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Transplantation of Human Chorion-Derived Cholinergic Progenitor Cells: a Novel Treatment for Neurological Disorders

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Abstract

A neurological disorder is any disorder or abnormality in the nervous system. Among different neurological disorders, Alzheimer's disease (AD) is recognized as the sixth leading cause of death globally. Considerable research has been conducted to find pioneer treatments for this devastating disorder among which cell therapy has attracted remarkable attentions over the last decade. Up to now, targeted differentiation into specific desirable cell types has remained a major obstacle to clinical application of cell therapy. Also, potential risks including uncontrolled growth of stem cells could be disastrous. In our novel protocol, we used basal forebrain cholinergic progenitor cells (BFCN) derived from human chorion-derived mesenchymal stem cells (hC-MSCs) which made it possible to obtain high-quality population of cholinergic neurons and in vivo in much shorter time period than previous established methods. Remarkably, the transplanted progenitors fully differentiated to cholinergic neurons which in turn integrated in higher cortical networks of host brains, resulting in significant improvement in cognitive assessments. This method may have profound implications in cell therapies for any other neurodegenerative disorders.

The original version of this article was revised: Affiliation 1 should be read as "Neuroscience Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran".

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Introduction

Neurological disorders are diseases of the nervous system caused by the loss of neurons. There have been several attempts to treat neurological diseases and disorders. Alzheimer's disease (AD) is a neurodegenerative disorder with progressive memory impairment which has noticeable economic and social impacts worldwide [1, 2]. Accumulation of amyloid β plaques and neurofibrillary tangles and loss of neurons in the hippocampus and cortex are specific characteristics of AD [3, 4]. Moreover, degeneration of cholinergic neurons in basal forebrain and impairment of cholinergic functions occur in patients with loss of memory [5]. Current therapeutic approaches, including acetylcholinesterase and glutamate inhibitors, at best delay clinical symptoms and do not compensate for the massive neuronal and synaptic losses in the cortex. Hence, current treatments providing significant improvements in cognitive performances of patients with AD, exclusively in the presence of well-established plaque and tangle pathology, are of great importance [6]. Cell replacement therapy, as one of the main alternatives, has been in the spotlight in recent years. In spite of primary achievements by different types of cells such as mesenchymal stem cells (MSCs) or neural stem cells (NSCs) which have been studied for their potential to replace deficient cell types, still there are many obstacles in the way to clinical application of these methods [7, 8].

Human stem cells need to be introduced into developing tissue or neurogenic areas of the mature brain to differentiate into specific neuronal phenotypes. Otherwise, these cells would persevere undifferentiated or develop predominantly into undesirable glial cell types. For instance, initial reports of MSC's ability to produce mature neural phenotypes have been essentially discredited and transplanted MSC has been shown not to differentiate be viable nor be in AD [9]. By the same token, application of NSCs for repairing neural structural defects has been accompanied by several difficulties and evidence for their appropriate functional assimilation in higher cortical networks in adult CNS is quite limited. In fact, regarding their essential multilineage competency and phenotypic plasticity, they may not necessarily differentiate into the phenotypes of interest [10]. On the other hand, embryonic stem cell-based therapy is limited by the hazardous potential of undifferentiated ES cells yielding teratomas or germinomas after transplantation [11]. In fact, deficient neuronal phenotypes or their immediate progenitors would need to be delivered in both high purity and large amounts as an effective measure to tackle neurodegenerative disorders affecting particular cell types. An effective cell therapy should address disease targets with appropriate donor phenotypes in order to attain structural repair especially in vivo [12–14].

Stem progenitor cells, with self-renewal properties and multilineage differentiation capacity, have attracted widespread interests in regenerative medicine recently [15, 16]. Other than cholinergic, dopamine and GABAergic neurons produced using progenitor cells have shown promise in Parkinson's and Huntington's animal disease models, respectively [17, 18]. Also, the neural progenitor cells are effective to regenerate hippocampal and cortical neurons in lower species, proposing the endogenous progenitor cells as a probable compensation for the lost neural circuits in neurodegenerative disorder [9]. To date, to the best of our knowledge, there is no evidence for generation of cholinergic progenitors from hC-MSCs and further differentiation of these progenitors into functional cholinergic neurons in the disease environment. In this project, we have used cholinergic neurons derived from hC-MSCs to improve the structural and functional impairments in an AD model.

Materials and Methods

Animal Modeling

Animals were kept and treated according to the ethical committee of Iran University of Medical Sciences (IUMS) guidelines. Adult male Wistar rats were from IUMS animal lab. They were maintained in standard housing conditions on a 12-h light/dark cycle with unlimited access to food and tap water. They were kept at constant temperature of 22 °C. We used a total number of 40 male Wistar rats, weighing about 220–250 g, with normal behavior test. Their skull was fixed in a stereotaxic device. A longitudinal incision was made along the sagittal line on the scalp. Aβ1–42 was prepared freshly and administered with a Hamilton microsyringe. Two micrograms of Aβ1–42 solution in 4 μl PBS was administered over 2 min into the dorsal hippocampus bilaterally at coordinates 3.6 mm posterior and ± 2 mm lateral to the bregma and 3.2 mm ventral to the skull surface [19]. The needle was remained in position for an additional 2 min after injection. The needle was slowly removed from the brain and the scalp was sutured. The animals were returned to their cages to recover. The animals were randomly divided into four groups: (1) control group, with no treatment; (2) sham group, treated with only cell vehicle injection; (3) hC-MSCs group, treated with MSCs 3); and (4) BFCN group, treated with BFCN progenitor cells. The control group received artificial CSF containing 2.9 mM KCl, 147 mM NaCl, 1.7 mM $CaCl_2$, 2.2 mM dextrose, and 1.6 mM $MgCl_2$ on the same days.

Isolation, Culture, and Characterization of Human Chorion-Derived MSCs

The hC-MSCs isolation protocol was initially approved by IUMS ethical committee. We employed a full-term placenta obtaining following maternal consent. Human chorion tissue was washed twice in phosphate-buffered saline (PBS; Gibco, Germany) and then cut into small pieces according to our established protocol [20]. The small fragments were digested using 0.3% type II collagenase solution (Gibco, Germany) at 37 °C for 30 min. The enzyme was deactivated using PBS upon cell filtration with 100 μm cell strainer. The cell suspension was centrifuged at 1200 rpm for 5 min. Then, the cells (5×10^3 cell/cm^2) were plated in tissue culture flasks (TPP, Switzerland) containing the expansion medium composed of DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 1000 μg/ml streptomycin (all from Gibco, Germany) and cultured at 37 °C and 5% CO_2. We employed third to fourth passage for subsequent experiments. We confirmed mesenchymal pluripotency properties of the isolated cells as below.

