FEBS Letters 586 (2012) 1472–1479

journal homepage: www.FEBSLetters.org

MiR-26 controls LXR-dependent cholesterol efflux by targeting ABCA1 and ARL7

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ARTICLE INFO

Article history:
Received 16 November 2011
Revised 12 February 2012
Accepted 30 March 2012
Available online 18 April 2012

Edited by Laszlo Nagy

Keywords:
MicroRNA
MiR-26
LXR
Cholesterol efflux
ABCA1
ARL7

ABSTRACT

Cellular cholesterol levels are tightly regulated and represent a balance of cholesterol uptake, endogenous synthesis and efflux. Although the classic transcriptional regulations of cholesterol metabolism by liver X receptors (LXRs) have been well studied, the potential effects of LXR-responsive microRNAs (miRNAs) still need to be unveiled. Here, we describe that miR-26, an LXR-suppressed miRNA, inhibits the expression of the ATP-binding cassette transporter A1 (ABCA1) and ADP-ribosylation factor-like 7 (ARL7), two LXR target genes which play critical roles in cholesterol efflux. These findings have not only figured out an alternative mechanism for LXR regulation, but also provided a potential therapeutic target for cholesterol metabolic disorders.

1. Introduction

Atherosclerosis is the leading underlying cause of death worldwide [1]. It is a progressive disorder wherein lipid-loaded macrophages initially accumulate in the subendothelial space. Subsequently, most of these macrophages convert to foam cells following uptake of modified lipoproteins and other cellular debris [2–4]. The inability of macrophages to efflux sufficient amounts of engorged cholesterol to the reverse cholesterol transport (RCT) pathway contributes significantly to foam cells formation [4,5]. Understanding the molecular mechanisms governing lesion development and macrophage cholesterol efflux will be essential to unraveling the pathogenesis of atherosclerosis.

Previous studies have shown that LXRs function as nuclear cholesterol sensors that are activated in response to elevated intracellular cholesterol levels in multiple cell types. Once activated, LXRs induce a battery of genes involved in cholesterol transport, including ABCA1, ABCG1, ABCG5, ABCG8, apolipoprotein (apo) E, LPL, and phospholipid transfer protein [6–9]. These LXR target genes mediate the cholesterol efflux from macrophages and protects against the development of atherosclerosis. For examples, ABCA1, a well known LXR target gene, promotes macrophage cellular cholesterol efflux and maintains cellular sterol homeostasis [10,11]. Deficiencies or mutations in this transporter lead to defects in cholesterol efflux and cholesterol ester accumulation in macrophages and increase the risk of developing cardiovascular disease [12–14]. ARL7, a recently identified LXR target gene, has previously been shown to transport cholesterol to the membrane for ABCA1-associated removal and thus may be integral to the LXR-dependent efflux pathway [15,16].

In addition to the classic transcription regulators of the cholesterol metabolism, a class of non-coding RNAs termed miRNAs has also emerged as critical regulators of gene expression, acting predominantly at the posttranscriptional level [17]. As noted, miRNAs have newly recognized influences on cholesterol homeostasis. miRNAs constitute a large family of small (18–25 nucleotides) non-coding RNA molecules, single stranded in the mature form, which are important posttranscriptional regulators of gene expression [17–19]. miRNAs typically control the expression of their target genes by imperfect base pairing with the 3′ untranslated regions (3′UTRs) of messenger RNAs (mRNAs) thereby inducing repression of the target mRNA. This inhibition can occur by transcripts destabilization, translational inhibition, or both [19–21]. Several miRNAs such as miR-33, miR-122, miR-370 and miR-758 have been implicated in regulating cholesterol metabolism [22–24].

Abbreviations: LXR, liver X receptors; miRNA, microRNA; qPCR, quantitative PCR; ABCA1, ATP-binding cassette transporter A1; ARL7, ADP-ribosylation factor-like 7; RCT, reverse cholesterol transport; 3′UTRs, 3′ untranslated regions.

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0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.03.068

Although it has been reported that miR-33 and miR-758 could regulate cholesterol efflux and high-density lipoprotein (HDL) biogenesis by downregulating ABCA1 [24–28], bioinformatic analyses indicated that *ABCA1* has a very long 3′UTR (3.3 kb) and more than 100 potential miRNA candidates. Our results also indicated that other miRNAs participated in the regulation of cholesterol metabolism by targeting ABCA1. Here, we report that miR-26, which is suppressed after LXR activation, could target two important regulators (ABCA1 and ARL7) of the LXR-dependent cholesterol pathway thus control cholesterol metabolism.

2. Materials and methods

2.1. Reagents

LXR agonist T0901317 was from Cayman Chemical Company (Michigan, USA). The specific LXR agonist GW3965 was provided by Peter Tontonoz (UCLA, USA). The miRNA mimics were from GenePharma (Shanghai, China). The miRNA inhibitors and LXR siRNAs were from Dharmacon (ThermoFisher Scientific). The antibodies against ABCA1 (clone AB1.G6) and ARL7 were purchased from Abnova (Taipei, Taiwan). The transfection reagents Jet-Endo™, Jet-PEI™ and INTERFERin™ were from Polyplus Transfection (Illkirch, FRANCE).

