

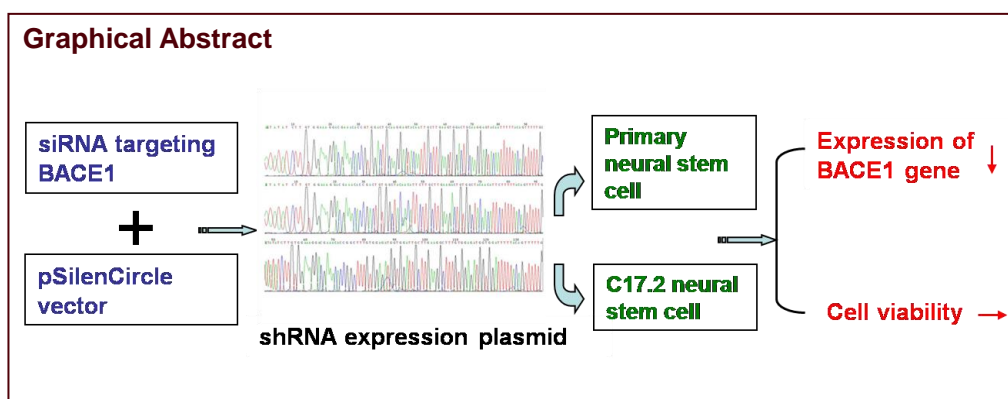
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Targeting β -secretase with RNAi in neural stem cells for Alzheimer's disease therapy

Zhonghua Liu, Shengliang Li, Zibin Liang, Yan Zhao, Yulin Zhang, Yaqi Yang, Minjuan Wang, Feng Li

Department of Neurobiology and Anatomy, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China



Zhonghua Liu, M.D.

Corresponding author: Feng Li, M.D., Ph.D., Professor, Department of Neurobiology and Anatomy, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China, lifeng@mail.sysu.edu.cn.

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Conflicts of interest: None declared.

Abstract

There are several major pathological changes in Alzheimer's disease, including apoptosis of cholinergic neurons, overactivity or overexpression of β -site amyloid precursor protein cleaving enzyme 1 (BACE1) and inflammation. In this study, we synthesized a 19-nt oligonucleotide targeting BACE1, the key enzyme in amyloid beta protein ($A\beta$) production, and introduced it into the pSilenCircle vector to construct a short hairpin (shRNA) expression plasmid against the BACE1 gene. We transfected this vector into C17.2 neural stem cells and primary neural stem cells, resulting in downregulation of the BACE1 gene, which in turn induced a considerable reduction in reducing $A\beta$ protein production. We anticipate that this technique combining cell transplantation and gene therapy will open up novel therapeutic avenues for Alzheimer's disease, particularly because it can be used to simultaneously target several pathogenetic changes in the disease.

Key Words

neural regeneration; neurodegenerative disease; β -secretase; neural stem cells; Alzheimer's disease; C17.2 neural stem cells; primary neural stem cells; shRNA; plasmid; genetic modification; grants-supported paper; neuroregeneration

Ethical approval: This experiment was approved by the Institutional Animal Care and Ethical Committee of the Sun Yat-sen University Institute for Life Sciences in China.

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INTRODUCTION

Alzheimer's disease is a common neurodegenerative disease. At present, drug therapy cannot reverse the course of Alzheimer's disease; it can only temporarily relieve symptoms. There are no effective treatments apart from acetylcholinesterase inhibitors, which only temporarily augment cholinergic function.

The combination of neural stem cell transplantation and genetic modification makes it possible to harness the dual functions of cellular replacement and gene therapy^[1-2]. Many successful experiments have been performed using neural stem cell transplantation, particularly for Parkinson's disease, for which the pathogenesis is relatively well known^[3-5]. For example, the degeneration or even loss of dopaminergic neurons in the substantia nigra results in a decrease in dopamine levels in the corpus striatum. Therefore, tyrosine hydroxylase gene-modified neural stem cells are transplanted into the corpus striatum, increasing dopamine neurotransmitter levels. This is an ideal treatment method because it is specifically aimed at the major pathogenetic change in Parkinson's disease^[6-11].

In contrast to Parkinson's disease, there are several pathological changes in Alzheimer's disease, including apoptosis of cholinergic neurons, an increase in levels and activity of β -secretase (beta-site amyloid precursor protein cleaving enzyme, BACE1), and inflammation^[12]. Some studies have indicated that transplantation of rat neural stem cells transfected with nerve growth factor, choline acetyltransferase or brain-derived neurotrophic factor can improve cognitive performance^[13-18]. However, it is usually insufficient to introduce a gene^[19] into cells to only upregulate the expression Alzheimer's disease-related proteins. Thus, in the present study, we synthesized a short hairpin RNA (shRNA) construct targeting BACE1, the key enzyme in amyloid beta protein (A β) production, downregulating its expression. The amyloid cascade hypothesis for the patho-

genesis of Alzheimer's disease postulates that memory deficits are caused by increased levels of both soluble and insoluble A β , which are derived from the larger amyloid precursor protein after undergoing sequential proteolytic processing. BACE1 is the rate-limiting enzyme in this A β synthetic pathway. Overexpression of A β exists only if there is overactivity or high levels of the enzyme.

A β is the key factor in Alzheimer's disease pathogenesis, because it not only has direct neurotoxicity, but can also combine with FPR-like-1 in mononuclear phagocytes, activating these small glial cells that gather in the lesion site and release inflammatory proteins. As a result, A β has indirect neurotoxic effects as well^[20]. The latest research shows that A β also affects the proliferation, survival and differentiation of neural stem cells, and may even reduce their ability to migrate toward the lesion site^[21]. Post-mortem analysis of brain samples from Alzheimer's disease patients has shown increased levels of BACE1 protein and A β levels in cortical regions^[22-23]. Thus, decreasing A β levels in the brain is a possible therapeutic approach for Alzheimer's disease, and many experiments indicate that proteins such as BACE1 and neprilysin could be used as therapeutic agents to reduce A β levels in Alzheimer's disease brain^[24-26].

Neural stem cell transplantation is designed to replace damaged nerve cells, and thereby restore cell loops and cell function. There are primarily two methods: (1) differentiation of endogenous neural stem cells; (2) exogenous neural stem cell transplantation. These two methods can both be applied for Alzheimer's disease therapy.

In most cases, it is insufficient to replace lost nerve tissue only by enhancing the production of endogenous neural stem cells, especially in the spinal cord or striatum^[27-28]. In this study, we chose the C17.2 cell line to use as exogenous neural stem cells, and primary neural stem cells to use as endogenous neural stem cells.

In the present study, we downregulated the expression of the BACE1 gene in C17.2 neural stem cells and primary neural stem cells *via* genetic modification for the first time. A short hairpin DNA oligonucleotide was introduced into the pSilenCircle plasmid to express a short hairpin RNA (shRNA) complementary to a sequence in the coding region of human BACE1 mRNA. The shRNA was able to inhibit the expression of the BACE1 gene in C17.2 neural stem cells and primary neural stem cells. Thus, when these cells were transplanted into the brain, they not only could replace the lost nerve cells through proliferation and differentiation, but also could downregulate the expression of BACE1 protein, resulting in a reduction in A β production. This can inhibit the inflammatory reaction, and promote the proliferation, survival, differentiation and migration of neural stem cells to the lesion area. Therefore, this technology simultaneously harnesses the power of cell transplantation and genetic therapy, and targets multiple pathological changes in Alzheimer's disease.

