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Inhibition of HES-1 might play a protective role in endothelial cells under cholesterol stimulation *via* PI3K/AKT signaling pathway

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Abstract. The occurrence of atherosclerotic cardiovascular disease (ASCVD) was closely related to low-density lipoprotein (LDL) cholesterol. HES-1 is critical for maintains of stem cells, quiescent cells or cancer cells, and contributes to drug resistance and metastasis of tumor cells. In this study, we established a cell model of HES-1 inhibition and overexpression in Ea.hy 926 cells, and firstly detected the proliferation rete of Ea.hy 926 cells under cholesterol stimulation using MTT assay, and apoptosis of Ea.hy 926 cells were detected using flow cytometry. Expression of HES-1, apoptosis related proteins and phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) signaling pathway were detected using Western blotting analysis. The expression of apoptotis related genes were detected using polymerase chain reaction (PCR) method. The concentration of angiogenesis cytokines was detected using enzyme-linked immunosorbent assay (ELISA) method. We found that proliferation of Ea.hy 926 cells was inhibited after stimulation of cholesterol, inhibition of HES-1 expression would reduce this effect. We also found that expression of apoptosis related molecules was increased and expressions of angiogenesis factors were decreased after cholesterol treatment. Besides, we revealed that these effects were mediated via PI3K/AKT signaling pathway, and HES-1 inhibition could increase the activity of this signaling pathway.

Key words: HES-1 — Cholesterol — Cardiovascular disease — Endothelial cells — Apoptosis

Introduction

One of the leading causes of morbidity and mortality worldwide is atherosclerotic cardiovascular disease (ASCVD) (Goldstein et al. 2015). Previous study found the direct relationship between concentrations of low-density lipoprotein (LDL)-cholesterol in plasma and the occurrence of cardiovascular disease. Dysfunction of endothelial cells might be a critical pathogenesis for cardiovascular disease, including hypertension and pregnancy-induced hypertension (Ference et al. 2017). Clinical trials proved that lipid-lowering drugs could significantly decrease the concentration of LDL-cholesterol, resulting in reduction in morbidity and mortality in cardiovascular disease patients and healthy people (Schwartz et al. 2018). Previous study indicated that transcriptional repressor of Hes family BHLH transcription factor 1 (HES-1) protects cells from differentiation, and some tumor cells even relay on HES-1 to protect against differentiation, and down-regulation of HES-1 leads to the reduction of cellular proliferation in ovarian cancer cells (Sang et al. 2008; Majidinia et al. 2017). There is also study found that almost all of undifferentiated cells is expressed HES-1 (Kageyama et al. 2005). HES-1 de-

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ficient mice presented some severe defects in brain, eye and pancreas (Ishibashi et al. 1995), HES-1 expression is disappeared when cells initate differentiation. However, there is also study indicated that promote the differentiation of neuronal and beta cell through silencing the repressor element silencer transcription (REST) factor (Abderrahmani et al. 2005).

In present study, we firstly constructed a HES-1 inhibition and HES-1 overexpression model in Ea.hy 926 cells, and explored the function of HES-1 on proliferation of endothelial cells under cholesterol stimulation. We further explored the effect of HES-1 in altering the expression of apoptosis and angiogenesis related molecules at gene and protein levels. And we also found that this effect might be mediated by PI3K/AKT signaling pathway, while reduction of HES-1 expression would enlarge this effect.

Material and Methods

Material

Fetal bovine serum (FBS) (12484-028) and High Glucose Dulbecco's Modified Eagle's Medium (H-DMEM) (11965-092) were purchased from Thermo (NY, USA). Cholesterol was purchased from Sigma (C4951). MTT Cell Proliferation and Cytotoxicity Assay Kit (C0009) were purchased from Beyotime (Shanghai, China). KpnI (R0142L), XhoI (R0146L), Quick Ligase (M2200S) and T4 PNK (M0201S) were purchased from NEB (NY, USA). FastDigest BsmBI (FD0454) was purchased from Fermentas (NY, USA). Hieff Trans transfection reagent (40802ES01) and G418 (60220ES03) were purchased from Yeasen Biotechnology Co (Shanghai, China). Protease Inhibitor Cocktail (CW2200), Ultrapure RNA Kit (CW0597) and Super TaqMan OneStep RT-qPCR Kit (CW2695) were purchased from CWbio Biological Technology Company (Beijing, China). Anti-HES-1 (ab108937), Wnt (ab15251), AKT (ab8805), p-AKT (ab38449), β-catenin (ab32572), c-Myc (ab32072), Bax (ab32503), Bcl-2 (ab196495), BIM (ab32158), p-BIM (ab17935), caspase-9 (ab32539), caspase-3 (ab13847) antibodies and TGF-β (ab119558), EGF (ab239424), VEGF-α (ab100786), PDGF (ab155464) ELISA kits were purchased from Abcam. Ea.hy 926 cells (No. GNHu39) and 293T cells (SCSP-502) were obtained from the cell library of the typical culture preservation committee of Chinese academy of sciences.

Vector construction

cDNA of HES-1 was cloned from cells using PCR methods using the primers listed: Forward: 5'-TCTACACCAGCAACAGTGG-3'; Reverse: 5'-TCAAACATCTTTGGCATCAC-3'. Blank pCNDA3.1-3×Flag vector and PCR product of HES-1 were digested with KpnI and XhoI, then HES-1 fragment was cloned into vector to construct pCDNA3.1-3×Flag- HES-1β overexpression vector. Ea.hy 926 cells were transfected using pCDNA3.1-3×Flag- HES-1 vector using Hieff Trans transfection reagent for 48 h under the guidance of manufacturer's protocol. Stable HES-1 overexpression cells were screened using 1000 μg/mL G418. HES-1 knockdown vector was constructed according to previous study (Canver et al. 2018). Briefly, CRISPR vector was firstly digested with BsmBI according to the protocol, and annealed oligoes was constructed using primers listed: Forward: 5'-CACCGC CGCTTACGGCGGACTCCATG-3'; Reverse: 5'- AAAC-CATGGAGTCCGCCGTAAGCGC-3'. Then ligation reaction was performed using annealed oligoes and digested CRISPR vector according to the protocol of Quick Ligase to construct the HES-1 knockdown vector. The lentivirus vector was constructed using 293T cells, and stable HES-1 knockdown cells were screened using 2 μg/ml puro.

