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Roles of notch signaling pathway and endothelial-mesenchymal transition in vascular endothelial dysfunction and atherosclerosis

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Abstract. – OBJECTIVE: To investigate the role of the Notch signaling pathway on the endothelial-mesenchymal transition (EndMT) during vascular endothelial dysfunction and atherosclerosis.

MATERIALS AND METHODS: Human coronary artery endothelial cells (HCAEC) were treated with the exogenous Notch homolog 1 (Notch1) factor to activate the Notch1 pathway, and cells were then observed under the microscope for morphologic changes. Changes in the expression of related proteins were detected by Western blot. *In vivo* experiments were performed using 18 Sprague Dawley® (SD) rats, and GSI factor was used to specifically inhibit Notch pathway activation. Rats were used and randomly divided into three groups: normal diet (ND) group, high-fat diet (HFD) group, and high-fat diet + GSI (HFD+GSI) group, 6 rats in each group. Hematoxylin and eosin (H&E) staining was used to examine the cardiac aortic morphology of the rats in each treatment group. Real-time polymerase chain reaction (RT-PCR) and Western blot were used to detect the expression of Notch1, Hes1, VE-cadherin and α-SMA in the aortic tissues of rats in each group at mRNA and protein levels, respectively.

RESULTS: After HCAECs were treated with Notch1, endothelial protein levels of VE-cadherin were significantly decreased and levels of the interstitial protein α-SMA were significantly increased. In the animal model, the rats fed with high-fat diet for two months presented obvious atherosclerosis spots in their aorta, but those fed with the same diet and treated with GSI inhibitor of Notch pathway showed significantly fewer atherosclerosis signs. Compared with ND group, mRNA and protein expression levels of Notch1, Hes1 and α-SMA were significantly increased, and the expression levels of endothelial marker VE-cadherin were significantly decreased in aortas of rats in HFD group. Compared with the rats in HFD group, the rats in HFD+GSI group showed significantly reduced expression levels of Notch1, Hes1 and α-SMA.

CONCLUSIONS: The activation of Notch signaling pathway can induce the EndMT progression and promote the development of atherosclerotic lesions.

Key Words:
Notch, Endothelial-mesenchymal transition, Atherosclerosis, Hes1, VE-cadherin, α-SMA, Endothelial dysfunction.

Introduction

Endothelial to mesenchymal transition (EndMT) is considered to be a source of fibroblasts and myofibroblasts[1], and it's also an important mechanism leading to vascular endothelial dysfunction and the development and progression of atherosclerosis[2]. During the process of EndMT, the basal membrane of endothelial cells will be degraded, and the connections between the cells will be lost to facilitate cell migration. At the molecular level this process is manifested by the reduction of endothelial cell markers (such as vascular endothelial cadherin, VE-cadherin) and increased protein expression of interstitial markers (such as alpha-smooth muscle actin, α-SMA)[3]. The Notch signaling pathway plays a key role in the development of the mammalian heart[4] by regulating the differentiation and proliferation of human vascular endothelial cells[5,6]. Notch1, as an important member of the Notch family, can par-

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ticipate in EndMT by inhibiting the expression of E-cadherin. Gamma-secretase inhibitor (GSI) can specifically inhibit the activation of the Notch signaling pathway[7]. Nasoohi et al[8] injected GSI into the lateral ventricle of living rats to examine the effect of GSI on the expression of pro-inflammatory markers in the acute and remission stages of neuroinflammation. In this work, we used exogenous recombinant Notch1 protein to activate the Notch signaling pathway in human vascular endothelial cells, and GSI was used to inhibit the activation of the Notch signaling pathway *in vivo*. This study investigated the effects of the Notch signaling pathway on the EndMT progression of endothelial cells and its regulatory role in the development of atherosclerosis in the animal model.

Materials and Methods

Materials

Experimental Cells

Human coronary artery endothelial cells (HCAEC) were purchased from the Cell Bank of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences). HCAEC were cultured in T-25 flasks (NEST, Wuxi, China) containing 5 mL of EBM-2 endothelial cell basal medium (Lonza, New York, NY, USA) supplemented with endothelial cell growth factor ECGS (100 ug/ml, Sigma-Aldrich, St. Louis, MO, USA), 100 U/mL penicillin sodium (HyClone Laboratories, South Logan, UT, USA) and 100 μg/mL streptomycin (HyClone Laboratories, South Logan, UT, USA). Cell culture flasks were placed in an incubator containing 5% CO_2 at 37°C. Notch1 factor (PeproTech US. Corporate, Rocky Hill, NJ, USA) was added to the medium at different concentrations to activate the Notch pathway and EndMT process. Cells were observed under an optical microscope, and relative protein expression levels were measured by Western Blot. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University.