We incubated 10^5 isolated cells with 10% goat serum at 4 °C for 1 h. The serum was then removed and cells were stained with fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated monoclonal antibodies against human CD44, CD45, CD34, CD73, and CD90 (all from BD Bioscience, USA) at 4 °C for 40 min. Isotype-matched antibodies were used as control. Fluorescent data were acquired using Becton-Dickinson flow cytometer and the results were analyzed using the Flowmax software. One of the most significant MSCs phenomena is their potency to differentiate into mesodermal lineages like osteocytes and adipocytes. Thus, we examine the potency as follows: Cells (5×10^4) were plated in a 35-mm culture dish containing expansion medium for 2 days. The medium was then replaced with the adipogenic one composed of DMEM supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 50 μM indomethacin (Sigma, USA), and 10–7 M dexamethasone (Sigma, USA).

We harvested the cells at day 17 followed by fixation in 4% paraformaldehyde and staining with oil red O solution for 30 min. The lipid droplets were observed under a phase contrast microscope (Olympus DP70 fluorescent microscope). We cultured cells (5×10^4) in a 35 culture dish in the presence of expansion medium to induce osteoblastic differentiation. The medium was replaced with osteogenic medium consisting of DMEM supplemented with 10% FBS, 50 μg/ml ascorbic 2-phosphate (Sigma, USA), 10 nM dexamethasone (Sigma, USA), and 10 mM β- glycerol phosphate (Sigma, USA) after 2 days. On day 17, the cells were fixed in 4% paraformaldehyde and stained with 2% Alizarin red solution for 20 min and observed with a phase contrast microscope (Olympus DP70 fluorescent microscope).

Human Chorion-Derived Mesenchymal Stem Cells Differentiation into BFCN Progenitor Cells

Human C-MSCs (24×10^5) were seeded in a 24-well plate containing the expansion medium and cultured for 2 days. Then, we replaced the medium with DMEM-F12, supplemented with 20% of fetal bovine serum, 10 ng/ml of basic fibroblast growth factor (FGF2, Sigma, USA), 2% B27 (Gibco, Germany), 250 mM of isobutylmethylxanthine, and 100 mM of β-mercaptoethanol for overnight. Afterward, the medium was changed with DMEM-F12, 0.2% B27 (Invitrogen, USA), 0.01 mM of all-trans retinoic acid (RA; Sigma, USA), and 500 ng/ml of Sonic hedgehog (Shh; R&D, USA). The medium was refreshed every 3 days. One week later, the cells were cultured in DMEM-F12, 0.2% B27 (Invitrogen, USA) for further 7 days. Respective protein expression levels were examined using real-time PCR on days 7 and 14. We extracted total cellular RNA using TRIzol reagent (Invitrogen, Germany). RNA purity was quantified employing a spectrophotometer (Applied Biosystems, USA) 280 upon DNA digestion. Complementary DNA (cDNA) was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentase, Canada). Real-time PCR reactions were carried out in 96-well optical reaction plates using a 7500 Real-Time PCR System (Applied Biosystems, USA). Target genes expression levels were normalized to GAPDH amounts (Delta Ct) and compared with human C-MSCs at passage 3 (delta Ct) (Supp. 1). Neuron-specific intracellular antigens were observed under an Olympus DP70 fluorescent microscope. The cells at day 14 were fixed, permeabilized, and incubated with primary antibodies against human nestin (Chemicon, USA), Islet-1 (Santacruz, USA), choline acetyltransferase (ChAT; Abcam, USA) and PAX6 (Abcam, USA) followed by FITC or phycoerythrin (PE)-conjugated secondary antibody (Sigma, USA) staining.

Functional Assessment of BFCN by ELISA

The quantitative ELISA test (Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit) was applied to measure the concentration of acetylcholine and the activity of acetylcholinesterase after treatment by BFCN progenitor cells both in vitro and in vivo. The brains were harvested right after rats were sacrificed by CO2 inhalation. The brain samples were preserved at 4 °C, then weighed and homogenized on ice in 150 mg/ml tissue protein extraction reagent (Thermo Fisher) supplemented with protease and phosphatase inhibitor cocktails (Sigma). Then, the mixture was centrifuged at 16,000*g* for 1 h at 4 °C and finally the supernatant was used for all ELISA analysis.

Cell Transplantation

We transplanted the cells upon 2 weeks of modeling. The cells were initially labeled with DiI (Invitrogen, USA). They were

detached and washed with PBS, then incubated for 3 min with a CM-DiI Dye (1:250). Next, cells were centrifuged and excess dye was removed. Resuspended cells were mounted on a stereotaxic frame (Stoelting Co, USA). The scalp went through a spindle-shaped median incision resulting in a dissected periosteum. We employed the bregma as reference point for stereotaxic coordination. Afterward, we transplanted the cells (4×10^5) through burr hole into CA_1: (AP: − 3/80, ML: 2, DV: 2/6). The holes were then sealed and washed with normal saline and sterilized using gentamicin (Iran).

Behavior Test

Passive Avoidance Apparatus

The device (PA) composed of a two-structure box having an illuminated room and a dark chamber (30 × 30 × 30 cm), equipped with a grid floor separated with a sliding door (30 × 15 × 25 cm). The dark chamber floor could be electrified.

The apparatus used for passive avoidance (PA) training consisted of a two-compartment box. The illuminated chamber (35 × 20 × 15 cm3) made from transparent plastic was connected by an 8 × 8-cm^2 guillotine door to the dark compartment with black opaque walls and ceiling. The floors of both chambers were made of stainless steel rods (3 mm diameter) spaced 1 cm apart. The floor of the dark chamber could be electrified. Shock was delivered to the animal's feet via a shock generator.

Training Procedure All experimental groups were first habituated to the apparatus ($n = 8$). The rat was placed in the illuminated compartment, and 5 s later, the guillotine door was raised. Upon entering the dark compartment, the rat was taken from the dark compartment into the home cage. The habituation trial was repeated after 30 min and followed after the same interval by the acquisition trial during which the guillotine door was closed and a 50-Hz, 1.2-mA constant current shock was applied for 1.5 s immediately after the animal entered to the dark compartment. After 20 s, the rat was removed from the dark compartment and placed into the home cage. The rats received a foot-shock each time it reentered the dark compartment. Training was terminated when the rat remained in the light compartment.

