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Phosphatase of regenerating liver-3 promotes migration and invasion by upregulating matrix metalloproteinases-7 in human colorectal cancer cells

Su-Kyung Lee[1]*, Young-Min Han[1]*, Jieun Yun[2], Chang Woo Lee[2], Dae-Seop Shin[1], Young-Ran Ha[1], Jina Kim[1], Jong Sung Koh[3], Su-Hyung Hong[4], Dong Cho Han[1] and Byoung-Mog Kwon[1]

[1] Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, University of Science and Technology, Yuseonggu, Daejon, Republic of Korea
[2] Bio-Evaluation Center, Korea Research Institute of Bioscience and Biotechnology, Chungbuk, Republic of Korea
[3] LG Life Science R&D Yoosung, Daejon, Republic of Korea
[4] Department of Oral Microbiology, School of Dentistry, Kyungpook National University, Daegu, Republic of Korea

Phosphatase of regenerating liver (PRL)-3, a member of a subgroup of protein tyrosine phosphatases that can stimulate the degradation of the extracellular matrix, is over-expressed in metastatic colorectal cancer (CRC) relative to primary tumors. To determine whether PRL-3-induced enhancement of migration and invasion is dependent on the expression of matrix metalloproteinases (MMPs), PRL-3 was expressed in DLD-1 human CRC cells. The motility, migration and invasion characteristics of the cells were examined, and metastasis to the lung was confirmed in a nude mouse using PRL-3-overexpressing DLD-1 cells [DLD-1 (PRL-3)]. Migration and invasion of the cells were inhibited by phosphatase and farnesyltransferase inhibitors. Expression of MMPs was enhanced 3- to 10-fold in comparison to control cells, and migration and invasion were partially inhibited by small interfering RNA (siRNA) knockdown of MMP-2, -13 or -14. Importantly, siRNA knockdown of MMP-7 completely inhibited the migration and invasion of DLD-1 (PRL-3) cells, whereas overexpression of MMP-7 increased migration. The expression of MMP-7 was also downregulated by phosphatase and farnesyltransferase inhibitors. It was found that PRL-3 induced MMP-7 through oncogenic pathways including PI3K/AKT and ERK and that there is a relationship between the expression of PRL-3 and MMP-7 in human tumor cell lines. The expression of MMP-13 and -14 was very sensitive to the inhibition of farnesyltransferase; however, the migration and invasion of DLD-1 (PRL-3) cells did not strongly depend on the expression of MMP-13 or -14. These results suggest that the migration and invasion of PRL-3-expressing CRC cells depends primarily on the expression of MMP-7.

Cancer Cell Biology

Phosphatase of regenerating liver (PRL)-1, PRL-2 and PRL-3 collectively make up a subgroup of VH1-like protein tyrosine phosphatases (PTPs) based on the amino acid sequences of their catalytic domains.[1] PRL PTPs (20 kDa) contain a characteristic PTP motif and catalytic residues and also contain a carboxyl-terminal prenylation motif (CAAX) similar to those found in Ras family small GTPases, which allows PRL proteins to be modified by farnesylation.[2] This feature is unique to the PRL PTPs, suggesting that they may have distinctive functions compared with other PTPs. PRL expression in normal tissues seems to correlate with terminal differentiation. For example, PRL-1 and PRL-3 expression in the intestine is limited to the terminally differentiated villus and is not found in proliferating crypt enterocytes.[3,4] In addition, expression of PRL-3 in heart tissue is primarily found in cardiomyocytes.[5]

Overexpression of PRL-1 and PRL-2 has been found to transform mouse fibroblasts and pancreatic epithelial cells *in vitro* and to promote tumor growth in nude mice, suggesting that they may play a role in tumorigenesis.[6,7] Migration and invasion are enhanced by PRL-1 and PRL-3 in Chinese hamster ovary cells, and overexpression of these proteins induced metastatic tumor formation in mice.[8] Saha *et al.*[9] first described PRL-3 overexpression in colorectal cancer (CRC) by comparing gene expression levels in metastases of

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*S.-K.L. and Y.-M.H. contributed equally to this work
Correspondence to: Byoung-Mog Kwon or Dong Cho Han, Laboratory of Chemical Biology and Genomics, Korea Research Institute of Bioscience and Biotechnology, University of Science and Technology, 52 Uendong, Yoosunggu, Daejeon 305-806, Republic of Korea, Tel.: 82-42-860-4557, Fax: +82-42-861-2675, E-mail: kwonbm@kribb.re.kr or Tel.: +82-42-860-4557, Fax: +82-42-861-2675, E-mail: dchan@kribb.re.kr

colon cancers that had spread to the liver with the levels in the primary tumors, and PRL-3 was nearly undetectable in normal colorectal epithelia. In addition, Bardelli *et al.* observed high PRL-3 mRNA expression in metastatic lesions derived from CRCs regardless of the site of metastasis (liver, lung, brain or ovary), and PRL-3 expression in metastatic CRC is significantly higher than in the primary tumor itself or in normal colorectal epithelia.[10] There is also evidence that PRL-3 expression positively correlates with progression in other tumor types, including gastric tumors, multiple myeloma and Hodgkin's lymphoma.[11,12] Moreover, a cause-and-effect relationship between PRL PTPs and tumor metastasis is supported by several recent observations in animal model systems.[10,13] Collectively, these observations strongly support a role for PRL PTPs, especially PRL-3, in tumor progression and particularly in the metastatic process. As a consequence, PRL-3 might be a promising target for small molecule inhibitors designed to prevent and/or treat metastasis.[14]

Decreases in cell adhesion and proteolytic degradation of collagen by matrix metalloproteinases (MMPs) promote the invasive migration of cells through the extracellular matrix.[15] PRL-3 has been recently described as one of the PTPs, which could also degrade the extracellular matrix. It has been shown to be expressed in liver metastases derived from CRC.[16] Among more than 20 MMPs, matrilysin (MMP-7) appears to be one of the most important MMPs in human colon cancers, because it is highly overexpressed in colon cancer.[17,18]

The mechanisms by which PRL PTPs promote these phenotypes have not yet been elucidated. We reasoned that introduction of PRL-3 into human colon adenocarcinoma cells might regulate other genes required for PRL-3-dependent migration and invasion. It was reported that MMP-2, -7 and -9 are strongly upregulated in colon cancers,[17,18] and MMP-2 and -14 are regulated by PRL-3; therefore, MMP-2, -7, -9, -13 and -14 were selected for further examination in this study.

Material and Methods

Reagents

The FPTase inhibitor (LB42908) and PRL-3 phosphatase inhibitor (benzylidene rhodanine: BR-1) were synthesized by reported methods.[19,20] The HA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MMP-7 antibody was purchased from Chemicon International (Temecula, CA). Antibodies against MMP-13, PRL-3, ezrin, actin, ERK, p-ERK, Akt, p-Akt and ERM pThr567 were purchased from Cell Signaling Technology (Danvers, MA).

Cell culture

Cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Human cancer cell lines were maintained in RPMI 1640 (SW620, DLD-1, LOVO, AsPC-1, MIA-PaCa2, DU145, MDA-MB-231 and B16BL6) or Dulbecco's Modified Eagle's Medium (DMEM) (A549 and HT1080). HCT116 (human colon cancer cell line) was maintained in McCoy's 5A. All culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). Cell cultures were maintained at 37°C under a humidified atmosphere of 5% CO_2.

Generation of stable DLD-1 cell lines expressing PRL-3 and mutant PRL-3

The cDNA containing full-length PRL-3 gene was obtained from human brain Quick-Clone cDNA (Clontech, Mountain View, CA). The forward primer (5′-CGA ATT CAT GGC TCG GAT GAA C-3′) and the reverse primer (5′-CCG CTC GAG CGG CCT GAG CTA CAT AAC-3′) were used for amplification of the PRL-3 gene. The polymerase chain reaction (PCR) fragments were digested with *Eco*RI and *Xho* and inserted into a modified pcDNA3 plasmid, which contains an HA epitope (10 amino acid residues) at the NH_2 terminus. The expression plasmid was confirmed by DNA sequencing analysis and introduced into DLD-1 human colon adenocarcinoma cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The DLD-1 cells were cultured in RPMI 1640 supplemented with 10% FBS. Stable cells were selected using culture media containing 1 mg/mL geneticin (G418, Invitrogen, Carlsbad, CA) for 20–30 days. Stable transformants were continuously maintained in RPMI 1640 media containing 200 μg/mL geneticin.

The point mutation C104S was introduced by PCR (MutaBest kit, Takara, shiga, Japan) using following primers for PRL-3 (C104S), 5′-CTTGTGCACT**C**TGTGGCGGGC-3′ (G to C, underlined) and 5′-TACGCAGCTTCCCGGGT-CATT-3′. The cDNA fragments containing the point mutation were cloned into the pcDNA3 vector constructed with the HA epitope. The constructs were confirmed by DNA sequencing. Cells (50–70% confluent) were transiently transfected with the recombinant plasmid (pcDNA3-HA-PRL3 C104S) using the Lipofectamine 2000.

