SNHG3 cooperates with ELAVL2 to modulate cell apoptosis and extracellular matrix accumulation by stabilizing SNAI2 in human trabecular meshwork cells under oxidative stress

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Abstract

Background: Glaucoma is the main reason for irreversible blindness, and pathological increased intraocular pressure is the leading risk factor for glaucoma. It is reported that trabecular meshwork cell injury is closely associated with the elevated intraocular pressure. The current study aimed to investigate the role of SNHG3 in human trabecular meshwork (HTM) cells under oxidative stress.

Methods: A series of experiments including real-time quantitative polymerase chain reaction (RT-qPCR), subcellular fractionation assay, western blot analysis, cell counting kit-8 (CCK-8) assay, RNA pull down, flow cytometry analysis, and RIP assay were employed to explore the biological function and regulatory mechanism of SNHG3 in HTM cells under oxidative stress.

Results: First, we observed that H_2O_2 induced SNHG3 upregulation in HTM cells. Then, we found that SNHG3 silencing alleviated H_2O_2-induced oxidative damage in HTM cells. Moreover, SNAI2 knockdown alleviated the oxidative damage induced by H_2O_2 in HTM cells. Mechanistically, SNHG3 bound with ELAVL2 to stabilize SNAI2. Finally, SNAI2 overexpression counteracted the effect of SNHG3 silencing on H_2O_2-induced HTM cells.

Conclusion: Our results demonstrated that SNHG3 cooperated with ELAVL2 to modulate cell apoptosis and extracellular matrix (ECM) accumulation by stabilizing SNAI2 in HTM cells under oxidative stress.

Introduction

Glaucoma is characterized by atrophy and depression of optic papilla, visual field defect and visual acuity decline [1,2]. Pathological elevation of intraocular pressure and inadequate blood supply of optic nerve are the main reasons for glaucoma [3,4]. Previous research has showed that trabecular meshwork (TM) cells exert crucial effect on aqueous humour circulation, and TM cell injury is closely related to the elevated intraocular pressure [5,6]. Hence, finding novel biomarkers that modulate human trabecular meshwork (HTM) cell injury in glaucomatous patients is of vital significance.

In primary open angle glaucoma, increased intraocular pressure results in deformation at the optic nerve head; especially, intraocular pressure elevation leads to the deformation at the lamina cribrosa region where extracellular matrix (ECM) molecules such as fibronectin and collagen tend to accumulate [7-9]. Changes in the levels of regulators of ECM homeostasis such as matrix metalloproteinases (MMPs) occur in the primary open angle glaucoma lamina cribrosa [10]. MMPs are zinc-dependent endopeptidases, which degrade ECM components such as fibronectin and collagen [11,12]. MMPs are crucial regulators of aqueous humor outflow for they can remodel TM ECM and keep a stable outflow resistance and ensuing intraocular pressure [13-15]. Therefore, MMPs are promising therapeutic targets for glaucoma treatment due to their capability to regulate aqueous humor outflow [13,14].

Long noncoding RNAs (lncRNAs) are a category of noncoding RNAs (ncRNAs), with over 200 nucleotides in size [16,17]. LncRNAs are related to multiple cellular functions, and most of the cellular functions involve the binding of lncRNAs with one or more RNA-binding proteins (RBPs) to increase the stability of target messenger RNAs (mRNAs) [18-21]. LncRNAs are active regulators in a variety of diseases, including glaucoma [22-24]. For example, lncRNA GAS5 knockdown relieves glaucoma in rat models via decreasing the apoptosis of retinal ganglion cells [25]. LncRNA MALAT1 regulates the apoptosis of retinal ganglion cells via the PI3K/Akt pathway in glaucomatous rats [26]. LncRNA MEG3 participates in glaucoma progression by facilitating the autophagy of retinal ganglion cells [27]. LncRNA SNHG3 (small nucleolar RNA host gene 3) has been found to participate in regulating the development of several diseases [28-30]. Importantly, SNHG3 has been found to be related to neurodegeneration [31], and it has been reported to be upregulated in H_2O_2-induced HTM cells [32]. Accordingly, we predicted that SNHG3 may exert certain effect on glaucoma.

DNA damage is related to various neurodegenerative diseases, including glaucoma [32,33]. In comparison with healthy controls, oxidative DNA damage is distinctly increased in the TM of patients with glaucoma [32]. In this study, a H_2O_2-induced oxidative damage HTM cell model was established and the role of SNHG3 in H_2O_2-induced HTM cells was explored. Our investigation may provide a novel direction for the treatment of glaucoma.

Materials And Methods

Cell lines and cell culture

HTM cells were acquired from cadaver eyes with the approval of the Ethics Committee of Nanjing Tongren Hospital (Jiangsu, China). HTM cells were incubated in Dulbecco's modified Eagle's medium (DMEM; USA) containing 10% fetal bovine serum (FBS; Invitrogen, USA), 100 U/ml penicillin (Sigma-Aldrich, USA) and 100 μg/ml streptomycin (Sigma-Aldrich, USA), and then stored in the humidified atmosphere with 5% CO_2 at 37℃.

Cell treatment

The cultured HTM cells were treated with disparate concentrations of H_2O_2 (0 – 300 μM) to establish an *in vitro* oxidative cell damage model. Non-disposed cells acted as a control.