2.2. Cell culture and transfection

RAW264.7, THP-1, HEK293T and HepG2 cell lines were purchased from American Type Culture Collection (ATCC) and maintained at 37 °C (5% CO_2, 95% air) in culture medium consisting of DMEM supplemented with 10% FBS. Plasmids expressing full-length murine LXRα were subcloned into the pBABE retroviral vector and packaged into retrovirus by transfection into Phoenix A cells to generate LXRα-HA-pBABE retrovirus. RAW264.7 cells were infected with pBABE or LXRα-HA-pBABE retrovirus and selected with 6 μg/ml puromycin for 1 week to generate stable-transfected cell line RAW-vector cells or RAW-LXRα cells, respectively. For ligand treatments, cells were cultured in DMEM supplemented with 1% FBS (low serum) and the synthetic LXR agonist T0901317 (1 μM) or GW3965 (1 μM). Jet-PEI™ was used to transfect plasmids into HEK293T cells and RAW264.7 cells according to the manufacturer's instructions. INTERFERin™ was used to transfect miRNA mimics, miRNA inhibitors or LXR siRNAs into RAW264.7 cells according to the manufacturer's instructions.

2.3. RNA quantification

Total RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. Real-time quantitative RT-PCR analysis was performed using the LightCycler (Roche) and SYBR RT-PCR kits (Takara). For miRNA analysis, reverse transcription primer for miR-26a was 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCTA-3′. Quantitative PCR (q-PCR) primers were 5′-ATGGCTTCAAGTAATCC-AGGA-3′ (forward) and 5′-GTGCAGGGTCCGAGGT-3′ (reverse). Similarly, U6 small nuclear RNA was quantified by using its reverse primer for RT reaction and its forward and reverse primers for q-PCR, which were 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGC-GT-3′ (reverse). The relative expression level of miRNAs was normalized to the internal control U6 by using $2^{-\Delta\Delta Ct}$ cycle threshold method [29]. For human and mouse ABCA1 and ARL7 mRNA analysis, as well as the human primary mir-26a mRNA analysis, the primer sequences are available upon request. The relative expression level of ABCA1 and ARL7 were normalized to the level of β-actin expression in each sample.

2.4. Plasmid construction

The primary mir-26a-1 promoter luciferase reporter construct was made by amplifying the 5′UTR region of human *CTDSPL* by PCR and cloning into the *Bgl* II and *Mlu* I sites of pGL3-enhancer construct (Promega). The primers for primary mir-26a-1 promoter were 5′-CCGGAAGTGTCCTTTAATACTTTGGAG-3′ (forward) and 5′-TTGGTCACCTGGGTGATGATGGC-3′ (reverse). The ABCA1 3′UTR and ARL7 3′UTR luciferase reporter constructs were made by amplifying the human ABCA1 and ARL7 3′UTR sequence by PCR and cloning into the *Xba* I site of pGL3-promoter construct (Promega). The primers for ABCA1 3′UTR were 5′-TGGAAGCTTA GAACTGTACACGTGTG-3′ (forward) and 5′-CACGTGGCAGAGT TTGTTGATTGT-3′ (reverse). The primers for ARL7 3′UTR were 5′-GGAAGAAGTCCTTTGGAAATC-3′ (forward) and 5′-CCTGCAGG CTGCTAACCACTGC-3′ (reverse). The mutant ABCA1 and ARL7 3′UTR luciferase reporter constructs were generated by converting miR-26a binding site "TACTTGA" into "TTCATCA".

2.5. 3′UTR luciferase reporter assay

HEK293T cells were cotransfected with plasmids and RNAs by Jet-Endo™ according to the manufacturer's instructions. Briefly, HEK293T were seeded into 96-well plates (15000 cells per well). 24 h later, the cells were cotransfected with 80 ng of *firefly* luciferase reporter plasmid, 10 ng of pRL-TK-*Renilla* luciferase plasmid, and the indicated RNAs (final concentration, 20 nM). After 24 h, luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Data was normalized for transfection efficiency by dividing *firefly* luciferase activity with that of *Renilla* luciferase.

2.6. Immunoblot

Cells were lysed using M-PER protein extraction reagent (Pierce) supplemented with protease inhibitor mixture (Calbiochem). Protein concentrations of the extracts were measured with a BCA assay (Pierce) and equalized with the extraction reagent. Equal amounts of the extracts were loaded and subjected to SDS–PAGE, transferred onto nitrocellulose membranes, and then blotted.

2.7. Cholesterol efflux assays

RAW264.7 cells were seeded in 24-well plates (1×10^5 cells per well) and transfected with control mimics or miR-26a mimics, or with control inhibitor or miR-26a inhibitor by using INTERFERin™ according to the manufacturer's instructions. After 24 h in complete media, the cells were washed and incubated for an additional 12 h in DMEM supplemented with 10% LPDS and [^{3}H]-cholesterol (1 μCi/ml). After 16 h, the cells were washed with PBS and then incubated in DMEM supplemented with 0.2% BSA for a 2 h equilibration period. Where indicated, the cells were incubated with T0901317 (1 μmol/l) during radiolabeling and equilibration. To determine cholesterol efflux, the cells were rinsed three times with PBS and then incubated for 6 h in DMEM supplemented with 0.2% BSA and, where indicated, either ApoAI (15 μg/ml) or FBS (10%). Subsequently, the media was removed, the cells were washed with PBS, and the radioactive content of the media and cells was determined by scintillation. Cholesterol efflux was determined by dividing the radioactive content of the media by the sum of the radioactivity in the cells and media.

