Potential role of TREM2 in high cholesterol-induced cell injury and metabolic dysfunction in SH-SY5Y cells

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Abstract. Triggering receptor expressed on myeloid cells 2 (TREM2) is an important member of the immunoglobulin family of inflammatory stimulating receptors and is involved in a number of pathophysiological processes. The present study aimed to investigate the role of TREM2 in neurotoxicity induced by high cholesterol levels in SH-SY5Y cells and explore the potential mechanism. SH-SY5Y cells were routinely cultured and stimulated with a range of cholesterol concentrations. Cell viability was assessed using an MTT assay, morphological changes were observed, and the cell cycle distribution was measured using flow cytometry. Lipid deposition was measured by Oil red O staining, and the mRNA and protein expression levels of SRBEP-1 and SRBEP-2 were detected by quantitative PCR and western blotting, respectively. Moreover, the protein expression levels of BDNF, Copine-6, TREM1, TREM2, and key molecules of the Wnt signaling pathways were detected by western blotting. Finally, TREM2 was overexpressed to investigate its potential role in high cholesterol-induced neurotoxicity. The results showed that cell viability was significantly decreased in SH-SY5Y cells stimulated with cholesterol (0.1~100 μM) in a dose- and time-dependent manner. Stimulation with 100 μM cholesterol for 24 h resulted in morphological injuries, increased the proportion of SH-SY5Y cells at G0/G1, the degree of lipid accumulation, and the protein expression levels of sterol regulatory element binding protein (SREBP)1 and SREBP2, markedly decreased the protein expression levels of BDNF, Copine-6, and TREM2, and the p-β-catenin/β-catenin ratio, and increased the expression levels of nesfatin-1, TREM1 and the p-GSK3β/GSK3β ratio. Furthermore, the imbalanced expression of BDNF, Copine-6, nesfatin-1, and p-GSK3β induced by high cholesterol levels was reversed after overexpression of TREM2. These results suggest that a high concentration of cholesterol could induce cell injury and lipid deposition in SH-SY5Y cells and that the underlying mechanism may be associated with imbalanced TREM2 expression.

Introduction

Cholesterol is a major component of brain lipids and plays a crucial role in the maintenance of neuronal cell function, neurotransmission, and synapse formation; however, an increasing number of studies have demonstrated the adverse effects of excessive plasma cholesterol levels (1-3). In 2018, the new version of the Blood Cholesterol Management Guidelines was jointly developed and released by the American College of Cardiology, American Heart Association, and other departments, and this version clearly notes that high levels of cholesterol, particularly low-density lipoprotein cholesterol (LDL-C), can increase the risk of cardiovascular and cerebrovascular diseases such as heart attack and stroke (4,5). Moreover, excessive cholesterol is reportedly closely related to the progression of cognitive impairment and even Alzheimer's disease (AD) (6-8). Cholesterol is normally maintained at low levels in neurons, which inhibits β-amyloid (Aβ) accumulation and enables astrocytes to regulate Aβ accumulation by cholesterol signaling (9). However, imbalanced plasma cholesterol levels, particularly elevated LDL-C and decreased high-density lipoprotein cholesterol levels, are negative factors affecting cognitive function (7,10,11) and are positively correlated with abnormal amyloid deposition in the brain (11). Moreover, disturbed neuronal cholesterol homeostasis has been observed in AD (12), and the accumulation of intracellular cholesterol reportedly reduces the clearance of defective mitochondria (13) and thus contributes to the pathogenesis of

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Abbreviations: LDL-C, low-density lipoprotein cholesterol; AD, Alzheimer's disease; Aβ, β-amyloid; TC, total cholesterol; BDNF, brain-derived neurotrophic factor; PFC, prefrontal cortex; SREBP, sterol regulatory element binding protein; TREM, triggering receptor expressed on myeloid cells; neuronal nucleus

Key words: cholesterol, BDNF, Copine-6, SH-SY5Y cells, TREM2, Wnt signaling pathway

AD. Furthermore, it has been reported that the targeted deletion of cholesterol synthesis in astrocytes decreases the burden of amyloid and tau in an AD mouse model (9). In our previous study, high-fat diet-fed rats showed not only hyperglycemia and hyperlipidemia with increased plasma concentrations of total cholesterol (TC), total triglycerides, and LDL-C, but also neuropsychiatric impairments, including decreased learning and memory abilities, as well as depression-like behavior, accompanied by reduced expression of brain-derived neurotrophic factor (BDNF) and related changes in synaptic plasticity in the hippocampus and prefrontal cortex (PFC) (14). These findings suggest a strong potency of high cholesterol against neuropsychiatric injuries. However, there remain other controversial results suggesting that high levels of cholesterol are a potential protective factor for cognitive decline (15) and are negatively correlated with the occurrence of dementia (16). Therefore, it is important to investigate the exact effects of cholesterol on neural cells and whether there is a clear connection and causality between turmoil lipids and cognitive impairment (17).

Sterol regulatory element binding proteins (SREBPs) are important regulators of cholesterol homeostasis that regulate several enzymes needed for the synthesis of cholesterol, fatty acids, triacylglycerol, and phospholipids (18). Dysregulation of SREBP-2 signaling has been observed in AD and related models, and this dysregulation is attributed to increased cholesterol levels and alterations in the tau protein (12). Nesfatin-1 is a peptide of 82 amino acids produced by hydrolysis of the N-terminus of nucleobindin2, which is widely distributed in central and peripheral tissues (19). Increased plasma concentrations of nesfatin-1 have been detected in diabetic patients (20) and rats with metabolic syndrome (21), and are involved in the regulation of glucose and lipid metabolism (22). It has been reported that chronic infusion of nesfatin-1 can prevent hepatic steatosis, partly by downregulating the expression of SREBP1 (23). Furthermore, nesfatin-1 has been demonstrated to be associated with the regulation of cognition and mood (24,25). In line with these findings, an increased plasma concentration of nesfatin-1 has been observed in rats with hyperlipidemia (26) or stress (27) in our previous studies (28,29), and the results showed that the intraperitoneal injection of nesfatin-1 induced anxiety and depression-related behaviors in rats accompanied by adaptive changes in the expression of BDNF and synaptic plasticity in the hippocampus and PFC. These findings suggest the potential role of nesfatin-1 in linking metabolic disorders with neuropsychiatric injuries.

The classic Wnt/β-catenin signaling pathway is a highly conserved signaling pathway and one of the most conserved regulators of evolution in embryonic development and adult tissue homeostasis (30-32). Dysfunction of the Wnt/β-catenin signaling pathway has been demonstrated to play an important role in the pathogenesis of AD, Parkinson's disease, depression, and other neuropsychiatric diseases (10,33), and is partly involved in controlling neuronal activity-regulated BDNF secretion (34,35). The results of our previous study showed that the impaired learning and memory abilities and decreased expression of BDNF found in the hippocampus and PFC of high-fat diet-fed rats were associated with imbalanced expression of key molecules in the Wnt/β-catenin signaling pathway, including increased expression of CyclinD1 and decreased expression of phosphorylated β-catenin (26). Taken together with the protective role of BDNF in regulating cholesterol homeostasis (36), these findings suggest that the Wnt/β-catenin signaling pathway may be involved in neuropsychiatric dysfunction and the changes in the BDNF abundance caused by glucose and lipid metabolism disorders (37).

