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miR-613 regulates cholesterol efflux by targeting LXRα and ABCA1 in PPARγ activated THP-1 macrophages

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ABSTRACT

Cholesterol efflux from macrophages is a critical mechanism to prevent the development of atherosclerosis. Although PPARγ is known to be a potent sterol sensor that play a fundamental role in cholesterol metabolism, the potential effects of PPARγ responsive miRNA still need to be revealed. In this study, we found that miR-613 is inversely correlated with LXRα and ABCA1 in PPARγ activated THP-1 cells. PPARγ negatively regulates the expression of miR-613 at transcriptional level, and miR-613 suppressed LXRα and ABCA1 by targeting the 3′-UTR of their mRNAs. Furthermore, downregulation of LXRα and ABCA1 by miR-613 inhibited cholesterol efflux from PPARγ activated THP-1 macrophages. These results revealed an alternative mechanism for PPARγ regulation and provided a potential target for the treatment of cholesterol metabolic diseases.

1. Introduction

Atherosclerosis is the major factor causing cardiovascular diseases [1]. Macrophage foam cells play a critical role in the occurrence and development of atherosclerosis [2]. The imbalance of cholesterol efflux from macrophages ultimately results in transformation into lipid-laden foam cells, which accumulation in the arterial wall become a hallmark of early stage of atherosclerotic lesions [3–5].

Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor that play pivotal roles in cholesterol homeostasis and key biological processes in atherogenesis [6]. Once activated, PPARγ induces a cascade of genes involved in cholesterol efflux in macrophages [7–9]. LXRα (liver X receptor α) is identified as one target gene of PPARγ [6]. One of the best-characterized effects of LXRα is to promote reverse cholesterol transport (RCT), which cholesterol delivery from the periphery to the liver for excretion. The first step in RCT is cholesterol efflux from macrophages, which intracellular cholesterol from macrophage is transferred to apoA-I and HDL via ABCA1 (ATP binding cassette transporter A1) and other transporters. ABCA1, one of the earliest identified LXRα target genes, promotes macrophage cellular cholesterol efflux and maintains cellular sterol homeostasis [10–12]. Mutation of ABCA1 abolishes cholesterol efflux and increase the risk of developing atherosclerosis [13].

MicroRNAs (miRNAs) are small (about 19–22 nt), non-coding RNAs that regulate gene expression primarily by binding with an imperfect complementarity to the 3′-UTR of target mRNAs, leading to mRNA degradation or translation repression [14]. miRNAs play important roles in cellular processes of in multiple normal and disease-related biological processes, including metabolic homeostasis [15], oncogenesis [16], and cardiogenesis [17]. Recently, a growing amount of evidences have proved that miRNAs are involved in the cholesterol metabolism.

Using bioinformatic analyses, several miRNAs were predicted to potentially target 3′-UTR of human LXRα and ABCA1 mRNA, and miR-613 was with a high score of probability to regulate LXRα and ABCA1. Previous reports have shown that miR-613 is associated with papillary thyroid cancer, gastric cancer, breast cancer, and lipogenesis. However, it is not clear whether miR-613 is involved in regulation of cholesterol efflux by targeting LXRα and ABCA1 in PPARγ activated THP-1. In this study, we reveal that miR-613 is inversely correlated with LXRα and ABCA1 in PPARγ activated macrophages. Treatment with miR-613 leads to downregulation of LXRα and ABCA1 and inhibits cholesterol efflux. These results suggest that miR-613, which is suppressed after PPARγ activation, may serve as a novel regulator for manipulating PPARγ-dependent cholesterol efflux by targeting LXRα and ABCA1.

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2. Materials and methods

2.1. Reagents

The miR-613 mimic, miR-421 mimic negative control (mimic NC), miR-613 inhibitor and miR-613 inhibitor negative control (inhibitor NC) were synthesized by Shanghai GenePharma. PPARγ agonist rosiglitazone was purchased from Sigma Chemical Company (St Louis, MO, USA).

