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Directed differentiation of basal forebrain cholinergic neurons from human pluripotent stem cells

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HIGHLIGHTS

- Robust generation of basal forebrain cholinergic neurons (BFCNs) from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs).
- Co-culture with human astrocytes enhances differentiation efficiency of BFCNs.
- Purmorphamine replaces SHH to produce BFCNs.
- Generation of human BFCNs under xeno-free condition.

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ABSTRACT

Background: Basal forebrain cholinergic neurons (BFCNs) play critical roles in learning, memory and cognition. Dysfunction or degeneration of BFCNs may connect to neuropathology, such as Alzheimer's disease, Down's syndrome and dementia. Generation of functional BFCNs may contribute to the studies of cell-based therapy and pathogenesis that is related to learning and memory deficits.
New method: Here we describe a detail method for robust generation of BFCNs from human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs). In this method, BFCN progenitors are patterned from hESC or hiPSC-derived primitive neuroepithelial cells, with the treatment of sonic hedgehog (SHH) or combination with its agonist Purmorphamine, and by co-culturing with human astrocytes.
Results: At day 20, ~90% hPSC-derived progenitors expressed NKX2.1, which is a transcriptional marker for MGE. Moreover, around 40% of NKX2.1+ cells co-expressed OLIG2 and ~15% of NKX2.1+ cells co-expressed ISLET1, which are ventral markers. At day 35, ~40% neurons robustly express ChAT, most of which are co-labeled with NKX2.1, ISLET1 and FOXG1, indicating the basal forebrain-like identity. At day 45, these neurons express mature neuronal markers MAP2, Synapsin, and VAChT.
Comparison with existing method(s): In this method, undefined conditions including genetic modification or cell-sorting are avoided. As a choice, feeder free conditions are used to avoid ingredients of animal origin. Moreover, Purmorphamine can be substituted for SHH to induce ventral progenitors effectively and economically.
Conclusion: We provide an efficient method to generate BFCNs from multiple hPSC lines, which offers the potential application for disease modeling and pharmacological studies.
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1. Introduction

Basal forebrain cholinergic neurons (BFCNs) are a major functional neuronal type in controlling learning and memory in the central nervous system (CNS) (Woolf, 1991). Anatomically, cholinergic projection neurons are mainly located in the medial septum

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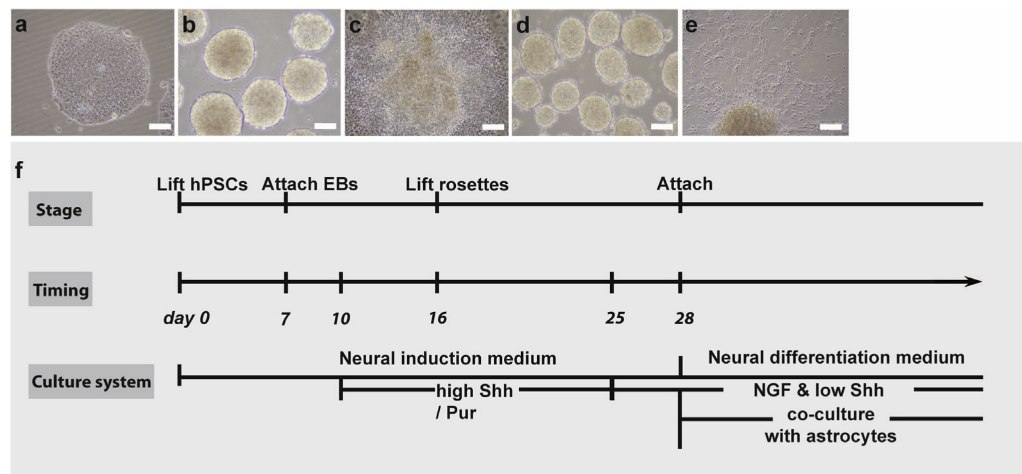

Fig. 1. Timeline of BFCN generation from hPSCs.
Cells exhibit developmental identities at each step: (a) hPSCs have defined edges without signs of differentiation, (b) embryonic bodies form from detached hPSCs, (c) neuroepithelial cells exhibit multilayered rosette-like columnar structure, (d) neural progenitors form neurospheres and differentiate to (e) neurons attached on coverslips. (f) Three major steps are composed of a total of 33 steps. Cell identity may be confirmed by immunostaining at the recommended time points. For regional patterning, cells are treated with a higher dose of SHH or Pur from days 10 to 24, and a lower dose of SHH starting at day 25. Scale bar = 250 μm.

