

## Original Article

# PCSK9 regulates apoptosis in human neuroglioma u251 cells via mitochondrial signaling pathways

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**Abstract:** Proprotein convertase subtilisin/kexin type 9 (PCSK9), belongs to a family of proprotein convertases (PCs), encodes a neural apoptosis-regulated convertase 1. However, the precise role of PCSK9 during glioma cells apoptosis has not been reported. Therefore, we examined the effects of knockdown and overexpression of PCSK9 on apoptosis of human neuroglioma U251 cells, and investigated the underlying mechanisms of apoptosis. We found that PCSK9 regulated cells proliferation as determined by CCK-8 and Hoechst staining analysis. In addition, western blot results showed that PCSK9 siRNA promote apoptosis via activation of caspase-3 and down-regulation of the anti-apoptotic proteins, XIAP and p-Akt, while PCSK9 overexpression inhibited apoptosis. Moreover, PCSK9 siRNA improved the ratio of Bax/Bcl-2 which leads to the release of cytochrome c, while PCSK9 overexpression decreased it. Taken together, these data demonstrate that PCSK9 may regulate apoptosis through mitochondrial pathway and is expected to be a promising therapeutic strategy for the malignant glioma.

**Keywords:** PCSK9, neuroglioma, apoptosis, mitochondrial pathway

## Introduction

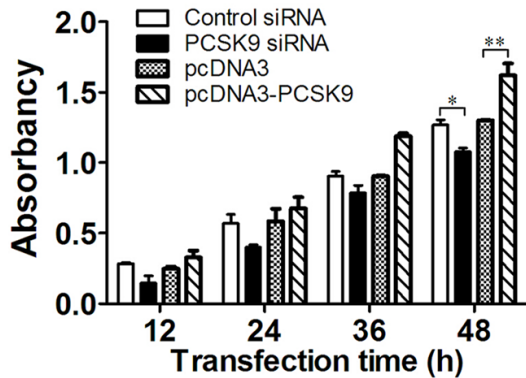
Human gliomas are one of the most lethal types of primary central nervous system tumors, are characterized by aggressive proliferation and expansion into surrounding brain tissue, and their biological features make successful treatment very difficult [1]. Currently, the standard approaches to treating for patients with glioma are surgical resection, irradiation, and conventional chemotherapy [2]. Due to the resistance of tumor cells to conventional therapies, it is very difficult to treat gliomas [3-5]. However, the identification of some genes or proteins that effectively inhibit tumor growth is expected to break through this bottleneck [6]. Despite great efforts to improve treatment, the survival time of patients remains 14-16 months after diagnosis [7]. Therefore, it is particularly important to find an effective treatment for gliomas. So far, it has been found that some genes are up- or down-regulated in tumorigenesis. Unfortunately, it has not found a truly effective treatment of gliomas.

Proprotein convertase subtilisin/kexin type 9 (PCSK9), a member of the subtilisin family of

proprotein convertases (PCs), encodes a neural apoptosis-regulated convertase 1 [8]. PCSK9 was initiated discovered to maintain plasma lipid homeostasis by regulating low-density lipoprotein receptors (LDLRs) [9-11]. The extensive investigation regarding PCSK9 revealed novel functions, including cell apoptosis, inflammatory response, neuronal development and tumor metastasis [12-15]. In vivo studies also showed that PCSK9 is implicated in these processes [16, 17]. One group revealed that PCSK9 deficiency reduced liver metastasis by its ability to lower cholesterol levels and by possibly enhancing TNF $\alpha$ -mediated apoptosis [13]. Ranheim *et al* demonstrated that PCSK9 significantly down-regulated the poly (ADP-ribose) polymerase (PARP) family in HepG2 cells through microarray analysis of D374Y-PCSK9 [18]. Furthermore, the pro-apoptotic factor TNF $\alpha$  levels were increased and the anti-apoptotic factor Bcl-2 levels were decreased [13], confirming the anti-apoptotic function of PCSK9.

