

LRP-1 Silencing Prevents Malignant Cell Invasion despite Increased Pericellular Proteolytic Activities[▽]

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The scavenger receptor low-density lipoprotein receptor-related protein 1 (LRP-1) mediates the clearance of a variety of biological molecules from the pericellular environment, including proteinases which degrade the extracellular matrix in cancer progression. However, its accurate functions remain poorly explored and highly controversial. Here we show that LRP-1 silencing by RNA interference results in a drastic inhibition of cell invasion despite a strong stimulation of pericellular matrix metalloproteinase 2 and urokinase-type plasminogen activator proteolytic activities. Cell migration in both two and three dimensions is decreased by LRP-1 silencing. LRP-1-silenced carcinoma cells, which are characterized by major cytoskeleton rearrangements, display atypical overspread morphology with a lack of membrane extensions. LRP-1 silencing accelerates cell attachment, inhibits cell-substrate adhesion, and induces the accumulation, at the cell periphery, of abundant talin-containing focal adhesion complexes deprived of FAK and paxillin. We conclude that in addition to its role in ligand binding and endocytosis, LRP-1 regulates cytoskeletal organization and adhesive complex turnover in malignant cells by modulating the focal complex composition, thereby promoting invasion.

Low-density lipoprotein (LDL) receptor-related protein 1 (LRP-1) is a large endocytic receptor that belongs to the LDL receptor superfamily (25, 52). This scavenger receptor is widely expressed in most tissues and mediates the rapid uptake and subsequent lysosomal degradation of various ligands from the extracellular environment through clathrin-coated pits or caveolae. First described as a receptor for alpha-2-macroglobulin (α 2M) (51), LRP-1 is now reported to interact with a variety of biological ligands at the cell surface, including viruses, lipoproteins, growth factors, matrix macromolecules, proteinases, and proteinase inhibitor complexes.

LRP-1-dependent endocytosis therefore emerges as a main mechanism controlling the extracellular amounts of various matrix metalloproteinase (MMP) family members (18). Such proteinases, which are well known to mediate extracellular matrix remodeling, were widely involved in cancer progression and found to have prognostic value for a variety of human cancers (13, 15, 26). The LRP-1-mediated internalization of MMP-2 was identified as a pivotal mechanism for controlling the extracellular activity of this proteinase. Yang and collaborators (62) first proposed that MMP-2 clearance occurs through formation of an MMP-2–thrombospondin-2 molecular complex. We recently demonstrated that LRP-1 is further able to mediate the internalization of pro-MMP-2 in complex with tissue inhibitor of metalloproteinases 2 (TIMP-2),

through a thrombospondin-independent mechanism (19). LRP-1 was also reported to bind with high affinity to MMP-9 and to mediate its cellular catabolism (24). Moreover, the internalization and catabolism of MMP-13 seem to require both LRP-1 and an unidentified receptor acting as a primary binding site on the plasma membrane (2).

Extracellular serine proteases, such as tissue-type and urokinase-type plasminogen activators (tPA and uPA, respectively), are involved in abnormal matrix remodeling occurring during tumor development and are frequently considered poor prognostic factors for patient survival and potential therapeutic targets for cancers (29, 38, 47). LRP-1 was also identified for interacting with such proteinases to remove excessive plasminogen activators from the pericellular environment by rapid endocytosis (25). Indeed, the level of tPA was shown to be regulated acutely by LRP-1 (4, 64). Furthermore, LRP-1 is able to mediate the clearance and catabolism of uPA when bound to its specific inhibitor PAI-1 and to its membrane receptor, uPAR (7). This requires uPAR distribution into clathrin-coated pits and tight physical interactions between uPAR and LRP-1 in the presence of the inactive and highly stable uPA–PAI-1 complex (9, 40). Recently, the uPA–PAI-2 complex was also shown to establish high-affinity molecular interactions with LRP-1, thus leading to an accelerated clearance of uPA from the cell surface (8).

Since removing excessive extracellular proteolytic activity may prevent tumor progression and spreading, LRP-1 has emerged as a promising therapeutic target against cancer cell invasion. Indeed, a small amount of membrane-anchored LRP-1 was previously closely related to the aggressive phenotype of human cancer cells from various tissues (14, 20, 27, 46, 52). In addition, neutralizing the endocytic function of LRP-1 commonly led to increased invasiveness of human malignant

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cells (46, 58). However, Li and colleagues (31) have conversely reported that exogenously added receptor-associated protein (RAP), an LRP-1 antagonist, decreased the capacity of breast cancer cells to invade. Similar apparently contradictory observations were also reported for non-tumor-cell mobility. Indeed, LRP-1-deficient cells and RAP-treated cells displayed increased normal cell migration in some studies (32, 57, 61), reduced cell migration in others (5, 30), and no effect in one (12). LRP-1 was recently proposed to modulate the cell motility processes by bridging with other cell signaling surface proteins (25, 52). This cargo receptor was mainly implicated in the regulation of the signaling pathway mediated by the uPA/uPAR system, which controls cell migration (6, 9, 12).

In order to narrow down the role of LRP-1 in cell adhesion and invasion, we developed a long-term vector-based short hairpin RNA (shRNA) strategy against LRP-1. Despite a prominent accumulation of pericellular proteolysis, LRP-1 silencing strongly inhibited malignant cell invasion by preventing focal adhesion disassembly and favoring cell spreading.

MATERIALS AND METHODS

Antibodies and chemicals. Anti-MMP-2 (Ab-6) and anti-uPA (Ab-1) were obtained from Merck Biosciences (distributed by VWR International, Strasbourg, France). Anti- α -actinin (BM-75.2), anti- β -actin (A2228), and chromomycin A3 (C2659) were purchased from Sigma (Saint-Quentin Fallavier, France) whereas antitalin (MAB1676) and antivinculin (MAB1674) were provided from Chemicon (Chandlers Ford, United Kingdom). Anti-focal adhesion kinase (anti-FAK) (3285) and anti-Y576/577-phosphorylated FAK (3281) antibodies were from Cell Signaling (Saint Quentin Yvelines, France). Antipaxillin antibodies used for immunoblotting (poly6007) and immunofluorescence (clone 349) were purchased from Ozyme (Saint Quentin Yvelines, France) and BD Biosciences (Le Pont de Claix, France), respectively. Anti-LRP-1 antibody (R2629), kindly provided by D. K. Strickland, was described elsewhere (43). Corresponding peroxidase-coupled anti-rabbit antibodies were from Cell Signaling, whereas anti-mouse and anti-goat secondary antibodies were from Amersham Biosciences (Orsay, France). Alexa Fluor 568-phalloidin (A12380) and secondary antibody labeled with Alexa Fluor 488 (A11001) or Alexa Fluor 568 (A11004 and A11036) were from Molecular Probes (Cergy Pontoise, France). Human α 2M and fluorescein isothiocyanate (FITC)-labeled human α 2M were purchased from BioMac (Leipzig, Germany). Plasminogen and purified human MMP-2 and uPA were also obtained from Calbiochem. The S-2251 (D-Val-Leu-Lys-p-nitroanilide) substrate was obtained from Chromogenix (Paris, France). Quenched fluorogenic casein (Bodipy FL casein) was from Molecular Probes. Other chemicals were from Sigma.

Generation of small interfering RNA (siRNA) and shRNA against LRP-1. LRP-1 knockdown was achieved by RNA interference using a vector-based shRNA approach. The plasmid pSuppressorNeo, in which the type III RNA polymerase promoter U6 drives shRNA expression, was purchased from Imgenex Corporation (Montrouge, France). Oligonucleotides were synthesized and annealed to generate linkers encoding shRNAs with a 21-mer sequence forming head-to-head repeats separated by an 8-mer spacer (CAGTACTC). The sense-loop-antisense sequence coding for LRP-1 shRNA was designed according to the method of Li and Bu (30). Oligonucleotides with the sequence AAGCAGTTT GCCTGCAGAGATCAGTACTCATCTCTGCGAGGCAAACTGCTT and its complementary sequence were synthesized and annealed. This sequence was confirmed by a BLAST search to be specific for LRP-1. As a control, we used a nonsilencing scrambled sequence, CTAAGTTCGCGATGTGGGCAACAGTACTCTTTGCCACATCGCAAGTTAG. Linkers containing the XhoI and XbaI restriction sites were added to the 5' and 3' ends, respectively, of the indicated sequences and then inserted into the XbaI/SalI sites of the pSuppressorNeo vector to generate transgenic shRNA constructs. The generated constructs were controlled by sequencing.

A second double-stranded RNA sequence that recognizes a different target site of the LRP-1 mRNA was used in each experiment to control that the observed effects were not due to off-target interactions. This sequence (named siLRP-1) was synthesized with the following primers: forward, GAC UUGCAGCCCCAAGCAGUU; reverse, CUGCUUGGGGCGUGCAAGU

CUU. The siLRP-1 sequence and its respective control were synthesized by Dharmacon (distributed by Perbio Science, Brebiere, France).

Cell culture and transfection. The FTC133 human follicular thyroid carcinoma cell line, first described by Goretzki and colleagues (22), was grown in Dulbecco's modified Eagle medium–Ham's F-12 medium (Invitrogen) with 10% fetal bovine serum, as previously described (46). siRNAs were transiently transfected using Oligofectamine (Invitrogen). Plasmids were stably transfected using Lipofectamine 2000 (Invitrogen), and clonal cell lines were selected using Geneticin (600 μ g/ml).

Internalization assays. Uptake experiments were adapted from the procedure reported by Goto and Mizunashi (23). Briefly, FTC133 cells were washed twice with phosphate-buffered saline (PBS) and incubated for 30 min in fresh serum-free medium containing FITC-labeled human α 2M, alone or together with a 100-fold excess of nonlabeled human α 2M, in the presence of 100 μ M chloroquine to inhibit lysosomal activity. Cells were then washed three times with ice-cold PBS, solubilized in PBS containing 0.05% Triton X-100, and sonicated. After precipitation with 10% trichloroacetic acid and centrifugation, the intracellular fluorescence was determined using a spectrofluorometer (LS-50; Perkin-Elmer) and expressed in relative units.

