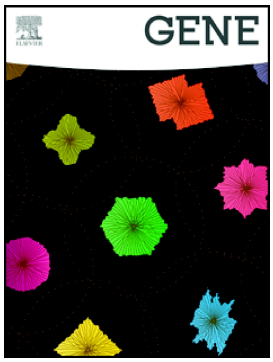

microRNA-212 promotes lipid accumulation and attenuates cholesterol efflux in THP-1 human macrophages by targeting SIRT1

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microRNA-212 promotes lipid accumulation and attenuates cholesterol efflux in THP-1 human macrophages by targeting SIRT1

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Abstract

Macrophage foam cell formation is a key initiating event in the pathogenesis of atherosclerosis. This work was conducted to determine the role of microRNA (miR)-212 in the transformation of foam cells from macrophages. We examined the expression of miR-212 in atherosclerotic lesions in an apoE-deficient (*apoE*$^{-/-}$) mouse model. The effects of miR-212 overexpression and knockdown on lipid accumulation and cholesterol homeostasis in THP-1 macrophages after exposure to oxidized low-density lipoprotein (oxLDL). The mechanism underlying the activity of miR-212 was explored. It was found that miR-212 was downregulated in atherosclerotic lesions and macrophages from *apoE*$^{-/-}$ mice fed high-fat diet, compared to the equivalents from *apoE*$^{-/-}$ mice fed standard diet. Overexpression of miR-212 promoted lipid accumulation in oxLDL-treated THP-1 macrophages, whereas miR-212 depletion exerted an opposite effect. Macrophage cholesterol efflux to apolipoprotein A-I was significantly reduced by miR-212, which was accompanied by reduced ABCA1 expression. Mechanistically, miR-212 targeted sirtuin 1 (SIRT1) to repress the expression of ABCA1 in THP-1 macrophages. Rescue experiments confirmed that co-expression of SIRT1 attenuated lipid accumulation and restored cholesterol efflux in miR-212-overexpressing THP-1 macrophages. Collectively, miR-212 facilitates macrophage foam cell formation and suppresses ABCA1-dependent cholesterol efflux through downregulation of SIRT1. Targeting miR-212 may provide a potential therapeutic strategy for atherosclerosis.

Key words: atherosclerosis; cholesterol efflux; foam cells; macrophages; microRNA.

Introduction

Macrophage subendothelial accumulation and transformation to foam cells is a key event in the initiation of atherosclerotic lesions [1]. The imbalance between cholesterol influx and efflux is responsible for macrophage foam cell formation [2]. Reduced macrophage cholesterol efflux capacity as a result of *Neil3* deficiency is associated with accelerated atherosclerotic plaque formation [3]. In an apoE-deficient (*apoE*$^{-/-}$) mouse model, pharmacological induction of cholesterol efflux in macrophages can prevent atherosclerotic lesion development [4]. Therefore, understanding the mechanism governing cholesterol efflux from macrophages is of importance in developing effective therapies for atherosclerosis.

ATP-binding cassette (ABC) transporters, such as ABCA1 and ABCG1 play a pivotal role in mediating the efflux of cellular cholesterol to extracellular acceptors apolipoprotein A-I (apoA-I) or high density lipoprotein (HDL) [5,6]. In support of this hypothesis, depletion of ABCA1 leads to reduced cholesterol efflux and excessive lipid deposition in macrophages [7]. Liver X receptor (LXR) is a member of the nuclear receptor family and functions as a important transcriptional activator of ABCA1 in macrophages [8]. Activation of LXR/ABCA1 signaling exerts protective effects against atherosclerosis through promotion of cholesterol efflux [9].

