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# Recombinant adenovirus-mediated overexpression of 3β-hydroxysteroid-Δ24 reductase

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## Abstract

3β-Hydroxysteroid-Δ24 reductase (DHCR24) is a multifunctional enzyme that localizes to the endoplasmic reticulum and has neuroprotective and cholesterol-synthesizing activities. DHCR24 overexpression confers neuroprotection against apoptosis caused by amyloid β deposition. The present study aimed to construct two recombinant adenoviruses driving DHCR24 expression specifically in neurons. Two SYN1 promoter DNA fragments were obtained from human (h) and rat (r). Recombinant Ad-r(h)SYN1-DHCR24 was transfected into AD-293, N2A (mouse neuroblastoma), and MIN6 (mouse pancreatic carcinoma) cells. Western blot analysis showed DHCR24 was specially expressed in 293 and N2A cells, but no specific band was found in MIN6 cells. This demonstrates that the recombinant adenoviruses successfully express DHCR24, and no expression is observed in non-neuronal cells. TUNEL assay results showed apoptosis was inhibited in adenovirus-transfected neurons. Detecting reactive oxygen species by immunofluorescence, we found that adenovirus transfection inhibits apoptosis through scavenging excess reactive oxygen species. Our findings show that the recombinant DHCR24 adenoviruses induce neuron-specific DHCR24 expression, and thereby lay the foundation for further studies on DHCR24 gene therapy for Alzheimer's disease.

***Key Words:*** *nerve degeneration; 3β-hydroxysteroid-Δ24 reductase; recombinant adenovirus; neuron; oxidative stress; synapsin-1; gene therapy; neuroprotection; NSFC grant; neural regeneration*

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## Introduction

Cholesterol plays an important role in brain tissue, particularly in signal transmission along neuronal axons, nervous system development, and regeneration[1-3]. Activation of neurons and emerging, development and maintenance of synapses are closely related to cholesterol[4-5]. Normal brain function depends on cholesterol stability[6-7]. Cells of surrounding tissues can intake cholesterol from low-density lipoproteins in the blood, but the brain cannot and must directly synthesize cholesterol[8]. Brain cholesterol levels are not related to blood cholesterol levels[9-10], and the only factors influencing brain cholesterol levels are its self-synthesis and secretion[11]. Alzheimer's disease patients are reported to show abnormal cholesterol metabolism[1, 12].

3β-Hydroxysteroid-Δ24 reductase (DHCR24) is a member of the family of flavin adenine dinucleotide-dependent oxidoreductases, which catalyze cholesterol synthesis from desmosterol[13]. Accumulating evidence demonstrates that DHCR24 located at the endoplasmic reticulum is a multifunctional enzyme that possesses anti-apoptotic and cholesterol-synthesizing activities[14]. DHCR24 mRNA expression levels are decreased in the affected hippocampus from Alzheimer's disease patients, compared with the unaffected side[15]. Biochemical and clinical studies show that DHCR24 is implicated in neuroprotective processes through several mechanisms[16-18]. DHCR24 can modulate membrane cholesterol levels, which are associated *in vivo* with amyloid precursor protein processing and amyloid β generation[17-18]. Furthermore, DHCR24 overexpression protects neuronal cells from oxidative stress-induced apoptosis[15-16, 19]. Conversely, DHCR24 also effectively inhibits caspase-3 activation, a key mediator of the apoptotic process[14-15]. Moreover, DHCR24 interacts with p53, increasing its stability and thereby regulating cell growth, senescence, and apoptosis[20-21]. We have previously demonstrated that DHCR24 plays an important role in the insulin-Akt cell survival signaling pathway, by maintaining cholesterol biosynthesis and cholesterol-rich caveolae structures. Therefore, we speculate that DHCR24 may also function similarly in neurons, because many neuronal growth factor receptors are located within caveolae or lipid rafts, which require cholesterol homeostasis[22-23]. Overall, findings to date demonstrate that DHCR24 possesses neuroprotective functions, suggesting that DHCR24 may be a new treatment target for gene therapy of Alzheimer's disease.

DHCR24 is widely expressed in many tissues, although specifically targeting neuronal DHCR24 is the preferred