2.8. Statistical analysis

Statistical significance was determined by Student's *t* test, with *P* values of $\leqq 0.05$ considered to be statistically significant.

3. Results

3.1. Inverse correlation between miR-26 and ABCA1, ARL7 in LXR-activated macrophages

Ligand activation of LXRs induced several genes that control the cholesterol metabolism, including the known LXR target genes ABCA1, Idol, SREBP-1c, FAS, AIM, PLTP and ARL7 [30].The induction of LXR target genes mainly depends on transcriptional activation, however, the posttranscriptional regulations also make contributions. We detected the reported miRNAs such as miR-33 [25–28], miR-146a [31], miR-122 [32,33], miR-370 [34], miR-378 [35], miR-27 [36] and miR-125a-5p [37], which either participated in the regulation of cholesterol metabolism, or related to the atherosclerosis. Meanwhile, several highly predicted miRNAs that may regulate LXR target genes were also detected. In order to increase the responsibility to LXR activation, we constructed an LXRα stable overexpressing cell line RAW-LXRα, which expressed high level of LXRα (Fig. 1A). Upon treatment with pan-LXR agonist T0901317, the LXR target genes ABCA1 and ARL7 were robustly induced, both in RAW-LXRα cells, a mouse macrophage cell line and in THP-1 cells, a human macrophage cell line (Fig. 1B–E). However, two of the candidate miRNAs, miR-26a and miR-26b, were downregulated significantly in both macrophage cell line (Fig. 1F and G and Fig. S1). However, the other candidates were comparable with or without LXR agonist treatment (Fig. S1). Meanwhile, by using other LXR/RXR agonists to activate the LXR pathway, we also found the downregulation of miR-26a expression in LXR-activated macrophages (Fig. S2). Taken together, we found an inverse correlation between miR-26 and two LXR target genes, ABCA1 and ARL7 in LXR-activated macrophages.

3.2. LXR-specific regulation of miR-26 was at transcriptional level

In order to determine whether the downregulation of miR-26 expression was LXR specific, we not only used a more specific LXR agonist GW3965, but also knocked down the endogenous LXR by using a mixture of siRNAs targeting LXRα and LXRβ. miR-26 expression was downregulated in GW3965-treated macrophages (Fig. S2). Higher miR-26a expression in LXR-silenced RAW264.7 cells, no matter treated with T0901317 or GW3965 (Fig. 2A). The above results indicated that miR-26 suppression was LXR specific in LXR-activated cells. To figure out the mechanism which mediated the downregulation of miR-26 expression in LXR-activated macrophages, we detected the primary *mir-26a* mRNA. Two loci align with miR-26a in the human genome [38,39]. *mir-26a-1* is located in an intron of C-terminal domain RNA polymerase II polypeptide A small phosphatase-like (*CTDSPL*) at chromosome 3, and *miR-26a-2* is located in an intron of C-terminal domain RNA polymerase II polypeptide A small phosphatase 2 (*CTDSP2*) at chromosome 12. Surprisingly, we found that only primary *mir-26a-1* transcription was inhibited rather than *mir-26a-2* in LXR-activated THP-1 cells (Fig. 2B and C). Given that *mir-26a-1* located in the intron of *CTDSPL*, we cloned about 2000 bp 5′UTR of CTDSP2 as a promoter of firefly luciferase. Stronger luciferase activity was found in the mir-26-1 promoter reporter-transfected RAW264.7 cells than in control vector-transfected cells, however, the luciferase activity was suppressed after treating with LXR agonist T0901317 (Fig. 2D). The above results indicated that the mir-26-1 promoter reporter worked very well and LXR regulated miR-26a expression transcriptionally. Taken together, the

