to the re-setting that occurs when generating induced pluripotent stem cells (Lapasset et al., 2011). In the next section, we will review successful attempts to generate very distinct neuronal subtypes (Fig. 2) with the aim of deciphering the molecular logic involved in establishing their identity (Fig. 3).

**Dopaminergic neurons**

Midbrain dopaminergic neurons are of great interest owing to their loss in Parkinson’s disease. They are generated in the floor plate of the mesencephalon (Fig. 1) and several genes have been implicated in their generation and specification. These include Otx2, which is involved in early patterning; FoxA1/2, which instructs the commitment of the progenitor cells; Lmx1a/b, which is important for progenitor cell differentiation; and Pitx3 and Nurr1 (Ntr4a2), which are involved in the maturation and long-term survival of midbrain dopaminergic neurons (Fig. 1; Arenas et al., 2015). Accordingly, many of these transcription factors have been successfully used to induce dopaminergic neurons from fibroblasts or astrocytes (Figs 2, 3; Addis et al., 2011; Jang et al., 2011; Pfisterer et al., 2011; Torper et al., 2013), with some of the resulting cells even capable of surviving when transplanted *in vivo* (Kim et al., 2011).

The molecular logic behind the different transcription factor combinations for reprogramming is such that factors active at different stages of specification and differentiation are usually combined. As mentioned above, Ascl1, Lmx1a and Nurr1 are active at different stages in the dopaminergic lineage and together can reprogramme fibroblasts into dopamine-releasing neurons (Caiazzo et al., 2011; Dell’Anno et al., 2014). However, none of these studies has been able to generate dopaminergic neurons belonging to the specific A8-10 clusters (Hegarty et al., 2013). Thus, it appears that a deeper level of understanding is required regarding the key regulators that specify the A8-10 clusters in order to generate...
different functional subtypes of midbrain dopaminergic neurons via
direct reprogramming (Kriks et al., 2011).

Striatal neurons
A further important neuronal subtype recently produced by direct
neuronal reprogramming are the medium-sized spiny neurons (MSNs).
These are the main output neurons of the striatum and the ones
that degenerate in Huntington’s disease (Zuccato et al., 2010).
They have been induced from human fibroblasts by combining pan-
neurogenic factors such as mir-124, mir-9/9* (Yoo et al., 2011)
and Myt1l (Vierbuchen et al., 2010), with Dlx1/2, a key patterning
factor of the lateral ganglionic eminence (Fig. 1A) and Bcl11B (also
known as Ctp2), a late postmitotic differentiation factor from this
region (Arlotta et al., 2008). Interestingly, some transplanted
induced MSNs survived for more than 6 months and projected to
their correct targets, the globus pallidus and substantia nigra (Victor
et al., 2014). Interestingly, Ctp2 is required for the normal
development of striatal patch neurons, but so far the patch identity
has not yet been detected in the induced MSNs.

Cerebral cortex projection neuron subtypes
Although glutamatergic pyramidal-like neurons have already been
generated in vitro (Heinrich et al., 2010; Maserdotti et al., 2015),
the crucial next step is to instruct distinct neuronal subtypes located
in different layers and projecting to distinct target sites, either within
the cerebral cortex or to subcortical targets (Lodato and Arlotta,
2015). Seminal work has demonstrated the conversion of one type
of cortical projection neuron, the callosal projection neurons of layer
II/III, into layer-V/VI subcortical projection neurons via the forced
expression of the transcription factor Fez2 at an early stage of
differentiation (Lodato and Arlotta, 2015; Rouaux and Arlotta,
2013). Interestingly, direct reprogramming from non-neuronal cells
using Neurog2 and Bcl2 generated deep layer pyramidal neurons,
based on the expression of Cht2 and FoxP2 and absence of the
upper layer markers Cux1 and Satb2 (Gascón et al., 2016).
Although much more work is required to identify the efferent
projections and afferent innervation of these neurons, this is yet
another example of how a transcription factor important for cell-type
specification during development can also specify that cell type
during reprogramming, as Neurog2 is involved in deep layer neuron
generation in vivo (Schuurmans et al., 2004).

