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# Therapeutic effects of human amniotic epithelial cell transplantation on double-transgenic mice co-expressing APPswe and PS1ΔE9-deleted genes

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Human amniotic epithelial cells (HAECs), which exhibit characteristics of embryonic and pluripotent stem cells, could be utilized for cell therapy without legal or ethical problems. Double-transgenic (TG) mice ($n$=20) and wild-type (WT) mice ($n$=20) were randomly assigned to two groups, respectively. The transplantation group was treated with HAECs and the control group with PBS. A six-radial arm water maze was used to assess spatial memory. Immunofluorescence was utilized to track HAEC survival. Immunohistochemistry was used to determine octamer-binding protein 4 (oct-4) and nanog expression in the HAECs. High-performance liquid chromatography (HPLC) was used to measure acetylcholine levels in the hippocampus. The density of cholinergic neurons in the basal forebrain and nerve fibers in the hippocampus was measured following acetylcholinesterase staining. Results showed that transplanted HAECs survived for at least eight weeks and migrated to the third ventricle without immune rejection. Graft HAECs also expressed the specific stem cell markers oct-4 and nanog. Compared with the control group, HAEC transplantation significantly ameliorated spatial memory deficits in TG mice, as well as increased acetylcholine levels and the number of hippocampal cholinergic neurites. Intracerebroventricular HAEC transplantation improved spatial memory in double-TG mice, and results suggested that increased acetylcholine levels in the hippocampus, released by surviving cholinergic neurites, were responsible for this improvement.

**Alzheimer's disease, human amniotic epithelial cells, spatial memory deficit, transgenic mice**

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by deposits of extracellular β-amyloid (Aβ) plaques, intracellular neurofibrillary tangles, and deficits in the cholinergic system [1]. The amyloid cascade and cholinergic hypotheses have been previously proposed for AD [2,3]. Transgenic mouse models of Alzheimer's disease have become indispensable tools for studying plaque formation, cholinergic deficits, and cognitive decline. Mutant mouse models have focused on two genes known to cause AD, namely amyloid precursor protein (APP) and presenilin 1 (PS1). Age-related amyloid deposition has been shown in the hippocampus and neocortex of APP/PS1 double-transgenic mice at 6–7 months, which is earlier than with single APP transgenic mice [4]. Previous results have shown that

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cholinergic nerve terminal degeneration precedes Aβ plaque deposition, as well as significantly less cholinergic nerve terminal varicosities in 4-month-old APP transgenic mice [5]. Cholinergic neurotransmission plays an important role in learning and memory, and the cholinergic hypothesis is supported by the fact that several cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) are currently marketed for AD treatment [6]. AchE inhibitors serve as a symptomatic therapy for AD, and a more restorative approach to AD therapy involves replacement of degenerating cholinergic neurons and nerve fibers through various possible cell sources [7]. While some success has been gained with fetal ventral mesencephalic (FVM) tissue transplants, the rapidly advancing stem cell field provides attractive alternative options to circumvent most ethical and practical problems inherent in trials using FVM tissue. Specifically, embryonic stem cells and induced pluripotent stem cells provide the most promise. However, ethical and political issues have limited the use of fetal stem cells, and cell resources and rejection issues have complicated the use of adult stem cells.

AD pathogenesis is complicated and the etiology of AD remains unknown. Treatment with traditional cholinesterase inhibitors has not resulted in great progress. However, it has been postulated that stem cell transplantation could provide novel methods for treating the disease [8]. In human beings, these therapies could create legal or ethical problems. Human amniotic epithelial cells (HAECs) are formed from epiblasts at eight days after fertilization, and these cells constitute the inner layer of the amnion, which suggests that they could maintain plasticity of pre-gastrulation embryo cells. HAECs lack major histocompatibility complex (MHC) antigen; therefore, there is a low risk for tissue rejection following transplantation [9]. HAECs secrete biologically active neurotrophins, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, insulin-like growth factors, epidermal growth factor, prostaglandin E2, laminin, nerve growth factor (NGF), and transforming growth factor-alpha [10,11], some of which exhibit trophic activities on dopaminergic neurons following transplantation[12]. In addition, HAECs do not express telomerase and are non-tumorigenic. Based on these biological characteristics, HAECs are suitable for transplantation. Most importantly, HAECs are derived from term placenta after live birth, which is a useful and noncontroversial source of stem cells for cell transplantation and regenerative medicine [13].