Cell migration assay and invasion assay

Cell migration assays were performed using 8.0-μm pore size Transwell inserts (Costar, Cambridge, MA) with some modifications. Cell invasion was investigated using Matrigel-coated 8.0-μm filter invasion chambers (BD Biosciences, CA). Cells were incubated for 12 hr (migration assay) or 48 hr (invasion assay) at 37°C in a humidified atmosphere of 5% CO_2. Cells on the upper surface of the membrane were removed using cotton tips after the indicated incubation times. The migrant cells attached to the lower surface were stained with crystal violet (500 μL of 5 mg/mL crystal violet dissolved in 20% methanol) and incubated for 30 min. The membrane was washed several times with phosphate-buffered saline, and the cells within the filter were counted under an optical microscope.

Cell motility assay into 3D collagen

Three-dimensional Collagen gels were made from collagen solution according to the manufacturer's recommendations

(Chemicon, Temecula, CA). The collagen solution was prepared on ice by mixing together collagen solution, 5× RIPA medium and neutralization solution in a ratio of 8:2:0.25 by volume. Cells were mixed with the chilled collagen solution in a ratio of 1:9 (by volume). 100 microL of the mixture (containing 6,000 cells) was transferred to a 96-well tissue culture plate. The plate was incubated at 37°C for 60 min to initiate polymerization of the collagen. The collagen gel was then covered with culture media and cultured for 2 weeks.

In vivo metastasis assays

For lung metastasis formation, 1×10^6 cells of DLD-1 (vector) or DLD-1 (PRL-3) were injected into the lateral tail vein of 6-week-old female S.P.F Balb/c nude mice in a volume of 0.1 mL (five mice per group). The mice lungs were harvested at 8 weeks after injection, and RNAs were isolated from the lung tissue using RNesay mini kit (Qiagen, CA). The isolated RNA was analyzed for human and mouse Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by real-time PCR with TaqMan Gene Expression Assay (Applied Biosystems, CA).

Reverse transcription-PCR and real-time PCR

cDNA was synthesized from 5 μg of total RNA using Superscript II reverse transcriptase (Invitrogen). PCR was performed under standard conditions in a 20 μL volume [10 mM Tris, pH 8.3, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 10 pM each primer (sense and antisense) and 1 U Taq DNA polymerase (Bioneer, Korea)]. Cycles for the PCR were as follows: one cycle at 95°C for 30 sec; 20–30 cycles of 95°C for 30 sec, 55–60°C for 30 sec and 72°C for 45 sec; one cycle at 72°C for 5 min. Primer sequences were designed using the primer3 software (http://frodo.wi.mit.edu/ primer3/primer3_code.html). The real-time PCR assays were performed on the iCycler iQ Real-Time PCR Detection System (Bio-Rad, CA) using the Iq SYBR Green Supermix (Bio-Rad). The denaturation step was the first regular cycling event and consisted of heating the reaction to 95°C for 5 min. Triplicate 25 μL PCR reactions were carried out with polymerase activation at 95°C for 10 min, followed by 55 cycles of denaturation at 95°C for 15 sec and a combined primer annealing/extension step at the specific annealing temperature for 20 sec. This cDNA was synthesized from 5 μg total RNA using Revert Aid First Strand cDNA synthesis kit (Fermentas, German). The primers for MMP-2 were MMP-2-F (5′-AGA ACC TGG ATG CCG TCG T-3-3′ and MMP-2-R (5′-TCA CGC TCT TCA GAC TTT GG-3′). The primers for MMP-7 were MMP-7-F (5′-TGG ACG GAT GGT AGC AGT CT-3′) and MMP-7-R (5′-TCT CCA TTT CCA TAG GTT GGA T-3′). The primers for MMP-9 were MMP-9-F (5′-ATC CGG CAC CTC TAT GGT C-3′) and MMP-9-R (5′-CTG AGG GGT GGA CAG TGG-3′). The primers for MMP-13 were MMP-13-F (5′-CCT GAA CAA GT A GTT CCA AAG G-3′) and MMP-13-R (5′-GCC GGT GTA GGT GTA GAT AGG A-3′). The primers for MMP-14 were MMP-14-F (5′-TAC TTC CCA GGC CCC AAC-3′) and MMP-14-R (5′-GCC ACC AGG AAG ATG TCA TT-3′).

Western blot analysis

Forty micrograms of protein was resolved by Sodium Dodesyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis (PAGE) on a 12 or 15% polyacrylamide gel and transferred to a Poly Vinylidene DiFluoride (PVDF) membrane (Roche, Mannheim, Germany). Membranes were blocked with 5% nonfat dry milk in TBST (50 mM Tris–HCl, pH 7.6, 150 mM NaCl and 0.1% Tween 20). The secondary antibodies used were horseradish peroxidase-conjugated goat antirabbit or antimouse IgG from Jackson Immunology Laboratory (Philadelphia, PA). Membranes were incubated with primary antibody for 2 hr at room temperature, washed five times with TBST, incubated with secondary antibody for 1 hr at room temperature and then visualized with Chemiluminescence horseradish peroxidas (POD) reagents (Roche, Mannheim, Germany).

Knockdown of MMP-2, -7, -13, -14 and PRL-3 using small interfering RNA in DLD-1 (PRL-3) cells

The MMP-2, -7, -13, -14 and negative control small interfering RNAs (siRNAs) were purchased from Samchully Pharmaceutical Company (Seoul, Korea). The sequences of each siRNA are as follows: MMP-2 (5′-CUG CAA ACA GGA CAU UGU A-3′), MMP-7 (5′-CUC GAC ACU UAC CGC AUA U-3′), MMP-13 (5′-CAG AUG UAU GUG CCC UUC U-3′), MMP-14 (5′-UCU UCG UUG CUC AGU CAG U-3′), PRL-3 (5′-GGA CAC CCG AAG GCA AUA A-3′) and the negative control (5′-CCU ACG CCA CCA AUU UCG U-3′). Cells were plated at a density of 8×10^4 cells/well in six-well plates and transfected with 50 or 100 nM MMP-2, -7, -13, -14, PRL-3 or negative control siRNA oligoduplexes after preincubation for 20 min with Oligofectamine in serum-free Opti-MEM I medium (Invitrogen, CA). RPMI 1640 medium containing 10% FBS (without antibiotics) was added 5 hr after the beginning of the incubation. After transfection for 48 hr, cells were collected and used for migration assays or for preparation of whole cell lysates.

Overexpression of MMP-7, -13 and -14 in the DLD-1 cell line (new experiment for MMP-14 overexpression)

The full-length MMP-7 gene was obtained by PCR using oligonucleotides 5′-CGG GAT CCA ATT GTC TCT GGA CGG CAG CT-3′ (forward) and 5′-GGA ATT CGA ATG GAT GTT CTG CCT GAA G-3′ (reverse) with an expressed sequence tag clone containing MMP-7 (Korea UniGene Clone ID hMU007325, cDNA clone MGC: 3913) as a template. The PCR product was digested with *Bam*HI and *Eco*RI and then inserted into the pcDNA3.1 mammalian expression vector, which had been digested with *Bam*HI and *Eco*RI. To generate cells expressing MMP-7, DLD-1 cells were transfected with the empty pcDNA3.1 vector or with pcDNA3.1-MMP-7 using Lipofectamine for 48 hr. The efficiency of transfection was confirmed by immunoblotting using an MMP-7 antibody. The full-length MMP-13 and -14 gene was

amplified by forward (5′-CCC AAG CTT AGG CAT CAC CAT TCA AGA T-3′) and reverse (5′-CTA GTC TAG ACA CTT AAC ACC ACA AAA TGG-3′) oligonucleotides and forward (5′-CGG GGT ACC CCA TGT CTC CCG CCC CAA G-3′) and reverse (5′-CGG AAT TCG ACC TTGT CCA GCA GGG AAC G-3′) oligonucleotides, respectively, using cDNA pools from mRNA of DLD-1 colon cancer cells. The PCR product for MMP-13 was inserted into *Hind*III and *Xba*I sites of pcDNA3.1-myc/His mammalian expression vector, the PCR product for MMP-14 was inserted into *Kpn*I and *Eco*RI sites of pcDNA3.1-myc/His mammalian expression vector, and MMP-13- or -14-overexpressing DLD-1 cells were generated using the same transfection protocol as for MMP-7.