Motor neurons
Direct reprogramming to spinal cord motor neurons has also relied
on the expression of key developmental regulators (Figs 1, 3;
Table 1; Son et al., 2011), again by combining common neurogenic
factors such as Ascl1, Neurog2, Myt1l and Brn2 (Pou3f2) with
transcription factors specific to spinal cord motor neuron
development, such as Lhx3 (Cho et al., 2014), Isl1 (Cho et al.,
2014; Ericson et al., 1992; Thaler et al., 2002) and Hb9 (Mnx1) (Lee
et al., 2009, 2005). The combination of these seven factors
generated functional motor neurons from mouse embryonic
fibroblasts, which were capable of forming functional
neuromuscular junctions with co-cultured myotubes and could
survive when transplanted in vivo (Son et al., 2011).

An alternative approach to generate induced motor neurons has
been the expression of two pan-neurogenic transcription factors,
Neurog2 and Sox11, together with forskolin, dorsomorphin and
Fgf2 treatment. This combination was sufficient to reprogramme
postnatal and adult human fibroblasts into cholinergic (as marked
by choline acetyltransferase, ChAT) Hb9+ induced motor
neurons (Fig. 2) (Liu et al., 2013). However, these neurons
were only partially specified, as they failed to express Isl1 and
Lhx3. Indeed, the co-expression of these two factors together
with Neurog2 and Sox11 significantly increased the proportion
of reprogrammed motor neurons: up to 84–95% of cells
expressing βIII-tubulin were also HB9 and ChAT positive (Liu
et al., 2015). These data therefore further support the requirement
of key region-specific transcription factors for the specification of
distinct neuronal subtypes. Of note, even the poorly specified
neurons formed functional synapses with muscle cells (Liu et al.,
2013), highlighting that more stringent assays are needed to
correct neuronal identity. Genome- and proteome-wide
analysis combined with electrophysiological analysis will be the
gold standard to determine unequivocally the identity of the
induced neuron. This is also important for future studies that aim
to generate specific subtypes of motor neurons, such as
branchial, visceral or somatic motor neurons of different
subtypes – alpha, beta or gamma. Correct specification of
neuronal subtypes is also crucial for disease modelling,
particularly for neurodegenerative diseases, which largely affect
only specific subtypes. Indeed, induced motor neurons generated
from fibroblasts of amyotrophic lateral sclerosis (ALS) patients
recapitulated some aspects of ALS pathology, like the
mislocalization of the ribonucleoprotein FUS, smaller soma,
lower firing frequency and higher susceptibility to cell death over
time (Lai et al., 2011; Liu et al., 2015).

Retinal photoreceptors
Photoreceptors are sensory neurons of the central nervous system
that develop after induction of the eye anlage as part of the
diencephalon. Their induction from human dermal fibroblasts has
been achieved by combining the neurogenic factor NeuroD1, which
plays key role in photoreceptor development (Hatakeyama and
Kageyama, 2004), with the patterning factor of the eye anlage retinal
homeobox protein RAX (also known as RX) (Zagozewski et al.,
2014) and the photoreceptor fate determinants Otx2 and Crx
(Zagozewski et al., 2014). The induced photoreceptors comprise
cells with rod or cone characteristics as indicated by the presence
of rhodopsin, S and M opsins, and significant responses to light
stimulus detected in some of the reprogrammed cells (Seko et al.,
2014). Although these results are encouraging, more work is
required to improve the efficiency of reprogramming and to ensure
their survival in vivo.

