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# Essential role of the low density lipoprotein receptor-related protein in vascular smooth muscle cell migration

Yonghe Li[a,*], Wenyan Lu[a], Guojun Bu[a,b]

[a] *Department of Pediatrics, Washington University School of Medicine and St. Louis Children's Hospital, St. Louis, MO 63110, USA*
[b] *Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, USA*

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**Abstract The low density lipoprotein receptor-related protein (LRP) is a multifunctional cell surface receptor highly expressed in human aortic smooth muscle cells. In the present study, we used the short interfering RNA (siRNA) technique to explore the role of LRP in smooth muscle cell migration. We identified an LRP-specific siRNA that selective silences LRP expression in human aortic smooth muscle cells. As a consequence, LRP-mediated ligand degradation was significantly reduced. More important, we found that platelet-derived growth factor-dependent cell migration was inhibited in cells transfected with LRP siRNA. These results demonstrate an important role of LRP in smooth muscle cell migration.**


*Key words:* Low density lipoprotein receptor-related protein; Short interfering RNA; Smooth muscle cell; Ligand degradation; Cell migration

## 1. Introduction

The low density lipoprotein receptor (LDLR)-related protein (LRP) is a large endocytic receptor that belongs to the emerging LDLR family. LRP binds and endocytoses over 30 structurally and functionally distinct ligands including apolipoprotein E/lipoproteins, proteinases, proteinase–inhibitor complexes, and extracellular matrix proteins [1,2]. Ligand interaction with LRP can be antagonized by a 39-kDa receptor-associated protein (RAP), a molecular chaperone that assists the folding and trafficking of LRP [3]. Increasing evidence has shown that LRP plays important roles in lipoprotein remnant catabolism, protease regulation, cell migration, neurotransmission, and signal transduction [1].

Smooth muscle cell proliferation and migration are essential features of vasculogenesis and blood vessel maturation and clearly play roles in the pathophysiology of several prominent cardiovascular disease states, such as atherosclerosis and restenosis [4,5]. LRP is present in macrophages and vascular smooth muscle cells from atherosclerotic lesions and from normal vessels [6–10]. Cellular uptake of aggregated low density lipoprotein by LRP may promote foam cell formation [11]. Internalization of apolipoprotein E, proteinase–inhibitor complexes, and α-defensin by LRP may alter vascular smooth muscle cell migration and contraction [12–15]. Furthermore, a compelling recent study has shown that inactivation of LRP in vascular smooth muscle cells in mice results in marked susceptibility to cholesterol-induced atherosclerosis [16].

Recent studies have revealed that RNA interference, a process of homology-dependent degradation of cognate mRNA by double-stranded RNA, is a powerful tool to inhibit gene expression [17]. This phenomenon is commonly present in fungi, plants, and nematodes. In mammalian cells, selective degradation of target mRNAs is achieved by transfection with short interfering (21 bp) duplex RNAs (siRNAs), leading to rapid and efficient degradation of the target [18,19]. Effective siRNA for members of the LDLR family, LRP in particular, has not been reported to date. To directly address the importance of LRP in smooth muscle cell migration, we designed several siRNAs against LRP and tested their effectiveness in suppressing LRP expression. We show that siRNA-mediated suppression of LRP expression results in a significant inhibition of LRP-mediated ligand degradation and smooth muscle cell migration.

## 2. Materials and methods

### 2.1. Reagents

Human α2-macroglobulin (α2M) was purified from human plasma and activated with methylamine (α2M*) as described [20]. Human recombinant RAP was expressed as a glutathione *S*-transferase fusion protein and was isolated as described previously [21]. Human two-chain urokinase plasminogen activator (uPA) was obtained by limited plasmin digestion of recombinant human single-chain uPA [22]. Platelet-derived growth factor-BB (PDGF-BB) was from Invitrogen. Recombinant human plasminogen activator inhibitor type 1 (PAI-1) was from American Diagnostica. Monoclonal antibody 8G1 against human LRP was from Research Diagnostics. Polyclonal anti-RAP antibody has been described before [23]. Monoclonal anti-actin antibody was from Sigma. Transwell cell culture chambers were from Costar. Carrier-free $Na^{125}I$ was purchased from Perkin-Elmer Life Sciences. Proteins were iodinated using the IODO-GEN method as described previously for RAP [24]. [$^{125}$I]uPA–PAI-1 complexes were prepared by the method described [25].