Zymography analysis

For the preparation of conditioned medium for zymography, cells were cultured for 24 hr in RPMI 1640 containing 10% FBS, 10 μM LB42908 and 30 μM BR-1 and then cultured for an additional 18 hr in serum-free medium containing LB42908 and BR-1 before collection of the conditioned medium for MMP assays. Proteins in the conditioned media were separated, without prior boiling, by SDS/PAGE under nonreducing conditions using a 10% polyacrylamide gel containing 1 mg/mL gelatin (Sigma–Aldrich, St. Louis, MO). After electrophoresis, the gel was rinsed with renaturing buffer (2.5% Triton X-100) for 30 min and then incubated overnight at 37°C in developing buffer (50 mM Tris, pH 7.5, 5 mM $CaCl_2$ and 1 μM $ZnCl_2$). The gel was stained with 0.5% Coomassie blue R250 and destained with 10% acetic acid in 30% methanol. The unstained bands corresponded to the areas of collagen digestion.

Statistical analysis

All data are presented as the mean ± SD. The statically significant differences in the experimental group compared with the untreated group were calculated by Student's *t* test.

Results

PRL-3 expression promotes migration and invasion of DLD-1 human colon adenocarcinoma cells

To investigate the molecular mechanism(s) by which PRL-3 promotes cell migration and invasion in human carcinoma cells, the DLD-1 human colon adenocarcinoma cells were selected, because these cells had the lowest basal level of PRL-3 expression in mRNA and protein level (Fig. 1*a*). As shown in Figure 1*b*, DLD-1 cells were stably transfected with a plasmid coding for WT PRL-3. DLD-1 cells transfected with PRL-3 [DLD-1 (PRL-3)] or the empty vector [DLD-1 (vector)] were subjected to migration assays or to invasion assays. DLD-1 (PRL-3) cells exhibited a 90% increase in migration and invasion compared with control vector cells (Fig. 1*c*). A collagen gel assay was performed to assess the effects of PRL-3 on cell motility in a 3D system. DLD-1 (PRL-3) cells could migrate into the collagen gel and formed spindle-like shapes (Fig. 1*c*). It was confirmed that the migration of DLD-1 (PRL-3) cells was inhibited by siRNA against PRL-3 (Fig. 1*d*).

PRL-3 induces lung metastasis in vivo

To clarify the role of PRL-3 in colon adenocarcinoma metastasis *in vivo*, DLD-1 (vector control) or DLD-1 (PRL-3) were injected into the lateral tail vein of Balb/c nude mice. At 6 and 8 weeks, mice were scarified, and the lungs were harvested. Metastatic nodules were not visible on the lung. To investigate and quantitate the tumor burdens in the lungs of the animals, RNA was extracted from lung of each mouse, and real-time PCR analysis was done using human-specific GAPDH primer, because the level of human GAPDH mRNA expression in each sample reflects the tumor burden in the lungs of the mice.[21,22] Mice lung tissues were lysed and analyzed for human (tumor) and mouse (control) GAPDH transcripts. Real-time PCR quantitation showed that the number of lung metastasis was significantly increased in the lungs of mice carrying PRL-3 cells ($p = 0.00135$; Fig. 1*e*). From this experiment, we could confirm that PRL-3 induced lung metastasis *in vivo*.

PRL-3 phosphatase inhibitor and FPTase inhibitor suppressed the migration and invasion of DLD-1 (PRL-3) cells

PRL-3 is a tyrosine phosphatase with a CAAX for farnesylation, and these modifications have been shown to be required for PRL-3 biological function. Therefore, we investigated whether a PRL-3 phosphatase inhibitor (BR-1) and FPTase inhibitor (LB42908) could modulate PRL-3-dependent localization, migration and invasion (Fig. 2*a*). First, we confirmed the specificity of the PRL-3 inhibitor by the rescue of phosphorylation on ezrin, a known PRL-3 substrate (Fig. 2*b*).[23] For the rescue of phosphorylation on ezrin, we used α-ERM pThr 567 antibody, because phosphor-ERM antibody is used to detect phosphor-Ezrin Thr567. Furthermore, it was found that the FPTase inhibitor also rescued the phosphorylation of ezrin, a constituent of α-ERM. BR-1 or LB42908 treatment in DLD-1 vector control cells did not affect the phosphorylation of ezrin (Fig. 2*b*). As we can see in Figure 2*b*, the levels of α-Ezrin, Radixin and Moesin (ERM) pThr 567 in 10 μM treated cells were 2-fold higher than control. PRL-3 actually localized to the plasma membrane in DLD-1 (PRL-3) cells in our system (Fig. 2*c*). As shown in Figure 2*c*, treatment with LB42908 (10 μM) to prevent farnesylation of PRL-3 in DLD-1 (PRL-3) cells resulted in the loss of PRL-3 from the plasma membrane and its accumulation in the cytoplasm and nucleus, whereas treatment with BR-1 (30 μM) did not change the localization of PRL-3. Previous studies showed that mutation of PRL-3 at the catalytic residue (C104S) completely abolished its metastatic activity.[13] To confirm the results obtained with the PRL-3 inhibitor, cells were also transfected with a catalytically inactive PRL-3 mutant (C104S). In these cells as well, PRL-3 protein was localized to the plasma membrane. Migration and invasion were also inhibited in the

