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MicroRNA-30c-5p inhibits NLRP3 inflammasome-mediated endothelial cell pyroptosis through FOXO3 down-regulation in atherosclerosis

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease involved in endothelial dysfunction. Pyroptosis is a pro-inflammatory form of cell death and plays pivotal roles in atherosclerosis. MicroRNAs (miRNAs) are implicated in atherosclerosis, however the mechanisms that underlie miR-30c-5p is required for endothelial cell pyroptosis remain elusive. In the present study, we probed the interaction of miR-30c-5p with forkhead box O3 (FOXO3) and investigated the effect of miR-30c-5p and FOXO3 on NLRP3 inflammasome and endothelial cell pyroptosis. Introduction of oxidized low density lipoprotein (ox-LDL) dose-dependently increased lactate dehydrogenase (LDH) release as well as pyroptosis in human aortic endothelial cells (HAECs). On the basis of ox-LDL treatment, we found the expression of miR-30c-5p was impaired and enrichment of miR-30c-5p protected HAECs from ox-LDL-induced pyroptosis. Moreover, addition of miR-30c-5p inhibited ox-LDL-activated NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, which was associated with HEACs pyroptosis. Nevertheless, miR-30c-5p failed to show efficacy of Toll-like receptor (TLR) signaling of NLRP3 inflammasome activation. Intriguingly, FOXO3 was suggested to be targeted by miR-30c-5p and addition of miR-30c-5p blocked FOXO3 expression, whereas miR-30c-5p depletion showed opposite effects. Furthermore, silencing of FOXO3 inhibited NLRP3-mediated pyroptosis and reversed anti-miR-30c-5p-induced activation of NLRP3 inflammasome and pyroptosis in HEACs with ox-LDL treatment. Our finding suggested that miR-30c-5p might play essential role in NLRP3 inflammasome-modulated cell pyroptosis by targeting FOXO3 in HAECs, providing a novel therapeutic avenue for atherosclerosis treatment.

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1. Introduction

Atherosclerosis is a key pathophysiological process of cardiovascular diseases with leading cause of death worldwide [1]. Endothelial dysfunction plays a pivotal role in development of atherosclerosis [2]. Cell death and inflammation are essential for cardiovascular diseases, including atherosclerosis process [3,4]. Pyroptosis is a form of inflammatory cell death and suggested to be involved in atherosclerosis process [5,6]. Besides, activation of NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome facilitates atherosclerosis via varying signaling pathway [7]. Uncovering the mechanisms of the process may provide a novel avenue for atherosclerosis treatment.

MicroRNAs (miRNAs), a class of short noncoding RNAs, have an evident role in driving endothelial dysfunction and may represent promising therapeutic approach to atherosclerosis [8]. MiR-21 is reduced and addition of miR-21 inhibits oxidized low density lipoprotein (ox-LDL)-induced Human aortic endothelial cells (HAECs) damage and autophagy dysfunction in atherosclerosis [9]. Increased miR-29b and interleukin-6 (IL-6) levels are associated with subclinical atherosclerosis and may be used as novel approach for atherosclerosis treatment [10]. Moreover, addition of miR-876 contributes to cell apoptosis in HAECs and improves atherosclerosis development [11]. Besides, many other miRNAs are reported to may be served as biomarkers and therapeutic avenue in atherosclerosis, such as miR-33, miR-548p, miR-15b-5p and miR-126 [12]. Notably, miR-30c-5p, as a suppressor in tumorigenesis, has been shown to regulate cell metastasis and epithelial to

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mesenchymal transition in gastric cancer [13]. Loss of miR30c-5p leads to epithelial to mesenchymal transition and poor prognosis for hepatocellular carcinoma cells [14]. Recently, miR-30c-5p has been showed great promise in atherosclerosis [15]. However, underlying mechanism that allows miR-30c-5p is required for atherosclerosis is still required.

The former finding suggests forkhead box O3 (FOXO3/FOXO3a), a member of FOXO with other subfamilies including FOXO1, FOXO4 and FOXO4, plays varying role in several types of diseases [16]. FOXO3 may inhibit endothelial progenitor cells proliferation by cell cycle arrest in many cardiovascular diseases [17]. Lacking FOXOs protect endothelial cells from vascular dysfunction and atherosclerosis in mice [18]. Likewise in vascular smooth muscle cell, FOXO3 decreases cell proliferation and migration, and results in apoptosis induction [19]. Since FOXO3 opens up a potential as a biomarker in atherosclerosis, the interaction between miR-30c-5p and FOXO3 in HAECs remains an open question. In the present study, we developed HAECs as cell model of atherosclerosis to investigate the effect of miR-30c-5p on pyroptosis. Here we demonstrated a link between miR-30c-5p and FOXO3 in HAECs. Functional analysis clarified and broadened our understanding of the function of miR-30c-5p and FOXO3 on NLRP3 inflammasome activation and pyroptosis in HAECS.

2. Materials and methods

2.1. Cell culture and treatment

HAECs were obtained from ScienCell Research Laboratories (ScienCell, Carlsbad, CA, USA) and cultured at 37 °C in a humidified incubator with 5% CO_2 using Endothelial Cell Medium (ECM) (ScienCell) containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) during the study. Functional ox-LDL (Yiyuan Biotechology, Guangzhou, China) assay was realized in HAECs by lactate dehydrogenase (LDH) release and Hoechst 33342/Propidium Iodide (PI) staining with different concentrations of ox-LDL (0, 1, 10, 25, and 50 μg/mL) for 24 h. And ox-LDL (50 μg/mL) was afforded in HAECs for further study.