2.2. Cell culture

THP-1 and HepG2 cell lines were purchased from American Type Culture Collection. THP-1 cells were maintained in RPMI 1640 and HepG2 cells were cultured in Dulbeco's Modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), and penicillin (100 U/mL) at 37 °C in 5% CO_2 humid incubator.

2.3. Plasmid construction

The promoter of miR-613 was amplified by PCR with the primers 5′-GGGGTACCGTATTATAAGTAATCTGGAG-3′ and 5′-CCAAGCTTTCA CCGAGAAATCAACCAATA-3′, using HepG2 cell-derived genomic DNA as template. The promoter sequence was then cloned into KpnI and HindIII sites of pGL3-basic luciferase reporter vector (Promega). The DNA fragments of human LXRα and ABCA1 3′-UTR containing the miR-613 recognition site were amplified by PCR and cloned into pMIR-REPORT vector (Invitrogen) at SpeI and HindIII sites downstream of the luciferase gene. The primers for LXRα 3′-UTR were 5′-GGACTAGTTCTTGGCCGGATGGCTGAGG-3′ (forward) and 5′-CCA AGCTTCACTGCTCAGTAGCCACAA-3′ (reverse). The primers for ABCA1 3′-UTR were 5′-GGACTAGTGGTATGCTATTATCCCTTCT-3′ (forward) and 5′-CCAAGCTTAACTTAGAAATCTGTTGTGA-3′ (reverse). The LXRα and ABCA1 3′-UTR mutant reporter was generated by site-directed mutagenesis using following pairs of primers (the mutated bases were underlined): LXRα 3′-UTR mutant primers, 5′-AGTGGAACA GACTGAGAAGGGCAAACGCCACTGGGAGCTG-3′ (forward) and 5′-CA GCTCCCAGTGGCGTTTGCCCTTCTCAGTCTGTTCCACT-3′ (reverse), AB CA1 3′-UTR mutant primers, 5′-CTTTGTTCATCA TTGGCCCTCGCCA CAAGCACTTTACGCT-3′ (forward) and 5′-AGCGTAAAGTGCTTGTGGC GAGGGCCAATGATGAACAAAG-3′ (reverse). The identities of all cloned sequences were verified by DNA sequencing.

2.4. Real-time quantitative reverse transcription PCR

For mRNA determination, total RNA was extracted from cells with TRIzol reagent (Invitrogen), and the first-strand cDNA was synthesized using M-MLV Reverse Transcriptase (Invitrogen). The quantities of LXRα and ABCA1 were determined by qRT-PCR using SYBR Green qPCR Master Mix (Promega) with the following pairs of primers: LXRα forward, 5′-TCTGGACAGGAAACTGCACC-3′, and reverse, 5′-ACATCTCTTCCTGGAGCCCT-3′, ABCA1 forward, 5′-GGGG TAGGAGAAAGAGACGC-3′, and reverse, 5′-ACAAGCCATGTTCCCTCA GC-3′. β-Actin was used as an internal control with the primers 5′-ACCCCGTGCTGCTGACCGAG-3′ (forward) and 5′-TCCCGGCCAGCCA GGTCCA-3′ (reverse). For the quantification of mature miR-613, small RNAs were prepared from cultured cells by using mirVana™ miRNA Isolation Kit (Ambion), miR-613 analysis were carried out with the All-in-One™ miRNA qRT-PCR Detection Kit (GeneCopoeia) according to the manufacturer's protocol. The forward primer for mature miR-613 determination were 5′-AGGAATGTTCCTTCTTTGC C-3′ and the reverse universal qPCR primer supplemented in the kit. The relative expression level of miR-613 was normalized with U6 small nuclear RNA (U6 snRNA), and its primers for qPCR were 5′-CGCTTCGGCAGCACATATACTAA-3′ (forward) and 5′-TATGGAAC GCTTCACGAATTTGC-3′ (reverse).

