Stem Cell Reports
Article

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ESC-Derived Basal Forebrain Cholinergic Neurons Ameliorate the Cognitive Symptoms Associated with Alzheimer's Disease in Mouse Models

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http://dx.doi.org/10.1016/j.stemcr.2015.09.010

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SUMMARY

Degeneration of basal forebrain cholinergic neurons (BFCNs) is associated with cognitive impairments of Alzheimer's disease (AD), implying that BFCNs hold potentials in exploring stem cell-based replacement therapy for AD. However, studies on derivation of BFCNs from embryonic stem cells (ESCs) are limited, and the application of ESC-derived BFCNs remains to be determined. Here, we report on differentiation approaches for directing both mouse and human ESCs into mature BFCNs. These ESC-derived BFCNs exhibit features similar to those of their in vivo counterparts and acquire appropriate functional properties. After transplantation into the basal forebrain of AD model mice, ESC-derived BFCN progenitors predominantly differentiate into mature cholinergic neurons that functionally integrate into the endogenous basal forebrain cholinergic projection system. The AD mice grafted with mouse or human BFCNs exhibit improvements in learning and memory performances. Our findings suggest a promising perspective of ESC-derived BFCNs in the development of stem cell-based therapies for treatment of AD.

INTRODUCTION

Alzheimer's disease (AD) is the most devastating neurodegenerative disorder (Tanzi and Bertram, 2005) and is characterized by progressive decline in cognitive functions (Bertram et al., 2010; LaFerla et al., 2007). The basal forebrain cholinergic system, including the nucleus basalis of Meynert (NBM), the horizontal and vertical diagonal bands of Broca, and the medial septal nucleus, provides the primary source of cholinergic innervation to the cerebral cortex, hippocampus, and amygdala (Mesulam et al., 1983) and plays critical roles in the processing of information related to cognitive function (Fodale et al., 2006). The degeneration and loss of cholinergic neurons and synapses throughout the basal forebrain, especially in the NBM, provides a pathological substrate for cholinergic deficiency in the brain of AD patients (Morrison and Hof, 1997). The early and progressive cholinergic deficiency of basal forebrain cholinergic neurons (BFCNs), characterized by a reduction in acetylcholine (ACh) synthesis, contributes substantially to the gradual cognitive decline of AD patients (Fisher, 2008; Perry et al., 1999). These studies imply that the transplantation of BFCNs might functionally restore cholinergic innervation in the basal forebrain of AD patients and repair cognitive impairments. Thus, the BFCNs hold potential in developing stem cell-based replacement therapy for the treatment of AD.

Embryonic stem cells (ESCs) or their derivatives, especially ESC-derived subtype-specific and functionally integrated neurons, have been proposed as promising therapies for neurodegenerative diseases (Lindvall and Kokaia, 2006; Lindvall et al., 2004). Assays that direct efficient differentiation of mouse and human ESCs into dopaminergic neurons have been established, and ESC-derived dopaminergic neurons were shown to correct functional deficits in animal models of Parkinson's disease (Kim et al., 2002; Kirkeby et al., 2012; Kriks et al., 2011; Yang et al., 2008). However, the mechanisms underlying BFCN generation in vivo are not well characterized. There is therefore a lack of knowledge on how to direct BFCN generation from ESCs in vitro. To date, the directed differentiation of BFCNs from mouse ESCs has not been achieved, and studies exploring the generation of BFCNs from human ESCs have emerged only recently. One report has used diffusible ligands, including retinoic acid (RA), to selectively direct the differentiation of human ESCs into BFCNs with an undefined regional identity (Bissonnette et al., 2011). Another study has demonstrated the generation of equal proportions of BFCNs and GABAergic interneurons from human ESC-derived medial ganglionic eminence (MGE)-like progenitors (Liu et al., 2013). Because the region-specific differentiation from

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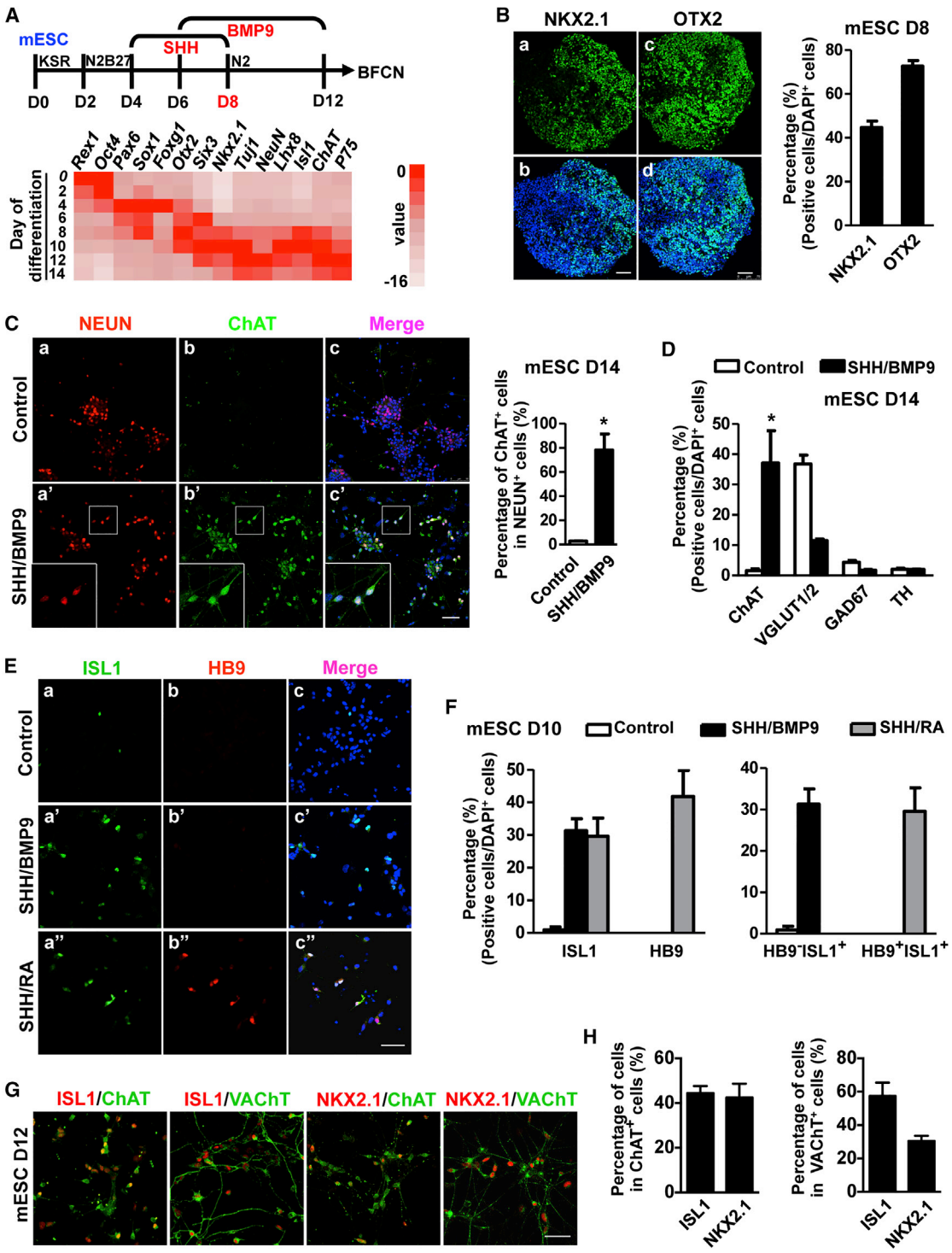

Figure 1. Efficient Derivation of BFCNs from Mouse ESCs

(A) Top: schematic representation of the methods used to direct the differentiation of mouse ESCs (mESCs) into BFCNs. Bottom: gene expression heatmap for different marker genes at the time indicated. The value indicates the log2-transformed fold change (relative to *Gapdh* and normalized to the highest value).

(B) Left: immunocytochemistry analysis of NKX2.1 and OTX2 expression in mouse EBs at day 8. Right: quantification of the data in the left panel.

