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Direct induction of functional neuronal cells from fibroblast-like cells derived from adult human retina

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ABSTRACT

Obtaining and manipulating neuronal cells are critical for neural biology basic mechanism studies and translational applications. Recent advances in protocol development and mechanism dissections have made direct induction of neuronal cells from other somatic cells (iN) a promising strategy for such purposes. In this study, we established a protocol to expand a population of fibroblast-like cells from adult human retinal tissues, which can be reprogrammed into iNs by forced expression of neurogenic transcription factors. Interestingly, the combination of *Ascl1*, *Brn2*, *Myt1l*, and *NeuroD1* transcription factors, which has been demonstrated to be sufficient to reprogram human embryonic and dermal fibroblasts into iNs, failed to reprogram the fibroblast-like cells from human retinas into iNs. Instead, supplementing *Ascl1* with *Pax6* sufficed to convert the cells into iNs, which exhibited a typical neuronal morphology, expressed neural marker genes, displayed active and passive neuronal membrane activities, and made synaptic communications with other neurons. Moreover, iNs converted from retina-derived fibroblast-like cells contained high ratios of γ-Aminobutyric acid- (GABA-) and tyrosine hydroxylase- (TH-) positive neurons. Thus, the present study proposes a protocol that makes use of discarded retinal tissues from eye banks for iN generation, and suggests that different sources of somatic cells require different iN induction recipes and may also affect the iN subtype outputs. Our study may also facilitate the future development of methods to convert resident cells *in situ* into retinal neurons for treating retinal degeneration disease purpose.

1. Introduction

Obtaining and manipulating neurons are critical for neural biology mechanism studies, drug discovery and cell therapy applications. To date, several methods have been established to derive neurons including direct isolation from neural tissues (Barker et al., 2013; Goldberg et al., 2002; MacLaren et al., 2006), differentiation from neural stem cells (NSCs) (Chen et al., 2016; Gage and Temple, 2013), or gradual generation from pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Kriks et al., 2011; Qian et al., 2016; Tao and Zhang, 2016). Each method has not only its advantages but also its caveats. For example, although primary neurons are naturally occurring cells that do not need further *in vitro* differentiation manipulations, their sources are very limited making them impractical for most neural biology translational applications. Although NSCs can be expanded *in vitro*, both their proliferation and differentiation capacities are far from ideal. Similarly, despite the rising interest in applying ESCs/iPSCs due to their tremendous amplification and differentiation capacities, their tedious preparation procedures and tumor formation concerns remain an issue in ES/iPS-based cell therapy applications (Fox et al., 2014; Steinbeck and Studer, 2015). Recently, it has been shown that terminally differentiated somatic cells can be directly reprogrammed to become functional neuronal cells, which not only demonstrated a much greater cell fate plasticity of terminally differentiated cells than previously thought, but also expanded the cell sources accessible for basic and translational neural biology studies (Pang et al., 2011; Vierbuchen et al., 2010).

In 2010, Wernig lab demonstrated that by overexpressing *Ascl1*, *Brn2* and *Myt1l* (BAM), mouse fibroblasts can be directly converted into functional neuronal cells, termed iNs for 'induced neuronal cells', that express multiple neuron specific genes, exhibit neural membrane activities, and establish synaptic communications with other neurons (Vierbuchen et al., 2010). By adding an additional neuron determining transcription factor-*NeuroD1*(BAMN), they later demonstrated that human iNs can also be generated from embryonic and postnatal fibroblasts, though at a lower efficiency than in mice (Pang et al., 2011). Based on this, iN direct reprogramming has been extensively explored by the field, and a number of direct reprogramming protocols for specific types of neuron have been established (Ambasudhan et al., 2011; Blanchard et al., 2015; Caiazzo et al., 2011; Hu et al., 2015; Kim et al.,

