Inhibition of Matrilysin Expression by Antisense or RNA Interference Decreases Lysophosphatidic Acid–Induced Epithelial Ovarian Cancer Invasion

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Abstract

Our previous reports show that matrilysin [matrix metalloproteinase (MMP)-7] is overexpressed in epithelial ovarian cancer (EOC) and recombinant MMP-7 promotes EOC invasion in vitro. In the present study, we further evaluated the correlation of MMP-7 expression to EOC invasiveness and examined its role in lysophosphatidic acid (LPA)-induced invasion. By sense and antisense gene transfection in vitro, we show that overexpression of MMP-7 in all MMP-7 stably transfected DOV13 clones significantly enhanced their invasiveness, although MMP-7 antisense transfection caused a 91% decrease of MMP-7 expression (P < 0.01) and 87% decrease of invasion (P < 0.05) in geneticin (G418)-selected DOV13 clone P47-M7As-3 compared with vector-transfected control. As assessed by MMP-7 ELISA, LPA treatment at 10 to 80 μmol/L significantly stimulated the secretion of total MMP-7 in DOV13 conditioned medium (P < 0.01). In addition, LPA apparently induced the activation of MMP-7 in DOV13 cells as detected by gelatin zymography. In the antisense MMP-7-transfected DOV13 clone (P47-M7As-3), LPA-increased invasion was significantly decreased compared with vector control. Moreover, knocking down of MMP-7 by small interfering RNA also suppressed LPA-induced invasion in two EOC cell lines (DOV13 and R182). Altogether, our results show that MMP-7 expression is correlated with EOC invasiveness and LPA-induced MMP-7 secretion/activation may represent a new mechanism that facilitates ovarian cancer invasion besides the well-known induction of MT1-MMP-mediated proMMP-2 activation by LPA. (Mol Cancer Res 2006;4(11):831–41)

Introduction

In the United States, epithelial ovarian cancer (EOC) accounts for more deaths than all other gynecologic malignancies combined (1). A key factor contributing to the high mortality is the widespread metastatic dissemination at time of initial presentation (2, 3). Therefore, to identify biomolecules that promote EOC metastasis and investigate the mechanisms involved in their modulation is critical in the search for effective treatment strategies.

Tumor invasion and migration through the degradation of basement membranes and extracellular matrix (ECM) as well as tumor neovascularization have been identified as essential features in EOC metastasis (4-7). Matrix metalloproteinases (MMP), a family of 24 structurally related zinc-dependent endopeptidases, are capable of directly degrading essentially all components of the ECM (8, 9) and thus are able to expose cryptic sites within the matrix molecules to activate other proteases, growth factors, and cytokines that may facilitate tumor invasion and metastasis. The role of several MMP family members, including MMP-2, MT1-MMP, and MMP-9, in EOC metastasis has been well documented (2, 5, 6, 10-15). However, the role of matrilysin (MMP-7, pump-1) in EOC remains unclear. Distinct from other MMPs, MMP-7 has the minimal domain organization required for secretion and activation and is one of the few MMPs that is overexpressed in ovarian carcinoma rather than stromal cells (16, 17). MMP-7 has broad proteolytic activity against a variety of ECM substrates and is recognized as an important regulator of cell surface proteolysis, binding to cell surface proteins, such as E-cadherin, β-integrin, tumor necrosis factor-α, Fas ligand, and heparin sulfate (18, 19). Overexpression of MMP-7 is seen in many malignancies, including prostate (20-22), stomach (23-26), colorectal (27-30), lung (31), ovary (32), esophageal (33), and squamous cell carcinomas of the head and neck (34). Transfection of the MMP-7 gene enhances the invasiveness of breast, prostate, and colon carcinoma cells in vitro and in vivo (35-37). MMP-7-specific antisense oligonucleotides inhibit liver metastasis of human colon cancer cells and gastric cancer invasion (38-41). We have previously shown that MMP-7 is overexpressed in EOC and that recombinant MMP-7 promotes EOC invasion in vitro and induces proMMP-2 activation in DOV13 cells (17).

