Article

Hsa-Let-7g miRNA Targets Caspase-3 and Inhibits the Apoptosis Induced by ox-LDL in Endothelial Cells

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Abstract: It has been well confirmed ox-LDL plays key roles in the development of atherosclerosis via binding to LOX-1 and inducing apoptosis in vascular endothelial cells. Recent studies have shown ox-LDL can suppress microRNA has-let-7g, which in turn inhibits the ox-LDL induced apoptosis. However, details need to be uncovered. To determine the anti-atherosclerosis effect of microRNA has-let-7g, and to evaluate the possibility of CASP3 as an anti-atherosclerotic drug target by has-let-7g, the present study determined the role of hsa-let-7g miRNA in ox-LDL induced apoptosis in the vascular endothelial cells. We found that miRNA has-let-7g was suppressed during the ox-LDL-induced apoptosis in EAhy926 endothelial cells. In addition, overexpression of has-let-7g negatively regulated apoptosis in the endothelial cells by targeting caspase-3 expression. Therefore, miRNA let-7g may play important role in endothelial apoptosis and atherosclerosis.

Keywords: hsa-let-7g; caspase-3; apoptosis; ox-LDL; endothelial cells

1. Introduction

It has been well confirmed of key roles of oxidized low-density lipoprotein (ox-LDL) in the development of atherosclerosis [1] Endothelial cells, macrophages, and smooth muscle cells, which are involved in atherosclerosis, exhibit atherosclerotic alterations, post exposing to ox-LDL *in vitro* [2].

Ox-LDL is known to induce apoptosis, monocyte adhesion, and reactive oxygen species generation [3–5] via binding to the lectin-like endothelial ox-LDL receptor (LOX-1) [4] on the vascular endothelial cells. The ox-LDL can also upregulate in endothelial cells, the LOX-1 expression, which can also be induced by cytokine tumor necrosis factor-α (TNF-α) [6], and angiotensin II [7] as well.

The ox-LDL-induced apoptosis via binding to LOX-1 in vascular endothelial cells [4,8], or in vascular smooth muscle cells [9], has been well determined. And it has shown that caspases, *i.e.*, caspase-9 and caspase-3, but not caspase-8, play roles in the ox-LDL-induced apoptotic signaling pathway [8] in human coronary artery endothelial cells (HCAECs). In addition, ox-LDL also induces apoptosis via inhibiting of NF-κB [10], or via modulating Bax/Bcl-2 in VSMCs. These findings provide new insights into the role of LOX-1 in ox-LDL-mediated endothelial injury.

MicroRNAs (miRNAs) are a novel class of endogenous, noncoding RNAs molecules (about 22 nucleotides long) that can bind the 3'-untranslated region of target messenger RNA (mRNA) and regulate gene expression [11]. The role of miRNAs in the cardiovascular system has just been revealed [12]. MiR-21 poses a proproliferative and anti-apoptotic effect on vascular smooth muscle cells (VSMCs) [13]. More effort needs to be made on this issue. More recently, a negative feedback regulation has been identified between microRNA let-7g and the ox-LDL receptor LOX-1: ox-LDL can reduce let-7g expression, while the let-7g can down-regulate the LOX-1 expression [14]. Furthermore, the down-regulation of the hsa-let-7g on LOX-1 expression has also been identified in human smooth muscle cells, in which, apoptosis is induced by the down-regulation [15].

Caspase-3 (CASP3) is a member of the cysteine-aspartic acid protease (caspase) family [16], which are sequentially activated and play a central role in the execution-phase of cell apoptosis. The CASP3 plays key role in apoptosis execution, post being activated from proCASP3, mainly by CASP3 itself, caspase-8, caspase-9, caspase-10, the CASP3, along with caspase-6, caspase-7, which are activated by it, executes the apoptotic cell death [17]. Sporadic reports show that microRNA let-7, *i.e.*, let-7a and let-7e, regulate the expression of CASP3 during apoptosis [18,19]. It's interesting to explore whether there are other microRNAs involved in the regulation of apoptosis induced by ox-LDL.

To further determine the anti-atherosclerosis effect of microRNA has-let-7g, and to evaluate the possibility of CASP3 as an anti-atherosclerotic drug target by has-let-7g, the present study determined the role of hsa-let-7g miRNA in ox-LDL induced apoptosis in the vascular endothelial cells.