Cell transfection

For overexpression of SNAI2, the SNAI2 sequence was synthesized and inserted into the pcDNA3.1 (Invitrogen, U.S.A.) plasmid to produce the pcDNA3.1/SNAI2. For knockdown of SNHG3, SNAI2 or ELAVL2, short hairpin RNA (shRNA) specifically targeting SNHG3, SNAI2 or ELAVL2 were devised and synthesized by GenePharma (Shanghai, China). Lipofectamine 2000 (Invitrogen, U.S.A.) was applied for cell transfection in line with manufacturer's recommendations.

CCK-8 assay

The viability of HTM cells were detected by using Cell Counting Kit-8 (CCK-8). The transfected cells with 1×10^4 cells per well were planted in 96-well plates. After incubation for 48 h, CCK-8 solution (10 μl) was supplemented into each well. After that, the incubation continued for 4 h. Cell viability was visualized using a microplate reader (EL340; BioTek Instruments, Hopkinton, MA) at a wavelength of 450 nm.

Flow cytometry analysis

The flow cytometry was conducted to assess HTM cell apoptosis with Annexin V Kit (Beyotime, China) with reference to protocols stipulated by the manufacturer. Cells transfected with indicated plasmid or negative control were collected 48 h later and washed twice with PBS. Subsequently, cells (1×10^6 cells/mL) were double stained by propidium iodide (PI) and Annexin V-FITC. The CELLQUEST program (Becton Dickinson, USA) was used to record cells.

Western blot

Transfected cells were lysed applying RIPA buffer (Thermo, USA), and cultured for 15 min at 4°C. Then, the lysate was centrifuged, and the concentrations of the proteins were measured using an BCA Protein Assay Kit (Thermo, USA). The proteins were isolated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and moved onto PVDF membranes (Thermo, USA). Then, 5% skim milk was used to block the membranes for 1 h. Next, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies were obtained from Abcam company (Shanghai, China): SNAI2 (ab51772), ELAVL2 (ab72603), collagen Ⅰ (ab34710), fibronectin (ab2413), collagen III (ab7778), MMP3 (ab52915), MMP9 (ab76003) and GAPDH (ab8245). Subsequently, secondary antibody was used to be incubated with the membranes for 2 h at room temperature. Finally, an enhanced chemiluminescence kit (GE Healthcare, Chicago, IL) was adopted to observe the signals.

Real-time quantitative polymerase chain reaction (RT-qPCR)

TRIzol reagent (ThermoFisher Scientific Invitrogen, USA) was employed to extract total RNA. Then, total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). RT-qPCR analysis was performed using a SYBR Premix Ex Taq II kit (Cat. #RR820A, TaKaRa) on the 7500 Real-Time PCR Systems (Applied Biosystems). U6 served as an endogenous control for miRNAs. The detection results for mRNAs were normalized to GAPDH. Expression fold changes were calculated adopting the $2^{-\Delta\Delta Ct}$ method.

Subcellular fractionation assay

Total RNA was extracted applying a DNA/RNA Isolation Kit (DP422, Tiangen Biotech, Beijing, China) in line with the instruction. The nuclear and cytoplasmic fractions were separated applying NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Total RNA was isolated from the nuclear and cytoplasmic fractions using Trizol (Invitrogen).

RNA pull down assay

The biotinylated RNAs were cultured with cell lysates, and then cultured with streptavidin beads (Invitrogen). Subsequently, the RNAs in complexes captured by streptavidin beads were washed, purified and assessed by western blot analysis.

RNA immunoprecipitation (RIP) assay

The RIP assay was carried out with a Millipore EZ-Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore, Bedford, MA, USA). The precipitated RNAs were evaluated using RT-qPCR. Antibodies against ELAVL2 and IgG were used. IgG served as the negative control.

Data statistics

Data analysis was conducted using SPSS 23.0 (IBM SPSS, Chicago, IL, USA). All data are exhibited as the means ± SD. Statistical significance among more than 2 groups was calculated using one-way ANOVA and Tukey's post-hoc test. Difference between 2 groups was evaluated using Student's t-test. $P < 0.05$ was regarded to be statistically significant.

Results

H_2O_2 induced SNHG3 upregulation in HTM cells

We first established a H_2O_2-induced oxidative damage model in HTM cells. Then, HTM cells were treated with different concentrations of H_2O_2 (0, 100, 200, and 300 μM). Next, the viability and apoptosis of H_2O_2-induced HTM cells were detected. CCK-8 assay revealed that HTM cell viability was inhibited by H_2O_2 at the concentrations of 100 μM, 200 μM and 300 μM (Fig. 1A). Since the IC50 of viability was exhibited at the concentration of 200 μM, we chose 200 μM H_2O_2 to dispose HTM cells in the subsequent assays. Flow cytometry analysis showed H_2O_2 induced the apoptosis of HTM cells compared with the control group (Fig. 1B). Additionally, the levels of extracellular matrix (ECM) proteins Collagen I, Collagen III, Fibronectin, MMP3, and MMP9 were obviously increased by H_2O_2 in HTM cells (Fig. 1C). Moreover, SNHG3 expression was upregulated in HTM cells (Fig. 1D). In summary, theses data showed that the H_2O_2-induced oxidative damage model in HTM cells was established successfully.