Western blot analysis. Whole-cell extracts were prepared as previously described (11). The plasma membrane-enriched fractions were prepared in an ice-cold lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-114). After extraction, the remaining pellet was separated by centrifugation (100,000 $\times$ g for 1 h at 4°C) and discarded. Sodium dodecyl sulfate (2%) and *N*-ethylmaleimide (6 mM) were then added to the supernatant. To measure the proteins secreted into the conditioned medium, serum-free culture medium was collected and concentrated 10-fold using a Vivaspinn concentrator (Vivascience, Palaiseau, France), and the samples were subjected to immunoblot analysis. The protein concentration in whole-cell extracts, membrane extracts, or conditioned media was quantified by the Bradford method (Bio-Rad Laboratories, Marne-la-Vallée, France). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane (Schleicher & Schuell GmbH, Mantes la Ville, France), and incubated overnight at 4°C with primary antibodies. Immunoreactive bands were revealed using an ECL Plus chemiluminescence kit from Amersham Biosciences (Orsay, France). β -Actin was used as a control to ensure equal loading.

Immunoprecipitation. Cell extracts were prepared in lysis buffer (10 mM Tris-HCl, pH 6.7, 0.75% Brij, 75 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). After centrifugation (10,000 $\times$ g, 10 min, 4°C), the supernatants were incubated for 4 h at 4°C with antitalin antibody. Immunoprecipitation was performed with protein G-Sepharose (Amersham Biosciences) for 2 h at 4°C. The samples were washed three times in lysis buffer, and protein complexes bound to beads were solubilized under reducing conditions and analyzed by immunoblotting, as described above.

RNA isolation and RT-PCR. Total RNAs were isolated and purified with an RNeasy isolation kit (Qiagen, Courtaboeuf, France), and reverse transcription (RT) was performed as recommended by the manufacturer (Promega, Charbonnières, France). Primers for human LRP-1 (33), uPA (48), MMP-2 (46), STAT-1 (53), oligoadenylate synthetase (OAS) (34), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (10) were previously described and were synthesized by Eurogentec (Seraing, Belgium). Numbers of cycles were adjusted to ensure that amplifications were in a linear range. The gels shown are representative of at least three separate experiments.

Gelatinase activity. Gelatinase activity in conditioned media and membrane extracts (see "Western blot analysis") was determined by gelatin zymography as previously described (46). Purified human MMP-2 was used as a positive control.

Gelatin-plasminogen zymography. Serum-free conditioned media were concentrated 10-fold by using a Vivaspinn concentrator. Proteins therein and in membrane extracts (see "Western blot analysis") were separated by electrophoresis under nonreducing conditions in a 10% polyacrylamide gel containing 1 mg/ml gelatin and 0.25 U/ml plasminogen. After electrophoresis, the gels were washed twice for 30 min in 2.5% Triton X-100, incubated with reaction buffer (100 mM glycine buffer, 5 mM EDTA, pH 8.3) for 24 h at 37°C to allow proteolysis, and stained with Coomassie blue G-250. Purified human uPA was used as a positive control.

Plasmin activity assay. Plasmin activity was measured by using a colorimetric assay, as previously described (46). Briefly, the assays were performed at 37°C in 100 mM Tris buffer (pH 7.8) containing 30 nM of Lys-plasminogen and 0.3 mM of the chromogenic plasmin substrate S-2251. Generation of *p*-nitroaniline upon S-2251 cleavage was proportional to plasmin activity and was measured at 405 nm.

In situ zymography with quenched fluorogenic substrate. Pericellular proteolytic activity was visualized using quenched fluorogenic casein as a substrate, as previously described (46).

Boyden chamber assay. A Matrigel invasion assay was performed using modified Boyden chambers in 24-well plates with filter inserts provided with 8- μ m pores (Transwell; Costar, Brumath, France), as described elsewhere (46). Matrigel-coated filters were used for invasion assays, and uncoated filters were used for migration assays. After 24 h of incubation, noninvasive cells on the upper surface of the filter were wiped out with a cotton swab, while the invaded cells on the lower surface of the filter were fixed with methanol and stained with crystal violet. Invasiveness was determined by counting cells in eight random microscopic fields per well.

Wound-healing assay. Cell migration was determined by wound-healing assay, as described previously (11). Briefly, cancer cells were seeded in gelatin-coated dishes, and after 24 h of quiescence, a wound was created across the surface of the cell monolayer with a pipette tip. The cells that moved into the scraped area to repair the injury were quantified by light microscopy for at least eight independent fields per plate.

Adhesion and trypsinization assays. Cancer cell adhesion to gelatin-coated surfaces was measured as already described (11). Trypsinization assays were carried out as described elsewhere (39, 60), by incubating cancer cells with 0.025% (wt/vol) trypsin for 10 min.

Immunofluorescence and confocal microscopy. FTC133 cells were seeded onto gelatin-coated glass coverslips for different times at 37°C and then fixed in acetone for 5 min on ice. After three washes in ice-cold PBS for 5 min, cells were incubated for 30 min at room temperature in blocking solution (PBS containing 2% bovine serum albumin). Coverslips were then incubated for 30 min with Alexa Fluor 568-phalloidin (red) or for 60 min with monoclonal antitalin or anti- α -actinin antibody, followed by three washes with PBS. Talin and α -actinin staining was revealed after 30 min of incubation with secondary antibodies conjugated to Alexa Fluor 488 (green). For p-FAK-paxillin colocalization, cells were incubated overnight at 4°C with mixed primary antibodies. Paxillin and p-FAK staining was revealed after 30 min of incubation with secondary antibodies conjugated to Alexa Fluor 488 (green) and Alexa Fluor 568 (red), respectively. Nuclei were stained with chromomycin A3 (red) or Hoechst reagent (blue). Nonimmune immunoglobulins G (IgGs) were used as controls. A Bio-Rad MRC 1024 system (Bio-Rad, Hercules, CA) mounted on an IX70 Axioplan optical microscope (Olympus, Shibuya-Ku, Tokyo, Japan) was used for acquisitions. All acquisitions were made using a UPlan FI 63 $\times$, 1.4-numerical-aperture objective. We used the 488-nm and 457-nm lines of an air-cooled 100-mW argon laser for excitations of Alexa Fluor 488 and chromomycin, respectively, and a 568-nm line of an air-cooled 60-mW krypton-argon laser for excitation of Alexa Fluor 568. Emitted fluorescence was detected through the use of the appropriate filter set, and images were captured with a 0.2- μ m z step. Images were treated with IMAGE J and Confocal Assistant software.

The percentage of cells positive for focal adhesions was determined as previously reported (43).

Densitometric analysis and statistical evaluation. Unstained areas corresponding to proteolytic degradation in zymography analysis and bands from immunoblots or agarose gels were quantified by using Quantity One image analyzer software (Bio-Rad Laboratories). The cell area was calculated by using IMAGE J image analysis software. All culture assays were normalized on the basis of cell viability by using the CellTiter-Glo assay from Promega. Each experiment was performed at least in triplicate, and data were expressed as means $\pm$ standard errors of the means. Comparisons were performed using Student's *t* test (Prism software; GraphPad Inc., San Diego, CA).

RESULTS

Specific silencing of endogenous LRP-1 by shRNA. To focus on the specific role of LRP-1 in tumor cell invasion, an RNA interference strategy against LRP-1 was conducted by using a vector-based shRNA approach. Two clonal cell lines that stably overexpress a specific shRNA for LRP-1 (shLRP-1-c1 and shLRP-1-c2) were selected, and a control cell line was established after transfection with pSuppressorNeo carrying the nonsilencing control shRNA (shCTRL). In parallel, to ensure that the biological effects observed were not due to off-target interactions of shRNA, a second independent double-stranded

RNA sequence (named siLRP-1) and its respective nonsilencing sequence (siCTRL) were used for each experiment.

The endogenous level of LRP-1 was assessed by both RT-PCR and immunoblotting (Fig. 1A and B). As expected, transfection with shCTRL had no effect on the LRP-1 expression level. Conversely, the LRP-1-specific shRNA plasmid was able to efficiently knock down the expression of LRP-1 at the mRNA level as well as at the protein level, by >90%. The same inhibition was observed for both shLRP-1 clonal cell lines, demonstrating that the observed effect was not clonal. Transfection of siLRP-1 led to about 70% inhibition of LRP-1 expression (Fig. 1A and B, right panels). Because it is known that double-stranded RNA can lead to the induction of a nonspecific interferon response (16, 50), we explored whether the interferon system was activated in transfected cells (Fig. 1C). Our data did not show any up-regulation of the interferon-stimulated proteins STAT1 and OAS, thus demonstrating the lack of interferon induction following expression of siRNA or shRNA. Altogether, these results validate the specific LRP-1 silencing.

LRP-1-mediated endocytosis is abrogated in shLRP-1-overexpressing cells. We first investigated whether shRNA-mediated inhibition of LRP-1 expression resulted in reduced uptake and degradation of specific ligands. Since LRP-1 was identified as the α 2M receptor (51), we used FITC-labeled α 2M as a test ligand (Fig. 1D). As expected, similar intracellular fluorescence levels were observed in shCTRL, siCTRL, and wild-type (WT) carcinoma cells. This indicates that overexpression of the nonsilencing RNAs had no significant effect on LRP-1-mediated endocytosis. In contrast, a drastic reduction of the intracellular fluorescence was measured in LRP-1-silenced cells (shLRP-1 and siLRP-1).