Peripheral sensory neurons
Peripheral sensory neurons are the afferent neurons that receive
external stimuli and transmit them to the central nervous system.
From a developmental perspective, these neurons differentiate from
neural crest cells (Pavan and Raible, 2012), and hence a promising
approach for generating them has been to reprogram human
fibroblasts into neural crest cells, which can then be further
differentiated into peripheral neurons (Kim et al., 2014). However,
direct neuronal reprogramming has proven to be more successful in
obtaining specific subtypes of peripheral neurons than the
neural crest cell route (Blanchard et al., 2015; Wainger et al.,
2015). As peripheral sensory neurons are different to those of the
central nervous system, an important question is how similar or different
the molecular requirements are for inducing these cells.
Interestingly, the same pan-neurogenic factors employed to generate central
nervous system neurons – namely Ascl1, Myt1l and Neurog1 – are
sufficient to induce an Aδ nociceptor phenotype in 14% of
transduced cells when combined with the terminal differentiation
factors Isl1 and Klf7 (Wainger et al., 2015). These latter two factors,
Is11 and Klf7, are important in maintaining the expression of TrkA (Ntrk1) in peripheral sensory neurons (Marmigere and Ernfors, 2007). Of the resulting induced nociceptor cells, about half were functional, as evidenced by their response to capsaicin stimulation and sensitization by prostaglandin E2 (Wainger et al., 2015).

A second pool of factors, Neurog1/2 and Pou4f1 (also known as Brn3), was successful in converting fibroblasts into sensory neurons, albeit with a low efficiency (4-6%; Blanchard et al., 2015). Single-cell expression analysis revealed non-overlapping expression of TrkA, TrkB (Ntrk2) and TrkC (Ntrk3), suggesting that the reprogramming protocol generated different sensory subtypes. This could imply that the relative expression of each of the two factors could determine the subtype of nociceptor generated, or alternatively that other factors are required to refine terminal differentiation further. Indeed, tuning the level and the length of the expression of Neurog1/2 and Pou4f1 has hardly been tested at all in reprogramming and will be crucial to investigate in order to understand the exact molecular logic of the underlying transcriptional events.

In summary, the identified combinations of factors tested so far uncover the instructive role of some common reprogramming factors, particularly Ascl1 and Neurog1/2, in direct neuronal conversion of different cell types. They also highlight the requirement for lineage-specific factors to generate different neuronal subtypes (Fig. 3). However, determination of neuronal subtype identity is still in its infancy and naturally limited in vitro, where neither the adequate input nor selective projection and output of the respective neuron can be identified. Thus, much more work is required in vivo, with a particular focus on the connectivity of the reprogrammed neurons. In addition, specific transcriptional signatures are becoming available for specific neuronal subtypes, as shown recently in pioneering work identifying molecular signatures of neurons at the single-cell level (Bikoff et al., 2016; Fuzik et al., 2016; Zeisel et al., 2015). These transcriptional signatures could provide a comprehensive molecular map of specific neuronal identities that could be used to classify reprogrammed neurons. These studies could also uncover novel selector factors for direct neuronal conversion. This would allow assessment of whether direct reprogramming can generate truly subtype-specific neurons in vitro, or whether the environment – that is the embryonic or adult brain, or organoids (Lancaster et al., 2013; Qian et al., 2016) – is required for complete phenotype maturation.

**Learning from neuronal reprogramming: transcriptional dynamics, the cell cycle and metabolic hurdles**

The ability of key developmental transcription factors to specify different neuronal subtypes highlights their instructive role towards a neuronal fate. In addition, direct neuronal reprogramming may also be used to understand better the molecular function of these factors in defined transcriptional networks. For example, Ascl1 can trigger different neuronal identities when expressed in either astrocytes or fibroblasts. How does the presence or absence of other transcription factors in the starting cell type affect the outcome of Ascl1 overexpression? Co-expression of Sox2, FoxG1, Ascl1, Dlx5 and Lhx6 (Figs 1, 3) converts murine and human fibroblasts into telencephalic, mainly parvalbumin-positive GABAergic interneurons (Colasante et al., 2015). Interestingly, Dlx2, a key regulator of GABAergic neuronal fate (Petryanik et al., 2007), was induced upon Sox2 and FoxG1 co-expression (Colasante et al., 2015). Sox2 was shown to interact with Ascl1, while FoxG1 was essential for Dlx2 upregulation both in vitro and in vivo. The identification of this regulatory network might explain why Ascl1 reprogrammes astrocytes into GABAergic neurons, as Sox2 and FoxG1 are already expressed in postnatal astrocytes and thus their exogenous expression is not required (Heinrich et al., 2010; Masserott et al., 2015; Zhang et al., 2014). Characterizing which factors are already present in the starting cell population may help to decipher the transcriptional networks required for neuronal specification in development and reprogramming. Indeed, proteomic changes not reflected in the transcriptome contribute significantly to the fate changes in other paradigms of direct reprogramming (Di Stefano et al., 2016).

