

# Effects of silencing leukocyte-type 12/15-lipoxygenase using short interfering RNAs

Shu-Lian Li,<sup>1</sup> Roopashree S. Dwarakanath,<sup>1</sup> Qiangjun Cai, Linda Lanting, and Rama Natarajan<sup>2</sup>

Gonda Diabetes Research Center, Beckman Research Institute of City of Hope, Duarte, CA 91010

**Abstract** The leukocyte-type 12/15-lipoxygenase (12/15-LO) has been implicated in the pathogenesis of atherosclerosis, hypertension, and diabetes. 12/15-LO and its products are associated with LDL oxidation, cellular growth, migration, adhesion, and inflammatory gene expression in monocytes/macrophages, endothelial cells, and vascular smooth muscle cells (VSMCs). Our objective, therefore, was to develop novel expression vectors for short interfering RNAs (siRNAs) targeting 12/15-LO to evaluate its functional relevance in macrophages and VSMCs. We used a PCR-based approach to rapidly identify effective siRNA target sites on mouse 12/15-LO and initially tested their efficacy on a fusion construct of 12/15-LO cDNA and enhanced green fluorescent protein. We then cloned these U6 promoter+siRNA PCR products into plasmid vectors [short hairpin siRNAs (shRNAs)] to knockdown endogenous 12/15-LO expression in mouse macrophages and also rat and mouse VSMCs. Furthermore, the functional effects of shRNA-mediated 12/15-LO knockdown were noted by the reduced oxidant stress and chemokine [monocyte chemoattractant protein-1 (MCP-1)] expression in a differentiated mouse monocytic cell line as well as by the reduced cellular adhesion and fibronectin expression in VSMCs. Knocking down 12/15-LO expression also reduced the expression of inflammatory genes, MCP-1, vascular cell adhesion molecule-1, and interleukin-6 in VSMCs. Our results illustrate the functional relevance of 12/15-LO activation in macrophages and VSMCs and its relationship to oxidant stress and inflammation.—Li, S-L., R. S. Dwarakanath, Q. Cai, L. Lanting, and R. Natarajan. Effects of silencing leukocyte-type 12/15-lipoxygenase using short interfering RNAs. *J. Lipid Res.* 2005. 46: 220–229.

**Supplementary key words** vascular smooth muscle cells • monocytes • macrophages • atherosclerosis • RNA interference • inflammatory genes • monocyte chemoattractant protein-1 • arachidonic acid

Atherosclerosis is a multicellular disease that results from a concerted action of multiple inflammatory and proliferative cytokines, growth factors, oxidized low density lipoprotein (OxLDL), and related lipids secreted by

various cells in the circulation and the vessel wall (1, 2). Endothelial cells (2, 3), smooth muscle cells (3, 4), and macrophages (5) all can oxidatively modify LDL to the atherogenic OxLDL, which can then bind to scavenger receptors, leading to foam cell formation. OxLDL is capable of recruiting and retaining monocytes into developing lesions (6–8). It interacts with innate immune receptors and also affects inflammatory gene expression in the vasculature (9), leading to changes in cytokine and chemokine responses (8, 10). One of the possible ways in which OxLDL is generated is through the action of a family of non-heme iron-containing dioxygenases called lipoxygenases (LOs) that insert molecular oxygen into polyunsaturated fatty acids such as arachidonic and linoleic acids (11, 12). These LOs can be activated when growth factors and cytokines bind to their receptors and activate phospholipases (13). They are classified as 5-, 8-, 12-, and 15-LOs on the basis of their ability to insert molecular oxygen at the corresponding carbon atom of arachidonic acid (11–14). 12-LO action can form oxidized lipids such as 12-hydroxy-eicosatetraenoic acid [12(S)-HETE] (11–13). Leukocyte or macrophage-type 12-LO has been cloned from porcine and mouse leukocytes (15, 16) and rat brain (17). Human and rabbit 15-LOs and leukocyte 12-LO and 15-LOs are highly homologous and are expressed in many cell types (12, 14). They are classified as 12/15-LO because of their high homology and the fact that they can form both 12-HETE and 15-HETE from arachidonic acid via their hydroperoxy precursors and mainly 9- and 13-hydroperoxy-octadecadienoic acids from linoleic acid (11–14).

Recent studies have indicated that leukocyte-type 12/15-LO plays a key role in the pathogenesis of atherosclerosis and diabetic vascular disease as a result of its ability to oxidize LDL as well as the inflammatory, chemotactic, and growth-promoting properties of its metabolites (18, 19). High levels of 12/15-LO are expressed in macrophages of atherosclerotic lesions (20–22). Convincing evidence that leukocyte-type 12/15-LO plays a key role in the pathogen-

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<sup>1</sup> S-L. Li and R. S. Dwarakanath contributed equally to this work.

<sup>2</sup> To whom correspondence should be addressed.

e-mail: rnatarajan@coh.org

esis of atherosclerosis comes from in vivo studies with 12/15-LO-deficient (12/15-LO<sup>-/-</sup>) and transgenic mice (23–25). Thus, there was a significant reduction in atherosclerotic lesions in apolipoprotein E-deficient (apoE<sup>-/-</sup>) mice that were cross-bred with 12/15-LO<sup>-/-</sup> mice (23), and this was attributed to reduced LDL oxidation and lipid peroxidation (18). On the other hand, 12/15-LO transgenic mice had evidence of increased endothelial cell activation as well as greater atherosclerotic lesions compared with control mice (24, 25). These data demonstrate the in vivo role of 12/15-LO and indicate that targeted inhibition of 12/15 LO may decrease the progression of atherosclerosis. 12/15-LO in macrophages was shown to colocalize with polymerized actin of emerging filopodia, promoting efficient phagocytosis of apoptotic cells. Phagocytosing macrophages derived from apoE<sup>-/-</sup>/12/15-LO<sup>-/-</sup> mice, on the other hand, had greatly reduced actin polymerization (26). The products of 12/15-LO are also agonists for the nuclear peroxisome proliferator-activated receptor- $\gamma$  and lead to subsequent increases in scavenger receptor CD36 expression (27). The proinflammatory role of macrophage 12/15-LO was further demonstrated by studies showing decreased lipopolysaccharide-induced interleukin-12 (IL-12) production in 12/15-LO<sup>-/-</sup> macrophages associated with reduced atherosclerosis in a mouse model of hypercholesterolemia (28).