So far, the precise mechanism through which PCSK9 inhibits neuronal apoptosis has not been fully elucidated. In this study, our goal is to identify the function of PCSK9 during apoptosis

## PCSK9 regulates apoptosis via mitochondrial pathways



**Figure 1.** Effect of PCSK9 on U251 cells proliferation. U251 cells were transfected with PCSK9 siRNA or pcDNA3-PCSK9 for indicated time. Cell proliferation was examined by CCK-8 assay. The OD value in each well was read at the wave lengths of 450 on a microtiter plate reader. Data represented means  $\pm$  SD of OD450 at 12, 24, 36, 48 h (each concentration was tested in triplicate) (\* $P < 0.05$ ; \*\* $P < 0.01$ ).

of glioma cells. We found that PCSK9 may regulate apoptosis through mitochondrial pathway in U251 cells.

### Materials and methods

#### Reagents and antibodies

Rabbit anti-human cleaved caspase-3 (c-caspase-3), Bcl-2, Bax, COXIV, XIAP antibodies and mouse anti-GAPDH antibody were purchased from Proteintech (Proteintech, Wuhan, China). Rabbit anti-human PCSK9 and cytochrome c antibodies were purchased from Abcam (Abcam, Cambridge, UK). Rabbit anti-human phosphor-Akt (p-Akt) and Akt antibodies were purchased from Cell Signaling (Cell Signaling, MA, USA). Anti-rabbit or mouse IgG-HRP were purchased from Proteintech. Cell counting kit-8 and Hoechst 33258 were purchased from Beyotime (Beyotime, Haimen, China). Other reagents were of analytical grade.

#### Plasmid construction, cell culture and transfection

To construct pcDNA3-PCSK9, human PCSK9 gene (NM\_174936) was PCR-amplified from human blood cDNA with gene-specific primers and cloned into the multiple cloning site of pcDNA3 (Invitrogen, USA). The construct was confirmed by DNA sequencing.

Human neuroglioma U251 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were routinely grown in Dulbecco's Modified Eagle Media (DMEM, Hyclone, Beijing, China) contained with 10% fetal bovine serum (FBS, Hyclone), 100 U/mL penicillin (Sigma, MO, USA) and 100  $\mu$ g/mL streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, and were fed every 2-3 d and were split 1 to 2 at 80% confluence.

U251 cells were transfected with 100 nM PCSK9 siRNA (Santa Cruz, sc-45482; Genbank ID for PCSK9: NM\_174936) or control siRNA (scrambled siRNA, a universal negative control) or pcDNA3-PCSK9 with Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions. At 48 h after transfection, the efficiency of PCSK9 expression was determined by western blot.

#### Cell proliferation assay

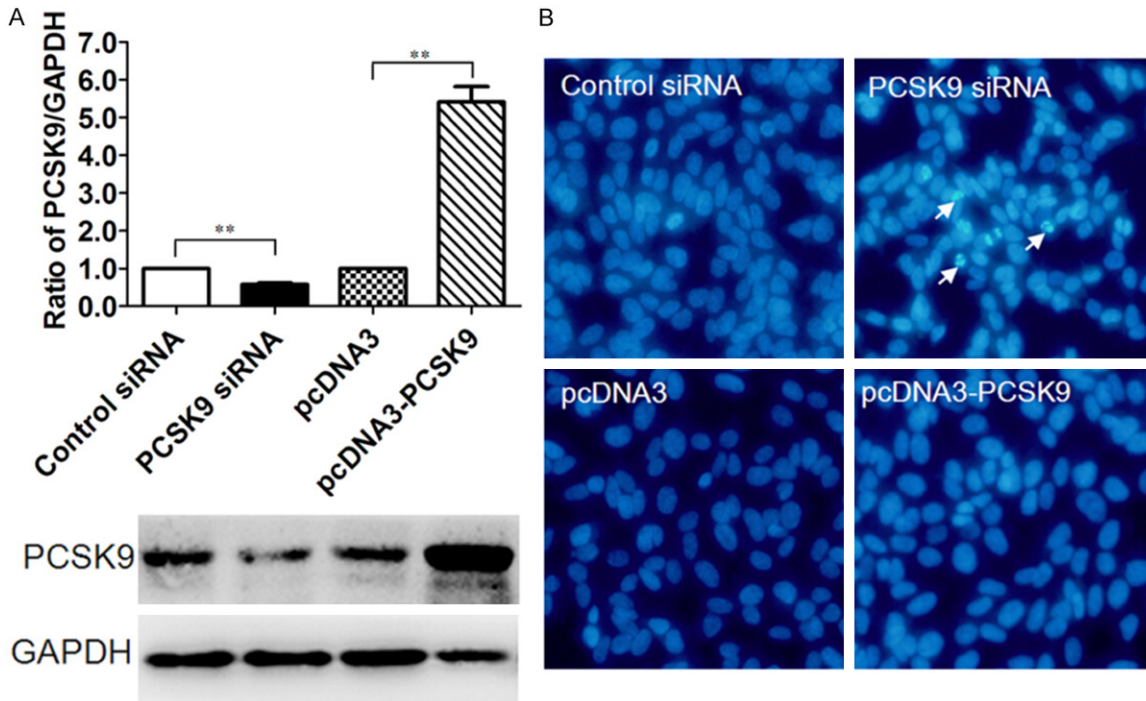
U251 cells were plated to each well of a 96-well plate at a density of  $5 \times 10^3$  cells/well in the culture medium. After 24 h of incubation, cells were transfected with PCSK9 siRNA or pcDNA3-PCSK9 or control for 12, 24, 36 and 48 h as described above, followed by the addition of 10  $\mu$ l CCK-8 solution. The cells were then incubated for 3 h at 37°C. Absorbance was measured at 450 nm in a spectrophotometer (Thermo, USA).

