

Low-Density Lipoprotein Receptor-Related Protein 1 Promotes Cancer Cell Migration and Invasion by Inducing the Expression of Matrix Metalloproteinases 2 and 9

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Abstract

The low-density lipoprotein receptor-related protein 1 (LRP1) is a multifunctional endocytic receptor involved in the metabolism of various extracellular ligands, including proteinases, that play critical roles in tumor invasion. Although several studies have shown an increased expression of LRP1 in cancer cells, its function in tumor development and progression remains largely unclear. Here, we reveal a novel mechanism by which LRP1 induces the expression of matrix metalloproteinase 2 (MMP2) and MMP9 and thereby promotes the migration and invasion of human glioblastoma U87 cells. Knockdown of LRP1 expression greatly decreased U87 cell migration and invasion, which was rescued by the forced expression of a functional LRP1 minireceptor. Inhibition of ligand binding to LRP1 by a specific antagonist, receptor-associated protein, also led to reduced cancer cell migration and invasion. Because MMPs play critical roles in cancer cell migration and invasion, we examined the expression of several MMPs and found that the expression of functional MMP2 and MMP9 was selectively decreased in LRP1 knockdown cells. More importantly, decreased cell migration and invasion of LRP1 knockdown cells were completely rescued by exogenous expression of MMP2 or MMP9, suggesting that these MMPs are likely downstream targets of LRP1-mediated signaling. We further show that the level of phosphorylated extracellular signal-regulated kinase (ERK) was significantly decreased in LRP1-silenced cells, suggesting that ERK is a potential mediator of LRP1-regulated MMP2 and MMP9 expression in U87 cells. Together, our data strongly suggest that LRP1 promotes glioblastoma cell migration and invasion by regulating the expression and function of MMP2 and MMP9 perhaps via an ERK-dependent signaling pathway. [Cancer Res 2009;69(3):879–86]

Introduction

Glioblastoma multiforme (GBM grade IV) is the most aggressive type of brain tumor with a strong ability to invade and migrate into surrounding normal brain tissue. These cells secrete various extracellular matrix degrading enzymes, including matrix metalloproteinases (MMP), to facilitate their migration and invasion (1, 2). In particular, MMP2 and MMP9 are highly expressed in

gliomas compared with normal brain tissues, and their mRNA and protein levels are further increased upon tumor progression (3–5). In addition, it is well established that MMP2 and MMP9 are closely associated with tumor invasion and metastasis in a variety of human tumors (6–8).

The low-density lipoprotein receptor (LDLR)-related protein 1 (LRP1) is a large endocytic receptor that belongs to the LDLR family. LRP1 binds and endocytoses over 30 structurally and functionally distinct ligands, including apolipoprotein, proteinases, proteinase inhibitor complexes, and extracellular matrix proteins, such as MMPs and urokinase-type plasminogen activator (uPA; refs. 9, 10). Although several studies have implicated LRP1 in tumorigenesis, its precise role and potential underlying mechanisms remain controversial. For example, several reports show that a low expression level of LRP1 is closely related to the aggressive phenotype of tumor cells derived from various tissues, such as human prostate, thyroid, and breast (11–13). LRP1-deficient fibroblasts, which have a decreased rate of uPA/PAI-1/uPAR complex catabolism, show an increased cell migration rate (14). In addition, inhibiting LRP1 expression and function is commonly reported to increase cell migration and invasion (15, 16). However, Li and colleagues showed that high LRP1 expression promotes breast cancer cell invasiveness (17), and LRP1 neutralization could abrogate cell motility in both tumor and nontumor cells (18, 19). Moreover, we previously reported that silencing LRP1 expression in human smooth muscle cells by LRP1 small interfering RNA (siRNA) resulted in significantly decreased cell migration (20). Recently, Dedieu and colleagues (21) reported that LRP1 silencing prevents the invasion of a follicular thyroid carcinoma cell line despite the increased pericellular proteolytic activities of MMP2 and uPA. Collectively, these results show the complexity of the LRP1 function in tumor cell migration and invasion, which likely depends on the tumor cell type and the specific extracellular proteins involved in these processes.

In this study, we hypothesized that LRP1 regulates tumor cell migration and invasion by altering the expression and function of MMPs. First, several MMPs, including MMP2, MMP9, and MMP13, directly or indirectly interact with LRP1, which could alter MMP-mediated pericellular proteolysis (22–25). Second, several microarray studies have shown that LRP1, as well as MMP2 and MMP9, is highly up-regulated in human glioblastoma, suggesting that their expression levels are likely coupled (4–6, 26). Herein, we present evidence that LRP1 regulates tumor cell migration and invasion by altering the expression of MMP2 and MMP9.

Materials and Methods

Materials and cDNA constructs. Human α 2-macroglobulin (α 2M) was purified from human plasma and activated with methylamine (α 2M*), as

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described (27). Human recombinant receptor-associated protein (RAP) was expressed as a glutathione *S*-transferase fusion protein and was isolated, as described previously (28). MMP inhibitors, OA-Hy and inhibitor IV, were obtained from Calbiochem. All tissue culture media and serum were from Sigma. Rabbit polyclonal anti-LRP1 antibody has been described previously (29, 30). Peroxidase-labeled antirabbit antibody and enhanced chemiluminescence (ECL) system were from GE Healthcare. Carrier-free Na¹²⁵I was purchased from Perkin-Elmer Lifescience. Minireceptor of LRP1 mLRP4 was described in a previous report (31). MMP2 and MMP9 promoter luciferase vectors were kindly provided by Dr. Christopher C.W. Hughes at the University of California-Irvine. MMP2-AAV and MMP9-AAV expression constructs were kindly provided by Dr. Jin-Moo Lee at Washington University.

