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Generation of H1 PAX6$^{WT/EGFP}$ reporter cells to purify PAX6 positive neural stem/progenitor cells

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ABSTRACT

Neural conversion from human pluripotent cells (hPSCs) is a potential therapy to neurological disease in the future. However, this is still limited by efficiency and stability of existed protocols used for neural induction from hPSCs. To overcome this obstacle, we developed a reporter system to screen PAX6^{+} neural progenitor/stem cells using transcription activator like effector nuclease (TALEN). We found that knock-in 2 A-EGFP cassette into PAX6 exon of human embryonic stem cells H1 with TALEN-based homology recombination could establish PAX6$^{WT/EGFP}$ H1 reporter cell line fast and efficiently. This reporter cell line could differentiate into PAX6 and EGFP double positive neural progenitor/stem cells (NPCs/NSCs) after neural induction. Those PAX6$^{WT/EGFP}$ NPCs could be purified, expanded and specified to post-mitotic neurons *in vitro* efficiently. With this reporter cell line, we also screened out 1 NPC-specific microRNA, hsa-miR-99a-5p, and 3 ESCs-enriched miRNAs, hsa-miR-302c-5p, hsa-miR-512−3p and hsa-miR-518 b. In conclusion, the TALEN-based neural stem cell screening system is safe and efficient and could help researcher to acquire adequate and pure neural progenitor cells for further application.

1. Introduction

In the past decade, various protocols have been developed to differentiate hPSCs into neural lineage cells to mimic or investigate the pathogenesis of central nervous system (CNS) related diseases [1–3]. The hPSCs-derived neural progenitor cells (NPCs) or neural stem cells (NSCs), a multipotent cell population, are capable of differentiation into various cell types in the CNS and could provide an unlimited source of cells for neural-related cell-based research [4]. In general, the cell fate transition from hPSCs to NPCs is orchestrated by sequential activation/inactivation of lineage-determining transcription factors. During hPSCs neural differentiation, the initial neural lineage cells express PAX6, a paired box (Pax) transcription factor expressed in region-specific neural progenitors after neural tube closure in mouse and human [5]. So PAX6 is believed to be the biomarker of neural progenitor or neural stem cells [6]. Hence, we developed a novel system to isolate PAX6 positive neural progenitor cells through knock-in reporter gene in endogenous PAX6 gene locus based on the method of gene editing tool, the transcription activator-like effectors-nuclease (TALENs). TALENs can be engineered to induce a double-strand break precisely at a predetermined position in the genome [7]. The double-strand break can be repaired through the homology-directed DNA repair pathway using an exogenous donor plasmid as a template [8]. With this repair reaction, endogenous PAX6 gene was targeted using a donor plasmid to integrate a 2 A-eGFP-loxP-PGKPuro-loxP cassette into the thirteenth exon of PAX6, resulting in expression of an EGFP protein under control of the endogenous PAX6 promoter. After neural induction, PAX6 positive NPCs/NSCs express EGFP simultaneously, which enables purification of NPCs efficiently by FACS. Purified PAX6^{+} NPCs could be maintained and expanded *in vitro*

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Abbreviations

ChAT	Choline O-acetyltransferase
CNS	Central nervous system
hPSCs	Human pluripotent stem cells
MAP2	Microtubule associated protein 2
MNs	Motor neurons
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PAX6	Paired box transcription factor 6
SYN	Synaptophysin
TALENs	Transcription activator-like effectors-nuclease chimeras
TH	Tyrosine Hydroxylase
Tuj	Neuronal Class III β-Tubulin

and differentiated into post-mitotic neurons for further application.

2. Material and methods

2.1. Human embryonic stem cell culture

The hPSCs used in this study were from H1 line (Wicell, Madison, WI, USA). H1 was cultured as described elsewhere on plates coated with Matrigel (BD Biosciences, San Jose, CA, USA) in mTesR1 medium (Stem cell Technologies, Vancouver, BC, Canada) [9]. The medium was changed every day and the hESCs were routinely passaged by EDTA dissociation every 4–6 days [10].