#### Morphological analysis after Hoechst 33258 staining

U251 cells were seeded in 24-well plates ( $6 \times 10^4$  cells per well) overnight, and were transfected with PCSK9 siRNA or pcDNA3-PCSK9 or control for 48 h. Then the cells were fixed and stained with Hoechst 33258. The apoptotic cells were visualized with fluorescence microscope (Leica, Germany).

#### Preparation of mitochondria and cytosol

Mitochondria/cytosol kit (Beyotime) was used to isolate mitochondria and cytosol according to the manufacture's protocol. After transfection as above, cells ( $5 \times 10^7$  cells) were collected by centrifugation at  $600 \times g$  for 5 min at 4°C, washed twice with ice-cold PBS and then resuspended in 500  $\mu$ l of isolation buffer containing protease inhibitors for 10 min on ice.



**Figure 2.** PCSK9 siRNA increased apoptosis of U251 Cells. U251 cells were transfected with PCSK9 siRNA or pcDNA3-PCSK9 for 48 h. A. The efficiency of PCSK9 expression was determined by western blot. GAPDH was included as a loading control. The bars indicated mean  $\pm$  S.D. (n = 3, \*\*, P < 0.01). B. After 48 h transfection, cells were incubated with Hoechst 33258 staining buffer. Healthy cells showed round and intact nuclei, whereas apoptotic cells exhibited nuclear karyopyknosis or fragmentation as the arrows showed.

The cells were mechanically homogenized with Dounce grinder. The unbroken cells, debris and nuclei were discarded by centrifugation at 800 g for 10 min at 4°C. The supernatants were centrifuged at 12000 g for 15 min at 4°C. The supernatant cytosol was collected and pellet fraction mitochondria was dissolved in 50  $\mu$ l of lysis buffer.

*Western blot*

U251 cells were transfected as described above and were lysed by using RIPA buffer. BCA Protein Assay Kit (Dingguo, Beijing, China) was used to measure the protein concentrations. The total protein was loaded onto 10% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed overnight at 4°C with indicated antibodies in TBST containing 1% BSA (w/v). Blots were then incubated for 1 h with anti-rabbit or mouse secondary antibodies. Immune complexes were detected using an ECL Detection Kit (Thermo, CA, USA) and quantified using a scanning densitometer with molecular analysis software (Bio-Rad, CA, U.S.A). Quantification of band density was done

using Quantity One software (Bio-Rad) with normalization to the GAPDH signal. Abundance of interested proteins in various groups was expressed relative to that under mock conditions.