2. Results

2.1. Ox-LDL Reduces EAhy926 Vascular Endothelial Cells Viability via Inducing the Cell Apoptosis

To observe the influence of ox-LDL on the EAhy926 cells viability, the MTT assay was conducted, it was shown in Figure 1a that the treatment with ox-LDL could reduce the cell viability with a dose of 30 μg/mL, post 12 or 24 h treatment ($82.97\% \pm 5.60\%$ for 10 μg/mL ox-LDL, or $72.00\% \pm 2.85\%$ for 30 μg/mL ox-LDL *vs.* $90.07\% \pm 3.37\%$ for control, $p = 0.29$ and $p = 0.021$ respectively; $72.3\% \pm 5.91\%$ for 10 μg/mL ox-LDL, or $59.63\% \pm 4.89\%$ for 30 μg/mL ox-LDL *vs.* $84.6\% \pm 5.38\%$ for control, $p = 0.20$ and $p = 0.023$ respectively). Ox-LDL-induced apoptosis has been reported in various cell models, *i.e.*, vascular endothelial cells [4,8] and vascular smooth muscle cells [9], and it was further determined whether the decreased EAhy926 cell viability was caused by apoptosis. Figure 1b–d

demonstrated significantly increased apoptosis after treatment with ox-LDL (30 μg/mL) for 24 or 48 h ($p < 0.01$ respectively). To further confirm the apoptosis is induced by ox-LDL, the expression of ox-LDL receptor, LOX-1, was measured in mRNA level and protein level. It showed that the ox-LDL induced significant high level of LOX-1 mRNA expression (Figure 2a,b) and significant high expression of LOX-1 in protein level (Figure 2c,d).