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ESCs to basal forebrain, especially to BFCNs, is not well established, efforts to explore the possibility of stem cell-based therapies for AD have mainly used other types of cells, such as adult neural stem cells (Blurton-Jones et al., 2009), ESC-derived neural precursor cells (Moghadam et al., 2009), human umbilical cord blood-derived mesenchymal stem cells (MSCs) and bone marrow-derived MSCs (Lee et al., 2010a, 2010b), astrocytes (Pihlaja et al., 2008), and sonic hedgehog (SHH)/RA-induced progenitors of ChAT$^+$ neurons (Moghadam et al., 2009). These cells have been transplanted into the hippocampus of AD animal models and demonstrated to rescue memory deficits of these AD animals. The functional properties of human ESC-derived BFCNs have been investigated after transplantation into murine hippocampal slice cultures (Bissonnette et al., 2011) or into severe combined immunodeficiency mice with a destroyed medial septum by muP75-saporin but not into AD model mice (Liu et al., 2013). Thus, it remains unknown whether mouse or human ESCs can efficiently differentiate into BFCNs and whether mouse and human ESC-derived BFCNs can restore cholinergic function and alleviate cognitive deficits in AD transgenic mice.

Here, we develop a differentiation approach that directs both mouse and human ESCs into mature and functional BFCNs. The differentiation process recapitulates all key features of BFCN specification in vivo and goes through a BFCN progenitor stage. The ESC-derived BFCN progenitors are transplanted into the basal forebrain, where the in vivo BFCNs are situated, of transgenic AD mice. Two months later, the transplanted BFCN progenitors are found to predominantly differentiate into mature cholinergic neurons that functionally integrate into the endogenous cholinergic projection system of these AD mice. Furthermore, these AD model mice show improved cognitive abilities upon transplantation.

RESULTS

Efficient Derivation of BFCNs from Mouse ESCs

Both SHH and bone morphogenetic protein 9 (BMP9) signals have been reported to play critical roles in specification and development of BFCNs during mouse neurogenesis (Dessaud et al., 2008; López-Coviella et al., 2000; Reilly et al., 2002). Here, we tested different combinations of sequential treatment with SHH and BMP9 in our serum-free, floating embryoid body (EB) system and finally established a method that allows efficient and preferential differentiation of mouse ESCs into BFCNs (Figure 1A). The progressive neural commitment from mouse ESCs to BFCNs was characterized by measuring the mRNA levels of various marker genes. While the expression of the pluripotency genes *Rex1* and *Oct4* decreased, the expression of the neural progenitor markers *Sox1* and *Pax6* increased and reached their peak level at day 4. This was followed by the highest expression of the ventral anterior forebrain progenitor markers *Foxg1*, *Otx2*, *Nkx2.1*, and *Six3* around day 8. Finally, the expression of the neuronal markers *Tuj1* and *NeuN*, together with the BFCN marker genes *Nkx2.1*, *Lhx8*, *Isl1*, *ChAT*, and *P75*, increased around days 12–14 (Figure 1A). Immunostaining analysis confirmed the neural fate commitment of mouse ESCs and showed that most cells in the day-8 EBs were SOX1$^+$ and NESTIN$^+$ neural progenitors, while OCT4$^+$ pluripotent cells were barely detectable at day 8, and TUJ1$^+$ immature neurons started to emerge at the border of EBs (Figure S1A). A high percentage of neural progenitors in day-8 EBs exhibited a ventral telencephalic identity by expressing the ventral telencephalic marker NKX2.1 and the anterior forebrain neuroectoderm marker OTX2 (Figure 1B). During mouse neurogenesis, BFCNs derive from the NKX2.1$^+$ ventral telencephalic progenitors, and continuous expression of *Nkx2.1* is essential for the maturation and maintenance of this subtype of neurons (Magno et al., 2011; Sussel et al., 1999). Most NKX2.1$^+$ ventral telencephalic progenitors are KI67$^+$ dividing cells in day-8 EBs (Figure S1B). Because the sequential treatment with SHH and BMP9 promoted the cholinergic fate commitment of NKX2.1$^+$ ventral telencephalic progenitors, the generation of ChAT$^+$ cholinergic neurons significantly increased compared with cultures without these factors, and most NEUN$^+$ mature neurons were ChAT$^+$ cholinergic neurons at day 14 (Figure 1C). In addition, the ChAT$^+$ cholinergic neurons (37%) were predominant in our system compared with other subtypes of neurons, such as VGLUT1/2$^+$ glutamatergic neurons (11.5%), GAD67$^+$ GABAergic neurons (1.7%), and TH$^+$ dopaminergic neurons (2%) (Figure 1D and Figure S1C). This was confirmed by the mRNA expression of corresponding subtype marker genes

(C) Left: double immunocytochemistry analysis of ChAT and NEUN expression on day 14. Right: quantification of the data in the left panel.
(D) Percentages of different subtypes of neurons among total cells on day 14.
(E) Double immunocytochemistry analysis of ISL1 and HB9 expression on day 10.
(F) Quantification of the data in (E).
(G) Double immunocytochemistry analysis of ISLl or NKX2.1 with ChAT and VAChT on day 12.
(H) Quantification of the data in (G).
Scale bars, 50 μm (B, C, E, G). n = 3 independent experiments; two-tailed t test. All data are presented as the mean ± SD. *p < 0.05.
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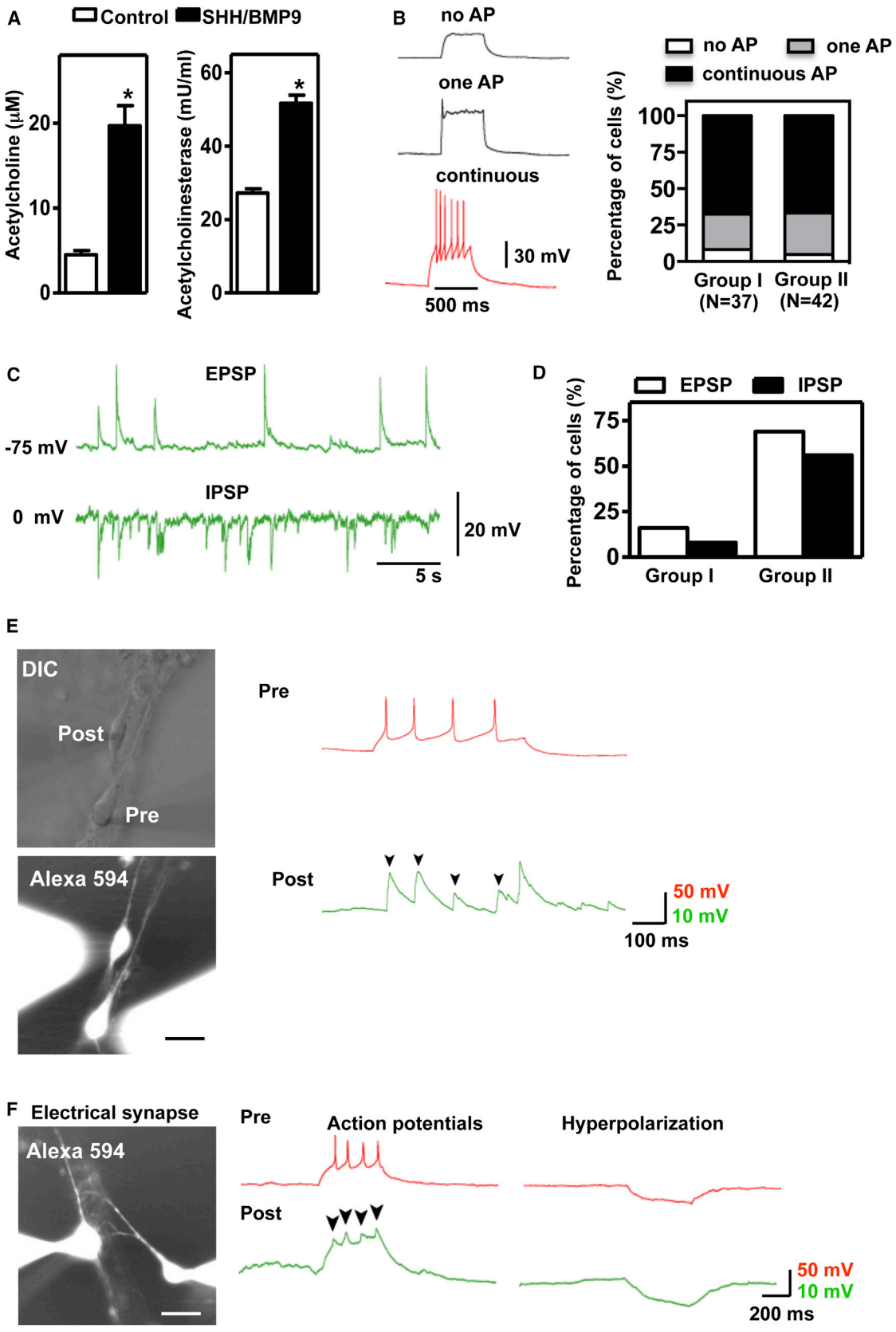