Nevertheless, the neuroprotective effects of intracerebroventricular HAEC transplantation on AD remain poorly understood. The present study transplanted EGFP-transfected HAECs into the lateral ventricle of double transgenic mice that co-expressed mutant APPswe and PS1ΔE9 genes to determine if transplantation improved cognitive functions.

# 1 Materials and methods

## 1.1 Experimental animals and groups

APPswe/PS1ΔE9 founder mice were obtained from Nanjing Institute of Experimental Model Animal and were bred in a heterozygote colony by pairing transgenic mice with normal wild-type (WT) littermates (C57BL6/J). The transgenic mice expressed a mutant form of human FAD variant of PS1ΔE9 (deletion of exon 9) and a chimeric mouse/human amyloid precursor protein APPswe (mouse APP695 harboring a human Aβ domain and mutations K595N and M596L linked to Swedish FAD kindred). The mice were allowed free access to food and water and were housed in a 12 h dark-light cycle. All experiments were performed during the light phase of the circadian cycle. Mice were genotyped using previously described methods [14], which were confirmed by slot blots shortly after weaning and at necropsy. Animal care and experimental procedures were conducted according to Chinese national guidelines for animal care.

The PS1ΔE9 transgenic mice and mutant APPswe transgenic mice were crossbred to generate hemizygous offspring with four possible genotypes: nontransgenic (wild-type); PS1ΔE9 transgenic; APPswe transgenic and double-transgenic APPswe+PS1ΔE9. Double-transgenic and WT mice were randomly assigned to two groups. The transplantation group was treated with EGFP-transfected HAECs, and the control group with an equal amount of PBS.

## 1.2 Tissue staining

Eight-month-old, female mice were anesthetized with pentobarbital (100 mg $kg^{-1}$ intraperitoneal) and were transcardially perfused with 25 mL normal (0.9%) saline. The brains were rapidly removed and fixed in freshly depolymerized 4% paraformaldehyde for 24 h. The right hemispheres were rapidly dissected into hippocampus+cortex regions on a cold stage, followed by freezing on dry ice and storage at −70°C. Fixed, cryoprotected hemispheres (left) were frozen and sectioned to 25-μm thick sections in a horizontal plane using a sliding microtome. The sections were then stored at 4°C in phosphate-buffered saline for immunocytochemistry and histology.

Sections were incubated with the primary antibody for 18 h at 4°C, and antisera specific for Aβ peptides ending at residue 42 (Aβ42) were obtained from Quality Controlled Biochemical (Hopkinton, USA), then incubated in biotinylated secondary antibody (120 min), followed by avidin-biotin-peroxidase complex using the Vectastain Elite kit.

Congo red and acid-silver methenamine histology was performed on sections mounted on slides. The sections were air-dried for a minimum of 12 h, then rehydrated for ap-

proximately 30 s prior to staining. For the Congo red staining, the hydrated sections were incubated in an alkaline alcoholic saturated sodium chloride solution for 20 min, then incubated in 0.2% Congo red in alkaline alcoholic saturated sodium chloride solution for 30 min. For the acid-silver methenamine staining, sections were stained for 15 min in a 1% solution and quickly rinsed with water. For both stainings, sections were rinsed in three rapid changes of 100% ethanol, cleared through three changes of xylene, and then coverslipped with DPX (Sigma, USA).

One week after behavioral analysis, samples were subjected to acetylcholinesterase staining. The sections were incubated (5 mg acetylcholine iodide, pH 6.0; 6.5 mL acetate buffer, 0.5 mL of 0.1 mol $L^{-1}$ sodium citrate; 0.5 mL of 0.03 mol $L^{-1}$ copper sulfate, 0.2 mL 0.005 mol $L^{-1}$ potassium fenicyanide, 1 mL deionized water) for 2 h, then rinsed through five changes of acetate buffer, cleared through 1% ammonia solution for 1 min, immersed in sulfide, then washed five times with 1% sodium nitrate.