We also reported that MMP-7 overexpression enhanced the invasiveness of breast cancer cells (35). However, the role and modulation mechanism of MMP-7 in EOC remains to be clarified. In the present study, we evaluated the correlation of MMP-7 expression to EOC invasiveness by transfection of the MMP-7 sense and antisense gene into DOV13 cells. We further

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investigated whether MMP-7 secretion and activity are regulated by lysophosphatidic acid (LPA), a bioactive lipid growth factor that is found to be elevated in the plasma and malignant effusions of the majority of women with EOC (98%) and also promotes EOC metastasis (42).

We previously reported that the proangiogenic molecules vascular endothelial growth factor and interleukin (IL)-8 induce the secretion/activation of MMP-7 in EOC (43, 44). Other reports also found that LPA could induce the expression of vascular endothelial growth factor and IL-8 (43, 45). Thus, we hypothesized that MMP-7 secretion and activation may be regulated by LPA. LPA-mediated signal transduction has shown myriad effects, including transient increase in cytosolic-free calcium, phosphorylation of focal adhesion kinase, activation of mitogen-activated protein kinases, and formation of focal adhesions (46-49). Among these effects, protease secretion and activation is one of the most important steps that ultimately facilitate EOC cells to invade and migrate through the basement membrane and ECM. We have previously reported that LPA increases EOC invasion by induction of MT1-MMP-mediated proMMP-2 activation and urokinase-type plasminogen activator activation (50). Because MMP-7 expression is closely correlated to EOC invasiveness, it may also play an important role in LPA-induced EOC invasion. To test this hypothesis, we assessed the effect of LPA on MMP-7 secretion/activation in DOV13 cells. Furthermore, we examined the effect of MMP-7 knocking down on LPA-induced EOC invasion by using antisense and small interfering RNA (siRNA) transfection.

**Results**

**MMP-7 Expression Is Correlated with DOV13 Invasion**

In vitro

To examine the effect of MMP-7 up-regulation and down-regulation on EOC invasion, the epithelial ovarian carcinoma cell line DOV13 was transfected with either a full-length MMP-7-expressing plasmid (pEXP47-M7-GFP; ref. 35) or an antisense MMP-7-expressing plasmid (pEXP-M7As-GFP; Fig. 1). Four clones that stably expressed pEXP47-M7-GFP were selected using geneticin (G418; 800 μg/mL), and their invasiveness was assessed by Matrigel invasion assay. All of the four clones showed a positive expression of green fluorescent protein (GFP) and significant increase of invasion in comparison with vector-transfected control (P < 0.001; Fig. 2A and B). Using gelatin zymography assay, conditioned medium from the four clones showed increased active form of MMP-7 over vector control (Fig. 2C). One clone (P47-M7As-3) that positively expressed GFP (Fig. 2A) was selected from the MMP-7 antisense-transfected DOV13 cells with geneticin (800 μg/mL). MMP-7 expression in one sense MMP-7-transfected clone (P47-M7-6) and one antisense-transfected clone (P47-M7As-3) was assessed by quantitative PCR. MMP-7 expression in clone P47-M7-6 was 2-fold over the vector control and clone P47-M7As-3 was only 9% of vector control (Fig. 3A and B). Accordingly, the number of cells invaded through Matrigel in the MMP-7 overexpression clone P47-M7-6 (1,750 ± 228, mean ± SD) is ~2-fold of the vector control (754 ± 158, mean ± SD; P < 0.01). In the antisense-transfected DOV13 clone P47-M7As-3, the number of cells that invaded through the membrane was significantly decreased to 13% (101 ± 3) of vector control (754 ± 158; P < 0.05; Fig. 3C). These data show that MMP-7 expression is correlated with EOC invasiveness, with the up-regulation of MMP-7 enhancing DOV13 invasion and down-regulation of MMP-7 attenuating DOV13 invasion.