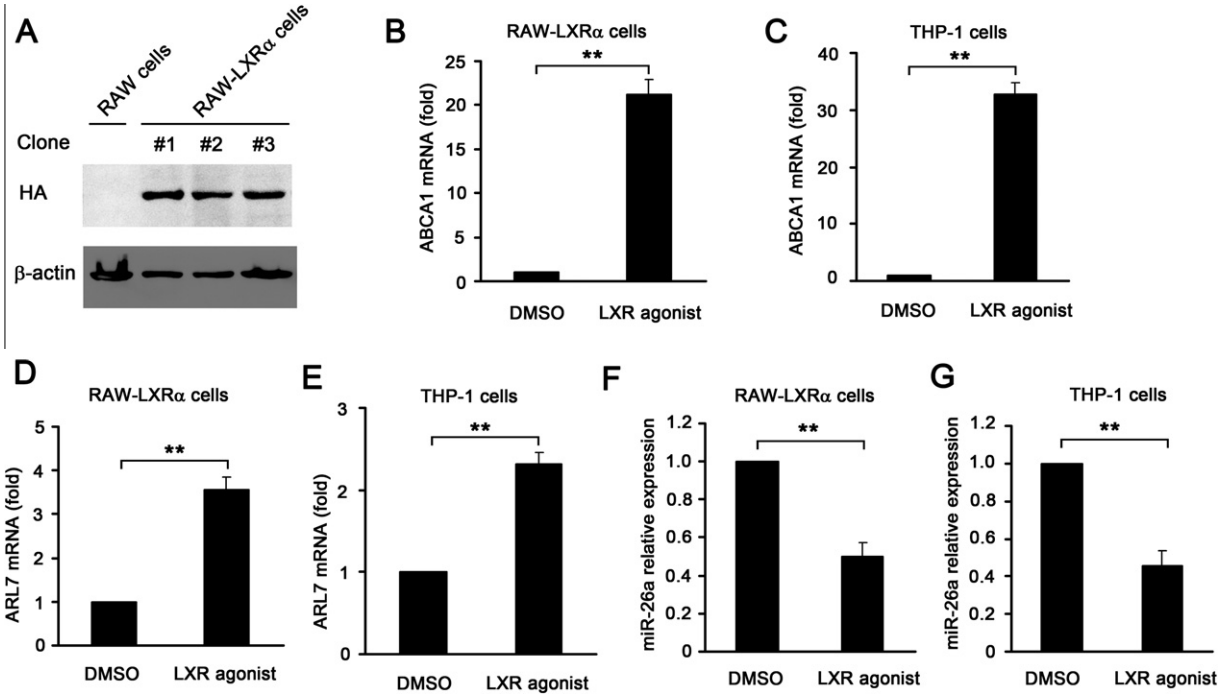

Fig. 1. miR-26 and LXR target genes expression in LXR-activated macrophages. (A) Western blot assay for HA in pBABE or LXRα-HA-pBABE retrovirus infected and puromycin selected RAW264.7 cells. The results of three clones of RAW-LXRα cells were shown. β-Actin was taken as a loading control, the data are one representation for three independent experiments. (B, C) Murine (RAW-LXRα) or human (THP-1) macrophage cells were treated with DMSO or LXR agonist T0901317 (1 μM) for 24 h, qPCR assays were performed to detect the ABCA1 mRNA expression. (D, E) ARL7 mRNA expression in cells described in (B, C) was detected by qPCR. (F, G) miR-26a relative expression in cells described in (B, C) was detected by qPCR. ABCA1 and ARL7 mRNA was normalized to β-actin mRNA, miR-26 was normalized to U6 mRNA. Data are mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$.

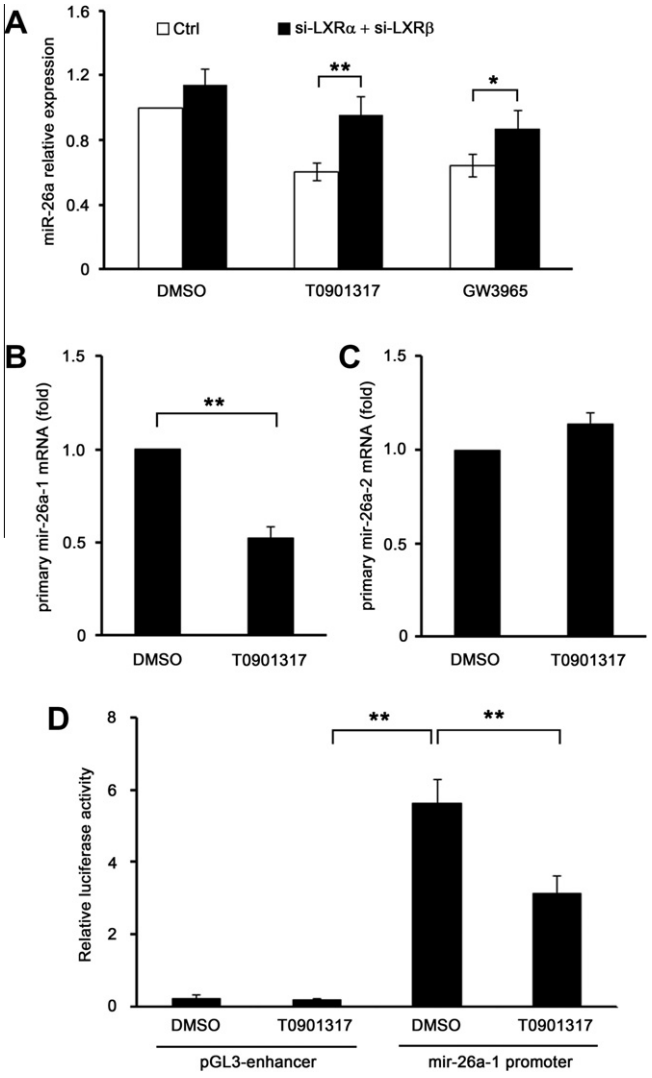

Fig. 2. LXR-specific regulation of miR-26 was at transcriptional level. (A) RAW264.7 cells were transfected with 10 nM mixture of si-LXRα and si-LXRβ, or 10 nM control siRNA. 24 h later, these cells were treated with DMSO or T0901317 (1 μM) or GW3965 (1 μM) for another 24 h, miR-26a expression was detected by qPCR assays and normalized to U6 mRNA. (B, C) THP-1 cells were treated with DMSO or T0901317 (1 μM) for 24 h, primary mir-26a-1 and mir-26a-2 were detected by qPCR and normalized to β-actin mRNA. Data of A–C are mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. (D) RAW264.7 cells were cotransfected with 500 ng indicated vector and 50 ng pTK-Renilla luciferase plasmids, 24 h later, these cells were treated with DMSO or T0901317 (1 μM) for another 24 h, the *firefly* luciferase was detected and normalized to *Renilla* luciferase. Data are shown as mean ± S.D. ($n = 6$) of one representative experiment. Similar results were observed in three independent experiments. $^{**}P < 0.01$.

suppression of miR-26 was LXR specific and the transcriptional inhibition of mir-26a-1 may be the reason for miR-26 downregulation in LXR-activated macrophages.