RESULTS

Identification and differentiation of C17.2 neural stem cells and primary neural stem cells

About 95% of C17.2 neural stem cells attached to the bottom 4 hours after passage. Furthermore, a monolayer of neural stem cells spread out and formed an almost confluent sheet after 3–4 days of passage. The neural stem cells were spindle or irregular in shape (Figure 1A, B).

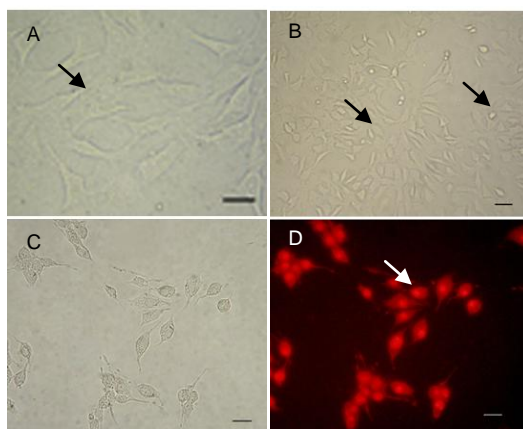


Figure 1 The identification of C17.2 neural stem cells.

(A, B) Cultured C17.2 neural stem cells 2 and 4 days after passage under an inverted microscope (black arrows). (C) Neural stem cells under an inverted microscope. (D) Nestin-positive neural stem cells (red fluorescence, white arrow) after immunofluorescence staining under an inverted microscope, which is an important marker for the identification and isolation of neural stem cells. (A, C, D) Scale bars: 50 μ m; (B) scale bar: 100 μ m.

After about 80% confluence, neural stem cells were passaged, surviving well in subsequent passages. Compared with C17.2 neural stem cells, the primary neural stem cells isolated from hippocampal tissue proliferated and aggregated into free-floating neurospheres in serum-free medium after 2–3 days of cultivation (Figure 2). The number of neurospheres increased with cultivation time.

However, when the neurospheres were passaged onto a culture plate covered with polylysine and supplemented with serum-containing medium after 7 days of culture, the neurospheres adhered to the bottom and a monolayer of primary neural stem cells emerged from the neurospheres 36 hours after inoculation (Figure 3A, B).

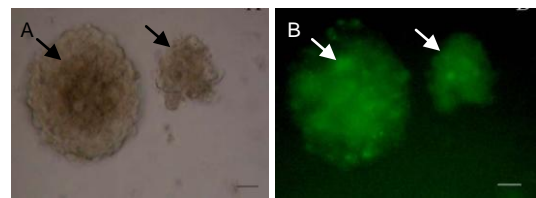


Figure 2 Primary neural stem cell proliferation and aggregation into neurospheres after 48 hours of culture.

Both pEGFP and psiEGFP expression plasmids were transfected into the neurospheres that developed from the primary neural stem cells. (A) Image under an inverted microscope (black arrows). (B) EGFP fluorescence within the neurospheres (white arrows). Scale bars: 100 μ m. EGFP: Enhanced green fluorescent protein.

Nestin is regarded as an important marker for the identification and isolation of neural stem cells. Therefore, immunofluorescence staining for nestin was conducted after 7 days of culture. Nestin was expressed in almost all C17.2 neural stem cells and primary neural stem cells (Figure 1C, D; Figure 3C, D), suggesting that these two cell types were characteristically neural stem cells. To investigate whether these nestin-positive cells possessed the pluripotent capacity to differentiate into neuronal cells as well as astrocytes and oligodendrocytes *in vitro*, the serum-free medium was replaced and the cells were cultured for an additional 7 days in the serum-containing medium. β -Tubulin is known to be a marker of neurons, while glial fibrillary acidic protein is a marker of astrocyte-like cells and oligodendrocytes. Immunofluorescence staining showed that the C17.2 neural stem cells and primary neural stem cells had spread out from the neurospheres and morphologically differentiated into neuron-like cells bearing long neurites or into astrocytic or oligodendrocytic cells (Figure 3E–H). This suggests that the C17.2 neural stem cells and primary

neural stem cells display a high capacity to proliferate and can phenotypically differentiate into functional neuronal and glial cell types *in vitro*.

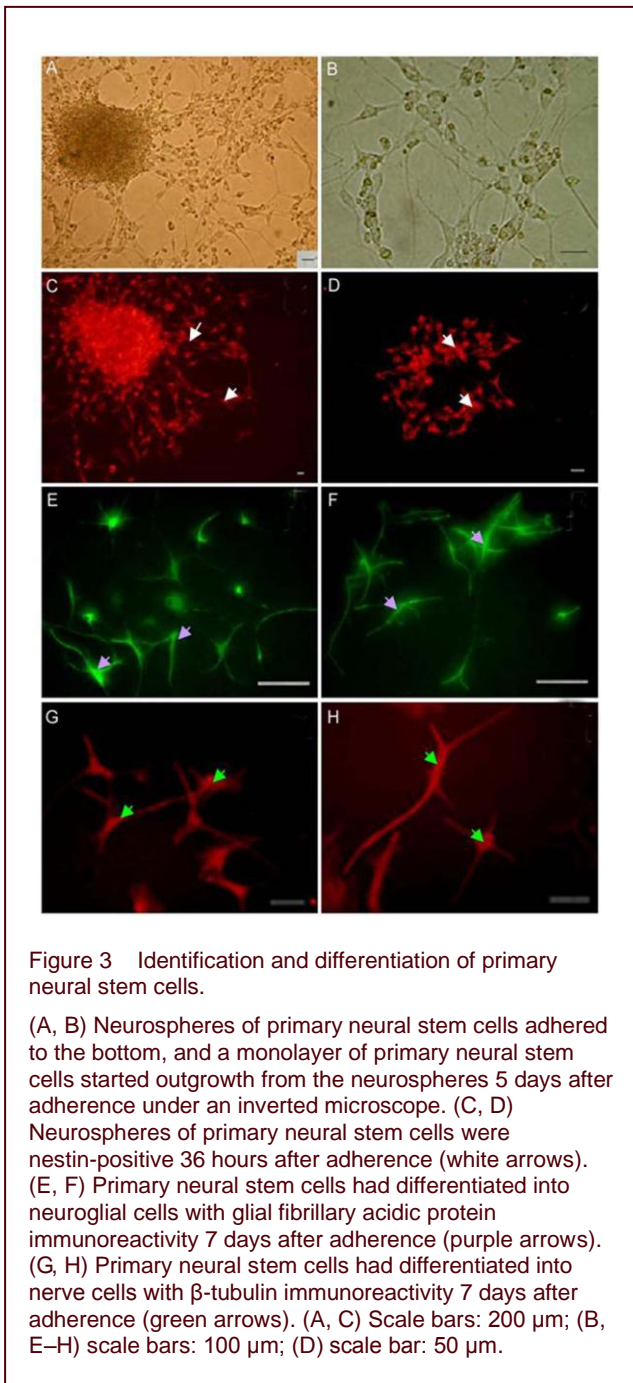


Figure 3 Identification and differentiation of primary neural stem cells.