A leukocyte-type 12/15-LO is also present in vascular smooth muscle cells (VSMCs) and plays a major role in their migration, matrix production, hypertrophy, and inflammatory responses and in mediating growth factor responses (29–31). We also recently demonstrated that VSMCs derived from 12/15-LO<sup>-/-</sup> mice had reduced protein and DNA syntheses, fibronectin levels, Platelet-derived growth factor (PDGF)-induced migration, and activation of p38 mitogen-activated protein kinase relative to genetic control mice (32). Furthermore, ribozymes targeting 12/15-LO could attenuate growth factor effects in VSMCs (33). 12/15-LO products could also increase nuclear factor  $\kappa$ B (NF- $\kappa$ B) activation and increase the expression of the NF- $\kappa$ B-dependent inflammatory gene in VSMCs (34, 35). Thus, owing to the evidence that leukocyte-type 12/15-LO plays a key role in the pathology of atherosclerosis and diabetic complications, in this study we developed novel RNA interference (RNAi) techniques with expressed short interfering RNAs (siRNAs) to evaluate the functional relevance of this 12/15-LO in macrophages and VSMCs.

RNAi is a process by which double-stranded RNAs induce posttranscriptional gene silencing by degrading homologous transcripts (36, 37). The double-stranded RNAs are first broken down into 21–23 nucleotide double-stranded fragments known as siRNAs. siRNAs can be delivered into cells by transfection of the double-stranded siRNA oligonucleotides or by endogenous expression using plasmid vectors. In mammalian systems, several groups have recently demonstrated that siRNAs can be transcribed in vivo using RNA Polymerase III (Pol III) promoters such as U6 or H1 (38–41). These short hairpin siRNAs (shRNAs) can be transfected into cells as plasmid vectors or by using viral vectors (38–42). We have used a novel PCR-based

strategy (35, 43) to rapidly identify effective siRNA target sites on mouse 12/15-LO and initially tested their efficacy on a fusion construct of 12/15-LO cDNA and enhanced green fluorescent protein (EGFP). We then cloned these U6 promoter+siRNA PCR products into plasmid vectors to knockdown endogenous and exogenous 12/15-LO expression in mouse macrophages and also rat and mouse VSMCs. These plasmid vectors were also designed to simultaneously express both EGFP and 12/15-LO shRNA to monitor transfection efficiency as well as selection. Furthermore, we have also shown the functional effects of shRNA-mediated 12/15-LO silencing by demonstrating reduced oxidant stress and chemokine expression in a differentiated mouse monocytic cell line as well as reduced cellular migration, adhesion, fibronectin, and inflammatory gene expression in VSMCs. Our results illustrate the functional relevance of 12/15-LO activation in macrophages and VSMCs and its relationship to oxidant stress and inflammation.

## MATERIALS AND METHODS

### Materials

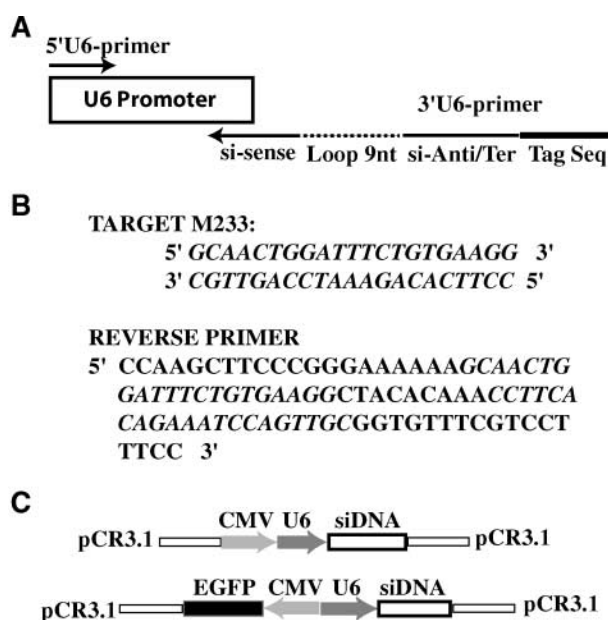
Oligonucleotides were obtained from the City of Hope's DNA synthesis facility and from Integrated DNA Technologies (Coraville, IA). Restriction and modification enzymes were from New England Biolabs (Beverly, MA). Taq Gold was obtained from Applied Biosystems (Foster City, CA). Lipofectamine 2000 and cell culture reagents were from Invitrogen (Carlsbad, CA). Fluorescently labeled 2',7'-bis-(2-carboxyethyl)-5-(and 6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) and phorbol 12-myristate 13-acetate (PMA) were from Sigma-Aldrich Chemicals (St. Louis, MO).

### Cell culture

Primary rat VSMCs (RVSMCs) and mouse VSMCs (MVSMCs) were isolated as described (32) and cultured in DMEM/F-12 medium (Invitrogen) containing 10% fetal calf serum (FCS), 2 mmol/l glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. HEK 293 cells (American Type Culture Collection) and the WEHI78/24 mouse monocytic cell line (WEHI; a gift from Dr. J. A. Berliner, University of California, Los Angeles) were maintained in DMEM (Irvine Scientific, Santa Ana, CA) containing 25 mmol/l glucose, 10% FCS, 2 mmol/l glutamine, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. The human monocytic cell line THP-1 was obtained from the American Type Culture Collection and grown in RPMI 1640 medium with 10% heat-inactivated FBS and 50  $\mu$ M  $\beta$ -mercaptoethanol.

### Transfection

RVSMCs, MVSMCs, and HEK 293 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. After 48 h of transfection, cells were harvested and used for Western blots. For evaluation of mRNA by RT-PCR, RVSMCs were selected with 600  $\mu$ g/ml G418 (Geneticin; Invitrogen) for 1 week, serum starved for 2 days in depletion medium (DMEM/F-12 medium plus 0.2% BSA), and then stimulated with 0.1  $\mu$ M Angiotensin II (AngII) for 4 h before harvesting the cells. For WEHI cells, the day before transfection, 0.1  $\mu$ g/ml PMA was added to induce differentiation. Cells were transfected by electroporation. Briefly,  $5 \times 10^6$  cells in 0.2 ml of cold  $1 \times$  PBS were mixed with 10  $\mu$ g of DNA in a 0.2 cm gap electroporation cuvette and electroporated in a Gene Pulser apparatus (Bio-Rad, Hercules, CA) set at 960  $\mu$ F and 170 V before being transferred to 2 ml of fresh medium on a six-well plate. After



**Fig. 1.** A: Schematic representation of the PCR strategy used to generate U6+shRNA (short hairpin RNA) expression cassettes. The forward primer is universal and complementary to the 5' region of the U6 promoter. The final product contains the U6 promoter followed by the sense and antisense (Anti) elements separated by a hairpin. B: An example of the design of the reverse primers for target M233. C: Expression vectors for introducing 12/15-lipoxygenase (12/15-LO) short interfering RNAs (siRNAs) into cultured cells. The first vector contains the U6+shRNA cassette alone, whereas the second one also contains a CMV (cytomegalovirus) +EGFP (enhanced green fluorescent protein) cassette.