Triggering receptor expressed on myeloid cells (TREM), which was first discovered in 2000, is a relatively novel type of immunoglobulin family inflammatory stimulating receptor (38). TREM1 and TREM2 are two subtypes of this receptor, and they have been demonstrated to be involved in not only inflammatory and immune responses but also the regulation of neuropsychiatric function, including learning and memory. In addition to the relationship between AD and the gene polymorphisms of TREM1 or TREM2 (39), it has also been reported that TREM2 can promote the survival of microglia by activating the Wnt/β-catenin pathway (40), accelerate the clearance of amyloid protein and reduce oxidative stress injury in hippocampal neurons (41). Upregulation of the expression of TREM2 can inhibit hippocampal neuronal apoptosis (12) and improve the spatial cognitive impairment in a mouse model of AD (42). In addition, inhibiting the expression of TREM2 can promote the abnormal accumulation of amyloid protein (43) and aggravate the impairment of spatial learning ability in P301S transgenic mice (44). However, Jiang *et al* (42) reported that the overexpression of TREM2 in microglia could effectively improve AD-like neuropathological changes and spatial learning and memory impairments in 7-month-old APPswe/PS1dE9 mice, including amyloid deposition, neuroinflammation, and neuronal and synaptic loss. However, their subsequent study revealed that the same intervention could not improve neuropathological damage and memory impairment in 18-month-old APPswe/PS1dE9 mice (45). These findings suggest that the role of TREM2 may be altered according to different pathological statuses. Consistently, in our previous study, differential expression levels of TREM2 were found in the hippocampus of rats of different ages and this was correlated with their performance in the Morris water maze. Moreover, imbalanced expression of TREM2 was also found in the hippocampus and PFC of rats fed a high-fat diet, suggesting that TREM2 may be involved in neuropsychiatric injury caused by abnormal glucose and lipid metabolism (46).

The aim of the present study was to investigate the effect of high cholesterol stimulation on the morphology and function of neuronal cells, and to explore the potential mechanism of action of TREM2 in neuronal cells.

Materials and methods

Cell culture. The neuroblastoma cell line SH-SY5Y was obtained from Wuhan Sunnbio Technology Co, Ltd. The cells were plated in DMEM (HyClone, Cytiva), 10% FBS (Biological Industries; Sartorius AG), and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology) in a humidified incubator at 37˚C and supplied with 5% CO_2.

Cholesterol challenge. 16 mg of water-soluble cholesterol (MilliporeSigma) was dissolved in 10 ml PBS buffer solution to prepare a cholesterol mixture of 1,000 μM, which was

successively diluted 10 times with DMEM before use. In the MTT assay, the cells were stimulated with 0.1-100 μM cholesterol for 3, 6, 9, 12, 24, or 48 h, and the effects of different concentrations and times on cell viability were observed. For all subsequent experiments, cells were treated with 100 μM for 24 h.

MTT assay. SH-SY5Y cells were plated in a 96-well culture plate at a density of $1x10^4$ cells per well. After treatment with cholesterol, 10 μl MTT (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well and the plate was incubated at 37˚C for 4 h. The supernatant was discarded, 150 μl DMSO was added per well, and the plate was incubated at 37˚C for 10 min. Net, the absorbance was measured with a microplate reader at 490 nm.

Oil red O staining. SH-SY5Y cells were plated in 6-well culture plates at a density of $2x10^5$ cells per well. At the end of the stimulation period, the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 10 min at 37˚C. Next, cells were washed twice, then stained with 0.5% Oil red O for 30 min at 37˚C. After staining, the cells were washed once with 60% isopropanol, washed with PBS until a colorless solution was obtained, and observed under a fluorescent inverted microscope at 50x magnification.

Cell cycle analysis. Cells ($5x10^6$) were collected and fixed in 70% ice-cold ethanol at -20˚C overnight. The cells were then collected and resuspended in PBS supplemented with 100 ng/ml RNase A and 50 ng/ml propidium iodide (PI) for 30 min at 37˚C. After staining, the distribution of cell cycle stage was assessed using a flow cytometer (CytoFLEX A00-1-1102, Beckman Coulter Biotechnology) and analysis software (CytExpert 2.4, Beckman Coulter Biotechnology).

Western blotting. SH-SY5Y cells were lysed using RIPA lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology) supplemented with containing protease inhibitors (cat. no. ST507-10mL, Beyotime Institute of Biotechnology) and phosphatase inhibitors (cat. no. ST019-10mM, Beyotime Institute of Biotechnology). The protein samples were prepared, resolved using 12.5% SDS gels by SDS-PAGE, and transferred to PVDF membranes (cat. no. IPVH00010; MilliporeSigma). The membranes were blocked using 5% skimmed milk for 2 h at room temperature and incubated with antibodies against β-catenin, p-β-catenin (1:800; Abcam), TREM1 (1:2,000; Abcam), TREM2 (1:1,000; Abcam), CyclinD1 (1:1,000; Abcam), nesfatin-1 (1:5,000; Technology), GSK3β, p-GSK3β (Ser9) (1:1,000; Cell Signaling Technology, Inc.), Copine-6 (1:1,000; Santa Cruz Biotechnology, Inc.), BDNF (1:1,000; Abcam), or β-actin (1:1,000; OriGene Technologies, Inc.) overnight at 4˚C and then incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000; OriGene Technologies, Inc.) at 37˚C for 2 h. Densitometry analysis was performed using ImageJ 1.53 (National Institute of Health).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and converted to cDNA using a RT kit according to the manufacturer's protocol (Takara Bio, Inc.). qPCR was performed in a thermal cycler with the following amplification program: 95˚C for 3 min followed by 40 cycles of 95˚C for 10 sec and 55˚C for 30 sec. The sequences of the primers used were: Human SREBP1 forward, 5'ACACAG CAACCAGAAACTCAA3' and reverse, 5'AGTGTGTCCTCC ACCTCAGTCT3'; human SREBP2 forward, 5'AGCAGAGTT CCTTCTGCCAT3' and reverse, 5'GACAGTAGCAGGTC ACAGGT3'; and human β-actin forward, 5'TTGCCGACA GGATGCAGAA3' and reverse, 5'GCCGATCCACACGGA GTACTT3'. Expression was calculated using the $2^{-\Delta\Delta Cq}$ method and normalized to the respective loading control.

Overexpression of TREM2. During the logarithmic growth phase, SH-SY5Y cells were counted and plated into 6-well culture plates with 2 ml cell suspension ($2x10^5$ cells/well). After the cells had adhered, the medium was discarded, and the cells were washed with PBS twice. A total of 1,600 μl Opti-MEM (Thermo Fisher Scientific, Inc.) was gently added to each well and the cells were then incubated at 37˚C for 12 h. Subsequently, 0.5 μg empty vector (pCMV-T7-3xFLAG-MCS-Neo, Wuhan MiaoLing Plasmid Company) or 0.5 μg TREM2 vector (pECMV-3xFLAG-TREM2, Wuhan MiaoLing Plasmid Company) were dissolved in 20 μl of sterile water, and 5 μl of the dissolved plasmid was added to 5 μl Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) and 200 μl Opti-MEM, and this solution was incubated at room temperature for 20 min. After incubation, the mixture was added to the corresponding wells in the 6-well plate, and the cells were incubated at 37˚C for a further 8 h. Next, the culture medium was removed, the cells were washed with PBS twice, and treated with either control (normal medium) or cholesterol-containing medium, and further incubated at 37˚C for 24 h. The experiment consisted of six groups: Control, cholesterol, control + empty vector, control + TREM2 vector, cholesterol empty vector, and cholesterol + TREM2 vector.