(A, B) Neurospheres of primary neural stem cells adhered to the bottom, and a monolayer of primary neural stem cells started outgrowth from the neurospheres 5 days after adherence under an inverted microscope. (C, D) Neurospheres of primary neural stem cells were nestin-positive 36 hours after adherence (white arrows). (E, F) Primary neural stem cells had differentiated into neuroglial cells with glial fibrillary acidic protein immunoreactivity 7 days after adherence (purple arrows). (G, H) Primary neural stem cells had differentiated into nerve cells with β -tubulin immunoreactivity 7 days after adherence (green arrows). (A, C) Scale bars: 200 μ m; (B, E–H) scale bars: 100 μ m; (D) scale bar: 50 μ m.

Construction and confirmation of siBACE1 plasmid

We constructed the shRNA expression plasmid, which was used directly in neural stem cell and primary neural stem cell RNAi experiments. The shRNA sequence contained the shRNA sense strand, antisense strand and a loop sequence. We introduced the shRNA sequence into the pSilenCircle plasmid to construct psiBACE1-1, psiBACE1-2, psiBACE1-3 and psiBACE1-4 shRNA expression plasmids (Figure 4).

The *Stu*I digestion sites in the pSilenCircle plasmid were lost in clones with successful fragment insertion, while plasmids without insertions retained the *Stu*I digestion sites. The pSilenCircle plasmids were cut by *Stu*I enzyme into 3.5 kb and 1.1 kb linear fragments, while psiBACE1-1, psiBACE1-2, psiBACE1-3 and psiBACE1-4 remained uncut (4.6 kb) (Figure 5A, B). We could select plasmids with successfully inserted shRNA sequences after restriction digestion with *Stu*I. The shRNA expression plasmids were then sequenced to confirm proper insertion (Figure 5C).

Each shRNA plasmid carried a BACE1 sequence cassette encoding a target-specific 19-nt shRNA with a 6-bp loop. When transfected into neural stem cells or primary neural stem cells *via* lipid-based transfection, the plasmids expressed shRNA from the U6 promoter. The shRNA offered an opportunity to potently and stably silence BACE1 gene expression.

Evaluation of target gene expression

The pEGFP expression plasmids were transfected into C17.2 neural stem cells at 0.6, 1.2, 1.8, 2.4 and 3.0 μ g (mixed with 50 μ L Lipofectamine 2000). 1.2 μ g of pEGFP displayed the most effective transfection rate, more than 85% (Figure 6).

In this study, siGFP was individually co-transfected with each of the four siBACE1 plasmids into C17.2 neural stem cells. The BACE1 activity assay was performed 48 hours after transfection of the expression plasmids, *i.e.*, psiBACE1-1, psiBACE1-2, psiBACE1-3 and psiBACE1-4, to identify the most efficient plasmid. Except for psiBACE1-4 (control plasmid), each of the other plasmids inhibited BACE1 activity by at least 72%, as assessed with real-time PCR assay (Figure 7). However, psiBACE1-2 displayed the greatest efficiency in knocking down BACE1 expression (over 87%) after 48 hours of transfection, which is ideal for accomplishing our goal. In addition, the siBACE1-2 plasmid was capable of stable inhibition of target gene expression; even 7 days after transfection, it still inhibited BACE1 activity by over 69% (Figure 8). This capacity for stable expression in neural stem cells is a major advantage of the shRNA system, especially when targeting a protein with slow turnover.

In the RNAi experiments with primary neural stem cells, the shRNA plasmids were the same as those used in the C17.2 neural stem cells, but only psiBACE1-2, which exhibited the strongest inhibition of BACE1 activity, was used (Figure 8).

siRNA1:
 5'acaccgactgtggctacaacattcttctgctgaagactgtggctacaacattct 3'
 3'gctgacaccgatgtgtaagaacgaactctgacaccgatgtgtaagaaaa 5'

siRNA2:
 5'acaccggcttggagatgggtgattgctgaaggcttggagatgggat 3'
 3'gccgaaacacctaccacctaacgaactccgaaacacctaccacataaaa 5'

siRNA3:
 5'acaccgtggactgcaaggagtacaattgctgaagtgactgcaaggagtacaat3
 3'gcacctgacgttctcatgtaacgaactcactgacgttcctcatgttaaaaa 5'

control siRNA:
 5'acaccgacctaggtactatcgcaattgctgaaggacctaggtactatcgcaat3'
 3'gttcgatagcttaggtcaacgaacttttcgatagcttaggtcaaaaa5'

psiRNA-BACE1-1:
 TTATATATCTTGTGGAAAGGACGAAACACCGACTGTGGCTACAACATTCTTGCTTGAAGACTGTGGCTAC
 AACATTCTTTTACAGTTTTTGTAGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGAT
 CAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCCCCCGTGCCTTCCTTGACCCTGG
 AAGGTGCCACTCCCAC

psiRNA-BACE1-2:
 CCCTTATATTCGCCTTTTTACAGTTGAGATTTCGATTCTTGGCTTTATATATCTTGTGGAAAGGACGAAAC
 ACCGGCTTGTGGAGATGGTGGATTGCTTGAAGGCTTTGTGGAGATGGTGGATTTTACAGTTTTTGTAG
 AGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGC
 CAGCCATCTGTTGTTGCCC

psiRNA-BACE1-3:
 TTAATATATCTTGTGGAAAGGACGAAACACCGTGGACTGCAAGGAGTACAATTGCTTGAAGTGGACTGC
 AAGGAGTACAATTTTTACAGTTTTTGTAGAGGGCCCTATTCTATAGTGTACCTAAATGCTAGAGCTCGC
 TGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGGCCCTCCCCCGTGCCTTCCTTGACC
 CTGGAAGGTGCCACTCC

Figure 4 Sequence of the short hairpin RNAs (shRNAs).

Upper: The three shRNA sequences that were complementary with the coding region of human β -site amyloid precursor protein cleaving enzyme 1 (BACE1) mRNA, and an irrelevant shRNA (control). Below: The three short hairpin RNA (shRNA) expression sequences were introduced into pSilenCircle plasmids to construct psiBACE1-1, psiBACE1-2 and psiBACE1-3 shRNA expression plasmids.

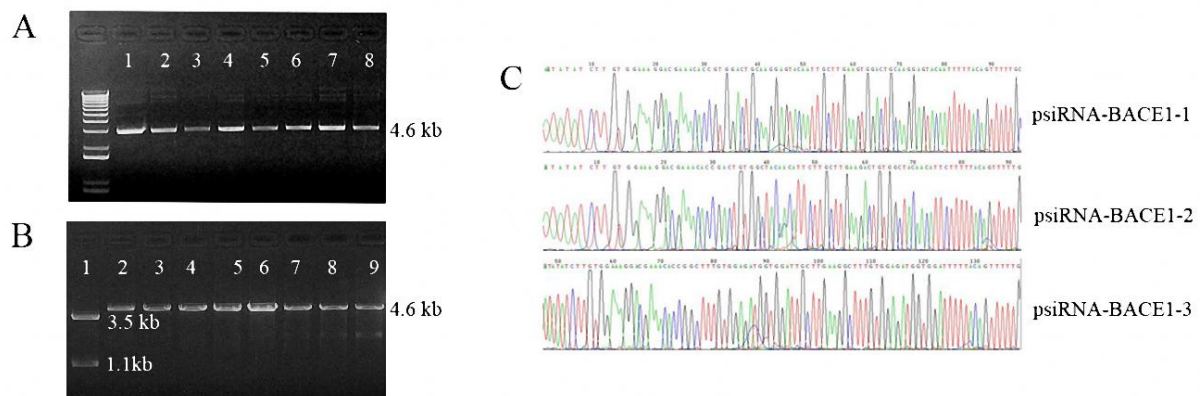


Figure 5 Successful construction of pSilenCircle plasmids.