3.3. Conserved miR-26 binding sites in ABCA1 3′UTR and ARL7 3′UTR

By using bioinformatic tools for miRNA target prediction (Targetscan [http://www.targetscan.org] and miRanada [http://www.microrna.org]), we found that there were binding sites in the 3′UTRs of ABCA1 and ARL7. The binding sites were highly conserved in mammals including humans, chimpanzees, mice, rats, rabbits and cows (Fig. 3A and B). Because of the high similarity of miR-26a and miR-26b, miR-26b was also found to be a potential miRNA which targets ABCA1 and ARL7. Therefore, the database prediction indicated that there were conserved miR-26 binding sites in ABCA1 3′UTR and ARL7 3′UTR. Considering the inverse correlation between miR-26 and ABCA1 (and ARL7), we assumed that miR-26 might target ABCA1 and ARL7, and thus mediate LXR-dependent cholesterol metabolism. What's more, we detected the miR-26a expression level in different mouse tissues and cells, and found relatively low expression of miR-26 in spleen, liver and macrophages comparing to the other tissues (Fig. 3C). Considering that macrophages and liver cells play critical roles in cholesterol metabolism, the expression pattern of miR-26 implied its potential functions in the regulation of cellular cholesterol level.

3.4. miR-26 suppressed ABCA1 and ARL7 via the binding sites located in their 3′UTRs

ABCA1 3′UTR, ARL7 3′UTR and their respective miR-26 binding site mutation luciferase reporters were constructed to verify the database predictions. By cotransfecting with these reporters and miR-26 mimics or inhibitors into HEK293T cells, we found that miR-26a mimics inhibited the luciferase activity of the ABCA1 3′UTR reporter. However, miR-26a mimics had no effect on the mutant ABCA1 3′UTR reporter, which has no binding site of miR-26a (Fig. 4A). Consistently, miR-26a inhibitor, a synthetic RNA which degrades the endogenous miR-26a, promoted the luciferase activity of ABCA1 3′UTR reporter without altering the luciferase activity of the mutant ABCA1 3′UTR reporter (Fig. 4B). ARL7 was also a predicted target of miR-26, similar to the ABCA1 reporter, we found that miR-26a mimics promoted and miR-26a inhibitors inhibited the luciferase activity of ARL7 3′UTR, while miR-26a mimics or miR-26a inhibitors did not alter the luciferase activity of mutant ARL7 3′UTR (Fig. 4C and D). In conclusion, the luciferase reporter system proved that miR-26 could target ABCA1 and ARL7 via the miR-26 binding sites located in their 3′UTRs.

3.5. miR-26 downregulated ABCA1 and ARL7 expression in LXR-activated macrophages

To further investigate the cellular effect of miR-26 on its potential targets ABCA1 and ARL7, we transfected the mouse and human macrophages with control mimics or miR-26a mimics, or relative inhibitors, and then treated these cells with LXR agonist. We found a robust upregulation or degradation of miR-26a after transfection with miR-26a mimics or miR-26 inhibitor, respectively (Fig. 5A and B). Consistent with the ABCA1 3′UTR reporter results, overexpression of miR-26a inhibited the ABCA1 protein expression in both human and mouse macrophages, while downexpression of miR-26a increased ABCA1 protein expression (Fig. 5C). For another LXR target gene ARL7, a potential target of miR-26a, was also inhibited after miR-26a overexpressing and upregulated after miR-26a downexpressing (Fig. 5D). Thus, miR-26 downregulated ABCA1 and ARL7 expression in LXR-activated macrophages, which further indicated that miR-26 could regulate cholesterol metabolism by targeting ABCA1 and ARL7.

What's more, miR-26 expression was also affected by dietary cholesterol in the liver and macrophages. We used LDL to mimics the dietary cholesterol. In LDL-loaded HepG2 and bone-marrow derived macrophages, we also found the upregulation of ABCA1 mRNA and downregulation of miR-26 (Fig. S3 A–D), which partially indicated that dietary cholesterol will affected miR-26 expression in the liver and macrophages. The results in HepG2 also implied that miR-26 might also regulate cholesterol metabolism by targeting ABCA1 in liver cells.

