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Stepwise, non-adherent differentiation of human pluripotent stem cells to generate basal forebrain cholinergic neurons *via* hedgehog signaling

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Abstract Basal forebrain cholinergic neurons (bfCNs) which provide innervation to the hippocampus and cortex, are required for memory and learning, and are primarily affected in Alzheimer's Disease (AD), resulting in related cognitive decline. Therefore generation of a source of bfCNs from human pluripotent stem cells (hPSCs) is crucial for *in vitro* disease modeling and development of novel AD therapies. In addition, for the advancement of regenerative approaches there is a requirement for an accurate developmental model to study the neurogenesis and survival of this population. Here we demonstrate the efficient production of bfCNs, using a novel embryoid body (EB) based non-adherent differentiation (NAdD) protocol. We establish a specific basal forebrain neural stem cell (NSC) phenotype *via* expression of the basal forebrain transcription factors NKX2.1 and LHX8, as well as the general forebrain marker *FOXG1*. We present evidence that this lineage is achieved *via* recapitulation of embryonic events, with induction of intrinsic hedgehog signaling, through the use of a 3D non-adherent differentiation system. This is the first example of hPSC-derived basal forebrain-like NSCs, which are scalable *via* self-renewal in prolonged culture. Furthermore upon terminal differentiation these basal forebrain-like NSCs generate high numbers of cholinergic neurons expressing the specific markers ChAT, VACht and ISL1. These hPSC-derived bfCNs possess characteristics that are crucial in a model to study AD related cholinergic neuronal loss in the basal forebrain. Examples are expression of the therapeutic target $p75^{NTR}$, the release of acetylcholine, and demonstration of a mature, and functional electrophysiological profile. In conclusion, this work provides a renewable source of human functional bfCNs applicable for studying AD specifically in the cholinergic system, and also provides a model of the key embryonic events in human bfCN development.

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Introduction

AD is the most common age-related neurodegenerative disease estimated to affect approximately 30 million people worldwide (Holtzman et al., 2011). Most prevalent symptoms are confusion and memory loss caused by synaptic dysfunction and neuronal death. One of the primary neuronal populations affected are the bfCNs, which are partly responsible for these cognitive deficits (Holtzman et al., 2011; Everitt and Robbins, 1997).

The progress of AD research has been inhibited by lack of accurate models that recapitulate the complex facets of AD (Han et al., 2011). Recent advances in hPSC technology have made it possible to produce regionally specified neuronal populations affected by various neurodegenerative conditions, providing a novel source of human neurons for *in vitro* disease modeling (Nat and Dechant, 2011; Liu, 2011). It is essential that these neurons are phenotypically accurate and functional, and that they can also be used to model the embryonic differentiation of these populations, which is applicable to their regenerative potential (Liu, 2011).

Previous studies have demonstrated the generation of cholinergic neurons, with a potential basal forebrain phenotype (Nilbratt et al., 2010; Bissonnette et al., 2011). However, high levels of specific extrinsic factors were used to direct differentiation, of which their exact role remains unclear. Our aim was to provide a method of generating high numbers of bfCNs in keeping with a requirement for a developmental model; a reductionist approach, where the cells establish their own developmental cues, in parallel to the developing embryo. Our reasoning for the advantages of this are two-fold; firstly we believe this approach provides a superior and more accurate developmental model to study the innate acquisition of basal forebrain cholinergic fate; furthermore by using intrinsic cues from development we would suggest that the resulting neuronal progeny would be more similar to those in the developing brain. We present evidence that a method of EB-based non-adherent differentiation (NAdD) is sufficient for the induction of basal forebrain fate. In the embryo, bfCNs differentiate in the ventral telencephalon, which will go on to form part of the basal forebrain (Marin et al., 2000). Hedgehog signaling is the master controller of dorso-ventral patterning, inducing ventral fate along the entire length of the developing neural tube (Briscoe and Ericson, 1999). We show that NAdD results in the production of the secreted hedgehog ligand SHH, which as in the embryonic ventral telencephalon, results in expression of the specific transcription factors NKX2.1 and *LHX8* (Marin et al., 2000; Ericson et al., 1995a; Shimamura et al., 1995; Pera and Kessel, 1997; Gunhaga et al., 2000; Sussel et al., 1999; Flandin et al., 2010; Zhao et al., 2003; Fragkouli et al., 2005). Optimization of the NAdD protocol generated an expandable population of NKX2.1$^+$/LHX8$^+$ NSCs, which retained this phenotype through long-term expansion. Upon terminal differentiation the NSCs generated TUJ1$^+$/ChAT$^+$ cholinergic neurons, demonstrating characteristics of bfCNs present in the adult human brain. Briefly they expressed high levels of p75^{NTR} protein, as well as cholinergic receptor subunit genes, essential for their function and upon transplantation the NSCs were also able to differentiate into cholinergic neurons in the adult rat brain. Furthermore, the hPSC-derived bfCNs were electrically active, released acetylcholine, and generated neuronal action potential firing as well as spontaneous activity. Therefore our work provides a model of the human bfCN population, which meets the criteria required for a multipurpose model of the basal forebrain cholinergic system, both developmentally and also in a mature functional context.