Cell culture and grouping

Cells were cultured in H-DMEM supplied with 10% FBS under a humid 37°C atmosphere supplied with 5% CO_2. Cells were grouped into four groups: control group (NC), cholesterol stimulation group (CH), HES-1 overexpression with cholesterol stimulation group (GO) and HES-1 knockdown with cholesterol stimulation group (GD).

Cellular toxicity of cholesterol on Ea.hy 926 cells

Cells in different groups were stimulated with different concentration of cholesterol for different period. Briefly, cells were seeded into each well of a 96-well plate at a concentration of 1×10^4 and cultured until confluence of cells reached 80–85%, cells in cholesterol stimulation groups were treated with 50 mmol/l, 100 mmol/l and 200 mmol/l cholesterol for 12, 24 and 48 h. Cells were incubated with 5 mg/ml MTT reagent for 4 h after washed with PBS, and then OD value at 560 nm was determined using SuPerMax 3100 microplate reader (Shanghai, China) after DMSO was added. Viability of cells = $(OD_{Experiment} - OD_{Blank}/OD_{Control} - OD_{Blank}) \times 100\%$.

RNA extraction and reverse transcription

RNA extraction was proceeded under the guidance of the protocol of Ultrapure RNA Kit. Briefly, cells were firstly lysed with TRIzol reagent followed with incubation at room temperature for 5 min, then mixed with chloroform followed by vigorous shake for 15 s. Then 70% ethanol was added after centrifuged for 5 min at 12,000 rpm, and then mixture was loaded onto an adsorption column and centrifuged for 1 min at 12,000 rpm. After washed with wash buffer, RNA

adsorbed in the adsorption column was eluted using elution buffer. Concentration of RNA was detected using Nanodrop ND-2000 (Thermo Fisher Scientifc, Inc.).

Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Reverse transcription and qPCR were performed according to the protocol of Super TaqMan OneStep RT-qPCR Kit. Primers used are listed as follows: cyclin D1: Forward: 5'-GGCTCCATGACTGTGGGATC-3', Reverse: 5'-TTCAGCTGCACAGCCCAGAA-3'; Bax: Forward: 5'-GAGGATGATTGCTGATGTG-3', Reverse: 5'-AGTTGAAGTTGCCGTCTG-3'; Bcl-2: Forward: 5'- GGCTACGAGTGGGATACTG-3', Reverse: 5'-GGCTGGAAGGAGAAGATG-3'; Caspase-3: Forward: 5'- GCAGTTTTGTGTGTGTGATT-3', Reverse: 5'- GAGTTTCGGCTTTCCAGT-3'. Reaction mixture was made up according to the protocol, and the reaction program was set as follows: reverse transcription at 45°C for 20 min, pre-degeneration at 95°C for 5 min, and degeneration at 95°C for 15 s and extend at 60°C for 45 s repeated for 45 cycles. The expression of relative genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001). GAPDH was used as an internal reference, and the quantification results for each target gene were normalized to GAPDH. Each experiment was repeated for three times.

Western blotting analysis

Cells in each group were lysed on ice for 30 min using lysis buffer (8 M Urea, 10 mM DTT, 50 mM IAA and protease inhibitor cocktail) after washed with pre-chilled PBS. After lysis, the supernatant was collected after centrifugation, and concentration of proteins were determined using BCA assay. Protein samples (60 μg) in four groups were separated with SDS-PAGE electrophoresis. Then proteins were then transferred onto a 0.22-μm nitrocellulose membranes using Trans-Blot Turbo system (Bio-Rad). Membranes were firstly incubate with 5% skim milk, followed with incubation with primary antibodies (1:1000) at 4°C overnight and incubation with secondary antibody (1:5000) for 1 h at room temperature. Chemiluminiscence was performed using ECL reagents to measure the expression levels of target proteins. The gray values of the bands in each group were quantified using Scion Image software. Gray values were normalized with GAPDH.

Flow cytometry

Cells in each group were seeded into 100 mm plate at a concentration of 1×10^6, and after the confluence reached 80–85%, cells were digested with 0.25% trypsin. After washed with pre-cool PBS, cells were incubated with Annexin V for 15 min at room temperature, followed with incubation with propidium iodide (PI) for 5 min. The apoptotic cells were detected using FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

Enzyme linked immunosorbent assay (ELISA)

ELISA experiment was proceeded according to the protocol. Briefly, 100 μl samples and standards were transferred into each well and incubated for 90 min at 37°C, followed with incubation with antibody at 37°C for 60 min. After washed with TBS, ABC working solution was added into each well and incubated 37°C for 30 min. TMB solution was added after washed with TBS and incubated at 37°C for 25 min. The OD value at 450 nm was measured after TMB stop solution was added using SuPerMax 3100 microplate reader (Shanghai, China). Un-cultured medium was set as a blank control.

Statistical analysis

Data are presented as the mean ± SD, each experiment was repeated for three times independently. One-way ANOVA was performed to compare differences between groups followed by Tukey's *post-hoc* test. $p < 0.05$ was set as a statistically significant difference. GraphPad (version 7) was used to analyze the data.

