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MiR-410 inhibition induces HUVECs proliferation and represses ox-LDL-triggered apoptosis through activating STAT3

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ABSTRACT

Ox-LDL-induced endothelial cells injury has been reported to play an important role in the development of atherosclerosis (AS). MicroRNAs have been identified to regulate their target genes post-transcriptionally and they are able to participate in the various diseases, including AS. However, the role of miR-410 in ox-LDL-triggered abnormal function of endothelial cells remains to be elaborated. Hence, our current study was to find out the underlying mechanism of miR-410 in AS. Here, we observed that ox-LDL can inhibit HUVECs growth and lead to a great cell apoptosis both dose-dependently and time-dependently. Meanwhile, it was exhibited that miR-410 expression was remarkably elevated in ox-LDL-indicated HUVECs. miR-410 knockdown was able to induce cell proliferation and alleviate HUVECs apoptosis subjected to ox-LDL. Reversely, signal transducer and activator of transcription 3 (STAT3) expression was greatly decreased in ox-LDL-incubated HUVECs in a time and dose dependent manner. Additionally, these findings exhibited that STAT3 was a target of miR-410, which was validated by a dual-luciferase assay in our study. Additionally, we observed that overexpression of STAT3 rescued ox-LDL induced AS events in vitro. Taken these together, our current study implied that miR-410 silence can inhibit the ox-LDL-induced HUVECs proliferation and rescue cell apoptosis through activating STAT3 in vitro.

1. Introduction

Atherosclerosis (AS) has been regarded as the leading cause of morbidity and mortality world [1]. It is well known that hyperlipidemia, inflammation and endothelial dysfunction contribute greatly to AS development [2]. Increasing evidence has indicated that endothelial dysfunction which is caused by vascular injury contributes to AS pathogenesis [3]. It is a candidate direct anti-AS therapy to improve endothelial function.

MicroRNAs are a class of 22 nts endogenous non-coding RNAs and they can act as important gene regulators by targeting 3′UTR of their target mRNAs [4]. Recently, many studies have reported that miRNAs can exert significant roles in AS progression and they can act as potential diagnostic or prognostic biomarkers [5,6]. For example, miR-155 can prevent inflammation progression in AS-correlated foam cell formation through targeting calcium-regulated heat stable protein 1 [7]. It has been demonstrated that miR-21, miR-210, and miR-34a are overexpressed in atherosclerotic plaques [8]. MiR-19a can induce inflammation and formation of foam cells by inhibiting HBP-1 expression in AS [9]. MiR-495 can regulate HUVECs growth and apoptosis capacity by inhibiting chemokine CCL2 [10]. miR-410 has been identified in multiple disease. For example, it has been reported that miR-410 can induce stemness by targeting Gsk3β in NSCLC [11]. MiR-410 is upregulated in liver and colorectal cancers, which can enhance tumor cell progression by targeting FHL1 [12].

Our current study was to explore the biological function of miR-410 in AS pathogenesis. miR-410 was dramatically upregulated and STAT3 was inactivated in ox-LDL-treated HUVECs. miR-410 silence can rescue ox-LDL-inhibited cell proliferation and alleviate apoptosis of HUVECs. We hypothesized that miR-410 could regulate AS progression through targeting STAT3.

2. Materials and methods

2.1. Cell lines

HUVECs and HEK-293T cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (Invitrogen Corp, Grand

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Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin was used to culture the cells. Cells were cultured in 5% CO_2 at 37 °C.

2.2. CCK8 assay

HUVECs were seeded in a 96-well plate at 37 °C incubator. 100 μL CCK8 reagents (Dojindo Molecular Technologies, Tokyo, Japan) were added to each well. After incubated for 4 h, a microplate reader (Bio-Tek, Winooski, VT, USA) was employed to detect the absorbance at 450 nm.

2.3. Cell transfection

According to the instructions of manufacturer, miR-410 mimics, inhibitors or their NCs were transfected into the cells for 48 h using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The human STAT3 plasmid pcDNA3.1-STAT3 or the control plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) was transfected into HUVECs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.4. qRT-PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen Corp, Grand Island, NY, USA). Reverse transcriptase (TaKaRa, Tokyo, Japan) was used to reverse transcribe total RNAs. Then, real-time PCR was conducted using SYBR Green Mastermix kit (Takara, Tokyo, Japan). Specific RT primers and real-time PCR primers were obtained from RiboBio (Guangzhou, China). Primer sequences were exhibited in Table 1. $2^{-\Delta\Delta Ct}$ method was used.