**LPA Promotes the Secretion/Activation of MMP-7 in DOV13 Conditioned Medium**

To determine whether MMP-7 contributes to LPA-induced EOC invasion, we examined the effect of LPA on MMP-7 secretion and activation by ELISA and gelatin zymography in the nontransfected DOV13 cells. LPA, at 10 to 80 μmol/L, significantly increased the secretion of total MMP-7 in DOV13 conditioned medium (Fig. 4A). At 20 μmol/L, LPA apparently stimulated MMP-7 activation in DOV13 and the activation of MMP-7 seems to be concentration dependent as detected by gelatin zymography (Fig. 4B).

**MMP-7 Antisense Transfection Reduced LPA-Increased DOV13 Invasion**

Because MMP-7 plays a critical role in facilitating EOC cells to invade and migrate through ECM and LPA has shown the ability to promote MMP-7 secretion and activation in DOV13 conditioned medium, LPA may regulate EOC invasion partially through the regulation of MMP-7. To test this hypothesis, we evaluated the invasive behavior on LPA treatment (20 μmol/L) in vector-transfected, pEXP47-M7-GFP-transfected, and P47-M7As-GFP-transfected DOV13 clones (vector, P47-M7-6, and P47-M7-As). In clone P47-M7-6, LPA treatment (20 μmol/L) caused ~2-fold more invasion over nontreated control than in vector-transfected control. In the antisense-expressing clone P47-M7-As, the increase of invasion induced by LPA treatment (20 μmol/L) decreased by 67% (from 1.2-fold to 0.4-fold increase over nontreated control) compared with vector control (Fig. 5A and B). No significant induction of invasion by LPA treatment was observed in the antisense-transfected DOV13 clone P47-M7As-GFP (Fig. 5A and B). Our previous results show that LPA-induced EOC invasion through proMMP-2 activation and MMP-7 promotes EOC invasion also partially through activation of proMMP-2. To test if the effect of MMP-7 antisense transfection on LPA-induced invasion is partially through the regulation of MMP-2 activity, total gelatinase activity was assessed by fluorogenic DQ-gelatin degradation assay. We report that MMP-7 antisense transfection almost completely blocked the LPA-induced increase of gelatinase activity, which may be due to the activation of both proMMP-2 and proMMP-7 (Fig. 5C).

**MMP-7 Knockdown by siRNA Suppresses LPA-Induced DOV13 Invasion**

As full-length MMP-7 antisense transfection may have nonspecific inhibitory effects on other members of the MMP family, such as MMP-2 and MMP-9 (as suggested in Fig. 5C), we further evaluated the effect of MMP-7 knockdown on LPA-induced DOV13 invasion by siRNA. MMP-7 siRNA transient transfection reduced the expression of MMP-7 mRNA in DOV13
cells by a range of 30% to 70% (Fig. 6A). In all three predesigned siRNA-transfected (siMMP-7-09, siMMP-7-31, and siMMP-7-20; Table 1) DOV13 cells, LPA treatment at 10 μmol/L induced considerably less invasion compared with the negative control siRNA (NC-siRNA)-transfected DOV13 cells (Fig. 6B). Zymographic analysis of conditioned medium collected from the invasion assay showed reduced activation of MMP-7 in siMMP-7-09- and siMMP-7-31-transfected DOV13 cells (Fig. 6C). Quantitative assay of total MMP-7 in siRNA-transfected DOV13 cells by ELISA showed a reduction of MMP-7 secretion by 25% to 50% when compared with NC-siRNA-transfected cells (Fig. 6D). The reduction of total MMP-7 correlated with the decrease of LPA-induced invasion, with siMMP-7-31-transfected cells showing the least knockdown of MMP-7 and least suppression of LPA-induced invasion among the three siRNAs. When the transfected cells were treated with LPA at a higher concentration (20 μmol/L), similar reduction of LPA-induced invasion was observed in siMMP-7-31- and siMMP-7-20-transfected DOV13 cells (Fig. 6E). The increase of invasion was down-regulated from 22.2-fold in negative control–transfected DOV13 cells to 4.9-fold in MMP-7 siRNA-transfected DOV13 cells, ~4-fold decrease in comparison with negative control (Fig. 6E). MMP-7 siRNA transfection completely or partially reversed the effect of LPA on MMP-7 activation without showing a significant decrease of MMP-2 secretion/activation (Fig. 6F), suggesting the high specificity of MMP-7 silencing, which was also confirmed by examining MMP-2 expression in MMP-7 siRNA-transfected DOV13 cells (data not shown).