### 2.2. Cell culture and transfection

Human glioblastoma U87 cells were cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and maintained at 37°C in humidified air containing 5% $CO_2$. Human breast cancer MCF-7 cells were cultured in the same

*Corresponding author. Fax: (1)-314-286 2894.
*E-mail address:* li_yo@kids.wustl.edu (Y. Li).

*Abbreviations:* α2M, α2-macroglobulin; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; PAI-1, plasminogen activator inhibitor type 1; PDGF, platelet-derived growth factor; RAP, receptor-associated protein; siRNA, short interfering RNA; uPA, urokinase plasminogen activator

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conditions as for U87 cells, except that its medium contains no sodium pyruvate. Human aortic smooth muscle cells were purchased from Clonetics (San Diego, CA, USA) and were cultured under standard culture conditions according to the manufacturer's protocol.

Single-stranded, LRP-specific sense and antisense RNA oligonucleotides were synthesized by Ambion (Austin, TX, USA). Double-stranded RNA molecules were generated according to the manufacturer's instructions. For transfection, cells were trypsinized and seeded into six-well plates ($2.0\times10^5$ cells/well) without antibiotics. After 24 h, cells were transfected with siRNA using Lipofectamine2000 (Invitrogen) according to the manufacturer's specifications. siRNAs were used at a concentration of 120 nM in transfections. The final volume of culture medium was 2.0 ml/well. Cells were harvested for analysis 48 h after transfection.

### 2.3. Western blotting

U87, MCF-7, and human aortic smooth muscle cells were lysed with 0.5 ml of lysis buffer (phosphate-buffered saline containing 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Equal quantities of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions. Following transfer to Immobilon-P transfer membrane, successive incubations with anti-LRP antibody 8G1, anti-RAP antibody, or anti-actin antibody and horseradish peroxidase-conjugated secondary antibody were carried out for 60 min at room temperature. The immunoreactive proteins were then detected using the ECL system. Films showing immunoreactive bands were scanned by Kodak Digital Science DC120 zoom digital camera and analyzed with Kodak Digital Science1D image analysis software.

### 2.4. Ligand degradation

Ligand degradation was performed using the methods as described [24]. Briefly, $2\times10^5$ cells were seeded into 12-well dishes 1 day prior to assays. Pre-warmed assay buffer (minimal Eagle's medium containing 0.6% bovine serum albumin (BSA) with radioligand, 0.6 ml/well) was added to cell monolayers in the absence or presence of unlabeled 500 nM RAP, followed by incubation for 4 h at 37°C. Thereafter, the medium overlying the cell monolayers was removed and proteins were precipitated by addition of BSA to 10 mg/ml and trichloroacetic acid to 20%. Degradation of radioligand was defined as the appearance of radioactive fragments in the overlying medium that were soluble in 20% trichloroacetic acid. The protein concentration of each cell lysate was measured in parallel dishes that did not contain LRP ligands.

### 2.5. Cell migration assay

Cell migration assays were carried out in 6.5-mm Transwell chambers as described [12], with minor modifications. Twenty-four hours after siRNA transfection, human aortic smooth muscle cells were made quiescent for 24 h with SmGM-2 medium (Clonetics) and 0.4% fetal bovine serum. Cells were harvested, washed, and resuspended in the same medium, and placed in the upper compartment of the Transwell chambers ($5\times10^4$ cells in 100 μl). The lower compartment was filled with 600 μl SmGM-2, 0.4% fetal bovine serum, and 0.1% BSA, with or without 30 ng/ml PDGF-BB. After incubation for 4 h at 37°C, cells on the lower surface of the filter were fixed and stained, and eight random fields/filter were counted at ×200 magnification.