48 h, 600  $\mu$ g/ml G418 was added to the cells and selected for 10 days. The selected cells were used for RT-PCR and Western blots.

### Construction of 12/15-LO shRNAs

We designed shRNAs to mouse leukocyte 12/15-LO by testing accessibility sites using a rapid PCR-based approach (35, 43) that generates sense and antisense siRNAs separated by a hairpin loop downstream of the U6 promoter (as shown in Fig. 1A, B). The plasmid pTZ/U6+1 was used as a template. The following universal U6 forward primer was used in all reactions: 5'-GGAA-GATCTGGATCCAAGGTCGGGCAGG-3'. The 3' primers, which contain a six nucleotide termination sequence (sense), a nine nucleotide loop (antisense), and a sequence complementary to the U6 promoter, are listed in Table 1. All PCR procedures were carried out for 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for 30 cycles using Taq Gold.

These PCR products generated as siDNAs were then cloned into the pCR3.1 vector (Invitrogen) to generate a series of shRNA expression vectors (Fig. 1C). These are termed pCR3.1/Mouse 233, pCR3.1/Mouse 826, pCR3.1/Porcine 594, and pCR3.1/Scrambled (Fig. 1C, top). A second generation of shRNA expression vectors was designed to incorporate an EGFP expression cassette and simultaneously express the 12/15-LO shRNA and EGFP, to check transfection efficiency and facilitate cell sorting (Fig. 1C, bottom). This was carried out by inserting the EGFP gene (from pEGFP-N1; Clontech, Palo Alto, CA) into the pCR3.1/shRNA vector. The vector pEGFP-N1 was digested at *AseI* and *AflII* sites followed by ligation of the Cytomegalovirus (CMV) promoter and EGFP fragments to *HincII* and *SacI* sites of pCR3.1-siRNA vector. These vectors are termed pCR3.1/EGFP-shRNA. All experiments used this shRNA-EGFP construct, except those shown in Figs. 2 and 5. In addition, we also constructed an expression cassette encoding a nonspecific scrambled shRNA sequence as an important control (Table 1).

### Preparation of a mouse 12/15-LO-EGFP fusion construct as a target substrate

The pcDNA1/12/15-LO vector containing the mouse 12/15-LO cDNA was obtained from Dr. Colin Funk (University of Pennsylvania). The 12/15-LO cDNA was digested at the *NdeI* site at the 3' end, and the end was flushed with Klenow to regenerate the last two nucleotides of the 12/15-LO sequence. The plasmid was then digested with *SpeI* to release the 12/15-LO cDNA. The plasmid pEGFP-N1 (Clontech) was digested with *BamHI*, and the ends were flushed with Klenow and then digested with *NheI* to generate compatible ends for cloning in the 12/15-LO fragment. The resulting vector pEGFP-N1/12/15-LO has the 12/15-LO cDNA fused to EGFP at the 3' end of the LO coding region. This was used to express exogenous 12/15-LO in cells.

### Immunoblotting

Cell lysates were electrophoresed on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Bio-Rad). Immunoblotting was performed as described previously (44). Detection of 12/15-LO was carried out using a polyclonal antibody raised to a peptide derived from 12/15-LO (31). Immunoblots were scanned using a model GS-800 densitometer, and protein bands were quantified with Quantity One software (Bio-Rad).

### RT-PCR

Total RNA from transfected cells was isolated with TRIzol Reagent (Invitrogen), and 1  $\mu$ g of RNA was used for the RT reaction using the Gene Amp RNA PCR kit (Applied Biosystems). Gene-specific primers (Table 2) were paired with Quantum RNA 18S Classic Internal Standards for the relative RT-PCRs performed as described (44). PCR products were fractionated on 2% agarose gels, photographed using Alpha Imager 2000, and quantitated with Quantity One software (Bio-Rad).

TABLE 1. Sequences of reverse primers used to generate siRNAs

Name	Sequence (5'→3')
Mouse 233	CCAAGCTTCCCGGGAAAAAGCAACTGGATTCTGTGAAGGCTACACAAACCTTCACAGAAATCC- AGTTTCGGGTGTTTCGTCCTTTCC
Mouse 826	CCAAGCTTCCCGGGAAAAAGCAAGCGGATTCTTCCTTCTGCTACACAAACAGAAAGGAAGAAATC- CGCTTCGGGTGTTTCGTCCTTTCC
Porcine 594	CCAAGCTTCCCGGGAAAAAGAACAGCGCTGGATAGCTTCAACTACACAAATGAAGCTATCCAGG- CTGTTTCGGGTGTTTCGTCCTTTCC
Scrambled	CCAAGCTTCCCGGGAAAAAGGATATATCCCGAACTAGACACTACACAAATGTCTAGTTCCGGAT- ATATCCGGGTGTTTCGTCCTTTCC

siRNAs, short interfering RNAs. Reverse primers used in the PCR procedures to generate the siRNAs to mouse or porcine 12/15-lipoxygenase. Sequences in boldface and italics are sense and antisense siRNA coding regions.



TABLE 2. Gene-specific primers used in the RT-PCR

Animal	mRNA	Forward Primer (5'→3')	Reverse Primer (5'→3')
Rat	Fibronectin	GCAAGCCTGAACCTGAAGAGACC	CCTGGTGTCTGATCATTGCATC
Rat	Monocyte chemoattractant protein-1	CCTGCTGCTACTCATTAC	TCTCACTTGGTTCTGGTCC
Rat	Interleukin-6	GGATACCACCCACAACAGAC	GAAACGGAAGCTCCAGAAGAC
Rat	Vascular cell adhesion molecule-1	ACACCTCCCCCAAGAATAC	CCAGATTCACTCCTTCACAC
Mouse	JE/monocyte chemoattractant protein-1	CAGCAAGATGATCCCAATGAG	TTGAGGTGTTGTGGAAAAG

Sequences of the primer pairs used to amplify the indicated mRNAs of interest in transfected rat or mouse cells.