Experimental Animals

A total of 18 Sprague Dawley® (SD) male rats (200 ± 20 g,) were purchased from Laboratory Animal Center of the School of Pharmacy, Fudan University. Rats were raised at room temperature (25 ± 2°C) under specific-pathogen-free (SPF) conditions and were allowed to access food and water freely.

Experimental Methods

Animal Grouping and Model Building

The 18 SD rats were randomly divided into the control group, which received a normal diet (ND group), high-fat diet group (HFD group), and high-fat diet plus GSI (HFD+GSI group), 6 rats in each group. The special high-fat diet[9] consisted of 100 g basal feed, 10 g milk powder, 10 g lard, 1 egg, 10 drops of concentrated cod liver oil (containing 17000 U Vitamin A and 1700 U Vitamin D), 250 g fresh bean sprouts, 10 g casein (protein ≥ 85) and 20 g sucrose. After feeding with high-fat diet for one week, SD rats in the HFD+GSI group were injected with GSI reagent diluted in saline (100 mg/kg/time, 3 times/week), GSI was bought from Selleck Chemicals (Houston, TX, USA). Rats were sacrificed by decapitation two months later, and aorta blood samples were collected, part of the blood samples was fixed in 4% paraformaldehyde overnight at to prepare slices, and the remaining samples were stored in liquid nitrogen for other experiments.

H&E Staining Analysis

Cardiac aortas of rats were fixed in 10-fold volume of formalin overnight and then dehydrated by passing a series of graded concentrations of alcohol. Then, tissues were embedded in wax and sliced into 0.4 μm sections. Tissue sections were baked in an oven at 65°C for 3 hours, and then they were dewaxed with xylene reagents and rehydrated by passing a series of graded concentrations of alcohol. H&E staining was performed to observe the morphology of tissues under an optical microscope (DM-5000B, Leica, Wetzlar, Germany).

RT-PCR Analysis

The cardiac aortas of rats were ground in liquid nitrogen. Then 1 mL TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added, and the liquid was extracted and transferred to a 2.5 ml Eppendorf (EP) tube. EP tubes were kept on ice for 15 min. Then 200 μL chloroform was added, and samples were centrifuged at 12,000 g for 15 min at 4°C. After that, the supernatant was transferred to a new EP tube and 200 μl isopropyl alcohol was added. Samples were shaken several times and then placed on ice for 10 min, followed by centrifugation at 12,000 g for 15 min at 4°C, and the supernatant was discarded. Then 2 mL 75% ethanol was added to wash RNA sample. RNA

samples were dried at room temperature. Finally, an appropriate amount of RNAse-free water was added to dissolve RNA, and RNA concentration was measured using a spectrophotometer. Based on the protocol provided by TaKaRa (Article No.: 2690A; Otsu, Shiga, Japan), 1 μg RNA was used in reverse transcription using the PrimeScript™ Kit (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA was stored at -20°C. The mRNA levels of each index were determined according to the descriptions of the All-in-One ™ qPCR Mix kit (GeneCopoeia, Guangzhou, China). Ct values were processed using 2-ΔCt, where ΔCt = Ct (target gene) – Ct (GAPDH)]. The corresponding primer sequences were shown in Table I.