(Ch1), diagonal band (Ch2, 3), and nucleus basalis of Meynert (Ch4) (Mesulam et al., 1983), while projecting to the cerebral cortex (Ch2, 3) and hippocampus (Ch1). Degeneration or dysfunction of BFCNs is relevant to memory loss, impaired spatial recognition, and disturbance in language, as manifested in Alzheimer's disease, Down's syndrome and dementia which do not have effective treatments to date (Oliveira and Hodges, 2005; Whitehouse et al., 1982). Availability of a large quantity of functional human BFCNs will offer promising insights into the development of novel cell-based therapeutics (Gage et al., 1984) and the pathogenesis of neurodevelopmental diseases.

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), hold the potential to differentiate to any cell type in the human body and provide a powerful tool for regenerative medicine. Over the past decade, breakthroughs have been made in commitment of hPSCs to certain type of neurons, such as cerebral or hippocampal glutamatergic neurons (Eiraku et al., 2008; Li et al., 2009; Yu et al., 2014), striatal neurons (Ma et al., 2012), cerebral cortical GABA interneurons (Liu et al., 2013a; Maroof et al., 2013; Nicholas et al., 2013), midbrain dopaminergic neurons (Kriks et al., 2011; Xi et al., 2012), and spinal motoneurons (Du et al., 2015; Li et al., 2005). Moreover, these hPSC-derived neurons have shown promises in improving behavioral deficits in disease animal models after transplantation, including cellular treatment for models of Parkinson's disease (Kriks et al., 2011; Yang et al., 2008), Huntington's disease (Ma et al., 2012) and learning and memory deficits (Liu et al., 2013b)

Recent advances in our and others' laboratories have led to the derivation of BFCNs from hPSCs (Bissonnette et al., 2011; Crompton et al., 2013; Liu et al., 2013a,b; Maroof et al., 2013). Nevertheless, the protocols are capricious and mostly depend on methods that introduce undefined conditions including genetic modification or cell-sorting. In addition, most reports were relied on the application of SHH (Bissonnette et al., 2011; Crompton et al., 2013; Liu et al., 2013b), which is a costly recombination protein. Thus, a well-described step-by-step method for generating BFCNs with defined identities from hPSCs is warranted.

In this method, we first convert hPSCs to a nearly homogeneous population of medial ganglionic eminence cells (MGE) (Puelles et al., 2000; Wilson and Rubenstein, 2000) that express the ventral forebrain homeodomain transcription factor NKX2.1, by adding a high concentration of ventralizing factor sonic hedgehog (SHH) or its substitute Purmorphamine (Pur) (Fig. 1f). MGE progenitors can be further specified to 40% BFCNs of total cells in the presence of human astrocytes. This method offers a robust approach to produce BFCNs from hPSCs, raising hopes for cell-based therapeutic studies and drug discoveries of neurological diseases with learning and memory deficits.

2. Materials

2.1. Reagents

2.1.1. Cells

Induced pluripotent stem cell lines. IMR90-4 (Wisconsin International Stem Cell Bank, cat. no. iPS (IMR90)-4).

hESC lines H9, H1 (Wisconsin International Stem Cell Bank, cat. nos. WA09 and WA01).

! CAUTION Manipulate human cells in a biosafety level-2 area.

2.1.2. Growth media and supplements

2-Mercaptoethanol (β-mercaptoethanol) (Sigma, cat. no. M3148).

! CAUTION Avoid direct contact or inhalation.

Ascorbic acid (Sigma–Aldrich, cat. no. A4403).

B27 Supplement (without vitamin A, 50×, Gibco, cat. no. 12587-010).

Brain derived neurotrophic factor (BDNF) (PeproTech, cat. no. 450-02).

Cyclic AMP (Sigma–Aldrich, cat. no. D-0260).

Compound E (EMD Biosciences, cat. no. 565790-500UG).

D-MEM/F-12 (1×), liquid, 1:1 (Gibco, cat. no. 11330032).

Essential 8 medium (E8) (Life Technology, cat. no. A1517001).

Fetal bovine serum (FBS) (Gibco, cat. no. 10099141).

Fibroblast growth factor 2 (FGF2) (R&D Systems, cat. no. 233-FB).

GlutaMAX^{TM} Supplement (Gibco, cat. no. 3505061).

Insulin-like growth factor (IGF1) (PeproTech, cat. no. 100-11).

DMH1 (Tocris Bioscience, cat. no. 4126).

Knockout Serum Replacement (KOSR) (Gibco, cat. no. 10828).

MEM Non-Essential Amino Acids Solution (100X), (NEAA) (Gibco, cat. no. 11140050).

N-2 Supplement (100×), liquid (Gibco, cat. no. 17502-048).

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