Retention Test Twenty-four hours after training, a retention test was performed to determine long-term memory. Each animal was placed in the light chamber for 10 s, then the door was opened, and the latency of entering to the dark compartment (step-through latency) and the time spent in the dark compartment were recorded as a measurement of retention performance. The ceiling score was 300 s. During these sessions, no electric shock was applied [21].

Training Procedure

All the animal groups were first habituated to the apparatus ($n = 6$). For this purpose, each rat was placed in the illuminated chamber for 10 s and the sliding door was then raised. Upon entering the dark compartment, the animal was taken from the dark box into the home cage. Each rat was placed in the illuminated compartment for 3 min. Then 50 Hz, 1.2 mA constant current shock was applied for 1.5 s through the floor grid of the dark box. After 20 s, the animals were moved back to cages.

Retention Trial

We performed a retention test to examine long-term memory 24 h after training. In this regard, we measured spent time in darkness while each animal was placed in the illuminated chamber for 10 s. The door was then opened and the latency of entering to the dark compartment and spent time in the dark were recorded as measurements of retention performance [21].

Morris Water Maze Test (MWM)

To test whether chorion derived-BFCN progenitor cells have positive effects on learning and memory, the MVM test was employed. The swimming tank was separated into four quarters with an escape platform hidden 1–2 cm under opaque water at room temperature. The hidden platform was changed in different places at the start of each time point. The automated video system was used to monitor swimming behavior of each rat. Rats were trained to find the hidden platform for the four following days. The time for finding the platform is 60 s each day, with 30 min of interval between the blocks. The latency to find the platform was recorded in order of training trials. After 4 days training, the hidden platform was omitted, and probe trial was served to record the prolonged and spent time as well as a number of platform crosses in each zone. Furthermore, a visible platform was employed for two following days to confirm that the water maze behaviors were not influenced by alterations in vision [22].

Histological Studies

Thirty days after transplantation of BFCN progenitor cells and 60 days after injection of hC-MSCs, animals were sacrificed for immunohistochemical staining of the hippocampal region. Animals' brains were perfused using 8% paraformaldehyde solution and sectioned. Then, the slides were treated with 2 N HCl and borate buffer to retrieve the antigens and were permeabilized with 0.3% Triton X for 30 min. Afterward, they were

incubated with 10% goat serum for 30 min. Primary antibodies including ChAT (Abcam, USA), Islet-1 (Santacruz, USA), nestin (Abcam, USA), Neun (Abcam, USA), VGLUT1.2 (Abcam, USA) GAD67 (Chemicon, USA), and TH (Abcam, USA) were diluted according to the manufacturer and applied to slides at 4 °C overnight and stained with the corresponding FITC (Abcam, UK) or PE (Abcam, UK)-conjugated secondary antibody at 37 °C for 90 min and observed under a Zeiss LSM 5 fluorescent microscope.

Statistical Analysis

All results were expressed as the mean ± SE and analyzed using GraphPad Prism software version 5 programs (Graph Pad Software, Inc., CA, USA). *P* values less than 0.05 were considered significant. One-way ANOVA was performed to analyze ELISA and visible platform water maze. Two-way ANOVAs with repeated measures were performed to analyze real-time PCR, protein marker expression in the both ICC and IHC, Aβ plaques, regular water maze, and passive avoidance data.

Results

Characterization of Human C-MSCs

As represented in Fig. 1, a viable population of adherent mesenchymal stem cells is obtained from the chorionic membrane. Morphological features of the cultured hC-MSCs using our method were similar to other types of MSCs. The cultured human C-MSCs exhibited a spindle-shaped morphology in the primary culture condition. Flow cytometry results revealed that the established cells were significantly positive for MSC cell markers including CD73, CD44, and CD90 with 98.56, 91.42, and 97.07%, respectively, while they were negative for hematopoietic markers, including CD45 (1.25%) and CD34 (0.99%) (Supp. 2). The differentiation potential of hC-MSCs were evaluated and showed that mineralized nodules formed in the hC-MSCs following the osteogenic induction. Also, adipogenic induction was determined following intracellular oil red O staining of the hC-MSCs after 14 days (Supp. 3).