2.5. Transient transfections and luciferase assays

HepG2 cells were transfected with reporter luciferase expression plasmids, internal control expression plasmid pMIR-β-gal, plus miR-613 mimic or corresponding mimic negative control (NC) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). After 24 h, cells were harvested and luciferase activities were measured using a luciferase assay system (Promega). For miR-613 promoter reporter assay, cells were transfected with miR-613 promoter reporter plasmid, pCMX–PPARγ, and pCMX-β-gal. Twenty-four hours after transfection, cells were treated with vehicle or rosiglitazone (1 μM) for 24 h, then, cells were harvested for luciferase and β-gal assays. All transfection experiments were performed in triplicate and repeated at least three times.

2.6. Western blot analysis

The whole cell proteins were extracted and the protein concentrations were determined by BCA assay (Pierce). One hundred micrograms of proteins were separated with 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with 5% fat-free dry milk in Tris-buffered saline tween-20 (TBST, 20 mM Tris–HCl, pH 7.6, 0.15 M NaCl, and 0.05% Tween-20) at room temperature for 3 h and then incubated with anti-LXRα antibody (Santa Cruz), anti-ABCA1 antibody (Santa Cruz), or anti-β-actin (Santa Cruz) at 4 °C overnight. After washed with TBST, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h before signal detection by using the enhanced chemiluminescence detection reagents (Pierce).

2.7. Cholesterol efflux assay

THP-1 cells were stimulated with 160 nmol/mL of PMA for 72 h to differentiated into macrophages and transfected with miR-613 mimic/inhibitor, or corresponding mimic/inhibitor NC. After 24 h, the cells were incubated in RPMI 1640 supplemented with 10% LPDS (lipoprotein deprived serum) and [^{3}H]cholesterol (1.0 μCi/mL) in the presence or absence of PPARγ agonist rosiglitazone (1 μM) for 24 h. The cells were washed with PBS and incubated in RPMI 1640 containing 0.2% BSA in the presence of ApoA-I (15 μg/mL), HDL (50 μg/mL) for another 16 h. Subsequently, the cells were lysed and the radioactive content of the media and cells was determined by liquid scintillation. The percentage of cholesterol efflux was calculated by dividing the radioactive content of the media by the sum of the radioactivity in the cells and media.

2.8. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the SPSS13.0 software. The differences between groups were determined by Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-613 is inversely correlated with LXRα and ABCA1 in PPARγ activated THP-1 cells

The expression of LXRα, ABCA1 and mature miR-613 were separately examined by Western blot and real-time RT-PCR. As shown

in Fig. 1, upon treatment with PPARγ agonist rosiglitazone, the PPARγ target gene LXRα and LXRα target gene ABCA1 were significant induced in THP-1 cells (Fig. 1A and B). However, the expression of mature miR-613 was decreased significantly in THP-1 cells (Fig. 1C). These results suggest that the LXRα and ABCA1 may be inversely correlated with miR-613 in PPARγ activated THP-1 cells.

3.2. PPARγ negatively regulates the expression of miR-613 at transcriptional level

To investigate whether PPARγ was involved in the downregulation of miR-613, the THP-1 cells were treated with PPARγ agonist rosiglitazone. As shown in Fig. 2A, rosiglitazone reduced the expression of mature and primary miR-613 in PPARγ activated THP-1 cells. Rosiglitazone-responsive suppression of miR-613 was abolished when the expression of PPARγ was knocked down by siRNA (Fig. 2B), suggesting that miR-613 downregulation was PPARγ dependent. To further inquire whether miR-613 was subjected to PPARγ regulation, we constructed the luciferase reporter plasmid pGL/pro-miR-613 that contain the promoter region (−495 to +5) of miR-613, and the reporter assay was conducted with pGL/pro-miR-613 construct. As shown in Fig. 2C, the luciferase activity was suppressed significantly after treatment with PPARγ agonist rosiglitazone. The above results indicated that suppression of miR-613 was PPARγ dependent and the negatively regulation at transcriptional level.