LRP1 siRNA. The sense and antisense sequences for LRP1 siRNA were reported in our previous studies (20). Single-stranded, LRP1-specific sense and antisense RNA oligonucleotides were synthesized by Ambion, and double-stranded RNA molecules were generated according to the manufacturer's instructions. The sequences of oligos are as follows: sense siRNA, GCAGUUUGCCUGCAGAGAUt and antisense siRNA, AUCUCUG-CAGGCAACUGCt.

Cell culture and transfection. Human glioblastoma U87 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate. For transfection, U87 cells were grown to 80% confluence and cells were transfected with LRP1 siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. After 48 h of transfection, cells were collected for migration and invasion assays, real-time PCR, and Western blotting.

Ligand degradation assay. The ligand degradation assay was performed, as previously described (32). Briefly, 2×10^5 cells were seeded into 12-well dishes 1 d before assays. Prewarmed binding buffer [DMEM containing 0.6% bovine serum albumin (BSA) with radioligand, 0.6 mL/well] was added to cell monolayers in the presence or absence of unlabeled 500 nmol/L RAP, followed by incubation for 4 h at 37°C. Thereafter, the media were collected and precipitated by addition of trichloroacetic acid (TCA; 20% of final concentration) and BSA (10 mg/mL). Degradation of radioligand was defined as the appearance of radioactive fragments in the overlying medium that were soluble in 20% of TCA. The protein concentration of each cell lysate was measured in parallel dishes that did not contain LRP1 ligands.

Quantitative real-time PCR. Quantitative reverse transcription-PCR was carried out using real-time PCR with the SYBR Green reporter. Total RNA isolated using the RNeasy Mini kit (Qiagen) was subsequently reverse transcribed to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). The reaction mix was subjected to quantitative real-time PCR to detect levels of the corresponding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LRP1, and several MMPs. GAPDH was used as an internal control for each specific gene. The relative levels of expression were quantified and analyzed by using Bio-Rad iCycler iQ software. Three independent experiments were performed to analyze the relative gene expression, and each sample was tested in triplicate. The real-time value for each sample was averaged and compared using the CT method. The amount of target RNA ($2^{-\Delta\Delta CT}$) was normalized to the endogenous GAPDH reference (ΔCT) and related to the amount of target gene in control sample, which was set at 1.0 on the calibrator.

Western blotting. U87 cells were lysed in lysis buffer (PBS containing 1% Triton X-100, protease inhibitor cocktail, and 1 mmol/L phenylmethylsulfonyl fluoride) at 4°C for 30 min. Equal quantities of protein were subjected to SDS-PAGE. After transfer to Immobilon-P transfer membrane, successive incubations with anti-LRP1 antibody or anti-actin antibody and horseradish peroxidase-conjugated secondary antibody were carried out. The immunoreactive proteins were then detected using the ECL system. Kodak Digital Science1D image analysis software was used for quantification.

Cell migration assay. Cell migration activities were examined by three-dimensional Boyden chamber assay and two-dimensional wound healing assay. Boyden chamber assay was carried out in 6.5-mm diameter transwell chambers with pore size of 8.0 μ m. At 24 h after LRP1 siRNA transfection, cells were resuspended in the migration medium of DMEM

containing 0.1% BSA and 2 mmol/L L-glutamine and placed in the upper compartment of the Transwell chambers coated with collagen I on the lower surface (5×10^4 in 100 μ L). The lower compartment was filled with 600 μ L of the same medium. After incubation for 6 h at 37°C, cells on the lower surface of the filter were fixed and stained, and five random fields per filter were counted at magnification of 200 \times . For wound healing assay, at 24 h after LRP1 siRNA transfection, a rectangular lesion was created using a cell scraper, and then the cells were rinsed twice with serum-free medium and incubated. After the designated times, three randomly selected fields at the lesion border were acquired using a CCD camera (Olympus) on an inverted microscope. In each field, the distance from the margin of the lesion to the 10 most migrated cells were measured, and the mean value of the distances was taken as the mobility of cells in each culture dish.

In vitro invasion assay. Matrigel invasion assays were used to assess the effect of the LRP1 knockdown on the invasiveness of cancer cells. Invasion of cells through Matrigel was determined using 24-well BD invasion chambers (8.0- μ m pore size with polycarbonate membrane; BD Biosciences) according to the manufacturer's instructions. BD invasion chambers were prehydrated with serum-free DMEM (500 μ L/well) for 2 h at 37°C in 5% CO₂. After harvest, cells were suspended in serum-free DMEM at a concentration of 1×10^6 cells/mL and immediately placed 100 μ L of the cell suspension onto the upper compartment of the plates. Subsequently, the lower compartment was filled with complete medium (500 μ L). After 24 h incubation, the noninvading cells remaining on the upper surface of the membrane were removed by wiping with cotton-tipped swabs. Cells on the lower surface of the membrane were stained with DiffQuick staining solution (IMEB, Inc.) according to the manufacturer's instructions. Five