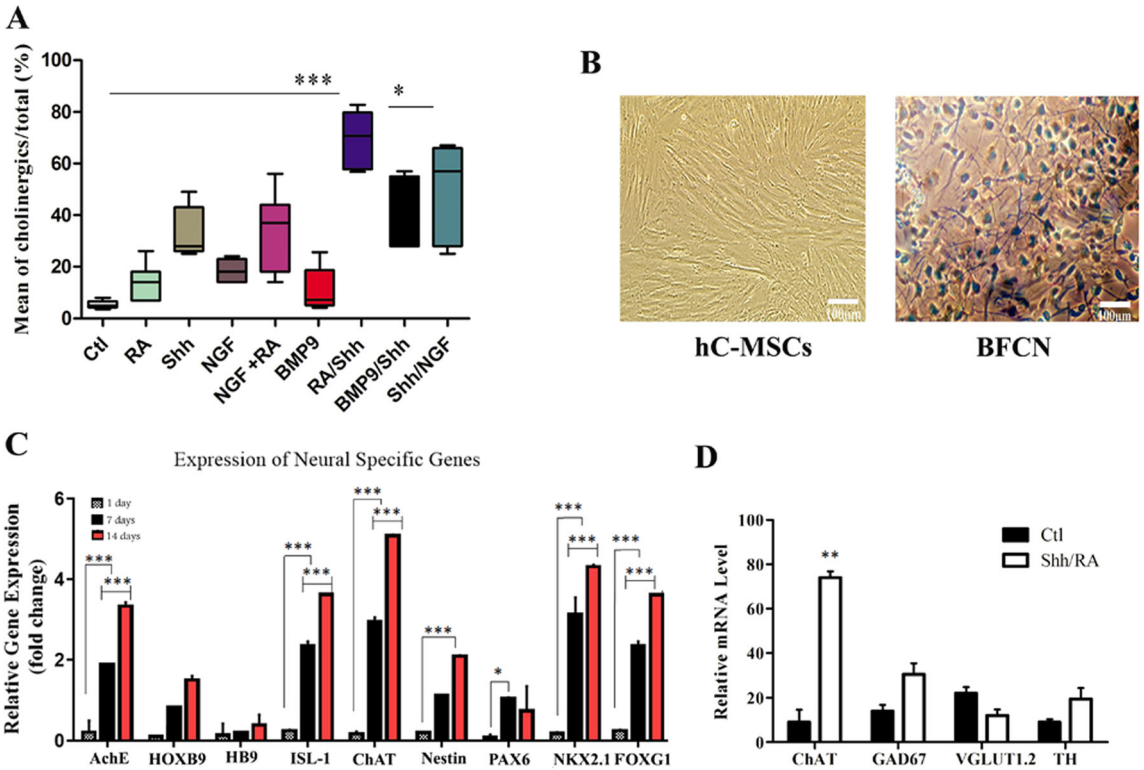

Fig. 1 Obtaining BFCN progenitor cells using tropic factors. **a** Mean percent of BFCN progenitor cells per total number of cells in treated and non-treated cell culture were measured by Dapi staining. The proportion of the BFCN progenitor cells significantly increased after treatment using combination of RA and Shh in comparison with non-treated cell cultures. **b** Light microscope images of the treated and non-treated cells showed that fibroblast-like cells successfully morphologically converted to the large multipolar neuron-like cells. **c** Gene expression profile was detected by quantitative RT-PCR analysis. In the analysis, cells were evaluated during 14 days after cholinergic induction. High amount of Ach E, FOXG1, ISL1, nestin, and NkX2.1 expression was detected after 14 days induction. **d** The mRNA levels expression of neuron subtype marker genes were determined by quantitative RT-PCR analysis at 14 days

hC-MSCs Convert to BFCN Progenitor Cells After a Differentiation Program

An alternative method for obtaining cholinergic neurons is using tropic factors. In order to choose suitable factor for induction, the hC-MSCs were subjected to different molecules including Shh, NGF, RA, BMP9, and their combination which are important for development of BFCNs during neurogenesis. The results, as shown in Fig. 1, indicate that the combination of trophic factors including RA and Shh had potentially significant effects on hC-MSCs. A clear benefit of BMP9 in the differentiation of hC-MSCs to cholinergic neurons could not be identified in this analysis.

Neural Phenotype

After exposure of hC-MSCs to isobutylmethylxanthine (IBMX) and beta-mercaptoethanol, the morphology of the cells changed and most of them became elongated at first and second week of induction. At day 14, the hC-MSCs showed typical characteristics of neural cells (i.e., large multipolar neuron-like cells, axon-like projects, prominent nucleoli (Fig. 1b).

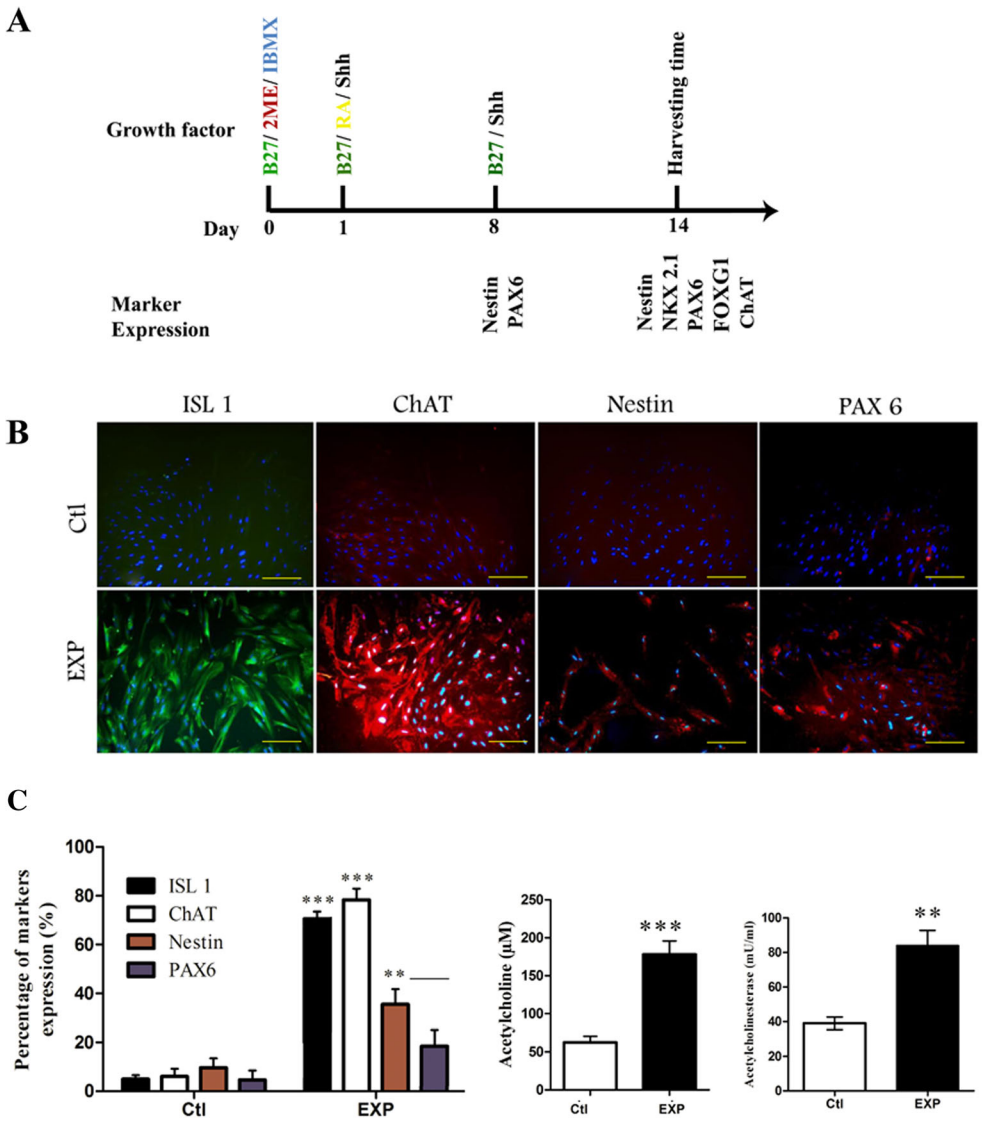

Fig. 2 Immunofluorescence analysis for the identification of the protein expression of ChAT, ISL-1, PAX6, and nestin. **a** Schematic showing specification of BFCN progenitor from hC-MSCs by RA and Shh for 14 days starting from day 1. **b**, **c** Nuclei were counterstained with DAPI (blue). The experimental group (BFCN) was found to be positive for ISL 1 and ChAT by immunofluorescence (scale bar = 200 μm) (**a**). The percentage of ISL 1 and ChAT with 71 and 80.66% expression was significantly higher than other markers in the treated cells ($P < 0.001$). The concentration of secreted Ach and activity of AchE were quantified by ELISA analyses for hC-MSCs derived BFCN on day 14

Cytofluorimetric Analysis

Cytofluorimetric analysis was performed to detect specific BFCN progenitor cell markers such as ChAT, nestin, PAX6, and Isl 1. Flow cytometry analysis exhibited that treated hC-MSCs expressed high levels of ChAT with 61.3% and also low level of nestin, PAX6, and Isl-1 with 14.9, 23.33, and 4.81%, respectively. Collectively, hC-MSCs were successfully differentiated into cholinergic lineage after 14 days treatment with tropic factors (Supp. 4).