Results

Effect of cholesterol on proliferation of Ea.hy 926 cells

In order to detect the effect of cholesterol on proliferation of Ea.hy 926 cells, we performed the MTT assay. The viability rate of Ea.hy 926 cells under 50 mmol/l cholesterol treatments for 12, 24 and 48 h were 95.3 ± 4.9%, 83.2 ± 4.1% and 63.3 ± 3.1%, respectively. The viability was significantly decreased in 24 and 48 h treatment groups compared with control and 12 h treatment groups ($p < 0.05$). Thus, we decided to treat endothelial cells for 24 h to perform the following experiments (Fig. 1A). The viability rate of Ea.hy 926 cells in CH, GO and GD groups under 50 mmol/l cholesterol stimulation for 24 h were 87.5 ± 5.8%, 81.5 ± 5.2% and 92.6 ± 6.4%, under 100 mmol/l cholesterol stimulation for 24 h were 75.3 ± 5.1%, 63.6 ± 4.1% and 85.3 ± 5.1%, and under 200 mmol/l cholesterol stimulation for 24 h were 62.1 ± 3.2%, 51.4 ± 3.6% and 74.5 ± 4.8%, respectively (Fig. 1B). Compared with NC group, the viability rate in GO group was significantly decreased under 50 mmol/l cholesterol stimulation ($p < 0.05$), significantly decreased in CH and GD group under 100 mmol/l cholesterol stimulation ($p < 0.05$) and significantly in CH, GO and GD groups under 200 mmol/l cholesterol stimulation ($p < 0.05$). The viability rate in GO group was significantly decreased under 100 mmol/l cholesterol stimulation ($p < 0.05$) and

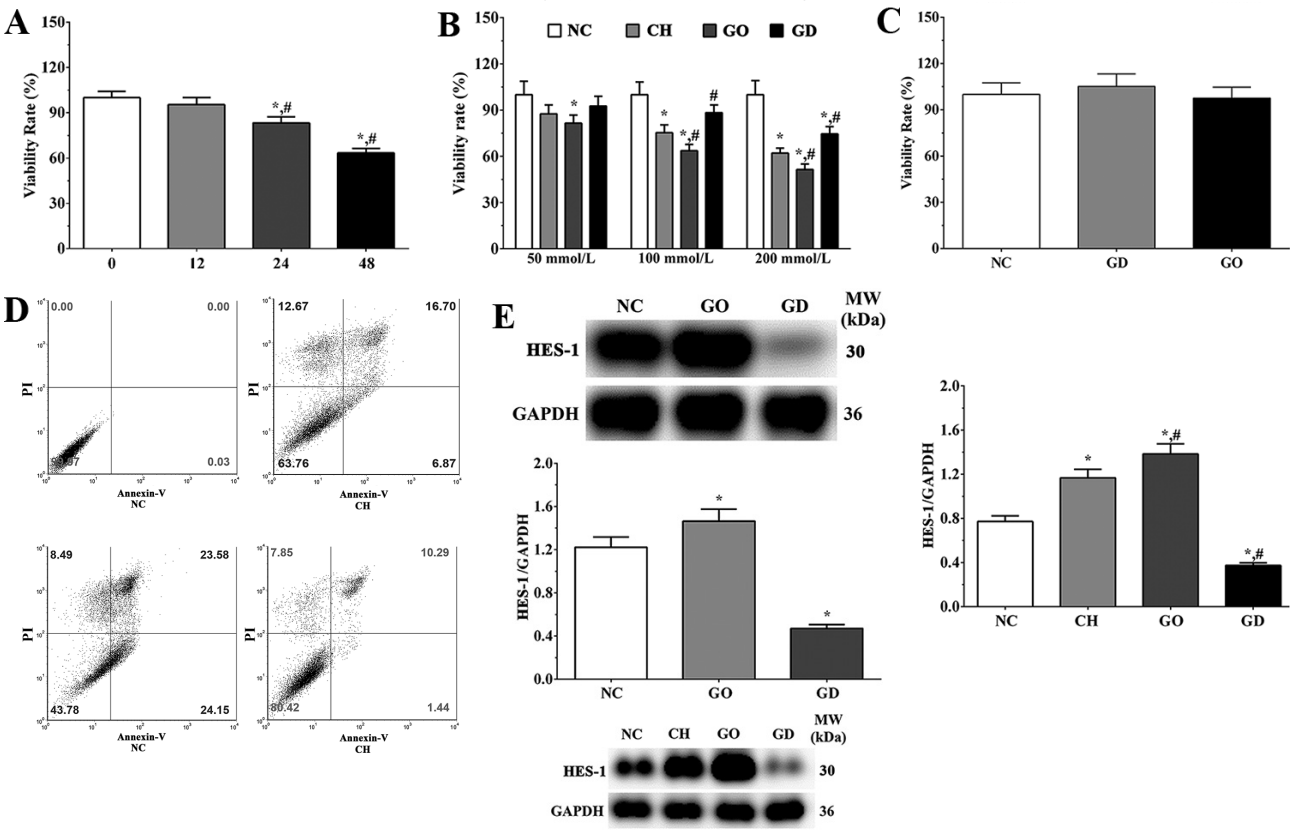

Figure 1. Effect of different cholesterol concentration on proliferation of Ea.hy 926 cells in each group. **A.** Effect of 50 mmol/l cholesterol on proliferation of Ea.hy 926 cells under different treatment period. * $p < 0.05$ *vs.* 0 h treatment group, # $p < 0.05$ *vs.* 12 h treatment group. **B.** Effect of different dose of cholesterol on proliferation of Ea.hy 926 cells for 24 h treatment. **C.** Viability rate in each group without cholesterol treatment. **D.** Effect of cholesterol on apoptosis of Ea.hy 926 cells. **E.** Expression of HES-1 in each group with and without cholesterol treatment. * $p < 0.05$ *vs.* NC group, # $p < 0.05$ *vs.* CH group. Data was presented as mean ± SD, each experiment was repeated for three times independently. NC, control group; CH, cholesterol stimulation group; GO, HES-1 overexpression with cholesterol stimulation group; GD, HES-1 knockdown with cholesterol stimulation group; PI, propidium iodide.

