Negative feedback regulation between microRNA let-7g and the oxLDL receptor LOX-1

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Summary
Lectin-like oxidized LDL receptor-1 (LOX-1) is a surface scavenger receptor for oxidized low-density lipoprotein (oxLDL). Several transcription factors have been reported to regulate LOX-1 expression. MicroRNAs are small noncoding RNAs that control gene expression, but there have been no reports of LOX-1 expression being regulated by microRNAs. Because the microRNA let-7g has been predicted to bind to LOX-1 mRNA, we investigated whether let-7g can regulate LOX-1 expression. Our experiments first demonstrated that oxLDL can reduce let-7g expression. We later confirmed that there is a let-7g binding site on the 3'-untranslated region of LOX-1 mRNA. We showed that intracellular Ca2+-activated protein kinase C is involved in the oxLDL–LOX-1–let-7g pathway. Bioinformatics predicted that the let-7g promoter has a binding site for the transcriptional repressor OCT-1. We used a promoter assay and chromatin immunoprecipitation to confirm this binding. Consequently, knockdown of OCT-1 was found to increase let-7g expression. Transfection of let-7g inhibited oxLDL-induced LOX-1 and OCT-1 expression, cell proliferation and migration. Mice fed with a high-fat diet showed a decrease in let-7g and an increase in LOX-1 and OCT-1. A study on humans showed the serum levels of let-7g are lower in subjects with hypercholesterolemia compared with normal controls. Our findings identify a negative feedback regulation between let-7g and LOX-1, and indicate that let-7g could be a target to treat cardiovascular disease.

Key words: Atherosclerosis, LOX-1, let-7g, microRNA, oxLDL

Introduction
Atherosclerosis is a chronic inflammatory disease characterized by lipid-rich plaques within arterial walls (Libby, 2002). The accumulation of oxidized low-density lipoprotein (oxLDL), which is modified from native LDL by reactive oxygen species, is an important pathological change during the development of atherosclerosis. OxLDL stimulates the endothelial cells to produce several adhesion molecules and proinflammatory mediators such as IL-8 and MCP-1, which recruit monocytes and lymphocytes into the subendothelium. The scavenger receptor, lectin-like oxidized-low-density lipoprotein receptor 1 (LOX-1), mediates the binding and internalization of oxLDL into cells. A previous study showed that LOX-1 can be upregulated by oxLDL (Mehta and Li, 1998). The activation of LOX-1 by oxLDL stimulates the expression of endothelial proinflammatory genes and superoxide radical formation, which is believed to play an active role in atherogenesis (Navarra et al., 2010).

LOX-1 is encoded by the oxLDL receptor 1 gene (OLR1; but for simplicity LOX-1 is used when referring to the gene and mRNA) and is a type II transmembrane glycoprotein (Sawamura et al., 1997). LOX-1 is expressed in the cell types involved in atherosclerotic pathogenesis, including endothelial cells, macrophages and smooth muscle cells. OxLDL and acetylated LDL, but not native LDL, can bind to LOX-1. Furthermore, LOX-1 is capable of recognizing some distinct ligands such as platelets, bacteria and phosphatidylserine (Kakutani et al., 2000; Shimaoka et al., 2001; Sakurai and Sawamura, 2003). In addition to oxLDL, several proinflammatory and proatherogenic stimulators, such as advanced glycation end product, angiotensin II, tumor necrosis factor-α, and transforming growth factor-β can markedly induce LOX-1 expression (Navarra et al., 2010). Serum level of LOX-1 has been shown to be associated with cardiovascular disease (Hayashida et al., 2005). Activation of LOX-1 is also a potent contributor to oxLDL-induced endothelial dysfunction, characterized by impaired vasorelaxation, increased adhesion to circulating leukocytes, superoxide generation and decreased nitric oxide release (Chen et al., 2007). In animal experiments, overexpression of LOX-1 results in intramyocardial vasculopathy in apolipoprotein-E-knockout mice (Inoue et al., 2005). Deletion of the LOX-1 gene reduces atherosclerosis formation in low-density lipoprotein receptors and knockout mice that were fed the high cholesterol diet (Mehta et al., 2007).