Real-Time-PCR

The progressive neural commitment from the human C-MSCs to BFCN progenitor cell was characterized by measuring the mRNA levels of various marker genes. Real-Time-PCR analysis confirmed that the expression of Pax6 as a neural progenitor marker increased and reached their peak level at day 4. Also, a significant upregulation of the ventral anterior forebrain progenitor markers Foxg1 and Nkx2.1 was shown following day 7. In addition, the expression of the Neun, Nkx2.1, BFCN, ChAT, AchE, and Isl1 was upregulated around day 14. As shown in Fig. 1c, the total number and cholinergic fate commitment (NKx2.1 ventral telencephalic progenitors) of the ChAT+ cholinergic neurons in the RA and SHH-treated cells significantly increased compared with other groups. Additionally, results showed that the ChAT+ cholinergic neurons with 75.9% expression was significantly high in comparison with other subtypes of neurons including VGLUT1/2+ glutamatergic neurons, GAD67+ GABAergic neurons and TH+ dopaminergic neurons with 10.8, 29.1, and 19.11%, respectively (Fig. 1d). Furthermore, the high expression of Isl and Nkx2.1 as specific anterior epencephalic markers and low expression of posterior epencephalic markers such as Hb9 and HOXb9 gene indicated that most differentiated cells showed BFCN of basal forebrain identity. Therefore, this data suggested that the combination RA/Shh induction procedure following 2 weeks treatment directed most of hC-MSCs in vitro to become functional BFCN progenitor cells.

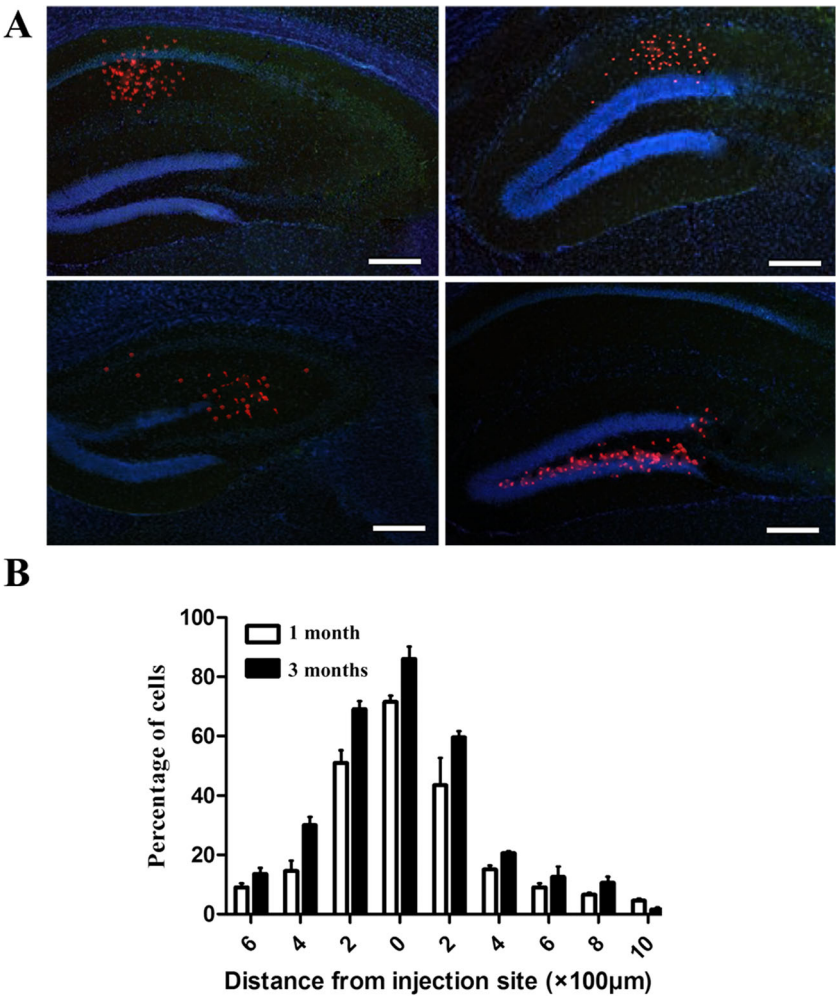

Fig. 3 **a**, **b** Immunohistochemical images of rat brain after 1 and 3 months cell transplantation. Red DiI-labeled cells were detected in injured sites in hippocampus. The total number of the NEUN, ChAT, ISL, and nestin-positive cells were counted in each field and data showed that BFCN efficiently engrafted in hippocampus compared with hC-MSCs posttransplantation. The majority of transplanted BFCN progenitor cells significantly express nestin on 1 month and NEUN and ChAT on 3 months nuclei were stained with DAPI. Scale bar = 100 μm

Immunocytochemistry

Immunocytochemistry analysis was used to determine whether differentiated hC-MSCs could express cholinergic neuron-specific proteins. Immunostaining for ISL-1, ChAT, nestin, and PAX6 were performed 2 weeks after the differentiation protocol. The results obtained from the analysis are represented in Fig. 2b. It has been found that most of the hC-MSCs differentiated into ISL-1+/ChAT+ BFCN in the treated group (Fig. 2a). Quantification analysis exhibited that the majority of cells remained ChAT+ and ISL-1+ with 82.3 ± 4.3 and 76.9 ± 2.55%, respectively, indicating their cholinergic phenotype. In addition, only 19.38 ± 6.7% of PAX 6 expression was detected in differentiated cells (Fig. 2c). No significant differences were found between cholinergic markers in the control group. These results showed that hC-MSCs could be effectively differentiated into BFCN by RA/Shh induction.