Western Blot

An appropriate amount of rat cardiac aortas was cut into pieces and transferred to a 2.5 ml EP tube. Tissues were mixed with 150 μL of radioimmunoprecipitation assay (RIPA) buffer and protease phosphorylation inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) was added. Samples in EP tubes were homogenized in a homogenizer for 10 min. Then a sonicator system was used for ultrasonic crushing (40A, 3 seconds/time; the process was repeated for 3 times). The resulting liquid was centrifuged at 12,000 g for 15 min at 4°C, and the upper layer of liquid containing soluble protein was extracted. Protein concentration was measured using BCA kit (Beyotime, Guangzhou, China). After denaturation, total protein was separated by 10% acrylamide SDS-PAGE gel (Beyotime Biotechnology, Shanghai, China) electrophoresis, followed by transmembrane to 0.22 μm nitrocellulose membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk for 1 hour, and incubated with Notch1 (1:1000), Hes1 (1:2000), VE-cadherin (1:1000), α-SMA (1:500) and GAPDH (1:2000) overnight. The next day, membranes were incubated with anti-mouse or anti-rabbit IgG secondary antibody for 1 hour. Signals were developed with the ECL system (Bio-Rad, Hercules, CA, USA). The relative protein content of the target protein was the ratio of the grey value of the target protein to that of the corresponding endogenous control. The following are the providers for each antibody used: anti-Notch1 (Abcam, Cambridge, MA, USA); anti-VE-cadherin (Cell Signaling Technology, Danvers, MA, USA), anti-α-SMA (Abcam, Cambridge, MA, USA); anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA); anti-rabbit and anti-mouse secondary antibodies (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

Results were analyzed using GraphPad Prism software (Version 5.01; GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used to compare differences among multiple groups. Expression levels of each factor were compared between every two groups using the least significant difference (LSD)-*t* test. $p < 0.05$ was considered to be statistically significant.

Results

Determination of the Optimal Action Concentration of Notch1 for in vitro Experiments by Western Blot

HCAEC were seeded in 6-well plates. When cell confluence reached 60%, different concentrations of exogenous human recombinant protein Notch1 (PeproTech US. Corporate, Rocky Hill, NJ, USA) were added to different wells to activate the Notch signaling pathway. Increases in the protein expression level of Hes1, which is a target of the Notch signaling pathway, was observed, reflecting the activation of the signaling pathway. As shown in Figure 1, 1000 ng/mL of exogenous Notch1 factor significantly enhanced the protein level of Hes1. Therefore, 1000 ng/mL was used as the optimal action concentration for the subsequent experiments.

Effects of Notch1 on HCAEC Morphology

As shown in Figure 2, HCAEC without exogenous Notch1 showed oval shape. However, morphological changes (spindle-shape and pseudopodia) were observed after incubation with 1000 ng/mL Notchl for 24 hours.

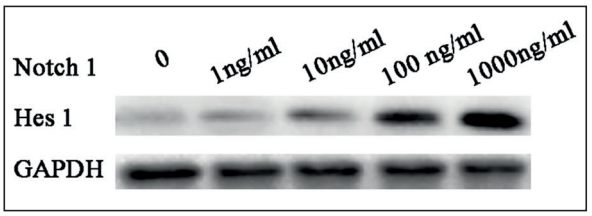

Figure 1. Determination of the optimal concentration of Notch1 to activate the Notch pathway by Western blot.