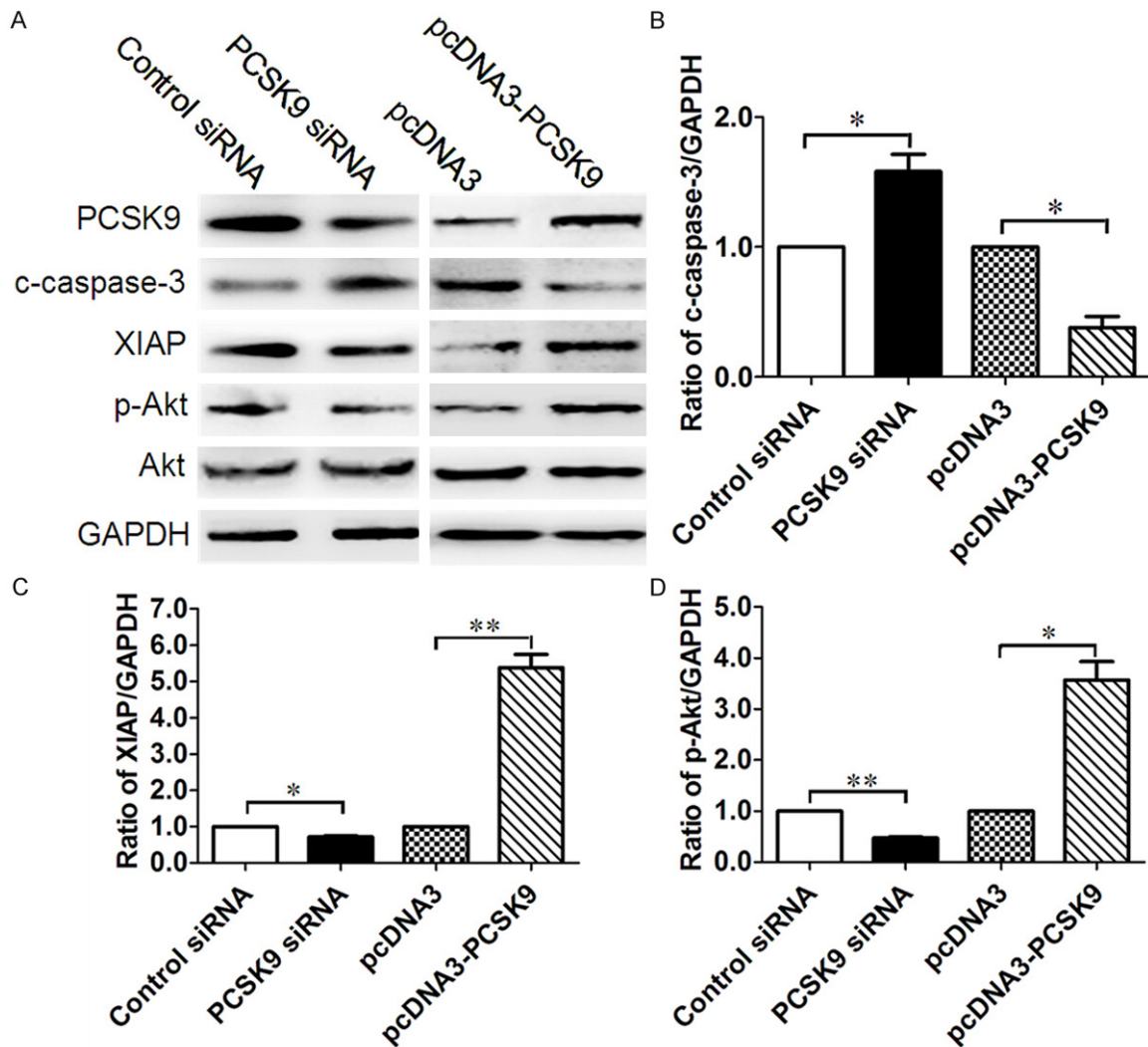
*Statistical analysis*

All data were presented as mean  $\pm$  S.D, and analyzed using Student's t-test and one-way ANOVA analysis to determine the levels of significance. A P value less than 0.05 or 0.01 was considered statistically significant. Statistical analysis was done with SPSS/Win11.0 software (SPSS Inc., Chicago, IL).