SNHG3 silencing alleviated H_2O_2-induced oxidative damage in HTM cells

To detect the biological function of SNHG3 in H_2O_2-induced HTM cells, SNHG3 was knockdown by transfection of sh-SNHG3#1 or sh-SNHG3#2 (Fig. 2A). Then, CCK-8 assay demonstrated SNHG3 knockdown promoted the viability of H_2O_2-induced HTM cells (Fig. 2B). Flow cytometry analysis showed that SNHG3 silencing suppressed the apoptosis of H_2O_2-induced HTM cells (Fig. 2C). Moreover, the levels of ECM proteins Collagen III, Collagen I, Fibronectin, MMP3, and MMP9 were reduced by SNHG3 silencing

in H_2O_2-induced HTM cells (Fig. 2D). Overall, SNHG3 silencing alleviated H_2O_2-induced oxidative damage in HTM cells.

SNAI2 knockdown alleviated H_2O_2-induced oxidative damage in HTM cells

SNAI2 has been widely reported to regulate ECM proteins [34,35], so we predicted that it regulated ECM proteins in HTM cells. To evaluate the biological function of SNAI2 in H_2O_2-induced HTM cells, SNAI2 was knockdown by transfection of sh-SNAI2#1 or sh-SNAI2#1. Western blot analysis demonstrated that SNAI2 protein level was increased by H_2O_2, but it was then decreased by transfection of sh-SNAI2#1 or sh-SNAI2#2 (Fig. 3A). Then, CCK-8 assay showed SNAI2 knockdown promoted the viability of H_2O_2-induced HTM cells (Fig. 4B). Flow cytometry analysis revealed that SNAI2 knockdown suppressed the apoptosis of H_2O_2-induced HTM cells (Fig. 3C). Moreover, the levels of ECM proteins Collagen III, Collagen I, Fibronectin, MMP3, and MMP9 were reduced by SNAI2 knockdown in H_2O_2-induced HTM cells (Fig. 3D). In summary, SNAI2 silencing alleviated H_2O_2-induced oxidative damage in HTM cells.

SNHG3 bound with ELAVL2 to stabilize SNAI2

Subsequently, we explored the molecular mechanism of SNHG3 in HTM cells. First, we found that SNHG3 mainly existed in the cytoplasm of cells by using online tool (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/) (Fig. 4A). Then, we further found that SNHG3 mainly existed in the cytoplasm of HTM cells (Fig. 4B). Next, as shown in Fig. 4C, by searching the website http://rbpdb.ccbr.utoronto.ca, one RBP, ELAVL2, was identified to bind with SNHG3 and SNAI2 (condition: binding score > 10). Based on the website http://pridb.gdcb.iastate.edu//RPISeq/, the accuracies of random forest (RF) and support vector machine (SVM) classifiers for SNHG3-ELAVL2 binding were 0.9 and 0.91 respectively, and the accuracies of RF and SVM classifiers for SNAI2 3'UTR-ELAVL2 binding were 0.65 and 0.96 respectively, suggesting ELAVL2 bound with SNHG3 and SNAI2 (Fig. 4D). RNA pull down assay demonstrated that ELAVL2 was enriched in the Bio-SNHG3 sense group and the Bio-SNAI2 sense group but not the Bio-SNHG3 antisense group and Bio-SNAI2 antisense group, confirming that ELAVL2 bound with SNHG3 and SNAI2 (Fig. 4E). Similarly, RIP assay suggested that both SNHG3 and SNAI2 were enriched in the anti-ELAVL2 group instead of the anti-IgG group (Fig. 4F). Subsequently, HTM cells were transfected with sh-ELAVL2, showing the decreased ELAVL2 expression (Fig. 4G). Then, western lot analysis revelated that ELAVL2 or SNHG3 silencing reduced the protein level of SNAI2 in HTM cells (Fig. 4H-I). Moreover, ELAVL2 silencing or SNHG3 silencing decreased SNAI2 mRNA half-life (Fig. 4J). Overall, SNHG3 bound with ELAVL2 to stabilize SNAI2.

SNAI2 overexpression counteracted the effect of SNHG3 silencing on H_2O_2-induced HTM cells

To detect whether SNAI2 overexpression counteracted the effect of SNHG3 silencing on HTM cells, rescue assays were conducted. First, SNAI2 protein level was elevated by the transfection of pcDNA3.1/SNAI2 in H_2O_2-induced HTM cells (Fig. 5A). Then, we found that SNAI2 overexpression counteracted the promotive effect of SNHG3 silencing on H_2O_2-induced HTM cell viability (Fig. 5B). SNAI2 overexpression offset the

inhibitive effect of SNHG3 silencing on H_2O_2-induced HTM cell apoptosis (Fig. 5C). Additionally, the suppressive influence of SNHG3 knockdown on ECM proteins (Collagen I, Collagen III, Fibronectin, MMP3, and MMP9) was abolished by SNAI2 overexpression (Fig. 5D). In conclusion, SNAI2 overexpression abrogated the effect of SNHG3 silencing on H_2O_2-induced HTM cells.

Discussion

In this research, we established a H_2O_2-induced oxidative damage model in HTM cells and explored the role of SNHG3 in H_2O_2-induced HTM cells. Our data suggested that SNHG3 cooperated with ELAVL2 to modulate cell apoptosis and ECM accumulation by stabilizing SNAI2 in H_2O_2-induced HTM cells.