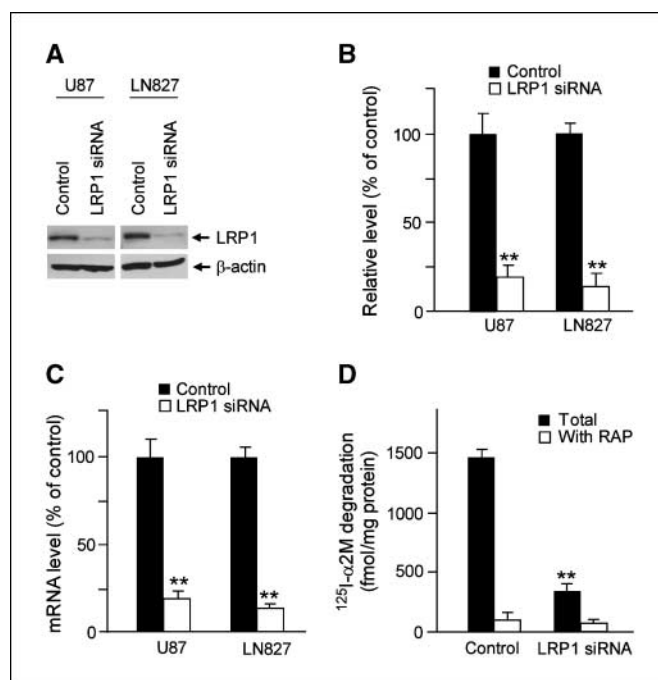
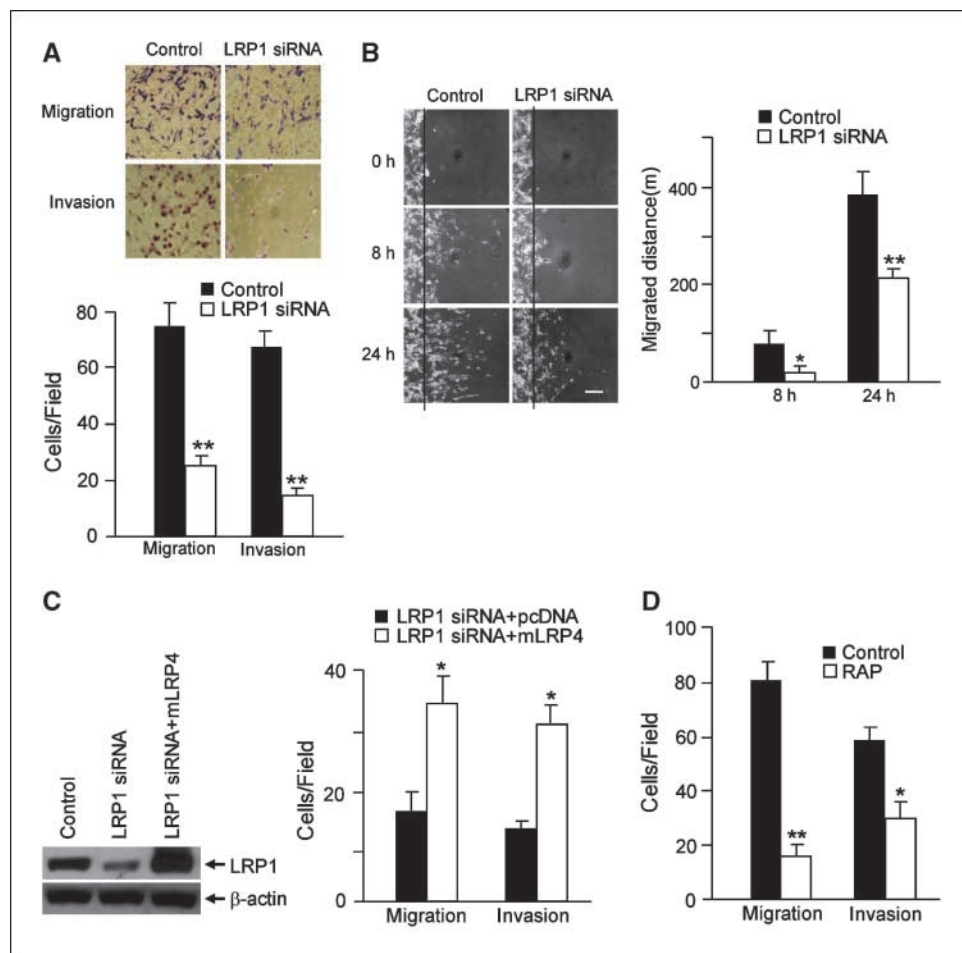


Figure 1. LRP1 silencing in glioblastoma cells. U87 or LN827 glioblastoma cells were transfected with LRP1 siRNA or control siRNA using Lipofectamine 2000. After 24 h transfection, cells were harvested for Western blotting, RNA extraction, or ligand degradation assays. *A*, LRP1 silencing by LRP1 siRNA in both U87 and LN827 glioblastoma cells. The same amounts of lysates were analyzed by Western blotting using an antibody against the 85-kDa subunit of LRP1. Anti-actin blot was used as a loading control. *B*, densitometric analysis of Western blots from triplicate samples was performed. *C*, mRNA levels of LRP1 were quantified by real-time PCR after treatment control or LRP1 siRNAs. *D*, ¹²⁵I- α 2M (1 nmol/L) uptake and degradation assays were performed at 37°C for 4 h in the absence or presence of RAP (500 nmol/L). The degradation of ¹²⁵I- α 2M was analyzed as described in Materials and Methods. Columns, average of triple determinations; bars, SD. **, *P* < 0.01.