**Results**

*Effect of PCSK9 on U251 cells proliferation*

U251 cell proliferation was examined by cell counting kit-8 assay (CCK-8) following transfection with PCSK9 siRNA or pcDNA3-PCSK9. As shown in **Figure 1**, PCSK9 siRNA inhibited cell proliferation, while PCSK9 overexpression promoted cell proliferation as compared to the



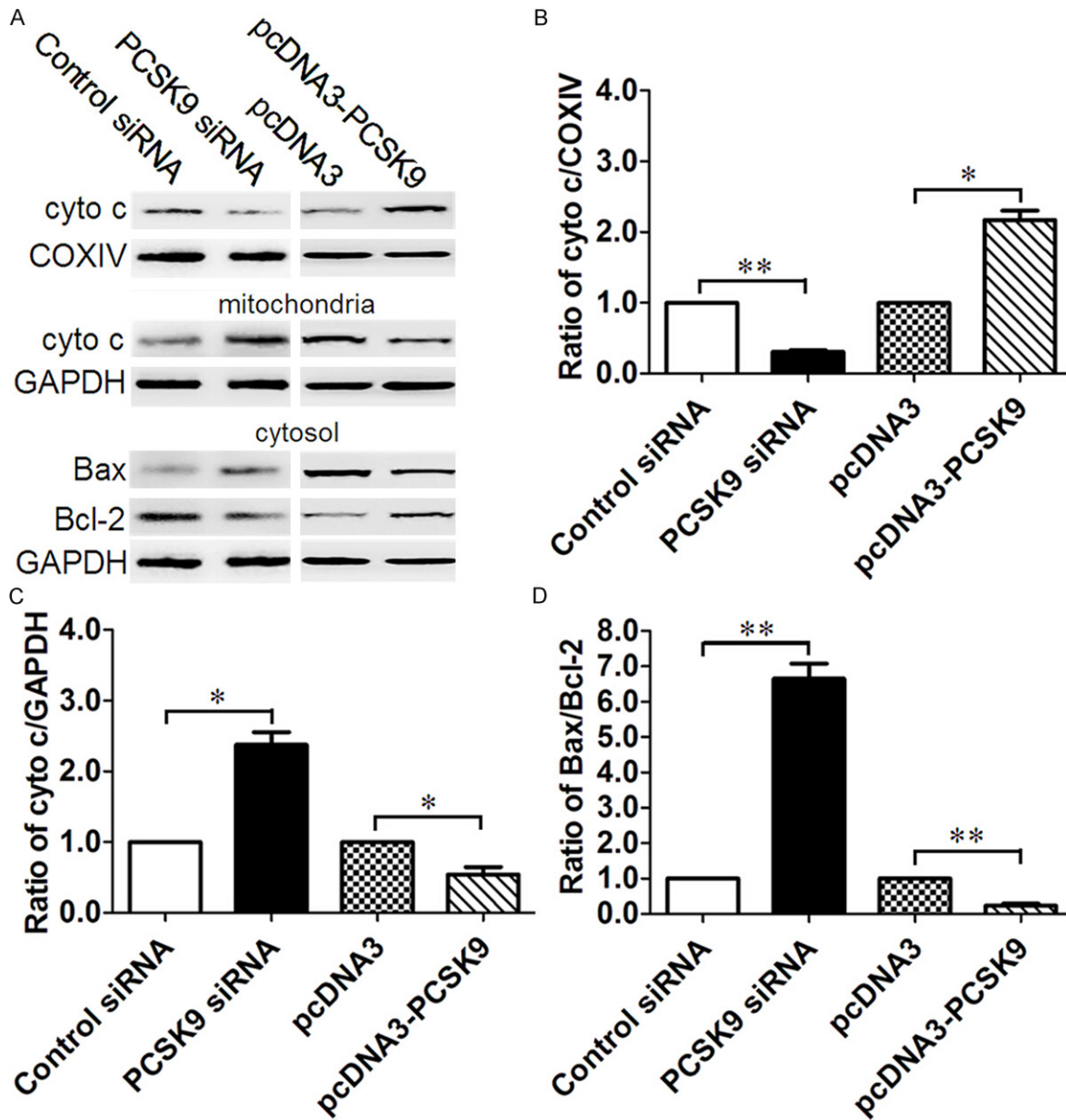
**Figure 3.** Western blot analysis of apoptotic-related proteins after silencing or overexpression of PCSK9. U251 cells were transfected with PCSK9 siRNA or pcDNA3-PCSK9 for 48 h before being subjected to protein extraction and western blot with the indicated antibodies. A. Representative western blot images. B-D. Three such experiments were quantified by measuring the intensity of apoptotic-related proteins relative to the GAPDH (loading) control (\*,  $P < 0.05$ , \*\*,  $P < 0.01$ ). The bars indicated mean  $\pm$  S.D. ( $n = 3$ ).