MicroRNAs (miRNAs) are a class of endogenous, small, non-coding RNAs that negatively control gene expression by binding to their target messenger RNAs (mRNAs) for degradation and/or translational repression (Linsen et al., 2008). The role of miRNAs in the cardiovascular system has recently been discovered. For example, miR-145 is a crucial modulator of vascular smooth
muscle cell (VSMC) phenotype and proliferation (Zhang, 2009). MicroRNA let-7 has nine family members in humans. The let-7 family plays a pivotal role in cell proliferation (Lan et al., 2011), cancer (Bussing et al., 2008; Schultz et al., 2008) and cardiovascular diseases (Zhang, 2008). Several studies have reported that LOX-1 activation could enhance downstream gene regulation including miRNAs (Li and Mehta, 2009; Huang et al., 2010). The LOX-1 3′-untranslated region (UTR) was predicted, using bioinformatic tools RNA22 (Miranda et al., 2006) and Targetscan (Friedman et al., 2009), to have a putative target site for let-7g. However, interaction between let-7 and LOX-1 has not been reported. In the present study, we aimed to investigate the role of human let-7g (hsa-let-7g) in the regulatory pathway of LOX-1. We tested the hypothesis that oxLDL upregulates LOX-1 expression by inhibiting let-7g gene expression. To further explore the cascade of the regulatory signaling pathway, we first demonstrated that LOX-1 influences the binding of transcriptional repressor OCT-1 (also known as POU domain, class 2, transcription factor 1; POU2F1) to the let-7g promoter. Then we showed that both protein kinase C (PKC) and intracellular Ca2+ are involved in the regulation of let-7g expression. Transfection of let-7g into VSMCs substantially inhibited oxLDL-induced LOX-1 and OCT-1 expression, cell proliferation and migration. These results substantiate the findings from cellular studies. We identified a new pathway and elucidated the signal transduction that causes a feedback regulation between oxLDL, LOX-1 and let-7g.

Results
OxLDL induced cell proliferation and migration through the LOX-1 receptor
The oxLDL–LOX-1 signaling pathway has been shown to be important in the regulation of endothelial cells in the context of atherosclerosis (Mattaliano et al., 2009), but its role in human aortic smooth muscle cells (HASMCs) has not been well explored. Our data showed that oxLDL induced LOX-1 upregulation in HASMCs, which is similar to the data on endothelial cells. OxLDL induced a dose- and time-dependent increase of LOX-1 mRNA and protein (Fig. 1A). We used HASMC proliferation and migration as a model for the development of atherosclerosis. To further investigate the effect of LOX-1 on HASMCs, we cloned the full length (822 bp) cDNA of LOX-1 into pEGFP-N3 plasmids without any fusion protein. We confirmed that pEGFP-LOX-1 could significantly (P=0.00007) increase LOX-1 expression and that LOX-1 shRNA could cause a decrease of LOX-1 expression (supplementary material Fig. S1). In the presence of oxLDL, overexpression and knockdown of LOX-1 significantly affected HASMC proliferation and migration (Fig. 1B,C; P<0.01). However, in the absence of oxLDL, LOX-1 levels did not influence HASMC proliferation or migration. Taken together, these results clearly suggested that oxLDL can enhance LOX-1 expression in HASMCs, which in turn affects HASMC phenotypes.

let-7g targeted the LOX-1 3′UTR and inhibited LOX-1 expression
Given that let-7g is predicted to bind to LOX-1 3′UTR (Fig. 2A), we first tested whether oxLDL could inhibit let-7g expression in HASMCs. Using both northern blotting and quantitative PCR, we found that oxLDL reduced let-7g expression in a dose-dependent manner (Fig. 2B). Similarly, oxLDL could reduce let-7g in oxLDL-treated human umbilical vein endothelial cells (HUVECs; supplementary material Fig. S2). To confirm LOX-1 is a let-7g target gene, the full length (1579 bp) LOX-1 3′UTR was cloned into a pMIR-reporter plasmid. As shown in Fig. 2C, oxLDL induced a dose-dependent increase of luciferase activity, which could be due to a decrease of let-7g expression and its influence on LOX-1 3′UTR. We further demonstrated that different concentrations of a let-7g mimic could decrease the luciferase activity (Fig. 2D). Supplying let-7g mimic significantly attenuated
the effect of oxLDL on luciferase activity (Fig. 2E; $P = 0.006$). This suggested that oxLDL induced the luciferase activity through the effect of let-7g on the LOX-1 3' UTR.

In addition, transfecting the let-7g mimic provided direct evidence of let-7g binding to the LOX-1 3' UTR. To further validate LOX-1 as a let-7g target gene, seven nucleotides located in the crucial binding region of the LOX-1 3' UTR were mutated by site-directed mutagenesis. This procedure would reduce or abolish let-7g binding to LOX-1. As shown in Fig. 2F, the let-7g mimic did not have an effect on luciferase activity after mutating the let-7g targeted site. We also directly tested the effect of let-7g on LOX-1 expression and found that transient transfection of the let-7g mimic into HASMCs significantly and dose-dependently decreased LOX-1 mRNA and protein expression (Fig. 2G; $P < 0.05$). Accordingly, the above experiments validated LOX-1 as a let-7g target gene.

