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Direct Conversion of Fibroblasts into Functional Astrocytes by Defined Transcription Factors

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SUMMARY

Direct cell reprogramming enables direct conversion of fibroblasts into functional neurons and oligodendrocytes using a minimal set of cell-lineage-specific transcription factors. This approach is rapid and simple, generating the cell types of interest in one step. However, it remains unknown whether this technology can be applied to convert fibroblasts into astrocytes, the third neural lineage. Astrocytes play crucial roles in neuronal homeostasis, and their dysfunctions contribute to the origin and progression of multiple human diseases. Herein, we carried out a screening using several transcription factors involved in defining the astroglial cell fate and identified NFIA, NFIB, and SOX9 to be sufficient to convert with high efficiency embryonic and postnatal mouse fibroblasts into astrocytes (iAstrocytes). We proved both by gene-expression profiling and functional tests that iAstrocytes are comparable to native brain astrocytes. This protocol can be then employed to generate functional iAstrocytes for a wide range of experimental applications.

INTRODUCTION

Direct cell-reprogramming technology is based on the dominant action of cell-lineage transcription factors (TFs) in converting adult somatic cells into different cell types (Graf and Enver, 2009). This technique represents a promising avenue in the field of regenerative medicine, with the potential to generate cellular sources suitable for cell-replacement therapies (Chambers and Studer, 2011). In fact, since the groundbreaking discovery of the induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka, 2006), increasing approaches of direct cell reprogramming have been established, culminating with the development of induced cellular types for neurons, cardiomyocytes, and hepatocytes (Vierbuchen et al., 2010; Ieda et al., 2010; Huang et al., 2011). In addition, we and others employed the forced expression of defined sets of TFs to generate specific induced neuronal sublineages for dopaminergic, cholinergic, and motor neurons (Caiazzo et al., 2011; Pfisterer et al., 2011; Kim et al., 2002; Son et al., 2011; Liu et al., 2013; Theka et al., 2013). More recently, two groups succeeded in the generation of induced oligodendrocyte precursors by direct conversion of fibroblasts (Najm et al., 2013; Yang et al., 2013). Surprisingly, to date, there is no report for the generation of astrocyte by means of direct cell reprogramming. Astrocytes are the most-abundant cell type in the CNS and a critical neural cell type responsible for the maintenance of brain homeostasis. Indeed, they play irreplaceable roles in neurotransmitter trafficking and recycling, nutrient and ion metabolism, regulation of blood supply, release of transmitters and growth factors, and protection against oxidative stress (Molofsky et al., 2012). Consistent with such a variety of fundamental functions exerted by astrocytes in supporting neuronal survival and function, astrocyte dysfunctions have been found to contribute to several neurological diseases, such as epilepsy, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, lysosomal storage diseases (Di Malta et al., 2012), and Rett syndrome (Molofsky et al., 2012). Conversely, recent data showed that transplanted astrocyte progenitors display robust survival and differentiation in the host brain and are able to decelerate the disease course in ALS and Alzheimer's disease models (Lepore et al., 2008; Pihlaja et al., 2008). However, current protocols rely on the isolation of astrocyte progenitors from neonatal brains with serious limitations for any therapeutic approach as the paucity of cell supply and unmatched immunoprofile with the host, leading to immune reaction and possible rejection after

transplantation. Cell-reprogramming approaches, by generating astrocytes starting from adult skin fibroblasts from an immunomatched or autologous source, can represent a promising alternative system for overcoming those bottlenecks. Notably, procedures of direct iPSC differentiation into astrocytes have been established only very recently (Krencik et al., 2011; Emdad et al., 2012; Juopperi et al., 2012; Roybon et al., 2013; Serio et al., 2013; Shaltouki et al., 2013). However, these approaches rely on the previous generation of stable and mutation-free iPSC lines, and the cell differentiation protocols are considerably time-consuming, complex, and required extensive time up to 180 days. We therefore considered that a direct reprogramming approach could have interesting advantages, providing a more practical procedure to generate astrocyte-like cells. Indeed, after the identification of the reprogramming cocktail composed by the astroglial TFs NFIA, NFIB, and SOX9, we defined a straightforward and fast (~2 weeks) protocol to generate induced astrocytes (iAstrocytes) derived from mouse embryonic and postnatal fibroblasts. Our experiments indicate that iAstrocyte molecular phenotype and biological functions closely recapitulate that of native astrocytes, thus validating the direct reprogramming technology as an alternative for the generation of astrocytes.