significantly decreased in GO and GD group and significantly increased in GD groupunder 200 mmol/l cholesterol stimulation ($p < 0.05$) compared with CH group. Thus, we used 100 mmol/l cholesterol treated for 24 h to perform the following experiment. And the viability rate in NC, GD and go group was 100.0 ± 7.4, 105.2 ± 8.1 and 97.6 ± 7.1, and the viability rate was not significantly changed in these groups (Fig. 1C). The expression of HES-1 in NC, GO and GD groups without cholesterol treatment were 1.22 ± 0.09, 1.46 ± 0.11 and 0.47 ± 0.04, respectively. The expression of HES-1 was significantly increased in GO group ($p < 0.05$) and significantly decreased in GD group ($p < 0.05$) compared with NC group, indicating the successfully establish of the cell model. The expressions of HES-1 were 0.77 ± 0.05, 1.17 ± 0.08, 1.38 ± 0.09 and 0.37 ± 0.02 in NC, CH, GO and GD group, respectively. The expression level of HES-1 was significantly up-regulated in CH and GO group compared with NC group ($p < 0.05$), and was significantly down-regulated in GD group ($p < 0.05$). The expression level of HES-1 was significantly up-regulated in GO group compared with CH group ($p < 0.05$) and was significantly down-regulated in GD group ($p < 0.05$, Fig. 1E). And the percentage of apoptotic cells in each group were 0.03 ± 0.01%, 36.24 ± 3.21%, 56.22 ± 5.48% and 19.58 ± 2.03% (Fig. 1D). Compared with NC group, the apoptic cells were significantly increased in all treatment groups ($p < 0.05$), and was significantly increased in GO group *vs.* CH group ($p < 0.05$). These results indicating that cholesterol treatment could significantly activate the apoptosis process in cells, and knockdown of HES-1 could reverse this change into a normal level.

Altering in expression of apoptosis related genes under cholesterol stimulation

Cytokines including TNF-α, would activate downstream molecular, such as NF-κB, further affects multiple downstream proteins, such as BAX, Bcl-2 and caspase cascades. As shown

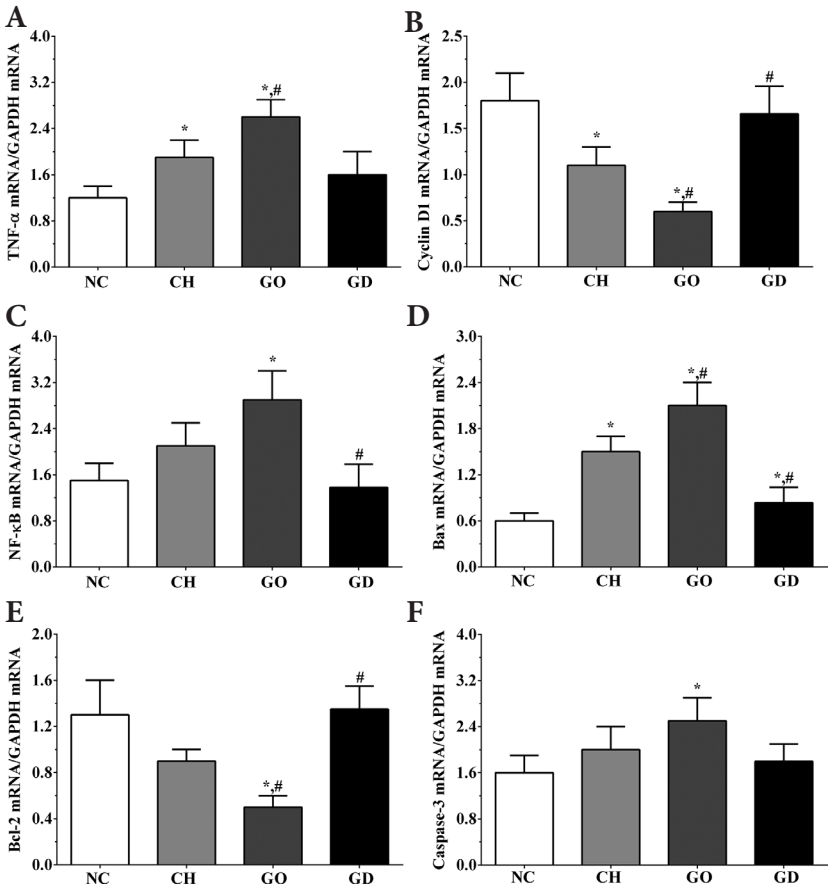

Figure 2. Effect of cholesterol on gene expression of apoptosis related molecules TNF-α (**A**), Cyclin D1 (**B**), NF-κB (**C**), Bax (**D**), Bcl-2 (**E**) and caspase-3 (**F**) of Ea.hy 926 cells in each group. GAPDH was used as an internal control. * $p < 0.05$ *vs.* NC group, # $p < 0.05$ *vs.* CH group. Data was presented as mean ± SD, each experiment was repeated for three times independently. For abbreviations, see Fig. 1.

in Fig. 2, the expression level of TNF-α in NC, CH, GO and GD groups were 1.1 ± 0.2, 1.8 ± 0.3, 2.6 ± 0.5 and 1.6 ± 0.5, respectively. The expression of TNF-α were significantly up-regulated in CH and GO groups ($p < 0.05$) *vs.* NC group, and compared with CH group, the expression of TNF-α was significantly up-regulated in GO group ($p < 0.05$). The expression level of cyclin D1 in these groups were 1.8 ± 0.4, 1.1 ± 0.2, 0.6 ± 0.1 and 1.7 ± 0.3. Compared with NC group, the expression of cyclin D1 was significantly decreased in CH and GO groups ($p < 0.05$), and compared with CH group, the expression of cyclin D1 was significantly decreased in GO group ($p < 0.05$) and significantly increased in GD group ($p < 0.05$). The expression level of NF-κB in these groups were 1.5 ± 0.3, 2.1 ± 0.4, 2.8 ± 0.5 and 1.3 ± 0.4. Compared with NC group, the expression of NF-κB was significantly up-regulated in GO group ($p < 0.05$). When compared with CH group, the expression of NF-κB was significantly down-regulated in GD group. The expression level of Bax in these groups were 0.6 ± 0.1, 1.4 ± 0.3, 2.2 ± 0.4 and 0.9 ± 0.2. Compared with NC group, the expression of Bax was significantly up-regulated in all cholesterol treatment groups ($p < 0.05$), and was significantly up-regulated in GO group and significantly down-regulated in GD group *vs.* CH group ($p < 0.05$). The expression level of Bcl-2 in these groups were 1.3 ± 0.3, 0.9 ± 0.1, 0.5 ± 0.1 and 1.4 ± 0.2. Compared with NC and CH group, the expression of Bcl-2 was significantly down-regulated in GO group ($p < 0.05$), and the expression of Bcl-2 was significantly up-regulated in GD group ($p < 0.05$) *vs.* CH group. The expression level of caspase-3 in these groups were 1.6 ± 0.3, 2.0 ± 0.4, 2.4 ± 0.4 and 1.8 ± 0.2. The expression of caspase-3 was significantly up-regulated in GO group *vs.* NC group ($p < 0.05$).