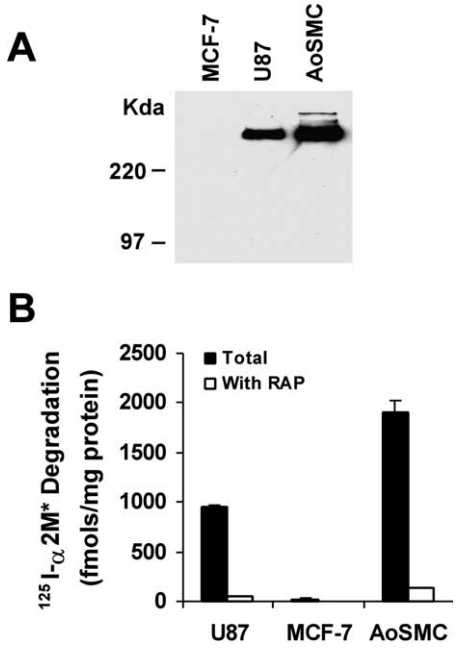


Fig. 1. LRP expression in human aortic smooth muscle cells. A: Western blotting analysis of LRP expression. Cell extracts from MCF-7 cells, U87 cells, and human aortic smooth muscle cells were separated via SDS–PAGE and analyzed by Western blotting with anti-LRP antibody 8G1. B: α2M* degradation in human aortic smooth muscle cells. MCF cells, U87 cells, and human aortic smooth muscle cells were incubated with [$^{125}$I]α2M* (1.25 nM) at 37°C for 4 h in the absence or presence of 500 nM RAP. The degradation of [$^{125}$I]α2M* was analyzed as described in Section 2. Values are the average specific degradation of triple determinations with the S.D. indicated by error bars. This experiment is representative of two such experiments performed with similar data.

## 3. Results

### 3.1. LRP is highly expressed in human aortic smooth muscle cells

LRP is highly expressed in vascular smooth muscle cells. The presence of LRP in human aortic smooth muscle cells was verified by Western blotting using LRP-specific antibody. In previous studies, we found that human breast cancer MCF-7 cells express only trace amounts of LRP [26], while human glioblastoma U87 cells express large amounts of LRP [27]. To assess the relative expression of LRP in human aortic smooth muscle cells, we used these two cell lines as controls. Fig. 1A shows a Western blotting analysis comparing the LRP expression in human aortic smooth muscle cells with MCF-7 cells

Table 1
LRP siRNA sequences

| | | |
|---|---|---|
| LRP siRNA#1 | Targeted region (cDNA) | $^{73}$AAGACTTGCAGCCCCAAGCAG$^{93}$ |
| | Sense siRNA | GACUUGCAGCCCCAAGCAGtt |
| | Antisense siRNA | CUGCUUGGGGCUGCAAGUCtt |
| LRP siRNA#2 | Targeted region (cDNA) | $^{88}$AAGCAGTTTGCCTGCAGAGAT$^{108}$ |
| | Sense siRNA | GCAGUUUGCCUGCAGAGAUtt |
| | Antisense siRNA | AUCUCUGCAGGCAAACUGCtt |
| LRP siRNA#3 | Targeted region (cDNA) | $^{114}$AACCTGTATCTCAAAGGGCTG$^{134}$ |
| | Sense siRNA | CCUGUAUCUCAAAGGGCUGtt |
| | Antisense siRNA | CAGCCCUUUGAGAUACAGGtt |
| LRP siRNA#4 | Targeted region (cDNA) | $^{222}$AAACGAGCATAACTGCCTGGG$^{242}$ |
| | Sense siRNA | ACGAGCAUAACUGCCUGGGtt |
| | Antisense siRNA | CCCAGGCAGUUAUGCUCGUtt |

and U87 cells. As seen in the figure, human aortic smooth muscle cells express abundant LRP. The LRP band intensity from human aortic smooth muscle cells was about two times greater than that from U87 cells. To characterize the function of LRP in human aortic smooth muscle cells, we examined ligand degradation mediated by LRP using a LRP-specific ligand, α2M* (Fig. 1B). As expected, human aortic smooth muscle cells displayed a high degree of α2M* degradation. The level of LRP-mediated α2M* degradation by human aortic smooth muscle cells was about 2.0 times greater than that by U87 cells.