Previous research demonstrated that lncRNAs have been implicated in neurodegenerative diseases, including Alzheimer's disease [36,37] and glaucoma [23,24]. SNHG3 has been reported to regulate a variety of diseases, such as hepatocellular carcinoma [29] and osteosarcoma [30]. Notably, SNHG3 has been found to be related to neurodegeneration [31], and it has been reported to be upregulated in H_2O_2-induced HTM cells [32]. In the present study, our data revealed that SNHG3 was upregulated in H_2O_2-induced HTM cells and SNHG3 silencing alleviated H_2O_2-induced oxidative damage in HTM cells by promoting cell viability, inhibiting cell apoptosis and decreasing ECM protein level.

SNAI2 (snail family transcriptional repressor 2) is also known as SLUG, SLUGH, SLUGH1, SNAIL2, or WS2D; it has been reported to regulate a wide range of human diseases [38–40]. Importantly, previous research has proved that SNAI2 is involved in the regulation of neurodegenerative diseases [41], and it may participate in the modulation of glaucoma [42]. However, the specific role and regulatory mechanism of SNAI2 in glaucoma is not clear.

LncRNAs can cooperate with specific proteins to increase mRNA stability or induce mRNA decay in cytoplasm [43]. For example, LncRNA LINC00707 interacts with mRNA stabilizing protein HuR to increase the stability of VAV3/F11R in gastric cancer [44]. LncRNA LAST interacts with CNBP to promote CCND1 mRNA stability in human cells [45]. Previously, the regulatory mechanism of SNHG3 has been explored in some human diseases [29,30,46]. However, the molecular regulatory mechanism of SNHG3 in glaucoma remains to be explored. In this study, we observed that SNHG3 bound with ELAVL2 to stabilize SNAI2 in H_2O_2-induced HTM cells. SNAI2 overexpression counteracted the effect of SNHG3 silencing on H_2O_2-induced HTM cells. In addition, we found that SNAI2 knockdown relieved H_2O_2-induced oxidative damage in HTM cells by promoting cell viability, inhibiting cell apoptosis and decreasing ECM protein level.

Conclusions

The main purpose of the present research was to investigate the biological role and regulatory mechanism of SNHG3 in H_2O_2-induced HTM cells. Our results illustrated that SNHG3 interacted with ELAVL2 to regulate cell proliferation, apoptosis and ECM accumulation by increasing the stability of

SNAI2 in H_2O_2-induced HTM cells. This finding may shed some light on the regulatory mechanism of SNHG3 in human diseases and advance our understanding on the pathogenesis of glaucoma. Future studies should be conducted on the biological functions of SNHG3 in other cell types and the regulatory mechanism of H_2O_2-induced dysregulated SNHG3 expression. In addition, *in vivo* experiments could be added to further validate the role of SNHG3 in glaucoma.

Abbreviations

HTM: human trabecular meshwork; RT-qPCR: real-time quantitative polymerase chain reaction; CCK-8: cell counting kit-8; ECM: extracellular matrix; TM: trabecular meshwork; MMPs: matrix metalloproteinases; lncRNAs: long noncoding RNAs; ncRNAs: noncoding RNAs; RBPs: RNA-binding proteins; mRNAs: messenger RNAs; RIP: RNA immunoprecipitation;

Declarations

Ethical Approval and Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of supporting data

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgement

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Conflicts of interest

The authors declare that there are no competing interests in this study.

Authors' contributions

Sizhen Li conceived and designed the experiments. Sizhen Li, Qingsong Yang, Zixiu Zhou, Min Fu, Xiaodong Yang, Kuanxiao Hao, and Yating Liu carried out the experiments. Sizhen Li and Qingsong Yang analyzed the data. Sizhen Li and Qingsong Yang drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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Figures