microRNAs (miRs) are a large family of small, non-coding RNAs that can repress

multiple target gene expression through partial complementary binding to the 3′-untranslated region (3′-UTR) of mRNAs [10]. Expression profiling studies have demonstrated that many miRs are aberrantly expressed in atherosclerotic plaques compared to healthy arteries, suggesting their implication in the progression of atherosclerosis [11]. A growing number of miRs have been found to participate in macrophage foam cell formation and atherosclerosis [12,13]. For instance, miR-150 shows the ability to accelerate cholesterol efflux from lipid-loaded macrophages [12]. miR-302a was reported to block cholesterol efflux to apoA-I in murine macrophages, and delivery of anti-miR-302a inhibitors was found to reduce atherosclerotic plaque size in a mouse model [13]. A recent study has demonstrated that miR-212 is upregulated in macrophages after stimulation with lipopolysaccharide (LPS) and can inhibit LPS-induced inflammatory response [14]. Another study showed that miR-212 inhibits the production of proinflammatory cytokines in peptidoglycan-stimulated macrophages [15]. These studies indicate an anti-inflammatory activity of miR-212 in macrophages. However, its role in the maintenance of cholesterol homeostasis of macrophages in atherosclerosis remains unclear.

In this study, we examined the expression of miR-212 in atherosclerotic lesions in an *apoE*$^{-/-}$ mouse model and explored the effects of miR-212 overexpression and knockdown on lipid accumulation and cholesterol metabolism in macrophages. The underlying molecular mechanism was further investigated.

Materials and methods

Animal studies

Male *apoE*$^{-/-}$ mice (6 weeks old) were purchased from the Laboratory Animal Center of Zhengzhou University (Zhengzhou, China) and housed in a pathogen-free environment under a 12-h-light/-dark cycle. An atherosclerotic mouse model was generated as described previously [16]. In brief, animals (n = 6) were fed standard rodent chow or high-fat diet (HFD) containing 20% fat and 1% cholesterol for 12 weeks. After dietary treatment, primary peritoneal macrophages were isolated from *apoE*$^{-/-}$ mice after stimulation with 3% thioglycollate solution (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1000 g for 5 min, cells were resuspended in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The cells were then plated onto 6-cm dishes and incubated for 4 h at 37°C to allow adhesion. Nonadherent cells were discarded and adherent macrophages were collected and subjected to gene expression analysis. For analysis of miR-212 levels in atherosclerotic lesions, animals were killed and aortas from the heart to the iliac arteries were dissected. The protocol involving animals was reviewed and approved by the Institutional Animal Care and Use Committee of Zhengzhou University.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated from tissues and macrophages using the miRNeasy Mini Kit

(Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. qRT-PCR was conducted using the TaqMan MicroRNA Assays (Applied Biosystems, Carlsbad, CA, USA). The expression of miR-212 was normalized against the level of U6 (used as an endogenous control).

miR-212 mimic, anti-miR-212 inhibitor, and SIRT1-expressing plasmid

miR-212 mimic, anti-miR-212 inhibitor, miR-148a mimic, and negative controls were purchased from Invitrogen (Carlsbad, CA, USA). For SIRT1 overexpression, a plasmid encoding full length SIRT1 open-reading frame lacking the 3′-UTR was purchased from OriGene (Gaithersburg, MD, USA).

THP-1 macrophage culture

Human monocytic THP-1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS; Sigma-Aldrich). THP-1 monocytes were incubated with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 72 h to differentiate into macrophages.

THP-1 cell transfection and oxLDL treatment

PMA-differentiated THP-1 macrophages were transfected with miR-212 mimic, anti-miR-212 inhibitor, and their corresponding controls (50 nM for each) using the FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). Twenty-four

hours later, transfected cells were subjected to gene expression or oxLDL treatment. In some transfection experiments, THP-1 macrophages were co-transfected with miR-212 mimic (50 nM) and SIRT1-expressing plasmid (0.5 μg). For oxLDL treatment, THP-1 macrophages were exposed to 50 μg/ml oxLDL (Biosynthesis Biotechnology Company, Beijing, China) for 48 h and then tested for lipid accumulation [12]. Each treatment was composed of three replicates and the experiment was repeated three times.