(A) Because of the insertion of exogenous fragments, the short hairpin RNA (shRNA) expression plasmids could not be cut by *StuI* enzyme. The psiBACE1-1 (1, 2), psiBACE1-2 (3, 4), psiBACE1-3 (5, 6) and psiBACE1-4 (7, 8) all were 4.6 kb in length. (B) The pSilenCircle plasmids without insertion of exogenous fragments (1) were cut by *StuI* enzyme into 3.5 kb and 1.1 kb linear fragments. The psiBACE1-1 (2, 3), psiBACE1-2 (4, 5), psiBACE1-3 (6, 7) and psiBACE1-4 (8, 9) still were 4.6 kb in length. (C) The three shRNA expression plasmids were sequence-verified.

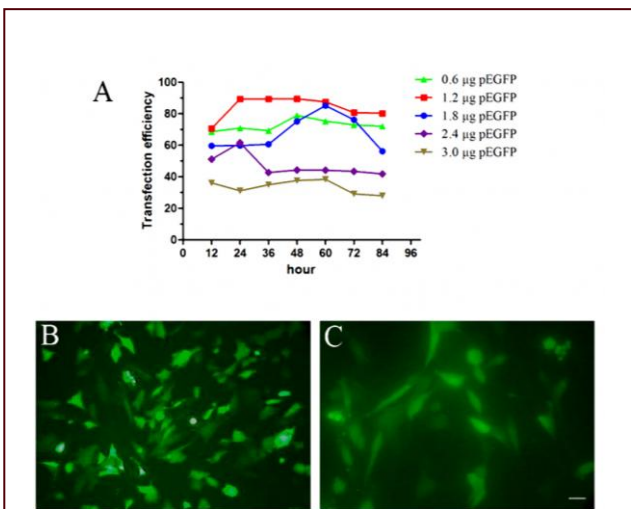


Figure 6 Green fluorescent protein (GFP) expression in C17.2 neural stem cells after transfection.

(A) C17.2 neural stem cells were transfected with 0.6, 1.2, 1.8, 2.4 or 3.0 µg pEGFP expression plasmid. 1.2 µg of pEGFP displayed the most effective transfection rate of more than 85% at different time points post-transfection. Results were assessed using GFP fluorescence intensity. (B, C) C17.2 neural stem cells abundantly expressed the GFP protein (green) 24 hours after transfection (visualized under an inverted fluorescence microscope). (B) Scale bar: 200 µm; (C) scale bar: 50 µm.

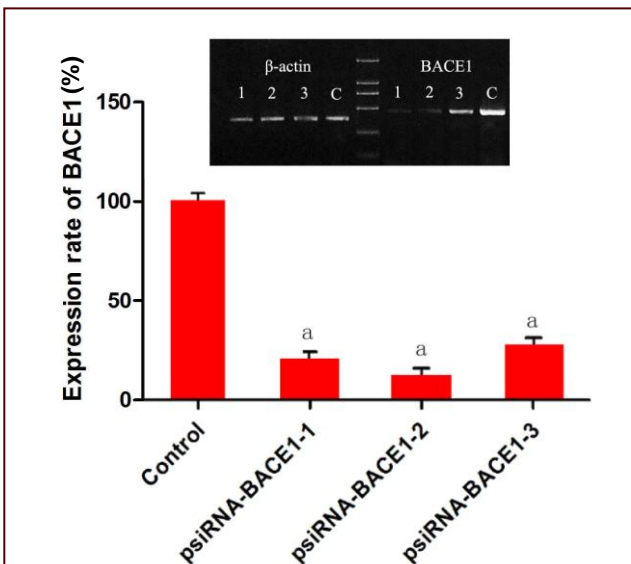


Figure 7 Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) expression in C17.2 neural stem cells 48 hours after transfection (real-time reverse-transcription PCR).

The psiBACE1-2 plasmid displayed the greatest efficiency in knocking down BACE1 expression, with an efficiency of more than 87% after transfection. The psiBACE1-1 and the psiBACE1-3 plasmids also reduced BACE1 expression. Each group consisted of three sections. Tests were repeated three times. Data were analyzed with one-way analysis of variance and least significant difference test. ^a*P* < 0.01, vs. control.

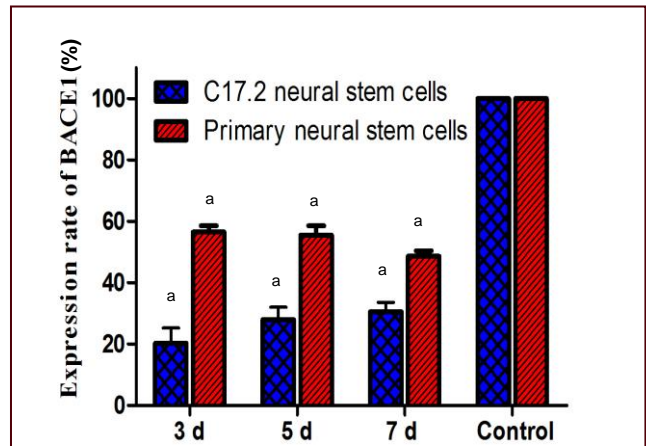


Figure 8 Effect of siBACE1-2 on BACE1-2 expression in C17.2 neural stem cells and primary neural stem cells (real-time reverse-transcription PCR).

The siBACE1-2 plasmids could inhibit BACE1 activity by over 69%, even 7 days (d) after transfection. psiBACE1-2 reduced BACE1 expression in C17.2 neural stem cells and primary neural stem cells 3, 5 and 7 days after transfection. Each group consisted of three sections. Tests were repeated three times.

All values are presented as mean ± SD. Data were analyzed by one-way analysis of variance and least significant difference test. ^a*P* < 0.01, vs. control. BACE1: β-Site amyloid precursor protein cleaving enzyme 1.

The primary neural stem cells isolated from hippocampal tissue were cultured for 7 days, and the resulting neurospheres were co-transfected with siGFP and si-BACE1-2 plasmids. The ability of psiBACE1-2 to down-regulate BACE1 was confirmed by PCR after 48 hours of transfection (data not shown). We also examined the long-term ability of psiBACE1-2 to downregulate BACE1 expression in the primary neural stem cells. As shown in Figure 8, we observed effective downregulation of BACE1 expression by psiBACE1-2 3–7 days after the shRNA treatment. The reduction in BACE1 transcript levels was stable during the course of RNAi treatment. These results demonstrate that the siBACE1-2 plasmid provides a stable and long-term means of inhibiting BACE1 gene expression.