Figure 1. Ox-LDL decreased the EAhy926 cell viability by inducing apoptosis. (**a**) Viability of EAHY926 cells significantly decreased 12, or 24 h post treatment with 30 μg/mL ox-LDL; (**b**) Apoptosis of EAhy926 cells without ox-LDL treatment; (**c**) Apoptosis of EAhy926 cells post 30 μg/mL ox-LDL treatments. And (**d**) significant upregulation in apoptosis of EAhy926 cells with ox-LDL treatment than of EAhy926 cells without ox-LDL treatment. The results are expressed as percentages of positive mean values ± S.E. for three independent experiments. * $p < 0.05$, ** $p < 0.01$.
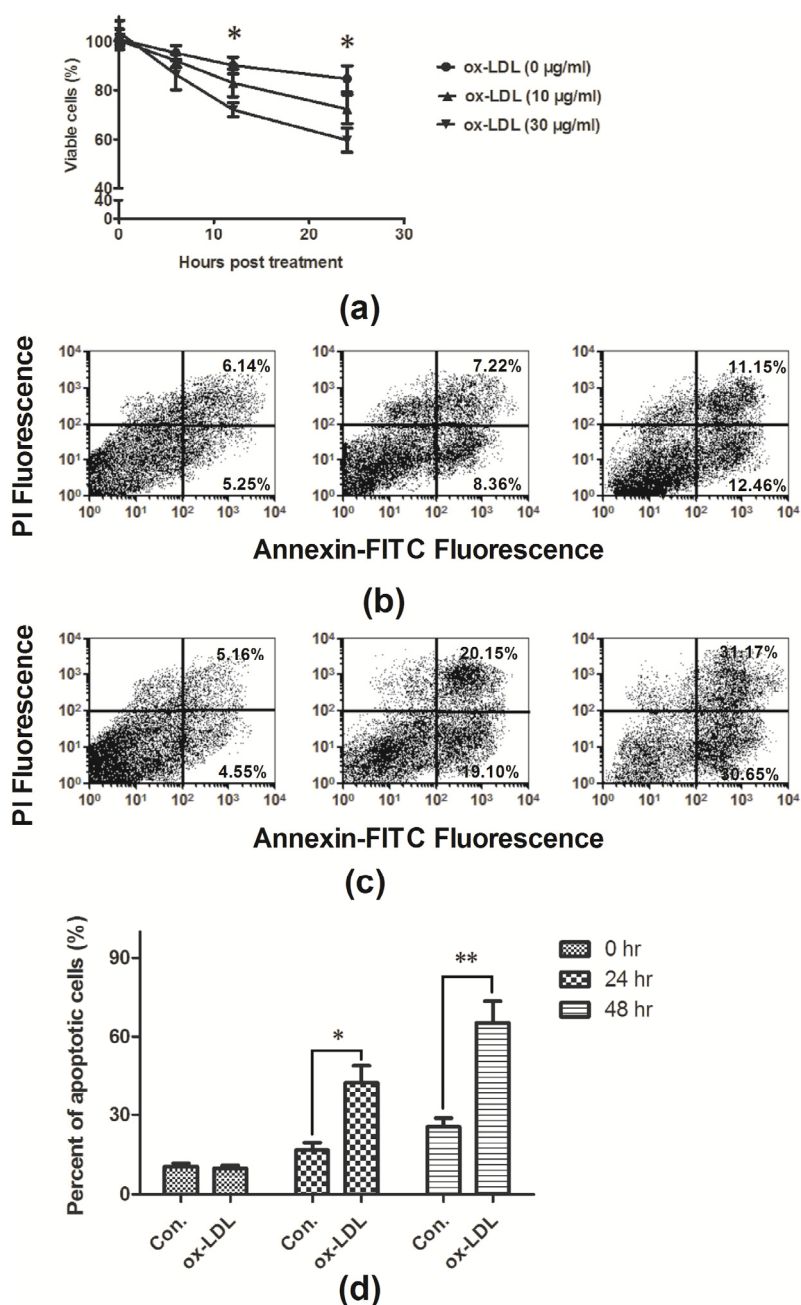
Figure 2. Ox-LDL upregulated LOX-1 and apoptosis-associated molecules. (**a**) The mRNA expressions of LOX-1, Bax in EAhy926 cells were significant upregulated 6 h post ox-LDL treatment. (**b**) The mRNA expressions of LOX-1, Bax and CASP3 in EAhy926 cells were significant upregulated 12 h post ox-LDL treatment. (**c**) and (**d**) The western blot analysis for LOX-1, BAX and CASP 3 of EAhy926 cells post ox-LDL treatment. All results are representative of at least three independent experiments. * $p < 0.05$, ** $p < 0.01$.
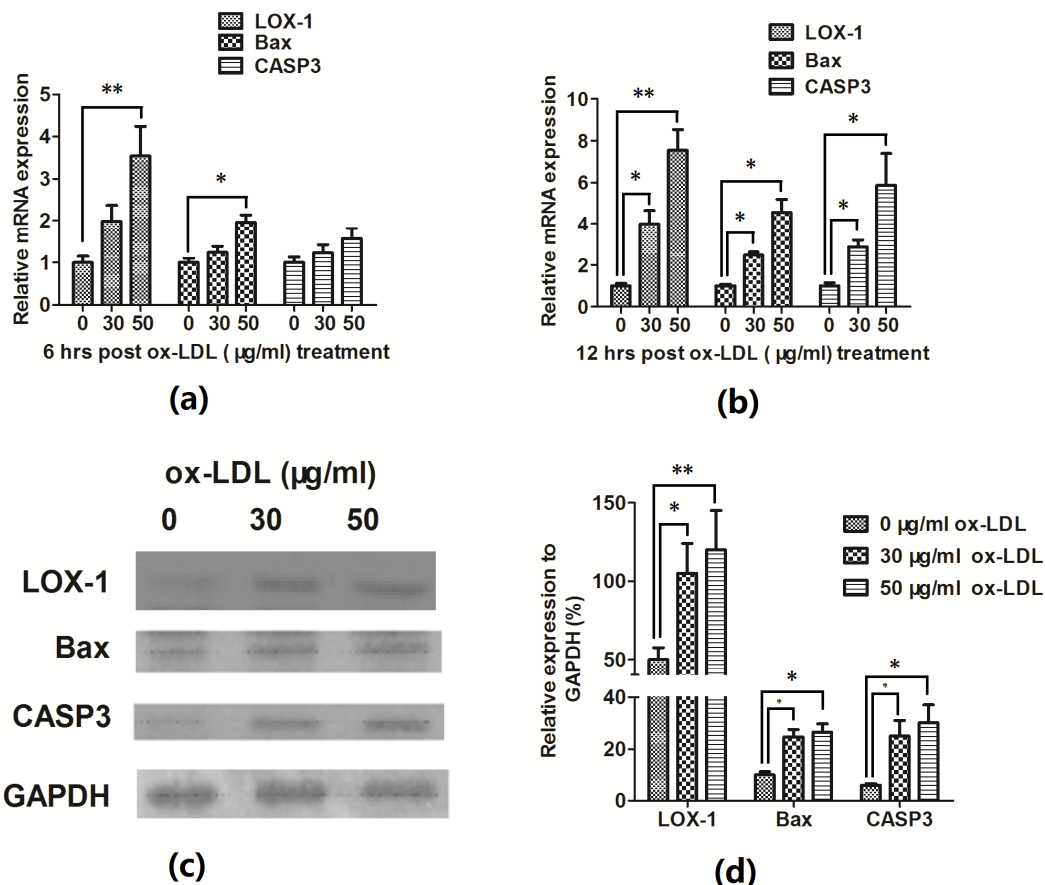

To further evaluate the ox-LDL-induced apoptosis in EAhy926 cells, the expression in mRNA and protein levels of proapoptotic protein Bax, apoptosis executioner CASP3 were also assayed by quantitative RT-PCR and western blotting assay; it was shown that both Bax and CASP3 were expressed significantly high in both mRNA (Figure 2a,b) and protein level (Figure 2c,d) in the EAhy926 cells post ox-LDL treatment.