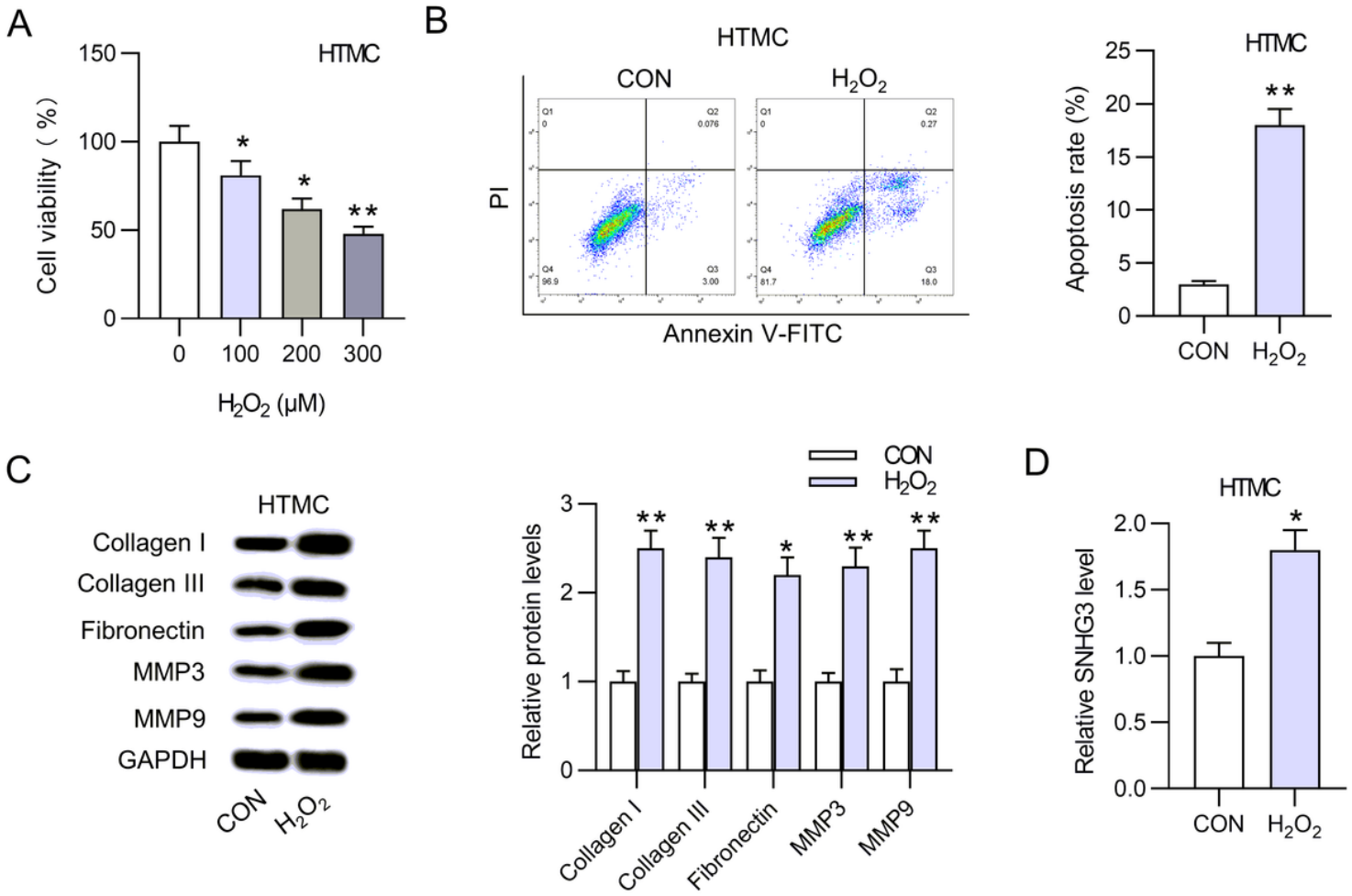

Figure 1

H2O2 induced SNHG3 upregulation in HTM cells. (A) HTM cell viability upon H2O2 at the concentrations of 100 μM, 200 μM and 300 μM was assessed by CCK-8 assay. (B) Flow cytometry analysis was used to detect the apoptosis of H2O2-induced HTM cells. (C) The levels of extracellular matrix (ECM) proteins Collagen I, Collagen III, Fibronectin, MMP3, and MMP9 were measured by western blot analysis. (D) The expression of SNHG3 was detected in H2O2-induced HTM cells *P< 0.05, **P< 0.01, ***P< 0.001.