Statistical analysis. All data were analyzed using SPSS (version 17.0, SPSS Inc.). Data are presented as the means ± SEM of at least three repeats. Data were compared using Student's t-test or a one-way ANOVA followed by a Tukey's post hoc test. GraphPad Prism 8 (GraphPad Software, Inc.) was used for Pearson correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Dose- and time-dependent effects of cholesterol treatment on the viability of SH-SY5Y cells. SH-SY5Y cells were cultured and treated with cholesterol (0.1, 1, 10, or 100 μM) for 24 h. As shown in Fig. 1A, compared with that of the control group, the viability of SH-SY5Y cells gradually decreased in a dose-dependent manner, and the viability of cells treated with 100 μM cholesterol was ~50% ($F_{0.05,4}$=70.344, P≤0.0001). Fig. 1B shows the time-dependent effect of cholesterol on cell viability; a plateau in the effect of time was observed at 24 h ($F_{0.05,6}$=24.095, P≤0.0001). Therefore, for subsequent experiments, cells were treated with 100 μM cholesterol for 24 h.

Effect of high cholesterol treatment on the cell cycle distribution of SH-SY5Y cells. As shown in Fig. 2, the number of SH-SY5Y cells in the G0/G1 after 24 h of stimulation with

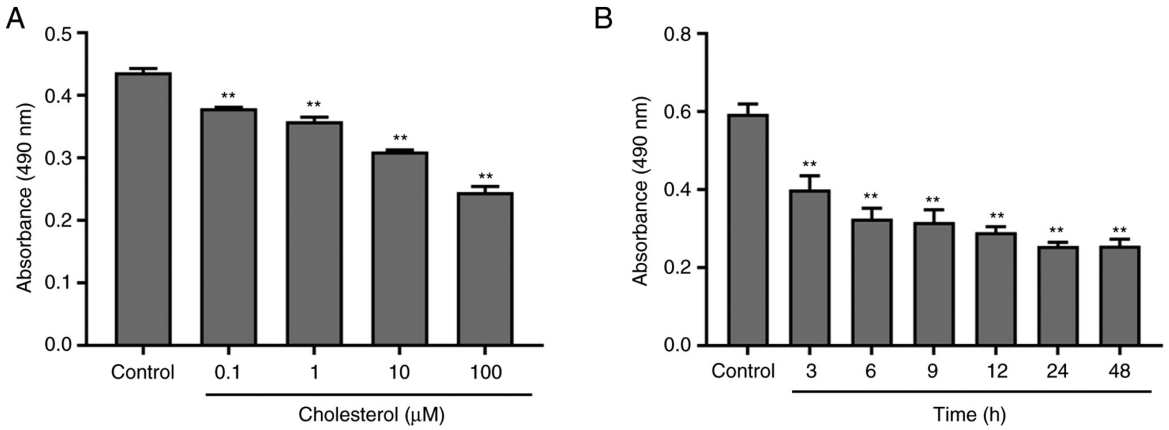

Figure 1. Dose- and time-dependent effects of cholesterol treatment on the viability of SH-SY5Y cells. Data are presented as the means ± SEM of three repeats each from two separate passages (n=6). SH-SY5Y cells were treated with (A) increasing concentrations of cholesterol (0.1-100.0 μM) or (B) for increasing lengths of time from 3-48 h. **P<0.01 vs. control.

100 μM cholesterol was significantly higher than that of the control group (t=4.354, P=0.022). However, cholesterol treatment had no significant effect on the proportion of cells in the S and G2/M phases.

Effects of high cholesterol treatment on lipid deposition and SREBP expression in SH-SY5Y cells. Fig. 3A and B show a typical graph of the Oil red O staining of each group. Compared with the results observed in the control group, high cholesterol treatment altered the morphology of SH-SY5Y cells from a spindle-like to an oval-like shape and increased the number of Oil red O-stained cells, with stained fat granules observed both inside and outside the cell. The protein (Fig. 3C-F) and mRNA (Fig. 3G and H) expression levels of SREBP1 (t=7.655, P≤0.0001 and t=6.994, P=0.020; respectively) and SREBP2 (t=5.643, P≤0.0001, t=1.582, P=0.254) in the cholesterol-stimulated SH-SY5Y cells were markedly higher than that in the control group.

Effect of high cholesterol treatment on the expression of BDNF, Copine-6, TREM1, and TREM2 in SH-SY5Y cells. Fig. 4A and B show the protein expression levels of BDNF, Copine-6, TREM1, and TREM2 in SH-SY5Y cells. Treatment with 100 μM cholesterol for 24 h markedly decreased the protein expression levels of BDNF (t=2.732, P=0.020), Copine-6 (t=6.144, P≤0.0001), and TREM2 (t=8.189, P≤0.0001) and markedly increased the protein expression levels of TREM1 (t=2.871, P=0.015) compared with that in the control group. Pearson correlation analysis showed that the protein expression levels of BDNF in SH-SY5Y cells were positively correlated with the expression of Copine-6 (r=0.888, P=0.003, Fig. 4C) and TREM2 (r=0.901, P=0.002, Fig. 4D) but negatively correlated with the expression of TREM1 (r=-0.715, P=0.046, Fig. 4E).

Effects of high cholesterol treatment on the protein expression levels of nesfatin-1 and key molecules in the Wnt/β-catenin signaling pathway in SH-SY5Y cells. As shown in Fig. 5A and B, the cholesterol-stimulated SH-SY5Y cells exhibited a lower p-β-catenin/β-catenin protein expression ratio and higher protein expression levels of nesfatin-1 (t=4.299, P=0.001) and CyclinD1 (t=5.619, P≤0.0001) and an increase in the p-GSK3β/

