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MicroRNA-101 overexpression by IL-6 and TNF-α inhibits cholesterol efflux by suppressing ATP-binding cassette transporter A1 expression

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ABSTRACT

Background: MicroRNAs play key roles in regulating cholesterol homeostasis. Here, we investigated the role of microRNA-101 (miR-101) in regulating ATP-binding cassette transporter A1 (ABCA1) expression and cholesterol efflux under non-inflammatory and inflammatory conditions in human THP-1-derived macrophages and HepG2 hepatoblastoma cells.

Methods: The cell lines were transfected with one of four lentiviral vectors: miR-101, miR-101 control, anti-miR-101, or anti-miR-101 control. A luciferase reporter assay was used to examine miR-101 binding to the 3' untranslated region (UTR) of ABCA1. Western blotting was conducted to assess ABCA1 protein expression. Cells were loaded with BODIPY-cholesterol and stained with oil red O to assess cholesterol efflux.

Results: The luciferase activity assay revealed that wild-type miR-101 binding at site 2 significantly repressed ABCA1 3' UTR activity, suggesting that miR-101 directly targets the ABCA1 mRNA at site 2. In both cell lines, Western blotting revealed that miR-101 expression negatively regulates ABCA1 protein expression and significantly suppresses cholesterol efflux to ApoA1 under both low-density lipoprotein (LDL) and non-LDL conditions, which was confirmed by pronounced lipid inclusions visible by oil red O staining. In HepG2 cells, both IL-6 and TNF-α treatments produced significant miR-101 overexpression; however, in THP-1-derived macrophages, only IL-6 treatment produced significant miR-101 overexpression. Anti-mir-101 transfection under both IL-6 and TNF-α treatment conditions led to ABCA1 upregulation, indicating that miR-101 expression represses ABCA1 expression under inflammatory conditions.

Conclusions: miR-101 promotes intracellular cholesterol retention under inflammatory conditions through suppressing ABCA1 expression and suggests that the miR-101-ABCA1 axis may play an intermediary role in the development of NAFLD and vascular atherosclerosis.

1. Introduction

Excessive intracellular cholesterol accumulation is a key process underlying the development of both non-alcoholic fatty liver disease (NAFLD) and vascular atherosclerosis [1,2]. Under inflammatory conditions, both hepatocytes and vascular macrophages increase their intake of cholesterol, which can eventually lead to the formation of pathological lesions in the liver parenchyma and vascular wall, respectively [1,2]. In vitro, inflammatory cytokines have been shown to promote intake of low-density lipoprotein (LDL) in HepG2 (human hepatoblastoma) cells, primary liver hepatocytes, THP-1-derived macrophages, and vascular smooth muscle cells [1–4]. Therefore, systemic inflammatory conditions contribute to a maladaptive cellular environment that facilitates cholesterol accumulation that, if unchecked, can lead to NAFLD, atherosclerosis, and more serious downstream conditions such as liver cirrhosis and coronary artery disease (CAD) [5,6]. Therefore, developing a better understanding of the mechanism (s) underlying cholesterol accumulation on a cellular level under inflammatory conditions is crucial.

Cholesterol homeostatic equilibrium on a cellular basis is tightly regulated through a balance of intracellular cholesterol biosynthesis, cholesterol intake from the extracellular environment, and cellular cholesterol efflux [2]. A protein named adenosine triphosphate (ATP)-binding membrane cassette transporter

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A1 (ABCA1, ABC1) was initially identified by Langmann et al. to be upregulated in the presence of acetylated LDL (AcLDL), which was reversed by LDL depletion through addition of high-density lipoprotein (HDL) [7]. Specifically, under conditions of intracellular cholesterol overload, ABCA1 is upregulated and mediates the cellular efflux of cholesterol and phospholipids to extracellular apolipoprotein A-I (apoA-I) [2,8,9]. Clinically, mutations in the ABCA1 gene produce Tangier disease – a rare, autosomal recessive disorder characterized by extremely low plasma HDL cholesterol levels, cholesterol accumulation in multiple tissues, peripheral neuropathy, and accelerated atherosclerosis [10]. Moreover, the interaction of apolipoproteins with ABCA1 is known to activate signaling pathways (e.g., JAK2/STAT3, protein kinase A, and Rho family G protein CDC42) that regulate ABCA1-mediated cholesterol efflux, making ABCA1 both a lipid exporter and a signaling receptor [11].

Notably, extracellular inflammatory conditions have been shown to inhibit ABCA1 expression, thereby decreasing ABCA1-mediated cholesterol efflux [2]. For example, LXR/RXR agonists that inhibit inflammatory cytokine production have been shown to induce ABCA1 expression, making these agonists potential therapeutic agents for mobilizing cholesterol from tissues under inflammatory conditions [11]. However, the precise mechanism (s) by which ABCA1 expression is regulated on a cellular level under inflammatory conditions remain unclear. Recent studies have shown that several microRNAs (miRNAs) – small, non-coding RNAs that negatively regulate target mRNAs by binding to complementary sites on their 3' untranslated regions (UTRs) – such as miR-26, miR-33, miR-122, miR-370, and miR-758 play key roles in regulating cholesterol homeostasis [12].