control group. Furthermore, the cell proliferation at 48 h in PCSK9 siRNA group was reduced significantly than control siRNA group ( $P < 0.05$ ), while cell proliferation at 48 h in PCSK9 overexpression group was increased significantly than vector control group ( $P < 0.01$ ). Thus, these data imply that PCSK9 may influence cellular proliferation in U251 cells.

#### PCSK9 regulates apoptosis in U251 cells

In order to evaluate whether the proliferation alteration regulated by PCSK9 in U251 cells was associated with apoptosis, we examined

the morphologic changes by Hoechst 33258 staining. The efficiency of PCSK9 expression was determined by western blot (Figure 2A). U251 cells were transfected with PCSK9 siRNA or pcDNA3-PCSK9 for 48 h, the apoptotic morphologic changes were observed as compared with the control group. In control group and pcDNA3-PCSK9 group, nuclei of U251 cells were round and homogeneously stained (Figure 2B). However, PCSK9 siRNA-transfected cells exhibited evident apoptosis characteristics including cell shrinkage and membrane integrity loss or deformation, nuclear fragmentation and chromatin compaction of late apoptotic



**Figure 4.** Regulation of mediators in the mitochondrial pathway in apoptotic U251 cells by PCSK9 siRNA. U251 cells were transfected as above. A. The levels of cytochrome c (cyto c) in mitochondria (COXIV as loading control) and cytosol (GAPDH as loading control) and the ratio of Bax/Bcl-2 were measured by western blot. B-D. The intensity of interested proteins was measured for quantitative analysis. GAPDH was included as a loading control. The bars indicated mean  $\pm$  S.D. (n = 3) (\* $P$  < 0.05, \*\* $P$  < 0.01).

appearance. Together, these data indicate that PCSK9 siRNA induces apoptosis in U251 cells.

*PCSK9 regulates apoptosis via the caspase-dependent pathway*

To further assess the role of PCSK9 in U251 cell apoptosis, we next evaluated expression of

apoptosis-related proteins. These include pro-apoptotic c-caspase-3, anti-apoptotic XIAP and p-Akt. Western blot results and statistical analysis indicated that the c-caspase-3 expression in PCSK9 siRNA group was evidently higher than that in the control siRNA group (Figure 3A,  $P$  < 0.05), and the c-caspase-3 expression in pcDNA3-PCSK9 group was significantly lower than that in vector group (Figure 3B,  $P$  < 0.05).

## PCSK9 regulates apoptosis via mitochondrial pathways

On the contrary, the levels of XIAP and p-Akt were significantly decreased in PCSK9 siRNA group as compared with the control siRNA group (**Figure 3C, 3D**,  $P < 0.05$  and  $P < 0.01$ ), while the levels of XIAP and p-Akt were significantly increased in PCSK9 overexpression group as compared with the vector control group (**Figure 3**,  $P < 0.01$  and  $P < 0.05$ ).

### *PCSK9 regulates apoptosis via mitochondrial signaling pathway*

In order to better understand the molecular mechanisms by which PCSK9 regulated apoptosis, we followed the protein expression of mediators in mitochondrial signaling pathway. Firstly, we determined whether PCSK9 involved in the release of cytochrome c into the cytosolic fraction in U251 cells. As expected, cytochrome c was redistributed after overexpression or silencing of PCSK9. In mitochondria, the level of cytochrome c in PCSK9 siRNA group was significantly decreased by 70% ( $P < 0.01$ ), and the level of cytochrome c in PCSK9 overexpression group was increased by 2.2-fold ( $P < 0.05$ , **Figure 4A and 4B**). Correspondingly, in cytosol, the level of cytochrome c in PCSK9 siRNA group was increased by 2.3-fold ( $P < 0.05$ ), while the level of cytochrome c in PCSK9 overexpression group was significantly decreased by 50% ( $P < 0.05$ , **Figure 4A and 4C**).