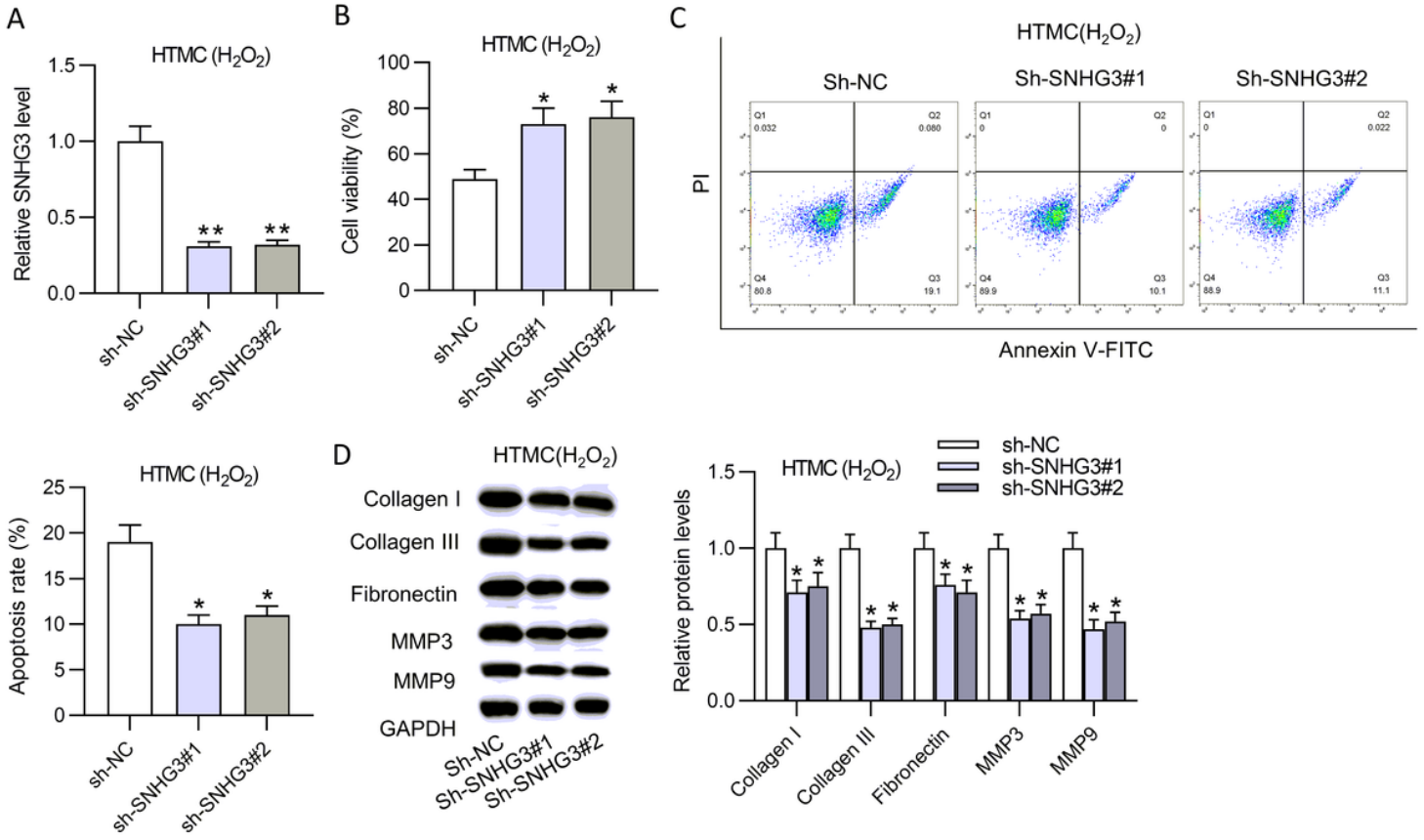

Figure 2

SNHG3 silencing alleviated H2O2-induced oxidative damage in HTM cells. (A) The knockdown efficiency of SNHG3 was assessed. (B) CCK-8 assay was used to evaluate the effect of SNHG3 silencing on the viability of H2O2-induced HTM cells. (C) Flow cytometry was performed to detect the apoptosis of H2O2-induced HTM cells after transfection of sh-SNHG3#1 or sh-SNHG3#2. (D) The Collagen I, Collagen III, Fibronectin, MMP3, and MMP9 proteins in H2O2-induced HTM cells after transfection of sh-SNHG3#1 or sh-SNHG3#2 were detected using western blot analysis. *P< 0.05, **P< 0.01, ***P< 0.001.