### 3.2. Specific inhibition of LRP expression by LRP siRNA

We next examined the effects of LRP siRNA on LRP expression in human aortic smooth muscle cells. We designed four double-stranded, 21-nucleotide-long siRNAs with TT dinucleotide 3′ overhangs against the coding sequence of human LRP (GI 4758685) (Table 1). None of the siRNAs shares homology with exons of other known human genes. Human aortic smooth muscle cells were transfected with these four different LRP siRNAs, and LRP expression was assessed by Western blotting. As seen in Fig. 2, LRP siRNA#4 had no effect on LRP expression, and LRP siRNA#1 and #3 suppressed LRP expression to a moderate degree. Significantly, LRP siRNA#2 silenced LRP expression by more than 90%, suggesting that LRP siRNA#2 is a powerful tool for modulating LRP expression. The differences in effectiveness of different LRP siRNAs were not due to the transfection efficiency. We found a similar LRP reduction pattern in LRP siRNA-treated human lung cancer cell lines (data not shown). The reduction of LRP expression was maintained for as long as 72 h after transfection with LRP siRNA (data not shown).

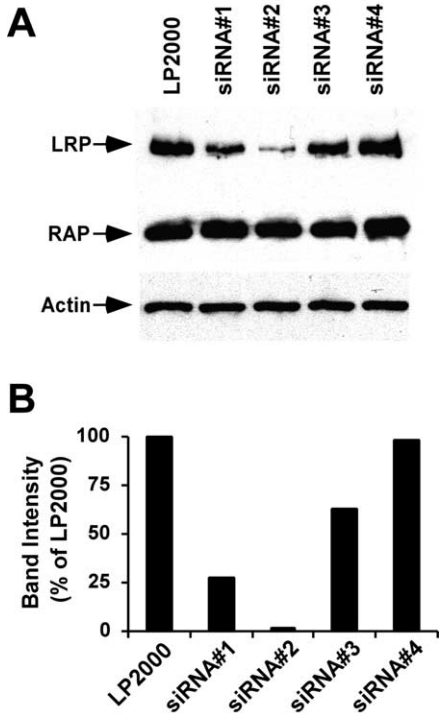


Fig. 2. Specific inhibition of LRP expression by siRNA. A: Effect of siRNAs, targeted toward four different sites (1–4) in the LRP coding region, on LRP expression. Human aortic smooth muscle cells were transfected with four different LRP siRNAs or treated with Lipofectamine2000 only (LP2000). LRP expression was measured by SDS–PAGE and Western blotting 48 h after transfection. The same amounts of lysates were analyzed by Western blotting with either anti-RAP antibody or anti-actin antibody, indicating that the same amounts of protein were loaded under each condition. B: Densitometric analysis of LRP expression presented in A. This experiment is representative of three such experiments performed with similar data.