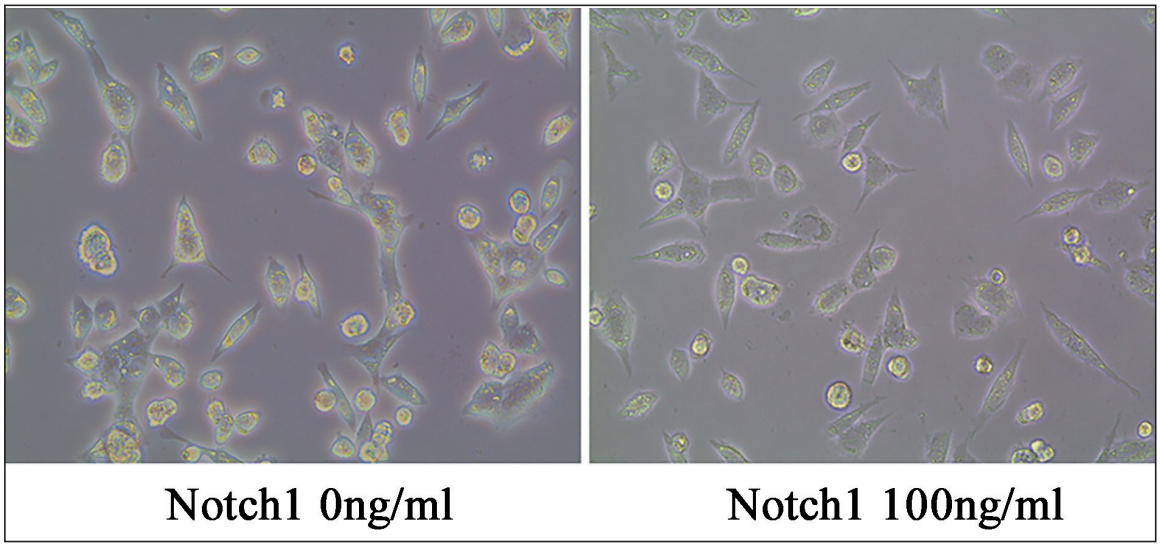

Figure 2. Microscopic view of effects of Notch1 on HCAEC morphology (200×). HCACEC incubated in the presence or absence of Notchl factors for 24 hours, note the appearance of spindles and pseudopodia in the 1000 ng/ml treated cells.

Effects of Notch1 on EndMT Markers by Western Blot

As shown in Figure 3, compared with the control group, treatment with 1000 ng/mL Notch1 significantly increased the protein content of Hes1 and α-SMA, while decreased the expression level of VE-cadherin.

Detection of Morphological Changes of Cardiac Aortas in Rats by H&E Staining

HE staining results showed that, compared with the ND group, the subendothelial layer of the cardiac aorta of rats in the HFD group was significantly thickened, as seen by the emergence of some small uniformly distributed plaques. In contrast, no apparent plaques appeared in the cardiac aorta of rats in the HFD+GSI group (Figure 4). These data suggest that inhibiting the activation of the Notch signaling pathway has a protective effect on the development of atherosclerosis.

Detection of the Notch Signaling Pathway Activation and the Expression Levels of EndMT Markers Measured by RT-PCR

Correlations between the Notch signaling pathway and EndMT markers in the rat models

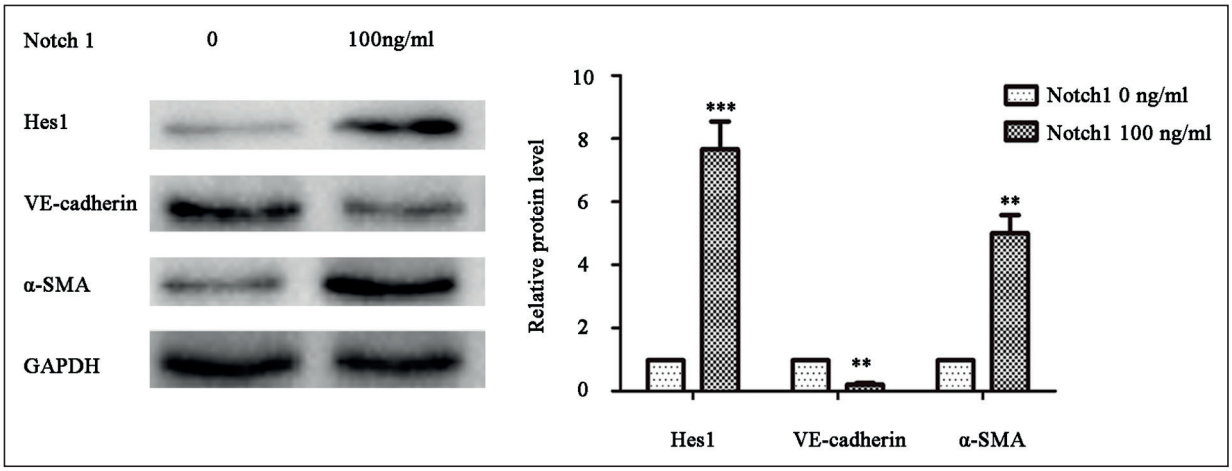

Figure 3. Effects of Notch1 on EndMT markers by Western blot.