The measurement of cell viability is very important in developing a safe shRNA expression plasmid for neural stem cells. We examined cell viability using the X-gal assay after transfection with the shRNA expression plasmid. Greater than 95% of neural stem cells survived after 2 days in control (untransfected) cultures. Cell viability was also quite high (up to 90%) 48 hours after shRNA treatment (Figure 9). Therefore, there was no significant difference between untransfected cells and those transfected with siBACE1-2 plasmid.

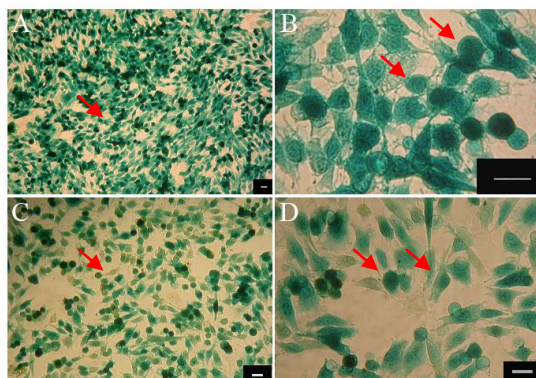


Figure 9 Cell viability of C17.2 neural stem cells 48 hours after transfection (X-gal staining).

The X-gal assay was used to assess cell viability after transfection with the shRNA expression plasmid under the inverted fluorescence microscope. C17.2 neural stem cells were used. There was no significant difference in cell viability between cells transfected with siBACE1-2 plasmid (A, B) and those that were untransfected (C, D). Red arrows show the stained C17.2 neural stem cells. (A, C) Scale bars: 200 μm ; (B) scale bar: 50 μm ; (D) scale bar: 100 μm . BACE1: β -Site amyloid precursor protein cleaving enzyme 1.

DISCUSSION

Cell transplantation has proven to be an effective method for treating diseases, especially when combined with genetic engineering; it can not only repair damaged tissue through cell replacement, it can also be used as a form of gene therapy^[2]. Among the neurodegenerative diseases, Parkinson's disease is often the first to be selected for cellular and genetic therapeutic studies, because there is a relatively clear pathogenesis. For example, the impairment of motor function is mainly related to the loss of the neurotransmitter dopamine. Thus, the transplantation of cells that secrete dopamine can increase neurotransmitter levels, and thereby have a substantial therapeutic effect^[8]. However, in contrast, there are numerous pathogenetic processes in Alzheimer's disease. In addition to the apoptosis of cholinergic neurons^[27-28], recent studies show that the levels and activity of β -secretase are considerably elevated in brain regions affected by Alzheimer's disease. The lack of a simple pathogenesis makes Alzheimer's more complex and difficult to treat through cell transplantation. However, the overactivity or overexpression of β -secretase likely leads to the overproduction of A β , ultimately inducing cognitive dysfunction in patients.

In a previous study, we inhibited the expression of the

BACE1 gene using RNAi in nerve cells, resulting in a robust reduction in A β production^[29]. However, the new challenge we faced in this study was how to simultaneously provide two or more therapeutic effects to tackle the complicated pathogenesis of Alzheimer's disease. We focused on the replacement of lost nerve cells and reducing the activity or levels of BACE1 protein. Although fibroblasts or Schwann's cells are often used as gene carriers, they cannot integrate with nerve tissues of the host or form synaptic connections after transplantation into the brain because they are not neuronal cells. Consequently, they cannot survive for long periods in the host or stably express the exogenous gene in the nervous system. Therefore, in this study, we used C17.2 neural stem cells as the carriers of the gene. C17.2 neural stem cells can proliferate and maintain their self-renewal and multipotent differentiation capacities *in vitro*. They can also migrate to the damaged area in the brain, where they may survive, proliferate and differentiate into neuron-like cells and form synaptic connections with host nerve cells. However, unlike primary nerve cells, neural stem cells are readily obtained and can be easily transfected with target genes^[30]. Studies have shown that the expression of the target gene can last for 22 months and substitute for the endogenous gene after transplantation of neural stem cells into the host^[31-32]. There are currently many reports on the treatment of neurodegenerative diseases with neural stem cell transplantation, in which researchers usually introduce neurotrophic factor genes to restore specific nerve functions. Thus, neural stem cells are ideal gene carriers for the treatment of neurodegenerative diseases, and they can have the dual function of cell replacement and gene therapy. The detailed understanding of neural stem cell transplantation has opened up novel therapeutic avenues for Alzheimer's disease.

In a growing number of studies, the nerve growth factor gene was introduced into neural stem cells, which were then transplanted into the brains of Alzheimer's disease animal models for preventing or delaying the onset of symptomatic Alzheimer's disease^[33-34]. However, the lack of nerve growth factor is only one of the several pathogenetic changes in Alzheimer's disease. The latest studies indicate that the level of BACE1 in the brain of patients with Alzheimer's disease is 40–60% higher than in normal aged persons, and the activity of BACE1 is relatively much higher as well. Although the basis for the higher BACE1 activity remains largely unknown, it is thought that downregulating the activity of the enzyme is the best strategy for the treatment of Alzheimer's disease^[35-39]. Hence, we considered it necessary to develop

a novel method of neural stem cell transplantation, aimed simultaneously at several pathogenetic changes in Alzheimer's disease. Unlike other studies in which genes were introduced to increase the expression of proteins, we introduced an shRNA expression plasmid to knock down BACE1 expression in neural stem cells and reduce A β protein levels. Such neural stem cells could have a greater therapeutic effect.

After transplantation, the neural stem cells generated in this study should proliferate and differentiate into neuron-like cells, and substitute for the lost nerve cells in the injured region, but without excessive BACE1 gene expression. Although it is not clear how BACE1 activity is increased in Alzheimer's disease patients, it is possible that the activity of BACE1 within neural stem cells might be increased in the microenvironment where they are transplanted into the Alzheimer's disease brain, because it has been found that there is a complicated interaction between exogenous neural stem cells and the brain microenvironment^[40]. Recent studies show that the proliferation of neural progenitor cells, and their survival and potential to differentiate into neuron-like cells are affected in the hippocampal dentate gyrus of Alzheimer's disease transgenic mice^[41]. *In vitro* studies show that A β in its pathological form (A β 42) may affect the proliferation, survival and differentiation of neural progenitor cells. It is thought that Alzheimer's disease patients gradually lose neurons and develop dementia because of neural progenitor cell damage induced by A β 42^[42]. Here we assumed that the inhibition of A β synthesis within neural stem cells through RNAi should reduce A β 42 aggregation. As a result, our strategy should promote the proliferation, survival and differentiation of the neural stem cells into neuron-like cells.