### 12(S)-HETE assay

RVSMCs were seeded onto six-well plates and transfected with 3  $\mu$ g of pCR3.1/Scrambled or pCR3.1/M233+M826 shRNA plasmids per well using Lipofectamine 2000. After 48 h of transfection, the cells were washed twice with PBS and subsequently incubated in depletion medium for another 48 h. Before harvesting the supernatants, 0.1  $\mu$ M AngII was added for 30 min. Levels of the 12/15-LO product, 12(S)-HETE, in the supernatants were assayed with a specific Enzyme Immunoassay kit (Assay Designs, Inc., Ann Arbor, MI). One hundred microliters of supernatant was used per assay.

### Dihydroethidium staining of WEHI cells to evaluate superoxide production

To detect reactive oxygen species (superoxide), WEHI cells were stained with dihydroethidium (DHE; Molecular Probes, Eugene, OR) as described (32). Images were captured using Viewfinder and processed using Adobe Photoshop 7.0.

### Binding assay with monocytic cells

THP-1 monocytic cells were preincubated in RPMI 1640 containing 2% FBS and 5  $\mu$ g/ml of the fluorescent dye BCECF/AM at 37°C for 30 min in foil-covered tubes. Fluorescently labeled cells were washed twice to remove unincorporated dye and then resuspended in medium containing 0.2% BSA. Loaded monocytic cells ( $5 \times 10^4$ ) were added to each well of RVSMCs that had been transfected with the empty vector, the 12/15-LO specific shRNAs, or the scrambled control and incubated at 37°C. After 30 min, unbound monocyte suspensions were withdrawn, and the VSMC layers with attached monocytes were gently washed twice with DMEM and lysed with 0.2 ml of 0.1% Triton X-100 in 0.1 M Tris buffer/well (pH 8.0). Fluorescence was measured with the fMax fluorescence microplate reader (Molecular Devices Corp., Sunnyvale, CA) (excitation, 485 nm; emission, 535 nm). Data were analyzed with SoftMax Pro Version 1.3.1. The number of adherent cells per well was expressed as percentage fluorescence of the control group.

### Data analyses

Values are expressed as means  $\pm$  SEM of multiple experiments. ANOVA with Dunnett's post tests was used for multiple groups, and Student's *t*-test was used for comparisons between two groups using PRISM software (Graph Pad, San Diego, CA).

## RESULTS

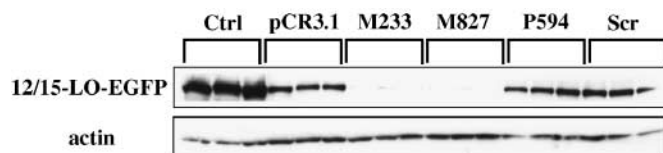
### Construction of U6 promoter+shRNA cassettes targeting 12/15-LO using the PCR-based method

The PCR approach to evaluate optimal shRNA accessibility sites was adapted from recent reports (35, 43). The PCR products included the U6 promoter sequence, the sense and antisense sequence with a nine nucleotide loop

between them, a terminator sequence, and the staffer tag sequence at the 3' end of the product (Fig. 1A, B). We identified two good target sites from the coding regions of mouse leukocyte 12/15-LO cDNA (GenBank accession number 467216) beginning at nucleotides 233 and 826, which were also compatible with rat 12/15-LO sequences (GenBank accession number 205212). We also identified a site on the porcine sequence beginning at nucleotide 594 (GenBank accession number 164536). As recommended for optimal siRNA efficacy, these sequences had  $\sim$ 50% GC content and were more than 100 bp downstream of the transcription start site. We constructed U6+shRNA expression cassettes to all of these sites. In addition, we also constructed an expression cassette encoding a nonspecific scrambled shRNA sequence as an important control. As described in Materials and Methods, the PCR products were incorporated into the pCR3.1 vector to generate plasmids pCR3.1/Mouse 233 (M233), pCR3.1/Mouse 826 (M826), pCR3.1/Porcine 594 (P594), and pCR3.1/Scrambled. The mouse 12/15-LO shRNAs were designed to work against both mouse and rat 12/15-LOs but not porcine 12/15-LO. We then generated second-generation expression vectors containing a second cassette of CMV promoter driving EGFP expression. These vectors simultaneously express both EGFP and the shRNA of interest (Fig. 1C). EGFP expression helps to monitor the transfection efficiency and to sort out pure populations of VSMCs expressing the shRNA using flow cytometry. In addition, cells were selected for G418 resistance using the selectable marker.

### 12/15-LO-EGFP protein is downregulated by cotransfection of HEK 293 cells with specific shRNAs

To evaluate the efficiency of these shRNA expression cassettes in reducing levels of 12/15-LO protein, we first used a target substrate construct in which mouse 12/15-LO is expressed as a fusion with EGFP. In cotransfection of HEK 293 cells, this target substrate plasmid was used in combination with pCR3.1/12/15-LO shRNA or pCR3.1/Scrambled shRNA separately. At 48 h after transfection, cells were analyzed for expression of the LO-EGFP fusion protein by immunoblotting with a 12/15-LO polyclonal antibody. As shown in Fig. 2, we noticed specific knock-down of mouse 12/15-LO-EGFP protein in cotransfections with the mouse M233 and M826 shRNAs ( $>95\%$  reduced protein compared with controls without shRNA). However, with porcine P594 and scrambled constructs, we saw no specific inhibition of mouse 12/15-LO-EGFP, as