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(Figure S1D). Then, ISL1/HB9 double immunostaining, a classical assay to distinguish the two major types of cholinergic neurons in the mammalian CNS (Woolf, 1991), was performed to determine whether ChAT$^+$ cholinergic neurons are ISL1$^+$/HB9$^-$ BFCNs of basal forebrain identity or ISL1$^+$/HB9$^+$ motor neurons of spinal cord identity. The ISL1/HB9 double staining was validated in the forebrain and spinal cord of E12.5 mouse embryos (Figure S1E). With the SHH and BMP9 treatment, almost all mouse ESC-derived ISL1$^+$ cells were HB9$^-$ BFCNs, and the HB9$^+$ cells were barely detected on day 10 (Figures 1E and 1F). In contrast, the SHH and RA treatment mainly induced ISL1$^+$/HB9$^+$ motor neuron-like cells (Figures 1E and 1F), which was consistent with previous reports (Wichterle et al., 2002). Double immunocytochemistry analysis for ISL1 or NKX2.1 with ChAT or VAChT showed that approximately half of ChAT$^+$ or VAChT$^+$ neurons expressed ISL1 or NKX2.1 on day 12 (Figures 1G and 1H), indicating that a relatively high percentage of SHH/BMP9-induced cholinergic neurons possessed a basal forebrain identity. Consistently, the expression of *Isl1*, *Nkx2.1*, and the anterior marker gene *Six3* increased and the expression of the posterior markers *HB9*, *En2*, *Gbx2*, and *Hoxb9* decreased on day 12 (Figure S1F). Taken together, we have established a method that directs region-specific differentiation from mouse ESCs to basal forebrain. Following our approach, the mouse ESCs mainly give rise to NKX2.1$^+$ ventral telencephalic progenitors that predominantly differentiate into BFCNs. The differentiation process recapitulates the expression dynamics of key marker genes during BFCN specification and via a BFCN progenitor stage.

Mouse ESC-Derived BFCNs Are Mature and Functional In Vitro

To test whether the mouse ESC-derived BFCNs acquired the ability to synthesize and hydrolyze ACh, we cultured these cells for 16 days and then measured the concentration of secreted ACh and activity of acetylcholinesterase (AChE) by ELISA. The data showed that the SHH and BMP9 treatment significantly increased ACh secretion, as well as AChE activity, compared with control cells (Figure 2A), suggesting that mouse ESC-derived BFCNs possessed similar functions to their counterparts in vivo in regards to the metabolism of ACh.

Whole-cell patch-clamp recordings were performed on randomly picked SHH- and BMP9-treated neurons differentiating for 15–16 days (n = 37, group I) or 20–22 days (n = 42, group II) from mouse ESCs, respectively. More than half of the cells in each group fired action potentials (APs) continuously in response to the 500 ms step currents, and approximately one fourth of the recorded neuron cells fired a single AP (Figure 2B). The mouse ESC-derived neurons displayed remarkable panoply of biophysical cellular profiles in vitro (Table S1). In addition, the group II neurons showed more hyperpolarized resting membrane potential, increased AP amplitude, and decreased AP half-width compared with group I neurons (Table S1). More than half of the neurons in group II expressed both excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs), while the percentages of EPSP- and IPSP-expressing cells in group I were much lower (Figures 2C and 2D), indicating that functional synaptic connections formed among the mouse ESC-derived neurons in a time-dependent manner. Paired whole-cell recordings on neurons from groups I and II successfully detected the postsynaptic responses that were triggered by presynaptic APs (Figure 2E) and the electrical synaptic connections between neurons (Figure 2F). Together, these data suggested that ESC-derived BFCNs possessed membrane properties similar to those of mature and functional neurons in vivo.

The same protocol allowed the predominantly neural differentiation of mouse induced pluripotent stem cells (iPSCs) into ChAT$^+$ cholinergic neurons (Figures S2A and S2B). Whole-cell recordings showed that the mouse iPSC-derived cholinergic neurons possessed functional membrane properties similar to those of neurons in vivo, such as APs and spontaneous postsynaptic currents (Figure S2C). Taken together, the mouse ESC-derived BFCNs can secrete ACh, fire APs, form mature pre- and postsynaptic specializations, and display appropriate electrophysiological properties in vitro.

Figure 2. Characterization of Membrane Properties Mouse ESCs-Derived BFCNs

(A) Quantification of ACh and AChE by ELISA analyses for mouse ESC (mESC)-derived BFCNs at day 16.

(B) Left: representative traces of no AP (upper), one AP (middle), and continuous AP (lower) from mESC-derived neuron cells on days 15–16 (group I) and days 20–22 (group II). Right: percentages of group I or II cells expressing different types of AP.

(C) Representative traces showing EPSPs and IPSPs received by group I and II neuron cells.

(D) Percentages of cells in group I or II receiving EPSPs or IPSPs.

(E) Left: representative differential interference contrast and fluorescent images showing dual whole-cell recordings. Right: representative traces showing that group II neuron cells formed synaptic connections. Arrowheads indicate single EPSPs evoked by presynaptic APs.

(F) Representative fluorescent images (left) and dual recording traces (right) showing that the mouse neurons formed electrical synapses. Scale bars, 25 μm (E, F). n = 3 biological replicates; two-tailed t test. Data are presented as the mean ± SD. *p < 0.05.

Related to Figure S2 and Table S1.

Transplanted Mouse ESC-Derived BFCN Progenitors Predominantly Differentiate into Cholinergic Neurons in the Basal Forebrain of the AD Model Mouse

To study whether ESC-derived BFCNs can function in vivo and replace lost neurons in diseased mice, we transplanted mouse ESC-derived BFCN progenitors into the NBM of two strains of transgenic AD model mice: 5XFAD and APP/PS1. Both AD model mice can recapitulate major neuropathological features of AD, especially the AD amyloid pathology, and development of AD symptoms, such as cognitive deficits (Jankowsky et al., 2004; Oakley et al., 2006). Before transplantation, we examined the neuropathological processes of AD mice by measuring the levels of Aβ deposits in the brain. Consistent with previous studies (Jankowsky et al., 2004; Oakley et al., 2006), the Aβ plaques in the brain of 5XFAD mice appeared in the subiculum and deep layers of the cortex as early as 2 months of age and accumulated robustly within most regions of the brain, including the NBM at 3 to 4 months of age (Figure S3A). In APP/PS1 mice, the deposited amyloid did not appear until 6 months of age and began to spread into most regions of the brain at 9 months of age (Figure S3B). We chose to perform cell transplantations at the age when each strain exhibits dramatic amyloid plaque but does not show detectable neuron degeneration or cognitive deficits (Jankowsky et al., 2004; Oakley et al., 2006), which is 4 months of age for 5XFAD mice and 10 months for APP/PS1 mice.

To trace the transplanted cells in vivo, day-8 BFCN progenitors derived from EGFP-labeled mouse ESCs (Adams et al., 2003) were used in this experiment and thereafter. The proliferation and differentiation of GFP^+ BFCN progenitors in the brain of 5XFAD mice were measured at different periods, such as 2 weeks, 1 month, 2 months, 3 months, and 6 months after transplantation. We found that the proliferation of grafted BFCN progenitors decreased gradually with the disappearance of $KI67^+$ cells while the neuronal differentiation among these grafted cells increased with the appearance of $NEUN^+$ neurons (Figure S3C). Two months after transplantation, the proliferating cells among the grafted cells were seldom detected, and most cells among the grafted cells were $NEUN^+$ neurons (Figure S3D), indicating that 2 months are sufficient and necessary for the neuronal differentiation of grafted BFCN progenitors. Thus, the grafted BFCN progenitors were kept for 2 months in the brain of AD mice in the following experiments.