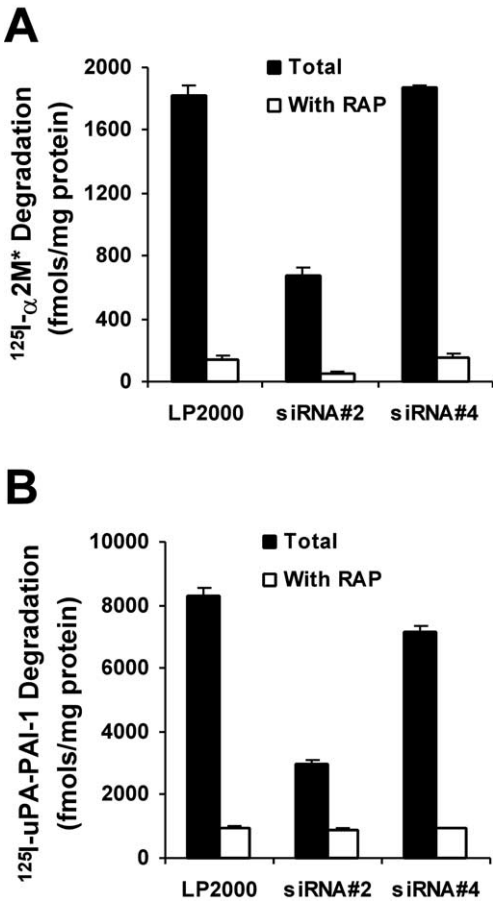


Fig. 3. LRP-mediated ligand degradation is inhibited in smooth muscle cells transfected with the LRP siRNA. Human aortic smooth muscle cells were transfected with LRP siRNA#2, siRNA#4, or treated with Lipofectamine2000 only (LP2000). Forty-eight hours after transfection, cells were incubated with [$^{125}$I]α2M* (1.25 nM) or [$^{125}$I]uPA–PAI-1 (5 nM) at 37°C for 4 h in the absence or presence of 500 nM RAP. The degradation of [$^{125}$I]α2M* and [$^{125}$I]uPA–PAI-1 complex was analyzed as described in Section 2. Values are the average specific degradation of triple determinations with the S.D. indicated by error bars. This experiment is representative of two such experiments performed with similar data.

### 3.3. LRP ligand degradation is inhibited in smooth muscle cells transfected with LRP siRNA

We then examined whether the siRNA-mediated suppression of LRP expression results in reduced ligand degradation. We used LRP siRNA#4 as the negative control, as the effect of this siRNA duplex on LRP expression is negligible. We chose two well-characterized LRP ligands, α2M* and uPA–PAI-1 complex, as test ligands. As seen in Fig. 3, when LRP siRNA#2 was applied in human aortic smooth muscle cells, inhibition of α2M* degradation and uPA–PAI-1 degradation was 63% and 72%, respectively. These results demonstrate that LRP siRNA#2 inhibits LRP-mediated ligand degradation.

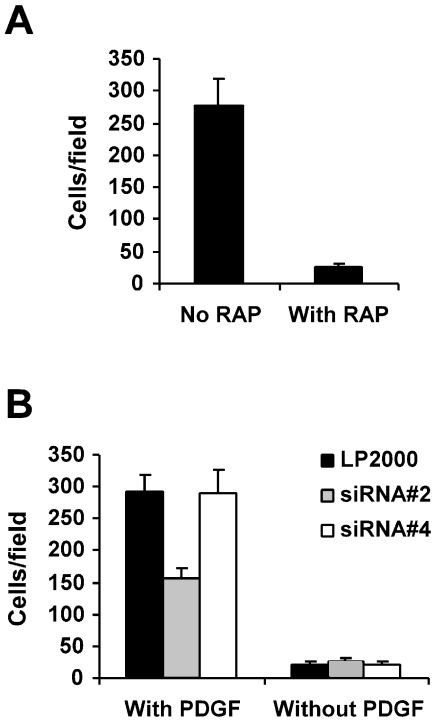


Fig. 4. Cell migration is inhibited in LRP siRNA-transfected smooth muscle cells. A: Effects of RAP on smooth muscle cell migration. Human aortic smooth muscle cells were made quiescent for 24 h with SmGM-2 medium and 0.4% fetal bovine serum. Cell migration was then analyzed with 30 ng/ml PDGF-BB in the lower chamber, and with or without 500 nM RAP in both chambers. B: Effects of LRP siRNA on smooth muscle cell migration. Human aortic smooth muscle cells were transfected with effective LRP siRNA#2, control siRNA#4, or treated with Lipofectamine2000 only (LP2000). After 24 h transfection, cells were made quiescent for 24 h with SmGM-2 medium and 0.4% fetal bovine serum. Cell migration was then analyzed with 30 ng/ml PDGF-BB in the lower chamber. Values are the average of triple determinations with the S.D. indicated by error bars. This experiment is representative of two such experiments performed with similar data.