Figure 2. LRP1 silencing and functional blocking by RAP significantly decreased U87 cell migration and invasion. **A**, after 24 h of control or LRP1 siRNA transfection, three-dimensional migration assays were performed using Boyden chambers. Cell invasion was analyzed using Matrigel precoated transwell chambers. **B**, two-dimensional migration assays were conducted with a modified wound healing assay. The migrated distance during the designated period was measured. Scale bar, 100 μ m. **C**, LRP1 minireceptor mLRP4 was cotransfected with LRP1 siRNA into U87 cells, and cellular properties of U87 cells were analyzed. LRP1 silencing and mLRP4 overexpression were analyzed by Western blotting. **D**, U87 cell migration and invasion were analyzed with or without 500 nmol/L RAP in both chambers. Representative photos of migration and invasion are presented, which were taken at a magnification of 200 \times under inverted microscopy. Columns, average of triple determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$.



fields of adherent cells were counted randomly in each well under an inverted microscope at magnification of 200 \times , and the results were numerically averaged and counted.

Luciferase assay. U87 cells were cotransfected with the appropriate cDNAs: MMP2 or MMP9 promoter-Luc and LRP1 siRNA or control siRNA. A β -gal reporter construct was also cotransfected for normalizing transfection efficiency. At 24 h after transfection, the luciferase activity and β -gal activity were measured by the Luciferase Assay System and β -gal Assay System, respectively, following the manufacturer's instructions (Promega).

Statistical analysis. All quantified data represent an average of at least triplicate samples. Error bars represent SD. Statistical significance was determined by Student's t test, and $P < 0.05$ was considered significant.

Results

LRP1 silencing inhibits U87 cell migration and invasion. To determine the specific role of LRP1 in U87 glioblastoma cell migration and invasion, we knocked down LRP1 expression by LRP1 siRNA. Previously, we had tested four double-stranded, 21-nucleotide-long LRP1 siRNAs with TT dinucleotide 3 overhangs against the coding sequence of human LRP1 (GI 4758685; ref. 20). Among them, we chose the LRP1 siRNA most effective at silencing LRP1 expression. As seen in Fig. 1A and B, LRP1 expression levels were clearly decreased by >80% by LRP1 siRNA, indicating that this LRP1 siRNA is a powerful tool to modulate LRP1 expression in U87 cells. We found a similar LRP1 reduction in LN827 cells, another

human glioblastoma, by the same LRP1 siRNA (Fig. 1A and B). We confirmed that the LRP1 siRNA significantly decreased LRP1 mRNA levels in both U87 cells and LN827 cells by quantitative real-time PCR (Fig. 1C). The reduction of LRP1 expression was sustained for >72 h after transfection with LRP1 siRNA (data not shown). We then examined whether the LRP1 siRNA-mediated suppression of LRP1 expression results in reduced LRP1 function in ligand degradation using the LRP1-specific ligand α 2M. As expected, LRP1 knockdown greatly reduced α 2M degradation compared with control cells (Fig. 1D). In addition, we examined the effect of LRP1 silencing on the survival and proliferative activity of U87 cells because a decrease in survival or proliferation after LRP1 silencing may also contribute to the observed decrease in migration and invasion of U87 cells. As shown in Supplementary Fig. S1, LRP1 silencing decreased the proliferation rate of U87 cells slightly, but significantly, compared with control cells. Survival of U87 cells were not changed significantly by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). Given this finding, we normalized the altered migratory and invasive activity of U87 cells in the following experiments against the altered proliferation rate.

To investigate whether LRP1 silencing affects U87 cell migration and invasion, we conducted three-dimensional cell migration assays using Transwell chambers and invasion assays with Matrigel-precoated Transwell chambers. We found that knockdown of LRP1 in U87 cells decreased cell migration and invasion at ~70% and 80%, respectively, compared with controls (Fig. 2A).

To confirm this result, a two-dimensional migration assay was performed. As shown in Fig. 2B, compared with control cells, the migrated distances of LRP1 siRNA-transfected U87 cells were decreased by 60% at 8 h and 40% at 24 h, after making the scratch. In addition, LRP1 siRNA significantly decreased both migration and invasion of LN827 cells by >60% (Supplementary Fig. S2A). To further confirm the effects of LRP1 silencing on cell migration and invasion, we also examined the effects of short hairpin RNAs