LRP-1 shRNA-mediated accumulation of extracellular MMP-2 and uPA activities. Since LRP-1-mediated endocytosis was reported to be a major process regulating the matrix-degrading activities involved in cancer progression, the two main proteolytic enzymes produced by FTC133 cells, i.e., MMP-2 and uPA (46), were explored in LRP-1-silenced cancer cells (Fig. 2). As revealed by gelatin zymography (Fig. 2A and B), two gelatinolytic bands, corresponding to latent MMP-2 (72 kDa) and active MMP-2 (62 kDa), were detected in medium conditioned by wild-type cells. An overall increase in the amount of extracellular MMP-2 was detected for shLRP-1-transfected cells compared to shCTRL cells or wild-type cells. Indeed, both the latent (72 kDa) and active (62 kDa) forms of MMP-2 were increased in the conditioned medium, by 1.5- and 2.2-fold, respectively. In the membrane extracts, MMP-2 was present only as the latent form, and no variation was observed with LRP-1 silencing (Fig. 2A and B). Similar results were obtained using the siRNA sequence (Fig. 2A and C).

As revealed by gelatin-plasminogen zymography, the shRNA-mediated inhibition of LRP-1 resulted in a twofold accumulation of extracellular uPA in conditioned medium (Fig. 2D and E). The amount of cell surface uPA was also increased, about 2.6-fold, when LRP-1 was silenced compared to the level in clonal control or wild-type FTC133 cells (Fig. 2D and E). Similar results, but to a lesser extent, were obtained with siLRP-1-overexpressing cells (Fig. 2D and F). Activated uPA catalyzes the conversion of plasminogen to plasmin, which is in turn able to degrade many extracellular matrix compo-

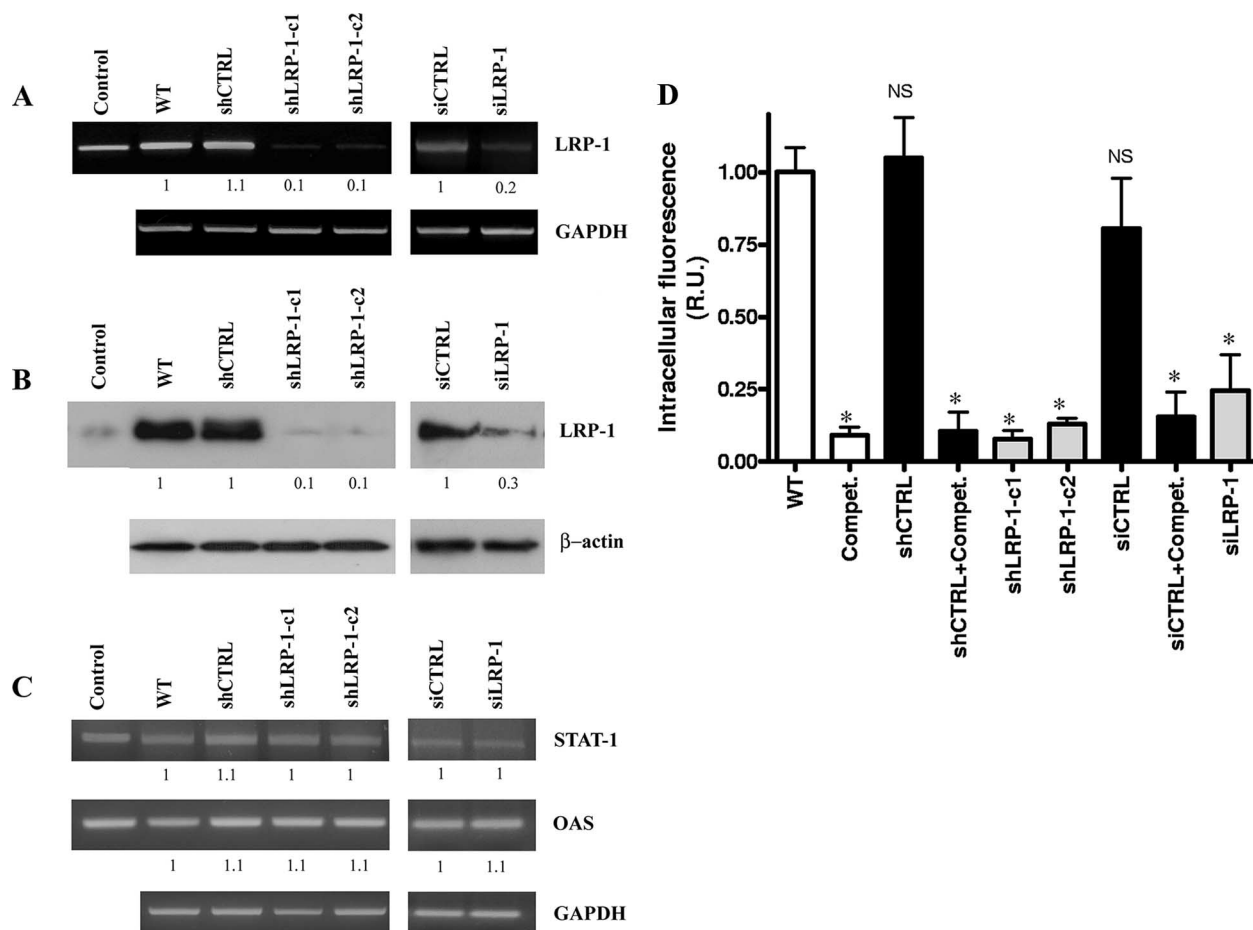


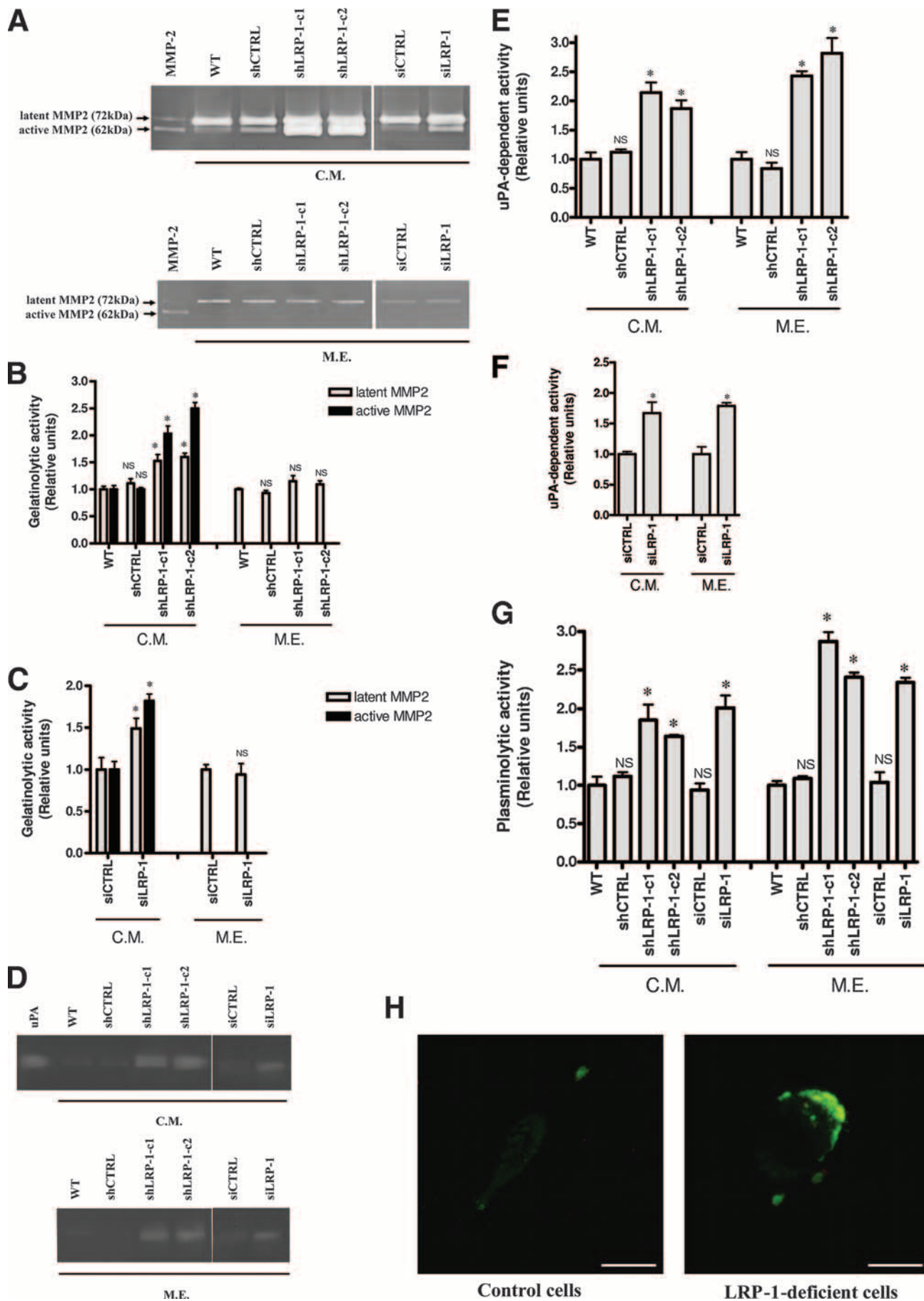
FIG. 1. The short hairpin LRP-1 approach leads to specific LRP-1 silencing and to abrogation of LRP-1-mediated endocytosis in carcinoma cells. Total RNAs were purified from FTC133 wild-type (WT) cells, the control clonal cell line (shCTRL), two clonal cell lines that stably overexpress specific shRNAs for LRP-1 (shLRP-1-c1 and shLRP-1-c2), and siRNA-transfected cells (siCTRL and siLRP-1). (A) The transcriptional level of LRP-1 was assessed by RT-PCR. GAPDH primers were used as a normalization control. (B) Whole-cell extracts from each cell line were subjected to immunoblot analysis with anti-LRP-1 antibody. β -Actin antibody was used for normalization. (C) Total RNAs were isolated from each cell line, and the expression of the interferon-stimulated proteins STAT1 and OAS was measured by RT-PCR. GAPDH primers were used as a control. (D) Each cell line was incubated for 30 min in serum-free medium containing FITC-labeled human α 2M in the presence of 100 μ M chloroquine. The specificity of internalization in WT and control cells was controlled by using a 100-fold higher concentration of the nonlabeled protein in a competition experiment (Compet.). The intracellular fluorescence was determined as described in Materials and Methods and expressed in relative units (R.U.) compared with the signal from WT cells. Each value is the mean $\pm$ standard deviation (SD) for three separate experiments. The gels and immunoblots are representative of three separate experiments. Numbers under the gels and immunoblots indicate the levels of induction (n -fold) by comparison with the WT (left panels) or siCTRL (right panels) cells. NS, differences from the WT are not significant; *, $P < 0.01$.