At present, there are two ways to treat neurodegenerative diseases with neural stem cells; (i) the induction of differentiation of endogenous neural stem cells and (ii) the transplantation of exogenous neural stem cells. The mechanism of migration of endogenous neural stem cells in the embryo is quite well understood. Numerous important signaling molecules and cytokines are involved in the dynamics of migration of neural stem cells in the embryo^[43]. However, the mechanism in the adult remains largely unknown. Even less is known about the process in pathologic conditions such as Alzheimer's disease^[44]. Recent studies show that A β 42 can affect the migration of neural stem cells, and *in vivo* experiments demonstrate that the transplanted neural stem cells can migrate to the region of A β injection where they may differentiate into neurons and astrocytes^[45]. A β is frequently aggre-

gated in the hippocampus and subventricular zone, where numerous neural stem cells are gathered^[40]. It has been recently shown that A β 42 is the ligand for the chemoattractant receptor FPR-like-1, which is expressed on the surface of neural stem cells^[20]. Zhang *et al*^[2] identified many receptors responsible for the migration of neural stem cells, including FPR, SDF-1 and CXCR-4, and found that the migration of neural stem cells was significantly affected after incubation with low concentrations of A β 42 for long periods. Notably, the expression of CXCR-4 on the surface of neural stem cells also showed a dose-dependent decrease. Therefore, they conjectured that A β 42 reduces CXCR-4 expression *via* FPR, leading to a reduction in neural stem cell migration. The upregulation of GRK2 on the surface of neural stem cells was also associated with the inhibition of neural stem cell migration.

In this study, the risk of overproduction of A β 42 was reduced by having introduced an shRNA expression plasmid targeting the BACE1 gene into neural stem cells. As a result, the neural stem cells should be much more efficient in migrating to the damaged area. Consequently, our study is vitally important in efforts for improving neural stem cell migration in pathological states. Studies have shown that successful implementation of siRNAs *in vivo* is hampered by the low bioavailability of these hydrophilic compounds and their inability to cross key biological barriers such as the blood-brain barrier^[46-48]. Thus, introducing a shRNA plasmid (such as ours) *via* intraventricular injection should be much more therapeutically effective.

The shRNA expression plasmid with the U6 promoter used in the present study stably expressed the shRNA for a long period of time, and significantly inhibited the expression of the BACE1 gene in neural stem cells. These neural stem cells should not only proliferate and differentiate into nerve cells to replace the lost neurons, they should also have reduced endogenous BACE1 activity, suggesting that they should successfully fulfill the dual objective of treatment and prevention. Therefore, we reasonably consider that the transplantation of neural stem cells transfected with a shRNA expression plasmid against BACE1 is an ideal form of targeted gene therapy, aimed at tackling the complex pathogenesis of Alzheimer's disease.

MATERIALS AND METHODS

Design

A randomized, controlled *in vitro* study.

Time and setting

Experiments were performed at the Department of Neurobiology and Anatomy, Zhongshan School of Medicine, Sun Yat-sen University, China from June 2010 to March 2012.

Materials

Sprague-Dawley (SD) neonatal male rats (newborn), weighing about 1.5 g, were purchased from the Experimental Animal Center of Sun Yat-sen University in China (license No. SYXK (Yue) 2007-0081). Rats were housed in a quiet room with a ventilation and air filtration system at 20°C and 50% humidity, and allowed free access to food and water. Bedding and drinking water were replaced daily. Animal procedures were conducted in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China^[49].

Methods

Cell culture

The C17.2 neural stem cell line was purchased from Kunming Institute of Zoology, Chinese Academy of Sciences in China. The C17.2 neural stem cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor, in a 5% CO₂ humidified atmosphere at 37°C. The primary neural stem cells were obtained from neonatal rats. Hippocampal tissue was dissected and digested with 0.1% trypsin-ethylenediamine tetraacetic acid (EDTA; Invitrogen) and 0.5 mg/mL DNase (Sigma, St. Louis, MO, USA) at 37°C for 40 minutes and then centrifuged at 1 000 r/min (radius: 13.5 cm) for 10 minutes. Then, primary neural stem cells were cultured in 83% DMEM with 10% fetal bovine serum, 5% horse serum (Gibco), 1% L-glutamine and 1% antibiotic solution in an incubator at 37°C and 5% CO₂. After culture, primary neural stem cells isolated from the hippocampal tissue of neonatal rats proliferated and aggregated into floating neurospheres in culture *in vitro*. The C17.2 neural stem cells and primary neural stem cells were cultured for another 7 days prior to transfection.

Immunocytochemical staining

The free-floating neurospheres of primary neural stem cells attached to the culture plate covered with polylysine. A monolayer of primary neural stem cells started outgrowth from the neurospheres 8 hours post-inoculation. Primary neural stem cells and C17.2 neural stem cells were fixed in 4% paraformaldehyde in 0.1 mol/L PBS pH

7.4 for 15 minutes at room temperature, and then washed three times with 0.1 mol/L PBS. Afterwards, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 minutes and blocked with 5% goat serum albumin for 30 minutes. The cells were incubated with anti-nestin (1:100), anti- β -tubulin (1:100) and anti-glia fibrillary acidic protein (1:100) monoclonal antibodies (Abcam Inc, Cambridge, MA, USA) for 1 hour at room temperature, and subsequently incubated with secondary antibodies; fluorescein isothiocyanate-conjugated goat anti-rat IgG and tetramethylrhodamine isothiocyanate-conjugated goat anti-rat IgG (1:1 000; Zhongshan Co., Ltd., Beijing, China) for 2 hours at room temperature. The fluorescence images of nestin (red), β -tubulin (red) and glial fibrillary acidic protein (green) staining in C17.2 neural stem cells and primary neural stem cells were captured using a Zeiss Axio Imager Z1 microscope (Carl Zeiss, Jena, Germany).

Plasmid construction

To screen for the most effective shRNA sequence, we designed three shRNA sequences that were complementary to the coding region of human BACE1 mRNA, and we designed an irrelevant (control) shRNA as well. The shRNA sequences were as follows: shRNA1 (sense) 5'-gcttgggagatggtgga-3'; shRNA2 (sense) 5'-gacgctcaa catcctggtg-3'; shRNA3 (sense) 5'-tggactgcaaggagtaca a-3' and irrelevant shRNA (sense) 5'-ttggcttctgtcagcgc- 3'. The shRNA DNA oligonucleotides were introduced into pSilenCircle plasmid (Allele Biotech & Pharmaceuticals, Inc, San Diego, CA, USA) to produce four recombinant plasmids; psi-BACE1-1, psiBACE1-2, psiBACE1-3 and psi-BACE1-4^[50]. All constructs were sequence-verified by Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China. The shRNAs were delivered as gene constructs composed of the U6 RNA-based polymerase III promoter, the short hairpin DNA oligonucleotide encoding the shRNA against the BACE1 gene and a modified terminator, which could synthesize shRNA duplexes 19 nt in length with 2 nt overhangs at the 3' end. Pol III-directed synthesis of shRNA should produce high levels of shRNA expression, which, in turn, should result in highly efficient gene silencing.

Cell transfection

Neural stem cells were cultured in 24-well plates at a density of 2×10^5 cells/well for 24 hours prior to transfection. Various concentrations of psiEGFP were diluted into 50 μ L DMEM. 10 μ L Lipofectamine 2000 (Invitrogen) was diluted into 50 μ L DMEM and mixed with psiEGFP plasmid at room temperature. After about 80% conflu-

ence, the GFP-expressing plasmid (pEGFP-C1) and psiEGFP plasmid (Clontech, Mountain View, CA, USA) encoding the shRNA against the EGFP gene were simultaneously transfected into neural stem cells to facilitate the evaluation of the effectiveness of the exogenously introduced shRNA. The recombinant psiBACE1-1, psiBACE1-2, psiBACE1-3 and psiBACE1-4 were also individually transfected into neural stem cells with Lipofectamine 2000 according to the manufacturer's instructions. The same procedure was used for the transfection of primary neural stem cells. The serum-free medium containing the DNA-liposome complexes was changed and replaced with fresh medium 4–6 hours post-transfection, and the neural stem cells and primary neural stem cells were continuously cultured for 12–84 hours. The fluorescence images of the C17.2 neural stem cells and primary neural stem cells were obtained using a Zeiss Axio Imager Z1 inverted fluorescence microscope (Carl Zeiss).