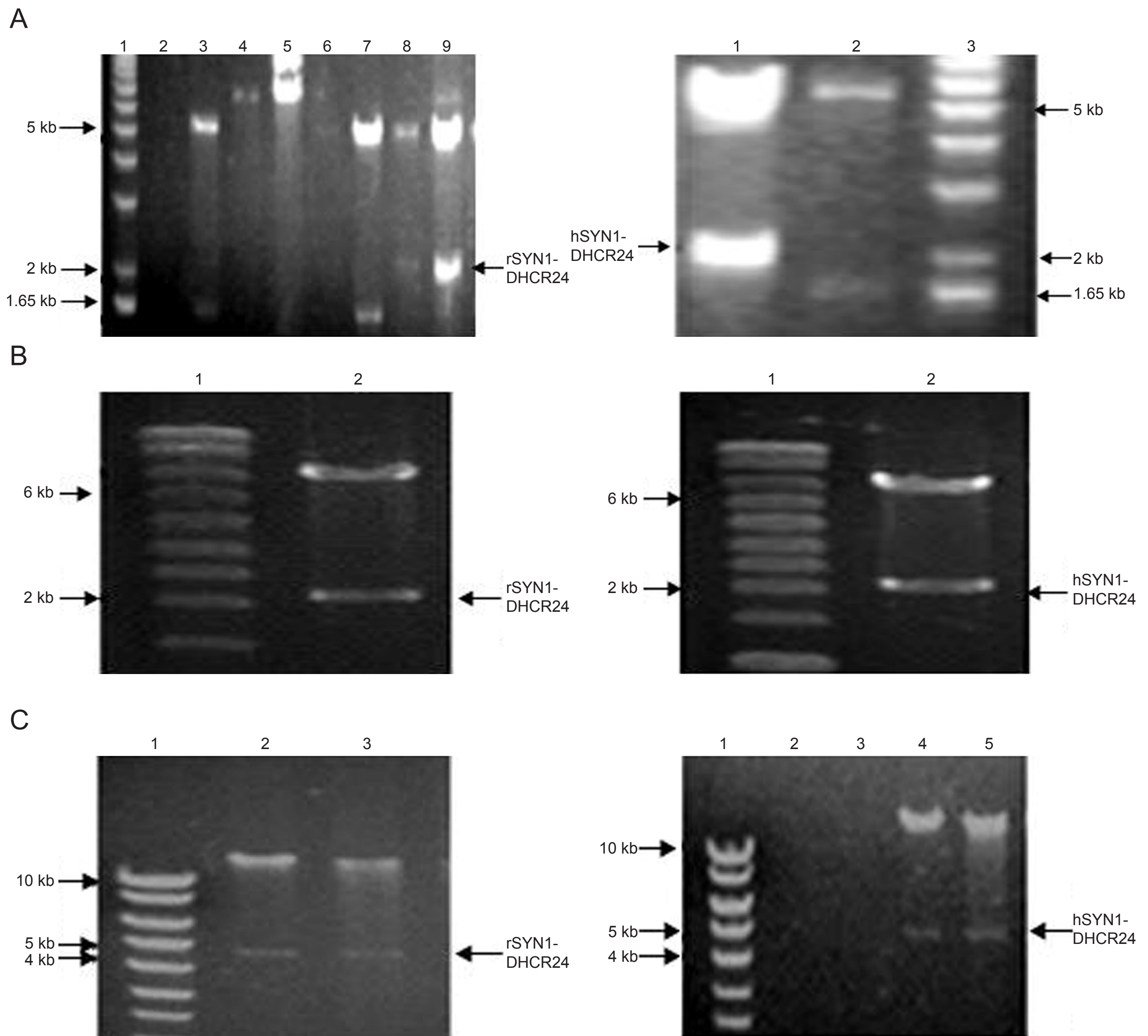


**Figure 1 Enzyme digestion identification of recombinant plasmids.**
(A) *Kpn*I and *Pme*I double digestion of pcDNA3.1A-SYN1-DHCR24-myc from several clones. Left: 1, DNA marker; 2–9, different clones. Right: 3, DNA marker; 1 and 2, different clones. (B) *Kpn*I and *Hin*dIII double digestion of pShuttle-SYN1-DHCR24 from several clones. 1: DNA marker; 2: positive clone. (C) *Pac*I digestion of pAdEasy-1-SYN1-DHCR24, from several clones. 1: DNA marker; 2–5: different clones. DACR24: 3β-Hydroxysteroid-Δ24 reductase; SYN1: synapsin-1.

treatment strategy. DHCR24 overexpression in tissues and cells outside the brain, may cause excessive cholesterol synthesis and generate unwanted side effects *e.g.,* hyperlipidemia[24-26]. To investigate the neuroprotective effects of DHCR24 and the possibility of DHCR24 as a gene target of AD therapy, we used the neuron-specific promoter, synapsin-1 (SYN1), to generate a recombinant adenovirus overexpressing DHCR24 specifically in neuronal cells.

## Results

### Design of recombinant adenovirus expressing the DHCR24 gene specifically in neuronal cells

Agarose gel electrophoretic analysis showed that clone 9 in the rat (r) SYN1 group and clone 1 in the human (h) SYN1 group both contained the SYN1-DHCR24 (2,000 bp) and pcDNA3.1A (5,500 bp) fragments (Figure 1A), demonstrating successful construction of the recombinant plasmid, pcDNA3.1A-SYN1-DHCR24-myc. Subsequently, the recombinant plasmid, pShuttle-SYN1-DHCR24, was identified by *Kpn*I and *Hin*dIII double digestion. We obtained r(h) SYN1-DHCR24 (2,000 bp) and pShuttle (6,600 bp) fragments in both rat and human SYN1 groups (Figure 1B). Finally, clones 2 and 3 in the rat SYN1 group and clones 4 and 5 in the human SYN1 group contained SYN1-DHCR24 (4,500 bp) and pAdEasy (30 kbp) fragments (Figure 1C), indicating they are positive homologous recombination clones. Overall, we successfully generated the recombinant plasmids, pAdEasy-1-r(h) SYN1-DHCR24.

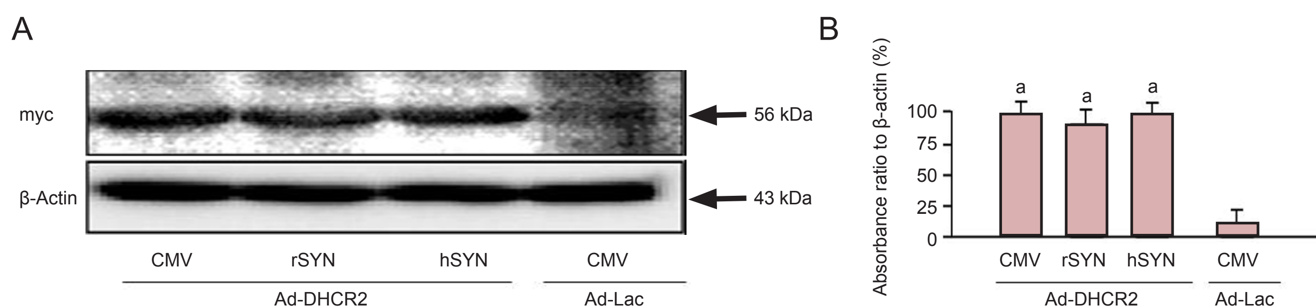


**Figure 2 Western blot analysis for the identification of DHCR24-myc in AD-293 cells.**
AD-293 cells were transfected with linearized pAd-r(h)SYN1-DHCR24-myc, and harvested after 7 days. Western blot analysis using a primary myc antibody was performed. Ad-CMV-DHCR24 and Ad-CMV-LacZ were used as positive and negative controls, respectively. Representative images are shown in A. Quantitative results are shown in B. Data are expressed as mean ± SD. The experiment was performed in triplicate. Differences among groups were compared using one-way analysis of variance followed by Bonferroni's multiple *t*-test. $^{a}P < 0.05$, *vs.* Ad-CMV-LacZ. DACR24: 3β-Hydroxysteroid-Δ24 reductase; SYN1: synapsin-1; h: human; r: rat; Ad: adenovirus.