nents (17). Therefore, uPA-dependent plasmin activation was quantified using a chromogenic assay, as previously reported (9, 46). As shown in Fig. 2G, plasmin generation was increased about 1.8-fold in the conditioned medium and 2.6-fold at the plasma membrane in LRP-1-deficient carcinoma cells compared to that in control cells. Increased pericellular matrix degradation in LRP-1-deficient cells was confirmed by an in situ zymography experiment using Bodipy FL casein as a substrate (Fig. 2H).

The extracellular accumulation of both MMP-2 and uPA in LRP-1-deficient cells was confirmed by Western blotting (Fig. 3A). Extracellular MMP-2 and uPA amounts were increased 2.1- and 2-fold, respectively, when LRP-1 was silenced by the shRNA strategy. Down-regulation of LRP-1 in siLRP-1-overexpressing cells led to 1.5- and 1.7-fold increases, respectively.

To ensure that protease accumulation was not due to transcriptional regulation, MMP-2 and uPA were also analyzed at the mRNA level (Fig. 3B). No change in either the MMP-2 or uPA transcript level was observed.

LRP-1 silencing impairs invasive capacity of malignant cells. We previously demonstrated that thyroid cancer cell invasion was not ameboid and was governed mainly by protease-dependent processes (46). The increased extracellular proteolytic activities upon LRP-1 silencing should therefore enhance the ability of the cells to invade the matrix network. To test this hypothesis, a cell invasion assay was carried out in which we assessed the ability of the LRP-1-deficient cells to migrate through Matrigel-coated Transwell invasion chambers (Fig. 4). The invasive properties of wild-type cells were unaffected by overexpression of the nonsilencing RNA sequences (Fig. 4A



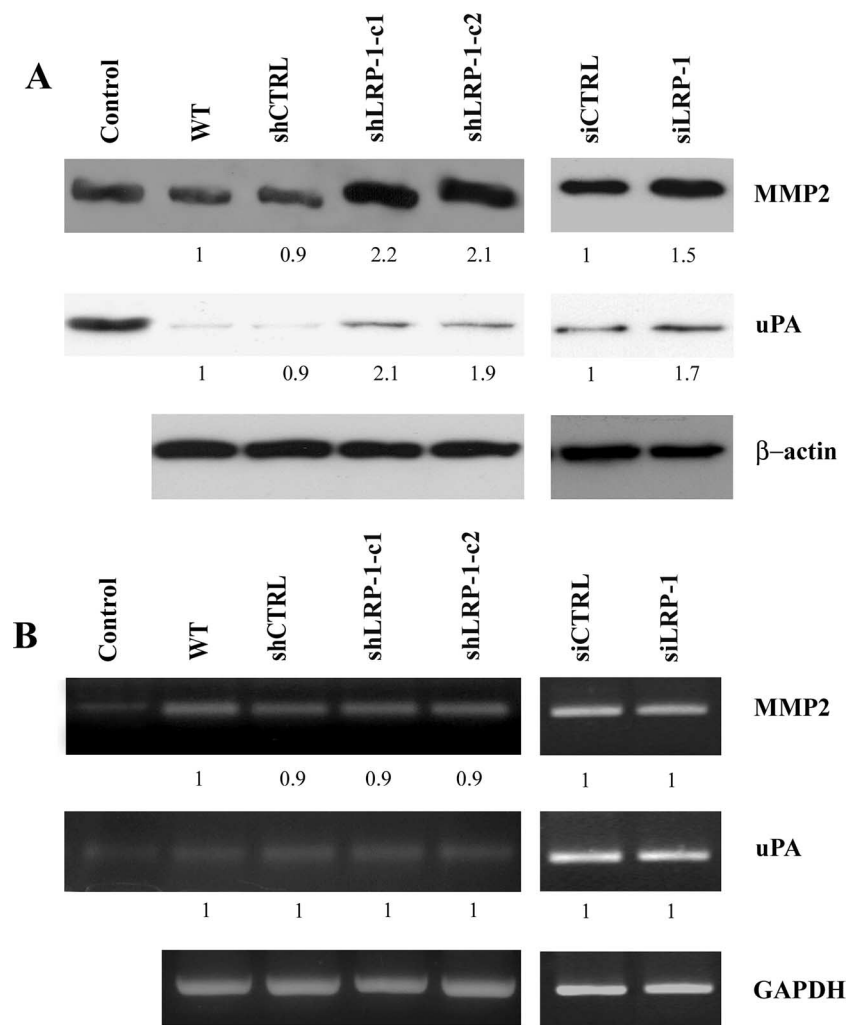


FIG. 3. LRP-1 silencing contributes to maintaining high levels of the soluble proteinases MMP-2 and uPA secreted into the extracellular environment. (A) Serum-free conditioned medium from WT, shCTRL, shLRP-1-c1, shLRP-1-c2, siCTRL, and siLRP-1 cells was collected after 24 h. The proteins were subjected to Western blotting analysis using anti-uPA, anti-MMP-2, and anti- β -actin antibodies. Purified human MMP-2 and uPA were used as positive controls. (B) Total RNAs were isolated from these cells, and the expression of the MMP-2 and uPA genes was assessed by RT-PCR. GAPDH amplification was used as a control. Representative gels and immunoblots are presented. Numbers under the gels and immunoblots indicate the levels of induction (*n*-fold) by comparison with WT (left panels) or siCTRL (right panels) cells.

and B). However, surprisingly, the number of cells that invaded the lower surface of the filter was reduced fourfold when LRP-1 expression was silenced by using the shRNA approach. When LRP-1 expression was inhibited to a lesser extent by using the siLRP-1 approach (Fig. 1), cellular invasion was inhibited only 2.5-fold.

LRP-1 is required for two-dimensional and three-dimensional carcinoma cell migration. To understand how LRP-1-deficient cells exhibited reduced invasive capacities despite the accumulation of extracellular MMP-2 and uPA-dependent activities, a three-dimensional cell migration assay was performed with uncoated Transwell chambers. As presented in

FIG. 2. LRP-1 silencing mediates the increase of MMP-2- and uPA-dependent pericellular proteolysis and the stimulation of plasmin generation in human malignant cells. (A to G) WT, shCTRL, shLRP-1-c1, shLRP-1-c2, siCTRL, and siLRP-1 cells were cultured for 24 h in gelatin-coated dishes in the absence of serum. Gelatinolytic (A to C) and uPA-dependent (D to F) activities were evaluated by gelatin and gelatin-plasminogen zymography, respectively, with conditioned medium (C.M.) and membrane extracts (M.E.). Purified human MMP-2 (A) or uPA (D) was used as a positive control. Representative zymograms from three independent experiments are shown. Quantifications of the zymograms are presented in panels B, C, E, and F. (G) Plasmin generation was quantified by a colorimetric assay with the same samples. For panels B, E, and G, values were normalized by comparison to those obtained with WT cells. For panels C and F, values were normalized by comparison to those obtained with siCTRL cells. Results are presented in relative units, as means $\pm$ SD for triplicate wells from three separate experiments. NS, not significant; *, significantly different from the corresponding control ($P < 0.01$). (H) Control and LRP-1-silenced cells were subjected to in situ zymography, using Bodipy FL casein as a fluorogenic substrate, to detect pericellular proteolytic activities (green). Bars, 20 μ m.