**OxLDL decreased let-7g promoter activity through the OCT-1 transcription factor**

To investigate whether a transcriptional binding site is present in the promoter of let-7g, a 1.5 kb upstream region of the let-7g promoter was cloned into the pGL3-luciferase reporter vector. Because no luciferase activity could be detected in HASMCs, we used CRL-1999 cells (human aorta smooth muscle cell line) to conduct this promoter assay. A dose-dependent decrease of let-7g promoter activity was noticed after oxLDL treatment (Fig. 3A), which suggested that a transcriptional factor is involved in the oxLDL–LOX-1–let-7g signaling pathway. To search for the core region determining let-7g promoter activity, various fragments of the promoter were cloned. As shown in Fig. 3B, deletion of the region between 2100 and 2150 bp yielded the most substantial change in luciferase activity. The TFSEARCH software (ver 1.3) (Heinemeyer et al., 1998) predicted three putative transcription factor binding sites (OCT-1, GATA-1 and C/EBPb) on the core region (Fig. 3C). OCT-1 is a transcriptional repressor and previous studies have reported that OCT-1 can be upregulated in the oxLDL-mediated LOX-1 pathway (Chen et al., 2006; Thum and Borlak, 2008). By contrast, GATA-1 and C/EBPb are both transcriptional activators (Boyes et al., 1998; Ruminy et al., 2001). GATA-1 could not be detected in HASMCs, but oxLDL-mediated C/EBPb downregulation was detected (supplementary material Fig. S3). Therefore, OCT-1 is more likely to be involved in oxLDL suppression of let-7g. After mutating five nucleotides of the putative OCT-1 binding site in the let-7g promoter, the inhibitory effect of oxLDL on let-7g promoter activity was abolished (Fig. 3D). By using the chromatin immunoprecipitation (ChIP) assay, we found that oxLDL induced more binding of OCT-1 protein to the let-7g promoter compared with the control treatment (without oxLDL; Fig. 3E). Therefore, our data indicate that OCT-1 is involved in the inhibition of let-7g by oxLDL.
OxLDL-mediated OCT-1 upregulation affected let-7g and LOX-1 expression

Recent studies have also reported that LOX-1 activates OCT-1 expression (Thum and Borlak, 2008). As shown in Fig. 4A, either overexpression or knockdown of the LOX-1 gene could consequently influence oxLDL-inhibited let-7g expression. A dose-dependent increase of OCT-1 mRNA and protein expression was demonstrated after oxLDL treatment (Fig. 4B). These results indicated that the oxLDL–LOX-1–OCT-1 pathway plays an inhibitory role on let-7g expression. To further identify the effect of OCT-1 on the regulation of the let-7g gene, OCT-1 shRNA was used to silence 50% of endogenous OCT-1 in HASMCs, which eliminated the oxLDL inhibitory effect on let-7g (supplementary material Fig. S4; Fig. 4C). Simultaneously, the oxLDL effect on OCT-1 mRNA and protein levels was attenuated because of the knockdown of OCT-1 (Fig. 4D,E). All the results suggested that the effect of oxLDL on OCT-1 expression is mediated by the LOX-1 receptor, which in turn downregulates let-7g expression.

PKC and let-7g expression

Because PKC has been reported to activate OCT-1 expression (Thum and Borlak, 2008), we sought to validate that PKC is involved in the oxLDL–LOX-1–OCT-1–let-7g pathway. First we used 1 mM chelerythrine, a commonly used PKC inhibitor, to inhibit PKC. As shown in Fig. 5A, chelerythrine significantly inhibited oxLDL effect on let-7g expression ($P < 0.023$). LOX-1 mRNA and protein levels, induced by oxLDL, were attenuated after chelerythrine treatment (Fig. 5B,C). Again, chelerythrine reduced the effect of oxLDL on OCT-1. These results indicate that PKC is involved in the oxLDL–LOX-1–OCT-1 pathway for let-7g down-regulation.