**DHCR24-myc expression induced by Ad-SYN1-DHCR24-myc in AD-293 cells**
At 7–10 days after transfection, western blot analysis showed bands of approximately 56 kDa present in all Ad-CMV-DHCR24-myc (positive control), Ad-rSYN1-DHCR24-myc and Ad-hSYN1-DHCR24-myc transfected groups. No obvious bands were detected in the Ad-CMV-LacZ control group (Figure 2), showing that Ad-SYN1-DHCR24-myc was successfully packaged and induced DHCR24-myc expression in AD-293 cells.

**Amplification, purification, and expression of recombinant adenovirus Ad-r(h)SYN1-DHCR24-myc**
We studied selective DHCR24-myc induction in neuronal cells, using MIN6 and N2A cells. Results of our western blot analysis are shown in Figure 3A, B. The positive control (Ad-CMV-DHCR24-myc) induced DHCR24 overexpression in both MIN6 and N2A cells. Strong bands were present in Ad-rSYN1-DHCR24 and Ad-hSYN1-DHCR24-myc infected N2A neuronal cells, but not in MIN6 cells. This suggests DHCR24-myc overexpression is only induced by Ad-r(h)SYN1-DHCR24-myc adenovirus in N2A cells.

To further examine DHCR24 expression induced by Ad-r(h)SYN1-DHCR24-myc in neuronal cells, we performed immunocytochemical analyses using an antibody against the myc peptide. In N2A cells, very weak fluorescent signals were present in the nuclei of negative controls (Ad-CMV-LacZ) (Figure 3C). However, strong green fluorescent signals were observed around the nuclei in both Ad-rSYN1-DHCR24 and Ad-hSYN1-DHCR24-infected N2A cells, suggesting strong neuronal DHCR24 expression induced by Ad-r(h)SYN1-DHCR24-myc. This result is consistent with a previous study[15, 22], and demonstrates that Ad-r(h)SYN1-DHCR24-myc can selectively drive DHCR24 expression in neuronal cells.

**Ad-r(h)SYN-DHCR24 protects neuronal cells from apoptosis caused by reactive oxygen ($H_2O_2$)**
To determine if Ad-r(h)SYN1-DHCR24-myc-induced selective DHCR24 expression can protect neuronal cells, we treated N2A cells with $H_2O_2$ and performed *in situ* apoptotic (TUNEL) assays. At 48 hours after $H_2O_2$ treatment, most adherent Ad-rSYN1-DHCR24-infected cells were positively stained (Figure 4A, C), while only a few TUNEL-positive cells were detected in the Ad-CMV-LacZ infected group. Similar results were obtained with the Ad-hSYN1-DHCR24 infected group (data not shown). These results suggest that exogenous DHCR24 induced by Ad-r(h)SYN1-DHCR24-myc may protect neuronal cells from apoptosis induced by $H_2O_2$.

In addition, we investigated the mechanism underlying the neuroprotective function of Ad-r(h)SYN1-DHCR24-myc. We measured intracellular reactive oxygen species using the fluorescent probe, 2′,7′-dichlorofluorescein diacetate (H2DCFDA). After $H_2O_2$ exposure for 3 and 12 hours, green fluorescent signals representing reactive oxygen species levels were much weaker in Ad-rSYN1-DHCR24 infected cells (Figure 4B) compared with Ad-CMV-LacZ control cells ($P < 0.05$; Figure 4D). These results demonstrate that Ad-r(h)SYN1-DHCR24-myc may protect neuronal cells from apoptosis under oxidative stress through reactive oxygen species-scavenging activities, consistent with our previous studies[19].

## Discussion

In the present study, we constructed two recombinant adenoviruses (Ad-rSYN1-DHCR24-myc and Ad-hSYN1-DHCR24-myc) that drive DHCR24 expression specifically in neuronal cells. We detected DHCR24 expression in neuronal cells infected with Ad-r(h)SYN1-DHCR24-myc, and confirmed a neuroprotective function of induced DHCR24.

The neuroprotective function of DHCR24 has generally been accepted[14-20], suggesting DHCR24 is a promising target for gene therapy of neurodegenerative diseases, such as Alzheimer's disease[18, 27-28]. Currently, adenoviruses are one of the most widely used vectors for gene transfer and gene therapy of nervous system diseases[29-30]. Adenoviral vectors have many advantages over other gene therapy vectors[31], *e.g.*, they have high transduction efficiencies, are capable of containing DNA inserts up to 8 kb, have extremely high viral titers (in the order of $10^{10}$–$10^{13}$), and can infect both replicating and differentiated cells[32-33]. Also, they do not cause mutagenic effects associated with random integration, as they do not integrate into the host genome[34]. Furthermore, they exhibit low pathogenicity in humans, and cause few, and only mild, symptoms associated with the common cold[35]. Therefore, we chose the adenovirus as the vector of gene therapy to deliver DHCR24 into cells and determine the feasibility of DHCR24 gene therapy for Alzheimer's disease[36].