Key components of the proteome include chromatin-modifying complexes, which can also affect the reprogramming process (Di Stefano et al., 2016). During reprogramming of mouse embryonic fibroblasts to neurons, for instance, Ascl1 occupies most of the genomic loci bound in NSCs and NPCs, even though in mouse embryonic fibroblasts these are covered by nucleosomes and therefore not easily accessible (Wapinski et al., 2013). A specific histone signature correlates with Ascl1-binding sites in mouse embryonic fibroblasts and NPCs that consists of monomethylation of histone 3 lysine 4, acetylation of histone 3 lysine 27 and trimethylation of histone 3 lysine 9 (Wapinski et al., 2013). Removing trimethylation of histone 3 lysine 9 via the expression of JmJD2d (Kdm4d) resulted in impaired efficiency of neuronal conversion. The ability of Ascl1 to act as a pioneer transcription factor (Iwafuchi-Doi and Zaret, 2016; Zaret and Mango, 2016) seems to be associated with this epigenetic signature, because in cells with low levels of these marks, such as human keratinocytes and osteoblasts, Ascl1 occupies almost completely different, off-target sites lacking the E-box motif. These cells, as well as adult human pericytes and human liver cells, can be poorly, if at all, reprogrammed by Ascl1 alone (Karow et al., 2012; Marro et al., 2011; Wapinski et al., 2013). Interestingly, co-transduction of adult human brain-derived pericytes with Ascl1 and Sox2 was sufficient to induce functional neurons (Karow et al., 2012). A possible scenario for the functional interaction between Ascl1 and Sox2 could be that their biochemical interaction is required for the activation of the key neuronal genes in cells otherwise refractory to neuronal reprogramming (see also Colasante et al., 2015). Chromatin remodelling is also required for Pax6-mediated reprogramming, as Brg1 (Smarc4)-deficient glia cannot be reprogrammed into neurons (Ninkovic et al., 2013), and the above-mentioned miR-9/9* and miR-124 regulate the composition of the Brg1-associated factors (BAF) complex during neural development (Yoo et al., 2009).

Data such as these prove the importance of chromatin status in direct reprogramming, and bring a fresh perspective to neurodevelopmental biology. One question is whether Ascl1 requires the same histone modifications to initially activate its in vivo targets as in reprogramming. It will also be interesting to understand whether other transcription factors require a different chromatin signature to work efficiently, and, conversely, if a given reprogramming cocktail could work in different cell types upon proper chromatin manipulation. To this end, direct reprogramming contributes essential new information about the mechanisms that stabilize cell fate, which also represent hurdles in reprogramming. For example, the histone chaperone LIN-53 limits the reprogramming of non-neuronal cells in larvae or adult worms (Caenorhabditis elegans) into neurons (Tursun et al., 2011) and the mammalian orthologue Rbp4 is part of the histone chaperone complex CAF-1 that has recently been shown to limit reprogramming towards several cell types, including neurons (Cheloufi et al., 2015).
It has been suggested that mitotic transition during the cell cycle provides a ‘window of opportunity’ during which the gene expression profile, and hence the fate, of a cell can be changed (Halley-Stott et al., 2014; Holtzer et al., 1975). However, direct reprogramming has clearly demonstrated that proliferation is not a prerequisite for the cell fate switch, as many cell types can convert into neurons without cell division (Fishman et al., 2015; Gascón et al., 2016; Heinrich et al., 2010; Karow et al., 2012; Marro et al., 2011). In fact, the overexpression of the cell cycle activator Myc actually reduced the efficiency of fibroblast conversion to neuronal cells (Fishman et al., 2015). This supports the observation that BET-bromodomain inhibitors enhance neuronal reprogramming mediated by small molecules (Hu et al., 2015; Li et al., 2015; Zhang et al., 2015), as these compounds cause cell cycle arrest and inhibit Myc expression at transcriptional levels (Cheng et al., 2007; Delmore et al., 2011; Gallagher et al., 2014).