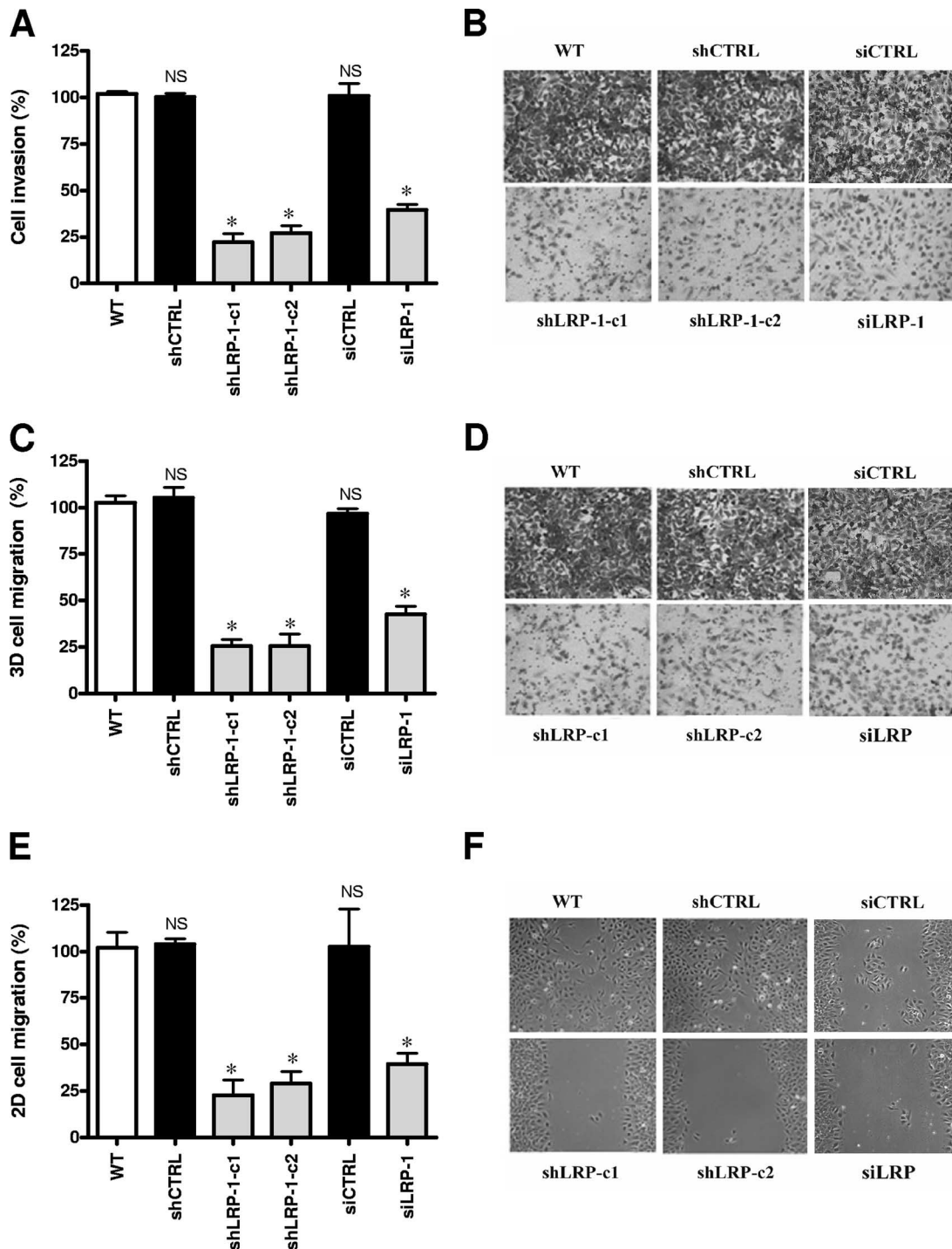


FIG. 4. Carcinoma cell invasion and migration processes are altered to the same extent by LRP-1 silencing. Cell invasion and cell migration assays were carried out with WT, shCTRL, shLRP-1-c1, shLRP-1-c2, siCTRL, and siLRP-1 cells. (A and B) Tumor cell invasion was measured on Matrigel-coated Transwell membranes. (C and D) Three-dimensional cell migration was assessed by using uncoated filters. (E and F) Two-dimensional cell migration was determined by a wound-healing assay. Representative images are shown. Results for invasion assays were obtained from eight separate experiments, with each performed in triplicate, and results for migration assays were obtained from three separate experiments, with each performed in triplicate. Invasion and migration were determined by counting cells in eight random microscopic fields per well. Results are expressed as means $\pm$ SD after normalization by comparison with WT cells. NS, differences from WT were not significant; *, $P < 0.01$.

Fig. 4C and D, the control cells (shCTRL and siCTRL) exhibited a migratory activity comparable to that of wild-type cells. The migration of siLRP-1- and shLRP-1-overexpressing cells was diminished 2.5- and 4-fold, respectively. To confirm this

result, a two-dimensional wound-healing assay was performed. As presented in Fig. 4E and F, the shLRP-1-overexpressing carcinoma cells exhibited 4-fold-repressed two-dimensional cell migration, whereas the siLRP-1 approach led to 2.5-fold

repression. These results emphasized the similarity of the effects of LRP-1 inhibition on two- and three-dimensional cell migration and cell invasiveness. They therefore support the concept that the altered invasive capacities of LRP-1-silenced cells should be related directly to migration defects.

Accelerated rate of cell attachment and inhibited cell-substrate deadhesion in LRP-1-silenced carcinoma cells. Because LRP-1 silencing inhibits carcinoma cell invasion and migration to the same extent, we hypothesized that the inhibition could be due to an LRP-1-dependent regulation of malignant cell adhesion and spreading. The different clonal cell lines were seeded in 96-well plates coated with gelatin, and nonadherent cells were discarded at different times. After 30 min, 50% of LRP-1-silenced cells were adherent, compared with only 12% of control cells (Fig. 5A). After 90 min of attachment, 100% of LRP-1-silenced cells were adherent, as opposed to only 40% of control cells. A time of 180 min was required to reach 100% attachment of control cells. Similar results were obtained with siLRP-1-overexpressing cells (Fig. 5B). In order to further investigate whether LRP-1 silencing increases the adherence of carcinoma cells and whether LRP-1 facilitates cell-substrate deadhesion, the numbers of both LRP-1-silenced cells and control cells that detached from their substrate were quantified after trypsin incubation (Fig. 5C). LRP-1-deficient cells on gelatin-coated dishes were twice more resistant to trypsin detachment than control carcinoma cells. Similar results were obtained with shLRP-1-c2 and by using fibronectin as a substrate (data not shown).

LRP-1-mediated control of carcinoma cell shape and spreading. To determine whether cell spreading is LRP-1 dependent, control clonal cells and LRP-1-silenced carcinoma cells were allowed to attach to gelatin-coated dishes for different times before examination of spreading (Fig. 6). After 1 h, control cells had spread and had begun to exhibit thin projections (Fig. 6C). Several distinct protrusions and filopodia consistent with migratory activity were detected after 90 min (Fig. 6E and G). In striking contrast, LRP-1-silenced cells displayed overspread morphology with membrane ruffles and failed to extend filopodia (Fig. 6D, F, and H). The area of LRP-1-silenced cells represented 180% and 160% that of control cells after 60 and 120 min of spreading, respectively (Fig. 7). Similar results were obtained with shLRP-1-c2 (data not shown) and by using siLRP-1-overexpressing cells (unpublished data).

Altogether, these results indicate that unlike fast-invasive carcinoma control cells, LRP-1-silenced cells display major morphological changes and develop strong cell-matrix interactions.

Actin cytoskeleton organization and focal adhesion complex disruption are controlled by LRP-1 in carcinoma cells. To determine whether LRP-1 is a regulator of the actin cytoskeleton and is required in the focal adhesion turnover in malignant cells, clonal cells were allowed to spread onto gelatin. The organization of actin filaments during spreading of shRNA-transfected cells was examined by staining with fluorescently labeled phalloidin. The distribution of alpha-actinin and talin was detected by immunolabeling with specific antibodies. Staining of control cells with phalloidin at different early stages of spreading revealed a highly polarized morphology, with the presence of prominent actin-rich fibers at the protrusion sites (Fig. 8A and C). Central stress fibers appeared in control cells

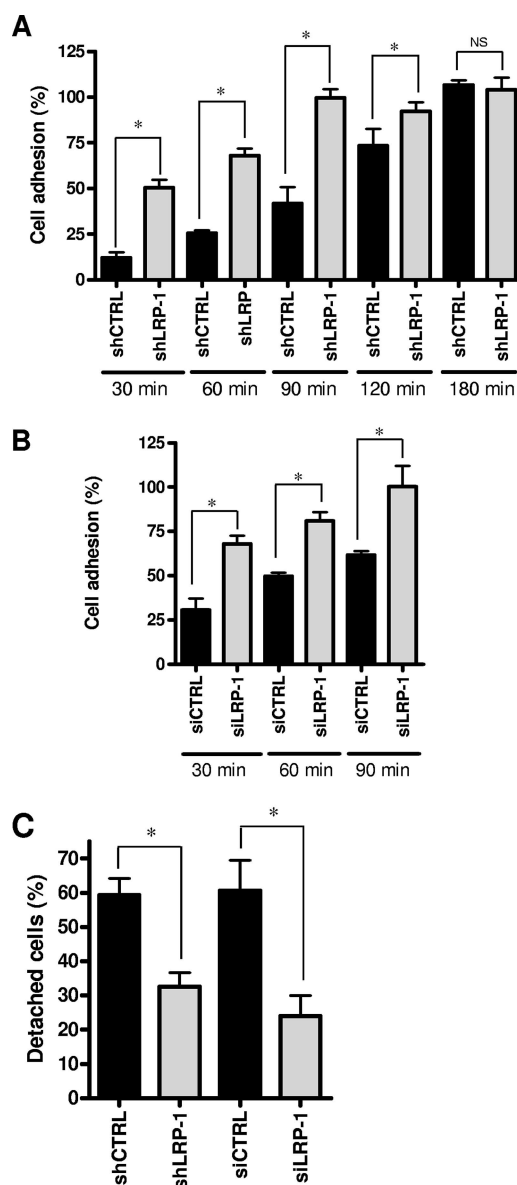


FIG. 5. LRP-1 silencing stimulates the rate of carcinoma cell attachment and inhibits cell-substrate deadhesion. (A) shCTRL and shLRP-1-c1 cells were seeded onto gelatin-coated plates, and the nonadherent cells were discarded after 30, 60, 90, 120, or 180 min. For each cell type, results are expressed as percentages of adherent cells. (B) The same experiment was performed using siCTRL and siLRP-1 cells. (C) Control (shCTRL and siCTRL) and LRP-1-silenced (shLRP-1 and siLRP-1) cells were grown in gelatin-coated dishes for 24 h and subjected to trypsinization assay by incubating cells with 0.025% (wt/vol) trypsin for 10 min. For each cell type, results are expressed as percentages of detached cells. Each value is the mean $\pm$ SD for four separate experiments, with each performed in triplicate. NS, differences from corresponding control were not significant; *, $P < 0.01$.

after 2 h of spreading but remained poorly detectable (Fig. 8C). In contrast, LRP-1-silenced cells exhibited prominent membrane ruffles and revealed numerous peripherally and centrally distributed stress fibers (Fig. 8B and D).