Although several vectors driving DHCR24 expression (in-

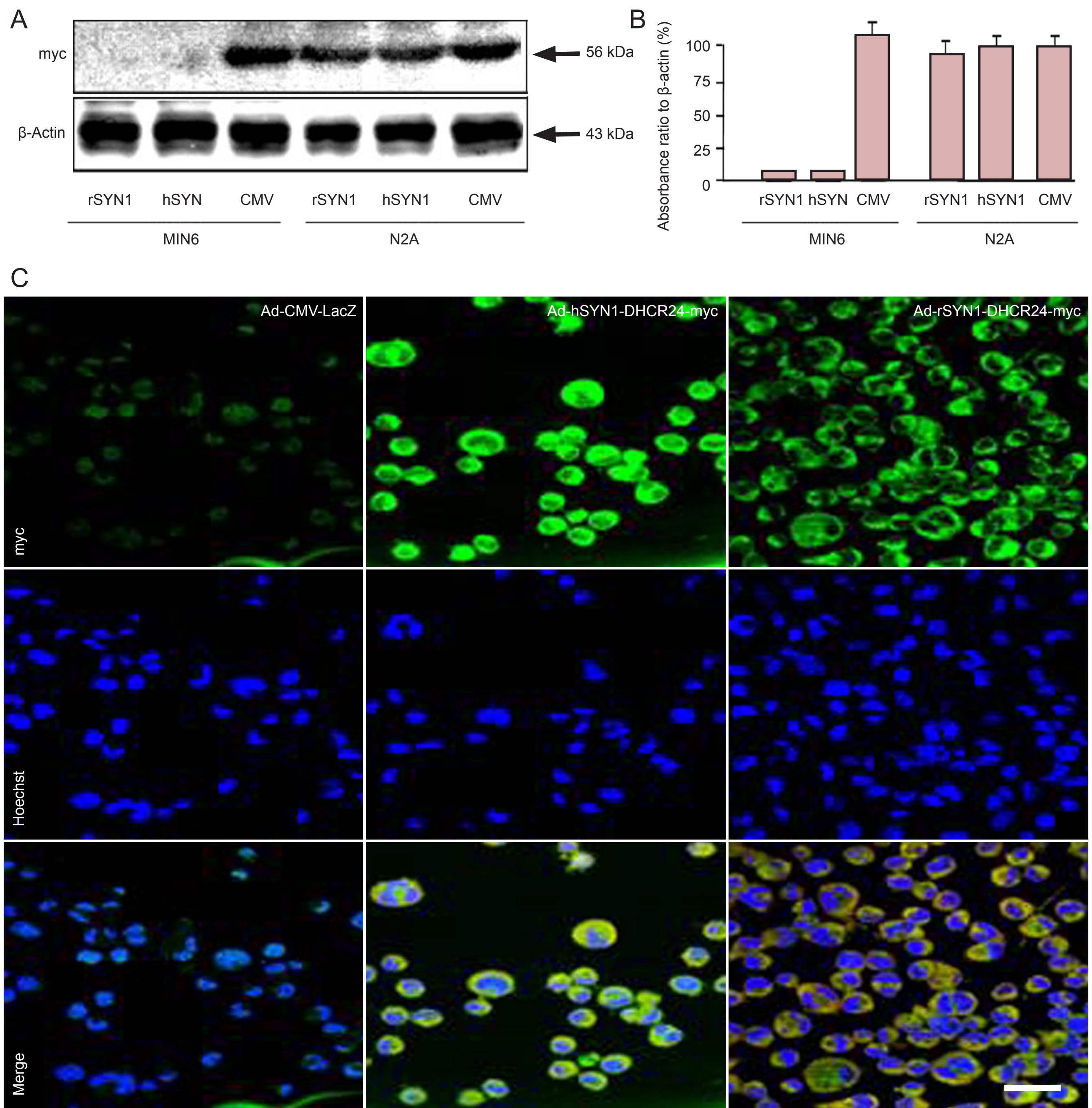


**Figure 3 Selective Ad-r(h)SYN1-DHCR24-myc expression in neuronal cells.**
Cells were infected with the indicated adenoviruses and subjected to western blot (A) or immunocytochemical (B) analyses. (A) Western blot analysis using anti-myc antibody was performed 48 hours after infection. (B) Quantitative results. DHCR24-myc expression levels were normalized to β-actin levels and expressed as percentages relative to maximal levels in MIN6 cells transfected with Ad-CMV-DHCR24. Data are expressed as mean ± SD. The experiment was performed in triplicate. Differences among groups were compared using one-way analysis of variance followed by Bonferroni's multiple *t*-test. (C) N2A cells were infected with adenovirus for 48 hours before immunocytochemical analysis. Hoechst staining identified nuclei (blue fluorescence). Merged images show predominant DHCR24 expression in the endoplasmic reticulum. Scale bars: 10 μm. DACR24: 3β-Hydroxysteroid-Δ24 reductase; SYN1: synapsin-1; h: human; r: rat; CMV: cytomegalovirus.

cluding Ad-CMV-DHCR24 generated by us previously) are reported and widely used for DHCR24 functional analysis, a vector driving neuron-specific DHCR24 expression has not been reported. Our preliminary experiments showed that in N2A cells, DHCR24 overexpression (delivered by Ad-CMV-DHCR24) caused elevation of intracellular cholesterol (data not shown). DHCR24 is ubiquitously expressed in many tissues, including brain, heart, lung, liver, and colon, suggesting DHCR24 functions in many tissues. This leads to the likelihood that side effects may occur in these other tissues if a non-specific vector like Ad-CMV-DHCR24 is used to target DHCR24 in gene therapy for Alzheimer's disease, as