Cell cycle and Myc expression are both strongly linked to the metabolic and redox state of cells (Bretones et al., 2015). In nervous system development, however, very little is known about the metabolic changes that occur during the transition from neural stem and progenitor cells to neurons or distinct glial cells (Diaz-Castro et al., 2015). Embryonic fate transitions such as the differentiation of NSCs into neurons often include progenitor intermediates, which afford the opportunity to have better control of all aspects of commitment and differentiation. Conversely, in direct reprogramming the starter cell fate and newly imposed cell fate transiently coexist, and this may create stressful conditions for the cell undergoing transdifferentiation. In line with this, cells undergoing reprogramming suffer from an overshoot in oxidative stress (Gascón et al., 2016). This may be due to the need to change from the metabolism of astrocytes and fibroblasts, which utilize primarily anaerobic glycolysis and β-oxidation (McKay et al., 1983; Tsacopoulos and Magistretti, 1996), to an oxidative metabolism, which is required by neurons. During this metabolic transition, the upregulation of the protective machinery may be delayed, thereby leading to excessive oxidative stress (Gascón et al., 2016; Quadrato et al., 2016), with subsequent death by ferroptosis (Gascón et al., 2016). Accordingly, protection from oxidative stress and cell death greatly improved reprogramming efficiency after brain injury in vivo (Gascón et al., 2016).

These findings prompt the question of why NSCs do not run into the same problem when generating neurons. A possible explanation is that their gradual differentiation, through successive rounds of proliferation and often an intermediate progenitor state, could allow them to acquire the most appropriate metabolism slowly. Indeed, recent RNA-seq data revealed that adult NSCs start to change their metabolism when they become activated and enter cell cycle (Llorens-Bobadilla et al., 2015; Shin et al., 2015). Even the recent identification of NSCs turning into neurons in the adult zebrafish forebrain without any apparent division seems to occur rather slowly (Barbosa et al., 2015), over the course of weeks, which would allow time for metabolic transition. Although endogenous glial cells in the adult neurogenic niches regulate metabolic transition apparently well, a key question is whether the metabolic switch is a consequence of the fate change or contributes to regulate it. Reprogramming experiments suggest that metabolic change is required for fate change, as neuronal conversion cannot occur when oxidative phosphorylation is blocked by oligomycin-A (Gascón et al., 2016). Strikingly, in this condition astrocytes do not die, but rather remain in their astrocytic state despite the constant expression of the neurogenic fate determinant (Gascón et al., 2016). In this context, as well as in fibroblasts, Myc may represent a major hurdle, as it is a metabolic regulator promoting a glycolytic programme (Dang, 2013; Wang et al., 2011). Indeed, mounting evidence suggests that changes in metabolism may play an instructive role in fate acquisition and conversion (Holmes and Terzic, 2016), and are not just a mere consequence of fate change. For example, during development there is a strict regulation of metabolic pathways along sequential phases of stem cell proliferation and differentiation, with glycolysis as the preferred metabolic state for rapid proliferation (Vander Heiden et al., 2009). Cells in the early embryo prior to the blastocyst stage rely almost entirely on glycolysis (Barbehenn et al., 1978; Shyh-Chang et al., 2013). When embryonic stem cells differentiate there is a dramatic decrease of glycolytic flux (Cho et al., 2006; Chung et al., 2007; Facucho-Oliveira et al., 2007; Wang et al., 2009) by enrichment of unsaturated lipids (Yanes et al., 2010). The oxidation of these unsaturated lipids by reactive oxygen species (ROS) generated during the emerging oxidative metabolism improved embryonic stem cell differentiation (Yanes et al., 2010). Thus, it is clear that dynamic changes in cellular metabolism can have a direct impact on fate decisions.