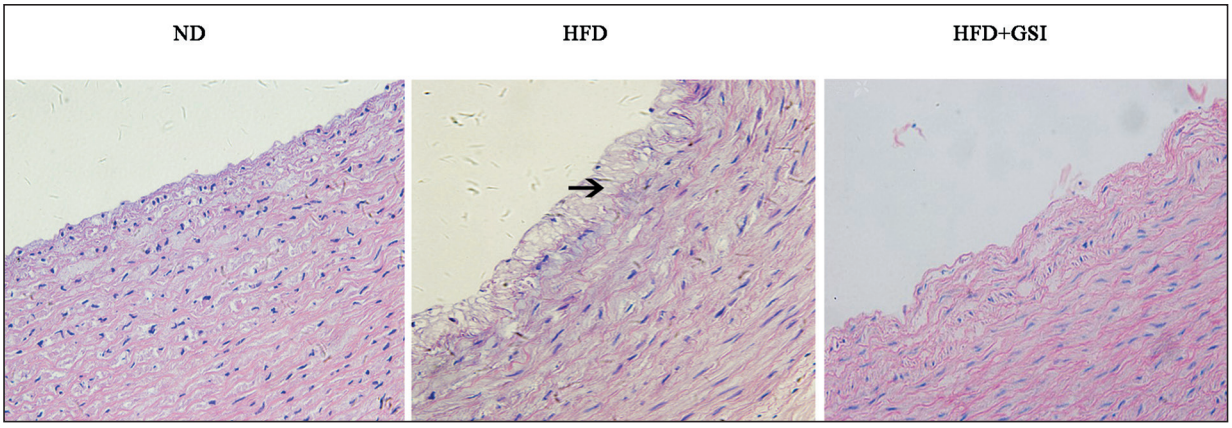

Figure 4. Detection of morphological changes of cardiac aortas in rats by H&E staining (200×). Note: the arrow shows the place where the subendothelial layer becomes thickened, some small uniformly distributed plaques are present.

were investigated. As shown in Figure 5, expression levels of Notch1, Hes1 and α-SMA in the cardiac aortas of rats in the HFD group were significantly higher than those in ND group (Notch1: HFD *vs.* ND, $p < 0.001$; Hes1: HFD *vs.* ND, $p < 0.01$; α-SMA: HFD *vs.* ND, $p < 0.01$). Meanwhile, the expression level of VE-cadherin was significantly decreased ($p < 0.05$). These findings suggest EndMT was significantly activated in HFD rats. After GSI intervention, expression levels of Notch1, Hes1 and α-SMA were significantly decreased (Notch1: HFD+GSI *vs.* HFD, $p < 0.01$; Hes1: HFD+GSI *vs.* HFD, $p < 0.05$; α-SMA: HFD+GSI *vs.* HFD, $p < 0.05$). While the expression level of VE-cadherin was significantly increased ($p < 0.05$), reflecting an inhibitory effect on EndMT markers

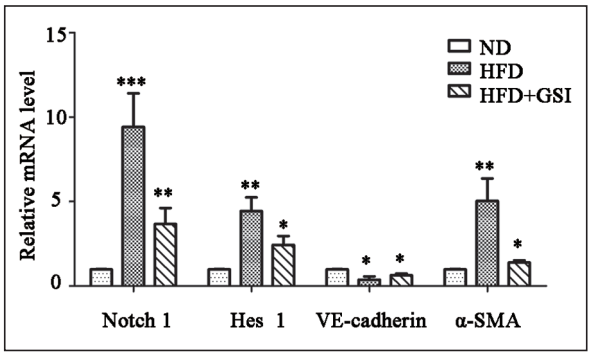

Figure 5. Detection of the Notch signaling pathway and the expression level of mRNA in EndMT indexes (Notch1, Hes1, VE-cadherin and α-SMA) by RT-PCR compared with the ND group, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Detection of EndMT Markers in Cardiac Aortas of Rats by Western Blot

Correlations between the Notch signaling pathway and EndMT markers were further studied at protein level. Results were consistent with the findings at mRNA level. As shown in Figure 6, high-fat diet could activate the Notch signaling pathway and promote the EndMT process. Protein expression levels of Notch1 and α-SMA were significantly increased, while the expression level of VE-cadherin was significantly reduced. In contrast, GSI inhibited the activation of the Notch signaling pathway and reversed EndMT.