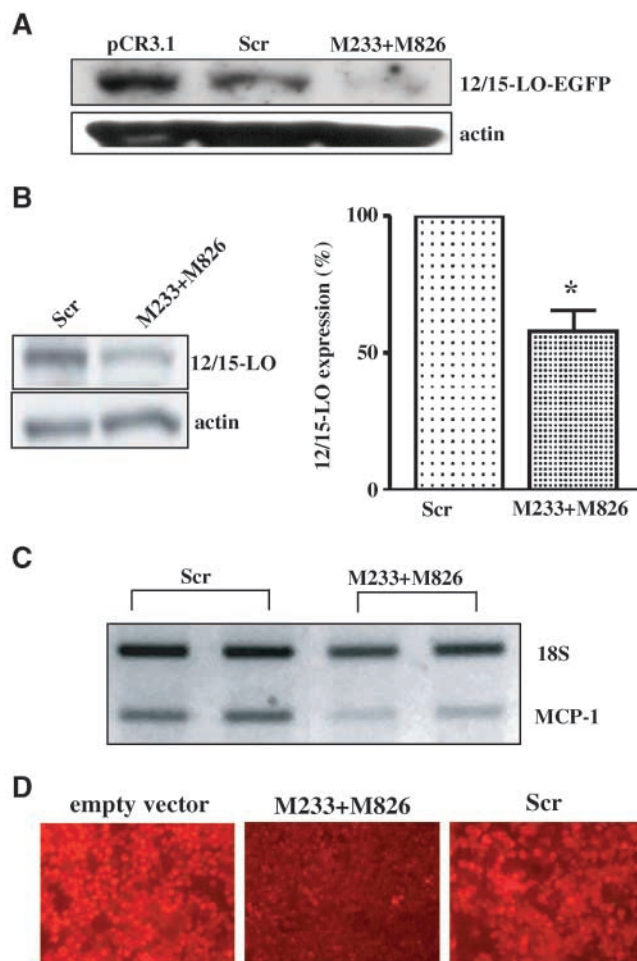
**Fig. 2.** 12/15-LO-EGFP fusion protein expression is blocked by M233 and M826 in HEK 293 cells. Cotransfections were performed on 24-well plates as described in Materials and Methods. The plasmid amounts used for transfection were 300 ng of pEGFP-N1/12/15-LO and 500 ng of pCR3.1/shRNA. Western blots of transfected cell lysates were probed with a 12/15-LO polyclonal antibody (top). Blots were then stripped and reprobed with an antibody to  $\beta$ -actin as an internal control for protein loading (bottom). Scr, scrambled.

anticipated, and these constructs produced results similar to the pCR3.1 empty vector.

**The 12/15-LO shRNAs, M233 and M826, can knockdown endogenous 12/15-LO and also reduce monocyte chemoattractant protein-1 mRNA expression and oxidant stress in differentiated WEHI murine monocytic cells**

Mouse macrophages exhibit high levels of 12/15-LO that are associated with atherogenic properties. Therefore, to determine whether these shRNAs could specifically knockdown endogenous 12/15-LOs in macrophages, we transfected differentiated murine WEHI monocytic cells with the mouse 12/15-LO shRNA plasmids followed by selection with G418. In cotransfections of WEHI cells using pEGFP-N1/12/15-LO target substrate and the shRNAs pCR3.1/EGFP-M233 plus pCR3.1/EGFP-M826, we saw a 60% decrease in levels of the fusion protein 12/15-LO-EGFP compared with cells cotransfected with pCR3.1/EGFP-Scrambled shRNA and a 75% decrease compared with pCR3.1/EGFP-M233 and -M826 versus pCR3.1/EGFP-Scrambled shRNA (Fig. 3A). We then analyzed endogenous 12/15-LO expression in these WEHI cells transfected with pCR3.1/EGFP-M233 and -M826 versus pCR3.1/EGFP-Scrambled shRNA. As revealed by Western blot (Fig. 3B, left) and bar graph quantitation (Fig. 3B, right), we noticed a significant decrease ( $P < 0.01$ ) in 12/15-LO protein levels in cells transfected with a combination of specific shRNAs M233 and M826. We observed that although M233 and M826 were each individually effective (data not shown), optimum gene knockdown was obtained by a combination of the two shRNAs.

As a result of reduced 12/15-LO levels, we noticed two important functional consequences in these cells. First, cells transfected with M233 and M826 also exhibited a 50% decrease in levels of the key inflammatory and chemotactic gene monocyte chemoattractant protein-1 (MCP-1) compared with cells transfected with scrambled controls (Fig. 3C). Second, we measured oxidant stress in these cells by comparing the changes in fluorescence resulting from the oxidation of DHE. DHE can enter the cell and be oxidized by reactive oxygen species such as superoxide to yield ethidium, which binds to DNA to produce bright red fluorescence. We observed a marked decrease in DHE fluorescence in WEHI cells transfected with M233 and M826 compared with controls (Fig. 3D). This decrease in fluorescence is suggestive of reduced reactive oxygen species production



**Fig. 3.** Expression of 12/15-LO shRNA in differentiated WEHI cells. The mouse 12/15-LO shRNAs M233 and M826 were transfected into differentiated WEHI cells, followed by selection with G418 as described in Materials and Methods. A: Western blot showing specific knockdown of transfected 12/15-LO-EGFP fusion protein by M233 and M826. B: Representative Western blot showing knockdown of endogenous 12/15-LO in WEHI cells by M233 and M826 (left). The bar graph at right shows that this knockdown of endogenous 12/15-LO is statistically significant (\*  $P < 0.01$ ;  $n = 3$ ). C: RT-PCR demonstrates knockdown of monocyte chemoattractant protein-1 (MCP-1) gene expression in WEHI cells transfected with M233+M826 compared with cells transfected with scrambled shRNA (Scr). Results shown are representative of two separate transfections with scrambled and M233+M826 run in duplicate. D: Measurement of superoxide production as determined by dihydroethidium staining of cells transfected with empty vector, a combination of M233 and M826, or scrambled shRNA. WEHI staining was performed using 5  $\mu$ M DHE at 37°C for 10 min followed by washing in a centrifuge. Results are expressed as mean  $\pm$  SEM.

and oxidant stress within these cells. This is supportive of data showing that 12/15-LO in macrophages is associated with oxidant stress and LDL oxidation (18, 23).

**Downregulation of both transfected 12/15-LO-EGFP and endogenous 12/15-LO proteins by specific shRNAs in MVSMCs and RVSMCs**

We next examined the effects of the 12/15-LO shRNAs in MVSMCs. VSMCs normally express lower levels of 12/

15-LO than macrophages. We carried out cotransfection experiments by introducing pEGFP-N1/12/15-LO and one of the shRNA plasmids per transfection set. In these cells, we saw a specific reduction in expression of the 12/15-LO-EGFP fusion protein in cells transfected with M233 and M826 [15% and 16% protein remaining compared with control untransfected and scrambled (100%) and pCR3.1 empty vector and P594 (73% each), respectively; Fig. 4A, top]. In addition, we also noticed a downregulation of the endogenous 72 kDa 12/15-LO protein using M233 and M826 (30% protein remaining compared with control; Fig. 4A, bottom). Similar results were obtained when these experiments were performed in RVSMCs (i.e., a specific and almost complete knockdown of both the transfected 12/15-LO-EGFP fusion protein and the endogenous 12/15-LO protein; Fig. 4B). The bar graph in Fig. 4C demonstrates that M233 and M826 can significantly knockdown endogenous 12/15-LO in RVSMCs. In the RVSMCs, this knockdown of 12/15-LO was also associ-