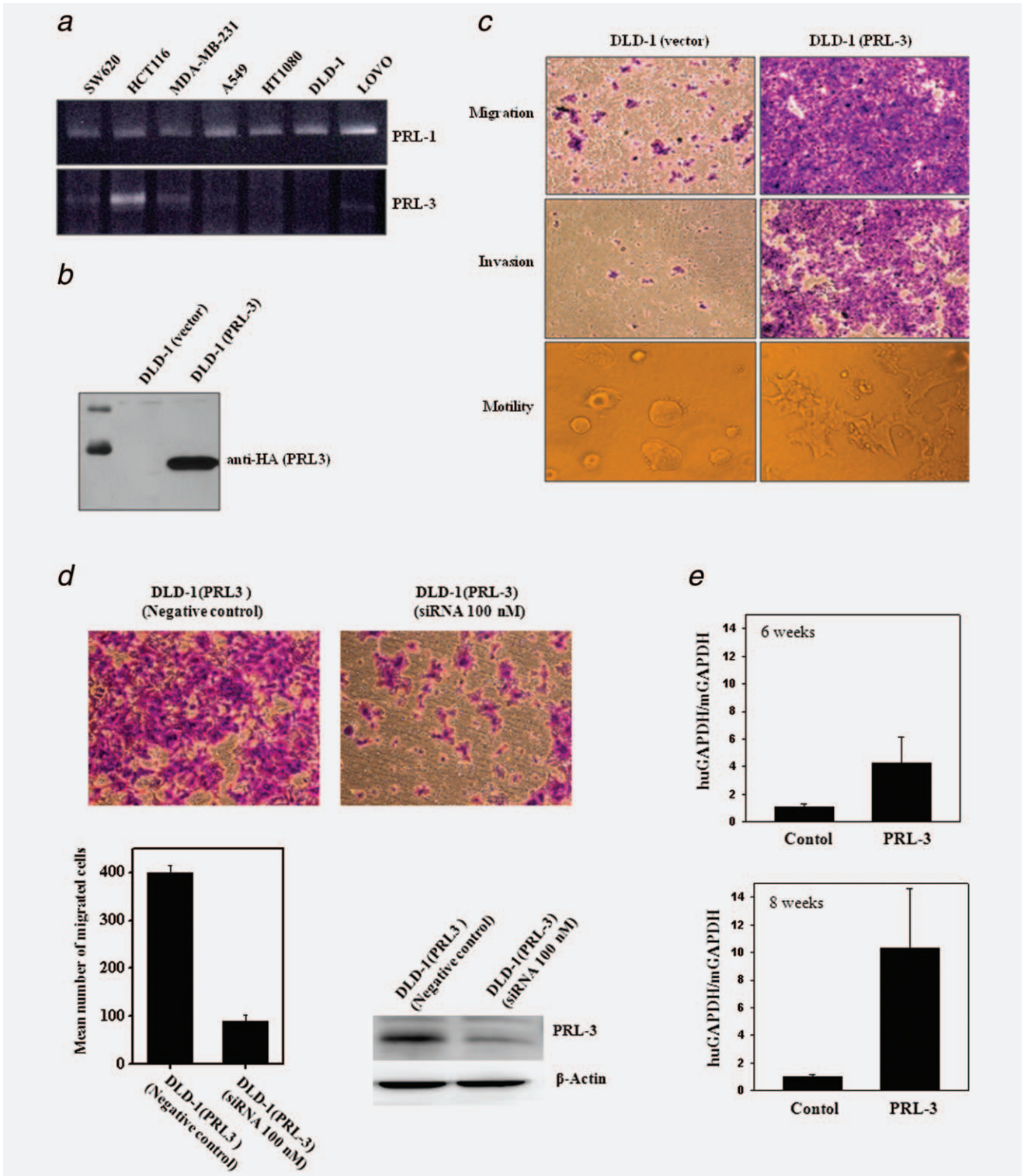

Figure 1. PRL-3 enhances migration and invasion of DLD-1 human colon adenocarcinoma cells. (*a*) PRL-3 expression level was demonstrated by RT-PCR and western blotting in human tumor cell lines including SW620 (colon), HCT116 (colon), MDA-MB231 (breast), HT1080 (melanoma) and LOVO (colon). (*b*) Expression of HA-tagged PRL-3 in DLD-1 cells was confirmed by immunoblot analysis. (*c*) Effect of PRL-3 overexpression in DLD-1 cells. Migration (upper panel) and invasion assay (middle panel) were performed with a Transwell system. The cells attached to the lower surface of the membrane were stained with crystal violet and visualized on an optical microscope. Cell motility into a 3D collagen gel is shown in the lower panel. (*d*) Inhibition of migration capacity by PRL-3 knockdown. Cells were incubated for 24 hr after transfection with siRNA (100 nM) at 37°C in a humidified atmosphere of 5% CO2 for 5 hr. Migrant cells were stained with crystal violet and counted under a light microscope. (*e*) For lung metastasis formation, 1×10^6 cells of DLD-1 (vector) or DLD-1 (PRL-3) was injected into the lateral tail vein of S.P.F Balb/c nude mice in a volume of 0.1 mL (five mice per group). The mice lungs were harvested at 6 and 8 weeks after injection, and RNAs were isolated from the lung tissue. The isolated RNA was analyzed for human and mouse GAPDH by real-time PCR with TaqMan gene expression assay.

PRL-3 mutant (C104S) cells. Next, the effect of BR-1 and LB42908 on PRL-3-induced cell migration and invasion was investigated. As shown in Figure 2*c*, migration (middle panel) and invasion (lower panel) of DLD-1 (PRL3) cells were strongly suppressed by treatment with BR-1 (30 μM) or LB42908 (10 μM) about 60–70% by phosphatase and FPTase inhibitor, respectively (Fig. 2*d*).

The effect of an FPTase inhibitor (LB42908) on MMP expression in DLD-1 (PRL-3) cells

MMPs are involved in many steps of tumor metastasis. In this study, the mRNA expression of several MMPs was measured in DLD-1 (PRL-3) cells by real-time quantitative PCR. When PRL-3 was expressed, the expression of MMP-2, -7, -13 and -14 increased 3- to 12-fold compared with levels in

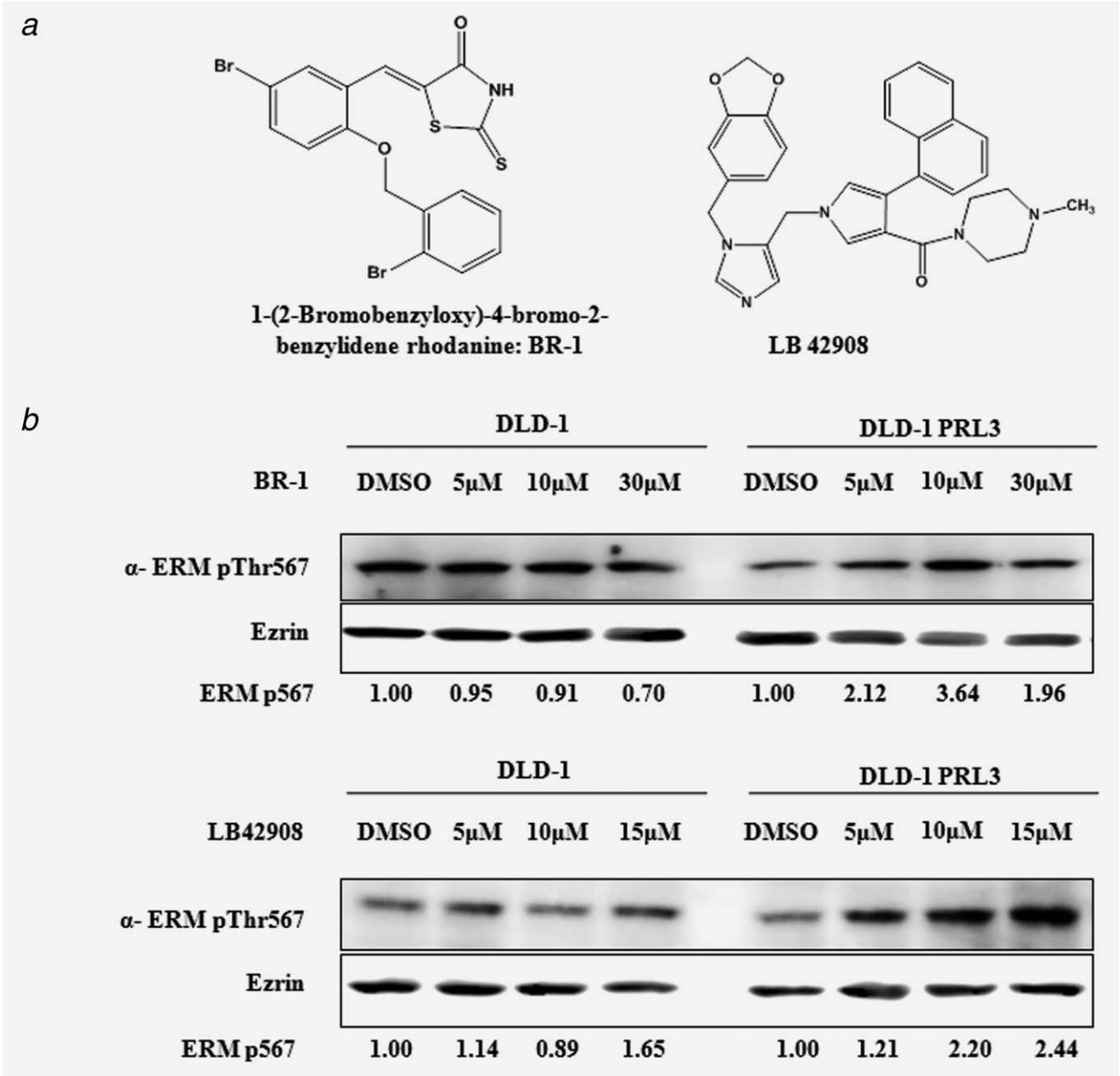

Figure 2. The PRL-3 phosphatase inhibitor (BR-1) and FPTase inhibitor (LB42908) suppress functional activity of PRL-3 in DLD-1 colon adenocarcinoma cells. (*a*) The structure of BR-1 and LB 42908. (*b*) Phosphorylation rescue of ezrin after treatment of BR-1 or LB42908 in DDL-1 or DLD-1 (PRL3). (*c*) PRL-3 localizes to the plasma membrane in DLD-1 cells (upper panel). DLD-1 cells transfected with vector (HA). WT PRL-3 or a catalytically inactive PRL-3 mutant DLD-1 (C104S) were treated with 10 μM LB42908, 30 μM BR-1 or DMSO for 24 hr and visualized live by fluorescence microscopy. Migrated (middle panel) or invaded (lower panel) cells were stained and counted under a light microscope. (*d*) Quantitative analysis of migrated and invaded cells.