Discussion

Atherosclerosis is the primary cause of coronary artery disease, cerebral infarctions and peripheral vascular diseases across the world. The sclerotic disease is characterized by the thickened and hardened endangium (intima) with reduced elasticity caused by cholesterol accumulation and changes in vascular endothelial cells[4,8]. The main clinical manifestations of atherosclerosis include ischemic heart disease, ischemic stroke and peripheral arterial disease, and the incidence of this disease showed an increasing trend in recent years[9]. However, the specific molecular mechanism of atherosclerosis remains unclear. Some studies have shown that endothelial dysfunction is the early stage of atherosclerosis[10]. Vascular endothelial cells as the main regulators of vascular homeosta-

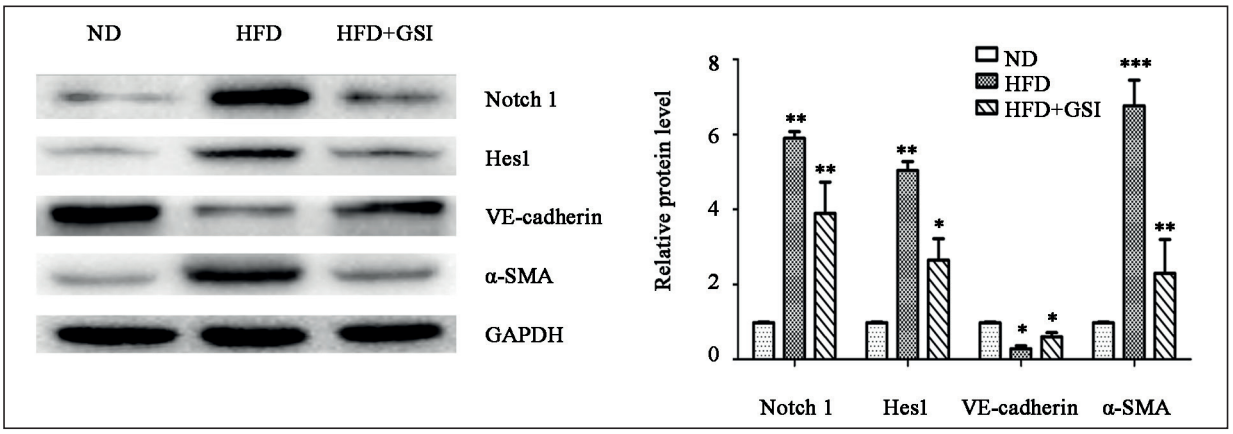

Figure 6. Detection of EndMT indexes of cardiac aortas in rats by Western blot compared with the ND group, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

sis can protect blood vessels by dilating them, inhibiting smooth muscle cell growth and reducing inflammatory responses[11-13]. Scholars[11,14] have shown that vascular endothelium-derived nitric oxide can regulate vasoconstriction, to prevent endothelial dysfunction. EndMT is the molecular basis of endothelial dysfunction and atherosclerosis[15].

Previous studies on EndMT mainly focused on cardiac development[18]. Zeisberg et al[19] first found that myocardial fibrosis is derived from endothelial cells. Solene et al[20] have shown that EndMT induces atherosclerosis and may be associated with complex plaques. During the early stages of atherosclerosis, various factors in the internal environment can induce a cascade of reactions, resulting in loss of connections between endothelial cells, skeleton remodeling, reduced expression levels of certain genes (E-cadherin) and increased the expression of mesenchymal genes (α-SMA, Vimentin, etc.) in the endothelial tissues. Finally, the endothelial cells are transformed into mesenchymal cells and migrated to intima to cause intimal hyperplasia[19,20].

In this report, we examined the effects of the Notch signaling pathway and EndMT on atherosclerotic diseases. We showed that the activation of the Notch signaling pathway *in vitro* cultured endothelial cells significantly promoted EndMT. In animal models, GSI was used to inhibit Notch signaling pathway. The rats treated with high-fat diet in the absence of the EndMT inhibitor GSI showed activated Notch signaling pathway and an EndMT process, while those treated with the GSI inhibitor showed lower levels of Notch signaling pathway and EndMT markers after rats were fed with high-fat diet. In addition, we carried out H&E staining to observe morphological changes of the cardiac aorta of rats, and our results showed that the endothelium of rats in the HFD group was thickened, and plaque spots on the cardiac aorta of some rats were observed.

Conclusions

Even though these findings were found in the rat model, it is likely that a very similar process takes place in humans. However, the correlations between the degrees of atherosclerosis in the heart and the degrees of up-regulation of Notch expression remain to be further studied. Notch and EndMT could become early diagnostic markers and potential therapeutic targets for atherosclerotic diseases.

Ethics Approval and Consent to Participate

The study was approved by the Second Affiliated Hospital of Wenzhou Medical University.

Statement of Interest

The study was supported by Natural Science Foundation of China (No.81704076).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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