[Ca$^{2+}$], increase in the oxLDL–LOX-1–OCT-1 signal transduction

Intracellular Ca$^{2+}$ is a well-known upstream regulator of PKC that participates in the development of atherosclerosis (Velarde et al., 2001). Therefore, intracellular Ca$^{2+}$ might also participate in the oxLDL–LOX-1–OCT-1 pathway. As expected, oxLDL increased the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) in a dose-dependent manner (Fig. 6A). Then we used MAPTAM, a cell-permeable intracellular Ca$^{2+}$ chelator, to inhibit the effect of Ca$^{2+}$. The results showed that MAPTAM abolished the effect of oxLDL on let-7g (Fig. 6B). In addition, MAPTAM reduced both mRNA and protein levels of LOX-1 and OCT-1 (Fig. 6C–E). All the data demonstrated that [Ca$^{2+}$] also plays a key role in oxLDL–LOX-1–OCT-1 signal transduction (the signaling pathway is depicted in Fig 8C).
let-7g mimic inhibited oxLDL-enhanced cell proliferation and migration

In order to test the effect of let-7g on the oxLDL-mediated LOX-1 pathway, 50 nM of let-7g mimic was transfected into HASMCs. As shown in Fig. 7A,B, let-7g mimic attenuated oxLDL-induced LOX-1 mRNA and protein production. let-7g mimic also caused a dose-dependent decrease of OCT-1 expression (supplementary material Fig. S5). Either the expression of OCT-1 or the intracellular [Ca2+]i changes were significantly abolished in let-7g mimic-transfected HASMC (Fig. 7A–C; P<0.05). Furthermore, let-7g mimic clearly inhibited oxLDL-induced HASMC proliferation and migration (Fig. 7D,E). All the results indicated that let-7g inhibits oxLDL-induced HASMC proliferation and migration through its effect on the LOX-1–OCT-1 pathway.

In vivo studies to confirm the effect of oxLDL on the LOX-1–OCT-1–let-7g pathway

We further validated the existence of the LOX-1–OCT-1–let-7g pathway

Fig. 4. OCT-1 is involved in oxLDL-regulated let-7g and LOX-1 gene expression. (A) Knockdown or overexpression of LOX-1 affects oxLDL-regulated let-7g expression. After cells were transfected with 1 μg pEGRF-N3, pEGRF-LOX-1, scrambled shRNA control or LOX-1 shRNA for 24 hours, the let-7g levels was measured by qPCR with oxLDL treatment for another 24 hours. (B) OxLDL induced a dose-dependent increase of OCT-1 expression. Cells were treated with increasing concentrations of oxLDL for 24 hours. The relative mRNA (lower panel) and protein (upper panel) levels of OCT-1 were analyzed using qPCR and western blotting assay, respectively. (C–E) The effect of OCT-1 knockdown on let-7g levels, LOX-1 mRNA and protein levels. After cells were transfected with 1 μg scrambled shRNA control or OCT-1 shRNA for 24 hours, the expression of let-7g or LOX-1 was measured by qPCR and western blotting assay. Data are means ± s.d. from three experiments; *P<0.05.

Fig. 5. PKC participates in oxLDL-mediated let-7g and LOX-1 gene expression. (A,B) The effect of the PKC inhibitor chelerythrine on oxLDL-regulated let-7g, and LOX-1 mRNA levels. (C) Chelerythrine inhibits oxLDL-induced LOX-1 and OCT-1 protein levels. After pre-treating with 1 μM chelerythrine for 1 hour, cells were further treated with oxLDL for 24 hours. The relative expression levels of let-7g or LOX-1 and OCT-1 was measured by qPCR and western blotting assay, respectively. Data are means ± s.d. from three experiments; *P<0.05.
Fig. 6. The [Ca\(^{2+}\)]\(_i\) is involved in oxLDL-mediated let-7g, LOX-1 and OCT-1 gene expression. (A) OxLDL induced a dose-dependent increase of [Ca\(^{2+}\)]\(_i\). After cells were treated with different concentrations of oxLDL, as indicated, for 15 minutes, cells were further treated with 1 \(\mu\)M Fluo-4 AM for 30 minutes and relative fluorescence values were determined. Data are means ± s.d. from three experiments; *\(P<0.05\). (B–E) The effect of the Ca\(^{2+}\)i inhibitor, MAPTAM, on oxLDL-regulated let-7g, LOX-1 and OCT-1 mRNA and protein levels. After pre-treating with 10 \(\mu\)M MAPTAM for 1 hour, cells were further treated with oxLDL for another 24 hours. The relative expression levels of let-7g, LOX-1 and OCT-1 was measured by qPCR and western blotting assay, respectively. Data are means ± s.d. from three experiments; *\(P<0.05\).