2.2. Cell transfection

MiR-30c-5p mimic, miR-NC, miR-30c-5p inhibitor (anti-miR-30c-5p), anti-miR-NC, siRNA duplex oligonucleotides targeting NLRP3 (siNLRP3), FOXO3 (siFOXO3) and negative control si-NC, were synthesized by Genepharma (Shanghai, China). Transfection was performed into HAECs using Lipofectamine 2000 (Invitrogen) referring to the manufacturer's protocol.

2.3. LDH release assay

Culture medium was collected for LDH release using commercial LDH Activity Assay Kit (Sigma, St. Louis, MO, USA) according to manufacturer's instructions. In brief, LDH substrate mix and assay buffer were added into cell culture supernatants in a 96 well plate. After the incubation at 37 °C for 15 min without light, the absorbance was recorded at 450 nm on a microplate reader (Molecular Devices, Palo Alto, CA, USA).

2.4. Hoechst 33342/PI staining

For Hoechst 33342/PI staining, HAECs were seeded in 6-well plate with varying treatment. Then cells were washed with PBS (Sigma) and incubated with Hoechst 33342 and PI solution (Sigma) for 20 min at room temperature. After the washes with PBS, stained cells were captured on a fluorescent microscope (Olympus, Tokyo, Japan) and evaluated using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was prepared using *mir*Vana™ miRNA Detection Kit (Thermo Fisher, Wilmington, DE, USA) following the manufacturer's instructions. Subsequently, 500 ng RNA was used for first strand cDNA syntheses by TaqMan Reverse Transcription Kit or TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Then diluted cDNA was used for qRT-PCR using SYBR Green Real time PCR Master Mix (Toyobo, Tokyo, Japan) detection following the amplification instructions. β-actin or U6 small RNA was used as housekeeping gene for normalization of mRNA or miR-30c-5p, respectively. All primers were obtained from Invitrogen: miR-30c-5p (Forward, 5′-GCCGCTGTAAACATCCTACACT-3'; Reverse, 5′-GTGCAGGGTCCGAGGT-3′), U6 (Forward, 5′-GCTTCGGCAGCACATA-TACTAAAAT-3'; Reverse, 5′-CGCTTCACGAATTT GCGTGTCAT-3′), Caspase-1 (Forward, 5′-GCACAAGACCTCTGACAGCA-3'; Reverse, 5′-TTGGGCAGTTCTTGGTATTC-3′), IL-18 (Forward, 5′-GACTCTTGCGT-CAACTTCA AGG-3'; Reverse, 5′-CAGGCTGTCTTTTGTCAACGA-3′), IL-1β (Forward, 5′-TTCAACA CGCAGGACAGGTACAG-3'; Reverse, 5′-CCAGGGACAGGATATGGAGCA-3′), NLRP3 (Forward, 5′-ACAGC-CACCTCACTTCCAG-3'; Reverse, 5′-CCAACCACAATCTCCGA ATG-3′), ASC (Forward, 5′-CTGACGGATGAGCAGTACCA-3'; Reverse, 5′-CAG-GATG ATTTGGTGGGATT-3′), MyD88 (Forward, 5′-GAGCGTTTC-GATGCTTCAT-3'; Reverse, 5′-CGGATCATCTCCTGCACAAA-3′), IRAK1 (Forward, 5′-ACTGGCCCTTGGCAGC TC-3'; Reverse, 5′-GGCCAGCTTCTGGACCATC-3′), TRAF6 (Forward, 5′-CCTTTGGC AAATGTCATCTGTG-3'; Reverse, 5′-CTCTGCATCTTTTCATGGCAAC-3′), FOXO3 (Forward, 5′-ACTTCAAGGATAAGGGCGACAGCA-3'; Reverse, 5′-CTTCATTCTGAA CGCGCATGAAGC-3′), β-actin (Forward, 5′-AGCAGCATCGCCCCAAAGTT-3'; Reverse, 5′-GGGCACGA AGGCTCAT-CATT-3′).

2.6. Western blots (WB)

Cell protein was isolated in lysis buffer with protease inhibitor and then quantified by BCA assay kit (Thermo Fisher) according to the instructions. After the denaturation at 100 °C for 10 min, equal amounts of samples were loaded onto SDS-PAGE gel, separated and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated in blocking reagent (Thermo Fisher) for 1 h at room temperature and then probed overnight at 4 °C with primary antibodies against Caspase-1, IL-18, IL-1β, NLRP3, ASC, FOXO3 or GAPDH (Cell Signaling Technology, Danvers, MA, USA). GAPDH was served as housekeeping protein in this study. After three washes with TBST, membranes were hatched with IgG secondary antibodies (CST) conjugated by horseradish peroxidase (HRP) for 2 h at room temperature. The enhanced chemiluminescence (ECL) chromogenic substrate (GE Healthcare, Amersham, UK) was used for visualization of protein bands and densitometry analysis for WB was conducted using Image Lab software.

2.7. Luciferase assays

Putative sites between miR-30c-5p and FOXO3 were predicted through the online software TargetScan. Luciferase reporters with wide or mutant sequence of 3' -untranslated regions (3′-UTR) sequences of FOXO3 were conducted using pGL3 vectors (Promega, Madison, WI, USA), respectively. Wt or Mut luciferase reporter plasmids with miR-30c-5p mimic, anti-miR-20c-5p or NC were co-transfected in 293T cells using Lipofectamine 2000 according to the

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