The bilateral transplantation was performed by the injection of 80,000 $EGFP^+$ cells into each NBM of AD mice brains. Two months later, the AD mice were subjected to a behavior test. After the behavior test, the numbers of $EGFP^+$ cells were counted. Among 42 analyzed mice, including both 5XFAD and APP/PS1 mice, 33 mice had numbers of $EGFP^+$ cells ranging from 60,000 to 80,000 per NBM, 4 mice had fewer than 60,000 $EGFP^+$ cells, and 5 mice had a little more than 80,000 $EGFP^+$ cells, showing that the average survival rate of transplanted cells is 75%. There was no detectable sign of tumor formation in host brains grafted with BFCN progenitor cells in the NBM.

Among both strains of AD mice grafted with mouse ESC-derived BFCN progenitor cells, approximately 80% of the surviving $EGFP^+$ cells remained in the NBM to which they were originally delivered (Figures 3A and 3B and Table S2). The characterization of surviving $EGFP^+$ cells in the NBM by immunostaining showed that approximately 75% of the $EGFP^+$ cells were $NEUN^+$ neurons, less than 15% of the $EGFP^+$ cells differentiated into $GFAP^+$ astrocytes and $O4^+$ oligodendrocytes, and only a few $EGFP^+$ cells expressed the neural progenitor cell marker NESTIN (Figures 3C and 3E), indicating that most transplanted BFCN progenitors had differentiated into mature neurons in the NBM of the host brain. In addition, approximately 50% of the $EGFP^+$ cells were $ChAT^+$ cholinergic neurons (Figures 3D and 3E). The number of $GAD67^+$ GABAergic or $VGLUT1/2^+$ glutamatergic neurons was much lower than that of $ChAT^+$ neurons (Figures 3D and 3E). Lastly, we measured the concentration of secreted ACh and the activity of AChE in the brain of 5XFAD mice with or without grafted BFCN progenitors by ELISA. We found that both the ACh secretion and the AChE activity significantly increased in the brain of 5XFAD mice with BFCN progenitors compared with the brain of 5XFAD mice without BFCN progenitors (Figure 3F). Together, these data indicate that the transplanted BFCN progenitors survive well in the NBM and can predominantly differentiate into mature cholinergic neurons in the basal forebrain of host AD mice. The mouse ESC-derived BFCNs exhibited functional properties that were characteristic of mature BFCNs in vivo.

Mouse ESC-Derived Neurons Functionally Integrate into the Endogenous Cholinergic Projection System of the AD Mouse

Previous studies indicated that reconstruction of lost neuronal circuitry may be an important prerequisite for the restoration of neural function in impaired brain regions (Lindvall and Kokaia, 2006; Lindvall et al., 2004). We thus sought to explore the electrophysiological properties and migration or integration of transplanted cells within the basal forebrain of both 5XFAD and APP/PS1 mice. After the behavior test, 200-μm-thick brain slices containing transplanted cells were prepared for whole-cell recording. Altogether, 18 $EGFP^+$ cells in the basal forebrain of 5XFAD or APP/PS1 mice were recorded, and subsequent immunohistochemistry confirmed that more than half of them (13/18) were $ChAT^+$ cholinergic neurons (Figure 4A). Among these cells, 15 cells fired repetitive APs (Figure 4B), indicating that they possessed membrane properties of mature neurons. In addition, these 15 neuronal cells

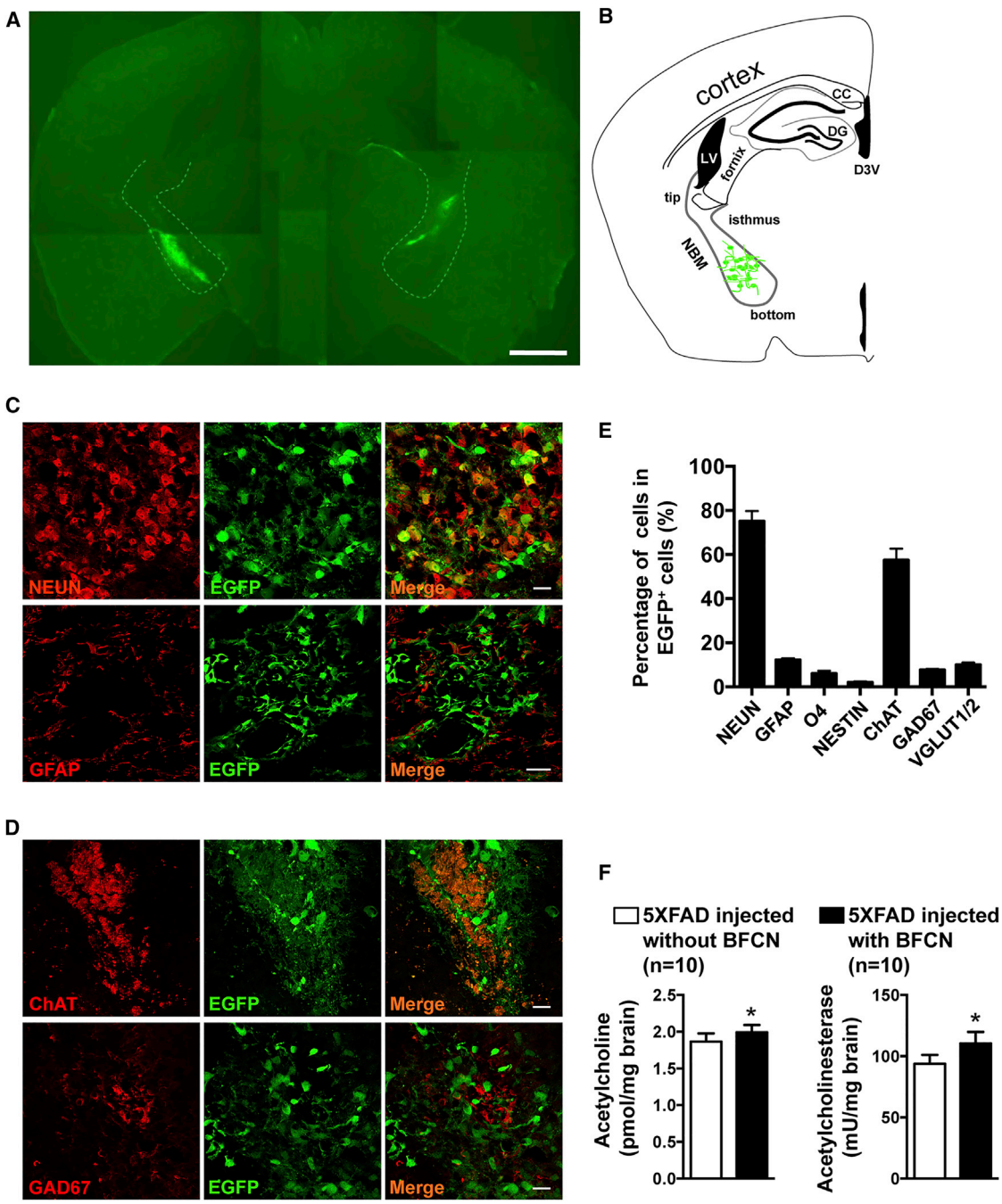

Figure 3. Differentiation of Mouse ESC-Derived BFCN Progenitors 2 Months after Transplantation into the NBM of AD Model Mice

(A) As shown on the coronal brain section of AD mice, most surviving EGFP$^+$ BFCN progenitors were found in the NBM.

(B) Schematic diagram of the distribution of EGFP$^+$ BFCN progenitors shown in (A).

(C) EGFP fluorescence and immunocytochemistry analyses of NEUN or GFAP expression in the NBM of the host brain.

(D) EGFP fluorescence and immunocytochemistry analyses of ChAT or GAD67 expression in the NBM of the host brain.

(E) Quantification of the data in (C) and (D).

(F) Quantification of ACh and AChE by ELISA analyses in the 5XFAD brain 2 months after transplantation (n = 10).

Scale bars, 1 mm (A) and 25 μm (C and D). n = 3 biological replicates; two-tailed t test. Data are presented as the mean ± SD. *$p < 0.05$.