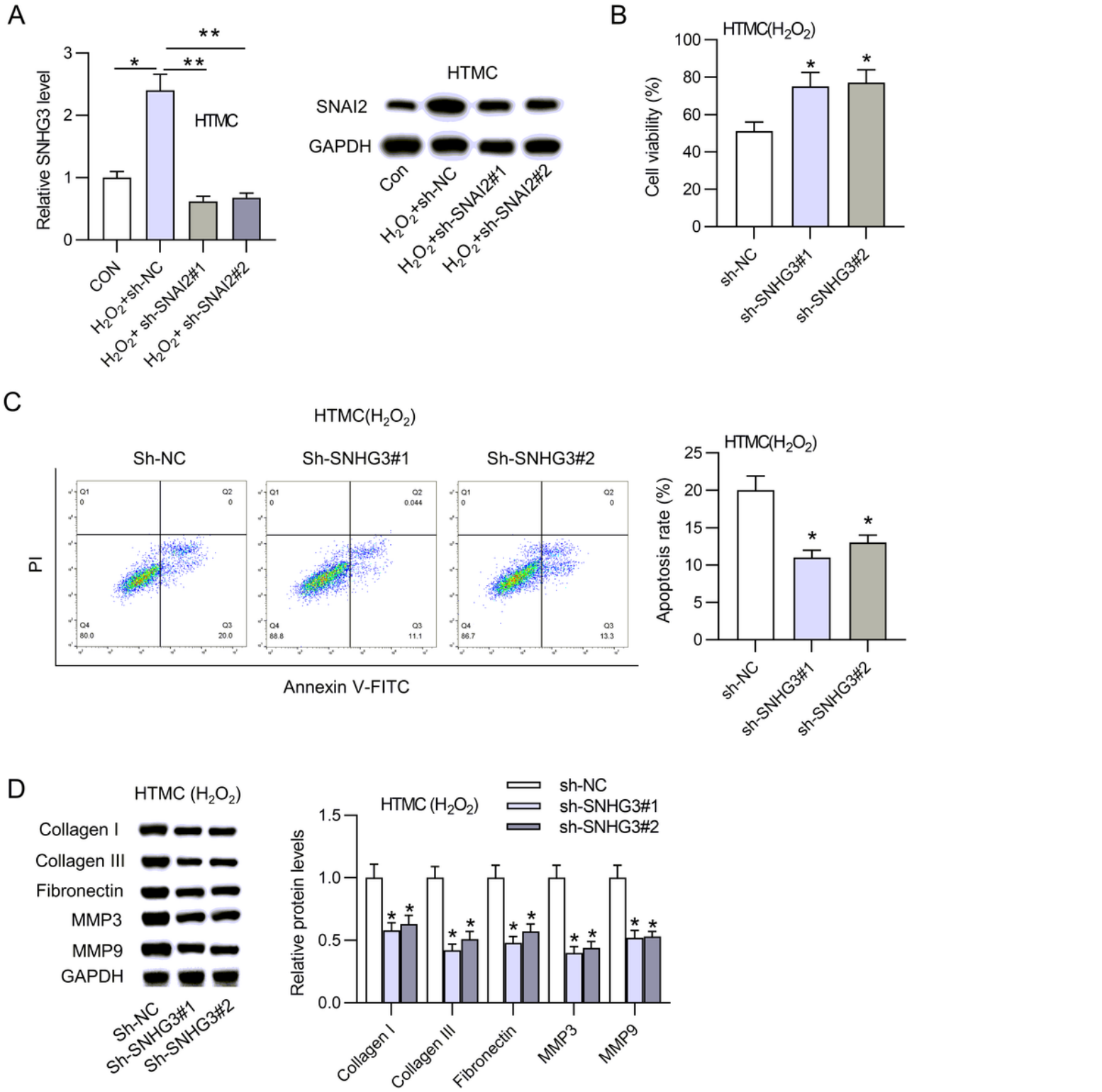

Figure 3

SNAI2 knockdown alleviated H2O2-induced oxidative damage in HTM cells. (A) The knockdown efficiency of SNAI2 was assessed. (B) CCK-8 assay was used to evaluate the effect of SNAI2 silencing on the viability of H2O2-induced HTM cells. (C) Flow cytometry was carried out to detect the apoptosis of H2O2-induced HTM cells upon sh-SNAI2#1 or sh-SNAI2#2. (D) The levels of ECM proteins (Collagen I,

Collagen III, Fibronectin, MMP3, and MMP9) in H2O2-induced HTM cells in response of sh-SNAI2#1 or sh-SNAI2#2.*P< 0.05, **P< 0.01, ***P< 0.001.

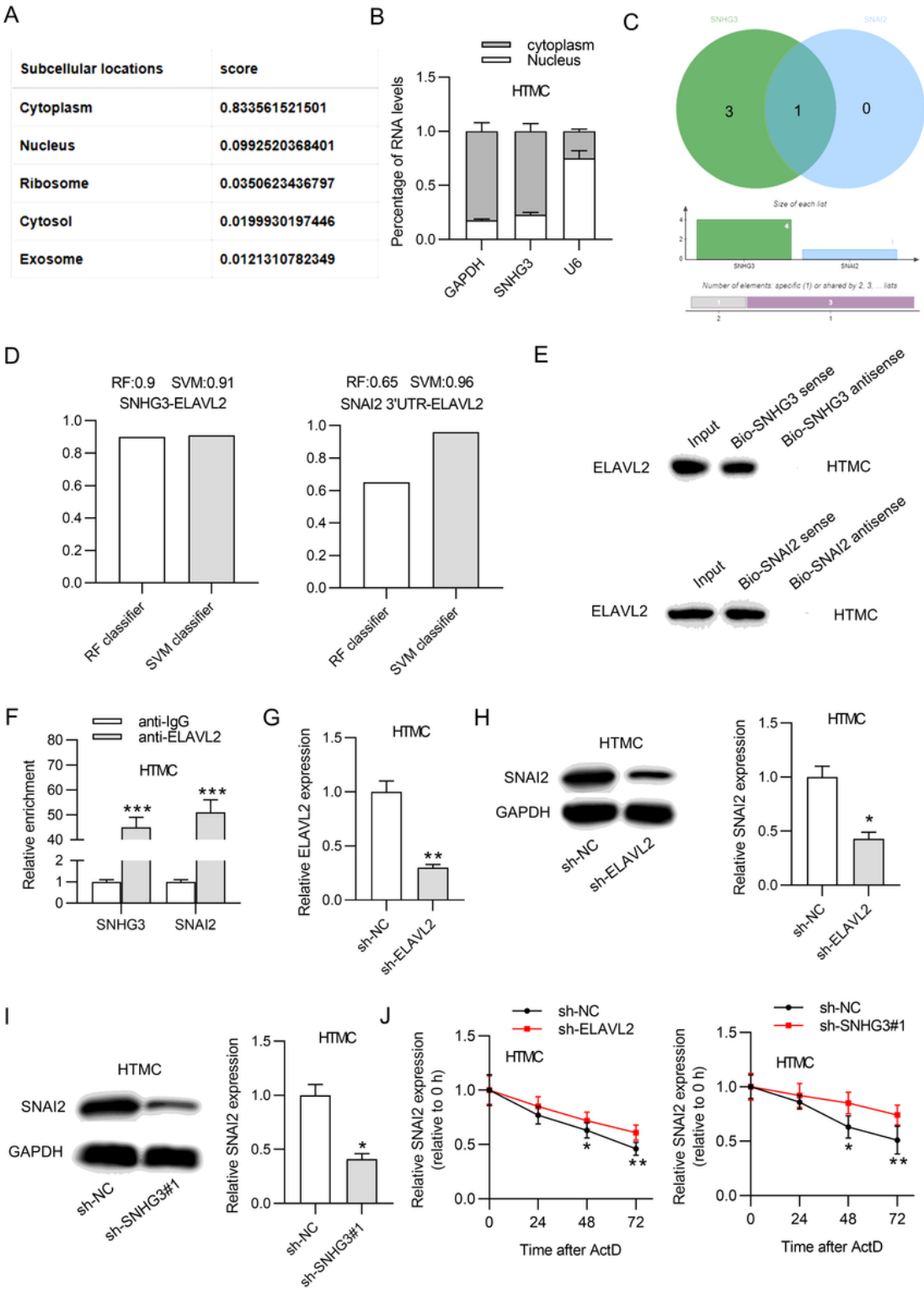

Figure 4

SNHG3 bound with ELAVL2 to stabilize SNAI2. (A) The location of SNHG3 in cells was identified by using the online tool (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/). (B) Subcellular fractionation assay was performed to localize SNHG3 in HTM cells. (C) Venn diagram exhibited the RBPs binding with SNHG3