Oil Red O staining

THP-1 macrophages were seeded onto coverslips and incubated with 50 μg/ml oxLDL for 48 h. The macrophages were then fixed with 4% paraformaldehyde for 2 h and stained with 0.5% Oil Red O (Jiancheng Bioengineering Institute, Nanjing, China) for 4 h. Cells were washed and imaged under a light microscope. For quantification of intracellular lipid droplets stained with Oil Red O, isopropanol was used to extract Oil Red O from THP-1 macrophages, as described previously [17]. Optical density was determined at a wavelength of 510 nm.

Cholesterol uptake and efflux assays

For assessment of cholesterol uptake, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylin docarbocyaninet (Dil)-oxLDL (Intracel, Frederick, MD, USA) was used. THP-1 macrophages were cultured in fresh media containing 50 μg/ml Dil-oxLDL for 3 h at 37°C, and the uptake of Dil-oxLDL was analyzed by a flow cytometer.

Cholesterol efflux assay was conducted as described previously [12]. In brief, THP-1 macrophages were cultured for 48 h in fresh medium containing 50 μg/ml oxLDL and [^{3}H]-cholesterol (2 μCi/mL; PerkinElmer, Waltham, MA, USA). The labeled cells were equilibrated for 4 h in fresh medium supplemented with 1 mg/ml bovine serum albumin (Sigma-Aldrich). Cells were then incubated with 25 μg/ml apoA-I (Sigma-Aldrich) for 2 h at 37°C. The radioactivity of [^{3}H]-cholesterol in the medium and cells was measured by liquid scintillation counting. Cholesterol efflux was expressed as percentage of radioactivity in the medium over total radioactivity (medium plus cell).

Luciferase reporter assay

The *ABCA1* 3′-UTR reporter construct (0.2 μg) was purchased from OriGene and co-transfected with miR-212 mimic, miR-148a mimic, or negative control miR (50 nM) into THP-1 macrophages. Forty-eight hours later, cells were lysed and tested for luciferase activity using the Dual-Luciferase Assay System (Promega).

Western blot analysis

Cells were lysed in a lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich). Protein extracts (50 μg per lane) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes. The membranes were incubated with primary antibodies

including anti-ABCA1 (catalog number, ab18180), anti-SIRT1 (catalog number, ab113426), and anti-β-actin (catalog number, ab8229; all from Abcam Inc., Cambridge, UK) overnight at 4°C. After incubation with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature, the blots were visualized by the enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Protein signals were quantified by densitometry using the Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All values are expressed as means ± standard deviation. The data were analyzed by the Student's *t* test and 1-way analysis of variance followed by Bonferroni post hoc test. *P* values <0.05 were considered significant.

Results

miR-212 is elevated in advanced atherosclerotic lesions in an *apoE*$^{-/-}$ mouse model

As determined by qRT-PCR analysis, the level of miR-212 was significantly higher in atherosclerotic lesions from *apoE*$^{-/-}$ mice fed the HFD than that in aortic tissues from control *apoE*$^{-/-}$ mice fed the standard diet (P = 0.008; Figure 1A). Moreover, macrophages isolated from *apoE*$^{-/-}$ mice fed the HFD had significantly greater levels of miR-212 compared to those from control mice (P = 0.003; Figure 1B). These results suggest that miR-212 may contribute to the pathogenesis of atherosclerosis.

miR-212 augments oxLDL-induced macrophage foam cell formation

Next, we assessed the role of miR-212 in macrophage foam cell formation. It was found that overexpression of miR-212 (Figure 2A) remarkably induced accumulation of lipid droplets in oxLDL-treated THP-1 human macrophages, as determined by Oil Red O staining (P < 0.05 *vs*. control miR-transfected cells; Figure 2B). Similar findings were observed in RAW264.7 mouse macrophages after the same treatment (data not shown). In contrast, oxLDL-induced lipid accumulation (Figure 2D) in THP-1 macrophages was significantly (P < 0.05) impaired when miR-212 was knocked down (Figure 2C).