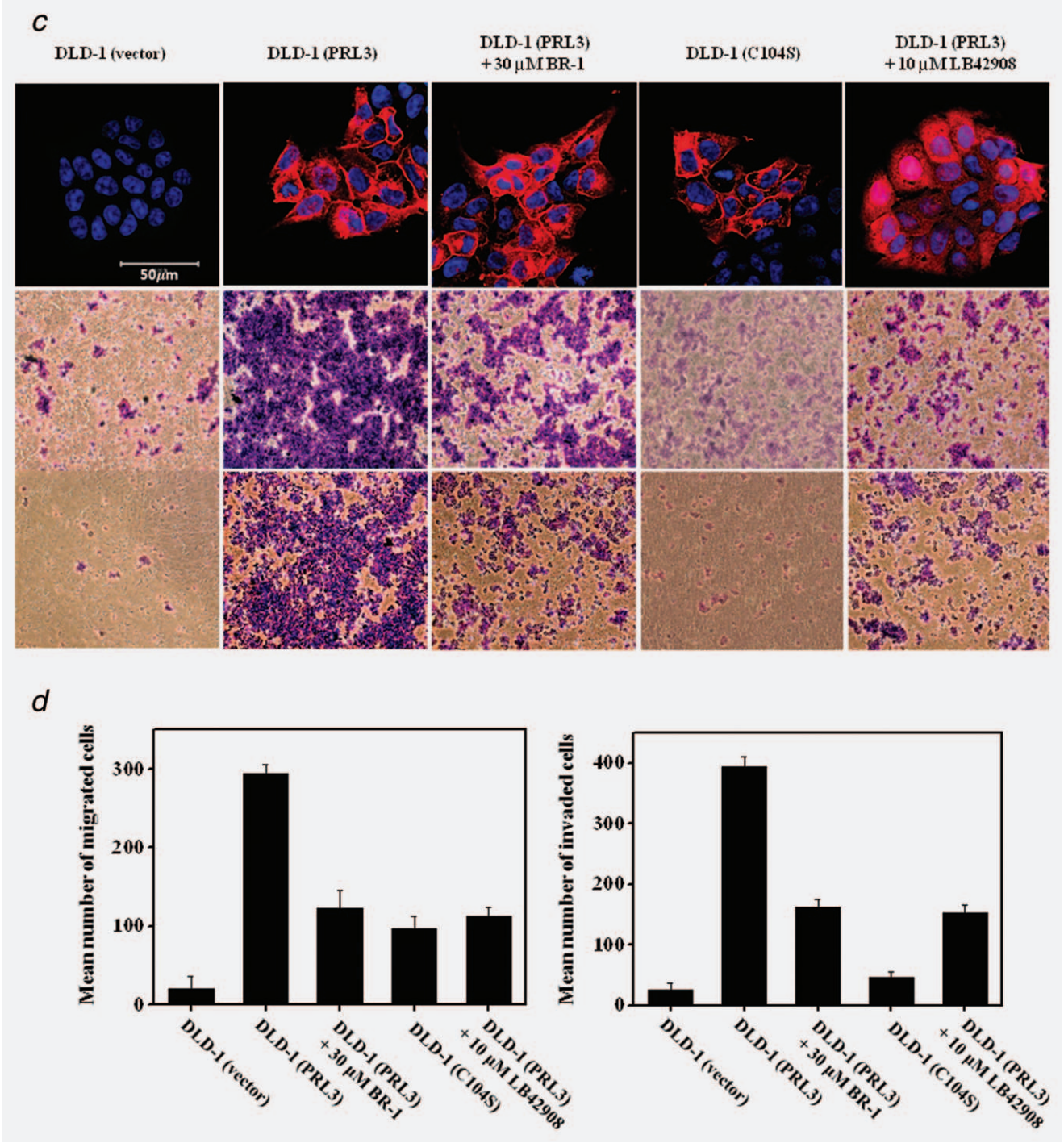

Figure 2. (*Continued*)

vector control cells (Fig. 3*a*). This result shows that MMP-2, -7, -13 and -14 expression correlates with the increased cell migration and invasion observed in PRL-3-expressing DLD-1 cells. It is difficult to understand the expression of MMPs in PRL-3 mutant (C104S) cells, because MMP-7 and -13 were also upregulated unexpectedly in comparison with that of DLD-1 (vector) cells. We investigated whether an FPTase inhibitor (LB42908) could inhibit MMP expression in DLD-1 (PRL-3) cells. As shown in Figure 3*b*, treatment with LB42908 (10 μM) in DLD-1 (PRL-3) cells resulted in the downregulation of MMP -2, -7, -13 and -14. In particular, MMP-13 and -14 were dramatically downregulated by LB42908. However, MMP-9 was upregulated in DLD-1 (PRL-3) and PRL-3 mutant (C104S) cells following treatment with LB42908, and MMP-13 was upregulated in DLD-1 (vector) and PRL-3 mutant (C104S) cells.

Suppression of cell migration and invasion of DLD-1 (PRL-3) cells by siRNA knockdown of MMPs

To determine whether enhancement of migration by PRL-3 was dependent on MMP expression, we knocked down MMP-2, -7, -13 and -14 with siRNA. After the transfection of MMP-2, -7, -13, -14 or negative control siRNA (100 nM) for 18 and 48 hr, migration and invasion assays were performed, respectively. As shown in Figures 3*c* and 3*d*, treatment with MMP-2, -13 and -14 siRNA blocked cell migration in comparison with DLD-1 (PRL-3) cells and negative control siRNA-treated cells. MMP-7 siRNA inhibited cell migration and invasion by more than 90% in comparison with negative control cells. These results suggested that MMP-2, -7, -13 and -14 involved in migration and invasion in human CRC cells expressing PRL-3. In particular, MMP-7 expression greatly contributed to the migration and invasion of DLD-1 (PRL-3) cells. As we can see in Figure 3*e*, MMP-7 much strongly modulated the migration and invasion of DLD-1 (PRL-3). To determine the relationship between PRL-3 and MMP-7, the expression of the two proteins was assessed in DLD-1, DLD-1 (migrated cells), DLD-1 (PRL-3), Mia-PaCa2, MDA-MB-231, AsPC-1, DU-145, B16BL6 and

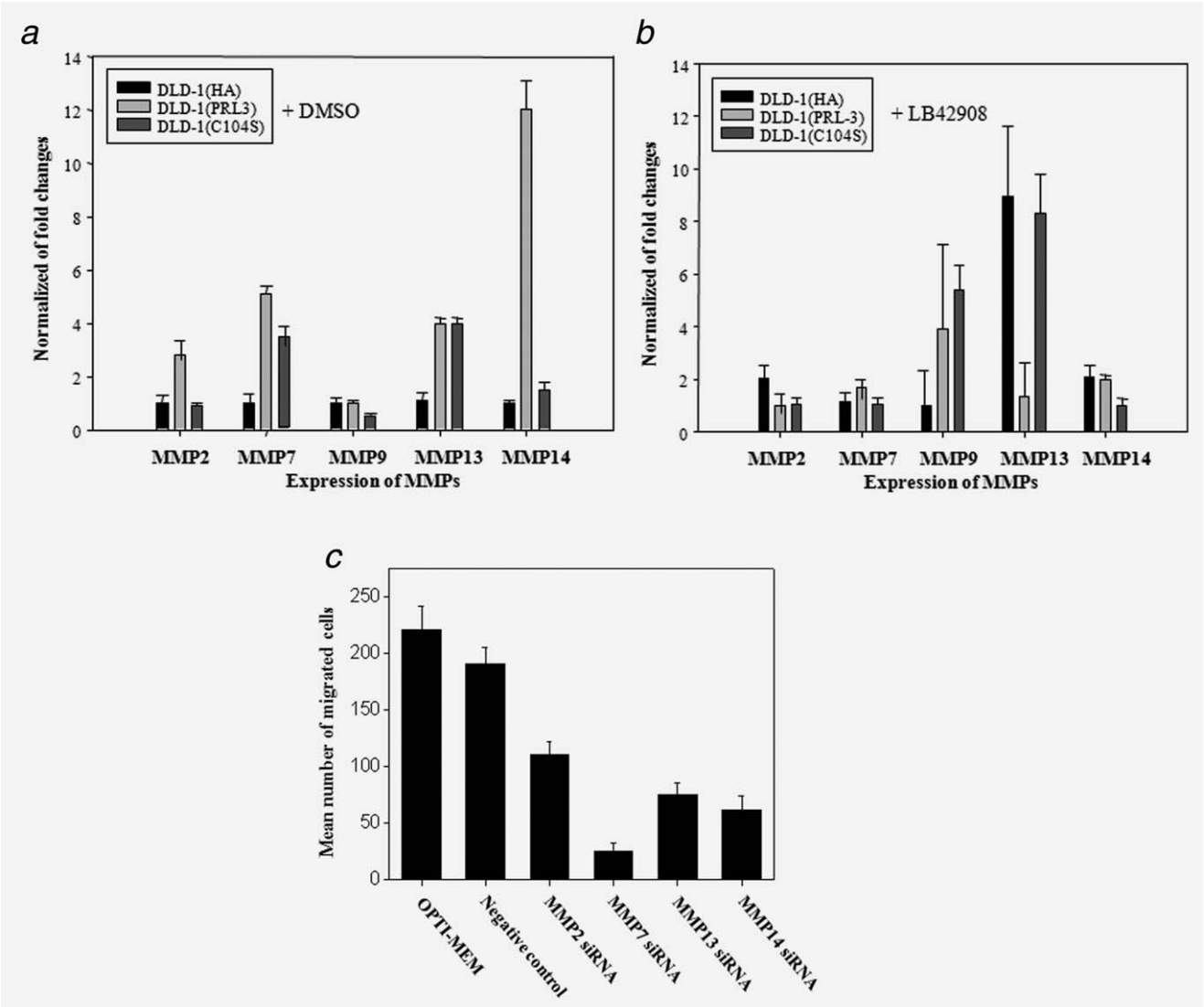

Figure 3. Expression of MMPs in DLD-1 (PRL-3) cells with or without LB42908 treatment, and suppression of cell migration and invasion of DLD-1 (PRL-3) cells by siRNA knockdown of MMPs. (*a*) mRNA expression of MMP-2, -7, -9, -13 and -14 in DLD-1 (vector), DLD-1 (PRL-3) and DLD-1 (C104S) cells. (*b*) To see effects of a farnesyltransferase inhibitor, on the expression of mRNA of MMPs, LG42908 was treated in DLD-1, DLD-1 (PRL-3) and catalytically inactive PRL-3 (C104S) mutant cells. (*c*) Migration of DLD-1 (PRL-3) cells after knockdown of the indicated MMPs with siRNA. (*d*) Effect on the migration and invasion of DLD-1 (PRL-3) cells of treatment with siRNA for MMP-7 or -14. (*e*) Quantitative analysis of the migration and invasion of DLD-1 (PRL-3) cells of treatment with siRNA for MMP-7 or -14. (*f*) The relationship between PRL-3 and MMP-7 expression in a various tumor cell lines.