### 1.3 Behavioral analysis

A total of 20 double-transgenic and 20 WT female mice were chosen for behavioral testing. A water maze with six radial arms was made from a black-painted steel pool with a one-meter diameter, which was filled with 20°C water. The pool contained a maze that consisted of six arms that radiated from the central arena. One of the arms contained a submerged escape platform. The testing room contained spatial clues consisting of posters and objects of different shapes that hung on the walls and ceiling. Prior to testing, the mice were pre-trained for 5 d. On the first day, the mice were placed in the water maze, and only two of the arms were open. The mice swam from one arm through the central arena to the escape platform on the other side of the pool. If the swimming time exceeded 120 s, the mice were gently guided to the escape platform. The mice were allowed to remain on the escape platform for 30 s before being returned to their cages. The mice performed five trials at 10-min intervals, and the trial complexity increased during the following four days of pre-training by opening a new arm each day. Memory testing was performed in the afternoon over 10 consecutive days. The mice were subjected to five trials, with 10-min intervals, each day and all arms were open. The mice were placed in a randomly chosen arm at each trial. The escape platform location was changed each day and remained at that location for the duration of the testing day. The platform location order was random, but all six arms were used as a target over a six-day testing period. An error was scored if a mouse entered more than halfway into an arm with no escape platform. The number of errors, search sequence, and time needed to locate the escape platform were noted for each trial. The mice were tested before and after transplantation.

### 1.4 HAECs cultures

HAEC cultures were prepared as previously described [15]. Briefly, the human amniotic membrane was mechanically peeled from the placenta chorion, which was obtained from an uncomplicated, elective, caesarean section with informed consent from each donor patient. The HAEC layer was thoroughly scraped out from underlying tissues, such as spongy and fibroblast layers. The HAEC layer was then treated three times with 0.125% trypsin for 20 min each to obtain dissociated HAECs. The cells were cultured in RPMI-1640 medium containing 10% fetal calf serum at 37°C in a 95% air/5% $CO_2$ humidified atmosphere. The culture medium was replaced every three days. The HAECs were transfected with EGFP as previously described [16].

### 1.5 Transplantation

Transgenic and WT mice were injected into the lateral ventricle with HAECs using a 10 μL Hamilton microsyringe (Hamilton) fitted with a steel cannula according to the following coordinates: AP –0.8 mm, ML –1.3 mm, and DV –4.8 mm. A total of 8 μL HAECs ($3\times10^4$ in 2 μL) were injected into each mouse. Following completion of injections, the cannula was left *in situ* for 5 min before being slowly retracted to minimize cell diffusion up the needle track. Mice from the control and model group received 2 μL basal medium. All experimental animals were immunosuppressed with FK-506 (Huijia Pharmaceutical, Beijing, China, 5 mg $kg^{-1}$, intraperitoneal, daily), starting at the day of transplantation surgery.

### 1.6 Immunofluorescence

At eight weeks after transplantation, the mice were sacrificed by $CO_2$. The brain sections were incubated overnight at 4°C with primary antibodies against octamer-binding protein 4 (oct-4) (1∶2000; Chemicon International, Temecula, CA, USA) and nanog (1∶2000; Sigma). The sections were then incubated for 1 h at room temperature with rodamine-conjugated secondary antibodies. After washing, cover slips were mounted onto the slides and digital images were recorded using an Olympus microscope.

### 1.7 High performance liquid chromatography assay for acetylcholine in hippocampus and cortex

To measure acetylcholine levels, a Metachem column (250 mm×4.6 mm, 18.5 μm) was used in combination with an electrochemical detector (500 mV (Ag/AgCl)). The mobile phase consisted of 50 mg $L^{-1}$ disodium EDTA, 4.1 g $L^{-1}$ sodium acetate, 140 mg $L^{-1}$ sodium octane-sulfonic acid, 140 mg $L^{-1}$ sodium octane-sulfonic acid, and 1.8% v/v methanol. The flow rate was 1.3 mL $min^{-1}$, and the detec-

tion limit was 0.5 fmol per sample.

### 1.8 Statistical analysis

Two-way ANOVA with the post-hoc Scheffe's test was utilized to compare escape latency and acetylcholine levels between groups. The significance level was set to $P<0.05$. SPSS 16.0 was used to perform statistical analysis.

## 2 Results

### 2.1 β-amyloid deposition in double-transgenic mice

A diffuse orange-red amyloid deposition was visible following the Congo red staining in the hippocampus and frontal cortex of the 8-month-old double-transgenic mice, but not in the WT mice, as showed in Figure 1. Neurofibrillary tangles, which were stained by acid-silver methenamine, were not observed in either the transgenic or the WT mice, as showed in Figure 2. These results demonstrated that 8-month-old double-transgenic mice co-expressing APPswe and PS1ΔE9 genes exhibited obvious amyloid plaques in the hippocampus and frontal cortex.