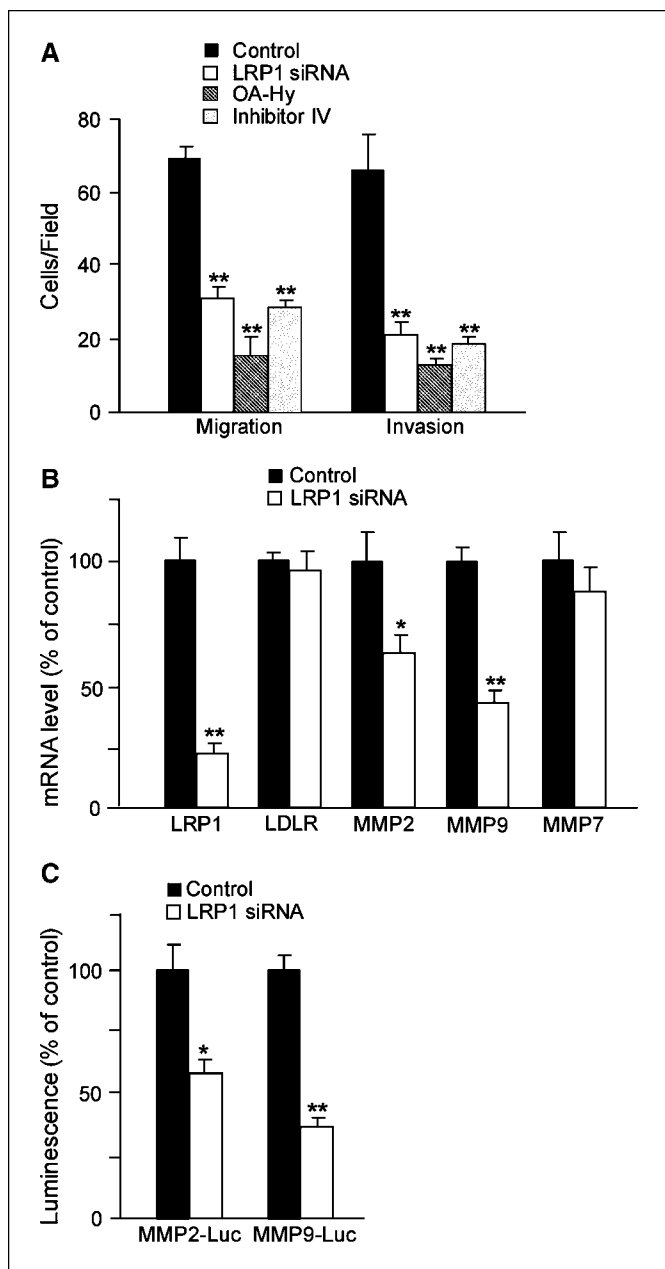


Figure 3. LRP1 silencing decreased MMP2 and MMP9 expression. *A*, migration and invasion assays were performed with U87 cells in the absence or presence of 10 μ mol/L MMP inhibitor OA-Hy or 10 nmol/L MMP inhibitor IV. *B*, mRNA levels of LRP1, MMP2, MMP9, LDLR, and MMP7 were quantified by real-time PCR 48 h after treatment with LRP1 siRNA. Levels of GAPDH mRNA were used as internal standards. *C*, U87 cells were cotransfected with control or LRP1 siRNA and MMP2-Luc or MMP9-Luc. Cells were harvested 24 h later and analyzed for luciferase expression. β -gal expression vector was cotransfected for normalization of transfection efficiency. Columns, average of triple determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$.

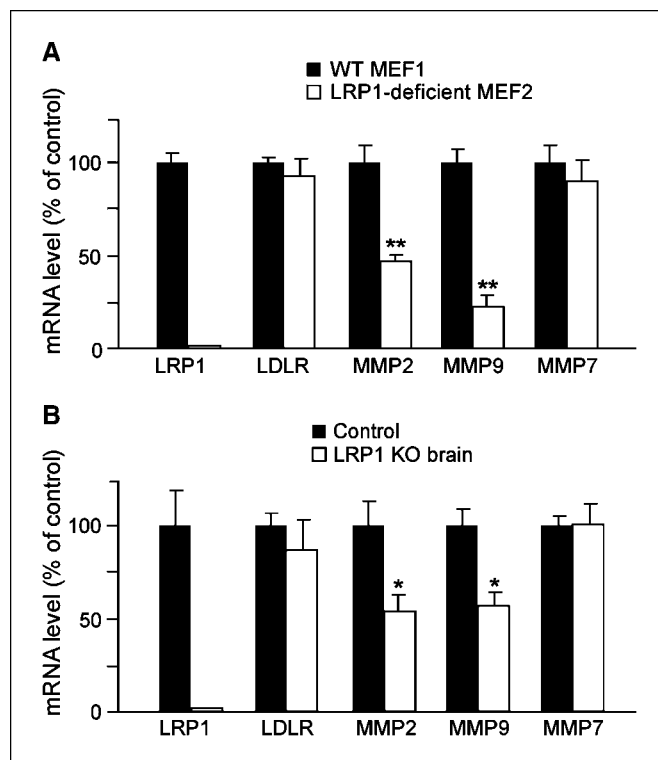


Figure 4. MMP2 and MMP9 are down-regulated in LRP1-deficient cells and brain tissues. Levels of MMP2 and MMP9 mRNA in MEF cells or mouse brain tissues were quantified by real-time PCR. *A*, mRNA levels of MMP2, MMP9, LRP1, LDLR, and MMP7 in WT MEF1 and LRP1-deficient MEF2 cells. *B*, mRNA levels of MMP2, MMP9, LRP1, LDLR, and MMP7 in brain tissues from control and LRP1-forebrain knockout mice. Columns, average of triple determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$.

(shRNA) for LRP1, which silenced LRP1 expression by >90% compared with the empty vector control. We found that LRP1 shRNA-transduced U87 cells exhibit significantly decreased 80% migration and invasion rates compared with control cells (Supplementary Fig. S2B).

To confirm that the effect of LRP1 silencing on the decrease of migration and invasion of U87 cells is specific, we restored LRP1 function by cotransfecting a minireceptor of LRP1 mLRP4, which possess the full functional activities of LRP1 (31). This mLRP4 does not contain LRP1 siRNA-targeting sequence and is therefore resistant to LRP1 siRNA knockdown. We observed that mLRP4 expression restored U87 cell migration and invasion (Fig. 2C). We have further tested the effects of LRP1 silencing on the invasion of several lung cancer cell lines (H292, H441, H520, and SK-LU-1) and found that the invasiveness of these lung cancer cells positively correlates with LRP1 expression levels (Supplementary Fig. S3A). Furthermore, the invasion rate was decreased by 50% in LRP1 knockdown SK-LU-1 cells, which express high level of endogenous LRP1. These results showed that the expression levels of LRP1 positively correlate with the migration and invasion rates of different cancer cell types.