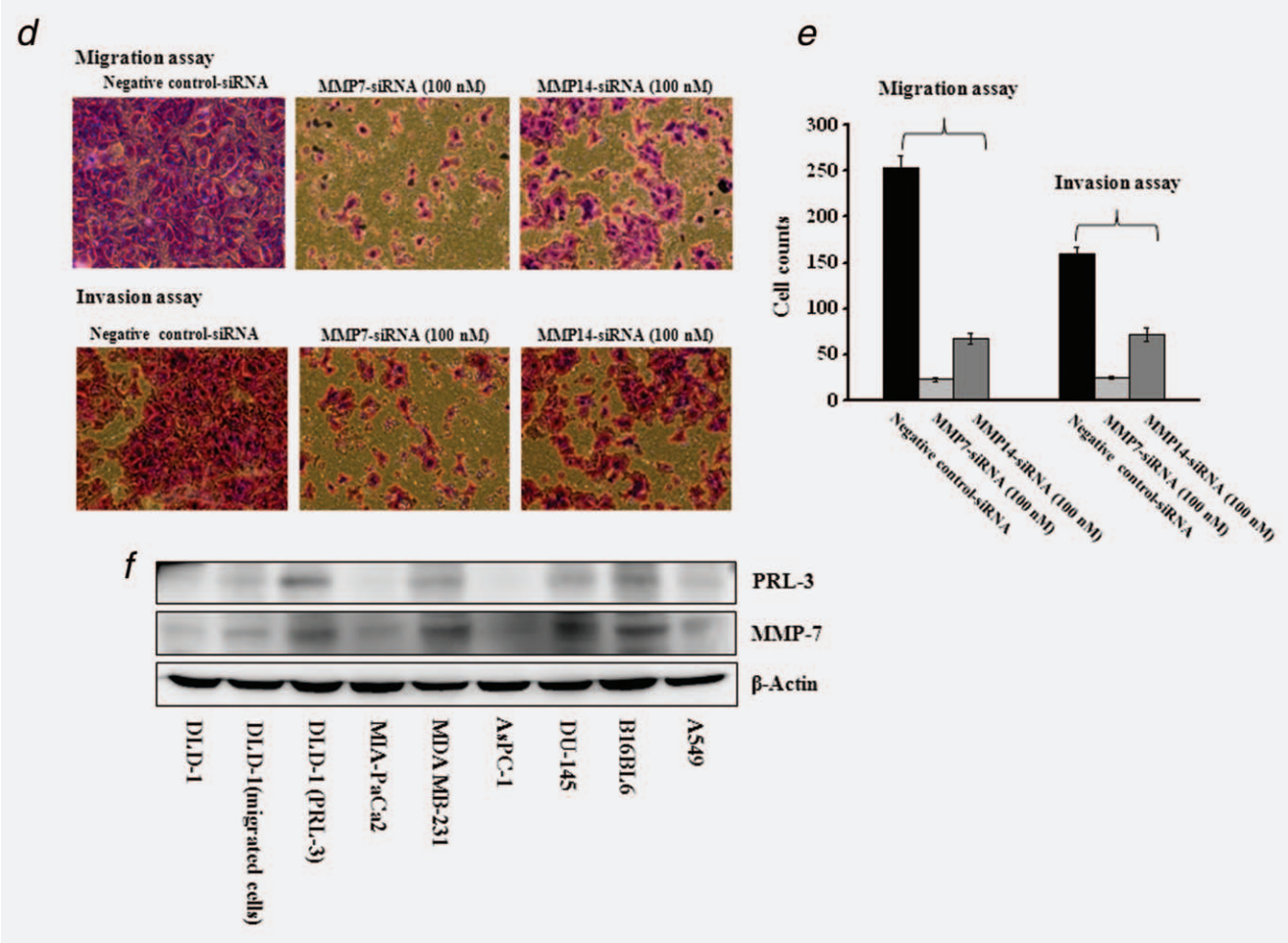

Figure 3. (*Continued*)

A549 cells by immunoblot. MMP-7 expression was increased in PRL-3-expressing cell lines (Fig. 3*f*).

MMP-7 is an important target molecule of PRL-3, but not expression of MMP-13 or -14 in migration of DLD-1 cells

siRNA experiment is not quite enough to conclude that PRL-3-induced migration and invasion are dependent on MMP-7, because effects of siRNAs depend on many factors. To determine whether the enhancement of migration by PRL-3 was dependent on MMP-7 expression, the mRNA and protein expression of MMP-7 in the cells were measured (Fig. 4*a*), and MMP-7 expression in DLD-1 (PRL-3) cells induced cell migration (Fig. 4*b*). Transfection of MMP-7 siRNA into DLD-1 (PRL-3) cells reduced MMP-7 expression at both the mRNA and the protein level (Fig. 4*c*), and these cells were therefore utilized in a cell migration assay. Because BR-1 and LB42908 suppressed PRL-3-dependent cell migration and invasion, the effect of these inhibitors on MMP-7 expression in the DLD-1 (PRL-3) cells was assessed by reverse transcription (RT)-PCR analysis. BR-1 and LB42908 each blocked PRL-3-dependent MMP-7 gene expression (Fig. 4*d*). These results suggest that MMP-7 is a PRL-3 target molecule and is critical to PRL-3-dependent cell motility and migration.

MMP-13 and -14 were upregulated (Fig. 3*a*) and sensitive to FPTase inhibitor (Fig. 3*b*) in DLD-1 (PRL-3) cells. To determine the effect of MMP-13 or -14 expression in DLD-1 cells, we constructed MMP-13- or -14-overexpressing DLD-1 cells and measured the migration of the cells in comparison with that of vector control DLD-1 cells. Overexpression of MMP-13 or -14 did not affect cell migration (Figs. 4*e* and 4*f*).

PRL-3 induces MMP-7 through PIK3/AKT and ERK pathway

Previous studies have shown that PRL-3 upregulates the oncogenic signaling pathways including Phosphatidylinositol 3-kinase (PI-3K)/AKT, MEK/Extracellular signal-regulated kinase (ERK), Signal Transducer and Activator of Transcription 3 (STAT3), Src and Rho family GTPases.[11] We therefore investigated whether PRL-3 induced MMP-7 expression through oncogenic signaling pathways including ERK and PI3K/AKT. DLD-1 cells were treated with the PI3K inhibitor LY-294002 (20 μM) or the ERK inhibitor U-0126 (10 μM). As shown in Figure 5*a*, PRL-3 increased the phosphorylation of AKT and ERK in DLD-1 (PRL-3) cells, which was

abolished by treatment with a specific PI3K inhibitor and ERK inhibitor, respectively. MMP-7 expression was also downregulated by each of the inhibitors. Migration of DLD-1 (PRL-3) cells was blocked by treatment of U-0126 or LY-294002 but not affected in DLD-1 cells (Fig. 5*b*). These data suggest that PRL-3 might regulate MMP-7 expression through oncogenic pathways including ERK and PI3K/AKT, leading to migration in DLD-1 (PRL-3) cells.

Discussion

Most cancer deaths result from tumor metastases rather than from primary tumors.[24,25] Yet, the specific molecular changes in a tumor cell that promote the metastatic process are largely unclear. PRL-3 is amplified and overexpressed in CRC metastases compared with primary tumors, and its expression correlates with metastasis in other tumor types.[8,11–16] In this report, PRL-3 induced cell migration and invasion in a human colon cancer-derived cell system (DLD-1 cells) and enhanced DLD-1 cell motility. It is consistent with previous studies with other cancer cell lines.[8,26,27] Furthermore, tumor metastasis to the lung was induced in mice injected with DLD-1 (PRL-3) cells.