3.3. miR-613 downregulates LXRα and ABCA1 expression through binding sites located in their 3′-UTRs

We used two different online database searches TargetScan (http://www.targetscan.org/) and miRanda (http://www.microrna.org/microrna/home.do) to predict the binding sites in 3′-UTR of human LXRα and ABCA1 mRNAs. Both bioinformatic tools predicted that there were miR-613 binding sites in the 3′-UTRs of LXRα (located at the 1680–1711) and ABCA1 (located at the 10,246–10,268) mRNA (Fig. 3A and B).

We constructed LXRα 3′-UTR, ABCA1 3′-UTR and their respective miR-613 binding site mutation luciferase reporter plasmids. Furthermore, cotransfection with these reporters and miR-613 mimics into HepG2 cells, we found that the luciferase activities of the LXRα and ABCA1 3′-UTR reporters were inhibited by miR-613 mimics (Fig. 3C and E). Whereas, cotransfection with miR-613 inhibitors in HepG2 cells led to significant restoration of the luciferase activities of LXRα and ABCA1 3′-UTR luciferase reporters (Fig. 3D and F). However, luciferase activities of miR-613 binding site mutation luciferase reporters were not affected by the cotransfection of miR-613 mimics or miR-613 inhibitors (Fig. 3C–F).

To investigate whether miR-613 could regulate LXRα and ABCA1 expression, human THP-1 macrophages were separately transfected with miR-613 mimic/miR-613 inhibitor or relative mimic NC/inhibitor NC. As shown in Fig. 3, the Western blot and real-time PCR showed that overexpression of miR-613 inhibited the LXRα and ABCA1 expression at both protein (Fig. 3G and H) and mRNA (Fig. 3I) levels, while downexpression of miR-613 resulted in a significant increase of LXRα and ABCA1 protein (Fig. 3G and H) and mRNA (Fig. 3I) levels in PPARγ activated THP-1 macrophages. These results indicated that miR-613 suppressed the expression of LXRα and ABCA1 via the miR-613 binding sites located in their 3′-UTRs in PPARγ-activated THP-1macrophages.

3.4. miR-613 suppressed cholesterol efflux from PPARγ activated THP-1 macrophages

To determine whether miR-613 was involved in the regulation of cholesterol efflux, THP-1 cells were transfected with mimic NC or miR-613 mimic. Then the transfected cells were incubated with [^{3}H]-cholesterol in the presence or absence of PPARγ ligand rosiglitazone. Cholesterol efflux assay shown that miR-613 clearly reduced the cholesterol efflux to apoA-I and HDL after activation of PPARγ in THP-1 macrophages (Fig. 4A). Conversely, inhibition of miR-613 with miR-613 inhibitor significantly increased PPARγ-dependent cholesterol efflux in THP-1 macrophages (Fig. 4B). Taken together, downregulation of LXRα and ABCA1 by miR-613 was associated with the suppressed cholesterol efflux of PPARγ activated THP-1 macrophages.

4. Discussion

PPARγ is known to be potent sterol sensors that play fundamental roles in cholesterol metabolism. PPARγ signaling pathways is involved in various key biological processes that are implicated in many conditions such as obesity, diabetes mellitus, atherosclerosis, and inflammatory diseases [18–20]. Upon ligand binding, PPARγ become activated and heterodimerize with RXR. The PPAR-RXR heterodimer binds to PPAR response elements (PPRE) within the promoter regions of their target genes, leading to gene transactivation or transrepression [21,22]. Here, we found that