LRP1 ligand-binding function is required for efficient cancer cell migration and invasion. To determine whether the ligand-binding ability of LRP1 affects U87 cell migration and invasion, we pretreated U87 cells with RAP, an antagonist that blocks all ligand binding to LRP1 (31). U87 cells treated with 500 nmol/L RAP showed significant decrease in migration and invasion at ~70% and 50%, respectively, compared with control

cells (Fig. 2D). Treating SK-LU-1 lung cancer cells with 500 nmol/L RAP also resulted in a significant decrease at ~80% of invaded cells compared with control cells (Supplementary Fig. S3B), suggesting that ligand binding to LRP1 is required for the LRP1 function in tumor cell migration and invasion.

LRP1 modulates cell migration and invasion by regulating MMP2 and MMP9 expression. To examine whether MMP activity is required for U87 cell migration and invasion, we tested the effect of OA-Hy, a broad spectrum MMP inhibitor, and inhibitor IV, a specific inhibitor of MMP2 and MMP9. Figure 3A shows that both inhibitors significantly blocked U87 cell migration and invasion, demonstrating that MMP activity is necessary for efficient migration and invasion of U87 cells.

To examine whether LRP1 regulates U87 cell migration and invasion by modulating MMP expression, we analyzed the mRNA levels of MMP2 and MMP9 upon LRP1 silencing. Quantitative real-time PCR results showed that the mRNA levels of MMP2 and MMP9 were decreased by >30% and 50%, respectively, in LRP1-silenced cells (Fig. 3B). As controls, the mRNA levels of the LDL receptor and MMP7 were not altered under these conditions, indicating that down-regulation of MMP2 and MMP9 expression by LRP1 silencing is specific. To confirm that LRP1 regulates MMP expression at the transcriptional level, we performed a luciferase reporter assay using constructs containing promoter regions of MMP2 and MMP9. As shown in Fig. 3C, knockdown of LRP1 decreased the transcriptional activity of both MMP2 and MMP9 promoters by 30% and 60%, respectively. These results suggest that LRP1 is involved in the transcriptional regulation of MMP2 and MMP9 in U87 cells.

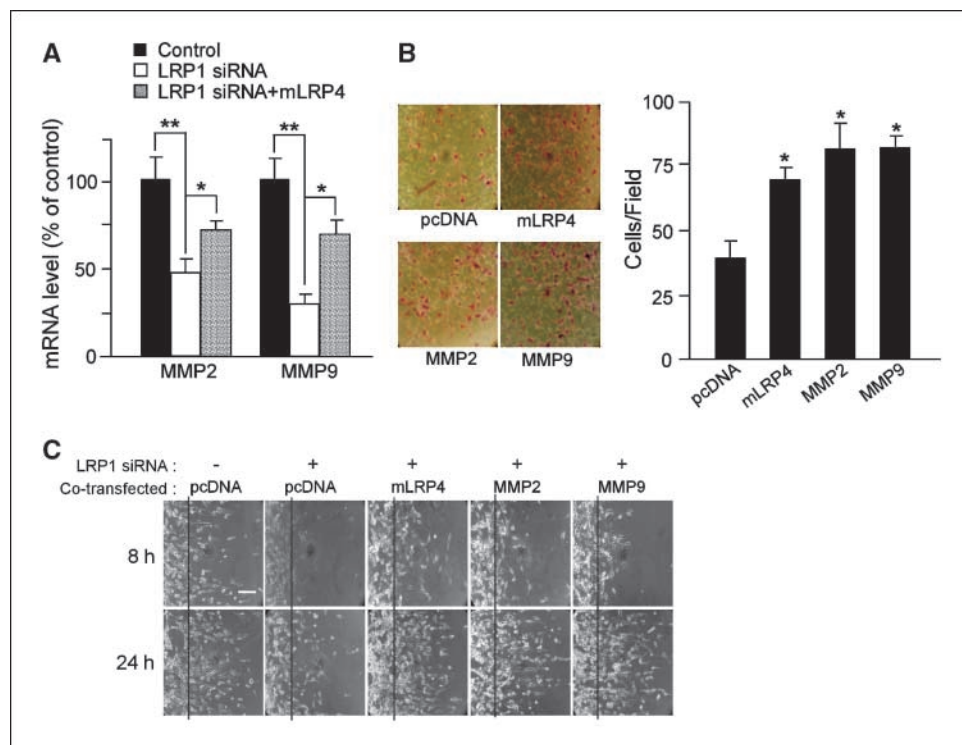
To confirm the finding that LRP1 regulates MMP2 and MMP9 expression, we examined the mRNA levels of MMP2 and MMP9 in LRP1-deficient mouse embryonic fibroblasts (MEF2) and LRP1-expressing MEF cells (MEF1) by quantitative real-time PCR.

Consistent with findings in LRP1 knockdown cells, mRNA levels of MMP2 and MMP9 in LRP1-deficient MEF2 cells are only ~50% and 30%, respectively, of WT MEF1 cells (Fig. 4A). Furthermore, we found that the mRNA levels of MMP2 and MMP9 in the brain of LRP1 forebrain knockout mice (33) are also significantly lower than those in the brain of littermate control mice (Fig. 4B). In both cases, the levels of LDLR and MMP7 were not changed, suggesting that LRP1 specifically regulates MMP2 and MMP9 expression.