Fig. 7. The effect of let-7g mimic on oxLDL-regulated LOX-1 and OCT-1 pathways, cell proliferation and migration. After transfection with 50 nM let-7g mimic for 24 hours, cells were further treated with oxLDL for another 24 hours. (A) qPCR and (B) western blotting assay of the expression levels of LOX-1 and OCT-1. (C) Changes in [Ca\(^{2+}\)]\(_i\), as determined using Fluo-4 AM. (D) Cell proliferation determined using the WST-1 assay. (E) Wound healing assay of cell migration. Data are means ± s.d. from three experiments; *\(P<0.05\).
expression of let-7g and an increased expression of LOX-1 and OCT-1 compared with mice fed a chow diet (Fig. 8A). Our animal studies confirm the findings in the cellular studies.

The let-7g levels in human serum
To determine the influence of hypercholesterolemia on circulating let-7g levels, we first measured the serum let-7g levels from 15 hypercholesterolemia patients and 14 healthy controls. Their data showed that let-7g levels were significantly reduced in hypercholesterolemic subjects compared with healthy controls. To determine the influence of hypercholesterolemia on circulating let-7g levels, we first measured the serum let-7g levels between stroke subjects without hypercholesterolemia compared with healthy controls (P = 0.79). The subsequent experiments identified a let-7g binding site in the LOX-1 3'UTR. We also discovered an OCT-1 binding site in the promoter of the let-7g gene. Through a series of experiments, we identified the signal transduction pathway where oxLDL binds to its receptor LOX-1 to initiate the Ca^{2+}–PKC–OCT-1–let-7g signal transduction. Our results provide an explanation of the results of a previous study, which found that oxLDL upregulates LOX-1 (Mehta and Li, 1998), let-7g mimic had a strong inhibitory effect on oxLDL-mediated cell proliferation and migration. Furthermore, the in vivo studies showed a lower level of let-7g in mice fed with a high fat diet than in mice fed a chow diet. A reduced expression of let-7g was accompanied by an increase of LOX-1, showing the existence of a negative feedback regulation between LOX-1 and let-7g. In human subjects, increased cholesterol levels have been associated with low let-7g levels. Owing to the pivotal role of LOX-1 in the cardiovascular system, our findings might offer a new therapeutic intervention to treat or prevent atherosclerosis. The role of LOX-1 in the pathogenesis of vascular disorders is well documented (Mehta et al., 2006). However, the detailed signaling pathway that regulates the LOX-1 gene has not been reported. Sequence analysis of the proximal 5' promoter region of the gene identified the existence of several potential cis-regulatory elements, such as the OCT-1-binding site, nuclear factor-kB (NF-kB), adaptor protein-1 and -2 sites and a shear–stress responsive element (Chen et al., 2002; Hofnagle et al., 2004). Studies have shown that OCT-1 activated by oxLDL and NF-kB mediated by angiotensin II could induce LOX-1 promoter activity in human coronary artery endothelial cells (Li et al., 1999; Chen et al., 2006). Glucose enhances LOX-1 expression in human macrophages and results in foam cell formation through NF-kB and AP-1 regulatory elements (Li et al., 2004). We demonstrated that let-7g could post-transcriptionally regulate LOX-1 gene expression. To our knowledge, this is the first study to reveal the role of microRNA in the oxLDL–LOX-1 pathway.

Negative feedback regulation is an important mechanism in homeostasis. In the context of atherosclerosis, the negative feedback can be found in several situations. For example, cholesterol synthesis in hepatocytes is under negative feedback regulation: increased cholesterol in the cell decreases the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis. Here, we discovered another feedback regulation.
between LOX-1 and let-7, which is potentially important in the development of atherosclerosis and for future strategies to treat or prevent atherosclerosis.

The regulation of the miRNA gene remains undiscovered, although epigenetic regulation (Toyota et al., 2008; Chen et al., 2011) and transcription factors (Taganov et al., 2006) have been reported to regulate miRNA expression. Because no CpG islands are located in the let-7g promoter, we searched for transcription factors and showed OCT-1 as an inhibitory factor for the let-7g gene. Recent results have linked a decreased expression of let-7 to tumorigenicity and poor prognosis in cancer patients (Bussing et al., 2008). The let-7 family has also been proposed as a tumor suppressive microRNA, especially let-7g (Kumar et al., 2008). To our knowledge, there are no reports to show the anti-atherosclerotic role of let-7g. We showed that a high fat diet can suppress let-7g expression, which not only provides a common mechanism for both cardiovascular disease and cancers, but also provides a potentially common therapeutic target for these two major diseases.