Since the Bcl-2 family proteins play a critical role in regulating the release of cytochrome c, we then investigated the possible involvement of Bax and Bcl-2 in the process of PCSK9-mediated U251 cells apoptosis. As shown in **Figure 4A and 4D**, the ratio of Bax/Bcl-2 in PCSK9 siRNA group was increased by 6.7-fold ( $P < 0.01$ ), while the ratio of Bax/Bcl-2 in PCSK9 overexpression group was significantly decreased by 70% ( $P < 0.01$ ).

### **Discussion**

Malignant gliomas are the most common and the most aggressive primary central nervous system (CNS) tumor of humans and are resistant to many kinds of conventional pro-apoptotic therapies, such as radiotherapy, chemotherapy and adjuvant therapies [1, 19-21]. Thus, it is particularly important to develop novel strategies in malignant glioma therapy.

PCSK9 encodes a neural apoptosis-regulated convertase 1 (NARC1), raising the possibility

that PCSK9 might be involved in apoptosis [8]. Since then, the implication of PCSK9 in apoptosis has been frequently evoked. A microarray study showed that PCSK9<sup>D347Y</sup> overexpression downregulate pro-apoptotic genes in HepG2 cells [18]. Additionally, the lack of PCSK9 enhances apoptosis during liver regeneration [17]. However, the accurate role of PCSK9 during neuroglioma cells apoptosis has not been completely known. In this study, we evaluated the effect of overexpression and silencing of PCSK9 on apoptosis in a neuroglioma cellular model. Herein, our results indicate that PCSK9 siRNA inhibits the proliferation of U251 cells and PCSK9 overexpression promoted cell proliferation (**Figures 1 and 2**). Based on the results of Hoechst 33258 staining and western blot, we conclude that PCSK9 regulates apoptosis likely via mitochondrial pathway in U251 cells. Thus, the present study constitutes the first evidence that PCSK9 has an anti-apoptotic effect in the neuroglioma cells.

A good strategy for killing cancer cells is to induce cell apoptosis. XIAP, a member of the inhibitor of apoptosis protein (IAP) family, contributes to apoptosis resistance of cancer cells [22]. Akt is a promoter of cell proliferation and survival and is found to be overexpressed in the tumor formation [23]. Thus, we investigated whether these apoptosis-related protein were involved in PCSK9 mediated apoptosis. Our investigation confirmed the role of PCSK9 in the apoptosis of U251 cells based on the following lines of evidence: PCSK9 siRNA promotes the c-caspase-3 expression and inhibits XIAP and p-Akt expression, while PCSK9 overexpression inhibits the c-caspase-3 expression and induces XIAP and p-Akt expression (**Figure 3**). Therefore, PCSK9 regulates apoptosis via the caspase-dependent pathway.

Bcl-2 family proteins play an important role in cancer cells apoptosis [24, 25]. The Bcl-2 family can primarily regulate mitochondrial membrane permeabilization [26]. Bax/Bcl-2 ratio is usually regarded as a criterion for apoptosis [27]. The results from the present study demonstrated that the level of cytochrome c in mitochondria was significantly decreased and in cytosol was increased after PCSK9 siRNA transfection, while in pcDNA3-PCSK9 overexpressed cells, the level of cytochrome c in mitochondria was higher and in cytosol was lower than vector group (**Figure 4**).

Meanwhile, the ratio of Bax/Bcl-2 in PCSK9 siRNA group was significantly increased, while the ratio of Bax/Bcl-2 in PCSK9 overexpression group was significantly decreased (**Figure 4**). The results indicate that PCSK9 may be able to influence mitochondrial membrane stability. Taken together, these data demonstrate that PCSK9 siRNA may exert its anti-tumor activity through mitochondrial signaling pathway in neuroglioma cells.

In conclusion, our data support a role of PCSK9 in regulating the apoptosis U251 cells. Further studies will be needed to define the exact mechanism by which PCSK9 regulates the apoptosis and proliferation of U251 cells. PCSK9 inhibitor may be useful in therapies directed against neuroglioma and possibly other types of cancer. PCSK9 expression and neuronal apoptosis requires further study and animal testing.

**Disclosure of conflict of interest**

None.

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