Alpha-actinin is an actin-binding protein that cross-links ac-

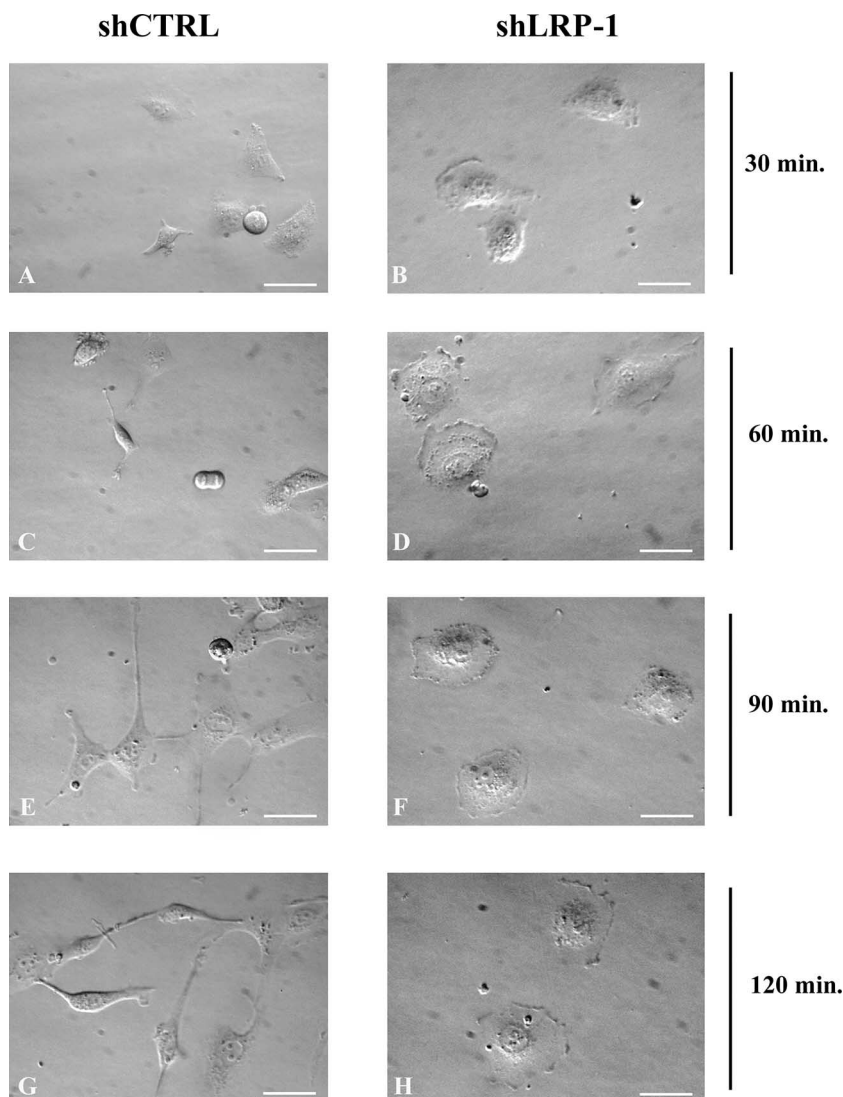


FIG. 6. LRP-1 silencing promotes human carcinoma cell spreading. shCTRL (A, C, E, and G) and shLRP-1-c1 (B, D, F, and H) cells were seeded onto gelatin-coated plates for 30 (A and B), 60 (C and D), 90 (E and F), or 120 (G and H) min and visualized by phase-contrast microscopy. Images are representative of three separate sets of cultures. Similar results were obtained with shLRP-1-c2 cells. Bars, 40 μ m.

tin filaments and adhesive complexes. At distinct stages of spreading, alpha-actinin was detected only at the cell periphery of control cells, mainly in protrusions and membrane projection sites (Fig. 8E and G). In LRP-1-silenced carcinoma cells, alpha-actinin was largely located in membrane ruffles and organized in a highly and broadly structured network extending out from the center to the periphery of the cell and supporting the overspread morphology (Fig. 8F and H).

Immunolabeling using antitalin antibody at distinct stages of spreading showed that LRP-1-silenced carcinoma cells exhibited more talin-containing focal adhesion complexes than did fast-invading control cells, and these were located at the cell periphery (Fig. 9, compare panels B, D, F, and H to panels A, C, E, and G). The accumulation of focal complexes in LRP-1-silenced cells was not modified upon cell-cell contact (Fig. 9, compare panels D and H to panels C and G). To confirm that LRP-1 mediates the disruption of adhesion complexes in ma-

lignant cells, the percentage of cells positive for focal adhesions was determined as recently reported (43). LRP-1 silencing increased that percentage about sixfold (Fig. 9I), suggesting that focal adhesion disassembly is inhibited when LRP-1 is silenced. Similar results were obtained with shLRP-1-c2 (data not shown) and with siLRP-1-transfected cells (unpublished data).

LRP-1-mediated regulation of talin amount in malignant cells. Western blot analyses were performed at different stages of spreading to detect talin and alpha-actinin (Fig. 10A). Although the cellular distribution of alpha-actinin in LRP-1-silenced carcinoma cells was different from that in control cells (Fig. 8F and H versus Fig. 8E and G), the expression levels were similar (Fig. 10A). On the other hand, talin was found to accumulate in control cells in correlation with the time course of cellular attachment. At each

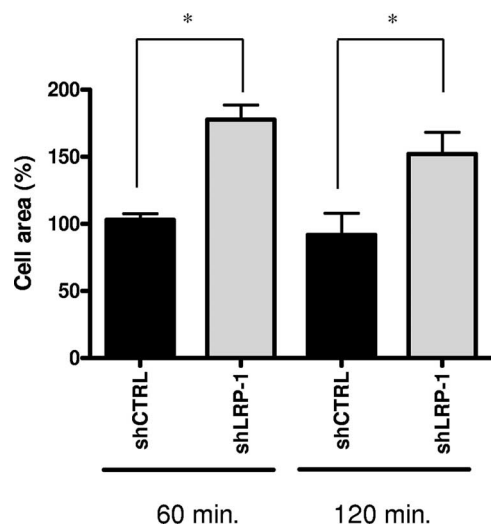


FIG. 7. LRP-1-silenced carcinoma cells exhibit a twofold increased cell area compared to control cells. shCTRL and shLRP-1-c1 cells were seeded onto gelatin-coated plates for 60 or 120 min. Cell area was calculated with Image J software by using 200 isolated cells for each cell line in two independent experiments. The shCTRL cell area was scaled up to 100%. *, $P < 0.01$. Similar results were obtained with shLRP-1-c2 cells.

stage of spreading, the native form of talin was accumulated when LRP-1 was lacking.

LRP-1 controls paxillin and FAK targeting to focal complexes in malignant cells. To understand how LRP-1 may control focal adhesion remodeling, we analyzed the expression level of the focal adhesion adaptor protein paxillin (56) (Fig. 10B). We demonstrated that paxillin expression was drastically inhibited in LRP-1-silenced cells at each stage of spreading. Two of the main paxillin-binding partners, i.e., vinculin and FAK (56), were likewise analyzed (Fig. 10B). The expression pattern of vinculin in LRP-1-deficient cells was similar to that in control cells. In contrast to the case for fast-invading carcinoma control cells, in cells lacking LRP-1, the amounts of total FAK and of FAK phosphorylated at Y576 were reduced to the same extent after 120 min of spreading.

To test whether LRP-1 was able to regulate the amounts of paxillin and FAK at focal complexes, the talin-containing focal contacts were immunoprecipitated. We established that LRP-1 silencing inhibited both paxillin and FAK association with these focal complexes (Fig. 10C). Immunolabeling using anti-paxillin and anti-p-FAK antibodies revealed that paxillin and p-FAK were colocalized at focal contact sites in control cells (arrows in merged image) but not in LRP-1-deficient carcinomas (Fig. 10D).

Altogether, our results clearly indicate that LRP-1 silencing induces major reorganization of the cytoskeleton and prevents the turnover of adhesive complexes, thus altering the ability of cancer cells to migrate and invade.

DISCUSSION

In this study, we used two independent and complementary strategies to silence LRP-1 expression. After thorough validation of efficiency and target specificity, we showed that LRP-1

silencing leads to a large decrease of cell invasive capacity. We evidenced that LRP-1 regulates cytoskeletal organization and focal complex composition and further demonstrated that reduced cell invasion is correlated with increased cell adhesion and spreading.

Invalidation of LRP-1 expression in human thyroid carcinoma cells resulted in increased levels of MMP-2 and uPA in the extracellular compartment. These results obtained by specific gene silencing confirm those previously reported by using LRP-1 blocking antibodies or exogenous RAP (19, 62). Furthermore, under our experimental conditions, we detected a strong accumulation not only of active MMP-2 but also of its latent form, without any change in the transcriptional level of the protease, suggesting that LRP-1 is also capable of mediating the endocytic uptake of pro-MMP-2. We previously reported that LRP-1 promotes endocytic clearance of pro-MMP-2 in complex with TIMP-2 (19). However, TIMP-2 expression was undetectable in our cellular environment (data not shown). This suggests that LRP-1 mediates the clearance of the TIMP-2-free pro-MMP-2, as previously reported by others (62). Moreover, our data evidenced an alternative TIMP-2-independent activation of pro-MMP-2, as previously suggested (35, 37).