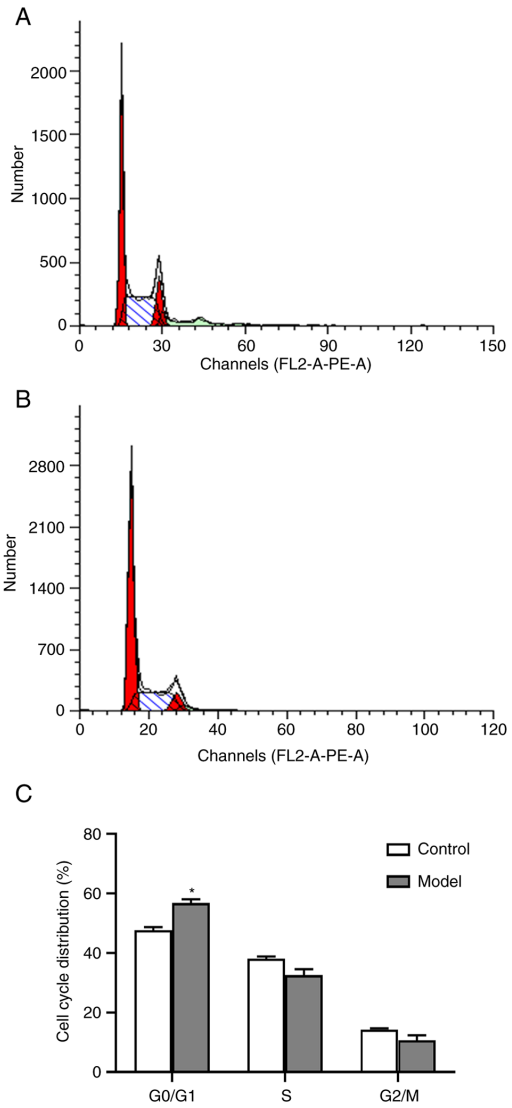

Figure 2. Effect of high cholesterol on the cell cycle distribution of SH-SY5Y cells. Data are presented as the mean ± SEM of two repeats each from two separate passages (n=4). The number of SH-SY5Y cells in the G0/G1 phase increased significantly after 24 h of stimulation with 100 μM cholesterol. (A) Control, (B) Model, (C) statistical analysis of the cell cycle distribution results. *P<0.05 vs. control. Model, SH-SY5Y cells treated with 100 μM for 24 h.

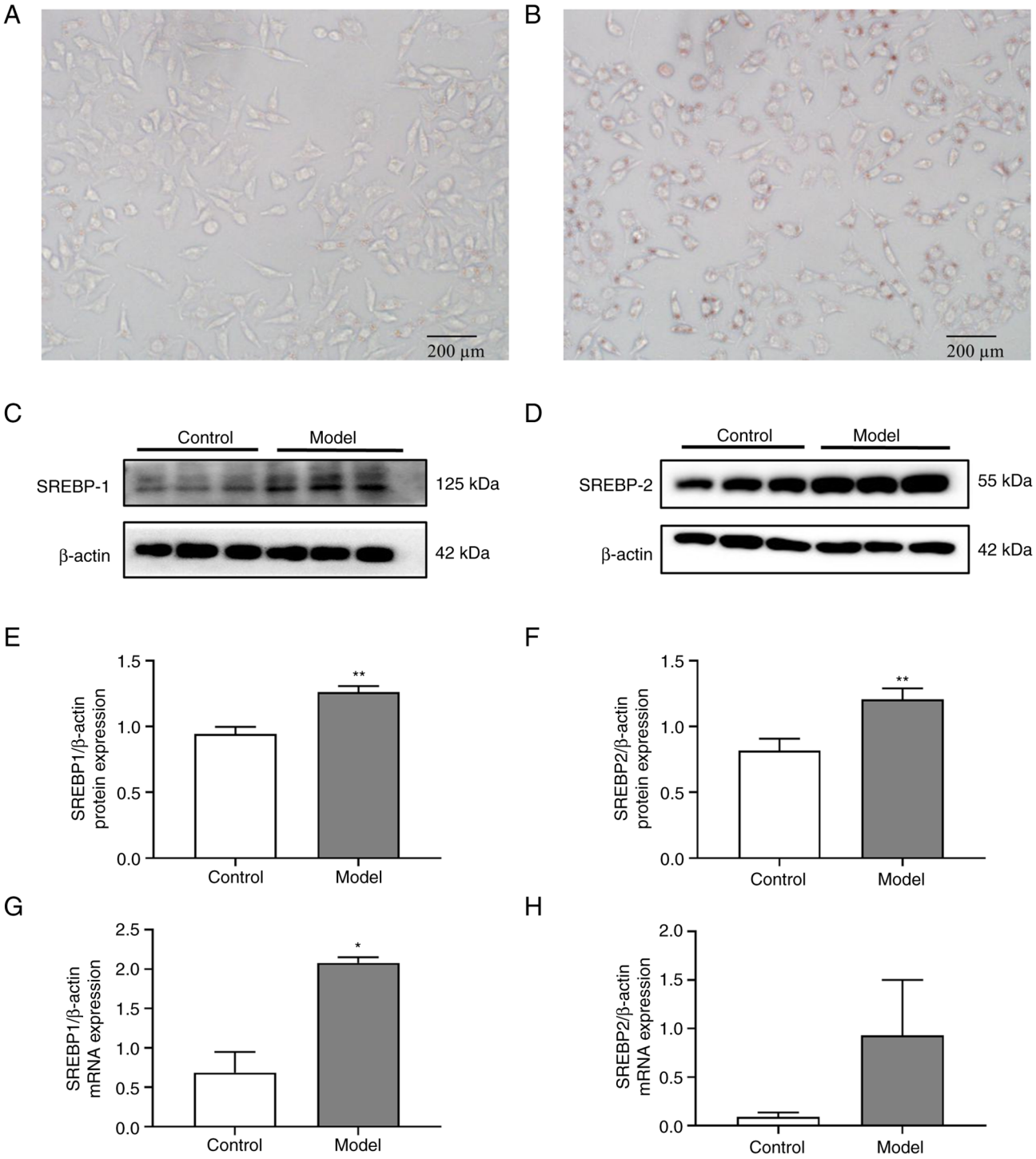

Figure 3. Effects of high cholesterol treatment on lipid deposition and SREBP expression in SH-SY5Y cells. Data are presented as the mean ± SEM of three repeats from two separate passages (n=6). (A and B) Morphological changes in SH-SY5Y cells and lipid accumulation were detected by Oil red O staining. Magnification, x50. (C and D) Representative blots of SREBP1 and SREBP2 protein expression. (E and F) Densitometry analysis of the western blotting results. (G and H) Quantification of SREBP1 and SREBP2 mRNA expression levels. *P<0.05, **P<0.01 vs. control. Model, SH-SY5Y cells treated with 100 μM for 24 h; SREBP, sterol regulatory element binding protein.

GSK3β ratio (t=2.883, P=0.015) compared with the control cells. No significant differences in CyclinD1 expression levels were observed between the two groups. Pearson correlation analysis showed that the protein expression levels of nesfatin-1 in SH-SY5Y cells was positively correlated with the expression of p-GSK3β (r=0.821, P=0.013, Fig. 5C), CyclinD1 (r=0.764, P=0.027, Fig. 5D) and TREM1 (r=0.706, P=0.050, Fig. 5E) but negatively correlated with the expression of TREM2 (r=-0.719, P=0.044, Fig. 5F), p-β-catenin (r=-0.869, P=0.005, Fig. 5G) and Copine-6 (r=-0.760, P=0.029, Fig. 5H).