Dysregulation of another human miRNA in particular – hsa-miR-101 – has been previously associated with NAFLD [13]. Moreover, miR-101 has been shown to inhibit autophagy, a process known to regulate the availability of free cholesterol for cellular efflux [14,15]. However, miR-101's role in regulating cellular cholesterol homeostasis remains unclear. Therefore, in this study, we investigated the role of miR-101 in regulating ABCA1 expression and cholesterol efflux under non-inflammatory and inflammatory conditions in human THP-1-derived macrophages and human HepG2 hepatoblastoma cells.

2. Materials and methods

2.1. Cell lines and reagents

The human monocyte cell line THP-1 and the human hepatoblastoma cell line HepG2 were obtained from American Type Culture Collection (ATCC, no. TIB-202 and no. HB-8065, respectively). THP-1 was cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum, 2 mmol/l glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. THP-1 was fully differentiated into macrophages after being triggered with 160 nmol/l phorbol-12-myristate-13-acetate (PMA) for 72 h, and the differentiated THP-1 macrophages were washed extensively with phosphate-buffered saline (PBS) prior to use.

LDL was isolated from fresh plasma of healthy human volunteers by sequential density gradient ultracentrifugation as described previously [16]. Written informed consents were obtained from all volunteers prior to sampling. Recombinant human interleukin (IL)-6 and tumor necrosis factor (TNF)-α were obtained from SinoBio (Shanghai, China) and PeproTech Asia (Rocky Hill, NJ, USA), respectively.

2.2. Lentiviral transfection

Two lentiviral vectors, GV259 (Con-miR) and GV159 (Con-anti-miR), were constructed as controls for their corresponding miR-101 and anti-miR-101 lentiviral vectors, respectively (Shanghai Ji Kai Gene Chemical Technology Co., Ltd., China). Then, HepG2 cells and THP-1 macrophages at 50–70% confluence were transfected with either miR-101, Con-miR, anti-miR-101, or Con-anti-miR (1×10^8 TU/ml lentivirus) using the manufacturers' protocols (Shanghai Ji Kai Gene Chemical Technology Co., Ltd., China). Cells were transfected for 48 h and treated with or without LDL (150 mg/ml) and with either IL-6 (20 ng/ml) or TNF-α (25 ng/ml) for an additional 24 h [17,18]. Then, Western blotting was conducted to assess ABCA1 protein expression with β-actin used as an internal control.

2.3. Western blotting

As previously described with minor modifications [19], identical amounts of protein from extracts or nuclear extracts of cultured THP-1 macrophages and HepG2 cells (with or without lentivirus infection) were denatured and then subjected to 6% or 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, UK). Electrophoretic transfer to nitrocellulose was performed at 85 V with 250 mA for 3 h. The membrane was then blocked with 5% skimmed milk for 2 h at room temperature and probed with rabbit anti-ABCA1 antibody (1:500 dilution; Abcam, USA) and mouse anti-human β-actin polyclonal antibody (1:2000 dilution; Abcam, USA) in 5% skimmed milk in PBS with 1% Tween at 4 °C overnight. After three 5-min washes in PBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:4000 dilution; ZSGB-BIO, Beijing, China) at room temperature for 1 h. After rinsing, the membranes were subjected to enhanced chemiluminescence (Amersham Biosciences, USA). With β-actin was used as loading control, the protein band intensities were analyzed by an imaging analysis system to assess relative protein expression.

2.4. 3' UTR luciferase reporter assays

cDNA fragments corresponding to the entire 3' UTR of human ABCA1 were amplified by reverse transcription-polymerase chain reaction (RT-PCR) from total RNA extracted from HepG2 cells with XhoI and NotI linkers (Jiang Su Beyotime Co. Ltd., China) The PCR products were directionally cloned downstream of the Renilla luciferase open reading frame (ORF) in a pc-DNA3.1 vector (Promega) that also contained a constitutively-expressed firefly luciferase gene used to normalize transfections. Site-directed mutations in the seed region of the predicted miR-101 sites within the 3' UTR of human ABCA1 were generated using Multisite-Quickchange (Stratagene) according to the manufacturer's protocol. All constructs were confirmed by sequencing.

HepG2 cells were plated onto 96-well plates and co-transfected with 0.2 μg of the indicated 3' UTR luciferase reporter vectors and the miR-101 vector. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). Renilla luciferase activity was normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control (Con-miR). All experiments were independently performed in triplicate.

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

As previously described with minor modifications [20], total RNA was isolated from THP-1 macrophages and HepG2 cells using a RNAiso kit (Takara, Dalian, China) according to the

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