By opposition to this first set of predictable data, our further observations that LRP-1 silencing induces a drastic reduction in cell migration and invasion appear as a paradox to previous reports showing that cell invasion is directly correlated with extracellular proteolytic activity and inversely correlated with LRP-1 expression. Indeed, through its aptitude to bind and internalize various proteolytic enzymes from the extracellular environment (18), LRP-1 is expected to prevent extracellular matrix remodeling and subsequently to limit cell invasiveness. Accordingly, low LRP-1 expression was repeatedly correlated with the invasive phenotype of tumor cells derived from various human tissues. For example, Desrosiers and colleagues (14) recently established that the LRP-1-mediated clearance of proteases was severely decreased in advanced stages of pediatric Wilms's tumors. Moreover, the amount of membrane-anchored LRP-1 was equally significantly reduced in endometrial carcinoma compared to that in the normal endometrium (20). We recently established that the aggressive behavior of thyroid carcinoma cells seemed inversely related to LRP-1 expression (46). Furthermore, LRP-1-deficient cells and RAP-treated cells exhibited increased migration and invasion (32, 57, 58, 61). On the other hand, in previous reports, accumulated membrane-interacting uPA and increased pericellular uPA-dependent plasminogen activation led to stimulation of cell mobility (1, 46). Consequently, uPA targeting is currently considered an attractive strategy for anticancer therapy (47). Nonetheless, it was previously reported that an excessive degradation of the extracellular environment may lead to decreased invasive capacities (13). In this report, we demonstrate that LRP-1 silencing in carcinoma cells reduced their migration in both two and three dimensions, as well as their invasiveness. In agreement with our results, RAP treatment previously decreased the invasive properties of a highly aggressive breast cancer cell line (31). In addition, LRP-1 neutralization was reported to reduce smooth muscle cell migration, although without altering invasive ability (30, 41, 54).

A putative explanation for this apparent paradox is that the

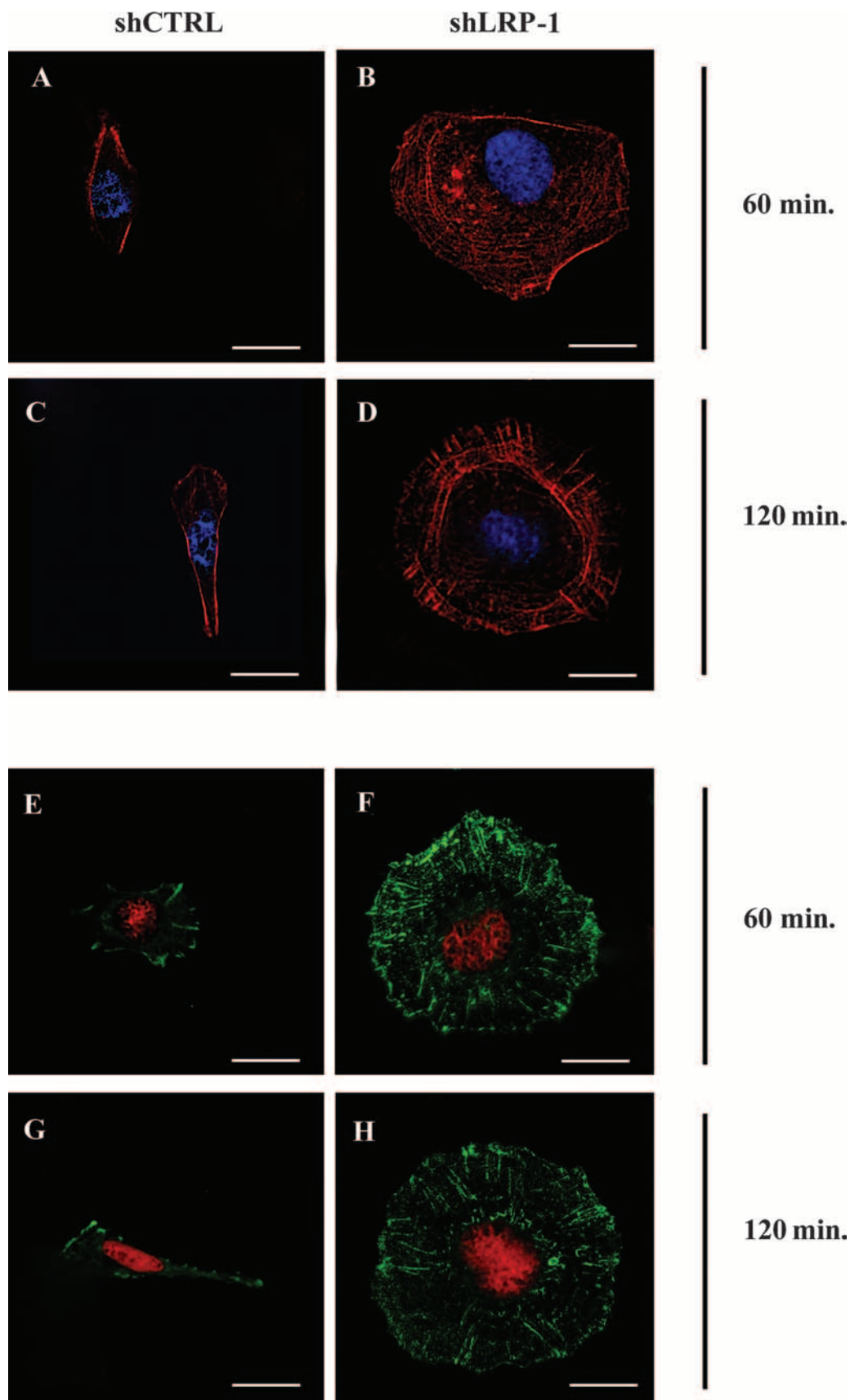


FIG. 8. LRP-1-silenced carcinoma cells exhibit striking differences in focal adhesion organization. shCTRL (A, C, E, and G) and shLRP-1-c1 (B, D, F, and H) cells were plated onto gelatin-coated coverslips for 60 or 120 min. Cells were stained for actin filaments (red) (A to D) or alpha-actinin (green) (E to H), and nuclei were counterstained in blue (A to D) or red (E to H). Images are representative of three separate experiments. Similar results were obtained with shLRP-1-c2 cells. Bars, 20 μ m.

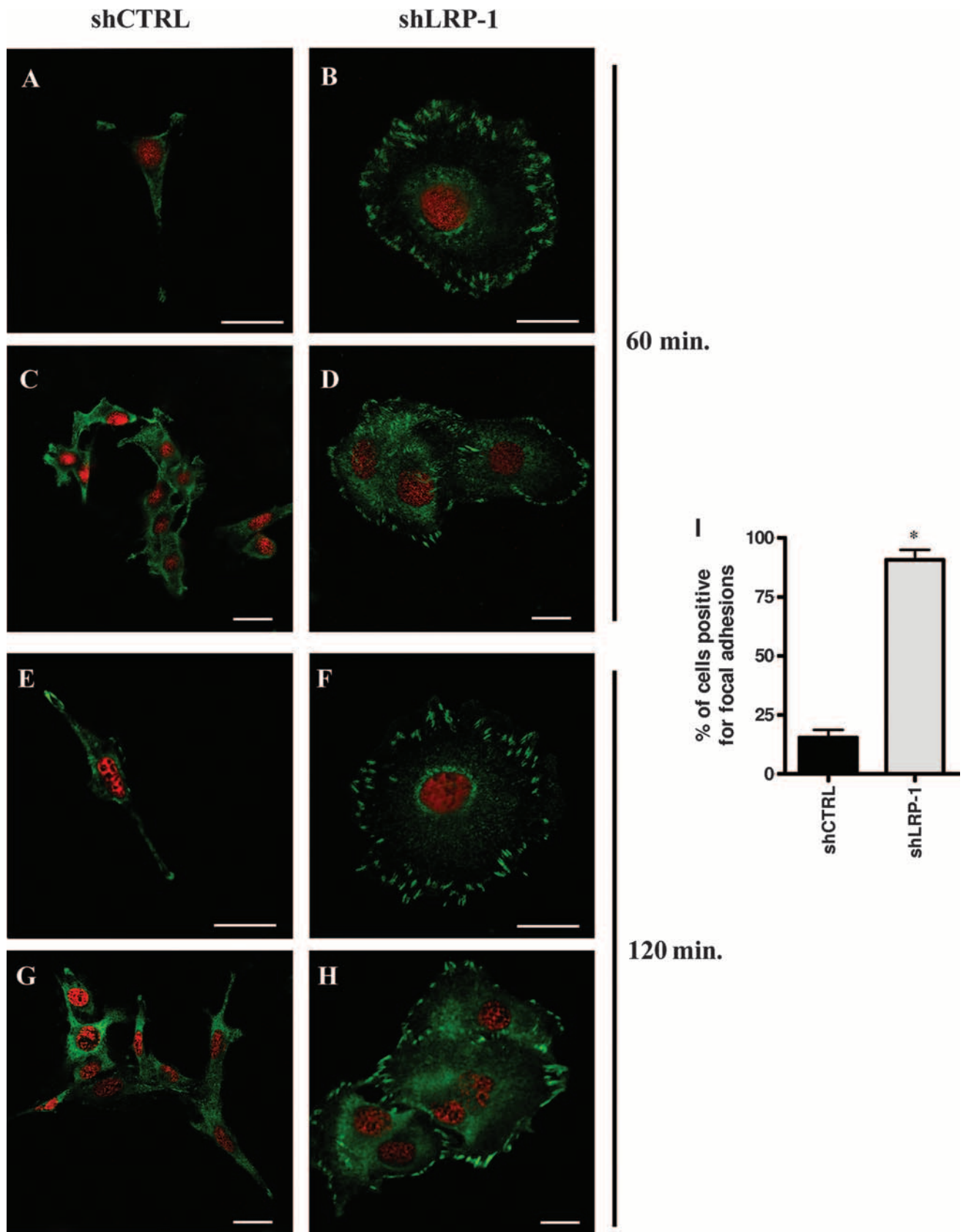


FIG. 9. Disruption of talin-containing focal complexes is inhibited in LRP-1-silenced carcinoma cells. shCTRL (A, C, E, and G) and shLRP-1-c1 (B, D, F, and H) cells were plated onto gelatin-coated coverslips for 60 or 120 min. Isolated cells (A, B, E, and F) and grouped cells (C, D, G, and H) were stained for talin (green), and nuclei were counterstained (red). Images are representative of three separate experiments. Bars, 20 μm. (I) Cells were assayed for the percentage of cells positive for focal adhesions. Two hundred fifty cells for each clonal cell line were evaluated from three separate experiments. *, $P < 0.01$. Similar results were obtained with shLRP-1-c2 cells.