Effect of TREM2 overexpression on dysregulation of BDNF, Copine-6, TREM1, and TREM2 protein expression induced by high cholesterol in SH-SY5Y cells. The mRNA (t=16.188,

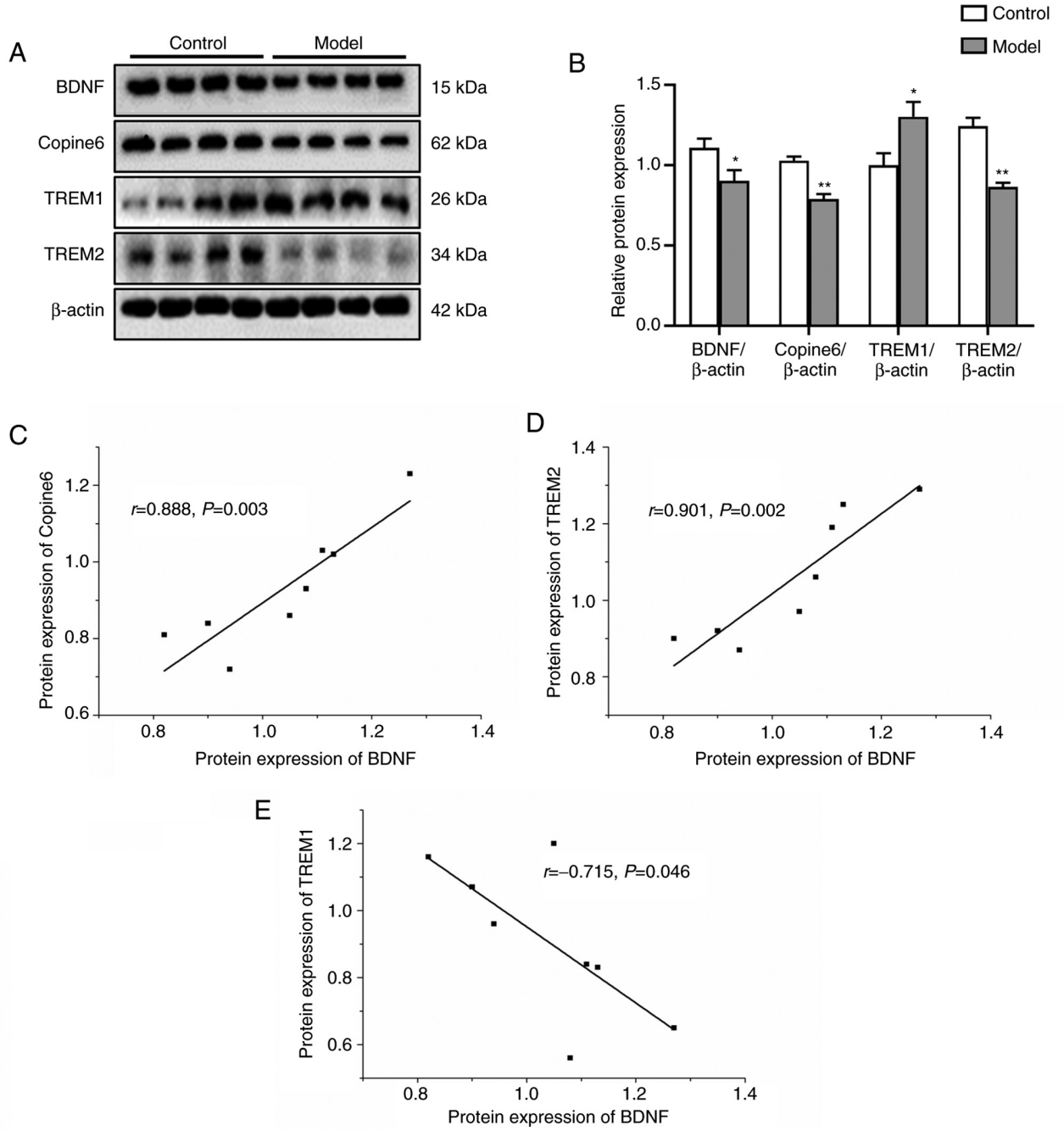

Figure 4. Effect of high cholesterol on the protein expression levels of BDNF, Copine-6, TREM1, and TREM2 in SH-SY5Y cells. Data are presented as the mean ± SEM of three repeats each from two separate passages (n=6). (A) Representative blots of BDNF, Copine-6, TREM1, and TREM2 expression in SH-SY5Y cells. (B) Densitometry analysis of the western blotting. (C-E) Pearson correlation analysis of BDNF with Copine-6, TREM1 and TREM2. *P<0.05, **P<0.01 vs. control. Model, SH-SY5Y cells treated with 100 μM for 24 h; TREM, triggering receptor expressed on myeloid cells.

P=0.004) and protein (t=9.849, P=0.010) expression levels of TREM2 after transfection of the TREM2-overexpressing plasmid were significantly increased (Fig. 6A-C) compared with those of the control group. These results indicated the successful establishment of the TREM2 overexpression model.

As shown in Fig. 7A and B, the cholesterol-stimulated SH-SY5Y cells exhibited lower protein expression levels of BDNF ($F_{0.05,5}$=7.497, P=0.033) and Copine-6 ($F_{0.05,5}$=4.565, P=0.044) and higher expression of TREM1 ($F_{0.05,5}$=5.597, P=0.024) compared with the control cells. However, this imbalance in expression was reversed after overexpression of TREM2 as shown in Fig. 7B.

Effects of TREM2 overexpression on the protein expression levels of nesfatin-1 and key molecules in the Wnt/β-catenin signaling pathway induced by high cholesterol in SH-SY5Y cells. As shown in Fig. 7C and D, cholesterol-stimulated SH-SY5Y cells showed higher expression levels of nesfatin-1 ($F_{0.05,5}$=2.824, P=0.015) and p-GSK3β ($F_{0.05,5}$=19.835, P≤0.0001) and lower expression levels of p-β-catenin ($F_{0.05,5}$=1.222, P=0.046) compared with the control cells. Overexpression of TREM2 reversed the increase in expression of nesfatin-1, and p-GSK3β induced by cholesterol treatment, and no significant change in the expression of CyclinD1 and p-β-catenin was observed in the TREM2 overexpression group.