2.5. Flow cytometry analysis

Cell apoptosis were detected using Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme Biotech, Nanjing, China). Cells were collected and then washed using PBS. After that 1x binding buffer (100 μL) was used to suspend cell pellet and then annexin APC and PE was used.

2.6. Western blot analysis

Equal cell protein samples were separated on 10% SDS-PAGE gels and PVDF membrane was used to transfer proteins. Primary antibodies against STAT3, and GAPDH (CST, Ala, USA) were used for western blotting analysis. The bonds were incubated with the primary antibodies overnight and next day secondary antibodies (anti-rabbit or anti-mouse) for 1 h at room temperature. Immunoreactive bands were observed with the ECL kit (Pierce, Rockford, IL, USA).

2.7. Luciferase activity assay

The WT or MUT of STAT3 was designed into pGL3 Basic vector (Promega, Madison, WI, USA). Mimics of miR-410 (RiboBio, Guangzhou, China) were cotransfected with pLUC-WT-STAT3 or pLUC-MUT-STAT3. Luciferase activity was evaluated by the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Table 1
Primers used for real-time q-PCR.

	Forward(5'–3')	Reverse(5'–3')
GAPDH	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
STAT3	ACCTGCAGCAA-TACCATTGAC	AAGGTGAGGGACTCAAACTGC

2.8. Statistical analysis

All data were indicated as mean ± SD and experiments were performed for at least three times. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used to do analysis. Comparisons between quantitative variables were analyzed using student's t test and One-Way ANOVA analysis. $P < 0.05$ represented a statistically significance.

3. Results

3.1. Ox-LDL inhibited HUVECs proliferation and induced cell apoptosis

Firstly, to observe the effects of ox-LDL on HUVECs proliferation and apoptosis, cells were subjected to ox-LDL at different concentrations (0–40 μg/ml) for 24 h or 20 μg/ml ox-LDL for various time durations (0–48 h). We observed that HUVECs proliferation was significantly inhibited by different doses of ox-LDL (Fig. 1A). Treatment of HUVECs with 20 μg/ml ox-LDL for various durations can lead to a time-dependent inhibition in cell proliferation (Fig. 1B). Next, it was shown in Fig. 1C and D, HUVECs cell apoptosis was greatly promoted by ox-LDL. All these data suggested that ox-LDL exerted an important role in the development of AS.

3.2. MiR-410 was elevated by incubation of ox-LDL

Furthermore, to test miR-410 expression in ox-LDL-indicated HUVECs, qRT-PCR was conducted. It was manifested in Fig. 2A that miR-410 was upregulated by different doses of ox-LDL. At various durations from 0 to 48 h, miR-410 was increased time-dependently in HUVECs (Fig. 2B). These findings indicated that miR-410 was involved in the progression of AS in vitro.

3.3. STAT3 was decreased by treatment of ox-LDL

Moreover, it was observed that mRNA expression and protein levels of STAT3 in HUVECs were down-regulated by ox-LDL dose-dependently (Fig. 3A and B). Consistently, Fig. 3C and D demonstrated a time-dependent decrease of STAT3 levels. These findings suggested that STAT3 also exhibited important roles in AS development in vitro.

3.4. MiR-410 down-regulation played an inhibitory role in AS development

Since miR-410 can be greatly increased by indicated ox-LDL, to investigate whether miR-410 can modulate HUVECs cell proliferation and apoptosis, miR-410 expression was modulated by transfection of mimics, inhibitors or their NCs in HUVECs. In Fig. 4A, we observed that miR-410 was strongly increased by miR-410 mimics and decreased by miR-410 inhibitors in vitro. Then, CCK8 assay was performed and it was shown in Fig. 4B that miR-410 inhibitors elevated cell proliferation ratios and miR-410 mimics exhibited a reverse effect after the cells were incubated with 20 μg/ml ox-LDL for 24 h. In addition, HUVECs cell apoptosis which was induced by 20 μg/ml ox-LDL were rescued by miR-410 inhibitors whereas miR-410 mimics exacerbated cell apoptosis in vitro (Fig. 4C). It was suggested that miR-410 inhibition can exhibit a suppressive role in the development of AS.

3.5. STAT3 was a target of miR-410

According to bioinformatics analysis miRDB database, a conserved binding region between miR-410 and STAT3 was demonstrated in Fig. 5A. To confirm the correlation between them, a wild-type binding site (WT-STAT3) or mutant binding site (MUT-STAT3) was constructed into the dual-luciferase vectors. In Fig. 5B, it was manifested that co-transfection of miR-410 mimics and the luciferase vector containing WT-STAT3 could decrease the relative luciferase activity in HEK-293T cells. Subsequently, to detect whether STAT3 can be regulated