To further confirm that the effect of LRP1 silencing on the decrease of MMP2 and MMP9 expression levels in U87 cells is specific, we restored LRP1 function by cotransfecting LRP1 minireceptor mLRP4. We observed that mLRP4 expression significantly restored mRNA levels of MMP2 and MMP9 in LRP1-silenced U87 cells (Fig. 5A). To further examine whether LRP1 modulates cell migration and invasion by regulating MMP expression and function, we tested whether ectopic expression of MMP2 or MMP9 can restore cell migration and invasion in LRP1-silenced U87 cells. We found that MMP2 or MMP9 overexpression successfully restored both migration and invasion (Fig. 5B and C), demonstrating that MMP2 and MMP9 are downstream targets of LRP1.

Extracellular signal-regulated kinase is a potential mediator of LRP1 regulation of MMP2 and MMP9 expression. The signal transduction pathways that modulate the activity of MMP transcription factors are highly diverse. Mitogen-activated protein kinase signal transduction pathways, including p38, extracellular signal-regulated kinase (ERK), and c-Jun NH₂ kinase (JNK), are well known mediators that stimulate or inhibit MMP expression depending on cell types (34–36). To understand the mechanism by which LRP1 regulates MMP2 and MMP9 expression, we analyzed the effects of LRP1-silencing on the activation of several potential signaling pathways. We found that the level of phosphorylated ERK was selectively decreased in LRP1-silenced U87 cells, which was

Figure 5. Decreased migration and invasion in LRP1 knockdown U87 cells were rescued by MMP2 and MMP9 overexpression. LRP1 minireceptor mLRP4, MMP2, or MMP9 expression vector was cotransfected with LRP1 siRNA into U87 cells, and cellular migration and invasion were analyzed. **A**, negative control or LRP1 siRNA, with or without mLRP4, were cotransfected into U87 cells. mRNA levels of MMP2 and MMP9 were quantified by real-time PCR. **B**, invasion assay was conducted after pcDNA, mLRP4, MMP2, or MMP9 was cotransfected with LRP1 siRNA. **C**, two-dimensional migration assay was performed after pcDNA, mLRP4, MMP2, or MMP9 was cotransfected with or without LRP1 siRNA. Scale bar, 100 μ m. Columns, average of triple determinations; bars, SD. *, $P < 0.05$; **, $P < 0.01$.



restored upon ectopic expression of mLRP4 (Fig. 6A and B). Phosphorylated JNK and p38 were not significantly changed in LRP1-silenced U87 cells. The levels of phosphorylated Akt were also not changed in LRP1-silenced U87 cells. These results show that ERK is likely a downstream target of LRP1-mediated signaling that regulates MMP2 and MMP9 expression and their functions in cell migration and invasion in U87 cells.

Discussion

In the present study, we show that LRP1 silencing leads to a significant decrease of glioblastoma cell migration and invasion and provide evidence that LRP1 regulates the transcriptional levels of MMP2 and MMP9 in U87 glioblastoma cells. We further show that LRP1 regulation of MMP2 and MMP9 expression is likely mediated by ERK signal pathways.

The finding that LRP1 silencing leads to a significant decrease of glioblastoma cell migration and invasion is consistent with Li and colleagues' previous report, which showed that high levels of LRP1 expression promoted breast cancer cell invasiveness (17). We obtained similar results using two different gene silencing techniques, siRNA and shRNA. Moreover, functional blocking of LRP1 by RAP also led to decreased cell migration and invasion, which is also consistent with reports that RAP treatment decreased migration of breast cancer cells, myogenic cells (18, 37), and nontumorous smooth muscle cells (19, 38). In contrast, there are reports that knockdown or deletion of LRP1 increased cell migration, likely by decreasing the catabolic rate of the uPA/PAI-1/uPAR complex (14, 16). It has also been shown that low endogenous LRP1 levels are often related to the aggressive phenotype of tumor cells, a phenomenon seen in various tissues (11–13), and neutralization of LRP1 function has been commonly reported to increase cell migration and invasion (15, 16). The

disparity of LRP1 function among these studies may reflect the diverse characteristics of the experimental systems, including cell types, LRP1 expression levels, expression levels of other members of the LDLR family, and signaling pathways that participate in cellular migration. Nevertheless, our observations that ectopic expression of mLRP4 successfully rescued cell migration and invasion in LRP1-silenced glioblastoma cells strongly suggest that LRP1 expression levels are positively correlated with cancer cell migration and invasion rates. Indeed, we found that LRP1-silenced LN827 cells, which is highly invasive *in vivo* (39), exhibit decreased migration and invasion rates. Although U87 cells showed migratory and invasive activity in the present *in vitro* results, a previous report showed that the intracerebral xenograft U87 cells form well-circumscribed tumors with no migration or invasion into normal brain tissue (40). This discrepancy of U87 cells between the *in vitro* and *in vivo* models may be due to their different microenvironment and/or host resistance *in vivo*. Therefore, in future studies, it will be interesting to examine the invasiveness of alternative glioblastoma cell lines using xenograft mouse models after LRP1 silencing. We further observed that the number of invaded cells positively correlates with endogenous levels of LRP1 in different lung cancer cells, suggesting that LRP1 regulates cancer cell migration and invasion via a similar mechanism among different cancer cell types. In fact, the invasion rate of SK-LU-1 cells, which express the highest level of LRP1 among the lung cancer cell lines tested, was greatly decreased upon LRP1 silencing.