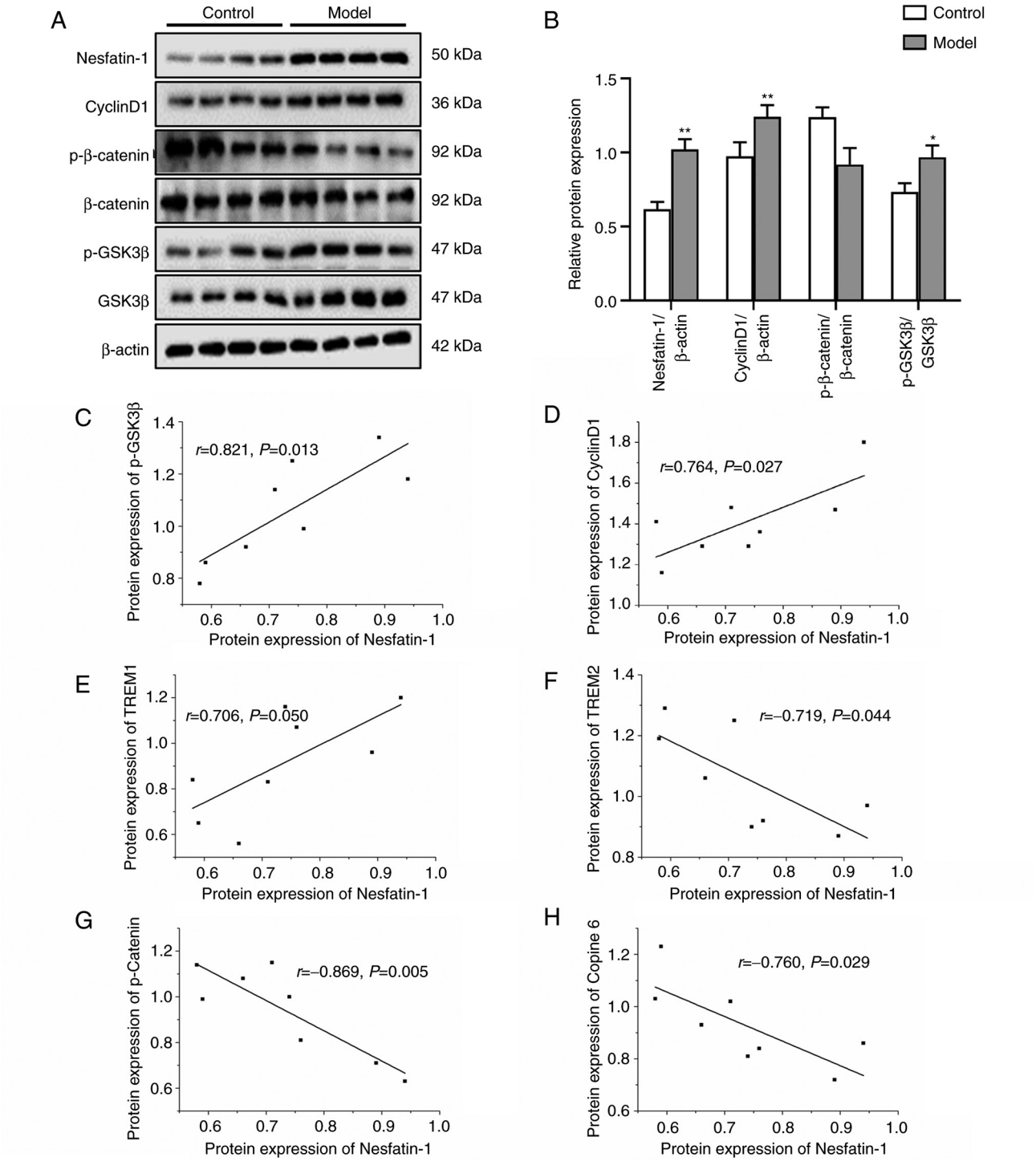

Figure 5. Effects of high cholesterol on the protein expression levels of nesfatin-1 and key molecules in the Wnt/β-catenin signaling pathway in SH-SY5Y cells. Data are presented as the mean ± SEM of three repeats each from two separate passages (n=6). (A) Representative blots of nesfatin-1, CyclinD1, p-β-catenin, β-catenin, p-GSK3β, GSK3β protein expression in SH-SY5Y cells. (B) Densitometry analysis of the western blotting results. (C-H) Pearson correlation analysis of nesfatin-1 with p-GSK3β, CyclinD1, TREM1, TREM2, p-β-catenin and Copine 6. *P<0.05, **P<0.01 vs. control. Model, SH-SY5Y cells treated with 100 μM for 24 h.

Discussion

In the present study, the potential role of TREM2 in cell injury and metabolic dysfunction induced by high cholesterol in SH-SY5Y cells was investigated and the potential mechanism was explored. The results showed that cholesterol could induce cell injury and lipid accumulation in SH-SY5Y cells, as indicated by the decrease in cell viability, changes in cell morphology from a spindle to an oval shape, an increase in the number of cells in the G0/G1 phase, an increase in the number of Oil-red O-positive cells, and higher protein expression levels of the SREBPs. Moreover, cholesterol-stimulated SH-SY5Y cells showed marked decreases in the protein expression levels of BDNF, nesfatin-1, Copine-6, TREM2,

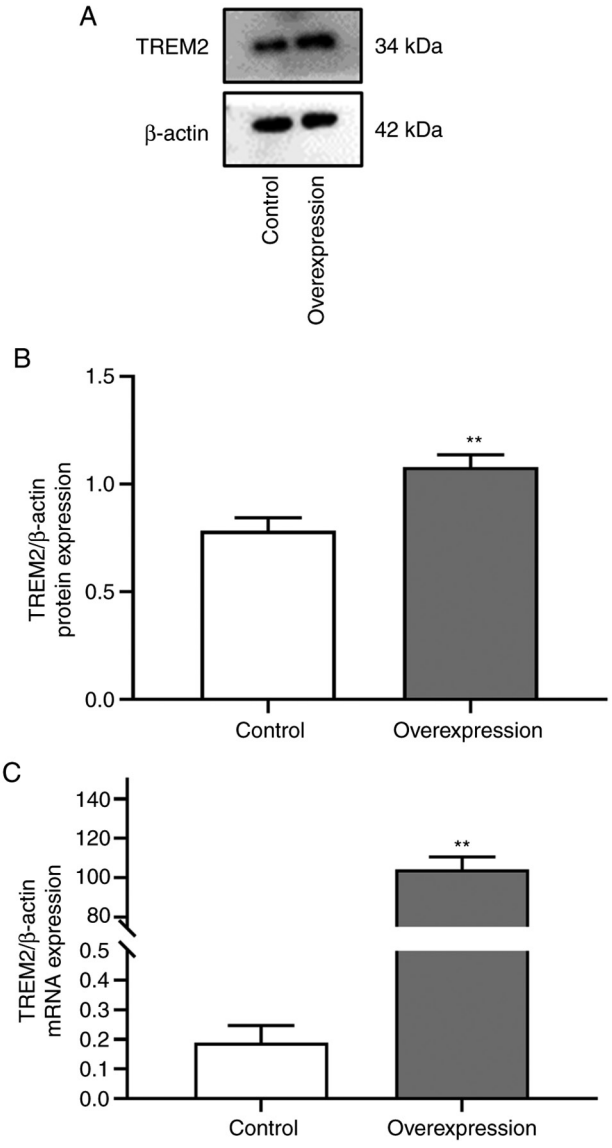

Figure 6. Protein and mRNA expression levels of TREM2 after transfection of the TREM2-overexpressing plasmid in SH-SY5Y cells Data are presented as the mean ± SEM of three repeats each from two separate passages (n=6). (A) Representative blots of TREM2 in SH-SY5Y cells. (B) Densitometry analysis of the western blotting results. (C) Quantification of TREM2 mRNA expression levels. **P<0.01 vs. control. Model, SH-SY5Y cells treated with 100 μM for 24 h; TREM, triggering receptor expressed on myeloid cells.

and p-β-catenin and increases in the expression of TREM1 and p-GSK3β. However, the imbalanced expression of BDNF, Copine-6, nesfatin-1 and p-GSK3β induced by cholesterol in SH-SY5Y cells was reversed after the overexpression of TREM2. These results suggested that TREM2 was associated with the neurotoxicity of cholesterol in SH-SY5Y cells.