Expression levels of apoptosis related molecules under cholesterol stimulation

The expression of Bax were 0.33 ± 0.02, 0.83 ± 0.06, 1.11 ± 0.07 and 0.56 ± 0.04 in NC, CH, GO and GD group, respectively (Fig. 3). The expression of BAX were significantly up-regulated in all treatment groups ($p < 0.05$) compared with NC group, and the expression of BAX was significantly up-regulated in GO group ($p < 0.05$) and significantly down-regulated in GD group ($p < 0.05$) *vs.* CH group. The expression of Bcl-2 were 1.02 ± 0.07, 0.88 ± 0.06, 0.68 ± 0.05 and 0.73 ± 0.05 in these groups. Compared with NC group, the expression of Bcl-2 were significantly down-regulated in all treatment groups ($p < 0.05$), and compared with CH group, the expression of Bcl-2

were significantly down-regulated in GO and GD group ($p < 0.05$). The expression of caspase-3 were 0.44 ± 0.03, 0.88 ± 0.06, 0.91 ± 0.06 and 0.30 ± 0.02 in these groups. The expression of caspase-3 were significantly up-regulated in CH and GO groups ($p < 0.05$) and significantly decreased in GD group ($p < 0.05$) *vs.* NC group, and the expression of Bcl-2 was significantly decreased in GD group *vs.* CH group ($p < 0.05$). The expression of caspase-9 were 0.12 ± 0.01, 0.68 ± 0.05, 0.96 ± 0.06 and 0.51 ± 0.03 in these groups. The expression of caspase-9 were significantly up-regulated in all treatment groups compared with NC group ($p < 0.05$), and was significantly up-regulated in GO group ($p < 0.05$) and significantly down-regulated in GD group ($p < 0.05$) *vs.* CH group. BIM is a downstream molecular of PI3K/AKT signaling pathway, which performs an improtant role in apoptosis process *via* regulation of Bcl-2/BAX. And the ratio of p-BIM/BIM were 0.76 ± 0.05, 1.52 ± 0.10, 1.51 ± 0.10 and 0.87 ± 0.06 in these groups. Compared with NC group, the ratio of p-BIM/BIM were significantly up-regulated in CH and GO groups ($p < 0.05$), and the ratio of p-BIM/BIM was significantly down-regulated in GD group compared with CH group ($p < 0.05$). Combined with the results of qPCR, the cellular apoptosis process was activated after cholesterol treatment, and knockdown of HES-1 would reverse these changes, presented a protective role.

Effect of cholesterol on secretion of angiogenesis related molecules

Maintainence of harmonious status of endothelial cells relys on the secertion of multiple factors in circulation blood, thus, angiogenesis process was critical for the proliferation of endothelial cells. As shown in Fig. 4, the concentration of TGF-β in cultured medium were 531.2 ± 23.1, 384.6 ± 18.4, 265.1 ± 12.2 and 486.2 ± 21.0 in NC, CH, GO and GD groups, respectively. Compared with NC group, the concentration of TGF-β was significantly down-regulated in CH and GO groups ($p < 0.05$), and was significantly down-regulated in GO group ($p < 0.05$), significantly up-regulated in GD group ($p < 0.05$) *vs.* CH group. The concentrations of IGF in cultured medium were 1321.2 ± 41.1, 984.6 ± 31.2, 841.2 ± 26.5 and 1287.1 ± 29.3 in these groups. The concentration of IGF was significantly down-regulated in CH and GO group compared with NC group ($p < 0.05$), significantly down-regulated in GO group ($p < 0.05$) and significantly up-regulated in GD group ($p < 0.05$) *vs.* CH group. The concentration of VEGF-α in cultured medium were 154.2 ± 10.1, 105.6 ± 9.2, 83.2 ± 5.5 and 134.1 ± 9.5 in these groups. Compared with NC group, the concentration of VEGF-α was significantly down-regulated in CH and GO group ($p <$