To determine how LRP1 promotes glioblastoma cell migration and invasion, we focused on delineating the relationship between LRP1 and MMPs, particularly LRP1 ligands, MMP2, and MMP9. It is well known that the mRNA and protein levels of MMP2 and MMP9 are highly increased upon glioblastoma progression (1–3). In this respect, it is interesting to note that several microarray studies have shown that the expression levels of LRP1 are highly up-regulated in human glioblastoma, suggesting that MMPs and LRP1 expression levels are likely coupled (4–6, 26). However, it is unknown how these genes regulate each other during tumorigenesis. Here, we found that LRP1 silencing significantly decreases the mRNA levels of both MMP2 and MMP9, but not the mRNA levels of the LDL receptor and MMP7, indicating that LRP1 specifically modulates the expression of MMP2 and MMP9. We confirmed that LRP1 regulates MMP2 and MMP9 expression at the transcriptional level by luciferase reporter assays using constructs containing the promoter regions of MMP2 and MMP9. Although ~90% of LRP1 expression was silenced by siRNA, the migration and invasion rates of U87 cells and the expression levels of MMP2 and MMP9 were reduced by only ~50% to 60%. A potential explanation for this discrepancy is the paradoxical function of some MMPs. Indeed, MMP2 has long been considered as a protumorigenic factor but MMP9 has a function of host resistance in cancer, as well as protumorigenic action (41). Additionally, other modulators may regulate MMP expression and U87 cell migration and invasion independent of LRP1. Further investigation will be needed to reveal the relationship of LRP1-dependent and LRP1-independent mechanisms in cancer cell migration and invasion. We further showed that the mRNA levels of MMP2 and MMP9 in both LRP1-deficient MEF2 and the brain of LRP1 forebrain knockout mice are significantly lower than those in the control samples. These results suggest that LRP1 is involved in the transcriptional regulation of MMP2 and MMP9 in U87 cells. Hu and colleague's previous report that tPA, an LRP1 ligand, regulates MMP9 expression in an LRP1-dependent manner (42) provides

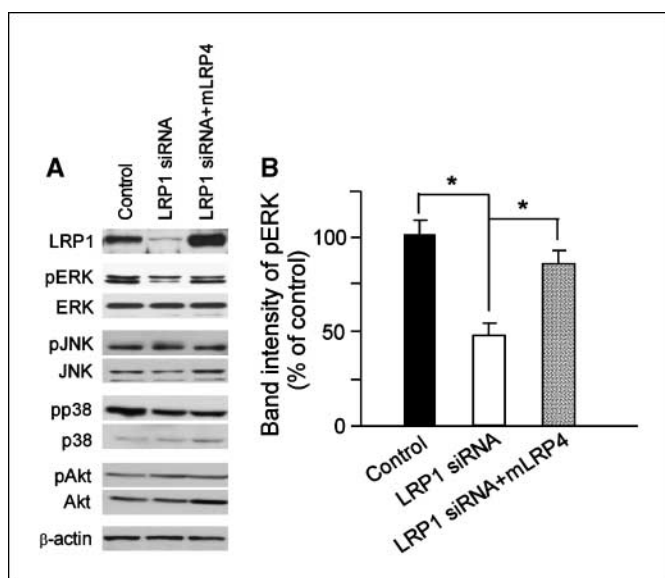


Figure 6. LRP1 silencing decreased phosphorylated ERK. Effects of LRP1 silencing on cellular signaling pathways were analyzed by Western blotting. A, negative control or LRP1 siRNA, without or with mLRP4, were cotransfected into U87 cells. The same amounts of lysates were analyzed by Western blotting for LRP1 expression and the levels of indicated signaling molecules. Actin blot serves as a loading control, and this blot is representative of three such experiments performed with similar data. B, densitometric analysis of Western blots for pERK from triplicate samples was performed. Columns, average of triple determinations; bars, SD. *, $P < 0.05$.

further support for our findings. We also showed that the ectopic expression of MMP2 or MMP9 successfully restores both migration and invasion of LRP1-silenced U87 cells, indicating that MMP2 and MMP9 are indeed downstream targets of LRP1.

LRP1 is also a signaling receptor that regulates several signal transduction pathways in different cell types (42–44). It is known that ERK is a signal mediator of tPA-induced MMP9 transcription through LRP1 (42) and that LRP1 is a regulator of ERK phosphorylation in HT 1080 fibrosarcoma cells (16). Indeed, we found that the level of phosphorylated ERK is selectively decreased in LRP1 knockdown cells. Our results also show that LRP1 regulates the transcriptional activity of MMP9 to a greater extent than MMP2. Dedieu and colleagues have shown that LRP1 effectively controls cell invasiveness via inhibition of focal adhesion complex disassembly, a process mediated by ERK signaling (21). Thus, ERK signaling pathways are likely associated with several mechanisms of cell motility mediated by LRP1, including regulation of the transcriptional levels of MMP2 and MMP9.

As a therapeutic target, LRP1 has long been considered as a promising possibility for cancer treatment. Despite a number of

contradicting reports, our results provide strong evidence that reducing LRP1 expression and function may well serve as a potential therapeutic strategy to treat cancer, particularly cancer metastasis. Further studies aimed at identifying the precise signal transduction pathways and transcription factors that mediate LRP1 regulation of MMP expression may provide a molecular understanding of the roles of LRP1 in cancer biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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