and SNAI2. (D) Online database (http://pridb.gdcb.iastate.edu//RPISeq/) was used to analyze the binding ability of ELAVL2 with SNHG3 or SNAI2. (E) RNA pull down assay was conducted to assess the binding of ELAVL2 with SNHG3 or SNAI2. (F) RIP assay was used to evaluate the binding of ELAVL2 with SNHG3 or SNAI2. (G) The knockdown efficiency of ELAVL2 was detected. (H) The protein level of ELAVL2 in HTM cells after transfection of sh-ELAVL2. (I) The protein level of ELAVL2 in HTM cells after transfection of sh-SNHG3#1. (J) The expression of ELAVL2 in HTM cells after transfection of sh-ELAVL2 or sh-SNHG3#1. *P< 0.05, **P< 0.01, ***P< 0.001.

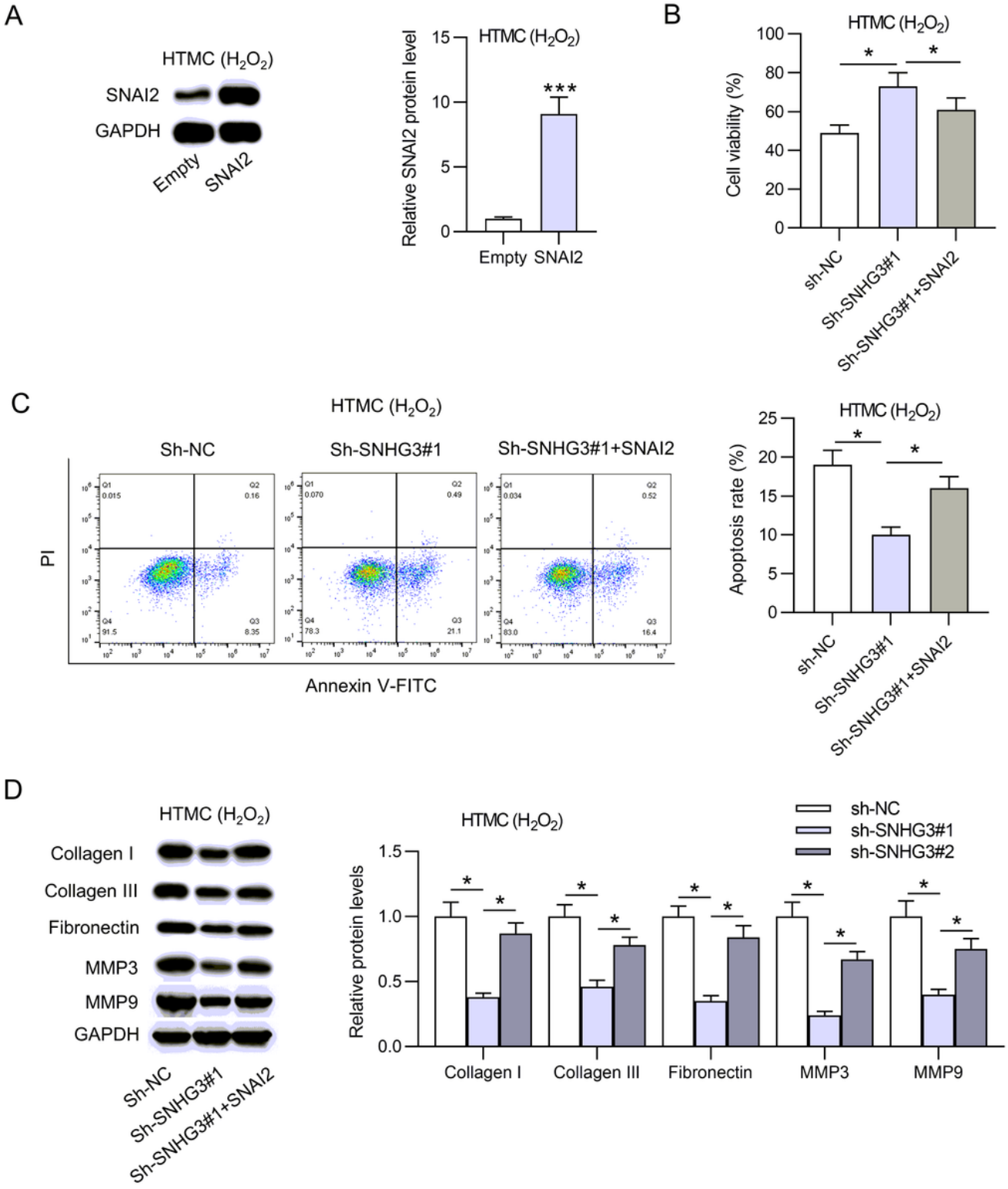

Figure 5

SNAI2 overexpression counteracted the effect of SNHG3 silencing on H2O2-induced HTM cells. (A) The overexpression efficiency of SNAI2 was assessed by western blot analysis. (B) The viability of H2O2-induced HTM cells after transfection of indicated plasmids was detected. (C) The apoptosis of H2O2-induced HTM cells after transfection of appointed plasmids. (D) The levels of ECM proteins (Collagen I, Collagen III, Fibronectin, MMP3, and MMP9) in H2O2-induced HTM cells in the different groups. *P< 0.05, **P< 0.01, ***P< 0.001.