2.2. Hsa-Let-7g miRNA Is Downregulated during the Ox-LDL-Induced EAhy926 Cell Apoptosis

It has been demonstrated that ox-LDL decreases let-7g promoter activity and downregulates the expression of the microRNA in the primary human aortic smooth muscle cells, and there is a negative feedback regulation between microRNA let-7g and the ox-LDL receptor LOX-1 [14]. To test the hypothesis that ox-LDL inhibited the let-7g gene expression during the ox-LDL-induced apoptosis in EAhy926 cells, we compared the Hsa-let-7g miRNA expression between the ox-LDL-treated and non-treated EAhy926 cells. As showed in Figure 3a, the hsa-let-7g expression in EAhy926 cells post ox-LDL treatment was significantly lower than in the non-treated cells ($p < 0.05$). To further evaluate the

downregulation of let-7g by ox-LDL, the mRNA expression of miRNA processing enzymes, Drosha and Dicer, was tested by the RT-qPCR, but surprisingly, the microRNA processing enzymes Drosha and Dicer were upregulated post the ox-LDL treated, in mRNA level (Figure 3b,c) and protein level (Figure 3d,e). It seems that more microRNAs being involved post ox-LDL treatment.

Figure 3. Let-7g was downregulated during the ox-LDL-induced apoptosis. (**a**) RT-qPCR for has-let-7g (**a**), Dicer (**b**) and Drosha (**c**) in EAhy926 cells treated with various concentrations of ox-LDL, all results were normalized to the GAPDH. (**d**) and (**e**) Western blot analysis of Drosha and Dicer in EAhy926 cells treated with various concentrations of ox-LDL. All results are expressed as mean values ± S.E. for three independent experiments. * $p < 0.05$, ** $p < 0.01$.
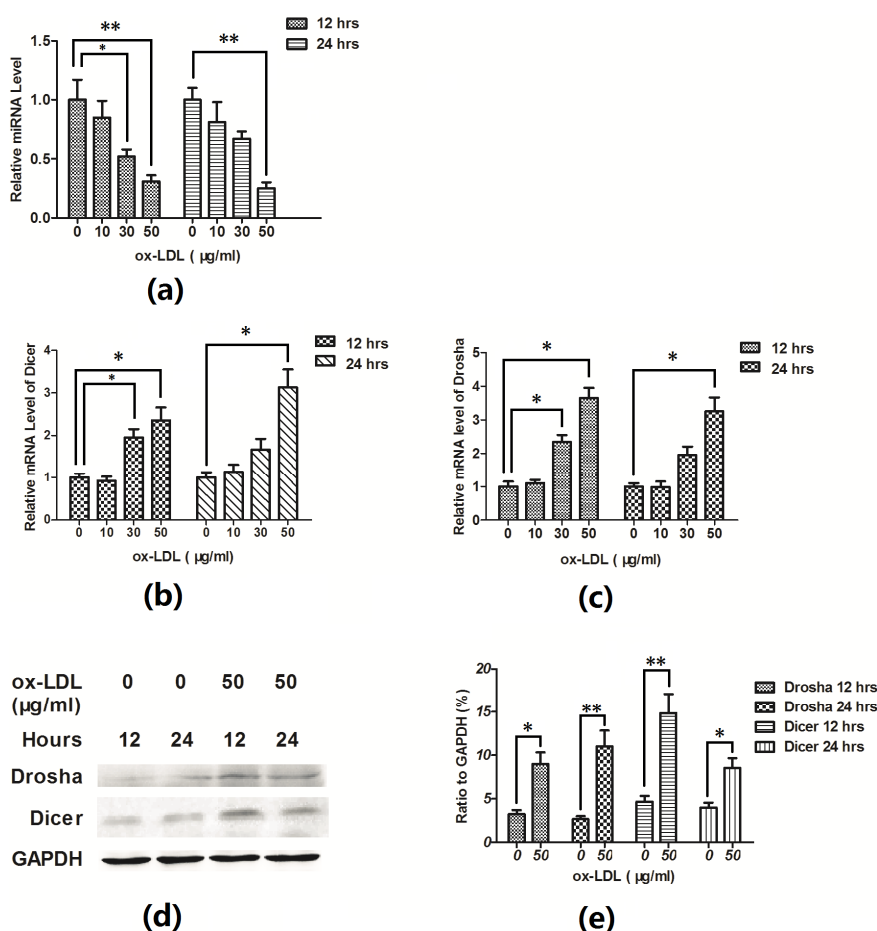

2.3. Hsa-Let-7g miRNA Inhibits the Apoptosis Induced by ox-LDL

An ox-LDL-induced apoptosis and has-let-7g downregulation were confirmed in EAhy926 endothelial cells. In addition, to investigate the association of has-let-7g downregulation with the apoptosis in the cells, we manipulated let-7g level in EAhy926 cells by transfecting with has-let-7g mimics. When 50 nM has-let-7g mimics was transfected into EAhy926 cells, the level of let-7g increased up to 850-fold 24 h post transfection or 615-fold 48 h post transfection (Figure 4a). It showed that let-7g miRNA mimics in the EAhy926 cells significantly diminished the ability of ox-LDL to induce apoptosis (Figure 4b). The regulation of let-7g on Bax and CASP3 were also assayed by quantitative RT-PCR and western blotting assay; it was shown that the ox-LDL induced Bax induction

was suppressed in mRNA level by the has-let-7g mimics (Figure 4c); and the upregulation of CASP3 by ox-LDL was significantly suppressed by has-let-7g mimics in both mRNA level (Figure 4d) and in protein level (Figure 4e,f). These data suggest that the specific repression of has-let-7g contributes to ox-LDL-induced apoptosis of EAhy926 cells *in vitro*.