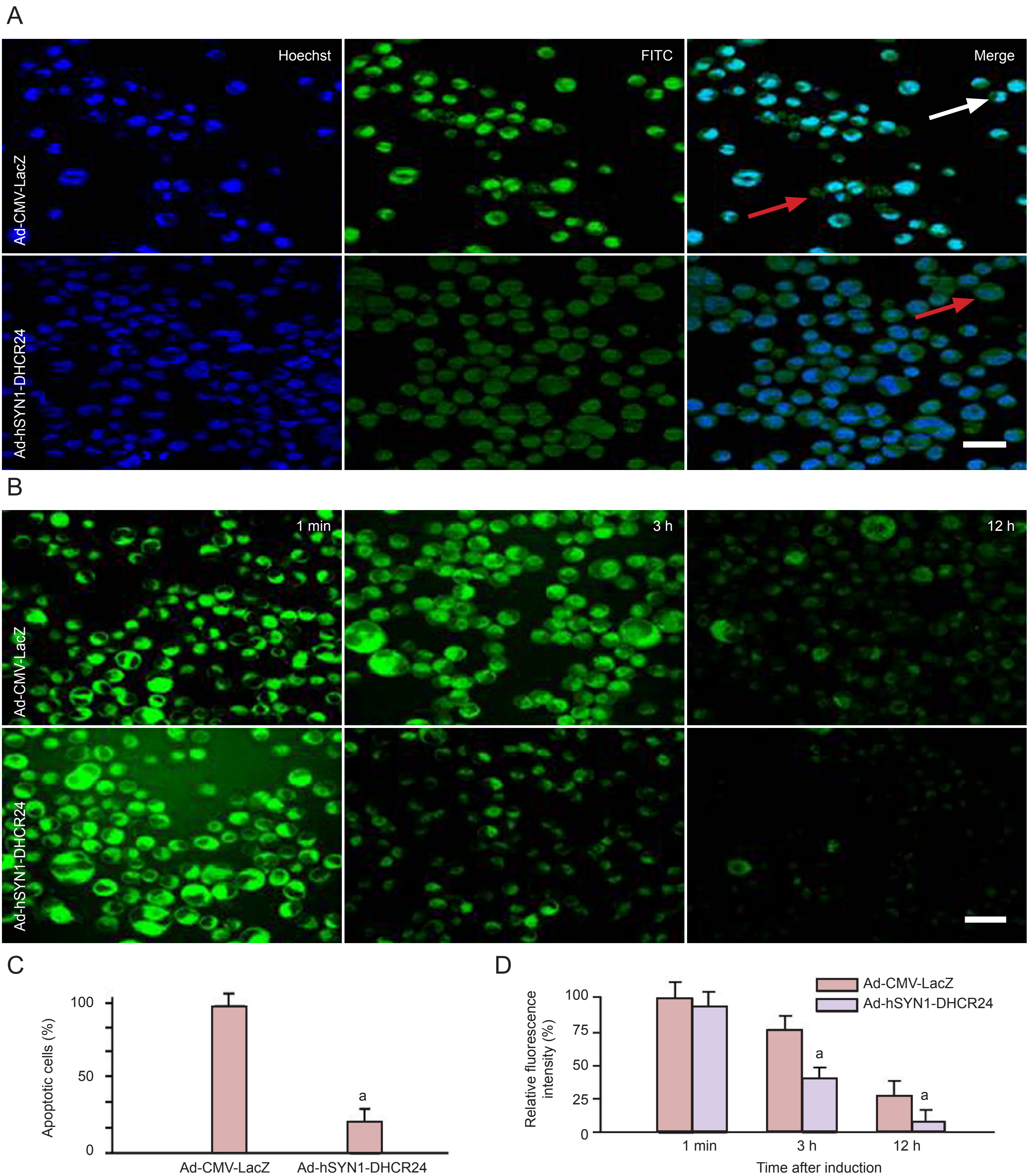


**Figure 4 Ad-rSYN1-DHCR24-myc protects neuronal cells from apoptosis induced by $H_2O_2$ and DHCR24 overexpression induced by Ad-rSYN1-DHCR24-myc, scavenged intracellular reactive oxygen species generated by $H_2O_2$.**

(A) N2A cells were infected with the indicated adenovirus for 48 hours (h), followed by $H_2O_2$ treatment for a further 48 h, and then *in situ* apoptotic (TUNEL) assays were performed. White arrows show apoptotic positive cells; red arrows show apoptotic negative cells. (C) Percentage of TUNEL-positive cells (apoptotic cells). [a]*P* < 0.05, *vs.* Ad-CMV-LacZ group. (B) N2A cells were infected with the indicated adenovirus for 48 h, followed by $H_2O_2$ treatment for a further 48 h, and then incubated with 20 μmol/L H2DCFDA for 30 minutes (min) at 37°C. Cells were then fixed and mounted. Cell images were obtained using a confocal laser microscope. (D) Fluorescent intensities of H2DCFDA are expressed as percentages of the maximal level at 1 min after $H_2O_2$ exposure in Ad-CMV-LacZ-infected cells. Scale bars: 10 μm. [a]*P* < 0.05, *vs.* levels in cells infected with Ad-CMV-LacZ. Data are expressed as mean ± SD. The experiment was performed in triplicate. Differences among groups were compared using one-way analysis of variance. DACR24: 3β-Hydroxysteroid-Δ24 reductase; SYN1: synapsin-1; h: human; r: rat; CMV: cytomegalovirus.

