Biochemical and Biophysical Research Communications 476 (2016) 42–48

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A combination of small molecules directly reprograms mouse fibroblasts into neural stem cells

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ARTICLE INFO

Article history:
Received 4 May 2016
Accepted 16 May 2016
Available online 17 May 2016

Keywords:
Reprogramming
Small molecules
Neural stem cell
Lineage conversion

ABSTRACT

The generation of induced neural stem cells (iNSCs) from somatic cells using defined factors provides new avenues for basic research and cell therapies for various neurological diseases, such as Parkinson's disease, Huntington's disease, and spinal cord injuries. However, the transcription factors used for direct reprogramming have the potential to cause unexpected genetic modifications, which limits their potential application in cell therapies. Here, we show that a combination of four chemical compounds resulted in cells directly acquiring a NSC identity; we termed these cells chemically-induced NSCs (ciNSCs). ciNSCs expressed NSC markers (Pax6, PLZF, Nestin, Sox2, and Sox1) and resembled NSCs in terms of their morphology, self-renewal, gene expression profile, and electrophysiological function when differentiated into the neuronal lineage. Moreover, ciNSCs could differentiate into several types of mature neurons (dopaminergic, GABAergic, and cholinergic) as well as astrocytes and oligodendrocytes in vitro. Taken together, our results suggest that stably expandable and functional ciNSCs can be directly reprogrammed from mouse fibroblasts using a combination of small molecules without any genetic manipulation, and will provide a new source of cells for cellular replacement therapy of neurodegenerative diseases.

1. Introduction

The generation of induced pluripotent stem cells (iPSCs) from somatic cells using defined transcription factors holds great promise for research and therapy in several diseases due to their ability to generate patient-specific cells for autologous transplantation [1]. However, the inefficiency of this process, the impurity of differentiated cell types, and the potential for tumor formation are obstacles to the clinical application of such cells [2]. To overcome these problems, alternative approaches have been developed to directly reprogram one somatic cell type into another bypassing the pluripotent state. The direct reprogramming approach can reprogram somatic cells into neurons, oligodendrocyte progenitor cells, cardiomyocytes, blood progenitor cells, hepatocytes, epiblast stem cells, and pancreatic β cells using lineage-specific transcription factors or Yamanaka factors combined with specific culture conditions [3].

Neural stem cells (NSCs) that can self-renew and differentiate into neural lineage cells are promising resources for pathological research and cell therapy in numerous neural diseases [4]. Recently,

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http://dx.doi.org/10.1016/j.bbrc.2016.05.080
0006-291X/

we and others directly converted somatic cells into NSCs or NSC-like cells by overexpressing neural lineage-specific transcription factors or Yamanaka factors combined with chemical compound treatment [3,5,6]. However, because of the potential for unexpected genetic modification following the introduction of exogenous genes, there remain open questions regarding clinical safety issues. Thus, direct reprogramming of somatic cells into NSCs using a combination of small molecules without the introduction of any genetic material should provide an alternative approach to recently available strategies.

Recently, researchers showed that some small molecules can enhance neural development from pluripotent stem cells, and support the self-renewal, proliferation, and survival of NSCs. For example, both a Wnt signal activator and a transforming growth factor-β (TGFβ) signal inhibitor can induce efficient neural differentiation from pluripotent stem cells, and ROCK inhibitors enhance the viability of neural cells by protecting them from apoptosis [7]. Sonic hedgehog (Shh) signaling activators such as Purmorphamine and oxysterol reportedly generate NSC-like cells through a reprogramming process [5]. Furthermore, neural progenitor cells can reportedly be induced from somatic cells using ESC culture medium combined with three chemicals, namely, valproic acid (VPA), CHIR99021, and RepSox, under physiological hypoxic conditions [8].

Here, we demonstrate that mouse embryonic fibroblasts (MEFs) can be directly reprogrammed into ciNSCs using a combination of four chemicals, namely, A-83-01, Thiazovivin, Purmorphamine, and VPA. These ciNSCs eliminate the potential for exogenous genetic mutations and can be expanded in vitro and differentiated into neuronal subtypes.

2. Results

2.1. Direct reprogramming of NSCs from MEFs using a combination of small molecules

In our previous report, we showed that Bmi1 can replace the functions of Sox2, Klf4, and c-Myc during reprogramming processes and can generate iPSCs with Oct4 alone. Furthermore, we found that activation of Shh signaling can replace the function of Bmi1 and induce several NSC markers [5,6]. However, this approach is not sufficient to obtain the complete characteristics of NSCs. Although these cells express some NSC markers, including Sox2 and Nestin, they do not express Sox1 or Pax6 (Supplementary Fig. S1A). Moreover, these cells do not exhibit complete epigenetic reprogramming (including DNA methylation patterns and histone modifications) and cannot be stably expanded (data not shown). The fact that expression of Sox2 is significantly lower in NSC-like cells than in NSCs prompted us to perform additional chemical screening to convert NSC-like cells into stably expanded NSCs.