Recently, extracellular miRNAs have been discovered in serum and plasma (Reid et al., 2010). The circulating microRNAs could serve as biomarkers. In the present study, we used data of serum let-7g to further validate our cellular and animal findings. We first showed that levels of serum let-7g are reduced in subjects with hypercholesterolemia. A decreasing let-7g level was noticed in normal subjects, hypercholesterolemic subjects and stroke subjects with hypercholesterolemia, which suggests let-7g levels are associated with the severity of cholesterol-induced atherosclerosis. Interestingly, the stroke subjects without hypercholesterolemia had a similar let-7g level to normal controls. Therefore, in humans, let-7g might play a role in dyslipidemia-induced atherosclerosis. Accordingly, increasing let-7g could reduce the risk for atherosclerosis.

In conclusion, the present study provides new insights into the role of the microRNA let-7g in oxLDL-induced atherosclerosis. Our data demonstrate an oxLDL-induced signaling pathway in HASMCS. More importantly, they suggest that let-7g has both tumor suppressive and anti-atherosclerotic functions. The illustrated regulation mechanism offers potential therapeutic targets for atherosclerotic cardiovascular disease.

Materials and Methods

Materials

Primary human aortic smooth muscle cells (HASMCS), medium 231 and smooth muscle cell growth supplement (SMGS) were purchased from Cascade Biologics (Portland, OR). Other cell culture-related reagents were purchased from Gibco-BRL. OxLDL was purchased from Biomedical Technologies Inc. (Stoughton, MA). Anti-LOX-1 antibody was purchased from Sigma-Aldrich. Anti-OC-1 antibody was purchased from Cell Signaling Technology, Inc. Anti-β-actin antibody, enhanced chemiluminescence (ECL) solution and WST-1 were purchased from Millipore. TRIZol® reagent, Lipofectamine and secondary antibodies were purchased from Invitrogen. SYBR® Green PCR Master Mix, MultiScribe™ reverse transcriptase kit, TaqMan® let-7g and U44 assays, and let-7g mimic were purchased from Applied Biosystems. cDNA northern blot assay kit, biotin labeled let-7g and U6 probes were purchased from Signosis, Inc (Sunnyvale, CA). The luciferase assay system was purchased from Promega Corporation. Primer sets were synthesized by Mission Biotech (Taipei, Taiwan). LOX-1 and OCT-1 shRNAs were purchased from National RNAi Core Facility (Nankang, Taipei, Taiwan). Unless otherwise specified, all other reagents were of analytical grade.

Cell culture, treatments and transfections

Primary human HASMCS were grown in medium 231 supplemented with SMGS at 37°C in a humidified atmosphere of 95% air and 5% CO2. Cells between passages 6 and 10 were used in all experiments. The normal VSMC cell line, CRL-1999, was obtained from ATCC (Manassas, VA). CRL-1999 cells used for the promoter assay were maintained in F12K Kaighn’s modification medium containing 0.3 mM TES, 0.3 mM L-aspartic acid, 0.001% Apo-transferrin, 58 mM sodium selenite, 0.003% endothelial growth supplement, 1% penicillin-streptomycin, 1% L-glutamine, 1% HEPES, 1% non-essential amino acids, 1% sodium pyruvate and 10% heat-inactivated FCS.

To conduct experiments related to oxLDL treatment, cells were seeded onto six-well plate at a density of 2 x 10^5 cells/well. Cells achieving 95% confluency were serum-starved in medium 231 without SMGS for 24 hours, and then co-cultured with 20 µg/ml oxLDL for another 24 hours. To validate the effect of PKC and (Ca2+), or oxLDL-mediated gene expressions, 1 µM chelerythrine or 10 µM MAPTAM [1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrasodium (tetraethyl ester)] was added for 1 hour before oxLDL treatment. LOX-1 shRNA, OCT-1 shRNA, pEGFP-LOX-1 (all 1 µg) or 50 nM let-7g mimic were each transfected with Lipofectamine 2000 (Invitrogen). After incubation for 24 hours, serum-free medium was replaced and the cells were harvested after oxLDL treatment for another 24 hours.

RNA isolation and quantitative real-time PCR (qPCR)

Total RNA extraction was carried out using TRIZol® according to the manufacturer’s instructions. RNA quality was confirmed using a Agro(A260/A280 readings. The cDNA was synthesized from 1 µg total RNA using a random primer and the MultiScribe™ Reverse Transcriptase Kit. For let-7g and U44 detection, cDNA was synthesized from TaqMan® MicroRNA assays. The cDNA was diluted 1:30 with PCR grade water and stored at –20°C.