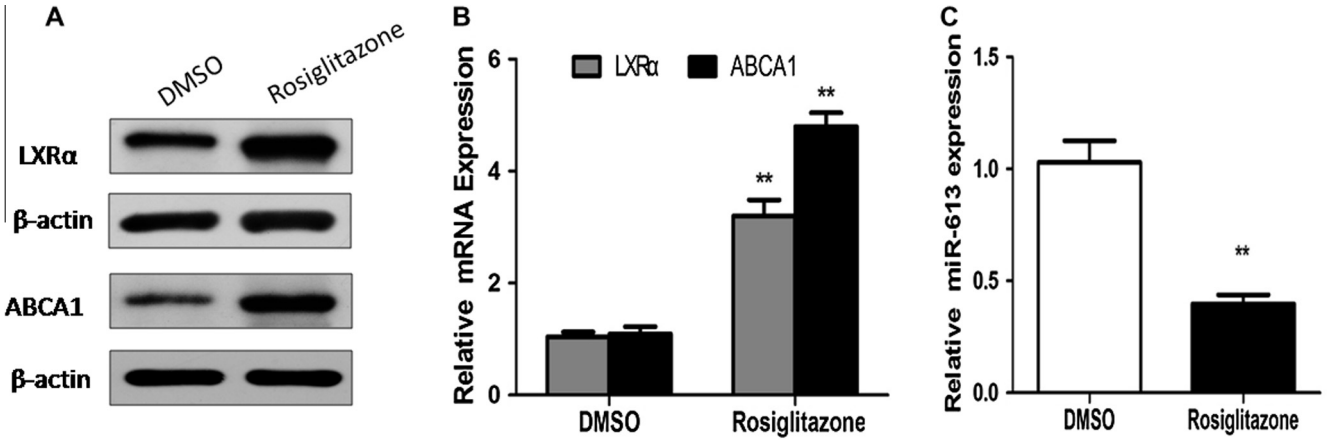

Fig. 1. miR-613 is inversely correlated with LXRα and ABCA1 in PPARγ activated macrophages. THP-1 cells were treated with vehicle DMSO or PPARγ agonist rosiglitazone (1 μM) for 24 h, Western blot (A) and real-time PCR analysis (B) were performed to detect the LXRα and ABCA1 protein or mRNA expression. Real time RT-PCR was conducted for mature miR-613(C). LXRα and ABCA1 expression was normalized to β-actin mRNA, miR-613 was normalized to U6 snRNA. Data are mean ± SD of three independent experiments. $^{**}P < 0.01$ *vs* DMSO.

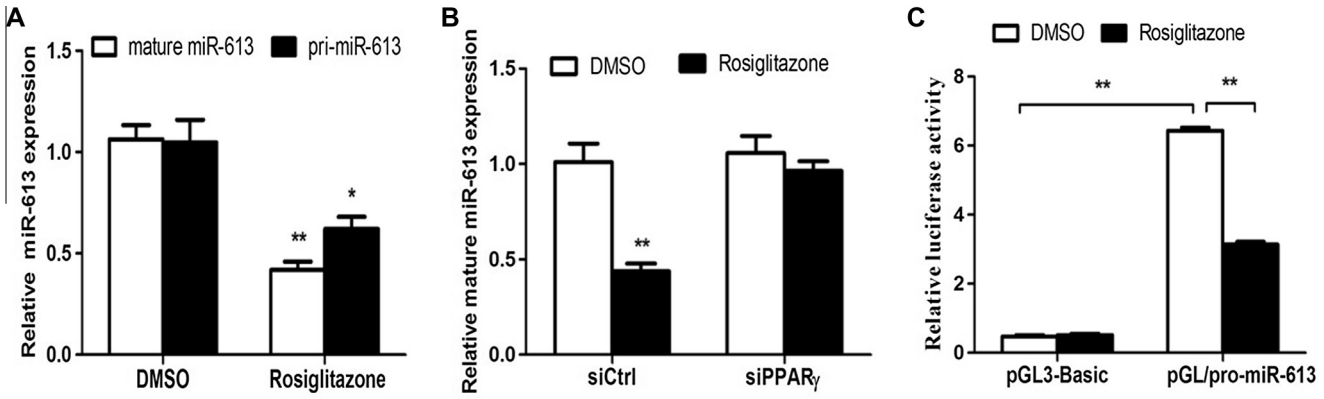

Fig. 2. PPARγ negatively regulates the expression of miR-613. THP-1 cells were treated with DMSO or PPARγ agonist rosiglitazone (1 μM) for 24 h, primary miR-613 and mature miR-613 were detected by real time RT-PCR (A). THP-1 cells were transfected with control siRNA or PPARγ siRNA (10 nM each). Twenty-four hours after transfection, cells were treated with vehicle DMSO or rosiglitazone (1 μM) for 24 h, then real time RT-PCR was performed to determine mature miR-613 expression (B). THP-1 cells were cotransfected with the reporter plasmid 0.5 μg pGL/pro-miR-613 and 50 ng pSV-β-gal, after 24 h, cells were treated with DMSO or rosiglitazone (1 μM) for another 24 h, then the luciferase and β-gal assays were conducted (C). The transfection efficiency was normalized against the β-gal activity. Data are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