As the basic structural component of the cell plasma membrane, cholesterol participates in the formation of dense myelin and plays an extremely important role in the structure and function of the central nervous system (47,48). An increasing body of evidence has demonstrated that hypercholesterolemia is an important risk factor for neuropsychiatric diseases, including AD (49,50). The results of a 13-year follow-up study (49) showed that higher levels of TC and LDL-C increased the risk of AD and accelerated the accumulation of β-amyloid molecules by 20-fold (51), the latter of which is a key factor in neuropathological damage in patients with AD (52,53). Consistent with the findings that excessive cholesterol levels can cause nerve cell injury and apoptosis (54), the results of the present study showed that stimulation with high cholesterol resulted in a decrease in the viability of SH-SY5Y cells in a dose- and time-dependent manner accompanied by morphological changes from a spindle-like shape to an oval-like shape, and a significant increase in the proportion of cells in the G0/G1 phase. Moreover, after stimulation with cholesterol, SH-SY5Y cells exhibited significantly higher lipid accumulation, as indicated by the increase in the number of Oil red O-positive cells, and in the protein expression levels of SREBP1 and SREBP2. These results indicated that cholesterol could induce dyslipidemia resulting in cell injuries and intracellular lipid deposition.

Synaptic plasticity is an important characteristic of the neural system that plays a significant role in maintaining the structure and function of the neural system. BDNF is a member of the neurotrophic factor family, and Copine-6 is a brain-specific, calcium-dependent protein. These molecules play important roles in regulating synaptic plasticity (55,56), and their secretion and abundance are involved in the activity of the Wnt/β-catenin signaling pathway (57). Moreover, BDNF participates in the regulation of cholesterol homeostasis (58,59) and improves nerve damage induced by high cholesterol levels (58). Consistently, the BDNF-related imbalance in the expression of Copine-6 and synapse-associated proteins in the hippocampus and PFC of stressed (60) or hyperlipidemic (14,46) rats has been demonstrated in our previous studies. In line with these findings, the results from the present study showed that cholesterol-induced downregulation of BDNF and Copine-6 protein expression in SH-SY5Y cells, and the expression of these molecules was positively correlated with each other. These results again suggest the role of BDNF-related Copine-6 expression in high cholesterol-related neural injury.

Nesfatin-1 may be involved in mediating metabolic disorders and neuropsychiatric injury. Our previous study showed that the plasma concentration of nesfatin-1 was markedly increased in high-fat diet-induced nonalcoholic fatty liver disease rats and was significantly correlated with impaired learning and memory abilities and imbalanced protein expression of BDNF in the hippocampus (46). In line with this finding, the protein expression levels of nesfatin-1 in SH-SY5Y cells were upregulated after stimulation with high cholesterol. In addition, SH-SY5Y cells stimulated with high cholesterol showed decreased expression of p-β-catenin, a key molecule in the Wnt signaling pathway, and increased expression of p-GSK3β. The results from the correlation analysis showed that the expression of nesfatin-1 was positively correlated with the expression of p-GSK3β and Cyclin-D1 and negatively correlated with the expression of p-β-catenin. This result was consistent with the previous finding that cholesterol metabolism is closely related to the activity and function of the Wnt signaling pathway (61).

Given the findings that the variants of TREM2 could markedly increase the risk of AD, this receptor has been a major focus in the neuroscience (62-64). Although studies have shown that TREM2 is exclusively expressed in microglia (62),

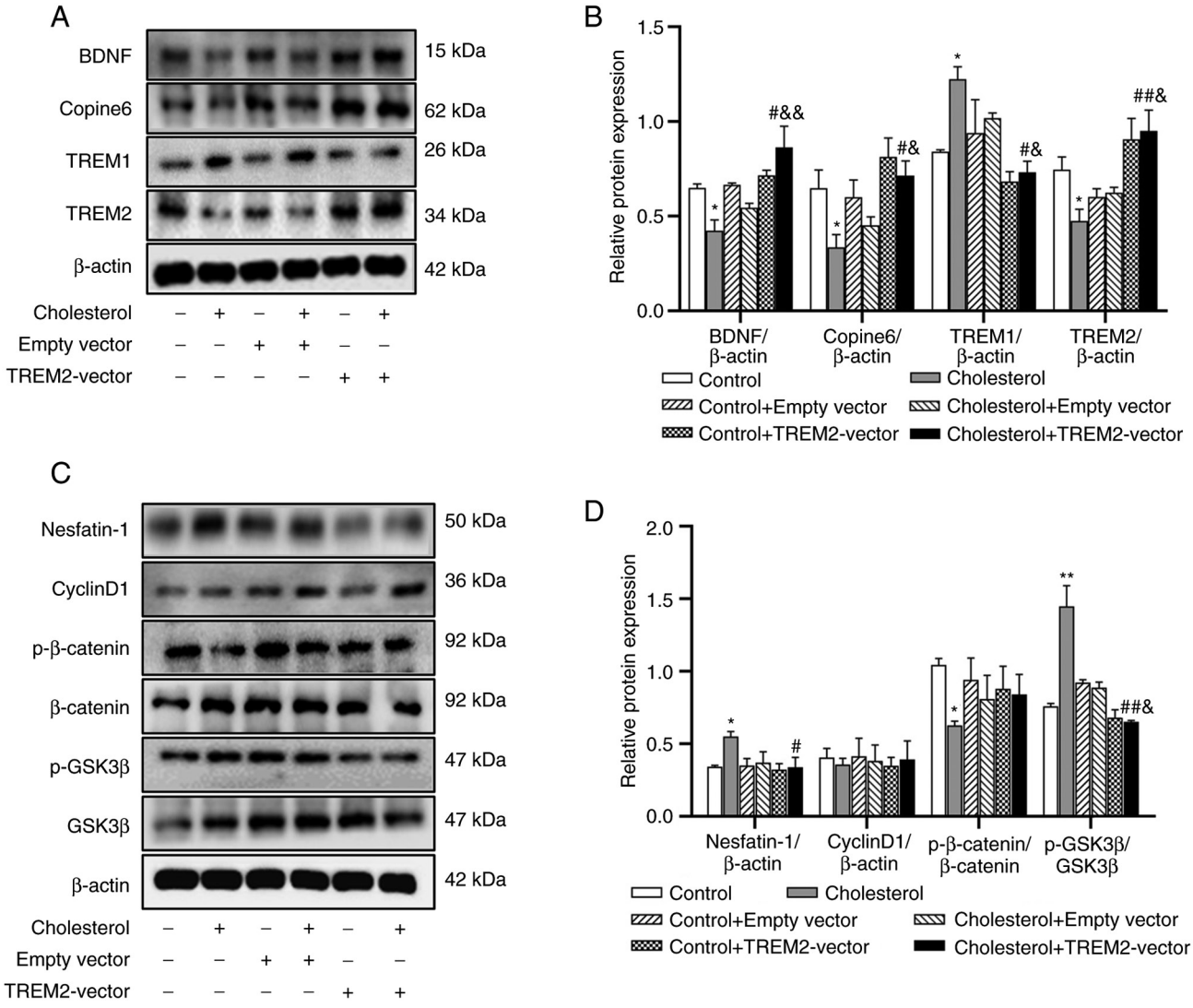

Figure 7. Effects of TREM2 overexpression on the protein expression levels of BDNF, Copine-6, TREM1, TREM2, nesfatin-1, and key molecules in the Wnt/β-catenin signaling pathway following treatment with 100 μM cholesterol in SH-SY5Y cells. Data are presented as the mean ± SEM of three repeats each from two separate passages (n=6). (A) Representative blots of BDNF, Copine-6, TREM1, and TREM2 expression in SH-SY5Y cells. (B) Densitometry analysis of the western blotting results. (C) Representative blots of nesfatin-1, CyclinD1, p-β-catenin, β-catenin, p-GSK3β, GSK3β expression in SH-SY5Y cells. (D) Densitometry analysis of the western blotting results. *P<0.05, **P<0.01 vs. control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. cholesterol. $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. cholesterol + empty vector.

double immunofluorescence staining has demonstrated that TREM2 is expressed in microglia (ionized calcium-binding adapter molecule 1), astrocytes (glial fibrillary acidic protein), and neurons (neuronal nucleus) in the brain (35,65).