Detection of RNAi activity using real-time reverse-transcription PCR

The RNAi activity was measured at different time points. We transfected pEGFP-C1 Vector (Clontech) into C17.2 cells and primary neural stem cells or neurospheres to optimize the conditions for transfection and observe the expression of the exogenous gene in these cells. The expression of the exogenous gene and the silencing effect were quantified using quantitative real-time reverse-transcription PCR (RT-PCR) on a Bio-Rad Chromo 4 instrument with a SYBR Green real-time RT-PCR master mix kit (Takara, Dalian, China). The procedure was as follows: 1×10^5 C17.2 cells or primary neural stem cells were plated into 35-mm glass dishes to achieve approximately 60–80% confluence in transfection experiments. An aliquot of pEGFP-C1 vector was diluted to 50 μ L with DMEM (serum-free) and incubated for 5 minutes at room temperature. Subsequently, Lipofectamine 2000 was diluted to 50 μ L with DMEM and incubated for 5 minutes at room temperature. Then, pEGFP-C1 vector and Lipofectamine 2000 diluent were mixed together and incubated for 20 minutes to produce the pEGFP-C1 vector- Lipofectamine 2 000 complexes. The complexes were added to each well of cells and incubated for 30 minutes at room temperature. The psiBACE1-1, psiBACE1-2, psiBACE1-3 and psiBACE1-4 (negative control) were separately transfected into C17.2 cells, primary neural stem cells or neurospheres as mentioned above. Reverse transcription was performed to produce cDNA, which was used to assess the silencing effect using

PCR. PCR was performed in triplicate. The expression of the gene was normalized to β -actin using the ddCt method^[51].

Cell viability assay using X-gal staining

Cell viability was measured using X-gal staining^[52]. The untransfected or transfected C17.2 neural stem cells were cultured at a density of 2×10^5 cells/well in 24-well plates and fixed in X-gal solution (Invitrogen) containing 2% paraformaldehyde for 10 minutes after washing with X-gal. Cells were then washed three times and incubated with X-gal solution for 14 hours at 37°C. Images were captured on a Zeiss Axio Imager Z1 inverted fluorescence microscope.

Statistical analysis

All data were analyzed using one-way analysis of variance and least significant difference test for comparisons between two groups using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). In all statistical analyses, $P < 0.05$ was regarded as statistically significant. All values are presented as mean \pm SD.

Research background: Preventing A β formation and deposition is the main strategy in Alzheimer's disease treatment. BACE1 is a key enzyme in A β metabolism. Reducing BACE1 gene expression is effective in preventing the production of A β .

Research frontiers: In this study, 19-nt constructs targeting the BACE1 gene were introduced into the pSilenCircle vector to construct shRNA expression plasmids against the BACE1 gene. This vector was transfected into C17.2 neural stem cells and primary neural stem cells, resulting in the downregulation of BACE1 gene expression.

Clinical significance: This study explored a new method to treat Alzheimer's disease using cell transplantation and gene therapy through the modification of neural stem cells.

Academic terminology: β -secretase 1, also known as BACE1, membrane-associated aspartic protease 2 (memapsin-2) and aspartyl protease 2, is an enzyme that in humans is encoded by the BACE1 gene.

Peer review: This study investigated targeting β -secretase with RNAi in neural stem cells for Alzheimer's disease therapy, and this work is interesting. However, some studies have shown that the cell membranes of nerve cells can prevent the penetration of siRNAs. Moreover, the blood-brain barrier can also restrict siRNAs from entering the central nervous system. Thus, the use of shRNA approaches for the treatment of central nervous system diseases is promising compared with siRNA.