### 2.2 HAEC characteristics and identification of EGFP-labeled cells

Under a light microscope, HAECs appeared round, oval, or triangular in shape, with a large nucleus and fat drops in the cytoplasm, as previously described [15]. Under a fluorescence microscope, EGFP-labeled HAECs exhibited blue fluorescence in the lesion, as shown previously [16].

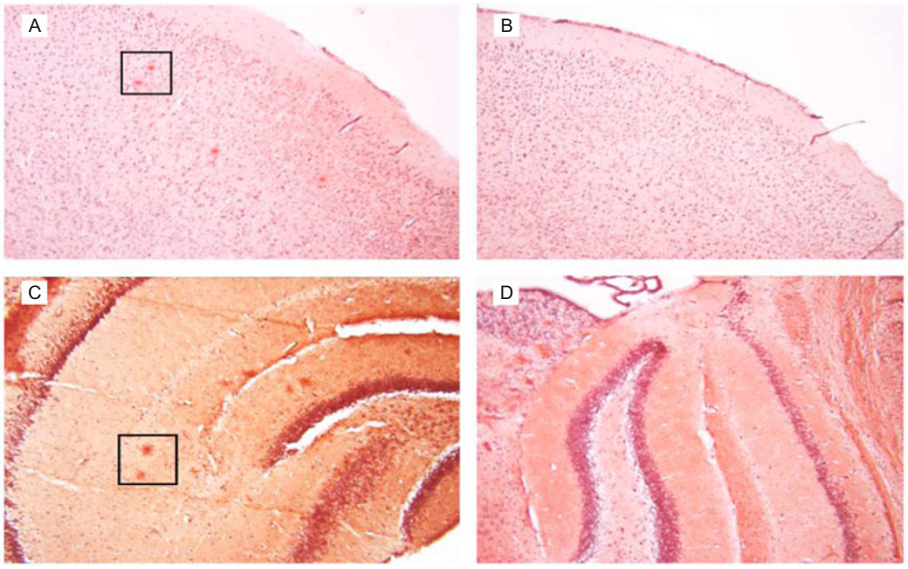


**Figure 1** Distribution of β-amyloid deposition stained by Congo red in double TG and WT mice after HAECs transplantation. Sections from frontal cortex and hippocampus in double TG mice (A and C) and WT mice (B and D) are compared. The orange-red amyloid deposition was found in double TG mice aged 8 months, and not found in WT mice aged 8 months.

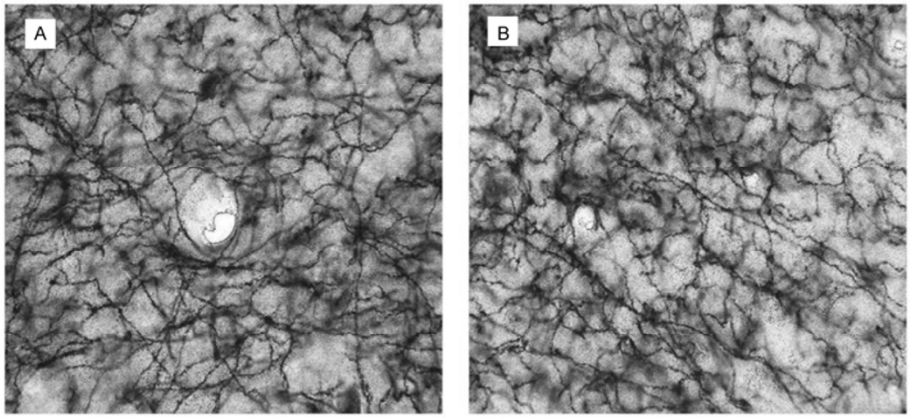


**Figure 2** Acid-silver methenamine staining in double TG (A) and WT (B) mice. No neurofibrillary tangles were found in them aged 8 months.

### 2.3 HAEC survival and expression following transplantation

At eight weeks after transplantation, surviving HAECs were observed in the lateral ventricle and third ventricle, and the cells expressed oct-4 and nanog. The surviving HAECs were not overgrown, which indicated that HAEC transplantation was safe for the AD mice. Oct-4 and nanog-positive cells were not detected in the control mice, as shown in Figure 3A and B.