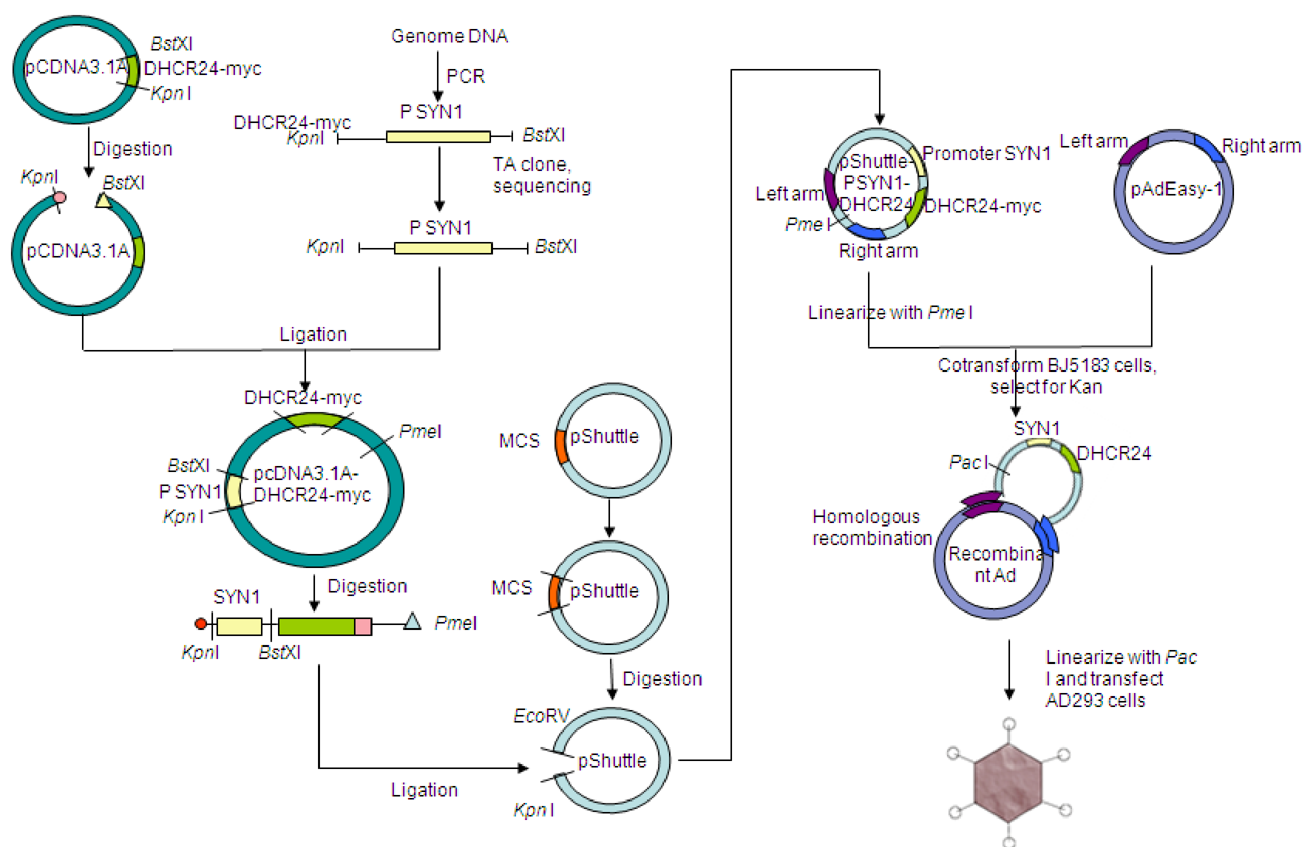


**Figure 5 Construction of Ad-r(h)hSYN1-DHCR24-myc.**
Promoter fragments from rat kidney and human HepG2 cells were PCR amplified and subcloned into pGEMT-easy by TA cloning, for amplification and sequencing confirmation. Next, positive pGEMT/r(h)SYN1 fragments were subcloned into pCDNA3.1A-DHCR24-myc to obtain r(h) SYN1-DHCR24-myc expression cassettes. Fragments containing the complete expression cassette were ligated into pShuttle by *Kpn*I and *Pme*I digestion. pShuttle-r(h)SYN1-DHCR24-myc was linearized with *Pme*I and transformed into ultracompetent BJ5183 bacteria containing pAdEasy-1, generating the recombinant plasmid pAdEasy-r(h)SYN1-DHCR24-myc. Finally, recombinant pAd-SYN1-DHCR24-myc was linearized by *Pac*I digestion and transfected into AD-293 cells for viral packaging. DACR24: 3β-Hydroxysteroid-Δ24 reductase; SYN1: synapsin-1; h: human; r: rat; CMV: cytomegalovirus; MCS: multiple cloning sites.

non-specific vectors will induce DHCR24 overexpression in many tissues, potentially increasing intracellular cholesterol levels in these cells or tissues. To avoid unnecessary side effects of DHCR24 expression in other tissues, and ensure neuron-specific DHCR24 expression, we cloned the recombinant neuron-specific promoter sequence (r(h)SYN1) fragment, upstream of DHCR24 cDNA in the adenovirus genome. This recombinant adenovirus drives neuron-specific DHCR24 expression, and is the first attempt to use this vector for tissue-specific DHCR24 expression. Our work will provide a reference for further animal model studies on DHCR24 functional analysis in other specific tissues, *e.g.*, liver, heart and lung.

We found Ad-r(h)SYN1-DHCR24-myc also induced DHCR24 expression in AD-293 cells, which were used initially to identify DHCR24 protein expression just after viral packaging. We did not investigate the reason for this, but it may be explained by a recent finding[37-39] showing that human embryonic kidney (HEK)293 cells (from which AD-293 cells are derived) express many neuronal specific proteins including neurofilament, suggesting this cell line has neuronal characteristics. In addition, other researchers have found similar results, for example the SYN1 promoter induced weak expression of downstream genes during adenoviral passage in HEK-293 cells[37-38].

We also confirmed the neuroprotective function of DHCR24 by Ad-r(h)SYN1-DHCR24-myc in N2A neuronal cells. We found DHCR24 overexpression induced by Ad-r(h) SYN1-DHCR24-myc could protect N2A cells from $H_2O_2$-induced oxidative stress and apoptosis, through the reactive oxygen species-scavenging activities of DHCR24.