TREM2 is required by microglia to respond to Aβ deposition and to limit neuronal degeneration, and microglia fail to colocalize with Aβ plaques in TREM2$^{-/-}$ mice (9). Moreover, TREM2 could regulate microglial cholesterol metabolism upon chronic phagocytic challenge, and loss of TREM2 or apolipoprotein E may result in dysregulated cholesterol transport and metabolism in microglia (13,66). TREM2 deletion not only causes cholesterol ester overload in microglia but also abrogates the recruitment of macrophages to enlarged adipocytes, leading to massive adipocyte hypertrophy, systemic hypercholesterolemia, inflammation, and glucose intolerance (67). These findings suggest that brain cholesterol metabolism and lipid signaling through TREM2 play major roles in age-related neurodegeneration processes (18,67). However, little is known regarding the role of TREM2 in cholesterol metabolism in neurons. Using SH-SY5Y cells, Huang *et al* (54) demonstrated that cholesterol overload in neuronal cells could result in an imbalance in cholesterol homeostasis and increase protein expression levels of truncated tyrosine kinase B, flotillin-2, Beta-Secretase (BACE) and β-amyloid (Aβ) and thereby inducing cell apoptosis, and the underlying mechanism involved the downregulation of BDNF. Consistent with these findings, the results of the present study showed that in addition to decreased viability, increased lipid accumulation, and imbalanced synaptic plasticity in SH-SY5Y cells, high cholesterol induced a decrease in the protein expression levels of TREM2. Moreover, the dysregulated expression of BDNF, Copine-6, and p-GSK3 in SH-SY5Y cells induced by cholesterol could be reversed by overexpression of TREM2. Taken together with the findings that microglia in the brains of TREM2$^{-/-}$ mice could phagocytose myelin debris but failed to clear myelin cholesterol,

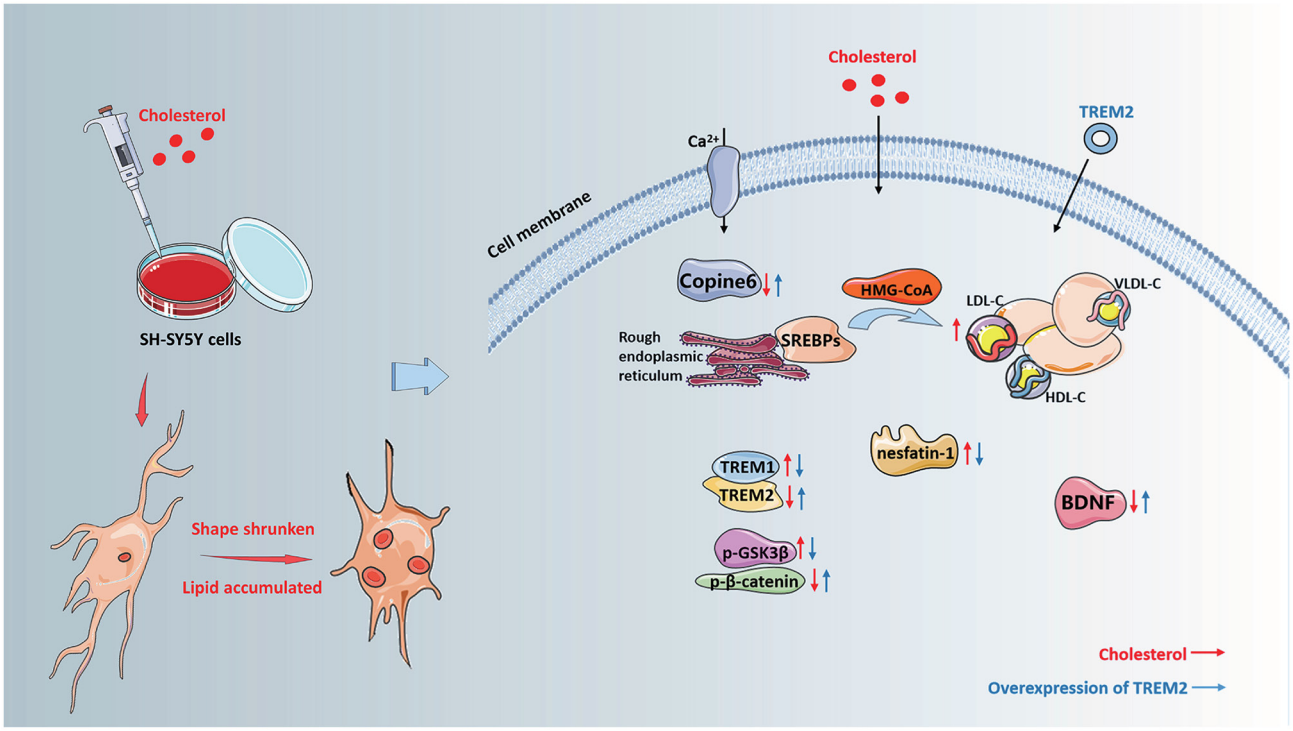

Figure 8. High cholesterol concentrations induced cell injury in SH-SY5Y cells, and the imbalance in TREM2 expression may serve an important role in injury.

resulting in cholesteryl ester accumulation (66), and the significant correlation between TREM2 protein expression and BDNF, Copine-6 and nesfatin-1, these results suggested that TREM2 also plays a major role in cholesterol metabolism in neuronal cells.

This study has several limitations that should be addressed in future studies. First, only one cell line was used in this study, and the role of TREM2 in nerve injury induced by high cholesterol should be verified using additional types of neuronal cells, particularly primary neurons. Second, the effect of high cholesterol on the protein expression levels of BDNF, Copine-6, and key proteins in the Wnt/β-catenin signaling pathway has been detected regardless of the overexpression of TREM2. In addition, an explanation for the fact that TREM1 and TREM2 are inversely correlated with each, as in other experimental models, could not be provided, and the accurate mechanism should be explored in detail in the future.

In conclusion, the results of the present study suggest that a high concentration of cholesterol can induce cell injury in SH-SY5Y cells, and that an imbalance expression in TREM2 may play an important role in these injuries. A summary schematic is shown in Fig. 8.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG, ZQ and YH conceived and designed the study. YH and QZ performed the experiments, analyzed the data, and drafted the manuscript. MF, MM, and JX analyzed and interpreted the data. XG and SL performed cell culture. JG, YH and QZ confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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