Figure 4. Let-7g inhibited apoptosis and apoptosis-associated molecules expression in the EAhy926 cells. (**a**) The expression of has-let-7g was significantly upregulated in EAhy926 cells post transfecting with has-let-7g mimics than with miR-control at a 50 nM concentration; (**b**) ox-LDL induced apoptosis was suppressed in EAhy926 cells transfecting with has-let-7g mimics. The results were expressed as percentage for apoptotic cells; (**c**) and (**d**) Bax and CASP3 mRNA were downregulated post 50 nM has-let-7g mimics transfection and 50 μg/mL ox-LDL treatment; Bax and CASP3 protein level post has-let-7g mimics (**e**) or miRNA control (**f**) and 50 μg/mL ox-LDL treatment, for 0, 12 or 24 h. All results are expressed as mean values ± S.E. for three independent experiments. * $p < 0.05$, ** $p < 0.01$.
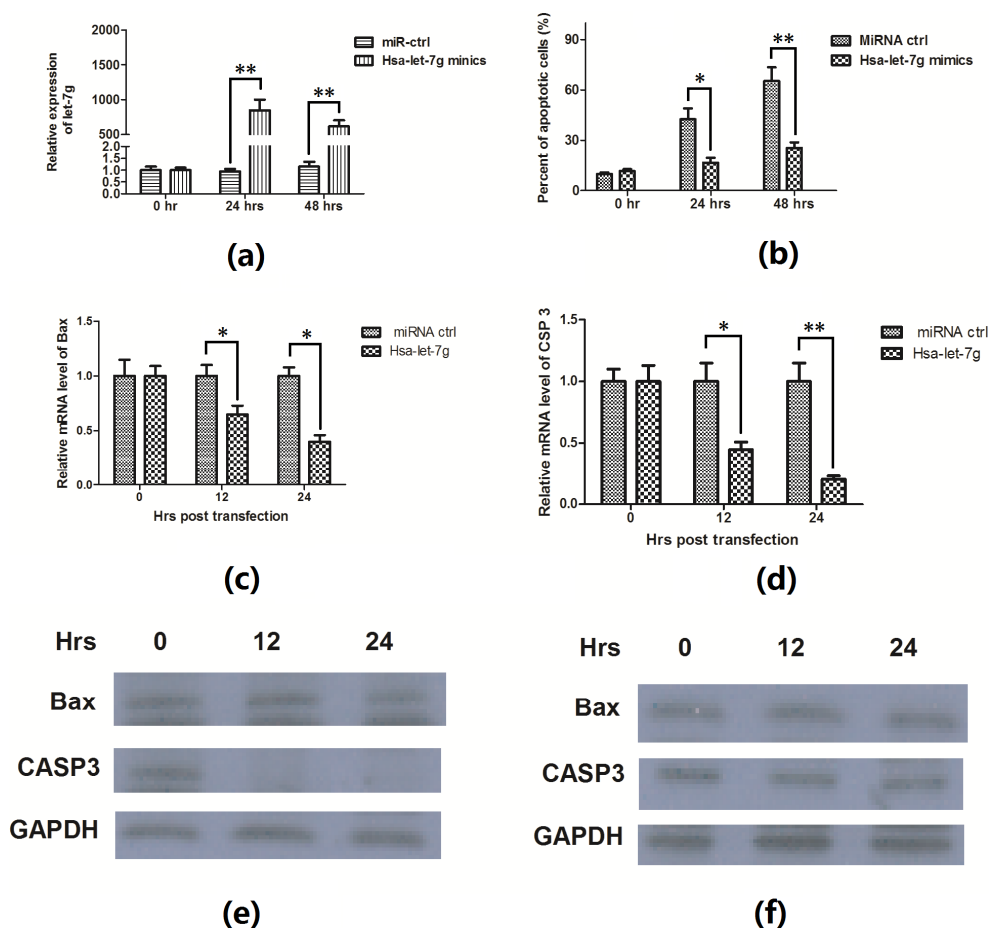
2.4. Hsa-Let-7g Targets 3' UTR of CASP3 and Inhibits CASP3 Expression

It was shown that during the apoptosis blocking by has-let-7g mimics in EAhy926 cells, CASP3 was more significantly inhibited than Bax ($p < 0.01$ *vs.* $p < 0.05$, Figure 4e,f). Analysis with software TargetScan demonstrated that the first eight nucleotides (seed sequence) from the 5' end of let-7g miRNA were complementary to the 153-159 nucleotides of the CASP3 3' UTR (Figure 5a). The CASP3 3' UTR mRNA has a perfect match with the seed sequence of has-let-7g. In order to evaluate the possible

targeting of CASP3 by let-7g in EAhy926 cells, the CASP3 expression in mRNA and protein levels was determined post the has-let-7g mimics or miRNA control transfection into the endothelial cells. It was shown that the pro-CASP3 protein was down-regulated after transfection with has-let-7g mimics while there was no effect for the control transfection (Figure 5b,e,f).