It should be noted that this study only examined the neuroprotective function of Ad-r(h)SYN1-DHCR24-myc under oxidative stress, as it is generally accepted that oxidative stress is a main mechanism of Alzheimer's disease, with many reports demonstrating that DHCR24 protects neuronal cells from oxidative stress induced by $H_2O_2$ or amyloid β[15-16, 19]. However, endoplasmic reticulum stress, cholesterol biosynthesis, and traffic disorders are also involved in Alzheimer's disease pathogenesis[1, 12, 24, 40-41]. We believe that DHCR24 can exert its neuroprotective function through multiple pathways, for example, the anti-endoplasmic reticulum stress function or facilitating cholesterol biosynthesis. We have also demonstrated that in N2A cells, DHCR24 overexpression (delivered by Ad-CMV-DHCR24) can protect cells from apoptosis through increasing intracellular cholesterol levels and facilitating caveolae structure in cholesterol-rich plasma membrane domains (data not shown). The exact mechanism by which Ad-r(h) SYN1-DHCR24-myc protects neuronal cells from apoptosis

requires further investigation.

In summary, we have for the first time successfully constructed an adenovirus that induces DHCR24 specifically in neuronal cells. Our results will lay the foundation for further studies on DHCR24 gene therapy and neuronal functional research in animal models.

## Materials and Methods

### Design

A contrast observation of cell biology.

### Time and setting

The experiments were performed at the School of Life Science, Liaoning University and School of Basic Medical Sciences, Shenyang Medical College, China from January 2010 to December 2012.

### Materials

*Cell lines*

HEK AD-293 cells were obtained from the Cell Resource Center of Life Sciences, Chinese Academy of Sciences (Shanghai, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and L-glutamine without sodium pyruvate, which is DMEM containing high glucose (Corning cellgro, Tewksbury, MA, USA), and supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin.

N2A cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 (high glucose) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

MIN6 cells were kindly donated from the University of Osaka (Osaka, Japan) and grown in DMEM (25 mmol/L glucose) supplemented with 10% fetal bovine serum, 50 mg/L streptomycin, and 75 mg/L penicillin sulfate.

All cell lines were equilibrated with 5% $CO_2$ and 95% $O_2$ at 37°C.

*Plasmids*

Recombinant plasmids pcDNA3.1a-DHCR24-myc, Ad-CMV-DHCR24, and Ad-CMV-LacZ were made previously in Lu's laboratory[22].

The vector, pGEMT-easy, was obtained from TaKaRa (Dalian, Liaoning Province, China).

*Vectors*

Competent cells (BJ5183) and the AdEasy™ Adenoviral Vector System were purchased from Agilent Technologies (La Jolla, CA, USA).

### Methods

*Construction of recombinant pShuttle-r(h)SYN1-DHCR24-myc*

Ad-CMV-DHCR24-myc and Ad-CMV-LacZ were generated as previously described[16]. Construction of the expression adenovirus Ad-r(h)SYN1-DHCR24 is shown in Figure 5. To express DHCR24 specifically in nerve cells, we cloned cDNA from a tissue-specific (SYN1) promoter (obtained from human HepG2 cells or rat tissue) upstream of DHCR24-myc cDNA, generating the expression cassette, SYN1-DHCR24-myc. To ensure and improve expression efficiencies, we extracted SYN1 promoter sequence from a male Sprague-Dawley rat with 250 g of body weight, or human transformed cells, and constructed independently two adenoviruses incorporating rSYN1-DHCR24-myc and hSYN1-DHCR24-myc, respectively (Ad-r(h)SYN1-DHCR24-myc). Human genomic DNA was extracted from the human hepatoma cell line, HepG2 (American Type Culture Collection; ATCC, HB8065). We designed specific SYN1 primers according to previous studies[37-38, 42]. Primers for rat SYN1 were: forward, 5′-GGT ACC TAG GAG CCT TAC GGG TCC-3′; reverse, 5′-CCA CCA CAC TGG GGT GGC AGC TTG GGG C-3′. Primers for nested PCR of human SYN1 were: first set forward, 5′-GCC TGT GTG GAT GTG GGA GAC TAA T-3′; reverse, 5′-TGC AGG TCT GTC ATG TAC CCA TTT G-3′; and second set forward, 5′-GGT ACC TGA CGA CCG ACC CCG-3′; reverse, 5′-CCA CCA CAC TGG GGC TGC GAC TTG GGG-3′.

Following gel purification of PCR products, PCR fragments were cloned into the vector, pGEM-T-easy, using TA Clone. Clones were confirmed by DNA sequencing and extracted to obtain plasmids, pGEMT/r(h)SYN1. These plasmids were digested with *Kpn*I and *Bstx*I enzymes, and r(h)SYN1 fragments cloned into corresponding sites of the prepared vector, pcDNA3.1A-DHCR24-myc. Enzyme digestion confirmed pcDNA3.1A-r(h)SYN1-DHCR24-myc plasmids, which were then digested with *Kpn*I and *Pme*I, and the fragments containing the complete expression cassette, r(h)SYN1-DHCR24-myc, ligated into pShuttle digested with *Kpn*I and *Eco*RV. The resulting positive plasmids were named pShuttle-r(h)SYN1-DHCR24-myc.

*Homologous recombination of recombinant plasmids*

The AdEasy™ Adenoviral Vector System was used to construct recombinant adenoviruses. pShuttle-r(h)SYN1-DHCR24-myc was linearized with *Pme*I and transformed into ultracompetent BJ5183 bacteria containing pAdEasy-1. Positive homologous recombination clones (pAdEasy-r(h)SYN1-DHCR24-myc) were identified by enzyme digestion. Plates containing transformation of linearized empty shuttle vector had uniform-sized colonies arising from uncut/recircularized vector. Compared with control shuttle vector plates, transformants on plates containing pAdEasy-1 recombinants appeared as two populations of normal and reduced size (at an approximate ratio of 3:1). Small colonies are potential recombinants and normal-sized colonies background from the shuttle vector. All protocols and procedures were performed according to the manufacturer's instructions.