For quantitative real-time PCR, specific primers for human or mouse LOX-1, OCT-1 and GAPDH were designed and are listed in supplementary material Table S1. Relative gene expression was determined using an ABI 7500 real-time PCR machine (Applied Biosystems) with pre-optimized conditions. PCRs were performed in duplicate using 5 µl 2× SYBR Green PCR Master Mix, 0.2 µl primer sets, 1 µl cDNA and 3.6 µl nucleotide-free H2O, to yield a 10 µl reaction. The expression ratios were calculated as the normalized Ct difference between the control and sample with the adjustment for the amplification efficiency relative to the expression level of the housekeeping gene GAPDH. To quantify let-7g expression, U44 was used as internal control.

Western blot analysis

Cells were harvested in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS in PBS) containing a protease inhibitor cocktail (Calbiochem) and centrifuged at 13,200 g for 10 minutes at 4°C. The supernatant was used as total cell lysate. Protein lysates (20 µg) were denatured in 2% SDS, 10 mM dithiothreitol, 60 mM Tris-HCl (pH 6.8) and 0.1% Bromphenol Blue, and loaded onto a 10% SDS polyacrylamide gel. The separated proteins were then transferred onto a PVDF membrane. The membrane was blocked for 1 hour at room temperature in PBS containing 5% nonfat dry milk and incubated overnight at 4°C in PBS-T (PBS with Tween 20) containing the primary antibody. The membrane was washed in PBS-T, incubated with the secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature, and then washed in PBS-T. The ECL non-radioactive detection system was used to detect the antibody–protein complexes by imaging with the Bio-Rad ChemiDoc XR System.

Construction of full-length LOX-1 cDNA

The full-length LOX-1 cDNA (NM_002543.3) was generated by PCR amplification using the primers listed in supplementary material Table S1. The following thermal profile was used for the PCR amplification of 500 ng cDNA on a GeneAmp PCR system 9700 (Applied Biosystems): an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. The PCR products were analyzed by agarose gel electrophoresis. All the PCR products were cloned into pEGM-T Easy (Promega) and sequenced. After BamH1–XhoI digestion, LOX-1 cDNA was cloned into pEGFP-N3 to form a construct pEGFP-LOX-1 without GFP fusion.

Northern blotting assay for let-7g

An miRNA northern blot assay kit (Signosis, Sunnyvale, CA) was used according to the manufacturer’s instructions to assay let-7g. RNA (5 µg/well) was fractionated using 15% TBE urea-PAGE, blotted on membranes, and hybridized with biotin labeled let-7g and U6 probes. The amount of RNA loaded in each well was normalized to the amount of U6.

Cell proliferation assay

Cell proliferation was determined using the WST-1 cell proliferation assay (Millipore) according to the manufacturer’s instructions. Briefly, the cells were seeded, in triplicate, in 12-well plates at 10^4 cells/well. After cells were treated with oxLDL or co-transfected with pEGFP-LOX-1, LOX-1 shRNA or let-7g mimic for 24 hours, they were further incubated with a 1:10 dilution of WST-1 reagent/medium for 4 hours, and the absorption of the samples (with a background control as a blank) was measured at 440 nm and 650 nm using a microplate reader.
Wound healing assay

The wound healing assay was carried out as described previously (Yoon et al., 2010). Cells (10^4) were cultured in a monolayer in the absence (control) and presence (experimental) of oxLDL for 24 hours. Wounds were then made by scraping through the cell monolayer with a sterile 200 μl pipette tip, followed by washing with the medium to remove cellular debris. Three wounds were made per dish, and the cells were left to grow for another 24 hours. Phase-contrast images (100 x magnification) were taken of the three wound sites using a Nikon phase-contrast microscope. The migration rate was measured by counting the number of monolayer cells and monitoring the decrease in area over 24 hours. Triplicate results were obtained in three separate experiments.