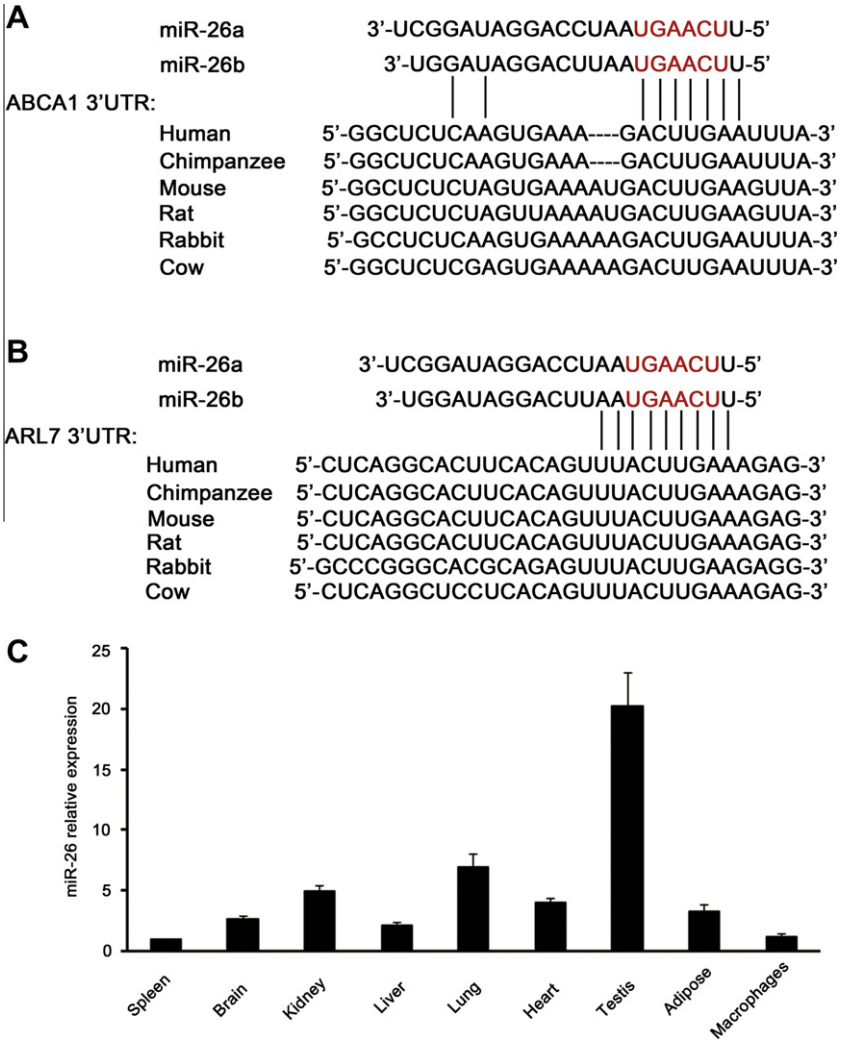

Fig. 3. miR-26 target prediction and miR-26 expression in different tissues. (A) The seed regions of miR-26a and miR-26b were complementary to the ABCA1 3′UTR. The binding site in ABCA1 3′UTR were very conserved among the mammals. (B) The seed regions of miR-26a and miR-26b were complementary to the ARL7 3′UTR. The binding site in ARL7 3′UTR were very conserved among the mammals. (C) miR-26 expression level in different mouse tissues and cells were detected by qPCR and normalized to β-actin mRNA. Data are shown as mean ± S.E.M. of three independent experiments.

3.6. miR-26 regulated cholesterol metabolism by suppressing cholesterol efflux

ABCA1 and ARL7 are important target genes of LXR and participate in the regulation of cellular sterol homeostasis. To determine whether miR-26 could modulate the efflux of cellular cholesterol, we transfected RAW264.7 cells with control mimics or miR-26a mimics. We then incubated the transfected cells with [^{3}H]-cholesterol in the presence or absence of ligands for LXR to induce ABCA1 and ARL7. Interestingly, altering miR-26a levels produced no differences in total cellular cholesterol content or [^{3}H]-cholesterol uptake (Fig. S4 A and B). The ability of the cells to efflux the radiolabeled sterol to BSA, ApoAI and FBS was analyzed 6 h later. We found that miR-26a overexpression resulted in reduced cholesterol efflux to ApoAI or FBS after activation of LXR (Fig. 6A). Thus, miR-26a overexpression decreases cholesterol efflux. Conversely, silencing miR-26a by miR-26a inhibitor resulted in enhanced LXR-dependent cholesterol efflux in RAW264.7 cells (Fig. 6B). Taken together, miR-26 could regulate cholesterol metabolism by suppressing cholesterol efflux, by targeting ABCA1 and ARL7.

In conclusion, we found an inverse correlation between miR-26 and two of the LXR target genes, ABCA1 and ARL7. miR-26 could control the LXR-dependent cholesterol efflux by targeting ABCA1 and ARL7.

4. Discussion

LXRs are nuclear receptors that play critical roles in the transcriptional control of cholesterol metabolism. Once activated, LXRs induce an array of genes involved in cholesterol absorption, efflux, transport, and secretion. LXRs are ligand-dependent transcription factors that form permissive heterodimers with the retinoid X receptor (RXR), and the complex can be activated by ligands of either partner. Ligand-binding to LXR induces a conformational change that facilitates coactivator-for-corepressor-complex exchange and transcription of target genes that have the LXR-responsive elements (LXRE) [40]. Ligand activation of LXRs also inhibits transcription from the promoter of certain genes (e.g., proinflammatory cytokines) that have no LXREs, a phenomenon referred to as *trans*-repression. Here, we found that miR-26, an LXR-suppressed gene, inhibited the two LXR target genes. We described an alternative mechanism: Ligand activation of LXRs not only induces a battery of their target genes, but also inhibit some suppressors such as miRNAs to enhance the LXR-dependent metabolic regulation.