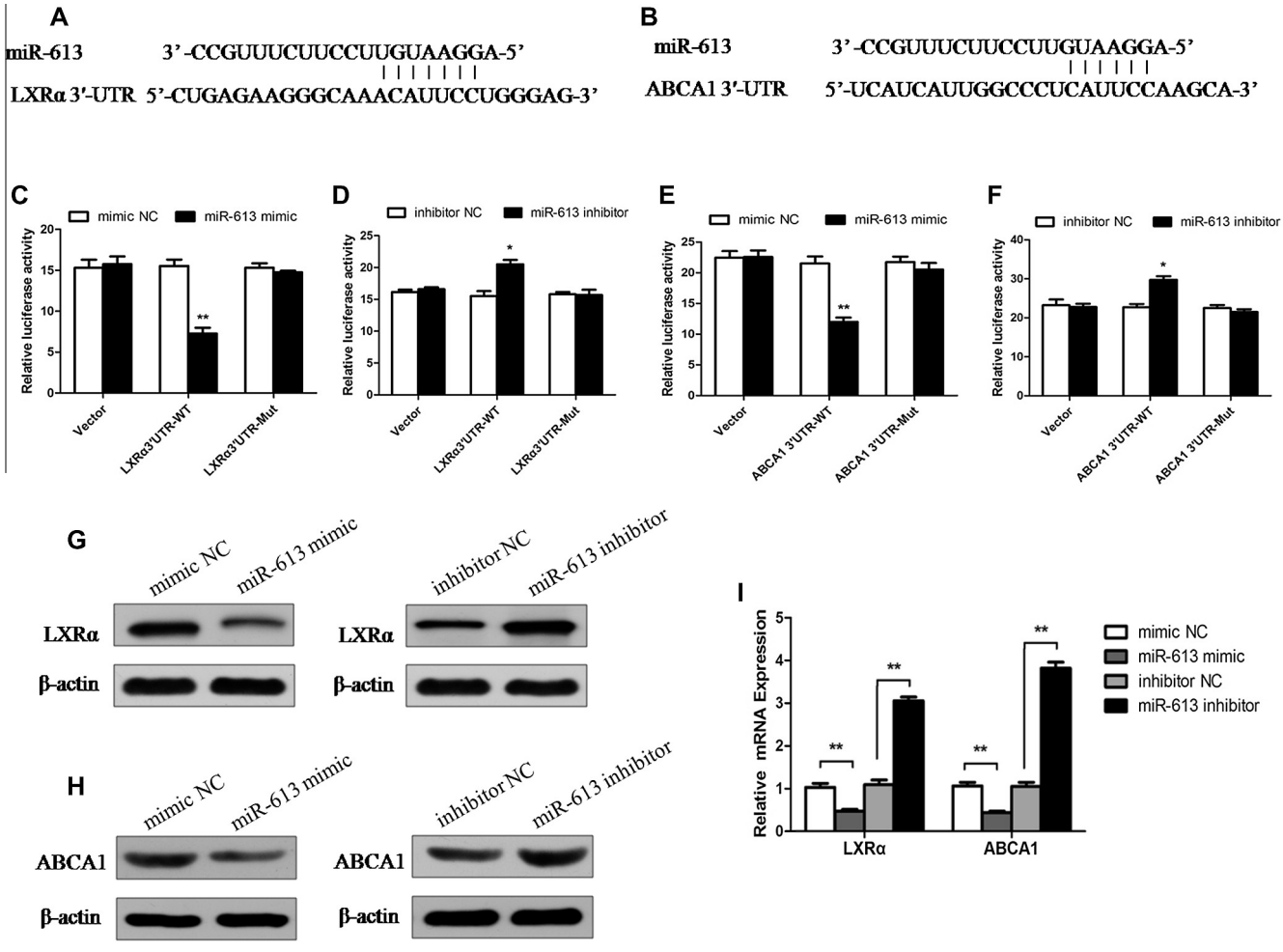

Fig. 3. miR-613 downregulate LXRα and ABCA1 expression in PPARγ activated THP-1 macrophages through binding sites located in their 3′-UTRs. Prediction of miR-613 binding sites on 3′-UTR of LXRα (A) and ABCA1 (B) mRNAs. The vertical lines indicated sequence complementarity between the seed region of miR-613 and its binding sites on LXRα and ABCA1 mRNAs. HepG2 cells were transiently transfected with the wild-type LXRα 3′-UTR-containing reporter pMIR/LXRα 3′-UTR wild-type (WT), the mutant LXRα 3′-UTR-containing reporter pMIR/LXRα 3′-UTR-Mut, or pMIR-REPORT (Vector), together with 50 nM miR-613 mimic or mimic NC (C), or with 50 nM miR-613 inhibitor or inhibitor NC (D) and the transfection efficiency control pMIR-β-gal. Cells were harvested and assayed for luciferase and β-gal activities 24 h after transfection. The pMIR/ABCA1 3′-UTR-WT, pMIR/ABCA1 3′-UTR-Mut, or pMIR-REPORT(Vector), were transiently cotransfected with 50 nM miR-613 mimic or mimic NC (E), or with 50 nM miR-613 inhibitor or inhibitor NC (F), and pMIR-β-gal into HepG2 cells for 24 h. Then Cells were harvested and assayed for luciferase and β-gal activities. THP-1 cells were transfected with 50 nM miR-613 mimic or mimic NC, or with 50 nM miR-613 inhibitor or inhibitor NC. After 24 h, the transfected cells were treated with PPARγ agonist rosiglitazone (1 μM) for another 24 h. Western blot analysis of LXRα (G) and ABCA1 (H) protein in transfected and treated cells. (I) Real-time PCR analysis of LXRα and ABCA1 mRNA in transfected and treated cells. The transfection efficiency was normalized against the β-gal activity. Data are mean ± SD of three independent experiments.$^{*}P < 0.05$, $^{**}P < 0.01$.