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2011; Ladewig et al., 2012; Li et al., 2015; Liu et al., 2012; Pfisterer et al., 2011; Son et al., 2011; Victor et al., 2014; Wainger et al., 2015; Xue et al., 2013; Yoo et al., 2011). Among a variety of iN induction transcription factors, *Ascl1* seems to be the most critical one: it is included in most iN induction protocols; it alone is able to induce iNs from fibroblasts, though at a lower efficiency, and requires the help from glial cells (Caiazzo et al., 2011; Chanda et al., 2014; Karow et al., 2012; Kim et al., 2011; Liu et al., 2012; Pang et al., 2011; Pfisterer et al., 2011; Son et al., 2011; Vierbuchen et al., 2010; Wainger et al., 2015). More importantly, Ascl1 has been reported to functions as an 'on target' pioneer factor that immediately occupies its cognate genomic targets to initiate the reprogramming process during iN induction, while other factors participate later to ensure the neural reprogramming route (Treutlein et al., 2016; Wapinski et al., 2013).

The fast advances in protocol and mechanism studies have made iN direct reprogramming a promising strategy to study and treat neural diseases. However, only a few human somatic cell types have been tested for iN induction. Whether the protocols for these somatic cells are generally applicable to other cell types remains unknown. In this study, we established a culture protocol that could easily expand a population of fibroblast-like cells from adult human retinas (hRFLC, for human Retina derived Fibroblast-Like Cell). Surprisingly, the 'classical' human iN induction cocktail-BAMN failed to convert hRFLCs to neuronal cells. Instead, by supplementing *Ascl1* with *Pax6*, RFLCs were effectively converted into neuronal cells that expressed mature neuronal marker genes, fired repetitive action potentials, and formed functional synapses. Thus, our study demonstrated that discarded retinal tissues from eye banks can be used as a raw material to generate functional iNs, and suggested that different sources of donor cells require distinct procedures for iN induction and may also affect the iN subtype outputs. Furthermore, our study may be the basis for future attempts to convert resident cells in the retina into retinal neurons *in situ* to treat retinal degeneration diseases.

2. Materials and methods

2.1. Human retinal cell culture

All experiments on human retinal tissues followed the guideline approved by the ethics committee of Zhongshan Ophthalmic Center. The retinal tissues were obtained from Zhongshan Ophthalmic Center eye bank within 24 h after cornea removal for keratoplasty surgery. Under sterile conditions, after removing the iris, lens and vitreous, the neural retinas were separated from the pigment epithelium and cut off at about 1 cm anterior to the optic nerve, washed 3 times with HBSS. After getting rid of surface blood vessels, retinas were cut into small pieces, dissociated with papain (20 units/ml) containing 40 U / ml DNAseI for 15 min at 37 °C. Digestion was stopped by adding 10 mg/ml ovomucoid. The cell suspension was centrifuged at 300*g* for 5 min, then resuspended in culture medium as specified below, plated onto matrix (gelatin, laminin, lysine, or metri-gel) coated cell culture plates at the density of 2×10^6 cells/ml. Cultures were kept at 37 °C in a humidified atmosphere, with 5% CO2 and 95% air, avoiding unnecessary disturbance for the first 3 days. Four days after plating, most non-adherent cells were washed away by HBSS and medium was replaced with fresh medium. Half medium was changed every 3 days. In primary culture, cell colonies usually emerged around 7–10 days after plating, when cell colonies became densely packed, cells were passaged using 0.25% trypsin, and continued to be passaged every 3–4 days at a ratio of 1:4. The culture medium for the first week is DMEF/F12 (ThermoFisher), 1%GlutaMax (ThermoFisher), 1% penicillin and streptomycin (ThermoFisher), 10–20% FBS (Biowest), 20 ng/ml FGF (Peprotech), 20 ng/ml EGF (Peprotech). One week later, FBS was either maintained in the medium, or replaced with various supplements, including B27, N2, ITS, Knockout serum replacement (all from ThermoFisher).