BR-1 and LB42908 inhibited the migration and invasion of the DLD-1 (PRL-3) cells (Fig. 2*c*) through modulation of ezrin phosphorylation (Fig. 2*b*), because when DLD-1 cells were treated with BR-1 or LB42908, ezrin phosphorylation

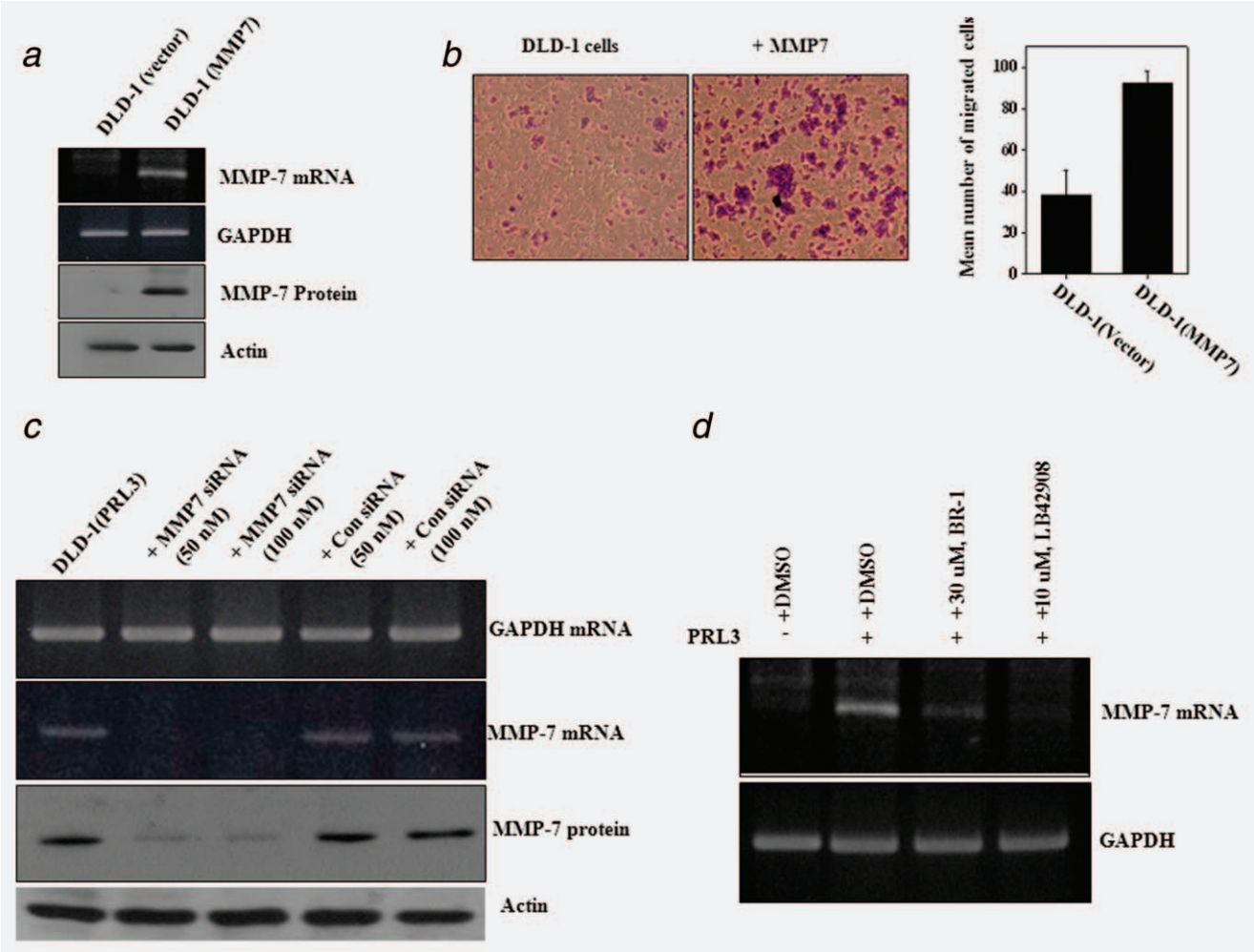

Figure 4. Overexpression of MMP-7 induces migration of DLD-1 cells; however, MMP-13 and -14 did not effect on cell migration. (*a*) MMP-7 expression is induced by PRL-3. RT-PCR and immunoblot analyses for MMP-7 and endogenous control genes in DLD-1 (PRL-3) cells. (*b*) Vector control or MMP-7-overexpressing DLD-1 cells were transiently transfected with MMP-7 for 48 hr and then used in an 18-hr cell migration assay. Migrating cells were imaged using a Nikon microscope after staining and counted. (*c*) DLD-1 (PRL-3) cells were transfected with negative control siRNA or MMP-7 siRNA. After 24 hr of siRNA transfection, whole cells lysates were prepared and analyzed by RT-PCR and western blotting. (*d*) Treatment of DLD-1 (PRL-3) cells with BR-1 or LB42908 downregulated MMP-7 expression. RT-PCR analysis was performed after cells were treated with the inhibitors for 24 hr. (*e*,*f*) DLD-1 cells were transiently transfected with vector control, MMP-13 or -14 for 48 hr, and an 18-hr cell migration assay was performed. Migrating cells were imaged using a Nikon microscope after staining and counted.

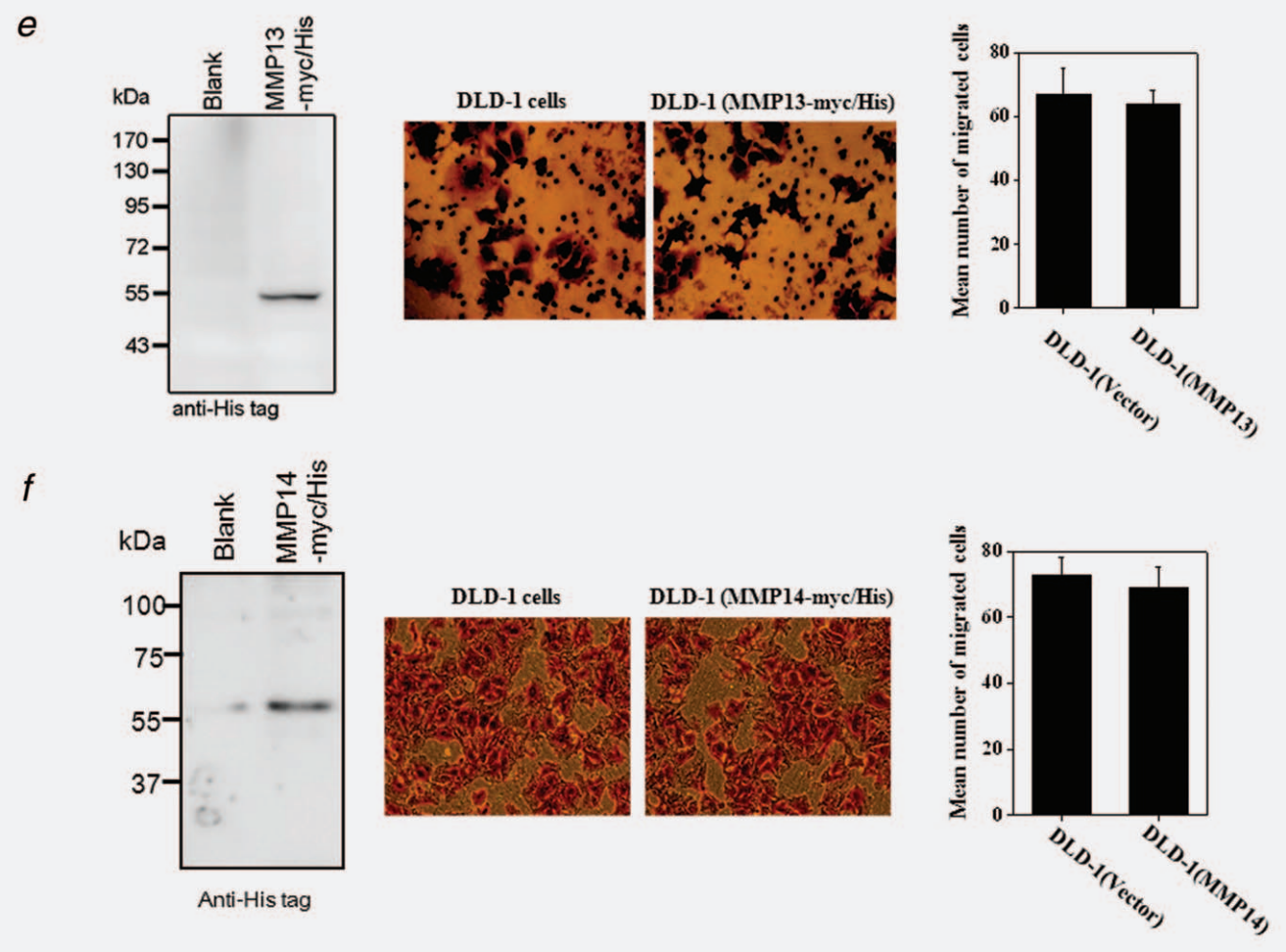

Figure 4. (*Continued*)

levels did not change. LB42908 also suppressed MMP expression (MMP-2, -7, -13 and -14) in DLD-1 (PRL-3) cells. This result is consistent with previous reports showing that the function and localization of PRL-3 can be inhibited by FPTase inhibitors and regulated by MMP expression in other cell types.[3] Although it has been reported that MMP-2 and -14 cooperatively contribute to PRL-3-dependent invasion of gliomas,[16] the expression level of MMP-14 mRNA was very high, and its expression was dramatically downregulated by the FPTase inhibitor in DLD-1 (PRL-3) cells. However, overexpression of MMP-14 in the cells did not effect on migration of the cells. Therefore, further studies are required to understand the functions of MMP-14 in tumor cell migration and invasion.