Materials and methods

hPSC culture

Shef hES lines were originally acquired from the UK-Stem Cell Bank. The NAS2 hiPS cell line was generated as previously described (Devine et al., 2011). The MSU0001 hiPS cells were a kind gift from Dr Jose Bernado Cibelli, Cellular Reprogramming Laboratory, Michigan State University (Ross et al., 2010). Cells were maintained upon mitotically inactivated mouse embryonic fibroblasts in growth media (80% KO-DMEM, 20% Knock-out Serum Replacer (KSR), 1% non-essential amino acids, 2 mM Glutamax, 0.1 mM ß-Mercaptoethanol, and 1% penicillin and streptomycin all Life Technologies), supplemented with either 10 ng/ml (Shef hES and NAS2 hiPS) or 20 ng/ml (MSU0001) FGF2 (Peprotech). Cells were passaged manually.

Neural differentiation optimized for generation of basal forebrain progenitors

A minimum of six 5 cm plates, containing approximately 25 colonies each were required to provide an adequate number of cells. Cells were washed with KO-DMEM (Life Technologies) and incubated in 1 mg/ml Collagenase IV (Life Technologies) for 30 min at 37 °C. Whole-colonies were remove and centrifuged at 50 ×*g* for 5 min. Colonies were chopped into uniform 150 μm pieces using a McIlwain tissue chopper (Mickle Engineering, Gomshall, U.K.), and resuspended in growth media (described above), without FGF2 but supplemented with 10 μM of the ROCK inhibitor Y27632 (Tocris). The pieces were transferred to a 25 cm (Everitt and Robbins, 1997) flask, coated with poly(2-hydroxyethyl methacrylate) (Sigma) to prevent cell attachment. Colony pieces formed spheres and differentiated into EBs over 4 subsequent days, after which they were transferred into modified chemically defined media (CDM) (Joannides et al., 2007), (BSA was substituted with 400 μg/ml Albumax-II, Life Technologies). CDM was supplemented with 10 μM Y27632 (Tocris) and 20 μM of the Nodal/TGF-β signaling inhibitor SB431542 (Tocris). During this period of neural enrichment EBs were regularly triturated with P1000 tip to prevent aggregation. After 8 days the neurospheres were transferred into NEM (7 parts KO-DMEM to 3 parts F12, 2 mM Glutamax, 1% penicillin and streptomycin, supplemented with 2% B27 (all Life Technologies), plus FGF2 and EGF [20 ng/ml] and heparin [5 μg/ml]) (Svendsen et al., 1998). NEM also contained 20 μM SB431542 and 10 μM Y27632, for the initial 10 day expansion period. We found that regular trituration of the EBs throughout enrichment, and passaging using the McIlwain tissue chopper (Mickle Engineering, Gomshall, U.K.) at day 10 of expansion were crucial to achieve a pure population of NSCs. Neurospheres were passaged every 2 weeks by chopping them into 200 μm pieces (Svendsen et al., 1998) (Anderson et al., 2007).

For monolayer differentiation hPSCs were seeded on Matrigel coated plastic-ware and differentiated as described (Chambers et al., 2009).