miR-212 inhibits ABCA1-dependent cholesterol efflux

To gain more insight into the regulation of macrophage foam cell formation by miR-212, Dil-oxLDL uptake studies were performed on THP-1 macrophages after transfection with miR-212 mimic. As shown in Figure 3A, the fluorescence intensity of internalized Dil-oxLDL was not altered by either miR-212 overexpression. Next, we questioned if miR-212 has the ability to induce cholesterol removal from macrophages. Of note, miR-212 overexpression decreased cholesterol efflux to apoA-I by 56%, compared to control miR-transfected cells ($P < 0.05$; Figure 3B). Consistent with the reduction in cholesterol efflux, a lower ABCA1 level was observed in miR-212-overexpressing THP-1 macrophages (Figure 3C).

miR-212 downregulates ABCA1 in THP-1 macrophages by targeting SIRT1

Having identified the downregulation of ABCA1 by miR-212, we next checked whether ABCA1 acts as a direct target of miR-212. To address this issue, we performed luciferase reporter assay using *ABCA1* 3′-UTR reporter constructs. In agreement with a previous study showing that miR-148a can repress ABCA1 expression by targeting the 3′-UTR of *ABCA1* [18], we found that the luciferase activity of the *ABCA1* 3′-UTR reporter was significantly suppressed by delivery of miR-148a mimic (Figure 4A). However, ectopic expression of miR-212 had no significant impact on the expression of the same *ABCA1* 3′-UTR reporter ($P > 0.05$ *vs.* control miR-transfected cells; Figure 4A). These results imply that miR-212 suppresses ABCA1 expression via an indirect mechanism.

It has been documented that SIRT1 controls the expression of ABCA1 through induction of LXR activity [19]. Since SIRT1 serves as a target gene for miR-212 in prostate cancer [20], we asked if miR-212-mediated downregulation of ABCA1 is ascribed to reduction of SIRT1. Unsurprisingly, miR-212 overexpression remarkably decreased the protein expression of SIRT1 in THP-1 macrophages (Figure 4B). Most importantly, ectopic expression of SIRT1 prevented the downregulation of ABCA1 by miR-212 (Figure 4C). These results suggest that targeting SIRT1 accounts for the inhibition of ABCA1 expression by miR-121 in THP-1 macrophages.

SIRT1 restores cholesterol efflux in miR-212-overexpressing THP-1 macrophages

Finally, we examined the effect of SIRT1 overexpression on miR-212-mediated suppression of cholesterol efflux from THP-1 macrophages. Cholesterol efflux from oxLDL-loaded macrophages was significantly ($P < 0.05$) restored by overexpression of SIRT1 even in the presence of abundant miR-212 (Figure 5A). Consistent with the enhancement of cholesterol efflux, enforced expression of SIRT1 significantly impaired miR-212-induced lipid accumulation in THP-1 macrophages in response to oxLDL treatment (Figure 5B). Collectively, SIRT1 attenuates miR-212-mediated macrophage foam cell formation through promotion of cholesterol efflux.

Discussion

miR-212 is deregulated in a variety of pathological processes [15,20,21]. For instance, miR-212 is downregulated in prostate cancer tissues and serves as an inhibitor of angiogenesis and cellular senescence [20]. This miR is overexpressed in non-alcoholic fatty liver and reduced after exercise intervention in a mouse model, suggesting its implication in hepatic lipid metabolism [21]. Previous studies have demonstrated that miR-212 participates in the regulation of macrophage response after inflammatory stimuli [14,22]. In this work, we demonstrated that miR-212 was upregulated in atherosclerotic lesions and macrophages in *apoE*$^{-/-}$ mice fed the HFD, suggesting its involvement in atherogenesis. In line with our results, a previous study has demonstrated that serum miR-212 levels are significantly increased in patients with atherosclerosis [23]. Given the importance of macrophages in the progression of atherosclerosis [1], we further investigated the role of miR-212 in the regulation of macrophage foam cell formation. The results showed that ectopic expression of miR-212 accelerated lipid accumulation in THP-1 macrophages in response to oxLDL treatment. Moreover, depletion of miR-212 significantly blocked oxLDL-induced lipid deposition in macrophages. These observations support the hypothesis that miR-212 contributes to macrophage foam cell formation and atherogenesis.