To further elucidate the hypothesis, the luciferase reporter constructs containing the let-7g recognition sequence of CASP3 mRNA immediately downstream of luciferase gene were utilized (Figure 5c). As shown in Figure 5d, transfection with the has-let-7g mimics suppressed while the miRNA control didn't regulate the activity of pGL-CASP3 reporter in EAhy926 cells. These results therefore agree with the fact that let-7g regulates CASP3 by targeting the 3' UTR and inducing translation repression of the gene.

Figure 5. Let-7g targeted on the 3' UTR of CASP3 and decreased the CASP3 expression. (**a**) Hsa-let-7g/CASP3 alignment by the Targetscan analysis; Hsa-let-7g suppressed CASP3 expression in mRNA level (**b**) or protein level (**e**) and (**f**). 24 h post treating with 50 μg/mL ox-LDL and transfecting with miRNA control or hsa-let-7g, EAhy926 cells were quantitatively determined for the CASP3 expression at mRNA level (**b**) and at protein level (**e**, **f**), compared to GAPDH. (**c**) Schematic diagram of pEZX-MT05 plasmid with CASP3 3' UTR. (**d**) Relative luciferase activity in EAhy926 cells 24 h post transfecting with miRNA control or has-let-7g mimics. All results were averaged with three independent experiments. Statistical significance was shown in the figure. * $p < 0.05$, ** $p < 0.01$.

Predicted consequential pairing of target region (top) and miRNA (bottom)	Seed match	Site-type contribution	3' pairing contribution	Position contribution	Context score	Context score percentile
5'…AGAACCACUAUGAAGCUACCUCA… 3' UGACAUGUUUGAU • GAUGGAGU	8mer	-0.310	0.003	-0.044	-0.36	96

(a)

(b)   (c)   (d)

(e)   (f)
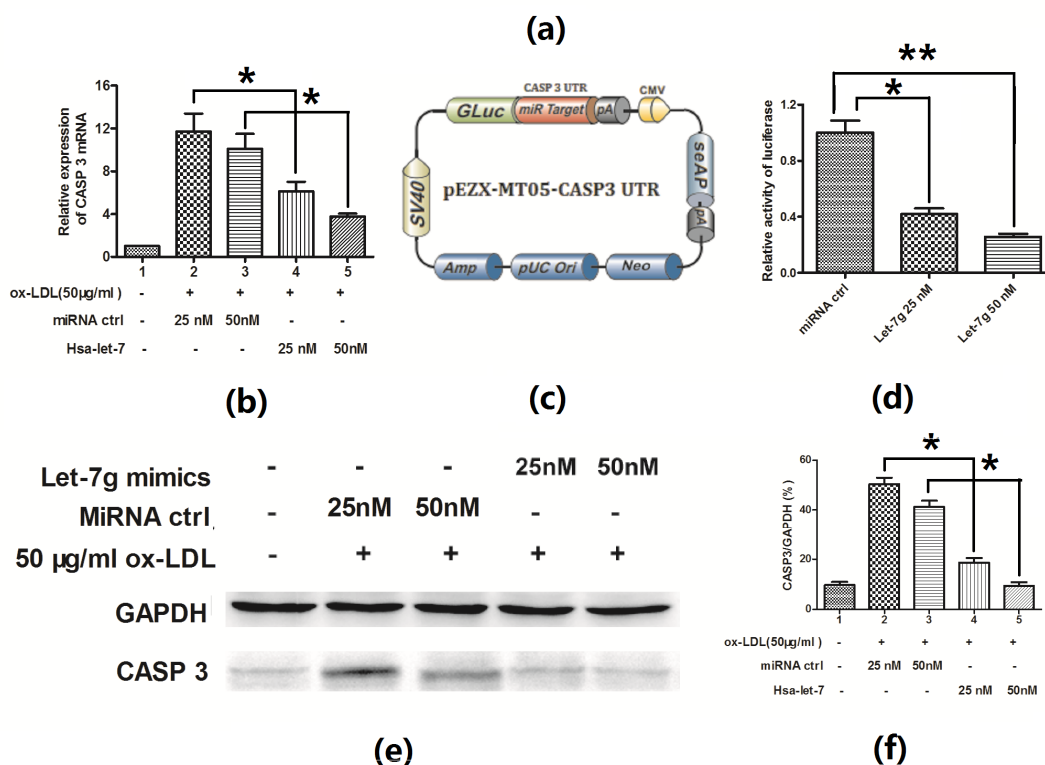
3. Discussion

Endothelial dysfunction, e.g., apoptosis, is an initiative stage of atherosclerosis. Particularly, the ox-LDL induced apoptosis in endothelial cells raises concern, because ox-LDL is a major risk factor in the development of atherosclerosis. We utilized a permanent endothelial cell line (EAhy926) to evaluate the ox-LDL-induced apoptosis. It was shown that ox-LDL could reduce the EAhy926 cell viability by inducing apoptosis, and the apoptosis was induced in a dose-dependent way (Figure 1).