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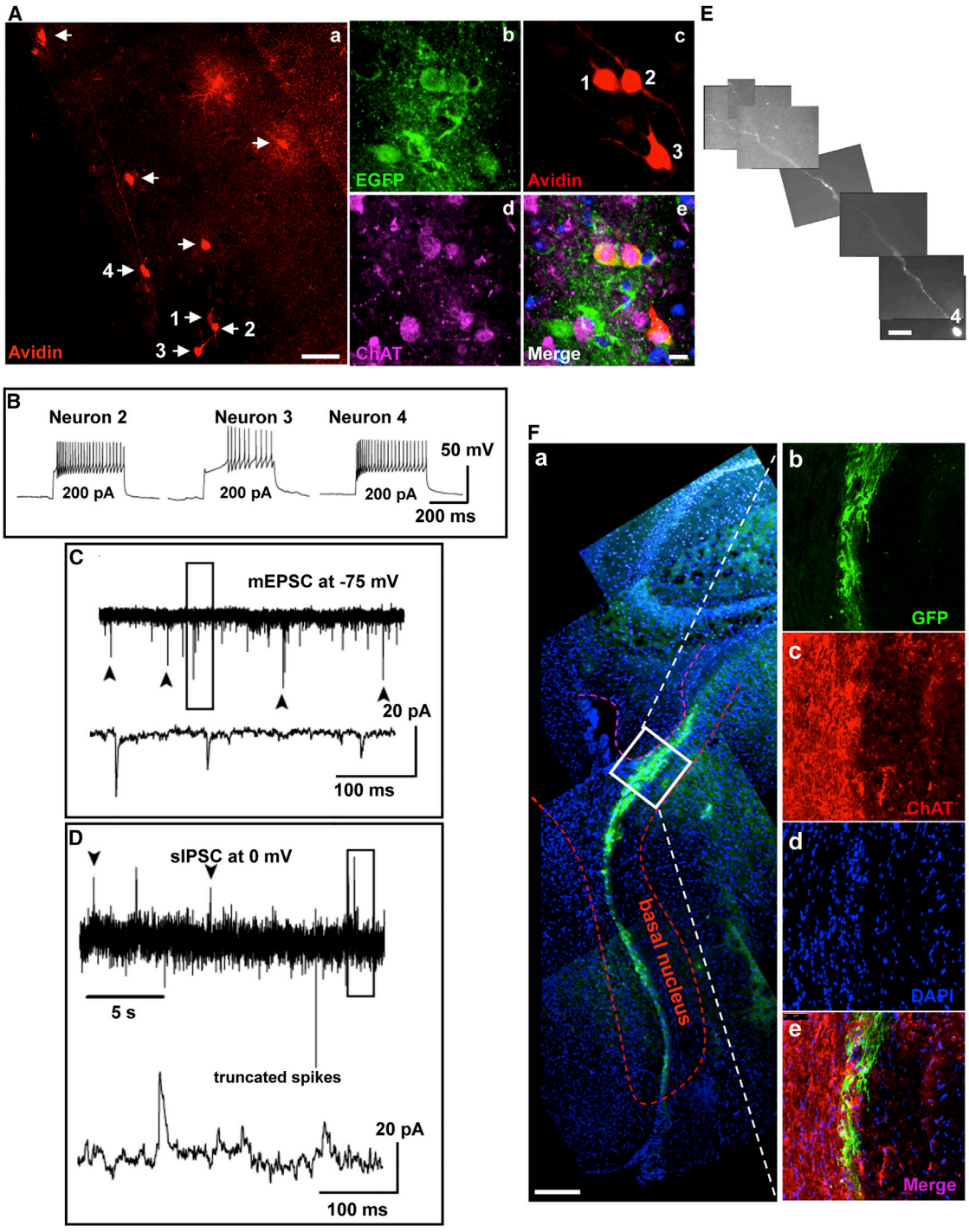

Figure 4. Mouse ESC-Derived Cholinergic Neurons Functionally Integrated into the Basal Forebrain of Host Mice

(A) Representative recorded cells labeled by Avidin-PE (a, red). Arrows indicate recorded cells involved in a basal cholinergic projection system. No. 1, 2, and 3 recorded cells were EGFP$^+$ (b), Avidin-PE$^+$ (c), and ChAT$^+$ (d), respectively, and appear in overlay images with these indicated markers (e).

(B) No. 1–3 EGFP$^+$/ChAT$^+$ cholinergic neurons fired continuous APs.

(C and D) No. 3 cholinergic neuron received functional synaptic inputs, as reflected by the occurrence of EPSCs (C) and IPSCs (D).

(E) No. 4 neuron in the basal forebrain displayed a long axon projecting along the NBM.

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exhibited a more hyperpolarized resting membrane potential and AP threshold, lower input resistance, higher cellular capacitance, increased AP amplitude, and decreased AP half-width compared to the recorded neurons differentiated in vitro (Table S1), suggesting that in vivo differentiated cholinergic neurons were functionally mature and the basal forebrain of host mice might provide a favorable environment for the differentiation of transplanted cells into mature neurons. Most recorded neurons (15/18) exhibited excitatory and inhibitory synaptic activities (Figures 4C and 4D). The average frequencies of the miniature excitatory postsynaptic current (EPSC) and spontaneous inhibitory postsynaptic current (IPSC) were 4.8 ± 0.5 and 1.3 ± 0.1 Hz, respectively, indicating that the ChAT$^+$ neurons were functionally integrated into the endogenous neuronal circuitry system and had maintained functional activity in vivo for up to 10 weeks after transplantation. After the EGFP$^+$ cells were recorded, post hoc immunohistochemistry was performed to trace the projection and migration of EGFP$^+$ cells. Most recorded EGFP$^+$ cells (14/18) showed the typical projection pattern of neurons of the basal nucleus, characterized by their axon-dendrite orientation, and axon projections up to 700 μm in length were detected (Figure 4E). Besides the recorded cells, most examined EGFP$^+$ cells projected or migrated along cholinergic projections throughout the NBM, from the bottom to the NBM tip or NBM isthmus and toward the cortex and hippocampus (Figure 4F and Table S2), indicating that transplanted cells or their progeny morphologically incorporated into the endogenous cholinergic projection system. In addition to whole-cell recording, electron microscopy using immunogold labeling anti-GFP antibody was performed. We observed typical synaptic structures between the green BFCN progenitor-derived neuron cell (pseudocolor green) and the endogenous neuron cell (pseudocolor yellow) (Figure S4A), which is indicative of the synapse formation between exogenous neurons and host neurons. Together, these data demonstrate that the mouse ESC-derived BFCNs functionally integrate into the endogenous basal forebrain neuronal circuitry and cholinergic projection system of both 5XFAD and APP/PS1 AD mice.

The correct connections between the grafted cells and the endogenous neurons were believed to be essential to the symptomatic relief of AD mice. In addition to the integration, trophic factor secretion and Aβ clearance caused by grafted cells were reported to induce the functional recovery of AD mice (Blurton-Jones et al., 2009; Lee et al., 2010a, 2010b; Pihlaja et al., 2008) . After the behavior test, we measure the levels of trophic factor BDNF and Aβ in the brain of 5XFAD mice with and without grafted BFCN progenitors. We found that levels of BDNF in 5XFAD mice with BFCN progenitors were significantly increased compared to those in 5XFAD mice without grafted cells (Figure S4B). The level of Aβ plaques in the brain of 5XFAD mice was measured by quantifying the Aβ plaque number and burden. The results showed that Aβ plaques between 5XFAD mice with and those without BFCN progenitors were similar (Figure S4C). These data indicate that the transplanted BFCN progenitors induce the secretion of trophic factors but do not alter the global level of Aβ plaques in the host brain.