REFERENCES

- [1] Abdel-Salam OM. Stem cell therapy for Alzheimer's disease. *CNS Neurol Disord Drug Targets*. 2011;10(4):459-485.
- [2] Zhang C, Wang ZJ, Lok KH, et al. Beta-amyloid42 induces desensitization of CXC chemokine receptor-4 via formyl peptide receptor in neural stem/progenitor cells. *Biol Pharm Bull*. 2012;35(2):131-138.
- [3] Park HJ, Shin JY, Lee BR, et al. Mesenchymal stem cells augment neurogenesis in the subventricular zone and enhance differentiation of neural precursor cells into dopaminergic neurons in the substantia nigra of a parkinsonian model. *Cell Transplant*. 2012;21(8):1629-1640.
- [4] Cho MS, Lee YE, Kim JY, et al. Highly efficient and large-scale generation of functional dopamine neurons from human embryonic stem cells. *Proc Natl Acad Sci U S A*. 2008;105(9):3392-3397.
- [5] Wernig M, Zhao J P, Pruszak J, et al. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A*. 2008;105(15):5856-5861.
- [6] Akerud P, Canals JM, Snyder EY, et al. Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. *J Neurosci*. 2001;21(20):8108-8118.
- [7] Lu P, Jones LL, Snyder EY, et al. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp Neurol*. 2003;181(2):115-129.
- [8] Lunn JS, Sakowski SA, Hur J, et al. Stem cell technology for neurodegenerative diseases. *Ann Neurol*. 2011;70(3):353-361.
- [9] Lynch WP, Sharpe AH, Snyder EY. Neural stem cells as engraftable packaging lines can mediate gene delivery to microglia: evidence from studying retroviral env-related neurodegeneration. *J Virol*. 1999;73(8):6841-6851.
- [10] Kriks S, Shim J W, Piao J, et al. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson's disease. *Nature*. 2011;480(7378):547-551.
- [11] Ganat YM, Calder EL, Kriks S, et al. Identification of embryonic stem cell-derived midbrain dopaminergic neurons for engraftment. *J Clin Invest*. 2012;122(8):2928-2939.
- [12] Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions. *J Clin Neurosci*. 2004;11(5):456-467.
- [13] Blurton-Jones M, Kitazawa M, Martinez-Coria H, et al. Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proc Natl Acad Sci U S A*. 2009;106(32):13594-13599.
- [14] Moghadam FH, Alaie H, Karbalaie K, et al. Transplantation of primed or unprimed mouse embryonic stem cell-derived neural precursor cells improves cognitive function in Alzheimerian rats. *Differentiation*. 2009;78(2-3):59-68.
- [15] Lee HJ, Lim IJ, Park SW, et al. Human neural stem cells genetically modified to express human nerve growth factor (NGF) gene restore cognition in the mouse with ibotenic acid-induced cognitive dysfunction. *Cell Transplant*. 2012;21(11):2487-2496.
- [16] Park D, Joo SS, Kim TK, et al. Human neural stem cells overexpressing choline acetyltransferase restore cognitive function of kainic acid-induced learning and memory deficit animals. *Cell Transplant*. 2012;21(1):365-371.
- [17] Park D, Lee HJ, Joo SS, et al. Human neural stem cells over-expressing choline acetyltransferase restore cognition in rat model of cognitive dysfunction. *Exp Neurol*. 2012;234(2):521-526.
- [18] Moghadam FH, Alaie H, Karbalaie K, et al. Transplantation of primed or unprimed mouse embryonic stem cell-derived neural precursor cells improves cognitive function in Alzheimerian rats. *Differentiation*. 2009;78(2-3):59-68.
- [19] Kim KS, Kim HS, Park JM, et al. Long-term immunomodulatory effect of amniotic stem cells in an Alzheimer's disease model. *Neurobiol Aging*. in press.
- [20] Le Y, Gong W, Tiffany HL, et al. Amyloid (beta)42 activates a G-protein-coupled chemoattractant receptor, FPR-like-1. *J Neurosci*. 2001;21(2): RC123.
- [21] Njie E, Kantorovich S, Astarly GW, et al. A preclinical assessment of neural stem cells as delivery vehicles for anti-amyloid therapeutics. *PLoS One*. 2012;7(4):e34097.
- [22] Santosa C, Rasche S, Barakat A, et al. Decreased expression of GGA3 protein in Alzheimer's disease frontal cortex and increased co-distribution of BACE with the amyloid precursor protein. *Neurobiol Dis*. 2011;43(1):176-183.
- [23] Yang LB, Lindholm K, Yan R, et al. Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat Med*. 2003;9(1):3-4.
- [24] Mueller-Steiner S, Zhou Y, Arai H, et al. Anti-amyloidogenic and neuroprotective functions of cathepsin B: implications for Alzheimer's disease. *Neuron*. 2006;51(6):703-714.
- [25] Schworer R, Zubkova OV, Turnbull JE, et al. Synthesis of a targeted library of heparan sulfate hexa- to dodecasaccharides as inhibitors of beta-secretase: potential therapeutics for Alzheimer's disease. *Chemistry*. 2013;19(21):6817-6823.
- [26] Wang CY, Xie JW, Xu Y, et al. Trientine reduces BACE1 activity and mitigates amyloidosis via the AGE/RAGE/NF-kappaB pathway in a transgenic mouse model of Alzheimer's Disease. *Antioxid Redox Signal*. 2013.
- [27] Valla J, Schneider L, Reiman EM. Age- and transgene-related changes in regional cerebral metabolism in PSAPP mice. *Brain Res*. 2006;1116(1):194-200.
- [28] Geula C, Nagykerly N, Nicholas A, et al. Cholinergic neuronal and axonal abnormalities are present early in aging and in Alzheimer disease. *J Neuropathol Exp Neurol*. 2008;67(4):309-318.
- [29] Li S, Liu Z, Ji F, et al. Delivery of quantum dot-siRNA nanoparticles in SK-N-SH cells for BACE1 gene silencing and intracellular imaging. *Mol Ther Nucleic Acids*. 2012;1:e20.

- [30] Park KI, Himes BT, Stieg PE, et al. Neural stem cells may be uniquely suited for combined gene therapy and cell replacement: Evidence from engraftment of Neurotrophin-3-expressing stem cells in hypoxic-ischemic brain injury. *Exp Neurol*. 2006;199(1):179-190.
- [31] Ryder EF, Snyder EY, Cepko CL. Establishment and characterization of multipotent neural cell lines using retrovirus vector-mediated oncogene transfer. *J Neurobiol*. 1990;21(2):356-375.
- [32] Snyder EY, Deitcher DL, Walsh C, et al. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. *Cell*. 1992;68(1):33-51.
- [33] Cao Q, Benton RL, Whittemore SR. Stem cell repair of central nervous system injury. *J Neurosci Res*. 2002;68(5):501-510.
- [34] Geuna S, Borriero P, Fornaro M, et al. Adult stem cells and neurogenesis: historical roots and state of the art. *Anat Rec*. 2001;265(3):132-141.
- [35] Chen CH, Zhou W, Liu S, et al. Increased NF-kappaB signalling up-regulates BACE1 expression and its therapeutic potential in Alzheimer's disease. *Int J Neuropsychopharmacol*. 2011:1-14.
- [36] Fukumoto H, Cheung BS, Hyman BT, et al. Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. *Arch Neurol*. 2002;59(9):1381-1389.
- [37] Holsinger RM, Mclean CA, Beyreuther K, et al. Increased expression of the amyloid precursor beta-secretase in Alzheimer's disease. *Ann Neurol*. 2002;51(6):783-786.
- [38] Li R, Lindholm K, Yang LB, et al. Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients. *Proc Natl Acad Sci U S A*. 2004;101(10):3632-3637.
- [39] Yang LB, Lindholm K, Yan R, et al. Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. *Nat Med*. 2003;9(1):3-4.
- [40] Haughey NJ, Liu D, Nath A, et al. Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal precursor cells in culture, by amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease. *Neuromolecular Med*. 2002;1(2):125-135.
- [41] Shruster A, Eldar-Finkelman H, Melamed E, et al. Wnt signaling pathway overcomes the disruption of neuronal differentiation of neural progenitor cells induced by oligomeric amyloid beta-peptide. *J Neurochem*. 2011;116(4):522-529.
- [42] Mazur-Kolecka B, Golabek A, Nowicki K, et al. Amyloid-beta impairs development of neuronal progenitor cells by oxidative mechanisms. *Neurobiol Aging*. 2006;27(9):1181-1192.
- [43] Serfozo P, Schlarman MS, Pierret C, et al. Selective migration of neuralized embryonic stem cells to stem cell factor and media conditioned by glioma cell lines. *Cancer Cell Int*. 2006;6:1.
- [44] Sun L, Lee J, Fine HA. Neuronally expressed stem cell factor induces neural stem cell migration to areas of brain injury. *J Clin Invest*. 2004;113(9):1364-1374.
- [45] Tang J, Xu HW, Fan XT, et al. Targeted migration and differentiation of engrafted neural precursor cells in amyloid beta-treated hippocampus in rats. *Neurosci Bull*. 2007;23(5):263-270.
- [46] Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. *Nature*. 2009;457(7228):426-433.
- [47] Davis ME, Zuckerman JE, Choi CH, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature*. 2010;464(7291):1067-1070.
- [48] Burnett JC, Rossi JJ, Tiemann K. Current progress of siRNA/shRNA therapeutics in clinical trials. *Biotechnol J*. 2011;6(9):1130-1146.
- [49] The Ministry of Science and Technology of the People's Republic of China. Guidance Suggestions for the Care and Use of Laboratory Animals. 2006-09-30.
- [50] Luo Y, Bolon B, Kahn S, et al. Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation. *Nat Neurosci*. 2001;4(3):231-232.
- [51] Karan R, Singla-Pareek SL, Pareek A. Histidine kinase and response regulator genes as they relate to salinity tolerance in rice. *Funct Integr Genomics*. 2009;9(3):411-417.
- [52] Zhang Q, Meng JH, Luo N, et al. The influencing elements and optimization of X-gal staining method in the study of transplantation. *Jiepo Xuebao*. 2003;33(3):312-316.

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