Consistent with the promotion of lipid accumulation, miR-212 overexpression significantly restrained macrophage cholesterol efflux to apoA-I. However, the uptake of oxLDL by macrophages was not altered by miR-212 overexpression. Therefore, miR-212-mediated macrophage foam cell formation is largely ascribed to reduction of cholesterol efflux. ABCA1 is required for lipid homeostasis in macrophages, as its ablation leads to a marked accumulation of lipid-loaded macrophages in animal models [7]. Several miRs such as miR-101 [24] have been reported to inhibit macrophage cholesterol efflux by targeting ABCA1. Consistently, Western blot analysis revealed that miR-212 markedly inhibited the expression of ABCA1 in THP-1 macrophages, which provides additional evidence for the impairment of cholesterol efflux by miR-212.

Mechanistically, miR-212 overexpression caused a marked decline in the expression of SIRT1 in THP-1 macrophages. Targeting SIRT1 by miR-212 has also been described in prostate cancer cells [20]. Since SIRT1 has the ability to induce LXR activity to promote ABCA1 expression [19], we assumed that targeting SIRT1 may be involved in miR-212-mediated downregulation of ABCA1. In support of this hypothesis, we found that overexpression of SIRT1 reversed the suppressive effect of miR-212 on ABCA1 expression in THP-1 macrophages. We also checked whether miR-212 exerted a direct effect on ABCA1 expression. As determined by luciferase reporter assay, miR-212 failed to repress the expression of the reporter harboring the *ABCA1* 3′-UTR. Taken together, miR-212 suppresses the expression of ABCA1 via an

indirect mechanism involving dowregulation of SIRT1.

Compelling evidence has indicated an antiatherogenic activity of SIRT1 [25-27]. Pharmacological induction of SIRT1 expression has been documented to suppress macrophage foam cell formation [25,27]. Concordantly, we showed that enforced expression of SIRT1 prevented miR-212-mediated lipid accumulation in THP-1 macrophages after oxLDL treatment. Moreover, cholesterol efflux from oxLDL-loaded macrophages was enhanced by SIRT1 overexpression, which is consistent with increased expression of ABCA1. These findings highlight SIRT1 as a direct target in the regulation of macrophage foam cell formation by miR-212.

In conclusion, we demonstrate that miR-212 is upregulated in atherosclerotic lesions and facilitates lipid deposition in macrophages through blockade of cholesterol efflux. Targeting SIRT1 is responsible for miR-212-mediated macrophage foam cell formation. These results warrant further investigation of the significance of targeting miR-212 in preventing atherosclerosis in animal models.

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Figure legends

Figure 1. miR-212 is elevated in atherosclerotic lesions and macrophages in an apoE$^{-/-}$ mouse model. apoE$^{-/-}$ mice were fed standard diet (used as a control) or high-fat diet (HFD; n = 6) for 12 weeks and miR-212 levels were measured in (A) aortic tissues and (B) macrophages.