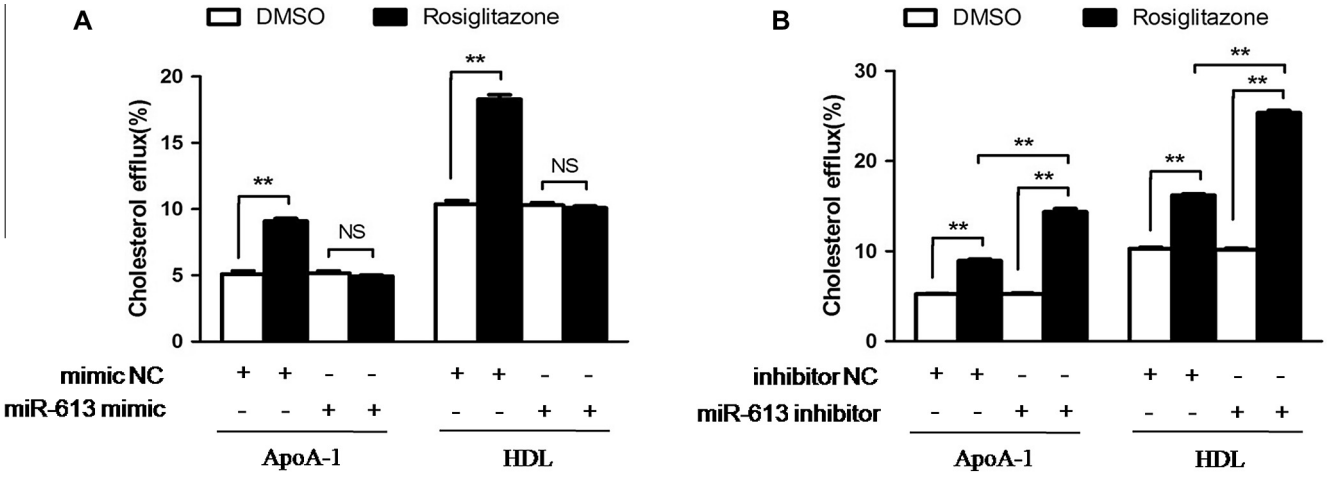

Fig. 4. miR-613 inhibited cholesterol efflux from PPARγ activated THP-1 macrophages. The differentiated THP-1macrophages transfected with miR-613 mimic (A), or miR-26 inhibitor (B). After 24 h, the cells were incubated in RPMI 1640 supplemented with [^{3}H]cholesterol (1.0 μCi/mL) in the presence or absence of PPARγ agonist rosiglitazone (1 μM) for 24 h and cholesterol efflux to ApoA-I and HDL was determined. The percentage of cholesterol efflux was calculated by dividing the radioactive content of the media by the sum of the radioactivity in the cells and media. Data are mean ± SD of three independent experiments. $^{**}P < 0.01$.

miR-613 is inversely correlated with LXRα and ABCA1 (PPARγ target genes) in PPARγ activated THP-1 cells, and the expression of miR-613 is also subjected to the regulation of PPARγ at transcriptional level, suggesting a new mechanism of miR-613 self-regulation.

miRNAs induce repression of their target mRNAs by imperfect base pairing to the 3′-UTR of mRNAs. This inhibitory effect can occur by either transcript destabilization, translational inhibition, or both. One miRNA often regulates multiple genes that are involved in a specific signaling cascade or cellular mechanism, thus making miRNAs potent biological regulators. Growing evidence suggests that faulty regulation of lipid metabolism promotes metabolic diseases. In addition to the classical transcriptional regulators, SREBPs and LXRs, several miRNAs have been shown to post-transcriptionally regulate the expression of key genes involved in lipid homeostasis, including miR-33, miR-122, miR-370, miR-378, miR-206, and miR-27 [23–28]. Recent researches indicated that miR-613 is considered as a tumor suppressors by negatively regulating oncogenes in human cancers [29,30] and miR-613 also suppresses lipogenesis [31]. However, its important role in cholesterol metabolism is not identified. In our study, we demonstrated that miR-613 plays a role in cholesterol efflux by targeting 3′-UTR of LXRα and ABCA1 mRNA.

LXRs are members of the orphan nuclear receptor superfamily. Two isoforms have already been characterized, namely LXRα and LXRβ, the expression of the former is more restricted in the liver, kidney, spleen, adipose tissue, lung, intestine, skeletal muscle, and macrophages, while the latter being ubiquitously expressed. LXRα, regarded as a key transcriptional regulator, participates in the regulation of cellular sterol homeostasis. ABCA1, one of the ATP-binding cassette (ABC) family, is the targets of PPARγ–LXRα signaling pathway. ABCA1 and ABCG1 play major roles in HDL-mediated and ApoAI-mediated cholesterol efflux [32,33]. Previous reports have shown that PPARγ directly upregulate the expression of LXRα by binding the PPRE within the promoter region of LXRα and LXRα directly upregulate ABCA1 expression [6]. In this study, we found that PPARγ also indirectly increase LXRα and ABCA1 expression by downregulating miR-613. Although it is not so clear how miR-613 was repressed by ligand activation of PPARγ, we have provided a new mechanism of PPARγ-dependent cholesterol metabolic regulation.

In conclusion, we demonstrated that PPARγ negatively regulates the expression of miR-613 at transcriptional level, and miR-613 downregulated LXRα and ABCA1 through directly targeting the 3′-UTR of LXRα and ABCA1 mRNA. Treatment with miR-613 suppressed cholesterol efflux from PPARγ activated THP-1 macrophages. This study provides new insights into the regulation of cholesterol metabolism and miR-613 may serve as a novel molecular target for the treatment of cholesterol metabolic diseases.

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