Accumulating evidence suggests important roles of miRNAs in vascular diseases through regulating target molecules expression in endothelial cells. Angiogenesis, migration, and proliferation of endothelial cells could be regulated by miR-424, hsa-let-7gcluster, miR-221 and miR-222 [20–22]; the vascular inflammation had been contributed by miR-155, miR-31, and miR-126 [23]. Further, microRNAs, such as miR-146a, miR-181a, miR-29b and miRNA let-7g, were reported to be involved in the ox-LDL-induced atherosclerosis [15,24–26]. Thus, the identification of miRNAs and their targets may cast new light on the mechanism and therapeutic strategies of (to) atherosclerosis. Previously, we utilized a miRNA microarray to analyze the miRNA expression profile in ox-LDL induced apoptotic EAhy926 cells. Let-7g was one of the most downregulated miRNAs post ox-LDL treatment (Data not shown). The preliminary results gave an important indication of miRNAs associated with atherosclerosis. In the present study, we confirmed the suppression of miRNA let-7g in the ox-LDL-treated EAhy926 endothelial cells by RT-qPCR (Figure 3a). In addition, the possible roles of let-7g in ox-LDL-induced atherosclerosis were identified.

The let-7 family is composed of 12 members (let-7-a1, a2, a3, b, c, d, e, f1, f2, g, i and miR-98) located on 8 different chromosomes [27]. It has been demonstrated that let-7 family is one of most expressed microRNAs in endothelial cells [28]. In addition, let-7 family members have been described to promote endothelial cell proliferation and angiogenesis by targeting antiangiogenic genes [20,29,30]. Furthermore, the let-7 family members were reported to be linked to hypertension and atherosclerosis [31,32]. Particularly, the let-7g could regulate the ox-LDL-induced atherosclerosis by targeting ox-LDL receptor LOX-1 [14]. The let-7 family members have also been described to exert anti-apoptotic effect in diverse biological processes [33]. In this study, TargetScan analysis showed a complementation of let-7g miRNA with the CASP3 3' UTR (Figure 5a). In addition, the CASP3 expression in protein level was inhibited by the transfection of the has-let-7g mimics, while no effect was found for the control transfection. Furthermore, the luciferase reporter constructs containing the 3' UTR of CASP3 mRNA reconfirmed the suppression of CASP3 by has-let-7g mimics in EAhy926 cells. Taken together, let-7g downregulates CASP3 by targeting the 3' UTR and inducing translation repression of the gene.

CASP3 is considered as the major executioner caspase in apoptosis. By targeting CASP3, has-let-7g mimics reduced the ox-LDL-induced apoptosis significantly in EAhy926 cells (Figure 4b). Therefore, let-7g may play an important role in the regulation of ox-LDL-induced apoptosis and atherosclerosis. In addition to apoptosis, CASP3 is also known to be important in non-apoptotic events, such as cell cycle regulation, cell migration, cell differentiation [34–36]. By targeting CASP3, let-7g might also be involved in the regulation of other cellular events involved in atherosclerosis. Therefore, the significance of the present finding about the association of let-7g with CASP3 is yet to be explored. Nevertheless, such kind of studies might lead to the future development of an alternative strategy that targeting at let-7g in treatment of atherosclerosis.

4. Method and Materials

4.1. Reagents and Cell Cultures

Ox-LDL was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA) and resolved in the RPMI 1640 medium supplemented with 2% FBS. Because of the nonspecific detergent activity of ox-LDL, especially at high concentration (>50 μg/mL), the maximal concentration of ox-LDL was 50 μg/mL. EAhy926 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA) and limitedly propagated in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (Gibco, Rockville, MD, USA) and 0.2% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C under 5% CO_2. The RPMI 1640 medium supplemented with 2% FBS replaced the medium supplemented with 10% FBS, after the EAhy926 cells grew to about 90% confluence, and were treated with ox-LDL of various concentration for various h. Post ox-LDL incubation, the cells were lyzed for the mRNA expression analysis or for the western blotting analysis.

4.2. Cell Viability Assay

Cell viability was evaluated by methyl thiazolyl tetrazoliym assay (MTT assay) (Invitrogen, Carlsbad, CA, USA). EAhy926 cells were cultured in 96-well plates and incubated at 37 °C for 24 h, then cells were changed with RPMI 1640 medium containing 2% FBS and ox-LDL (0, 10, or 30 μg/mL), and were continued incubating for up to 0, 6, 12 or 24 h. After that, cells were incubated with 50 μL MTT solution at 37 °C for another 2 h, and were dissolved by 150 μL DMSO completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer (Bio-Rad, Hercules, CA, USA).