Construction of the LOX-1 3'UTR reporter plasmid and mutagenesis

PCR was performed using sets of primers (supplementary material Table S1) specific for the LOX-1 3' UTR, of which the forward primer was SpeI-site-linked and the reverse primer MluI-site-linked. HASMC genomic DNA was used as the template. PCR products were digested with SpeI and MluI and cloned downstream of the luciferase gene in the pmir-REPORT luciferase vector (Ambion). This vector was sequenced and named pMIR-LOX-1-3UTR. Site-directed mutagenesis of the luciferase gene in the pMIR-REPORT luciferase vector was sequenced and named pMIR-LOX-1-3UTR. Site-directed mutagenesis kit (Stratagene) and named pMIR-LOX-1-mutant 3UTR, in which pMIR-LOX-1-3UTR was used as a template. For reporter assays, the cells were transiently transfected with wild-type or mutant reporter plasmid and treated with oxLDL using Lipofectamine 2000 (Invitrogen). pEGFP plasmids were co-transfected and acted as the internal control. Reporter assay was performed at 24 hours post-transfection using the Luciferase Assay System (Promega).

Construction of the let-7g promoter reporter plasmid and mutagenesis

To test the ability of the human let-7g promoter several reporter constructs were made. A 1.5-kb fragment of let-7g promoter was isolated by PCR using the primers listed in supplementary material Table S1. After digestion of the PCR product with MluI and HindIII, the insert was cloned into the reporter vector pG3L (Promega), creating the expression vector pG3L-let7g-1.5K. After sequencing, pG3L-let7g-1.5K was used as template, and pG3L-let7g-1K or pG3L-let7g-0.5K was isolated by PCR. Site-directed mutagenesis of the OCT-1 binding-site in the let-7g promoter was carried out using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and named pG3L-let7g-mutant, in which pMIR-LOX-1-3UTR was used as a template. For reporter assays, the cells were transiently transfected with different sizes of wild-type or mutant reporter plasmid and treated with oxLDL using Lipofectamine 2000. pEfpG plasmids were co-transfected and acted as the internal control. The reporter assay was performed at 24 hours post-transfection using the Luciferase Assay System.

ChIP assay

ChIP assays were performed according to the manufacturer’s instructions (EZ-ChIPTM, Millipore). Briefly, 10⁶ cells with or without oxLDL treatment were fixed with 1% formaldehyde, washed with cold PBS and lysed in buffer. Nuclei were sonicated to shear DNA, and the lysates were pelleted and preclared. The protein–DNA complexes were incubated with 1 μg antibodies overnight and then incubated with protein G beads followed by elution in 1% SDS and 0.1 M NaHCO₃, and cross-links were reversed at 65°C. DNA recovered from samples containing OCT-1 antibody was compared with negative controls (mouse IgG) and positive controls (anti RNA-PoI antibody) provided by the manufacturer. DNA was subjected to PCR analysis after being recovered. The PCR primers are listed in supplementary material Table S1.

Intracellular Ca²⁺ concentration measurement

Intracellular Ca²⁺ was quantified by fluorescence with Fluo-4 AM. After treating with different concentrations of oxLDL for 15 minutes, the cells were washed with PBS and re-suspended in 1 ml PBS. The cells were incubated for 1 hour with reporter plasmid and let-7g mimic or treated with oxLDL using Lipofectamine 2000 (Invitrogen). pEGFP plasmids were co-transfected and acted as the internal control. The reporter assay was performed at 24 hours post-transfection using the Luciferase Assay System (Promega).

Let-7g in human serum

We enrolled 14 health subjects and 39 patients suffering from hypercholesterolemia (defined as the total cholesterol >240 mg/dl; n = 15), stroke without hypercholesterolemia (n = 16) and stroke with hypercholesterolemia (n = 8). All participants provided written informed consent and the study protocols and methods were approved by the local Institutional Review Board (IRB). Total RNA, which included miRNA in 400 μl of serum, was isolated using a MasterPureTM complete RNA purification kit according to the manufacturer’s protocol (Epicentre Technologies, Madison WI). Optical densities at 260 and 280 nm were measured in triplicate to estimate RNA concentration and quality. The serum levels of let-7g and microRNA-16 (miR-16) were determined using the TaqMan miRNA RT-PCR kit (Applied Biosystems) with an ABI 7900HT Fast Real-Time PCR system. The miR-16 level was used as an internal control. Data were analyzed using the sequence detection system software version 2.4 (ABI) with the automatic C_I for assigning baseline and 0.2 manual threshold for C_t determination. The fold changes of miRNAs were quantified using the 2^-ΔΔCt transformation. The 2^-ΔΔCt means 2^(-CtControl-CtTarget). The significance of the difference between the serum let-7g levels of control subjects and patients was determined using Student’s t-test. A two-tailed P-value less than 0.05 was considered statistically significant. All statistical calculations were performed using JMP software (version 9, SAS Institute Inc., Cary, NC).

Statistical analysis

Student’s t-test was used to compare all experimental results. A P-value less than 0.05 was considered significant.

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