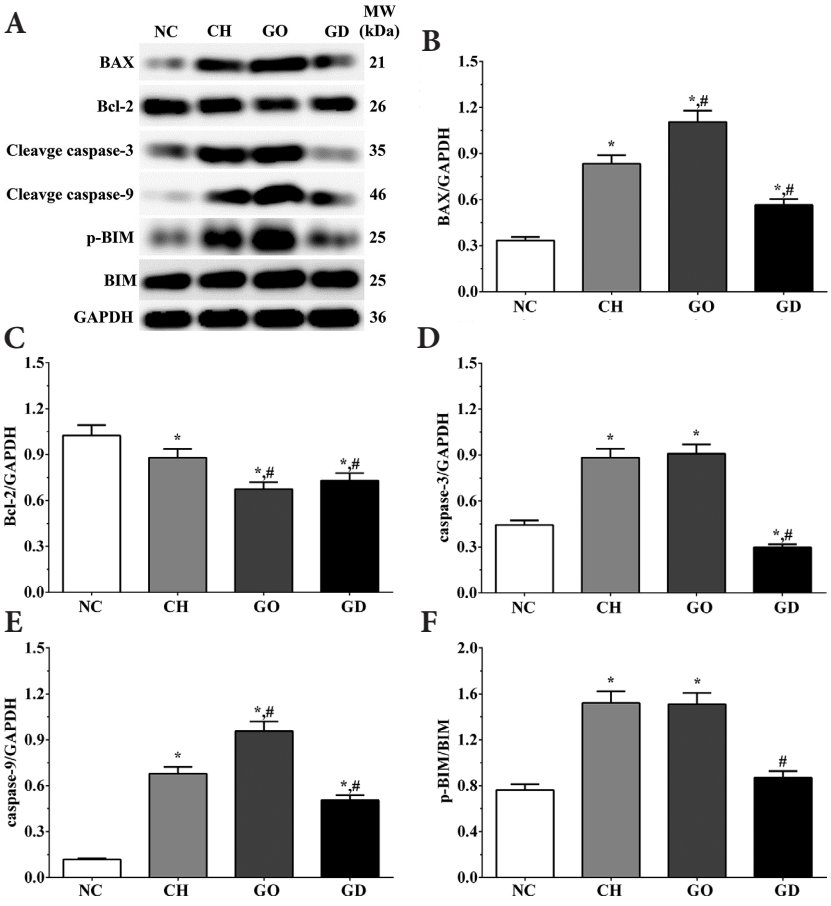

Figure 3. Effect of cholesterol on expression of apoptosis related proteins of Ea.hy 926 cells in each group. **A.** Western blotting analysis of Bax, Bcl-2, cleavge caspase-3, cleavge caspase-9, p-BIM and BIM. Quantitative analysis of Bax (**B**), Bcl-2 (**C**), cleavge caspase-3 (**D**), cleavge caspase-9 (**E**) and p-BIM/BIM (**F**). GAPDH was used as an internal control. * $p < 0.05$ *vs.* NC group, # $p < 0.05$ *vs.* CH group. Data was presented as mean ± SD, each experiment was repeated for three times independently. For abbreviations, see Fig. 1.

0.05), and significantly down-regulated in GO group *vs.* CH group ($p < 0.05$). The concentrations of PDGF in cultured medium were 3520.2 ± 60.5, 2965.1 ± 41.2, 2136.5 ± 35.0 and 3294.1 ± 37.3 in these groups. Compared with NC group, concentration of PDGF was significantly down-regulated in cholesterol treatment groups ($p < 0.05$), and compared with CH group, concentration of PDGF was significantly down-regulated in GO group ($p < 0.05$) and significantly up-regulated in GD group ($p < 0.05$). These results indicate that knockdown of HES-1 expression might promote the proliferation *via* promoting angiogenesis process.

Effect of cholesterol on expression levels of Wnt/PI3K/AKT/β-catenin signaling pathway

As shown in Fig. 5, the expression of Wnt were 1.11 ± 0.07, 1.14 ± 0.08, 0.55 ± 0.04 and 0.75 ± 0.05 in these groups. The expression level of Wnt was significantly down-regulated in GO and GD group ($p < 0.05$) compared with NC and CH group. The ratio of p-AKT/AKT were 1.19 ± 0.08, 0.98 ± 0.07, 0.63 ± 0.04 and 1.43 ± 0.10 in these groups. The ratio of p-AKT/AKT was significantly down-regulated in all treatment groups ($p < 0.05$) *vs.* NC group, significantly down-regulated in GO group and significantly up-regulated in GD group *vs.* CH group ($p < 0.05$). The expression of β-catenin was 1.06 ± 0.07, 1.06 ± 0.07, 0.63 ± 0.04 and 0.76 ± 0.05 in these groups. The expression of β-catenin was significantly down-regulated in GO and GD groups ($p < 0.05$) compared with NC and CH group. And the expression of c-Myc were 0.73 ± 0.05, 0.36 ± 0.02, 0.19 ± 0.01 and 0.51 ± 0.03 in these groups. The expression of c-Myc was significantly in all treatment groups compared with NC group ($p < 0.05$), significantly down-regulated in GO group ($p < 0.05$) and significantly up-regulated in GD group *vs.* CH group.

Discussion

Atherosclerosis is a basic pathology process of cardiovascular disease (CVD), leading to the occurrence of myocardial infarction, stroke and peripheral artery disease (Mozaffarian et al. 2016). Formation of atherosclerotic lesions is the main characteristic of atherosclerosis which is induced by excessive deposition of cholesterol in arterial intima. Research on clinical trials and animal models have found the direct correlation between concentration of cholesterol in plasma and the incidence of CVD (Barton et al. 2013). Meanwhile, cholesterol is critical for cellular biology and maintenance of cholesterol in mammals is important for systemic mechanisms. HES-1 plays an important role in cell proliferation and differentiation processes of multiple cell types, and also related to development of aorta and ventricular septal defects as well as CVD. In present study, we constructed a cell model in Ea.hy 926 cells of HES-1 overexpression and knockdown model, observed the effect of cholesterol on growth of Ea.hy 926 cells and explored possible mechanism. We found that the activation of Wnt/β-catenin/c-Myc signaling pathway after HES-1 overexpressed, while the activity of PI3K/AKT signaling pathway was inhibited. Besides, we also found that the cellular apoptosis process was activated