Human ESCs Efficiently Differentiate into Mature and Functional BFCNs In Vitro and In Vivo

After successfully generating BFCNs from mouse ESCs, we sought to generate BFCNs from human ESCs. To this end, we tried different dosages and time points of SHH and BMP9 treatment and developed the most effective approach for human ESCs (Figure 5A). During human ESC neural differentiation, the expression of *OCT4* decreased first and the expression of the neural epithelial markers *PAX6* and then *SOX1* increased substantially thereafter (Figure 5A). The addition of SHH decreased the expression of *PAX6* and increased *SOX1* expression continuously (Figure 5A). Most cells in day-26 neurospheres were SOX1$^+$ and NESTIN$^+$ neural progenitors (Figure S5A), and more than half of day-26 neural progenitors were NKX2.1$^+$/OTX2$^+$ BFCN progenitors (Figure 5B). In addition, most NKX2.1$^+$ BFCN progenitors were KI67$^+$ dividing cells (Figure S5B). The expression of *ChAT* was dramatically induced by sequential treatment with SHH and BMP9, similar to that of *NEUN* (Figure 5A). At day 40, more than half of the NEUN$^+$ cells were VAChT$^+$ mature cholinergic neurons (Figure 5C). Similar to the mouse ESC BFCN differentiation, human ESCs predominantly differentiated into VAChT$^+$ cholinergic neurons (38%) in our culture system and rarely into other neuronal subtypes (Figure 5D and Figure S5C), which was confirmed by the mRNA expression of subtype marker genes (Figure S5D). On day 35, almost all human ESC-derived ISL1$^+$ cholinergic neurons were HB9$^-$ BFCNs and few HB9$^+$ cells were observed (Figures 5E and 5F). On

(F) The track of migration or projection of transplanted EGFP$^+$ BFCN progenitors in the NBM. Transplanted EGFP$^+$ cells (a) migrated or projected along the endogenous NBM cholinergic projection track, which is long and regular, representative of the projection from basal nucleus to the hippocampus. Close-ups of the outlined region in (a) show EGFP$^+$ exogenous BFCN progenitors and their progeny (b), ChAT$^+$ endogenous and exogenous cholinergic neurons (c), nuclei stained with DAPI (d), and overlay images (e).
Scale bars, 50 μm (Aa, F), 10 μm (Ab–Ae), and 200 μm (E).
Related to Figure S4 and Tables S1 and S2.

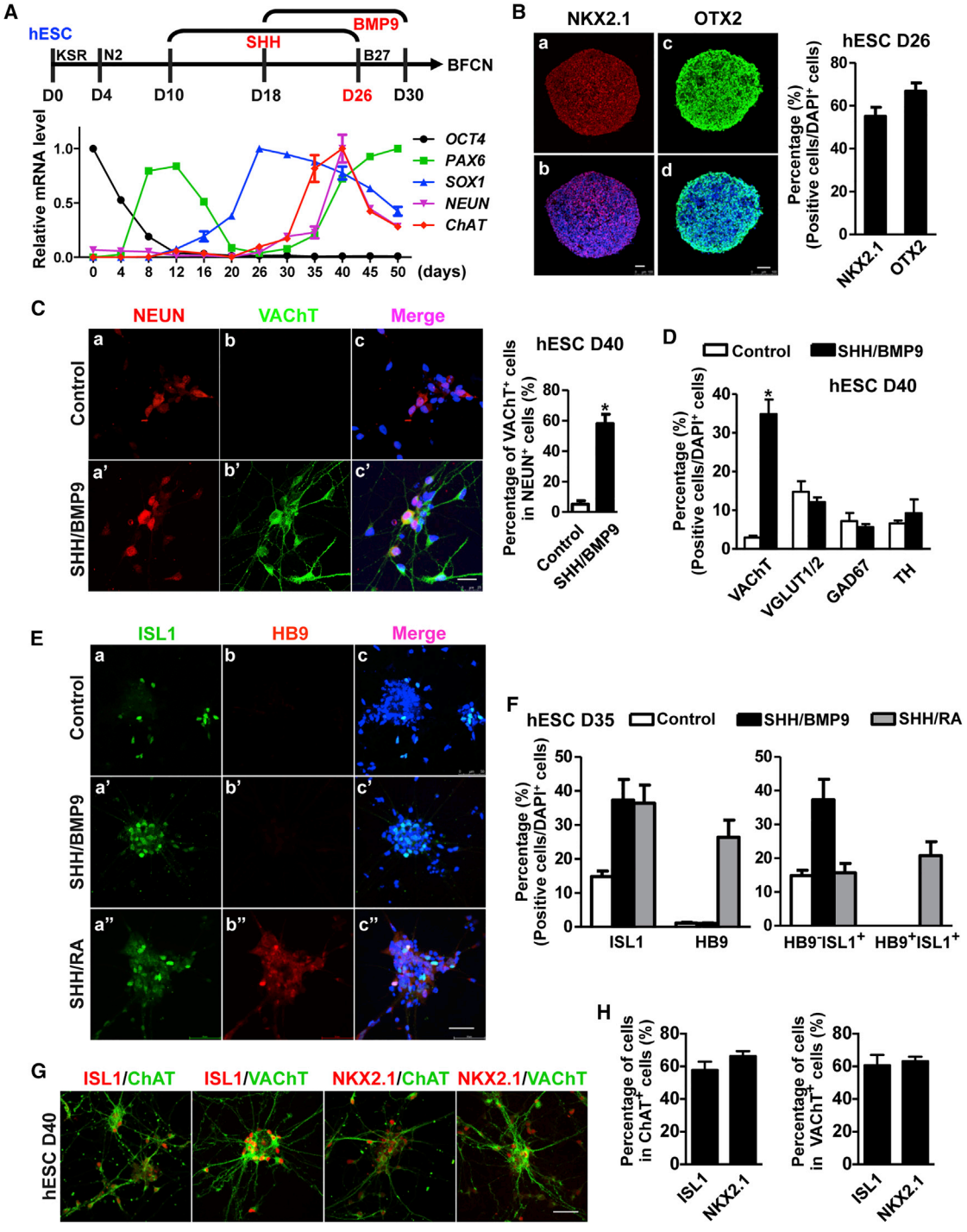

Figure 5. Efficient Derivation of BFCNs from Human ESCs

(A) Top: schematic representation of the method used to direct the differentiation of human ESCs (hESCs) into BFCNs. Bottom: qPCR analysis of the expression level of different marker genes at the time indicated.

(B) Left: immunocytochemistry analysis of NKX2.1 and OTX2 expression in human neurospheres at day 26. Right: quantification of the data in the left panel.

(C) Left: double immunocytochemistry analysis of VAChT and NEUN on day 40. Right: quantification of the data in the left panel.

(D) Percentages of different subtypes of neurons among total cells on day 40.

(legend continued on next page)

day 40, more than half of human ESC-derived ChAT$^+$ or VAChT$^+$ neurons were ISL1$^+$ or NKX2.1$^+$ BFCNs (Figures 5G and 5H). This was confirmed by the increased expression of anterior ventral marker genes and the decreased expression of posterior marker genes (Figure S5E). On day 40, the SHH/BMP9-induced human neurons secreted a higher level of ACh and showed higher AChE activity than did control cells (Figure S5F). On day 65, 30 SHH/BMP9-induced neuron cells were randomly picked and measured by whole-cell patch-clamp recordings, and most of them (23/30) were mature neurons that fired APs (Figure S5G). In addition, approximately half of the neurons expressing continuous AP (7/15) exhibited spontaneous postsynaptic currents. These neurons also expressed the synaptic marker SYNAPSIN (Figure S5H). Together, these data indicate that human ESCs, like mouse ESCs, predominantly give rise to mature and functional BFCNs through a BFCN progenitor stage in our culture system.

Similar to the bilateral transplantation with mouse ESC-derived BFCNs described earlier, 80,000 human ESC-derived BFCN progenitors were transplanted into each NBM of 5XFAD AD model mice. Immunostaining for human nuclear protein (hNuclei) indicated that the transplanted human cells survived and migrated within the mouse NBM after the behavior test, which was 7 weeks after transplantation (Figure S6A). Among 11 analyzed mice, the average number of surviving hNuclei$^+$ human cells in each NBM was 6,000. Double immunostaining showed that approximately 60% of these surviving hNuclei$^+$ cells were NEUN$^+$ mature neurons and less than 10% of human cells differentiated into GFAP$^+$ astrocytes or OLIGO$^+$ oligodendrocytes (Figure S6B). Among the human cells, approximately 20% of the hNuclei$^+$ cells were cholinergic neurons, expressing both ChAT and VAChT (Figure S6C). The GAD67$^+$ GABAergic or VGLUT1/2$^+$ glutamatergic neurons among hNuclei$^+$ cells were not detected. Together, these data indicate that the transplanted human BFCN progenitor cells can survive in the NBM and predominantly differentiate into cholinergic neurons in the basal forebrain of host AD mice.