Recently, a novel method, cell activation and signaling-directed (CASD), was developed for direct reprogramming of somatic cells into multipotent somatic precursor cells [9]. Somatic cell activation is based on transient overexpression of iPSC generation-related transcription factors, and lineage-specific soluble signals (signaling-directed) are used for fate determination in direct reprogramming. Thus, we hypothesized that some chemical cocktails may function in a similar mechanism to CASD for direct reprogramming. Various chemicals such as VPA (histone deacetylase (HDAC) inhibitor), E-616452 (TGFβ inhibitor), CHIR99021 (Wnt agonist), SB431542 (TGFβ inhibitor), A-83-01 (TGFβ inhibitor) Purmorphamine (Shh agonist), and Parnate (Par; LSD1 inhibitor) can reportedly induce expression of endogenous Sox2, which can directly reprogram fibroblasts into NSCs (iNSCs) [5,10,11]. In addition, inhibition of TGFβ not only enhances the reprogramming of somatic cells into iPSCs, but also promotes the neural induction of pluripotent stem cells [12,13]. Thus, we hypothesized that a combination of these chemicals, including Purmorphamine, can promote NSC induction. We first compared the abilities of different combinations of chemicals, including Purmorphamine, to directly reprogram MEFs into NSCs. No compact neuroepithelial colonies were observed when MEFs were cultured in the presence of VPA, Par, A-83-01, RG108, or CHIR99021 alone, or together with Purmorphamine (Fig. 1A and data not shown), suggesting that single small molecules combined with Purmorphamine are insufficient to convert MEFs into neuroepithelial-like colonies. Next, we tested more chemicals combined with Purmorphamine to determine which combinations directly reprogram MEFs into NSCs. Neither compact colonies nor NSC marker expression was observed in MEFs treated without chemicals (Supplementary Fig. S1B). However, four chemicals, namely, A-83-01, Purmorphamine, VPA, and Parnate, termed APV + Par, induced the formation of compact, monolayer neuroepithelial-like colonies and expressed NSC markers including Sox2, Sox1, and Nestin (Supplementary Fig. S2A and B). To test whether all four of these chemicals are required for compact neuroepithelial colony formation, we treated MEFs with various combinations of APV + Par lacking one chemical. Withdrawal of A-83-01, VPA, or Purmorphamine from the APV + Par cocktail significantly reduced the number of compact colonies (Supplementary Fig. S2C). Colony formation was slightly increased when Par were removed, suggesting it is dispensable (Supplementary Fig. S2C). There was no additive effect of RG108 (DNA methyltransferase inhibitor), which replaces Sox2 and enhances the reprogramming of somatic cells into iPSCs [14] (Supplementary Fig. S2D). Recently, researchers showed that Thiazovivin (ROCK inhibitor) can enhance NSC survival and the reprogramming efficiency [7,13]. Thus, we investigated whether a combination of A-83-01, Purmorphamine, and VPA (APV) together with Thiazovivin, termed ATPV, enhanced neuroepithelial-like colony formation. The number of Nestin$^+$ neuroepithelial-like colonies was significantly higher in ATPV-containing culture medium than in APV-containing culture medium (Supplementary Fig. S2D). MEFs in ATPV-containing medium started to aggregate at day 3, and approximately 149 ± 18 compact colonies, of which 96 ± 2.5% were Nestin$^+$, formed at day 12 (Fig. 1B-C). These colonies consistently expressed numerous NSC markers including Pax6, Nestin, Sox2, Sox1, and PLZF (Fig. 1C-D).

Other than NSCs, neural crest stem (NCS) cells also have ability to differentiate into neurons [15]. To investigate whether primary cultured MEFs contamination with small population of NCS cells, we isolated skin-derived precursor cells (SKPs) from mouse skin as a positive control, which reported have similar properties to embryonic NCS cells [16]. Then, we performed RT-PCR and observed that both SKPs and MEFs expressed fibroblast markers (Col1a1 and twist) (Supplementary Fig. S2D). However, only SKPs showed expression of NCS specific marker (Sox10) (Supplementary Fig. S2D). It is suggest that there were not any NCS cells contaminated with MEFs and ATPV-induced neuroepithelial-like colonies were not expanded from a small portion of NCS cells.

To further optimize the chemical combination for direct reprogramming of MEFs into NSCs, we induced MEFs with various combinations of ATPV lacking one chemical and quantified the reprogramming efficiency. The number of Nestin$^+$ colonies was reduced by the absence of any one chemical compound, and no Nestin$^+$ colonies were detected in the absence of all four chemical compounds (Supplementary Fig. S3A and B).

We further investigated whether combinations of other chemical inhibitors or activators of the same pathways could directly reprogrammed MEFs into NSCs. We used the same induction protocol as was used for ATPV induction. A-83-01, Thiazovivin,