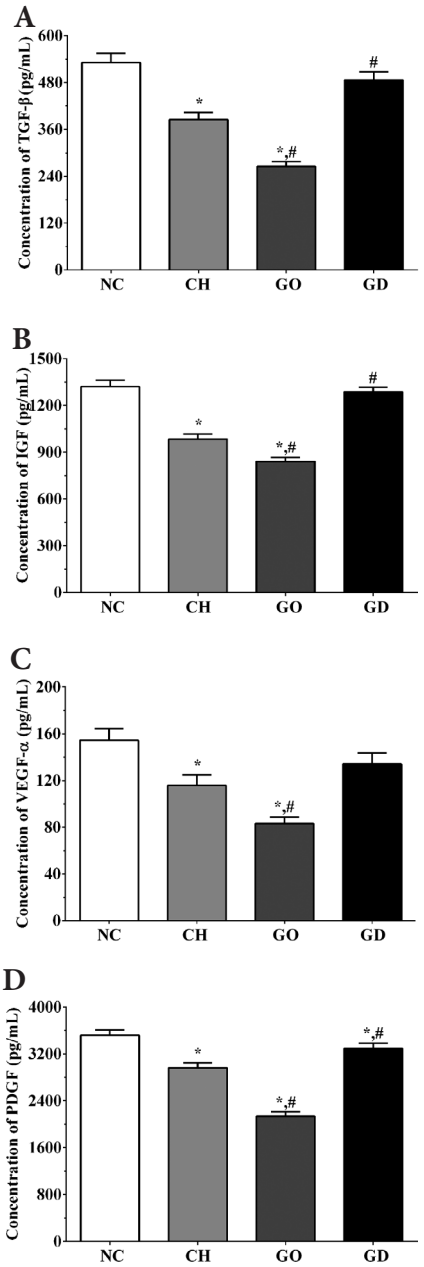

Figure 4. Effect of cholesterol on secretion of angiogenesis related molecules TGF-β (**A**), IGF (**B**), VEGF-α (**C**) and PDGF (**D**) in cultured medium. * $p < 0.05$ *vs.* NC group, # $p < 0.05$ *vs.* CH group. Data was presented as mean ± SD, each experiment was repeated for three times independently. For abbreviations, see Fig. 1.

after cholesterol treatment, and this effect was enlarged after HES-1 overexpressed. The concentration of key cytokines associated with angiogenesis was also down-regulated after cholesterol stimulation and HES-1 knockdown would reverse the phenomena, thus we speculated that HES-1 might be a new threptic target.

Wnt signaling pathway is a critical pathway not only in development biology, but also in vasculogenesis (Clevers et al. 2012). Vasculogenesis is the initial step of vasculature formation, which needs differentiations of endothelial cells from mesodermal precursors. And this step is regulated by multiple pathways, including Wnt signaling pathway. Previous study found that the differentiation process of embryonic stem cells into endothelial cells was under the regulation of Wnt/β-catenin signaling pathway (Yang et al. 2009). Besides, R-spondin-3 also contributes to vasculogenesis and angiogenesis. Previous study found the role of R-spondin-3 in Xenopus and mice embryos could promote the differentiation process of endothelial cells toward angioblasts and activation of β-catenin pathway, leading to the increasing in concentration of vascular endothelial growth factor (VEGF) (Kazanskaya et al. 2008). In Wnt signaling pathway, phosphorylation process of β-catenin was commonly undergo via suppression of CK1 and GSK-3 which allows formation of complex of β-catenin with various transcription factors including VEGF factor, c-Myc and c-Jun, further inducing the increased expression of target genes (Clevers et al. 2012). However, we noticed that the expression of Wnt and β-catenin was not significantly changed after inhibition and overexpression of HES-1, indicating that Wnt/β-catenin signaling pathway was not significantly affect after inhibition of HES-1 expression under cholesterol stimulation. Expression of c-Myc is commonly seen in endothelin (ET)-stimulated vascular smooth muscle cells (VSMC) (Chen et al. 2006). Previous study found that increased expression of ET in experimental model of atherosclerosis was associated with endothelial dysfunction and neovascularization, which is critical for initiation of atherosclerosis (de Nigris et al. 2003). Changing in expression of c-Myc would induce the oxidative stress and DNA damage in cells, besides, previous studies found that c-Myc participates in early atherogenesis *in vitro* and *in vivo* experiments (de Nigris et al. 2001;, Cárcamo et al. 2006). These effects were also regulated by AKT, p38 mitogen-activated protein kinase (MAPK) and Ras signaling pathways, which are critical for cellular survival, proliferation and apoptosis (Griendling et al. 2000). The expression of c-Myc was significantly increased after inhibition of AKT, a critical number of PI3K/PTEN/AKT/mTORC1 signaling pathway (Toker et al. 2014). Previous study also found that activation of PI3K/AKT would increase the expression of c-Myc while inhibition of PI3K/AKT would reduce the expression of c-Myc (Kumar et al. 2016). PI3K/AKT signaling pathway performs an important role in cellular survival, endothelial cell migration and angiogenesis (Huang et al. 2016). Activation of phosphatase and tensin homolog protein (PTEN) could reduce the activation of PI3K/AKT signaling pathway, dysfunction of PTEN was commonly seen in cancers for it could promote migration

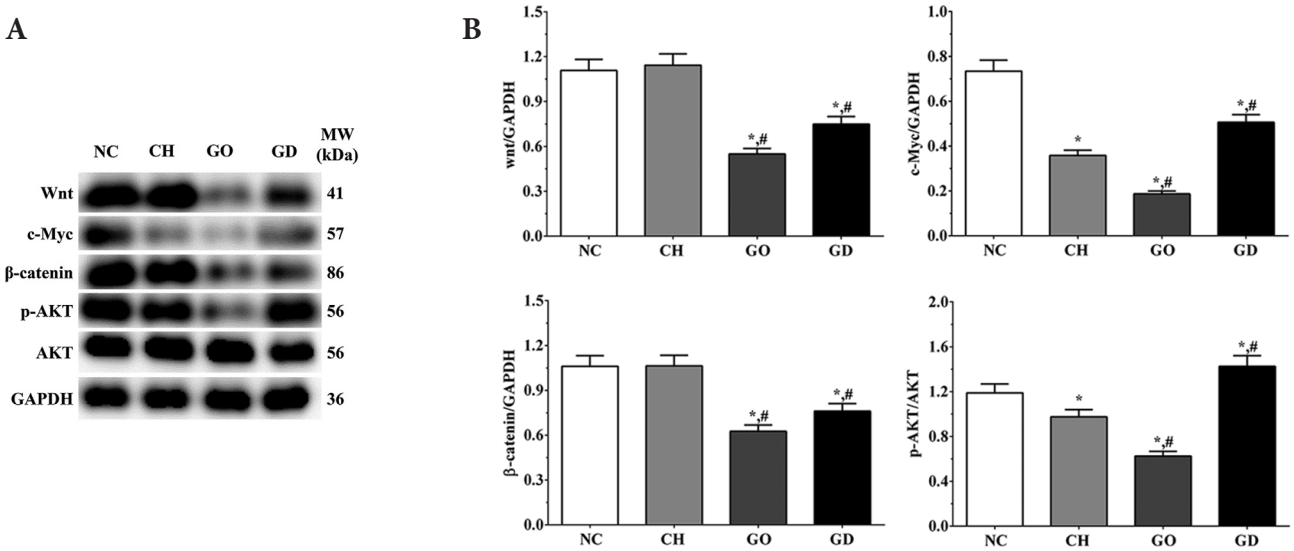

Figure 5. Effect of cholesterol on activation of Wnt/PI3K/AKT/β-catenin signaling pathway. **A.** Western blotting analysis of HES-1, Wnt, c-Myc, β-catenin, p-AKT and AKT. **B.** Quantitative analysis of Wnt, c-Myc, β-catenin and p-AKT/AKT. GAPDH was used as an internal control. * $p < 0.05$ *vs.* NC group, # $p < 0.05$ *vs.* CH group. Data was presented as mean ± SD, each experiment was repeated for three times independently. For abbreviations, see Fig. 1.