Transplanted Mouse and Human ESC-Derived BFCN Progenitors Ameliorate the Cognitive Decline of AD Model Mice

To assess cognitive function, particularly the spatial learning and reference memory abilities of 5XFAD and APP/PS1 mice with transplanted mouse ESC-derived BFCN progenitors, the Morris water maze (MWM) test was performed 2 months after transplantation. For each strain of AD model mice, four groups of mice were subjected to MWM: AD mice with transplanted ESC-derived BFCN progenitors (referred to as BFCN mice), AD mice receiving vehicle (sham) injections, age-matched AD mice, and wild-type mice. In a control experiment involving a visible platform, all groups of mice showed no differences in the speed and latency period to locate the platform, and the transgenic modification or cell transplantation had no effect on sensorimotor or motivational performance. Spatial learning abilities were assessed by escape latency toward a hidden platform. Intriguingly, both strains of mice with mouse ESC-derived BFCN progenitors exhibited gradually and significantly shorter escape latencies compared to their corresponding sham control and AD control, respectively, and BFCN mice showed a steady improvement in learning (Figures 6A and 6B). The performance of the AD mice transplanted with BFCN progenitors was comparable to that of wild-type mice in learning training, and vehicle delivery had no effect on the learning performance of AD mice (Figures 6A and 6B). These data indicate that transplanted mouse ESC-derived BFCN progenitors rescued the learning deficits of AD mice. The probe test was performed 24 hr after the final learning trial on day 7 to assess the spatial memory ability of these mice. Both 5XFAD-BFCN and APP/PS1-BFCN mice spent a significantly longer time in the target quadrant and showed a greater number of platform-site crossovers than the AD and sham-control mice, indicating that the spatial memory decline of AD mice was ameliorated by transplantation of mouse ESC-derived BFCN progenitors (Figures 6C and 6D).

5XFAD mice grafted with human ESC-derived BFCN progenitors were also subjected to behavior assays. Six weeks after transplantation, 5XFAD mice with human ESC-derived BFCN progenitors also showed improvement in learning (Figure 6E) and memory (Figure 6F) abilities. Taken together, these results indicate that the cognitive deficiency of AD model mice can be rescued by transplantation of both mouse and human ESC-derived BFCN progenitors.

DISCUSSION

In this study, we established an approach that efficiently directed both mouse and human ESCs into mature and

(E) Double immunocytochemistry analysis of ISL1 and HB9 on day 35.
(F) Quantification of the data in (E).
(G) Double immunocytochemistry analysis of ISLl or NKX2.1 with ChAT and VAChT on day 40.
(H) Quantification of the data in (G).
Scale bars, 50 μm (B, E, G) and 25 μm (C). n = 3 independent experiments; two-tailed t test. All data are presented as the mean ± SD. *p < 0.05. Related to Figures S5 and S6.

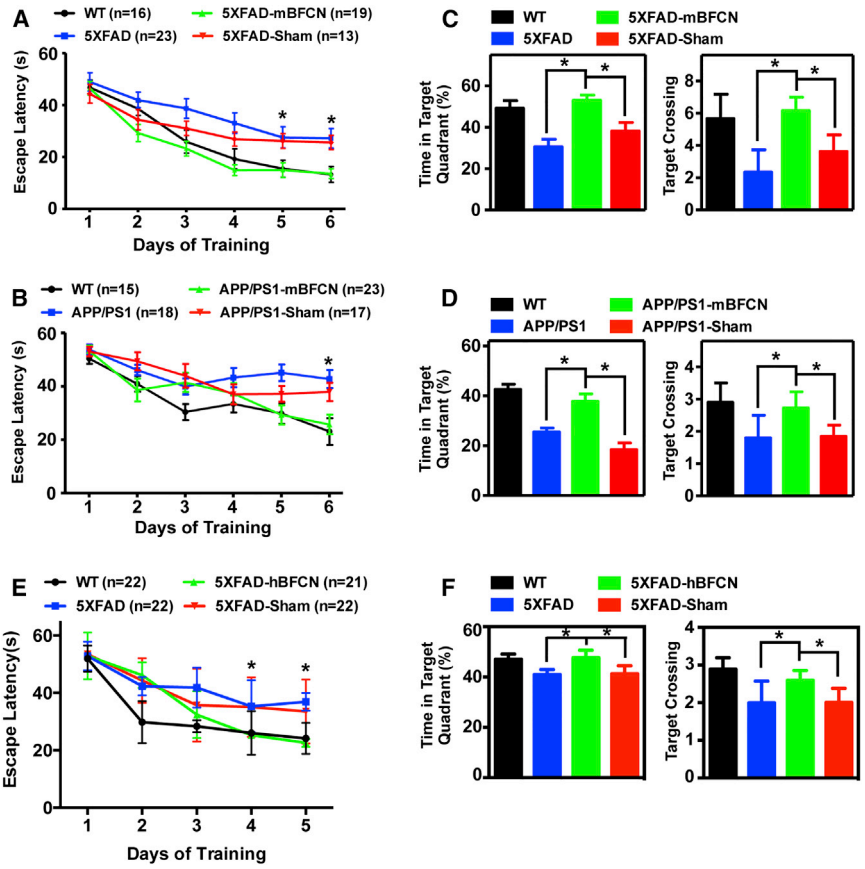

Figure 6. Transplantation of Mouse or Human BFCN Progenitors Rescues the Cognitive Impairments of AD Model Mice

(A and B) Training trials revealed that 5XFAD mice (A) and APP/PS1 mice (B) grafted with mouse ESC (mESC)-derived BFCN progenitors exhibited significantly shorter escape latencies from day 5 compared to sham controls and transgenic controls and performed as well as wild-type controls.

(C and D) In the probe test, 5XFAD mice (C) and APP/PS1 mice (D) grafted with mESC-derived BFCN progenitors spent significantly longer amounts of time in the target quadrant and had a greater number of target crossings compared to both sham controls and transgenic controls.

(E) Training trials revealed that 5XFAD mice with human ESC (hESC)-derived BFCN progenitors exhibited significantly shorter escape latencies from day 4 compared to controls.

(F) The probe test revealed that the 5XFAD mice with hESC-derived BFCN progenitors spent longer amounts of time in the target quadrant and had a greater number of target crossings compared to controls.

n = 3 independent experiments; two-way ANOVA test. Results are presented as mean ± SD. *p < 0.05.

functional BFCNs. The differentiation process recapitulated expression dynamics of key factors involved in BFCN development in vivo and went through a BFCN progenitor stage. After transplantation into the basal forebrain of AD transgenic mice, the ESC-derived BFCN progenitors predominantly differentiated into mature cholinergic neurons that functionally integrated into the endogenous cholinergic projection system. These AD model mice with grafted mouse or human BFCNs showed improved cognitive abilities. Together, we develop a region- and subtype-specific neural differentiation approach for both mouse and human ESCs, and the neurons generated from this approach ameliorate the cognitive symptoms of AD model mice upon transplantation.