Although several observations strongly argue for a causative role for PRL-3 in colorectal carcinoma invasion and metastasis, they do not provide information about the biochemical mechanisms by which PRL-3 promotes these processes. Among the many downstream genes encoding cell adhesion and motility, MMP-7 mRNA and protein were strongly upregulated by PRL-3, an effect which was reversed by treatment of DLD-1 (PRL-3) cells with the PRL-3 phosphatase inhibitor or the FPTase inhibitor. Experiments with MMP-7 siRNA or overexpression vectors confirmed that MMP-7 was essential for PRL-3-induced migration in human CRC cells (Figs. 3*d* and 4*b*). MMPs are thought to play an important role in extracellular matrix degradation during tumor growth, tumor invasion and tumor-induced angiogenesis.[28] In addition, MMP-7 is implicated in the transition to metastasis and is overexpressed in 90% of colonic adenocarcinomas.[29,30]

In addition to MMP-7, other MMPs are also thought to be involved in diverse cellular processes such as proliferation, migration, angiogenesis and apoptosis.[31] The ability of malignant tumor cells to aggregate platelets confers a number of advantages to the successful metastasis of cancer cells.[32] Platelets facilitate the adhesion of tumor cells to the vascular endothelium during tumor cell extravasation. MMPs are found to stimulate platelet aggregation; therefore, the tumor cell metastatic potential increases.[33] In addition to the well-known functions of MMPs in extracellular matrix turnover, this role may be of importance in cellular responses such as migration and invasion.[34] MMP-13 and -14 were also highly expressed and strongly downregulated by the FPTase inhibitor in the DLD-1 (PRL-3) cells (Figs. 3*a* and 3*b*). However,

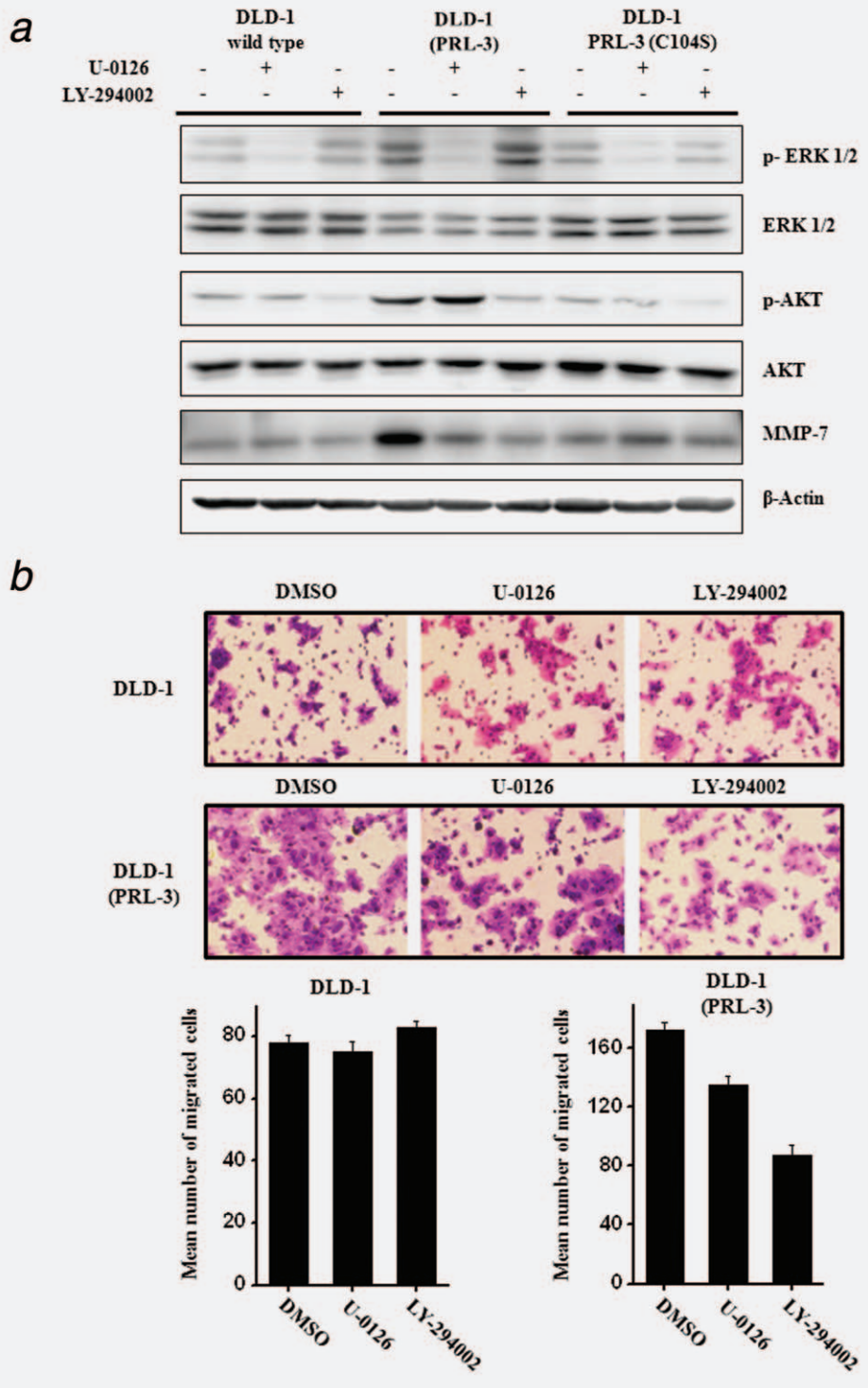

Figure 5. PRL-3 modulates expression of MMP-7 through AKT and ERK activation. (*a*)DLD-1 (vector), DLD-1 (PRL-3) and DLD-1 (C104S) cells were treated with a PI3K inhibitor (LY-294002, 10 μM) or an ERK inhibitor (U-0126, 10 μM) for 12 hr. Immunoblot analysis of p-ERK, p-AKT and MMP-7 was performed using specific antibodies. (*b*) Inhibition of AKT or ERK pathway by treatment of PI3K inhibitor (LY-294002, 20 μM) or an ERK inhibitor (U-0126, 10 μM) suppressed migration of DLD-1 (PRL-3) cells, but DLD-1 cells had no effect in the presence of AKT or ERK inhibitors. Migrant cells were stained with crystal violet and counted under a light microscope.

cell migration was not enhanced by MMP-13 or -14 overexpression in DLD-1 cells (Figs. 4*e* and 4*f*), and mRNA of MMP-13 and -14 was also highly expressed in DLD-1 (C104S) mutant cells. Therefore, MMP-13 and -14 may have the other functions instead of migration and invasion of the cells. Expression level of MMP-2 mRNA in DLD-1 (PRL-3) cells was relatively higher than in DLD-1 or DLD-1 (C104S) mutant cells, although MMP-2 was not strongly involved in the migration of DLD-1 (PRL-3) cells (Fig. 3*c*). These results showed that the relationship between MMP expression and enhancement of migration and invasion is very much dependent on cell type.

We have demonstrated that PRL-3 induced MMP-7 expression and led to cell migration in a number of tumor cell lines, including DLD-1 (PRL-3), MDA-MB-231, DU-145 and B16BL6 (data not shown). However, several tumor cell lines, such as A549, AsPC-1 and MIA-PaCa2, migrated readily despite the absence of PRL-3 or MMP-7 expression. These data demonstrate that tumor cell migration can occur through multiple signaling pathways.

It has been reported that PRL-3 induces oncogenic signaling pathways including PI3K/AKT, MEK/ERK, STAT3, Src and the Rho family GTPases.[35] In this study, it was confirmed using U-0126 (an ERK inhibitor) and LY-294002 (a PI3K inhibitor) that PRL-3 upregulated the expression of MMP-7 through these oncogenic signaling pathways. The results suggest that the PI3K/AKT and ERK pathways are involved in the induction of MMP-7 by PRL-3. As shown in Figure 5, AKT pathway might be one of key pathways, because cell migration is more strongly regulated by AKT inhibitor than ERK inhibitor. Finally, these data suggest that MMPs are directly or indirectly associated with PRL-3 and oncogenic signaling pathways to promote cell motility. In particular, MMP-7 plays a key role in enhancing migration and invasion in DLD-1 (PRL-3) cells.

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