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Neurogenin 2 enhances the generation of patient-specific induced neuronal cells

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ABSTRACT

Direct lineage reprogramming of human fibroblasts into functional neurons holds great promise for biomedical applications such as regenerative medicine and cellbased disease modelling. However, clinical applications must consider how to increase neuronal conversion efficiency and at the same time reduce the number of required transcription factors. Here, we investigated whether Neurogenin 2 (Ngn2), which is a proneural gene that directs neuronal differentiation of progenitor cells during development, can enhance the generation of patient-specific induced neuronal cells. In this study, we transfected Ascl1, Sox2 and Ngn2 into human fibroblasts from the patients' scalp by lentivirus. Morphological analysis, immunocytochemistry, gene expression and electrophysiological analysis were performed to identify the similarity of induced neuronal cells (iNCs) to human neuronal cells. Ngn2 increase the conversion efficiency from 4% to 13.4%. iNCs express neuronal cell markers and resemble wild-type neurons in their morphology, gene expression profiles and exhibit functional membrane properties of mature neurons. Implanted iNCs can survive and integrate in mouse brains and, unlike iPS cell-derived neural cells, do not generate tumours.

1. Introduction

Takahashi and Yamanaka (2006) demonstrated that cells could be reprogrammed into induced pluripotent stem cells (iPSCs) by over-expression of four transcription factors (Takahashi, K et al., 2006). Since then, accumulating studies have demonstrated cell reprogramming could be achieved by using different transcription factor cocktails and many optimisation techniques were gradually developed (Beyene and Boockvar, 2008; Maehr et al., 2009; Moretti et al., 2010; Park et al., 2008; Soldner et al., 2009; Yu et al., 2007). Rather than switching the fate of differentiated cells going back and forth, there may have been a direct shortcut to enable conversion of one adult cell type into another without going through the pluripotent state. Recent pioneering works show that this method is quite feasible. The first successful direct conversion of murine fibroblasts into

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functional induced neurons was achieved in 2010 (Vierbuchen et al., 2010), which display typical neuronal morphologies, express multiple neuronal markers and could generate action potentials, and this was followed by a number of studies describing similar switches between developmental remote cell types (Berry et al., 2011; Marro et al., 2011; Qiang et al., 2011; Ring et al., 2012; Wang et al., 2014; Yashar et al., 2012). Within 2 years, successful direct conversion of mesoderm into ectoderm and endoderm as well as endoderm into ectoderm was reported for murine and human cells. This rapidly evolving field abounds with new questions that aim to exploit these discoveries, for example to induce distinct cellular subtypes and expand progenitor populations, as well as to enhance conversion efficiency.

In a series of elegant studies, it was demonstrated that pre and postnatal astrocytes can be converted into functional neurons using forced expression of the transcription factors Ngn2 (Berninger et al., 2007). It would be interesting to analyse whether Ngn2 is sufficient to enhance a cortical conversion of human fibroblasts into functional neuronal cells.

In the present study, we directly reprogrammed human fibroblasts from patients undergoing neurosurgery into functional iNCs by using three transcription factors (Ascl1, Sox2 and Ngn2) which lead to distinct neuronal subtypes which were GABAergic and glutamatergic neurons. We also demonstrated that these iNCs could express human neuronal cell markers, and express the voltage-gated ion channels, generate spontaneous action potentials, express functional neurotransmitter receptors, form excitatory or inhibitory postsynaptic currents. Moreover, we have confirmed the safety of human iNCs in vivo by observing them over a period of time.

2. Results

2.1. *Conversion of human fibroblasts into iNCs with three transcription factors*

We collected the scalps of patients with traumatic brain injury (TBI) under informed consent. After three passages, we checked the morphology and antigenic expression pattern of cultured cells, which exhibited a typical fibroblast-like morphology and a reliable fibroblast marker Vimentin but without Nestin staining. Since the cells express Vimentin without Nestin, we concluded they were indeed fibroblasts. Moreover, the cultured cells expressing collagen I without the neuroepithelial marker Pax6, the neural crest marker Sox10 and the stem cell marker Nanog, confirming their fibroblastic identity which were distinct from neural crest derivatives or stem cells. Besides, the absence of neuronal markers Tuj1 showed the starting cells contain no neurons (Fig. 1).

We select three transcription factors (Ascl1, Sox2 and Ngn2), cloned into the lentiviral vectors harbouring a GFP reporter, and then we virally transduced fibroblasts with different unique combinations of these three vectors. The cells in the colonies proliferated more rapidly than the original fibroblasts.

After two rounds of viral infection, the cells were cultured in N3 neural induction medium. About 7 days later, transduced GFP+ cells presented as simple neuronal morphology with small cell body and mono- or bipolar neurite. On the 14th day after transduction, we observed GFP+ cells exhibiting more complex neuronal morphologies similar to that of human neuronal cell with long fine processes resembling neuronal axons. After 21 days, cells with more mature neuronal morphologies were positive for neuronal markers, including Tuj1 (also known as βIII-tubulin) and Map2. The GFP+/Tuj1+ neuronal cells were quantified at indicated time points after transduction, the number of double-positive cells peaked at 21 days (Fig. 2A–D).

Although most of our combinations can induce a neuron-like phenotype, including expression of Tuj1 and Map2, we observed that the addition of Ngn2, improved the conversion efficiency threefold than Ascl1+ Sox2 and Sox2 or Ascl1 alone. Thus, based on number of cells and morphological criteria, the combination of Ascl1, Sox2 and Ngn2 was the most efficient for iNCs induction, which gave the highest conversion efficiency of 13.40±1.24% (Fig. 2C).

Although the cells cultured in neural induction media without viral infection were not Tuj1-positive, some morphologic changes occurred, and the lentiviral expression of GFP alone in human fibroblasts was not capable for reprogramming in spite of treating them alike. Both controls confirmed that human fibroblasts lack spontaneous neuronal differentiation potential in the abscence of transcription factors.

2.2. *Human iNCs express Human neural subtype markers*

It would be of high interest if the presented combination leads to a distinct neuronal subtype. We sought to characterise the neurotransmitter phenotype of human iNCs. After being cultured for more than 30 days, we detected vGluT1-positive puncta outlining GFP-positive cells, indicating the presence of excitatory, glutamatergic neurons. In addition, we found iNCs labelled with antibodies against GABA, the major inhibitory neurotransmitter in brain. 4.7% of our GFP-positive cells expressed GABA, and 2.1% were vGluT1-positive, whereas we were unable to detect tyrosine hydroxylase, choline acetyltransferase, or serotonin expression (Fig. 3A and B).

Nevertheless, there were non glial fibrillary acidic protein (GFAP)-positive or MBP-positive cells in iNCs cultures (Fig. 3C and D), revealing that human fibroblasts cannot be converted into astroglia or oligodendroglia under this culture condition.

2.3. *Characterisation of protein and gene expression in human iNCs*

We tested the expression status of introduced foreign genes and some markers of neural lineages by western blot analysis of iNCs. The results showed a tendency toward up-regulation of Ascl1, Ngn2 and Sox2 from day 7 to day 28. Nevertheless, at day 28, all the three transcripts had a lower expression level than previous levels. So, transcription factors might play an initiate role in a reprogramming process. Protein levels of Map2, NeuN and Synapsin with a disparity in expression time and quantity reflected a conversion of fibroblasts to neurons. These results were also confirmed by immunofluorescence analysis. We also analysed the P53 protein expression level, its consistent low level showed some degree of safety