In Vivo Assessment

Effects of Transplanted Cells on Rat Model of Alzheimer's Disease

To assess the in vivo function of our produced BFCN progenitor cells, we used a previously described AD rat model, which is generated by Aβ. Histological evaluation was carried out to follow up the transplanted cells in vivo. According to our results obtained from IHC staining, the DiI-labeled hC-MSCs and BFCN could survive and distribute 3 months after transplantation in the hippocampus proper. Data showed that most of the BFCN progenitors tended to concentrate in the corona amonis 1 (CA1) and some of them were also distributed in other areas of the hippocampus (Fig. 3).

In the BFCN-treated group, 73.2 and 15.7% of the cells were nestin+, 34.13 and 84.02% were neun+, and 43.12 and

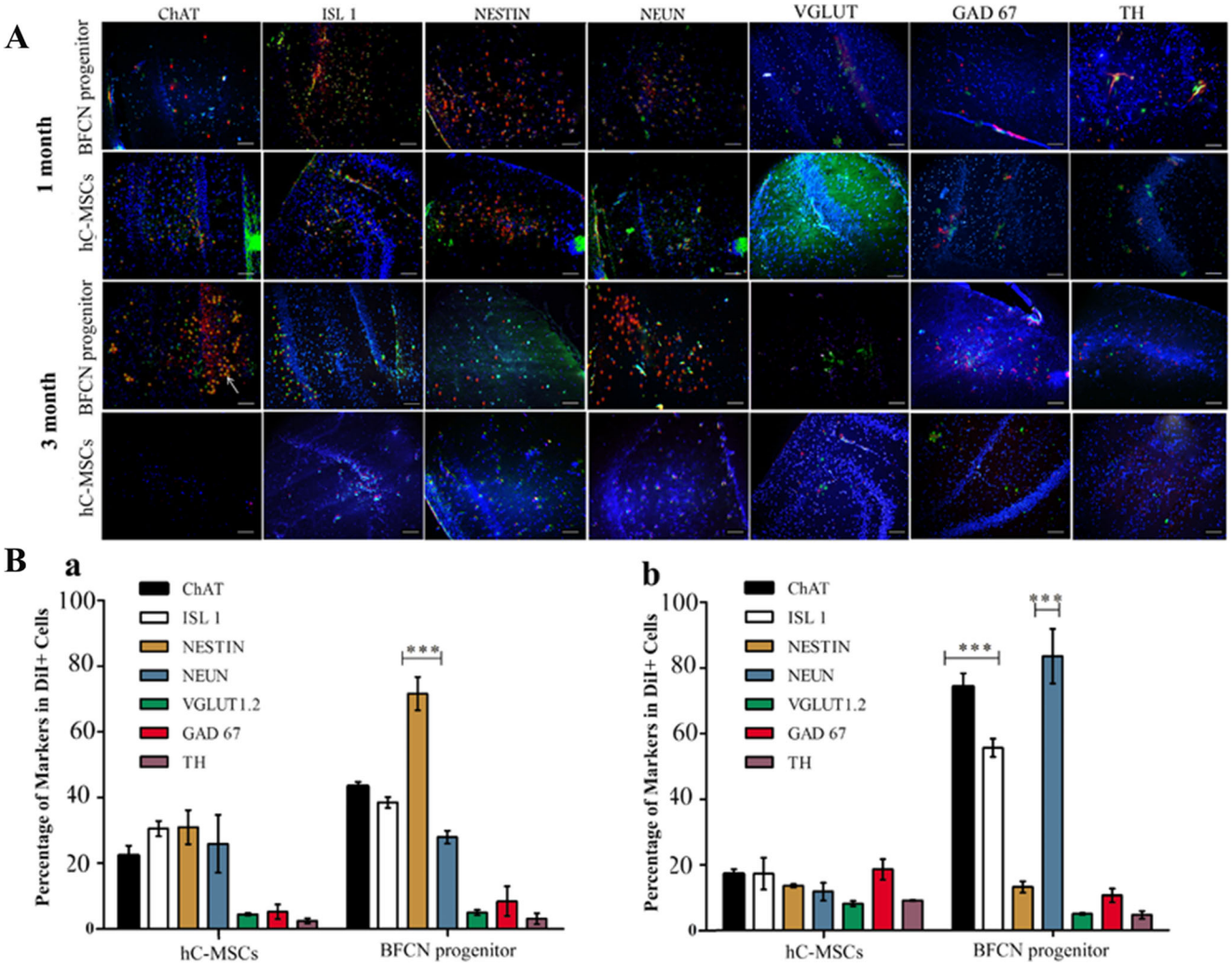

Fig. 4 Effects of BFCN progenitor cell transplantation on beta amyloid plaques formation. **a** Beta amyloid was detected in rat hippocampus (red). GFAP was identified in activated astrocytes (green). **b** There were no significant differences in total number of beta amyloid plaques between groups before and after treatment. **c** The concentration of secreted Ach and activity of AchE were quantified by ELISA analyses for hC-MSC-derived BFCN on 3 months after transplantation (***$P < 0.001$, *$P < 0.05$). Scale bar = 100 μm

71.33% were ChAT+ 1 and 3 months after transplantation, respectively (Fig. 3b). With regard to low expression of VGLUT, GAD67, and TH which are neuronal subtype markers, ChAT neurons increased to large numbers in the BFCN progenitor group and to a much lesser extent in the hC-MSCs group over time. By 3 months after transplantation, there were 71.33% ChAT+ cells on average in BFCN grafts but only 18.45% ChAT+ cells on average in hC-MSCs grafts (Fig. 4).

One of the most significant current issues in the development of Alzheimer's disease is amyloid beta plaque. The application of anti-β amyloid antibody revealed that there were no significant differences between groups in total number of amyloid β plaques both before and after transplantation (Fig. 5a, b). However, the findings of the current study are not consistent with [23] who found that amyloid β plaques formation was reduced after transplantation of MSCs derived from umbilical cord blood. Furthermore, the activity of ChAT+ cells in the BFCN group enhanced substantially from 1 month after graft, by 3 months after transplantation($p < 0.01$ and $p < 0.001$, respectively), as measured by Ach and AchE, compared with hC-MSCs. The BFCN progenitor cells exhibited typical properties of the functional cholinergic cells (Fig. 5c, d).