2.2. Virus preparation and hRFLC reprogramming

Ascl1, *Brn2*, *Myt1l*, and *NeuroD1* cDNA expressing lentivirus plasmids were from Dr. Marius Wernig lab through Addgene. Mouse Pax6 cDNA was amplified from mouse brain total RNA, human PAX6 and ASCL1 cDNAs were amplified from human retina total RNA, and subsequently cloned into home-modified TetO-pSicoR vector (Addgene). Lentivirus plasmids were co-transfected with packaging plasmids, psPAX2, and pMD2.G (Addgene) into HEK293 cells to produce virus. Two days after transfection, virus supernatants were collected, concentrated, and used to infect hRFLCs. The hRFLCs from passage 3–4 were used for reprogramming and plated at approximately 80% confluency. One day after virus infection, doxycycline (Dox) was added into the medium to induce transgene expression. Three days after transgene expression, the culture medium was replaced by the neural medium: DMEM/F12 containing neurobasal (1:1), N2, B27, penicillin, and streptomycin (all from ThermoFisher). The reprogramming phenotypes were analyzed at various time points between 1 and 4 weeks.

2.3. Co-culture with mouse glial cells or cortical neurons

For co-culture with mouse glial cells or cortical neurons, mCherry-expressing lentiviruses were included in the infection virus cocktails to label hRFLCs.

For the mouse glial cells preparation, brains of postnatal days (P) 3–5 mice were isolated and cut into small pieces, and dissociated by 0.25% trypsin (ThermoFisher) with 40 U/ml DNaseI at 37 °C for 15 min. The dissociated brain single cell mixture was collected by centrifugation and resuspended in DMEM + 10% FBS + penicillin and streptomycin. Mouse glial cells were passaged three times before being used for co-culture with iNs. Seven days after induction, iNs were dissociated with accutase (Sigma) and replated onto P3 mouse glia cells. The co-culture medium was DMEM/F12: neurobasal (1:1), N2, B27, and 2 μM Ara-C (Sigma). The medium was half-changed every other day.

For the mouse primary cortical neurons preparation, P0–1 mouse brains were cut into small pieces, digested with 10 U/ml papain (Worthington) for 10 min at 37 °C, collected by centrifugation, and resuspended in DMEM/F12 containing neurobasal (1:1), B27, 5% FBS (Biowest), and 20 ng/ml BDNF (PeproTech). Mouse primary cortical neurons were cultured for 2 days before receiving iNs. iNs were dissociated 7 days after induction and subsequently plated onto mouse cortical neurons. The co-culture medium was DMEF/F12: neurobasal (1:1), N2, B27, BDNF, and 2 μM Ara-C (Sigma). The medium was half-changed every other day.

2.4. Immunofluorescent staining

Cells were fixed with 4% formaldehyde (Sigma) for 10 min at room temperature and subsequently washed with phosphate-buffered saline containing 0.1% Triton X-100 (Sigma) (PBST). After blocking with 5% normal serum (Jackson ImmunoResearch) in PBST for at least 30 min, cells were incubated with the primary antibody for 2 h at room temperature, washed with PBST, and finally incubated with the secondary antibody for 1 h at room temperature. Stained cells were observed and imaged using a ZEISS Axio Observer Z1 inverted microscope or ZEISS confocal microscope. The primary antibodies used in this study were mouse anti-TUJ1 (Promega), rabbit anti-TUJ1 (Covance), mouse anti-MAP2 (Sigma), Rabbit anti-synapsin I (Millipore), guinea pig anti-vGLUT1 (Millipore), rabbit anti-vGLUT2 (Cell Signaling), rabbit anti-vGLUT3 (Synaptic System), guinea pig anti-Piccolo (Synaptic System), rabbit anti-Homer1/2/3 (Synaptic System), mouse anti-TH (Sigma), rabbit anti-GABA (Sigma), mouse anti-BrdU (Armersham), and rabbit anti-GFAP (DAKO). The secondary antibodies consisted of Alexa 488- and 568-conjugated antibodies (ThermoFisher).