### 2.4 Radial water maze testing

Body weight differences were not significant between the double-transgenic mice (36.2±1.3, $n$=20) and the WT mice (37.6±1.2, $n$=20, $P$>0.05). The numbers of errors per trial decreased according to trial number in both experimental groups. At 8 months of age, learning curves of transgenic mice were different than the WT mice. A Wilcoxon-Mann-Whitney test revealed differences between individual trials in 8-month-old WT and transgenic mice; trials 4 and 5 were significantly different (trial 1, $P$>0.92; trial 2, $P$>0.09; trial 3, $P$>0.06; trial 4, $P$<0.03; trial 5, $P$<0.02).

At 8 weeks after transplantation, learning curves of WT and double-transgenic mice with HAEC transplantation varied from control mice. A Wilcoxon-Mann-Whitney test between individual trials revealed significant differences in the 8-month-old transgenic and WT groups in trials 3–5 (trials 1–2, $P$>0.05; trials 3–5, $P$<0.05, respectively), as shown in Figure 4. At this age, the WT mice systematically committed fewer errors than the transgenic mice, whereas behavior of transgenic mice was not altered. Both WT and transgenic mice learned the escape platform position in the water maze test on the day of testing. Double-transgenic mice and WT mice with HAECs transplantation were more efficient than controls in locating the escape platform.

### 2.5 Acetylcholine levels in the hippocampus and frontal cortex following HAEC transplantation

Within the control group, acetylcholine levels were significantly less in the transgenic mice than in the WT mice. Compared to double-transgenic mice injected with PBS, hippocampal acetylcholine concentrations in the WT mice

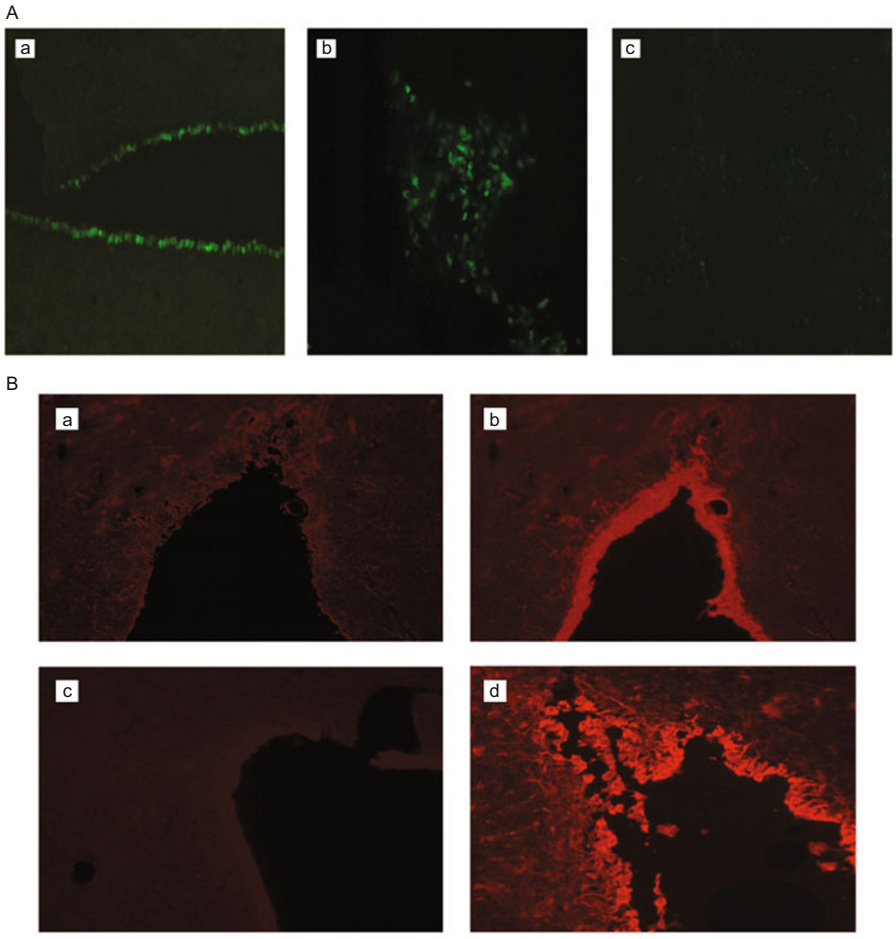


**Figure 3** A, The survival of transplanted grafts. Eight weeks after transplantation, HAECs were alive, not overgrown and found in the lateral ventricle (a) and the third ventricle (b). The control group (c) has not found the survived cells. B, The expression of transplanted grafts. Eight weeks after transplantation, HAECs expressed oct-4 (b) and nanog (d) in transplantation mice. No oct-4 or nanog-positive cells were found in controls (a and c).