BFCN Progenitor Cell Transplantation Restored Cognitive Function in Alzheimer Model

The MVM analysis and passive avoidance was carried out before and after cell transplantation to assess whether BFCN progenitor cell transplantation enhanced spatial learning and memory in Alzheimer's disease rat model. Swimming pattern of rats after modeling and before cell grafting which is traced by a digital camera showed that there are no differences between groups. After training, the AD model rats took much longer (***$P < 0.001$) to find a hidden platform compared with the sham group 1 month after modeling, indicating that the lesion reduced learning memory (Fig. 6b). Also, the BFCN-treated rats began to show a diminish latency in finding the hidden platform compared with the other groups after 3 months transplantation (Fig. 6b). The trend was statistically significant by 1 and 3 months after treatment (*$P < 0.05$ and ***$P < 0.001$, respectively, Fig. 6c). Moreover, at 3 months, the BFCN group represented preferential crossings and spent significantly ($P < 0.001$) more time in a compartment with the hidden platform than in other compartment, whereas other groups did not show compartment preference (Fig. 6d–f). Also, in a visible platform water maze test, sham-treated rats found the platform faster, all treated rats showed a similar latency in reaching on the platform, indicating that the water maze behaviors were not affected by motor or vision exploration (Fig. 6g). The passive avoidance test showed that the BFCN-treated rats had a similarly increased latency as did hC-MSCs and sham groups to enter a dark quadrant, whereas control rats showed a significantly shorter latency ($P < 0.001$; Fig. 6h, i). Step-through latency and time spent in dark area between the BFCN group and control group indicated significant differences. These data suggest that the chorion-derived BFCN progenitor cells can improve behavioral functions (Fig. 6h, i).

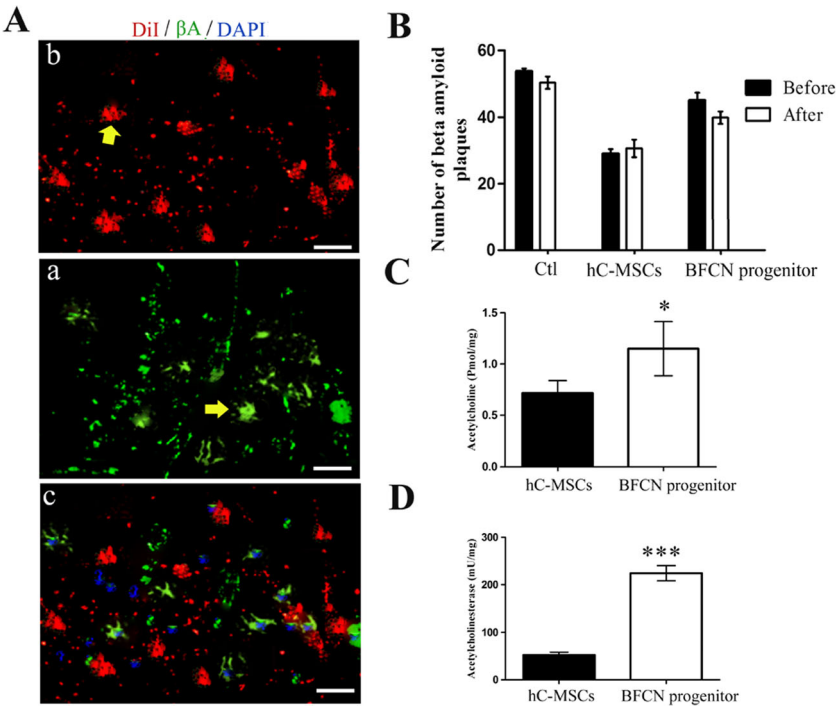

Fig. 5 **a**–**d** Transplanted of BFCN progenitor cells migrate into the adult hippocampus and express markers of cholinergic neurons. **a** Serial sections of 500 μm of the hippocampus (nuclei and grafted BFCN progenitor cells were stained with DAPI (blue) and DiI (red), respectively) and **a** distribution of transplanted BFCN progenitor cells expressing DiI after 1 and 3 months were determined by IHC which is showed that transplanted BFCN progenitor cells dispersed after grafting into recipient rats in spite of wide-ranging of hippocampal cell death resulting from Aβ injection. Scale bar = 200 μm