Figure 2. miR-212 augments oxLDL-induced macrophage foam cell formation. (A) Measurement of miR-212 levels by qRT-PCR analysis in THP-1 macrophages transfected with control miR or miR-212 mimic. (B) THP-1 macrophages transfected with control miR or miR-212 mimic were exposed to 50 μg/ml oxLDL for 48 h, and levels of lipid droplets were measured by Oil Red O staining. *Upper*, representative images of cells stained with Oil Red O. Scale bar = 50 μm. *Bottom*, quantification of lipid droplets. Intracellular Oil Red O was extracted from lipid-loaded THP-1 macrophages and measured colorimetrically. (C) qRT-PCR analysis of miR-212 levels in THP-1 macrophages transfected with anti-miR-212 or negative control (anti-control). (D) THP-1 macrophages transfected with anti-miR-212 or negative control (anti-control) were treated with 50 μg/ml oxLDL for 48 h and tested for lipid accumulation by Oil Red O staining. $^{*}P < 0.05$.

Figure 3. miR-212 inhibits ABCA1-dependent cholesterol efflux. (A) Dil-oxLDL uptake assay. THP-1 macrophages transfected with control miR or miR-212 mimic

were exposed to 50 μg/ml Dil-oxLDL for 3 h, and the uptake of Dil-oxLDL was analyzed by flow cytometry. N.S. indicates no significance. (B) Analysis of cholesterol efflux to apoA-I. THP-1 macrophages transfected with control miR or miR-212 mimic were cultured for 48 h in the presence of [^{3}H]-cholesterol (2 μCi/mL) and incubated with 25 μg/ml apoA-I for 2 h before liquid scintillation counting. $^*P <$ 0.05. (C) Western blot analysis of ABCA1 protein levels in THP-1 macrophages transfected with control miR or miR-212 mimic. Numbers below the blots indicate fold-change.

Figure 4. miR-212 downregulates ABCA1 in THP-1 macrophages by targeting SIRT1. (A) Luciferase reporter assay. The *ABCA1* 3′-UTR reporter construct was co-transfected with control miR, miR-148a mimic (used as a positive control), and miR-212 mimic, and after incubation for 48 h, luciferase activities were determined. N.S. indicates no significance; $^*P < 0.05$. (B) Western blot analysis of SIRT1 protein levels in THP-1 macrophages transfected with control miR or miR-212 mimic. (C) THP-1 macrophages were transfected with miR-212 mimic or together with the SIRT1-expressing plasmid or vector and measured for indicated proteins. Numbers below the blots indicate fold-change in protein levels.

Figure 5. SIRT1 restores cholesterol efflux in miR-212-overexpressing THP-1 macrophages. (A) THP-1 macrophages were transfected with miR-212 mimic or together with the SIRT1-expressing plasmid or vector and subjected to cholesterol

efflux assay as described in *Materials and methods*. (B) Quantification of lipid droplets in THP-1 macrophages treated as in (A) by Oil Red O staining. $^{*}P < 0.05$.