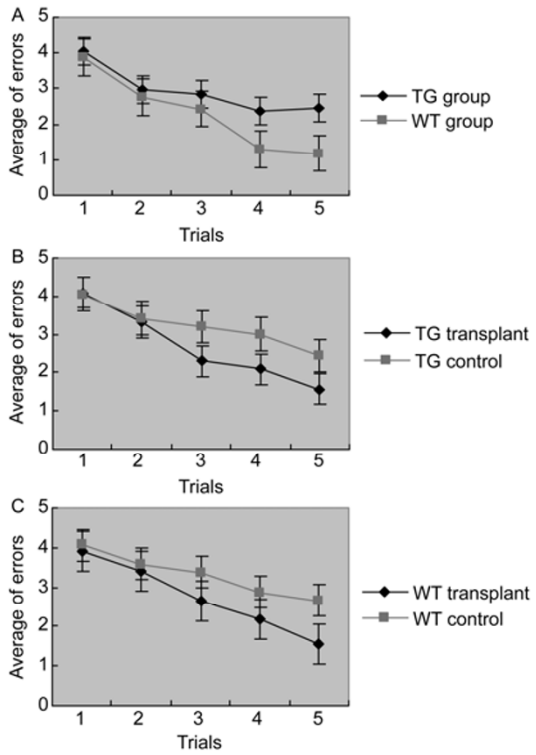


**Figure 4** Spatial memory testing in the water maze. A, Number of total errors obtained in 8 months old mice in double TG and WT mice before transplantation on the five trials. Each point represents the average number of errors per trial over 10 days of testing. B, Same as A at 8 months in TG mice in transplantation and controls. C, Same as A at 8 months in WT mice in transplantation and controls. Error bars represent in all cases the SEM.

**Table 1** Hippocampal acetylcholine concentrations of the different groups (each group $n$=10)[a)]

| Group | Peak area | Concentration (μg mL$^{-1}$) |
|---|---|---|
| TG transplant | 12.6 | 0.112▲ |
| TG control | 9.18 | 0.090★ |
| WT transplant | 19.1 | 0.169△ |
| WT control | 12.7 | 0.113* |

a) Compared with TG control, *, $P$<0.01; △, $P$<0.01; ▲, $P$=0.01; compared with WT control, △, $P$>0.05; ▲, $P$>0.05; ★, $P$<0.01.

and double-transgenic mice injected with HAECs were significantly increased, as shown in Table 1.

### 2.6 Cholinergic neurons in the basal forebrain and nerve fibers in the hippocampus, as detected by AChAT

Compared with control transgenic mice, the number of cholinergic neurons significantly increased in the basal forebrain of TG mice injected with HAECs ((37.7±2.7) μm$^{-2}$ vs. (24.4±2.8) μm$^{-2}$, $P$<0.01), but not in the WT mice. The size of cholinergic neurons in the WT mice injected with HAECs significantly increased compared with control WT mice ((53.1±4.2) μm$^{-2}$ vs. (44.2±3.8) μm$^{-2}$, $P$<0.05), as shown in Figure 5.

Hippocampal cholinergic nerve fibers in the double-transgenic mice were irregularly arranged. The number of hippocampal cholinergic nerve fibers significantly increased in the transgenic mice injected with HAECs compared with control transgenic mice ((653.1±162.2) μm$^{-2}$ vs. (554.2±