*Package, amplification, identification and purification of recombinant adenoviruses*

The recombinant pAd-SYN1-DHCR24-myc was linearized by *Pac*I digestion and transfected into AD-293 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions for packaging virus. After transfection, linearized recombinant adenoviral DNA is repaired in the cells and packaged into the normal viral style, with protein coats and infecting ability. After detachment of most of the cells, they were harvested into 10 mL EP tubes and freeze-thawed three times to destroy the cells and release the virus. Next, the cells were centrifuged at 1,500 × *g* for 5 minutes at room temperature. Cell pellets were subjected to western blot analysis to identify DHCR24 expression. Supernatants were used to infect more 293 cells for amplification. Approximately 90% confluent 293 cells in 500 $cm^2$ flasks were cultured in 50 mL growth medi-

um per flask (at least six flasks). After cell detachment, all the cells were harvested into 50 mL tubes and centrifuged at 800 × *g* for 5 minutes at room temperature. Cell pellets were resuspended in a total of 10 mL solution A (10 mmol/L Tris pH 8.0, 1 mmol/L $MgCl_2$) and collected in one tube. Next, they were freeze-thawed three times, and then centrifuged at 8,200 × *g* for 5 minutes at room temperature to release row viruses.

We purified Ad-hSYN1-DHCR24 and Ad-rSYN1-DHCR24 by CsCl density-gradient ultracentrifugation[43-44]. In the bottom of a tube, 4.2 mL heavy CsCl (1.45 g/mL), 4.2 mL light CsCl (1.20 g/mL) and 3 mL sample were added, and centrifuged at 160,000 × *g* for 2 hours at 20°C. The white band between heavy and light CsCl was collected into a fresh tube using a 22G needle, ultracentrifuged at 280,000 × *g* overnight at 20°C, and then the white band was collected into dialysis membrane. After dialyzing for 6–8 hours, we changed the solution and dialyzed overnight. Absorbance was measured using a BioSpec-nano (Shimadzu Corporation, Tokyo, Japan), and the solution sterilized through a 0.22 μm filter and stored at –80°C.

We produced adenovirus stock solutions with titers of 1 × $10^9$ pfu/μL (Ad-rSYN1-DHCR24-myc) and 3.5 × $10^9$ pfu/μL (Ad-hSYN1-DHCR24-myc). MIN6 cells are a pancreatic cell line that secrete insulin in response to glucose and other secretagogues. N2A cells are a semi-adherent, fast-growing, mouse neuroblastoma cell line, and are frequently used in biological research for Alzheimer's disease.

*Detection of apoptosis by TUNEL staining*

N2A cells were infected with adenovirus for 48 hours, followed by $H_2O_2$ treatment for a further 48 hours, and then in situ apoptotic assays were performed. DNA fragmentation due to apoptosis was analyzed by the TUNEL method using an *in situ* apoptosis kit (Takara, Otsu, Japan), according to the manufacturer's protocol. Cells were stained with Hoechst 33342, mounted using a mounting agent resistant to fluorescent quenching (Beyotime, Haimen, Jiangsu Province, China), and observed by laser confocal fluorescence microscopy (Carl Zeiss, Jena, Germany).

*Detection of myc-tagged DHCR24 expression by western blot analysis*

Procedures for western blot analysis have been described previously[19, 22]. In brief, we used equal amounts of N2A and MIN6 cells, and infected them with adenoviruses in six-well plates. Forty-eight hours after transfection, cells were harvested. Whole-cell lysates (40 μg/lane) were separated by 10% SDS-PAGE, and transferred onto polyvinylidene difluoride membrane (Amersham Pharmacia, Piscataway, NJ, USA). Blots were probed with mouse anti-myc antibody (1:1,000; BD Biosciences, Bedford, MA, USA) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1:2,000; Cell Signaling Technology, Danvers, MA, USA) for 1 hour at room temperature. Proteins were visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). DHCR24-myc expression levels were normalized to β-actin levels, and expressed as percentages of Ad-CMV-DHCR24.

*Measurement of intracellular reactive oxygen species using a fluorescent probe*

Intracellular reactive oxygen species were measured using a fluorescent dye technique coupled with laser confocal fluorescence microscopy[14, 45-46]. N2A cells were cultured on glass coverslips and adenovirus-transfected for 48 hours, followed by $H_2O_2$ exposure for the indicated time. Cells were then treated for 30 minutes with 20 μmol/L H2DCFDA (Molecular Probes, Eugene, OR, USA) in PBS. Coverslips were fixed and mounted. For detection of H2DCFDA fluorescence, the main beam splitter for excitation, the secondary beam splitter for emission, and barrier filter were 488 nm, 570 nm, and 505 nm long, respectively. Several images were captured under the same set of optical parameters. Densitometric analysis was performed using Multi-Gauge software in LAS-1000 (Fuji Film Co., Fuji, Japan).

**Statistical analysis**

Statistical analysis was performed using one-way analysis of variance followed by Bonferroni's multiple *t*-test, or without Bonferroni correction (in the reactive oxygen species test). A $P$ value < 0.05 was considered statistically significant. All analyses were performed using SPSS 15.0 statistical analysis software (SPSS, Chicago, IL, USA).

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**Conflicts of interest:** *None declared.*

**Peer review:** *The present study aims to verify the role of DHCR24 in gene therapy for Alzheimer's disease, which has been rarely reported. Using genetic engineering technology, we constructed replication-defective adenovirus recombinant adenovirus containing DHCR24 and SYN1 gene promoter of neuron-specific expression, and found that the constructed recombinant adenoviruses could delay and reduce neuronal apoptosis after DHCR24 was transfected into neurons.*

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