Region-Specific Differentiation from ESCs to BFCN Progenitors

Pluripotent stem cells hold potential to give rise to region-specific neural progenitors or functional neurons, and both mouse and human ESCs have been widely studied to assess their potential for ESC-based cell-replacement therapy for neurodegenerative diseases. Dopaminergic neurons (Kim et al., 2002; Kirkeby et al., 2012; Kriks et al., 2011; Yang et al., 2008), motor neurons (Harper et al., 2004; Kim et al., 2002; Wichterle et al., 2002), GABAergic neurons (Ma et al., 2012), and glutamatergic neurons (Eiraku et al., 2008; Li et al., 2009) have also been generated from human and mouse ESCs and demonstrated to be functional in transgenic animal models related to different neurodegenerative diseases. However, the directed differentiation of BFCNs from mouse ESCs has not yet been achieved. We show here that cholinergic neurons with a basal forebrain regional identity can be efficiently and rapidly derived from mouse ESCs in a serum-free system. With sequential treatment by SHH and BMP9, *ChAT* expression is robustly induced and approximately 40% of total cells have become $ChAT^+$ neurons, whereas other neuron subtypes, such as GABAergic, dopaminergic, and glutamatergic neurons, are not significantly induced, indicating that this method preferentially directs the differentiation of mouse ESCs into cholinergic neurons. Further analysis showed that the mouse ESC-derived cholinergic neurons are $ISL1^+$/$NKX2.1^+$ and $HB9^-$, suggesting that they possess a basal forebrain identity and are not motor neuron-like cells (Figure 1). Recent studies reported that human ESCs can differentiate into BFCNs (Bissonnette et al., 2011; Liu et al.,

2013). Bissonnette et al. (2011) showed that sequential treatment with RA, SHH, and BMP9 can efficiently induce the differentiation of human ESCs to BFCNs. It remained unknown, however, whether early RA treatment established a caudal positional identity or whether human ESC-derived ChAT$^+$ or VAChT$^+$ neurons expressed basal forebrain markers NKX2.1 or ISL1. Liu et al. (2013) showed that a nearly uniform population of NKX2.1$^+$ MGE-like progenitors can be derived from human ESCs. These MGE-like progenitors subsequently gave rise to equal amounts of ChAT$^+$ BFCNs (38% among TUJ1$^+$ cells) and GABAergic neurons (40% among TUJ1$^+$ cells). Here, we show that human ESCs can be predominantly differentiated into BFCNs expressing VAChT, ChAT, NKX2.1, and ISL1 and that other neuronal subtypes, including GABAergic neurons, were not induced to the same extent as BFCNs (Figure 5 and Figure S5). Together, these data indicated that region- and subtype-specific differentiation from both mouse and human ESCs to BFCNs has been successfully established.

Subtype-Specific Replacement and Functional Integration by ESC-Derived BFCNs Ameliorate the Cognitive Symptoms of AD Model Mice

Recently, multiple efforts have been made to relieve neuropathological symptoms and restore cognitive function in transgenic AD animal models using stem cell-based approaches. Astrocytes were transplanted into the hippocampus of a transgenic AD mice and showed a capacity to internalize Aβ deposits (Pihlaja et al., 2008). The hippocampal transplantation of adult neural stem cell was reported to induce the formation of new synapses and to rescue cognitive deficits of AD mice without altering Aβ deposits (Blurton-Jones et al., 2009). The hippocampal transplantation of human umbilical cord blood-derived MSCs was reported to rescue memory deficits in AD mice by reducing neuronal apoptosis (Lee et al., 2010a). The same group reported that bone marrow-derived MSCs could reduce Aβ deposits and rescue memory deficits in AD mice (Lee et al., 2010b). The NBM transplantation of ESC-derived cholinergic precursors by SHH/RA treatment rescued memory deficits of NBM-lesioned rats (Moghadam et al., 2009). Similarly, human iPSC-derived neuronal precursors by SHH/RA treatment were shown to restore spatial memory of AD mice upon hippocampal transplantation (Fujiwara et al., 2013). The early and substantial degeneration of BFCNs in AD patients is the key factor in cognitive deficits associated with the disease (Morrison and Hof, 1997), but it remains unknown whether ESC-derived BFCNs can restore cholinergic function and alleviate cognitive deficits in AD model mice. In addition, the differentiation, migration, or integration of grafted cells in the host brain was unclear. In the present study, we successfully transplanted both mouse and human ESC-derived BFCN progenitors into the basal forebrain of transgenic AD model mice: 5XFAD and APP/PS1. After the behavior test, the transplanted mouse ESC-derived BFCN progenitors were demonstrated to survive well and predominantly differentiate into mature cholinergic neurons in the basal forebrain of host mice (Figure 3 and Figure S6), suggesting that the grafted BFCN progenitors terminally differentiated into the correct neuronal subtype with regional specificity. More importantly, the BFCN progenitor-derived cholinergic neurons fired continuous APs, produced EPSCs and IPSCs in vivo, and formed synapses with host neuron cells (Figures 4C and 4D and Figure S4A), indicating that they functionally integrated into the endogenous neuronal circuitry. These cholinergic neurons also exhibited typical projection and migration patterns along the host NBM and toward the hippocampus (Figures 4E and 4F), indicating that they morphologically incorporate into the endogenous basal forebrain cholinergic projection system. In addition, the ACh secretion and AChE activity increased in the brain of 5XFAD upon transplantation, indicating that the grafted cell-derived cholinergic neurons functioned similarly to their in vivo counterparts. The secretion of neurotrophic factor BDNF also increased in the brain with grafted BFCN progenitors, which is consistent with the previous findings (Blurton-Jones et al., 2009). All these changes, the integration of ESC-derived BFCNs and the increased secretion of cholinergic transmitters or neurotrophic factor, in the host brain upon transplantation might synergistically contribute to ameliorate the cognitive symptoms of AD model mice.

Together, our findings demonstrate that both mouse and human ESCs efficiently differentiated into mature and functional BFCNs and these ESC-derived BFCNs integrated into the basal forebrain of AD mice and alleviated the cognitive deficits of AD. These findings suggest that ESC-derived BFCNs replacement and partial reconstruction of damaged cholinergic neuron circuitry of AD model mice might be possible, and they support the notion that stem cell-based therapies could be developed to restore function in the AD brain.

EXPERIMENTAL PROCEDURES

Mouse and Human ESC Culture and Neural Differentiation

Mouse ESC lines R1/E, E14, and CE3; human ESC line H9; and one mouse iPSC line were maintained in standard medium supplemented with 10 ng/mL of leukemia inhibitory factor (Millipore) for mouse ESCs and iPSCs and 8 ng/ml of fibroblast growth factor (R&D Systems) for human ESCs. Subsequent steps in the neural differentiation procedure are detailed in the Supplemental Experimental Procedures.

Immunofluorescence Staining

Immunocytochemistry staining on cell cultures was performed as previously described. Additional details are included in the Supplemental Experimental Procedures.

Cell Transplantation

The animal experiments were carried out following protocols approved by the Animal Ethics Committee of the Shanghai Institutes for Biological Sciences. Two strains of AD model mice, 5XFAD (Jackson No. 006554) and APP/PS1 (Jackson No. 004462), were purchased from Jackson Laboratory. Subsequent steps in the cell transplantation procedure are detailed in the Supplemental Experimental Procedures.

MWM Test

The MWM test was performed using hidden platform procedures as described previously (Vorhees and Williams, 2006). For mouse ESC-derived cells, the MWM test was performed 8 weeks after transplantation and the training trials were conducted for 6 days. For human ESC-derived cells, the MWM test was performed 6 weeks after transplantation and the training trials were conducted for 5 days.

Electrophysiology

Whole-cell patch-clamp recording was performed in mouse ESC-derived neuron cells on differentiating days 15–16 (group I) or days 20–22 (group II), mouse iPSC-derived neurons on day 18, human ESC-derived neurons on day 65, and $EGFP^+$ BFCN progenitor-derived neuron cells in the coronal brain slices of AD mice. The whole-cell recordings were performed with Multiclamp 700B (Molecular Devices). Additional details are included in the Supplemental Experimental Procedures.

Statistical Analysis

Student's t test (two-tailed) was performed for statistical analysis between two groups. ANOVA tests were used when three or more groups were compared. The statistical analyses were obtained from three independent experiments. Statistical significance was set at $p < 0.05$. All data are presented as the mean ± SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.09.010.

AUTHOR CONTRIBUTIONS

N.J. conceived and designed the study; C.Y. designed the experiments and wrote the manuscript. W.Y., Y.L., T.Z., M.J., Y.Q., M.Z., N.S., S.F., K.T., X.Y., and Y.S. performed the experiments and analyzed the data. All authors commented on the manuscript.

ACKNOWLEDGMENTS

This work was supported in part by the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA01010201), the National Key Basic Research and Development Program of China (2015CB964500 and 2014CB964804), and the National Natural Science Foundation of China (91219303, 31430058, and 31401261). We thank Dr. Weiyang Cheng for help in counting Aβ plaques, Dr. Lan Bao and Hui Liu for help in electron microscopy, and Dr. Francois Guillemot for critical reading of the manuscript.

Received: October 12, 2014
Revised: September 11, 2015
Accepted: September 11, 2015
Published: October 15, 2015

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