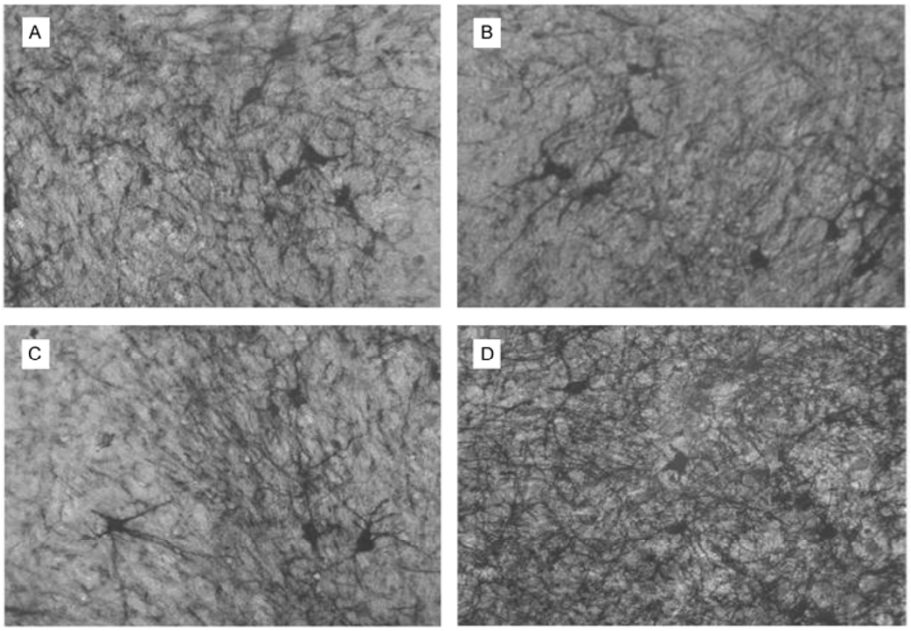


**Figure 5** The numbers of cholinergic neurons in basal forebrain stained by AChAT among the four groups were compared. A, WT transplantation. B, TG transplantation. C, WT control. D, TG control.

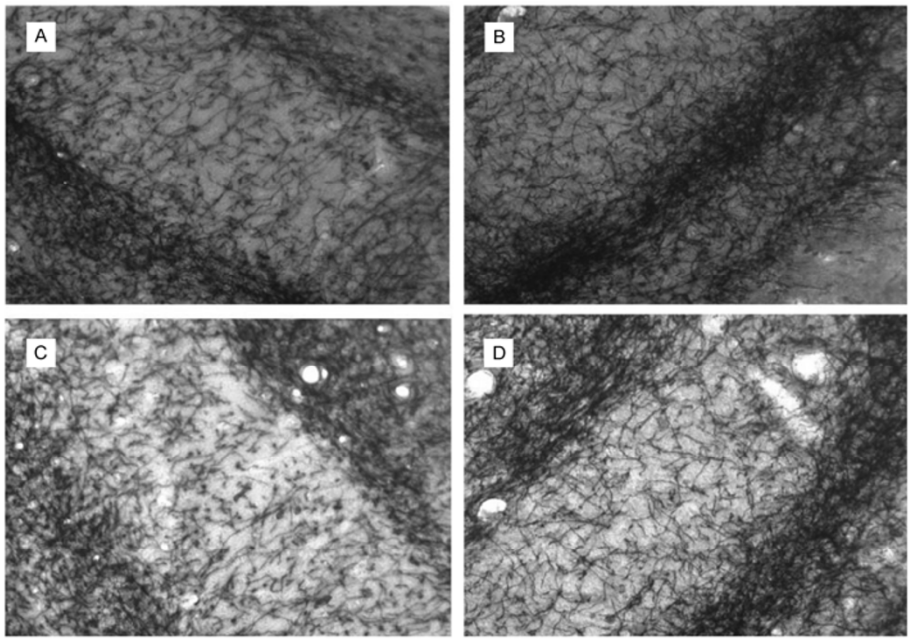


**Figure 6** Hippocampal cholinergic nerve fibers stained by AChAT among the four groups were compared. A, WT transplantation. B, TG transplantation. C, WT control. D, TG control.

133.8) $\mu m^{-2}$, $P<0.01$). The size of hippocampal cholinergic nerve fibers in the WT mice injected with HAECs was significantly greater than in control WT mice ((762.1± 194.2) $\mu m^{-2}$ vs. (689.2±173.8) $\mu m^{-2}$, $P<0.05$), as shown in Figure 6.