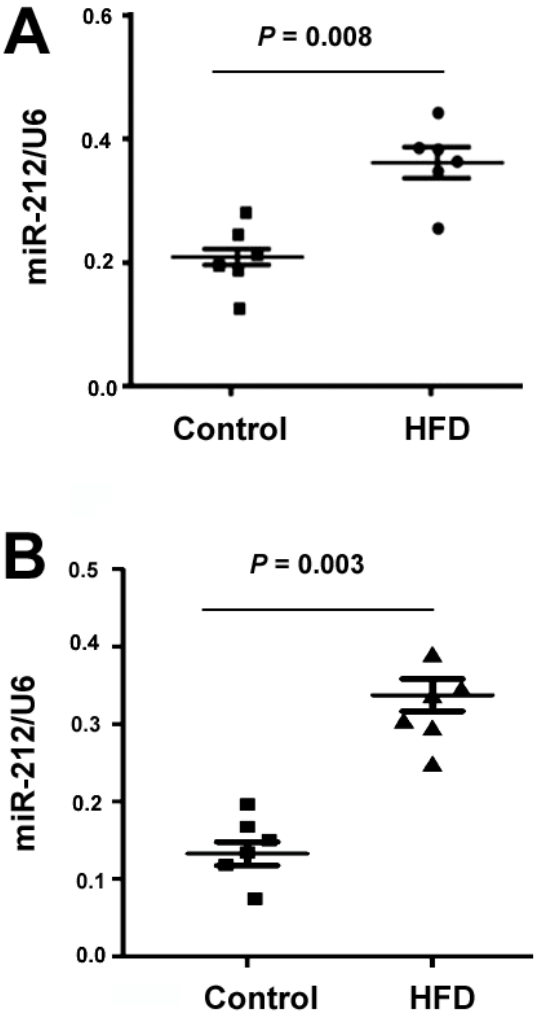

Fig. 1

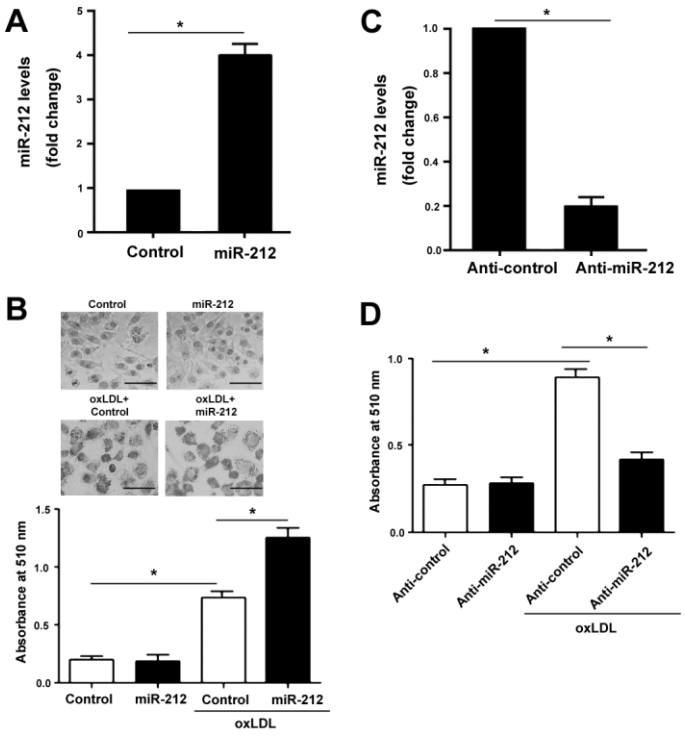

Fig. 2

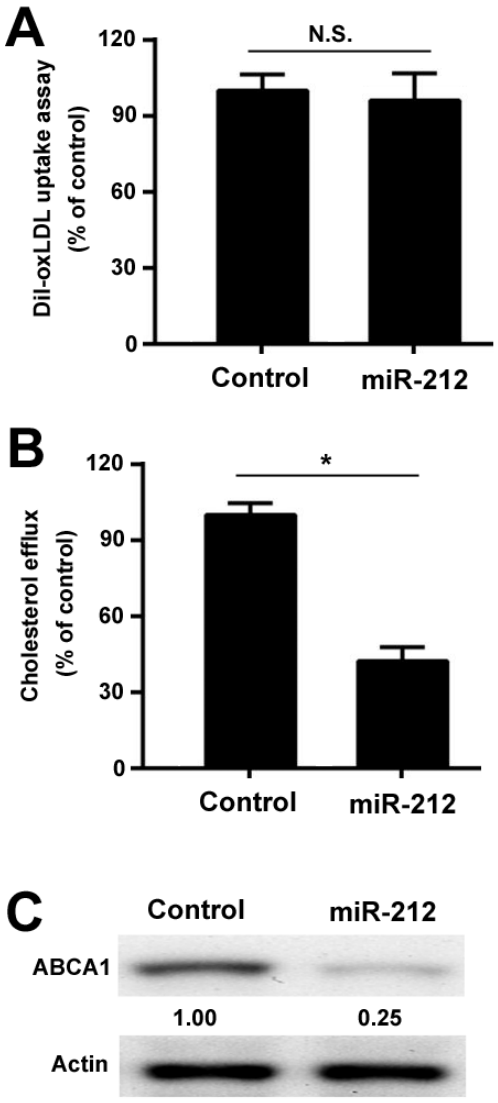

Fig. 3

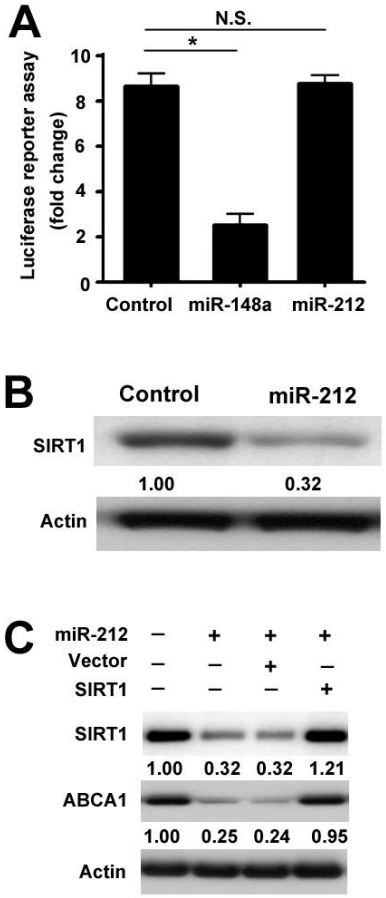

Fig. 4

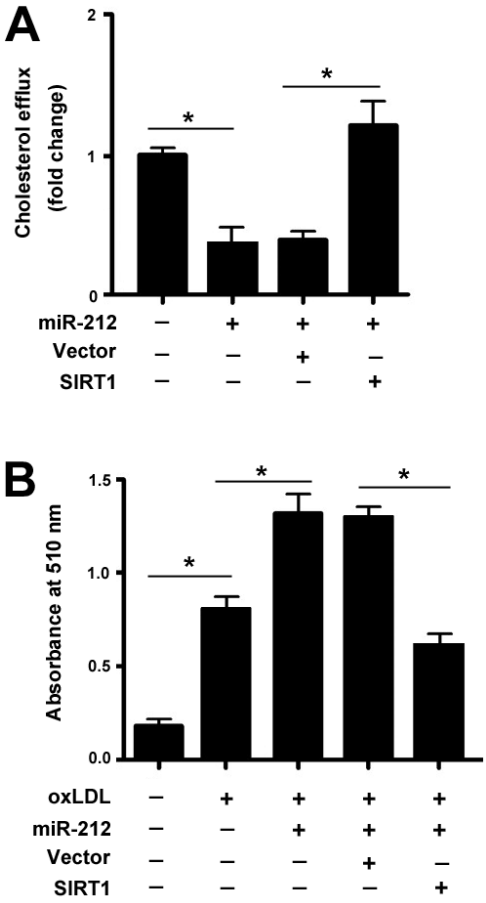

Fig. 5

Abbreviations list

miR-212: microRNA -212

apoE$^{-/-}$: apoE-deficient

oxLDL: oxidized low-density lipoprotein

SIRT1: sirtuin 1

ABC transporters: ATP-binding cassette transporters

apoA-I: apolipoprotein A-I

HDL: high density lipoprotein

LXR: Liver X receptor

MiR: microRNA

UTR: untranslated region

HFD: high-fat diet

FLSs: Fibroblast-like synoviocytes

qRT-PCR: Quantitative real-time polymerase chain reaction

HRP: horseradish peroxidase

PI: propidium iodide

Highlights

miR-212 promotes lipid accumulation in oxLDL-treated THP-1 macrophages.

Macrophage cholesterol efflux to apolipoprotein A-I was reduced by miR-212.

miR-212 targeted SIRT1 to repress the expression of ABCA1 in THP-1 macrophages.