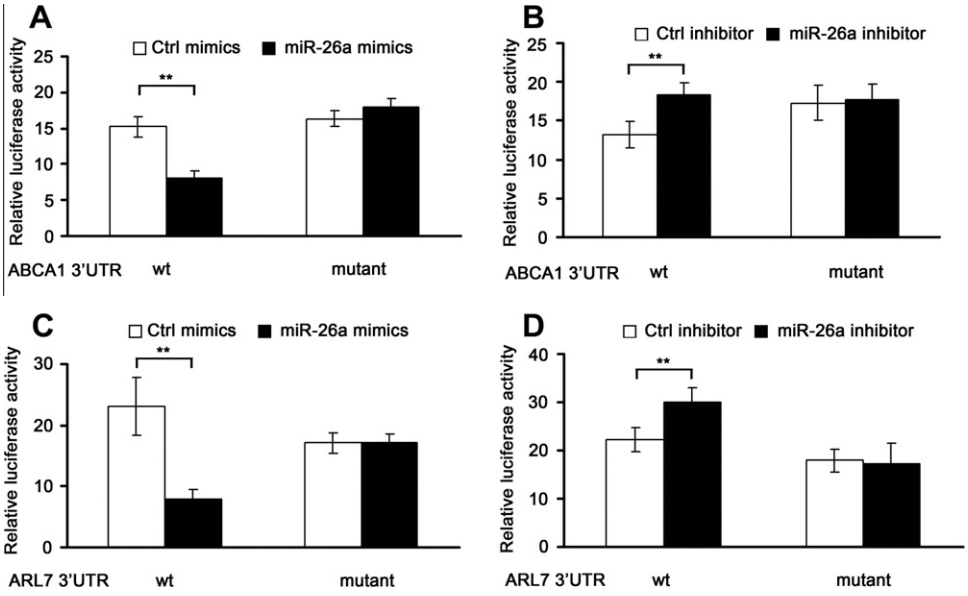

Fig. 4. miR-26 targeted ABCA1 and ARL7 3′UTR in reporter system. (A, B) HEK293T cells were cotransfected with 80 ng wild type (wt) or mutant ABCA1 3′UTR firefly luciferase reporter plasmids, 10 ng pTK-Renilla luciferase plasmids, together with control (Ctrl) mimics or miR-26a mimics, control (Ctrl) inhibitor or miR-26a inhibitor (final concentration: 20 nM) as indicated. After 24 h, *firefly* luciferase activity was measured and normalized to *Renilla* luciferase activity. (C-D) HEK293T cells were cotransfected with 80 ng wild type (wt) or mutant ARL7 3′UTR *firefly* luciferase reporter plasmids, 10 ng pTK-Renilla luciferase plasmids, together with control (Ctrl) mimics or miR-26a mimics, control (Ctrl) inhibitor or miR-26a inhibitor (final concentration: 20 nM) as indicated. After 24 h, *firefly* luciferase activity was measured and normalized to *Renilla* luciferase activity. Data are mean ± S.D. ($n = 6$) of one representative experiment. Similar results were observed in three independent experiments. $^{**}P < 0.01$.