and survival of cancer cells. This effect was indirect mediated by induced the activation of mammalian target of rapamycin (mTOR) activity by PTEN (Mao et al. 2012). HES-1 is a target gene of Notch signaling pathway, the expression of HES-1 would be increased at transcription level with the activation of Notch pathway, which would result in reduction of PTEN activation. Accumulation of PIP3 leads to the phosphorylation of AKT kinase at site of Ser473 or Thr 308, followed with phosphorylated at Ser473 by mTORC2 resulting in fully activation of AKT (Hubbard et al. 2014). Activation of Notch leads to the expression of HES-1, resulting in reduction of PTEN expression. These processes would activate PI3K/AKT/mTOR signaling pathway, promoting cellular proliferation, survival and angiogenesis (Maccario et al. 2007; Palomero et al. 2008).

TNF-α is an important cytokine in regulation of inflammation and cytokine network, maintaining its homeostasis (Balkwill et al. 2009), and was found participated in hemorrhagic necrosis process of tumor cells in both human and mice (Carswell et al. 1975). The releasing of TNF-α would also lead to the occurrence of inflammatory bowel disease, psoriasis and rheumatoid arthritis (Ma et al. 2016). Inflammatory diseases inducing by the release of TNF-α were not associated with its ability to induce NF-κB and MAPK-dependent signaling pathway, but for its ability to induce cell death. Previous study found that the inflammatory cell death in mice model induced by TNF-α could be reduced by genetic deletion of cell death components, including cytokines, chemokines and DMAPs (Rickard et al. 2014). Bcl-2 protein family contains plenty numbers, including pro-survival proteins (Bcl-2, Bcl-B, Mcl-1, Bfl-1/A1, Bcl-w and Bcl-xl), pro-apoptotic BH3-only proteins, and pro-apoptotic effectors (BAK and BAX) (Ke et al. 2018). Cytotoxic stimuli including DNA damage, chemotherapeutic agents and cytokines would induce down-regulation of the expression of pro-survival Bcl-2 protein and the up-regulation in BH3-only proteins expression (Czabotar et al. 2014). BAD specific bind with Bcl-w, Bcl-xl and Bcl-2, while NOXA selectively inhibits Bfl-1 and Mcl-1. BID, BIM and PUMA could bind with all pro-survival proteins. Followed with pro-survival proteins sequestered by BH3-only proteins, BAX and BAK could disrupt the outer membrane of mitochondrial, leading to the release of cytochrome c, activation of caspase cascade (including caspase-3) and finally cause the execution of apoptosis (Wong et al. 2011). Besides, some BH3-only proteins, including BIM, PUMA and BID, could directly bind with BAX or BAK, leading to the conformational changes and activation of BAX/BAK (Czabotar et al. 2013).

Previous study found that angiogenesis function of PI3K/AKT signaling pathway was mediated by increasing the expression of VEGF factor further increase the activity of endothelial nitric oxide synthase in endothelial cells (Manning et al. 2007). Besides, activation of Wnt/β-catenin signaling pathway could directly stimulate the expression of EGF and its downstream pathway, including PI3K/AKT/mTOR signaling pathway (Popescu et al. 2016). Secretion of platelet-derived growth factor (PDGF) could induce the aggregation of pericytes at the site of newly formed vessels, as well as promoting the formation of basement membrane *via* ANG/TIE pathway, a downstream pathway of Wnt/β-catenin (Ferrara et al. 2005). Previous study has found that TGF-β could enhance the process of angiogenesis *via* increasing the function of endothelial cells and the expression of VEGF (Pertovaara et al. 1994). Increasing expression of VEGF could promote angiogenesis through recruitment of endothelial cells (Petersen et al. 2010). Effect of TGF-β on endothelial cells could be contradictory through relationship between TGF-β and ALK1 and ALK5 receptors. Low concentration of TGF-β could interact with ALK1 receptors and increase the expression of metalloproteases-2 (MMP-2) and MMP-9, further activate endothelial cells. While higher concentration of TGF-β would interact with ALK5 receptor, further hinder angiogenesis (Goumans et al. 2002).

In this study, we firstly established HES-1 overexpression and knockdown cell model in Ea.hy 926 cells and observe the effect of HES-1 on proliferation of Ea.hy 926 cells under cholesterol stimulation. Then we found the down-regulated expression of pro-apoptosis factors and increased expression of anti-apoptosis factors in Ea.hy 926 cells at protein and gene levels. We further found that the activity of Wnt/β-catenin/AKT signaling pathway was inhibited after cholesterol stimulation and the effect was enlarged after HES-1 overexpression, while alleviated after knockdown of HES-1. Besides, we found that the concentration of angiogenesis factors in cultured medium was also down-regulated after cholesterol stimulation, and was increased after the expression of HES-1 inhibited. Inhibition of activity of Wnt/β-catenin leads to the inhibition of the activity of PI3K/AKT/c-Myc signaling pathway, resulting in activition of angiogenesis process and inhibition of cellular apoptosis process, further promotes the proliferation of cells. Thus, we speculated that HES-1 might be a new therapeutic strategy for cardiovascular disease. However, more experiments on endothelial cells and animal models would be helpful for us to explore the mechanism of cholesterol and cardiovascular diseases, and human trial was also critical in our following experiments.

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