### 3.4. *Cell migration is inhibited in LRP siRNA-transfected smooth muscle cells*

Several members of the LDLR family are involved in vascular smooth muscle cell migration. Incubation of human aortic smooth muscle cells with 500 nM RAP resulted in approximately 90% inhibition of PDGF-directed cell migration (Fig. 4A). To investigate the role of LRP in human aortic smooth muscle cell migration, we employed LRP siRNA. As seen in Fig. 4B, treatment of human aortic smooth muscle cells with LRP siRNA#2 resulted in about 50% inhibition of PDGF-directed cell migration. The 24 h quiescence with SmGM-2 medium and 0.4% fetal bovine serum had no effect on LRP reduction upon siRNA treatment (data not shown). Cell migration in the absence of PDGF was minimal and was not significantly affected by LRP inhibition (Fig. 4B).

## 4. Discussion

The very large size of LRP (~600 kDa) limits molecular manipulations at the cDNA level and the expression of this protein via transfection. In addition, LRP knockout in mice results in early embryonic lethality [28]. The recent development of the siRNA technique in mammalian cells has prompted us to explore the use of siRNA to moderate LRP expression. In the present study, we identified that, of the four LRP siRNAs tested, LRP siRNA#2 is the most effective in inhibiting LRP expression.

Human aortic smooth muscle cells express high level of LRP, which endocytoses and degrades over 30 different ligands [1]. In the present study, we chose α2M* and uPA–PAI-1 to study ligand degradation. LRP siRNA#2 suppressed more than 90% of LRP expression in human aortic smooth muscle cells, and resulted in 60–70% inhibition of ligand degradation. Smooth muscle cells express several members of the LDLR family including LRP1B (our unpublished data) and the very low density lipoprotein receptor [8,9,29]. When LRP expression is suppressed, human aortic smooth muscle cells could still maintain some levels of uPA–PAI-1 complex degradation via the very low density lipoprotein receptor and LRP1B, and of α2M* degradation possibly via LRP1B.

In the present study, we found that treatment with LRP siRNA or RAP inhibits human aortic smooth muscle cell migration in response to PDGF. This is consistent with results reported previously by other studies [12–14]. It has been demonstrated that inhibition of LRP function by either anti-LRP antibody or RAP reduces smooth muscle cell migration in response to uPA [13,14]. Despite these mounting studies, the mechanism underlying LRP modulating of smooth muscle cell migration is still not clear. LRP may promote uPA-induced smooth muscle cell migration by enhancing regeneration of unoccupied uPA receptor at the cell surface [13,30,31]. Alternatively, LRP may modulate the functional level of the PDGF receptor, thereby regulating the signal transduction pathways downstream of the PDGF receptor that participate in smooth muscle cell migration [16,32,33]. Both apolipoprotein E and RAP could interference with the binding of other ligand(s) (e.g. uPA–PAI-1 complexes) to LRP, and subsequently inhibit LRP function and smooth muscle cell migration.

The roles of LRP in regulation of cellular migration are not limited to smooth muscle cells. LRP antisense RNA-transfected HT1080 fibrosarcoma cells and LRP-deficient fibroblasts demonstrate increased cell migration [34,35]. However, RAP-treated breast cancer cells and myogenic cells display decreased cell migration [26,36,37]. Furthermore, LRP-null fibroblasts and RAP-treated endothelial cells do not migrate in response to thrombospondin stimulation [38]. The disparity of LRP function from these studies may reflect diverse characteristics of the experimental systems, including cell types, LRP expression levels, expression levels of other members of the LDLR family, and signaling pathways that participate in cellular migration. Nevertheless, all these studies point out an important role of LRP in cellular migration.

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