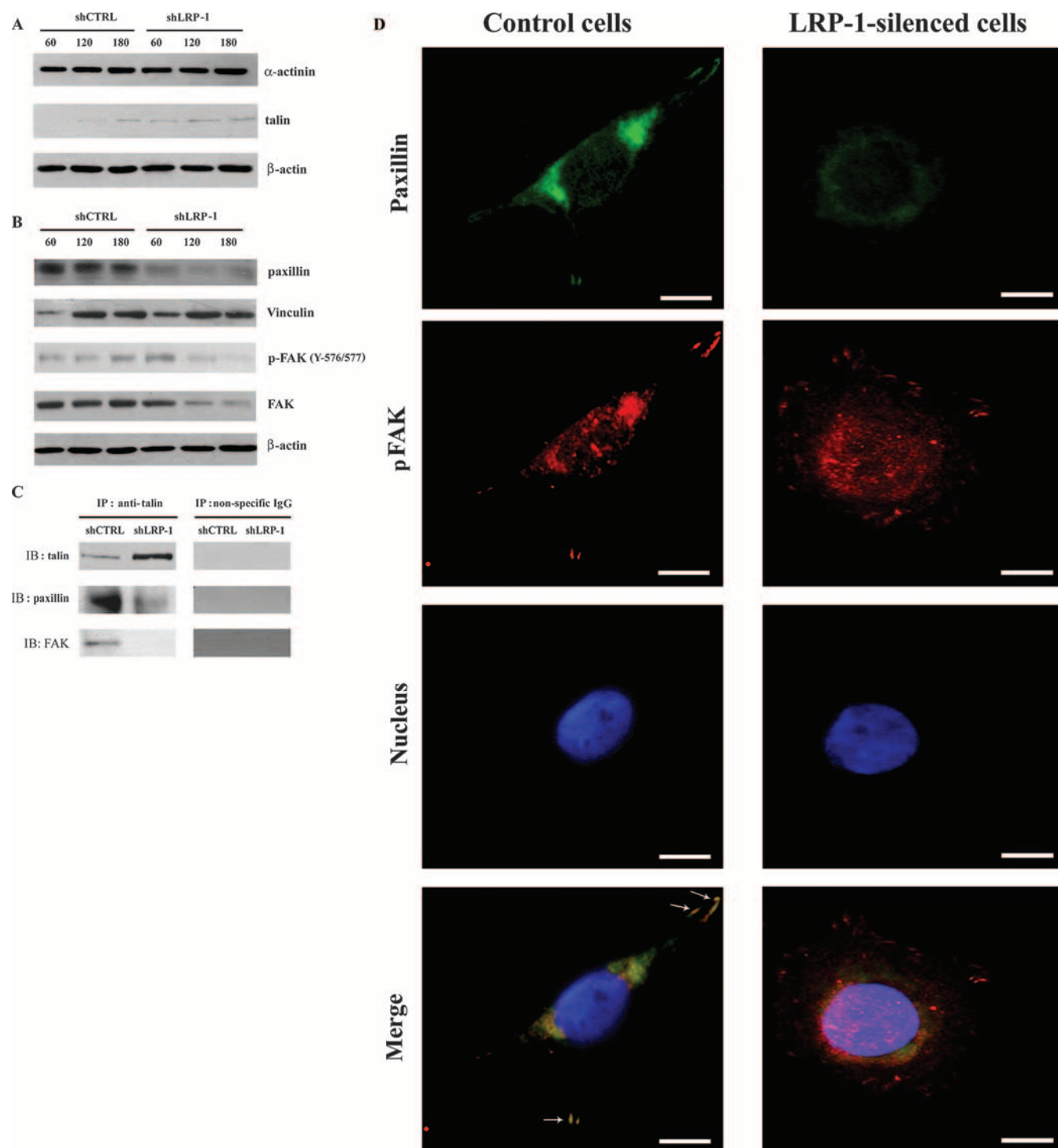


FIG. 10. LRP-1 mediates the control of focal complex composition in malignant cells. (A and B) shCTRL and shLRP-1-c1 cells were plated onto gelatin-coated coverslips for 60, 120, or 180 min. Whole-cell extracts were subjected to Western blot analysis. (A) Anti- α -actinin and anti-talin antibodies were used. (B) Antipaxillin, antivinculin, anti-phospho-FAK (Y576/577), and anti-FAK antibodies were used. β -Actin labeling was used for normalization. (C) Immunoprecipitation of talin-containing focal complexes was performed (IP: anti-talin), and the immunocomplexes were immunoblotted (IB) by using antitalin, antipaxillin, and anti-FAK antibodies. Nonspecific IgG was used as a negative control for immunoprecipitation. Immunoblots are representative of three separate experiments. (D) Control and LRP-silenced cells were stained for paxillin (green) and p-FAK (red), and nuclei were counterstained (blue). Representative images are shown. Bars, 10 μ m. Similar results were obtained with siLRP-1-transfected cells.

lack of LRP-1 at the cell surface may disrupt LRP-1's capacity to control cell motility through interactions with cell signaling receptors, such as platelet-derived growth factor (PDGF) or the uPA receptor. An abnormal activation of PDGF receptor signaling was recently detected after inactivation of LRP-1 in fibroblasts and smooth muscle cells (3, 55). Webb and collaborators (58) also reported that LRP-1 can influence cell motility by controlling the activity of the uPA/uPAR system and the associated signaling pathway. Binding of uPA to the membrane-activated signaling targets, such as Src, extracellular signal-regulated kinase (ERK), and the AP-1 complex, leads to increased motility (29, 47). In addition, molecular interactions between LRP-1 and the urokinase trimolecular complex uPAR-uPA-PAI-1 were necessary for cancer cell migration (6, 9), and the PAI-1-mediated stimulation of cell migration likely requires an LRP-1-dependent signaling pathway (12). However, such changes in signal transduction due to a lack of uPAR-LRP-1 interaction seem not to be sufficient to explain the inhibition of cell mobility detected in our cellular environment upon LRP-1 silencing. Indeed, blocking LRP-1 interactions with all other signaling receptors and extracellular ligands through RAP addition to FTC133 cells resulted in increased, not reduced, cell motility (46).

Our data strongly suggest that delayed migration and invasion exhibited by LRP-1-silenced carcinoma cells are not correlated with a defect in ligand binding and endocytic activity and may depend on an additional mechanism(s). Montel and colleagues recently suggested that LRP-1 can facilitate metastasis *in vivo* through the stimulation of cancer cell survival (36). Interestingly, we shed light for the first time on another mechanism, i.e., the capacity of LRP-1 to decrease native talin levels in carcinoma cells. Talin is well known to strengthen integrin/cytoskeleton connections and to stabilize focal complexes at adhesion sites (21). A defect in talin down-regulation, and its subsequent accumulation at the cell periphery, may explain why LRP-1-deficient carcinoma cells exhibit abnormal cytoskeletal architecture and display stronger adhesive properties than do parental cells. Furthermore, we established that LRP-1 is required for both paxillin and FAK association with focal contacts. Paxillin, a scaffold protein for recruitment of signaling molecules to the plasma membrane, functions as a main regulator of the actin cytoskeleton (56). FAK is a signaling molecule highly implicated in the regulation of focal adhesion disassembly and is found to be overexpressed in a range of tumor cells (59). Increased expression of FAK was recently directly associated with the development of thyroid carcinogenesis (28, 44), thus supporting our findings.

Altogether, our results identify LRP-1 as a crucial mediator of cancer cell de-adhesion. The capacity of LRP-1 to regulate the amount and localization of structural and signaling markers of focal complexes improves our understanding of the mechanism by which LRP-1 controls the adhesive properties of cancer cells. Our results differ from recent data showing that the blockade or absence of LRP-1 at the cell surface strongly decreases spreading and abrogates cell attachment (6, 45, 49, 62). However, in agreement with our results, induction of focal adhesion disassembly of endothelial cells was recently reported to occur in a RAP-dependent manner (43). Moreover, Chazaud and colleagues (6) localized LRP-1 in filopodia, at the leading edge of migrating cells, where focal complex dis-

assembly occurs. Consequently, LRP-1 appears as a major scaffold receptor mediating adhesion turnover through intracellular signaling. Focal adhesion disassembly may be mediated by the ERK signaling pathway (42), and LRP-1 was proposed as a regulator of ERK phosphorylation in HT1080 fibrosarcoma cells (58). However, it remains to be assessed whether LRP-1 can induce cancer cell de-adhesion by activating the ERK pathway.

Targeting the proteolytic activities associated with invasive cancer cells has long been regarded as a promising option for the development of potent anticancer therapies (13, 15). However, by disregarding other pivotal events influencing three-dimensional cell invasion, in particular the balance between cellular adhesion and contractile forces, such strategies could remain inadequate and ineffective. Indeed, a recent study elegantly demonstrated that three-dimensional mobility requires a subtle balance between at least proteolysis, matrix stiffness, traction forces, and cell-matrix adhesiveness (63). In agreement with this report, we demonstrate that the ability of cancer cells to invade can be altered effectively by targeting the LRP-1-mediated control of cell adhesiveness, in spite of increased extracellular proteolysis. Our results clearly indicate that strategies aiming at LRP-1 silencing, not enhancement, could provide new therapeutic approaches against metastasis.

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