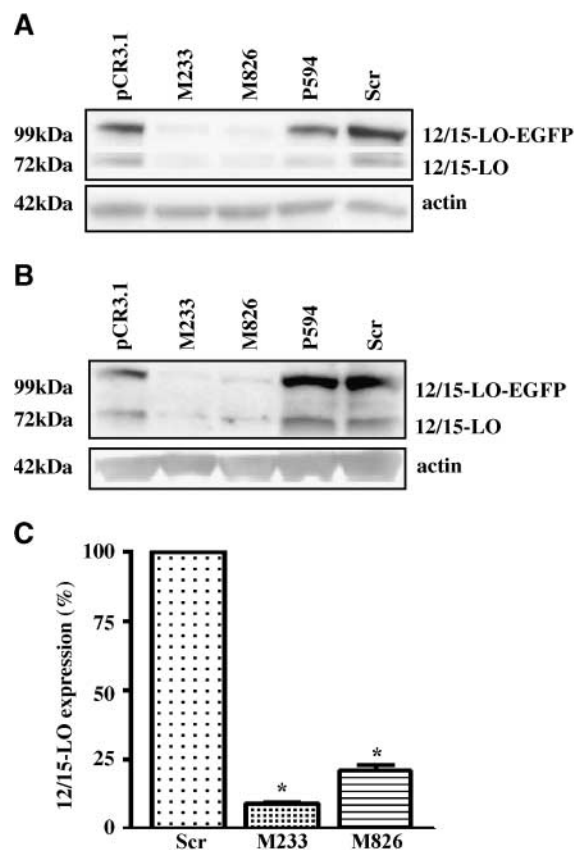
ated with a nearly 30% reduction in the production of the 12/15-LO product, 12(S)-HETE, as measured by a specific enzyme immunoassay (pCR3.1/Scrambled,  $859.7 \pm 13.6$  pg/100  $\mu$ l versus the shRNA pCR3.1/M233+M826,  $626.3\text{--}31.3$  pg/100  $\mu$ l;  $P < 0.002$ ).

#### Direct immunofluorescence reveals specific knockdown of the 12/15-LO-EGFP fusion protein in MVSMCs

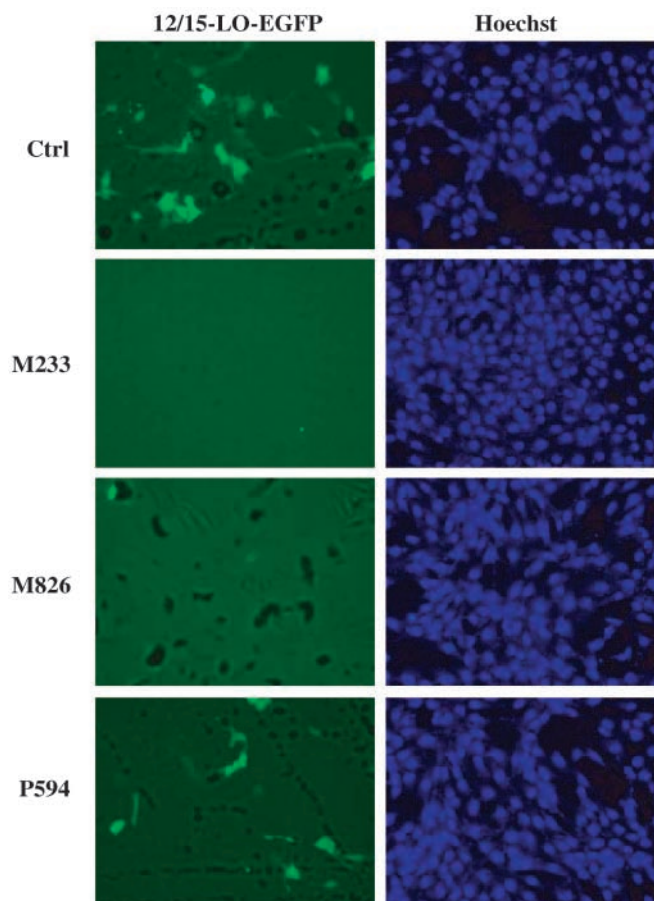
To directly visualize the effect of the shRNAs, we cotransfected MVSMCs with the plasmid 12/15-LO-EGFP and the shRNA vectors. Analysis of the transfected cells with a fluorescence microscope (FITC filter) demonstrated specific inhibition of 12/15-LO-EGFP fusion protein expression in cells cotransfected with M233 or M826 shRNA but not P594 shRNA (Fig. 5).

#### Inflammatory genes stimulated by AngII are specifically downregulated by the 12/15-LO shRNAs M233 and M826 in RVSMCs

Because 12/15-LO has been implicated in inflammation and in the hypertrophic effects of AngII in VSMCs, we hypothesized that 12/15-LO may also mediate the AngII-induced expression of proinflammatory genes in VSMCs. We therefore examined the effects of 12/15-LO



**Fig. 4.** Western blots showing that 12/15-LO expression is reduced by M233 and M826 in mouse vascular smooth muscle cells (MVSMCs; A) and rat VSMCs (RVSMCs; B). Approximately  $4 \times 10^4$  cells were seeded onto 12-well plates, and the next day 500 ng of pEGFP-N1/12/15-LO and 1.6  $\mu$ g of pCR3.1/EGFP-shRNA were transfected into cells with Lipofectamine 2000 as described in Materials and Methods. The sizes of the fusion protein 12/15-LO-EGFP and the endogenous 12/15-LO are indicated. C: Bar graph quantitation demonstrating that the inhibitory effects of M233 or M826 on endogenous 12/15-LO levels in RVSMCs are significant relative to scrambled shRNA (Scr) (\*  $P < 0.001$ ;  $n = 5$ ). Results are expressed as mean  $\pm$  SEM.