2.2. TALEN and donor vectors for gene targeting

TALENs were designed as described [7,11,12]. For donor DNA, left and right homology arms were amplified from genomic DNA of H1 cell. A loxP-flanked PGK-puromycin cassette was cloned between two homology arms in the pMD-18 T vector. For targeting, 1×10^6 H1 cells were electroporated with 2 μg of donor DNA and 4.5 μg of each TALEN plasmid. Then the cells were plated onto Matrigel-coated 6-well plates in the presence of Y-27632 (10 μm; Sigma) for 1 day. Positive clones were selected by puromycin (0.5 μg/ml) in mTeSR1.

2.3. PCR detection of corrected clones

PCR was performed using High Fidelity Platinum Taq (Invitrogen) according to the manufacturer's instructions. 50–100 ng of genomic DNA templates were used in all reactions. Primer set including P1 (on 5′ homology arm) and P2 (in the 3′ UTR region of PAX6 gene) was used to amplify a 2.8-kb product of the 5′ junction of a targeted integration. Primer set including P1 (on 5′ homology arm) and P4 (in the 3′ UTR region) was used to amplify a 1.8-kb product of the 5′ junction of a targeted integration. Primer set including P3 (on *PAX6* locus, upstream of 5′ homology arm) and P4 (in the reporter gene cassette) was used to amplify a 2.2-kb product or absent product to identify whether random integration occurs or not. A primer set including P3 and P5 was used to amplify a 3.2-kb product or absent to identify whether random integration occurs. And the PCR products were sequenced to identify the corrected clones. All of the primers used are listed in Table 2.

2.4. Teratoma formation and analysis

1×10^6 cells were harvested by 0.5 mM EDTA digestion, resuspended in Matrigel, and injected subcutaneously into NOD-SCID mouse. Eight weeks after injection, teratomas were dissected, fixed in 4% paraformaldehyde, and stained by HE. All of the animal experiments were performed according to the guidelines of the Animal Care and Use Committee of the Guangzhou Institutes of Biomedicine and Health.

2.5. Neural induction of hPSCs

For neural induction in adherent cultures, hPSCs were dissociated with EDTA, and then re-plated in 6-well plates (Corning, Corning, NY, USA) in mTesR1 medium. The next day, the mTesR1 medium was replaced with N2B27 medium with 2 inhibitors (SB431542 and compound C) and cultured for 2 weeks. On d15, the cell clusters were dissociated to single cells with accutase and replated onto Matrigel-coated plates in droplets of 5–15,000 cells/ml in Neurobasal, B27 supplement with vitamin A (1:50), brain-derived neurotrophic factor (BDNF) (20 ng/ml), glial cell line-derived neurotrophic factor (GDNF) (10 ng/ml), and ascorbic acid (200 mM). From d18 and onward, db-cAMP (0.5 mM) or DAPT (2.5 mM) was added to the medium for terminal differentiation. All cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.6. FACS analysis and cell sorting

Cell sorting was performed by FACS Aria II cell sorter (BD Biosciences). FACS analysis was performed by ACCURI C6 or LSR Fortessa system (BD Biosciences).

2.7. Motor neuron differentiation

The NPC clusters were dissociated with Dispase (1 mg/ml) and passaged with the same medium described above. RA (0.1 μM, Stemgent) and 0.5 μM Purmorphamine (Stemgent) were added in combination with 1 μM Compound C (ALK2, ALK3 and ALK6 inhibitor), and 2 μM SB431542 (ALK5 inhibitor). The medium was changed every other day. NPC cells maintained under this condition for 6 days differentiated into $OLIG2^+$ motor neuron progenitors (MNPs). The $OLIG2^+$ MNPs were then dissociated with Accumax (eBioscience) into single cells and plated on Matrigel coated plates. The MNPs were cultured with 0.5 μM RA, 0.1 μM Purmorphamine for 10 days to mature into CHAT positive MNs. Insulin-like growth factor 1 (IGF-1), brain-derived neurotrophic factor (BDNF), Glial cell-derived neurotrophic factor (GDNF), Ascorbic acid (AA) were added.

2.8. Functional analysis of mature MNs

Whole-cell patch-clamp recordings of Hb9: GFP positive neurons were performed at Day28 after neural differentiation as mentioned before [13].

2.9. Immunostaining and microscopy

Immunohistochemical staining was performed according to Liu et al. [13].

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