During adult neurogenesis, metabolism and related ROS signalling play a crucial role in cell fate decisions (Prozorovski et al., 2015). For example, maintenance of progenitors requires de novo lipogenesis (Knobloch et al., 2013), while commitment and differentiation are associated with reduced expression of glycolytic genes (Llorens-Bobadilla et al., 2015; Shin et al., 2015). This in turn is influenced by the activity of mTOR (Amiri et al., 2012; Kim et al., 2009), a metabolic rhesostat involved in the integration of nutrients and regulation of growth factor signalling (Mihaylova et al., 2014). The extent to which metabolic components regulate the fate and activation of NSCs, both during development and in the adult brain, remains not only a fascinating area of research but also has great relevance for understanding NSC behaviour in disease when metabolic pathways are often altered, for example by inflammation. Identification of the causal relationships between metabolic pathways and fate change is crucial for understanding both endogenously programmed fate decisions in vivo and forced fate changes by reprogramming.

Conclusions

Accumulating evidence has made it clear that cells do not revert back to an earlier developmental stage during direct neuronal reprogramming. Thus, the Waddington’s landscape model no longer accommodates many of the observations made in direct reprogramming. In addition, it makes further assumptions that are not necessarily met: it suggests that a cell is naturally fated to differentiate, like a marble rolling down a mountain to the valley because of gravity; it does not consider direct lineage conversions, unless one introduces ‘tunnels’ below the surface of the hills and valleys; and, last, it does not represent the energy required for cell fate specification and conversions to happen. A new model has been recently proposed that reconciles these aspects by incorporating the concept that a cell can move from one stable state to another with little preferred directionality (Sieweke, 2015). Called the Cook Islands model (Fig. 4), a boat, which represents a cell, can sail from island to island rather freely (Huang, 2009). In this case, the islands represent different stable cell fates: however, major constraints, such as high waves, reefs, shallow passages or storms, block certain routes. These constraints represent actual barriers to cell fate acquisition, such as different germ layer origin between the starter cell and the reprogrammed entity (and thus a different epigenetic

2505

DEVELOPMENT

state), or different energetic demands during and after the conversion [due to transcriptional changes, synthesis of new proteins, as well as a profound metabolic switch (Mathieu et al., 2014)]. Each of these constraints influences the probability that a given cell will change fate, sending the sailing boat towards different shores, depending on the type of constraint that is encountered. Identifying such constraints, investigating how they are established during development, and how they may be overcome either in vivo or in vitro during reprogramming are essential tasks for the future.

Reprogramming studies highlight the state of our knowledge of developmental biology, profiting from what is known and casting light on the still rather dark spots. Generation of the diverse array of neuronal subtypes is still in its infancy and shows us the limitations of our knowledge in this regard. As revealed by recent single-cell knowledge from development and reprogramming holds many promises for answering these crucial questions.

Acknowledgements
We thank Beatriz Gascón for providing support for the figures, and Olof Torper, Stefan Stricker, Jovica Ninkovic, Kalina Draganova, Sven Falck, Vidya Ramesh, Gianluca Russo and Pia Johansson for insightful comments on the manuscript and discussions.

Competing interests
The authors declare no competing or financial interests.

Funding
This work was supported by an Advanced European Research Council grant [ChroNeuroRepair GA no. 340793]; Sonderforschungsbereich (SFB) 870; the Bavarian Research Network ForIPs; the Helmholtz Association; and the Deutsche Forschungsgemeinschaft.

References


reprogrammed human neurons retain aging-associated transcriptomic signatures and reveal age-related nucleocyttoplasmic defects. Cell Stem Cell 17, 705-718.