**Fig. 5.** Fluorescent images of MVSMCs expressing the fusion protein 12/15-LO-EGFP to show that M233 and M826 (plasmids without EGFP) can specifically block mouse 12/15-LO but not P594. Ctrl, control.



shRNAs on AngII-induced proinflammatory gene expression. RVSMCs were transfected with plasmids containing the shRNA cassettes and selected with G418 at 600  $\mu\text{g}/\text{ml}$  for 1 week. The expression of three inflammatory cytokine mRNAs [MCP-1, IL-6, and vascular cell adhesion molecule-1 (VCAM-1)] after stimulation with AngII was analyzed by relative RT-PCR using 18S as an internal control. **Figure 6** shows the data from these experiments. All cells were treated with AngII. It is seen that AngII-induced MCP-1 gene expression was markedly blocked by a combination of pCR3.1/EGFP-M233 and pCR3.1/EGFP-M826 ( $\sim 50\%$  MCP-1 mRNA compared with untransfected con-

trol cells and cells transfected with either empty pCR3.1/EGFP vector or scrambled shRNA; Fig. 6A). In the case of IL-6 mRNA, we similarly noticed a specific knockdown using this M233+M826 combination (40% transcripts compared with cells transfected with empty vector pCR3.1, P594, or scrambled shRNA; Fig. 6B). This shRNA combination resulted in even greater knockdown of VCAM-1 mRNA (22% transcripts compared with control pCR3.1 and 30% relative to P594 or scrambled; Fig. 6C). The bar graph quantitation in Fig. 6D demonstrates that the inhibitory effects of the 12/15-LO shRNAs on the expression of these three inflammatory genes are statistically significant. These results demonstrate that blockade of 12/15-LO can attenuate the proinflammatory effects of AngII in VSMCs and support the notion that 12/15-LO and its products are associated with inflammation in the vessel wall.

#### Inhibition of fibronectin mRNA levels by 12/15-LO shRNAs in RVSMCs

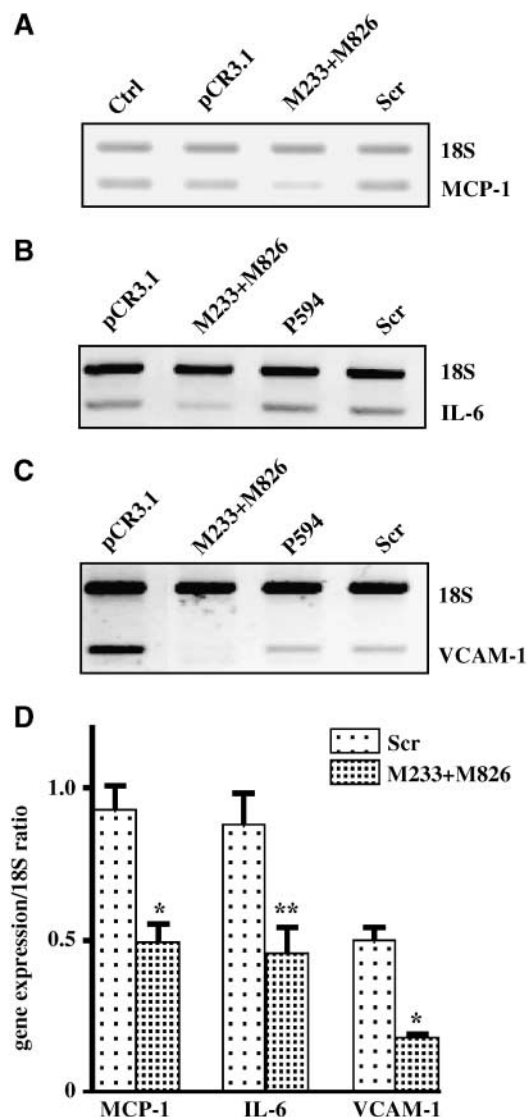
Because LO products have hypertrophic and matrix-inducing effects in VSMCs, we next examined whether the 12/15-LO shRNAs can block AngII-induced expression of the key matrix protein, fibronectin, in RVSMCs. Total RNAs were isolated from RVSMCs transfected with shRNA constructs and serum starved for 48 h before they were stimulated with AngII for 4 h. The cells were then analyzed for fibronectin mRNA levels by RT-PCR. As shown in **Fig. 7**, we noticed 2-fold lower levels of fibronectin mRNA in cells transfected with pCR3.1/EGFP-M233 or pCR3.1/EGFP-M826 compared with control pCR3.1 empty vector or those transfected with pCR3.1/EGFP-P594 or scrambled shRNA.

#### Involvement of 12/15-LO in the binding of monocytes to VSMCs

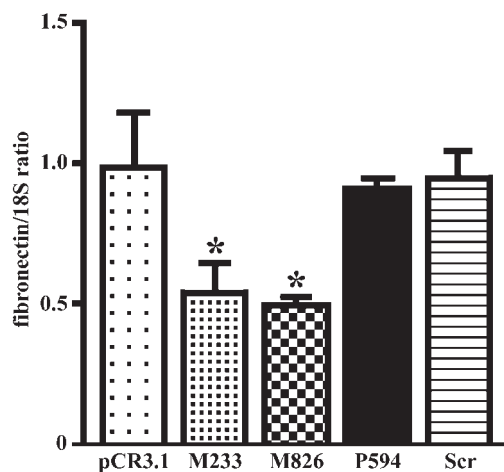
We recently showed that growth factors such as AngII can increase the binding of monocytes to VSMCs and that this could be a potential mechanism for monocyte retention in the vessel wall in the pathogenesis of atherosclerosis (45). Furthermore, we also noted that LO products such as 12(S)-HETE could also increase monocyte binding to VSMCs (45). To confirm the role of endogenous 12/15-LO as a key enzyme regulating monocyte binding, siRNA technology was used to knockdown endogenous 12/15-LO. As shown in **Fig. 8**, RVSMCs transfected with vector expressing M233 shRNA showed significantly decreased monocyte binding (40%;  $P < 0.01$ ) compared with cells transfected with pCR3.1 empty vector. M826 also significantly reduced monocyte binding ( $P < 0.05$ ), although less effectively than M233. The scrambled nonspecific shRNA had no effect on binding. These results suggest that 12/15-LO is an important mediator of VSMC/monocyte interactions.

#### DISCUSSION

12/15-LO and its oxidized lipid products have physiological and pathological effects, including cell growth,



**Fig. 6.** RT-PCR data showing that M233 and M826 shRNAs, but not P594 or scrambled shRNA (Scr), can inhibit Angiotensin II (AngII)-induced inflammatory gene expression in RVSMCs. A: MCP-1 mRNA level. B: Interleukin-6 (IL-6) mRNA level. C: Vascular cell adhesion molecule-1 (VCAM-1) mRNA level. D: Bar graph quantitation of gene expression normalized to 18S levels. The data show that M233+M826 significantly reduced inflammatory gene expression. Ctrl, control. \*  $P < 0.001$ ;  $n = 3$  for MCP-1. \*\*  $P < 0.01$ ;  $n = 3$  for IL-6. \*  $P < 0.001$ ;  $n = 3$  for VCAM-1. Results are expressed as mean  $\pm$  SEM.