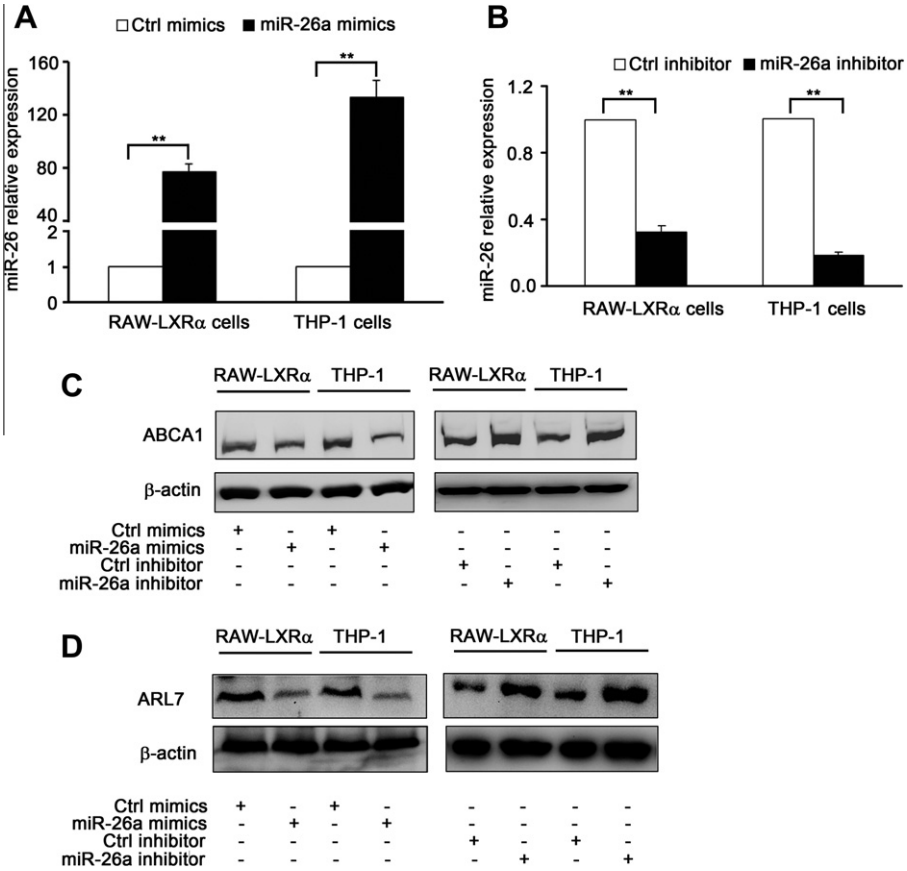

Fig. 5. miR-26 targeted ABCA1 and ARL7 in LXR-activated macrophages. RAW-LXRα cells or THP-1 cells were transfected with control mimics or miR-26 mimics (A), control inhibitor or miR-26a inhibitor (B), After 24 h, the transfected cells were treated with LXR agonist T0901317 (1 μM) for another 24 h. (A, B) qPCR detection of miR-26a in transfected and T0901317 treated cells. miR-26a was normalized to U6. Data are mean ± S.E.M. of three independent experiments. $^{**}P < 0.01$. (C-D) Western blot assay for ABCA1 (C) and ARL7 (D) in transfected and T0901317 treated cells. β-Actin as a loading control, and the data are one representation of three independent experiments.

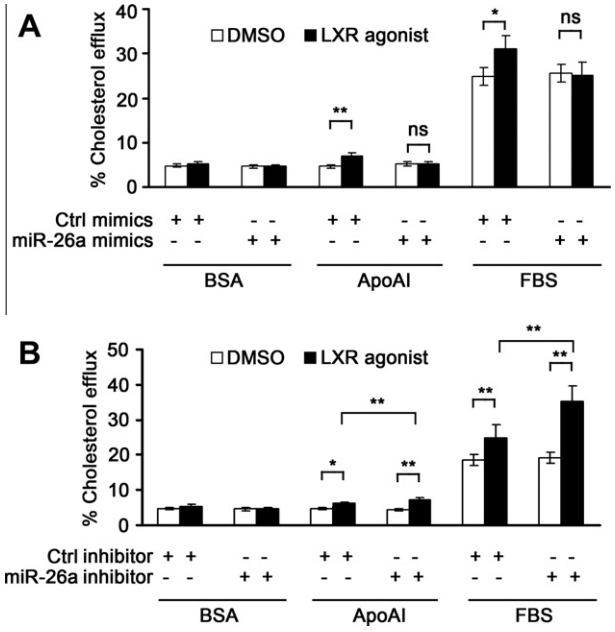

Fig. 6. miR-26 inhibited cholesterol efflux *in vitro*. Cholesterol efflux assay in RAW264.7 cells transfected with control (Ctrl) mimics or miR-26a mimics (A), or with control (Ctrl) inhibitor or miR-26 inhibitor (B). After 24 h, the cells were washed and incubated for 16 h in media supplemented with [^{3}H]-cholesterol (1 μCi/ml) in the presence or absence of LXR agonist T0901317 (1 μM) for 24 h. 16 h later, fresh media supplemented with BSA (0.2%), ApoAI (15 μg/ml), or FBS (20%) was added to the cells. Radioactivity in the media and in cell lysates was measured 6 h later. The percentage of efflux is expressed as dpm in the media versus total dpm (media + cells). Data are mean ± S.D. ($n = 6$) of one representative experiment. Similar results were obtained in three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

miRNAs have now been identified to be potent posttranscriptional regulators of genes involved in cholesterol metabolism. Several reports have shown that miR-33 regulates cholesterol efflux and HDL biogenesis by downregulating the expression of the ABC transporter, ABCA1 and ABCG1 [25–28]. It is very intriguing that the miR-33a and miR-33b are embedded in Srebp2 and Srebp1, two critical transcription factors in regulating fatty acid and cholesterol synthesis and uptake, respectively. Ramirez et al. recently reported miR-758 as a novel miRNA that posttranscriptionally controls ABCA1 and regulates macrophage cellular cholesterol efflux to apoA1, opening new avenues to increasing apoA1 and raising high-density lipoprotein levels [24]. In our study, we have also found that miR-26, an LXR-suppressed miRNA, plays a role in cholesterol efflux by targeting ABCA1 and ARL7. Although it is not so clear how miR-26 was repressed by ligand activation of LXR, we have provided a new member that mediates the LXR-dependent cholesterol metabolic regulation.

miR-33 can target multiple genes such as ABCA1, ABCG1, and NPC1 in cholesterol metabolism, CROT, APT1A, HADHB and AMPK in fatty acid oxidation, as well as SIRT6 and IRS2 in glucose metabolism [41]. It seems that a single miRNA may regulate a physiological or pathological activity by targeting several related genes in the same pathway or in the same process. Another example is miR-146, which can regulate the innate immune response by targeting TARF6, IRAK1 and IRAK2 simultaneously [42,43]. Thus, miR-26 targeting ABCA1 and ARL7, the important regulators of cholesterol efflux, may have its physiological significance. The in vivo function of miR-26 in the regulation of cholesterol metabolism should be further investigated. The role of miR-26 in liver cells is also a very interesting issue which needs to be unveiled.

miR-26, a newly identified LXR responsive miRNA, may confer the signal from LXR activation, or feedback regulate the LXR downstream signaling, as well as participate in other lipid metabolism processes. All the above issues need to be further determined. Meanwhile, given that miR-26 is a regulator of cholesterol efflux, miR-26 may serve as a potential therapeutic target of cholesterol metabolic disorders.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (30971505), Zhejiang Provincial Natural Science Foundation of China (Y206036) and Zhejiang Provincial Health Bureau Science Foundation of China (2006A080).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.03.068.

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