## 3 Discussion

Several attempts have been made to transplant stem cells into the brain to treat a variety of neurodegenerative disorders. Recent studies using implanted cells, including adult stem cells and fetal transplants, provide promising results in experimental models of neurodegenerative disease, such as Alzheimer's disease [17,18]. HAECs express surface markers typically present on embryonic stem and germ cells. In addition, under certain culture conditions, HAECs form spheroid structures that retain stem cell characteristics [19], evidenced by the fact that HAECs express the pluripotent stem cell-specific transcription factors oct-4, nanog, SSEA-4, TRA 1-60, and TRA 1-81 [20]. These results suggest that HAECs exhibit putative multipotential differentiation towards neurons, astrocytes, and oligodendrocytes [21]. Furthermore, HAECs synthesize and release BDNF, glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3, NGF, and other factors [21,22]. BDNF has been shown to exert trophic effects on cholinergic neurons [23], and GDNF and NGF provide protection to degenerating cholinergic neurons in AD, as well as promote regeneration of the hippocampal cholinergic system [24]. Therefore, HAEC transplantation could serve as a useful and noncontroversial source of stem cells [25]. Our previous study showed that some EGFP-labeled HAECs survive and migrate out of the injection site following transplantation [16]. In addition, HAEC transplantation has been shown to ameliorate impaired functions in a dopamine-degenerated mouse model [26].

In the present study, intracerebroventricular transplantation of HAECs in transgenic and WT mice resulted in improvement of spatial memory deficits compared to control mice. Transplanted HAECs survived for at least eight weeks in the transgenic and WT mice, and the numbers of errors were less in transplantation mice than control mice. HAECs also prevented decreased hippocampal acetylcholine levels in the transplantation mice. AChAT staining revealed an increase in the number of cholinergic neurons in the basal forebrain, as well as nerve fibers in the hippocampus, in the transplantation mice compared with the PBS group. The double-transgenic mice exhibited spatial memory deficits, which suggested an effective inhibitory effect of Aβ on acetylcholine release. It was assumed that improved spatial memory in the transplantation group correlated with enhanced levels of nutritional factors, such as NGF, BDNF, and GDNF [21–24]. Neurotrophins protect and nourish degenerating neurons, as well as induce HAECs to differentiate into cholinergic neurons. Cognitive symptoms of AD do not correlate with amyloid load. However, the symptoms do significantly correlate with a decreased number of hippocampal cholinergic neurons and nerve fibers. It has, therefore, been suggested that the increased acetylcholine secre-

tion by cholinergic neurons could improve cognitive declines of the disease [1,8]. Results have shown that the degree of dementia positively correlates to the number of degenerated cholinergic neurons [27]. The degree of learning and memory handicaps positively correlates with the number of cholinergic neurons and ChAT activity [28]. Previous results have also shown that cholinergic nerve terminal degeneration precedes Aβ plaque deposition [5]. Therefore, increased acetylcholine levels in the hippocampus, as a result of a greater number of surviving cholinergic neurites, is likely involved in this improvement. These results supported the hypothesis that HAECs could serve as useful cell resource for cell therapy in degenerative disease.

Results from the present study revealed β-amyloid deposition in the hippocampus and frontal cortex in the double-transgenic mice co-expressing APPswe and PS1ΔE9 genes at 8 months of age. These results were in accordance with previous results [29]. In addition to detection of amyloid plaques, learning and memory was analyzed in double-transgenic and WT mice using the six-arm radial water maze. The water maze test is dependent on hippocampal functions and is generally accepted as a sensitive test for short-term episodic memory. Visiting the platform location from the previous day reflects long-term reference memory [4]. Results demonstrated that performance of the 8-month-old, double-transgenic mice was affected compared to WT controls; the double-transgenic mice revisited the arm with the escape platform from the previous testing day more often. However, the double-transgenic mice made more errors than the WT controls. These results were similar to results from earlier studies using other types of behavioral tests that indicated deficits in spatial learning and memory in the APPswe/PS1ΔE9 mice [30]. The present results suggested that APPswe/PS1ΔE9 mice are suitable for testing possible replacement therapeutics.

Immunological rejection is a common problem associated with substitutive therapy with stem cells. In the present study, transplanted HAECs survived for at least eight weeks, which could correlate with a weak brain immune response to HAECs. Studies have shown that HAECs express major histocompatibility complex (MHC) I, but lack MHC II [19], resulting in a relatively weak immune response.

In summary, following transplantation into the lateral ventricle, HAECs survived for 8 weeks without inducing an immune rejection. HAEC transplantation increased hippocampal acetylcholine levels, improved spatial memory functions, and ameliorated damage to the surviving cholinergic neurons. These results provide a theoretical basis for clinical application of HAECs. The therapeutic future of HAECs as a reliable and efficient therapeutic tool for AD, as well other degenerative diseases, depends on methodological development of isolation, proliferation, storage, and easy access of a highly homogeneous cell population. However, this approach could provide promise and a novel method for AD treatment.


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