RESULTS

Defining the Minimal Set of TFs Able to Convert Fibroblasts to an Astrocytic Cell Fate

To generate iAstrocytes, we initially performed a literature data mining for selecting a first pool of eight candidate TFs known to play relevant roles in astrocyte differentiation and maintenance during nervous system development (Rowitch, 2004; Deneen et al., 2006; Rowitch and Kriegstein, 2010). In addition, we added six additional candidate TFs that exhibit a selective expression in astrocytes when compared to the global gene-expression profiles of neurons and oligodendrocytes (Lovatt et al., 2007; Cahoy et al., 2008; Doyle et al., 2008; Najm et al., 2013). Thus, we selected a total of 14 TFs (SOX2, SOX9, PAX6, NKX6.1, OLIG1, OLIG2, NFIA, NFIB, NFIX, HES1, HES5, NICD, TAL1, and PRDM16). Their coding regions were individually cloned into doxycycline (dox)-inducible lentiviral vectors, and each lentivirus was used to infect mouse embryonic fibroblasts (MEFs). The day after the infection, MEF culture medium was replaced with fresh medium supplemented with dox. After 12 days of dox exposure, the activation of the well-known astrocyte markers GFAP and S100B was assessed by immunostaining in the infected cell culture. Interestingly, only NFIA, NFIB, and SOX9 among all 14 candidate TFs induced a significant amount of S100B-positive cells, as well as a relative smaller number of GFAP-positive cells. On the contrary, GFP infected or not infected (control) MEFs did not show any relevant expression of these two astrocyte-specific markers (Figure S1A available online). In order to confirm the presence of GFAP-positive cells and develop a system for isolating them, we set up a genetic cell-fate-tracing method selective for the astrocyte cell lineage by using MEFs derived from the hGFAP-Cre;ROSA26-stop-flox-yellow fluorescent protein (YFP) double-transgenic mice. Fluorescence-activated cell sorting (FACS) analysis confirmed the presence of YFP-positive cells after infection with NFIA, NFIB, or SOX9 (data not shown). We then combined the three factors NFIA, NFIB, and SOX9 (hereafter abbreviated as A, B, and S9) in different groups for identifying the most-performing combination in giving the highest efficiency of astrocyte conversion. Among all the possible factor combinations (AB, AS9, BS9, and ABS9), ABS9 cocktail generated the largest number of GFAP- and S100B-positive cells, as shown in Figures S1B–S1D. We confirmed this finding also by FACS analysis using the hGFAP-Cre;ROSA26-stop-flox-YFP double-transgenic MEFs, estimating in ~15% the total number of YFP-positive cells (Figures 1A and S1D). Importantly, no other factor added to this combination enabled any evident increase of the reprogrammed cell number (data not shown), concluding that ABS9 is the minimal and sufficient set of TFs eliciting a sustained conversion of MEFs into iAstrocytes. Closer inspection of ABS9 iAstrocytes confirms their round cell shape, resembling somatic astrocyte morphology and the coexpression of both the GFAP and S100B proteins in a vast fraction of them (Figures 1B, 1C, 1E, 1F, and 1H). Conversely, very few (<0.2%) S100B-positive cells and no GFAP-positive cells were detected in the GFP-transduced cell population (Figures 1D and 1G). As shown in Figure 1I, iAstrocytes also expressed high levels of the main astrocytic glutamate transporter GLT-1 (National Center for Biotechnology Information: SLC1A2). Quantitative studies determined that infected MEFs activated S100B in high numbers (21%) and that GFAP (16%) and GLT-1 (11%) were found in large subgroups of cells always colocalizing with S100B (Figure 1J; data not shown).

In order to characterize the dynamics involved in iAstro reprogramming, we analyzed the conversion at different time points by immunostaining and quantitative RT-PCR (qRT-PCR). The results of this experiment showed that neither OCT4 nor SOX2 proteins are ever expressed (data not shown) and also the transcriptional analysis did not detect any other embryonic stem cell (ESC) or neural stem cell (NSC) marker, with the only exception of *Tlx* and *Pax6* that are minimally increased only at day 1 of the reprogramming protocol (Figures S2A–S2I). These results indicate that iAstro-reprogramming process is