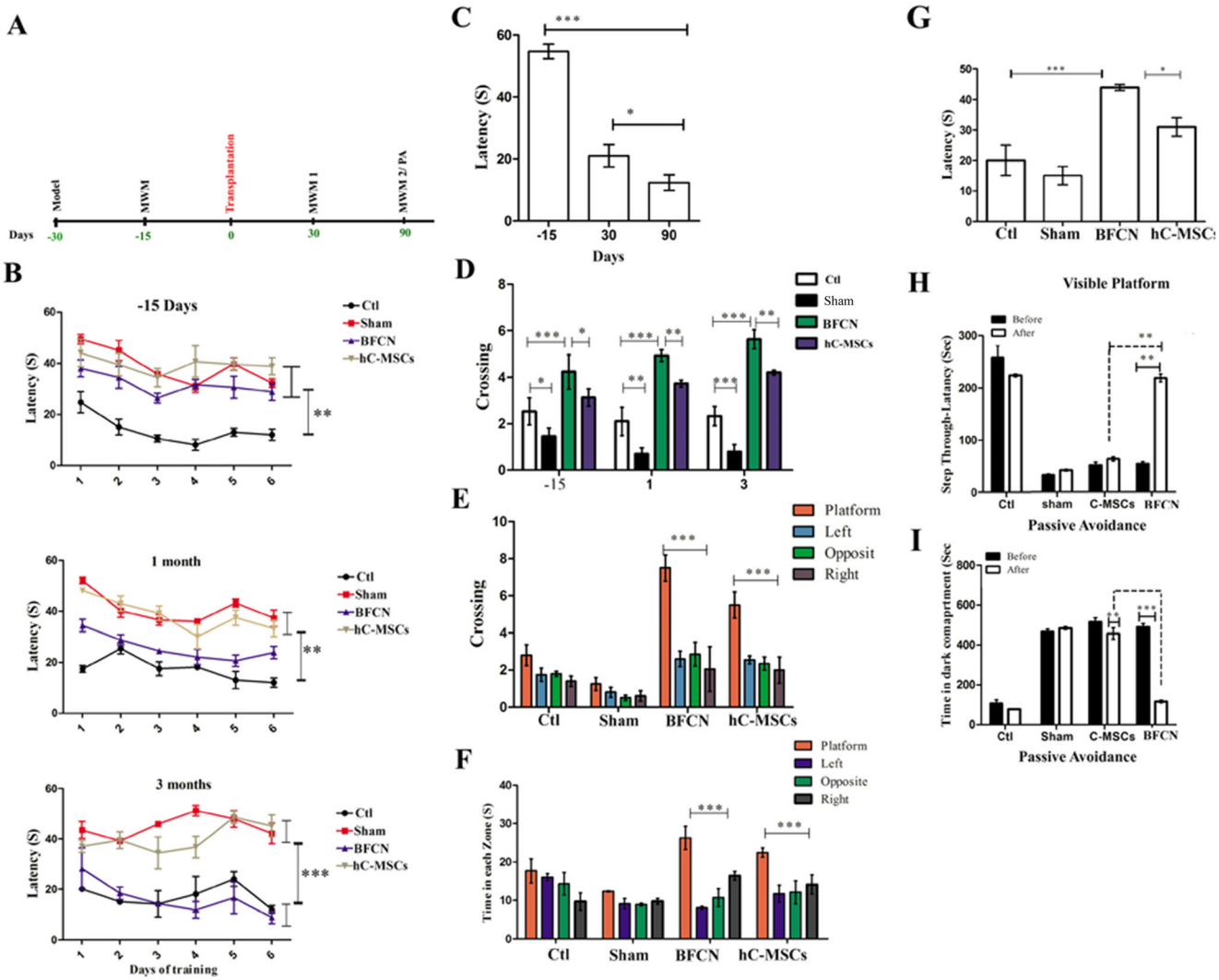

Fig. 6 Transplantation of the BFCN progenitor cells contributes to functional recovery. **a** Timeline of disease model creation and behavior analysis before and after treatment. **b** MVM analysis of latency showed that the BFCN-treated group, but not the control, model, and hC-MSC groups, progressively reduced the latency in finding the platform. **c** Summary of the latency in finding the hidden platform for the BFCN progenitor group over time. **d**–**f** Graphs of differential crossing and time spent in each zone. Rats in the BFCN group remained a longer time in target zone compared to the control, model, and hC-MSC groups at different time points after treatment. The BFCN progenitor-treated rats crossed the removed platform significantly more than the other groups at different time points after treatment. (***$P < 0.001$). **g** Latency in landing on the visible platform. **h**, **i** Latency in passive avoidance test. $n = 10$ per group

Discussion

This study set out with the aim of developing a method for efficiently differentiating hC-MSCS in vitro to ChAT + BFCN progenitor cells (75.9% of all cells). The findings of the current study are contradicted with those of Liu et al. who found that only 38% of cells were ChAT+ derived from human embryonic stem cells after 45 days transplantation [22]. A strong relationship between Shh and Wnt concentration has been reported in the literature. High concentrations of sonic hedgehog (Shh) induce ventralization [24], and low concentrations of Wnt induce caudalization after neural tube formation [25]. Furthermore, prior studies have noted retinoic acid (RA) and bone morphogenic protein play an essential role in telencephalon development [26]. Therefore, in this study high concentrations of Shh (500 ng/ml) and RA (0.01 μM) were used to produce BFCN progenitor cells from the chorionic mesenchymal stem cells. This is similar to hESCs in that ChAT+ cells were produced in the presence of Shh, RA, and NG, although only 65% of the sorted cells became ChAT+ [27]. The results showed that this protocol efficiently differentiated cells to ChAT+ progenitor cells morphologically in vitro. Remarkably, after grafting into AD rats model, the BFCN progenitor cells survived and integrated with host tissue that corrected learning and memory deficits.

Loss of the BFCN cells is a constant feature of Alzheimer's disease (AD) and has also been shown deficits in spatial learning and memory [28]. [29] found that hESC-derived

MGE progenitor transplantation ameliorated the cognitive symptoms in septo-hippocampal lesioned model. They suggested that transplanted hESCs successfully differentiated to cells such as BFCNs which may facilitate learning and cell memory [22]. Here, we showed that hippocampal transplantation of hC-MSCs-derived BFCN progenitors into Aβ induced rat corrected memory loss, indicating that in vitro-produced BFCN progenitors act similar to their in vivo counterparts. However, in our study, we found that there were no significant changes in the total number of Aβ plaques before and after CNL transplantation, as well as PBS treatment. Similar results were also found in a research reported by Zhang et al. [30]. The possible reasons may be the following: (1) we use different sources of stem cells or different animal models. (2) During the 12 weeks in our study, there was no change in the number of Aβ plaques, but the long-term effect remains to be observed. (3) Our transplanted CNLs migrated throughout the hippocampus and replaced the lost neurons around Aβ plaques. Also, long-term evaluation of beta amyloid plaque formation may show different results but it was not focus of our study [30].

The findings from prior studies suggest the grafted cells make several contributions: first, BFCN progenitors improve functional and structural deficits which may result in releasing trophic factors [31, 32]. Second, the BFCN progenitors create synaptic integration that is important for neural regeneration. The cognitive recovery we observed appeared mainly at 3 months after cell transplantation, when the BFCN grafted cells had become mature neurons, but fully differentiated neurons may also release trophic factors (Fig. 4). MSCs have been shown to further play roles in activating proinflammatory cytokines that are beneficial to the recovery of damaged neuronal environments or may differentiate into neural linages forming synapses and enhancing the recovery of function [33].

Several studies have documented neural transplantation of hESC derivatives is associated with increased risk of tumor formation [34–37]. One unanticipated finding was that our grafted cells scattered throughout the hippocampus and tumors were not identified (Fig. 3).

Our findings suggest that hC-MSCs derived BFCNs substituted and partially reconstructed injured cholinergic neuron circuitry of AD rat model, supporting the notion that progenitor cell-based therapies may rescue cognitive capacities of patients with AD. To test whether the human ESC-derived BFCNs acquired the ability to function as cholinergic neuron, 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 both the ACh secretion and AChE activity increased in the brain upon transplantation, indicating that the grafted cell-derived cholinergic neurons functioned similarly to their in vivo counterparts which is consistent with the previous findings [38]. 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 [33].

Taken together, we established an effective method for differentiating human chorion mesenchymal stem cells to BFCN progenitors. The BFCN progenitor transplantation into the rat brain improved learning and memory deficits. With appropriate quality and manufacturing control, this method provides clinical-grade BFCN in sufficient quantity for biopharmaceutical research and developmental studies, as well as therapeutic strategies addressing diseases and conditions characterized by loss of neurons such as Alzheimer's, Parkinson's, or Huntington's diseases. More broadly, research is also needed to determine clinical translation of this approach. It may be suitable for some criteria based on K-MMSE and DSM-V including, probably, AD and also amyloid +PET.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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