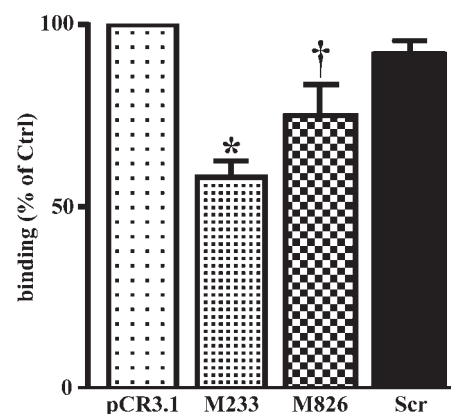


**Fig. 7.** Fibronectin mRNA levels in RVSMCs stimulated with AngII after transfection with shRNA expression vectors as indicated. Results shown are means  $\pm$  SEM from three experiments. \*  $P < 0.01$ ;  $n = 3$ . Fibronectin mRNA levels were quantitated by relative RT-PCR with 18S as an internal control. Scr, scrambled shRNA.

chemotaxis, adhesion, and inflammatory gene expression (19, 31–35). Furthermore, studies with 12/15-LO<sup>-/-</sup> mice and transgenic mice provide *in vivo* support for the role of 12/15-LO in the pathogenesis of atherosclerosis (18, 23–25). Evidence also suggests that high glucose culture mimicking diabetic conditions can increase 12/15-LO activity and expression in VSMCs and endothelial cells (19, 29), whereas animal models of diabetes show evidence of increased 12/15-LO (21, 46). These data suggest that 12/15-LO is also involved in the pathogenesis of diabetic vascular disease. Therefore, in this study, to develop modalities to block 12/15-LO, we developed a new generation of U6 promoter-expressed shRNAs to determine the functional effects of silencing 12/15-LO in both macrophages and VSMCs.

One limitation in the use of siRNAs as a therapeutic reagent is that, until recently, their delivery to target cells was accomplished by the use of synthetic, duplexed RNAs delivered exogenously to the cells. However, RNAi was recently induced by transfecting plasmids that “express” siRNAs within cells (38–41). These methods use plasmids with a sequence encoding a small hairpin RNA under the control of an RNA Pol III promoter. The U6 and H1 Pol III promoters were chosen because Pol III initiates synthesis at a defined distance from these promoters and termination occurs when a string of four to five uridines is encountered. When transfected into mammalian cells, these siRNA expression vectors (shRNAs) can reduce the levels of both exogenous and endogenous gene products. These significant findings have created new opportunities for expression of siRNAs and stable silencing of genes in mammalian cells. However, poor transfection efficiencies can limit the usefulness of shRNAs in cells such as macrophages and VSMCs.

Choosing the best accessible sites using rapid methods is critical to the success of siRNA efficacy. Target site accessibilities for siRNA pairing have been tested with antisense




**Fig. 8.** Binding of THP-1 monocytes to RVSMCs transfected with shRNA expression vectors shows reduced binding to cells expressing M233 or M826, but not scrambled shRNA (Scr) or control (Ctrl) pCR3.1. Results shown are means  $\pm$  SEM from three experiments. \*  $P < 0.001$  (M233 versus pCR3.1). †  $P < 0.05$  (M826 versus pCR3.1).

oligonucleotides and ribozymes (38, 47). However, multiple plasmid transfections can be tedious. Hence, very recently, a rapid PCR method of screening optimal accessible sites and siRNA testing was devised to block HIV Rev expression (43). We have modified this method to demonstrate that the PCR products cloned into the pCR3.1 expression vector do indeed express the 12/15-LO siRNA in HEK 293 cells. We then proceeded to generate second-generation siRNA vectors that express both shRNAs and EGFP to directly test transfection efficiency. Using these vectors, we set up transfection conditions with cationic liposomes, electroporation, and selection markers and showed that these shRNAs can effectively knockdown transfected as well as endogenous 12/15-LO expression in the differentiated mouse macrophage cell line WEHI. We showed for the first time that this 12/15-LO knockdown was associated with marked reduction in the expression of the inflammatory gene, MCP-1, as well as reduced levels of oxidant stress in WEHI cells. We then proceeded to test the specific 12/15-LO shRNAs M233 and M826 in MVSMCs and RVSMCs. These shRNAs were designed to be effective against both rat and mouse 12/15-LO sequences. In these two VSMC primary cell cultures, we observed that the shRNAs could decrease endogenous 12/15-LO protein levels. Furthermore, this was associated with decreased levels of AngII-induced expression of three inflammatory genes, MCP-1, VCAM-1, and IL-6. This agrees with our recent findings of reduced JE/MCP-1 expression in MVSMCs derived from 12/15-LO knockout mice (34) and that VSMCs treated with the 12/15-LO product 13-hydroperoxy-octadecadienoic acid increased the transcription and expression of VCAM-1 and MCP-1 (34, 35).

AngII as well as the LO product, 12(S)-HETE, can induce the transcription of the key extracellular matrix protein fibronectin in VSMCs through activation of the transcription factor CREB (44). AngII-induced fibronectin gene expression was also reduced in VSMCs derived from



12/15-LO knockout mice (32). Results obtained in this study using 12/15-LO shRNAs further support this, because there was a greater than 2-fold reduction in fibronectin expression levels in AngII-treated RVSMCs. In a transgenic mouse model of 12/15-LO, Reilly et al. (25) showed that 12/15-LO activity directly mediates monocyte adhesion to endothelial cells. The LO product 12(S)-HETE can also directly induce the binding of monocytes to endothelial cells as well as VSMCs (25, 45). In the present study, we noted that the specific shRNAs M233 and M826 could significantly decrease monocyte adhesion to RVSMCs in this coculture system. This further demonstrates the role of 12/15-LO in mediating subendothelial monocyte retention as well as the functional utility of these shRNAs in vascular cells.

The use of siRNAs as therapeutic agents is being actively pursued in the field of gene therapy. Because 12/15-LO and its bioactive oxidized lipid metabolites have been implicated in the pathogenesis of diseases such as atherosclerosis, hypertension, and diabetes, 12/15-LO is an attractive target for therapeutic intervention and drug design. Our studies have amply demonstrated the efficacy of shRNAs in knocking down the expression and functional effects of 12/15-LO. Furthermore, the methods we have used can be adapted to other pathological targets in vascular and blood cells. Therefore, it is attractive to speculate that these shRNAs may provide a novel approach to combat cardiovascular diseases resulting from the activation of 12/15-LO and related pathways. 

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