4.3. Apoptosis Assays

Cell apoptosis was determined by an annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, $1–5 \times 10^5$ EAhy926 cells post being treated with 0 or 30 μg/mL ox-LDL, resolved in RPMI 1640 medium containing 2% FBS, were collected and resuspended in 0.5 mL of binding buffer and incubated with annexin V-FITC and propidium iodide for 10 min in the dark at room temperature. A FACScan flow cytometer (Bio-Rad, Hercules, CA, USA) equipped with a FITC signal detector FL1 (excitation 488 nm, green) and a phycoerythrin emission signal detector FL3 (excitation 585 nm, red) was used to analyze cellular apoptosis. The results were calculated using the CellQuest™ Pro software (BD Biosciences) and expressed as the percentage of apoptotic cells from the total cells. To determine the effect of has-let-7g on the apoptosis induced by ox-LDL, the synthetic has-let-7g mimics or miRNA control (GenePharma, Shanghai, China) were utilized. EAhy926 cells were transfected with 50 nM has-let-7g mimics or miRNA control, 6 h later; the cells were inoculated with RPMI 1640 medium supplemented with 2% FBS, and with 50 μg/mL ox-LDL for 0, 24 or 48 h. Then the cell apoptosis was determined.

4.4. RNA Isolation, Reverse Transcription, qPCR

Total cellular RNA from about 10^5 cells was prepared with PureLink® RNA Mini Kit (Invitrogen, Carlsbad, CA, USA); miRNAs was isolated using mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA). In addition, reverse transcription-PCR (RT-PCR) was performed with Takara One Step RT-PCT kit (Takara, Dalian, China). For quantitative analysis of mRNA expression of LOX-1, Bax, CASP3, Drosha, Dicer, The resulting cDNAs were amplified using primer/probe sets specific for the genes of interest on a Lightcycler 480 II (Roche, Mannheim, Germany). Relative quantification was determined using the $\Delta\Delta Ct$ method using GAPDH as reference genes. The primers used were available upon a request.

4.5. Immunoblotting

About 10^5 cells were lyzed with cell lysis reagent (Promega, Madison, WI, USA), and supplemented with Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland). After protein concentration determination using Bradford Reagent (Bio-Rad, Hercules, CA, USA), the protein samples were separated onto a 4%–20% gradient SDS-PAGE gel, transferred to PVDF membrane and blocked in 5% skimmed milk. The following primary antibodies were utilized: LOX-1 rabbit polyclonal antibody, 1:300 (Sino Biological, Beijing, China), CASP3 rabbit polyclonal antibody, 1:500 (Sigma-Aldrich, St. Louis, MO, USA), Dicer rabbit polyclonal, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Drosha rabbit polyclonal, 1:500 (Santa Cruz Biotechnology); and GAPDH rabbit polyclonal, 1:1000 (Sigma-Aldrich, St. Louis, MO, USA). All immunoblots are representative of at least three independent experiments.

4.6. Overexpression of Hsa-Let-7g

Hsa-let-7g miRNA mimics (QIAGEN, Valencia, CA, USA) were utilized to overexpress the has-let-7g. For analysis of hsa-let-7 suppressing apoptosis, 50 nM miRNA mimics or miRNA control was transfected into EAhy926 cells with lipofectamine 2000. Post six hours' transfection, cells were treated with ox-LDL for 0, 24 or 48 h for let-7g expression and apoptosis analysis; or were treated with ox-LDL for 0, 12 or 24 h for Bax, or CASP3 expression analysis.

4.7. Luciferase Activity Assay

Reporter plasmid with CASP3 3'UTR (pEZX-MT05) was purchased from GeneCopoeia, Inc (Rockville, MD, USA). Briefly, 3.5×10^5 EAhy926 cells were transfected with 25 or 50 nM of miRNA control or has-let-7g mimics, and 0.5 μg pEZX-MT05 reporter plasmid with Lipofectamine 2000 according to the manufacturer's instructions. The medium was replaced with RPMI 1640 containing 10% FBS at 6 h post-transfection. At 24 h post-transfection, cells were harvested for analysis. Luciferase activity was determined by using a Promega luciferase assay system. Experiments were done in triplicate, and means were determined.

5. Conclusions

We found that miRNA let-7g was suppressed during the ox-LDL-induced apoptosis in EAhy926 endothelial cells. In addition, overexpression of let-7g negatively regulated apoptosis in the endothelial cells by targeting CASP3 expression. Therefore, miRNA let-7g may play an important role in endothelial apoptosis and atherosclerosis.

Acknowledgments

Conflicts of Interest

The authors declare on conflict of interest.

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