MMP-7 Knockdown by siRNA Suppresses LPA-Induced R182 Invasion

To determine whether the above observed results in DOV13 cells are cell line specific, we evaluated the effect of MMP-7 knockdown by the same siRNAs on LPA-induced invasion in another malignant EOC cell line, R182, which endogenously expressed high levels of MMP-7 (17). In all three siRNA-transfected (siMMP-7-09, siMMP-7-31, and siMMP-7-20) R182 cells, LPA treatment at 10 μmol/L induced a much smaller degree of invasion (2- to 9-fold of nontreated control) than in NC-siRNA-transfected R182 cells, where LPA (10 μmol/L) treatment induced an ~12-fold increase of invasion compared with untreated control (Fig. 7A). The suppressing effect of MMP-7 siRNA on LPA-induced invasion correlated with the decrease of total MMP-7 (pro and active form) secreted in R182 conditioned medium (Fig. 7B), with 50% reduction of MMP-7 secretion almost completely suppressing the effect of LPA on R182 invasion. Gelatin zymographic analysis of the conditioned medium from the above invasion assay revealed a decrease of MMP-7 activation in siMMP-7-transfected R182 cells similar to that in DOV13 cells (data not shown).

Selective Induction of LPA Receptor (LPA3 and LPA4) Correlates with Increased Invasion by LPA Treatment in DOV13 Cells

Although the exact mechanism involved in LPA-stimulated DOV13 invasion and MMP-7 secretion/activation remains unknown, LPA receptors are the main mediators of the effect of LPA. Thus, we first examined the expression levels of the

FIGURE 1. The construct of human MMP-7 mammalian cell expression plasmid pcDNA3.1/NT-GFP-MMP7-As. The coding region of MMP-7 was amplified by PCR from pOTB7-MMP-7 (MGC-3913) using the primer pairs as described in Material and Methods to generate a 910-bp MMP-7 fragment containing single 3′ adenine overhangs. The PCR product was then subcloned into pcDNA3.1-NT-GFP-TOPO vector according to the instruction of NT-GFP Fusion TOPO TA Expression kit (Invitrogen). The antisense orientation of the MMP-7 insert in the vector was verified by sequencing.
four known LPA receptors (LPA1-LPA4; ref. 51) in DOV13 cells and then evaluated the effect of LPA on the expression of those LPA receptors. We show that LPA1, whose expression is 85-fold of LPA2, 32-fold of LPA3, and 112-fold of LPA4, was predominantly expressed in DOV13 cells (Fig. 8A). However, treatment with LPA at 1 to 20 μmol/L significantly induced the expression of only LPA3 and LPA4 (Fig. 8B) without significantly affecting the expression of LPA1 and LPA2 (data not shown), suggesting there may be a correlation between LPA3 and LPA4 expression and LPA-induced DOV13 invasion.

Discussion

The high mortality of EOC is mainly attributed to the difficulty in diagnosing the disease at an early stage with the usual presentation of widely metastatic dissemination observed at initial presentation (52). The EOC metastatic cascade consists of cellular migration, ECM degradation, proliferation, adhesion, membrane vesicle formation, invasion, and angiogenesis, which are uniquely influenced by the bioactive lipid LPA (42, 43, 46, 47, 49, 50, 53-55). LPA has been proposed as a biomarker for ovarian cancer, with several studies showing that plasma levels were elevated in patients with ovarian cancer (49, 56, 57). LPA is produced by ovarian cancer cells but not normal ovarian surface epithelium (58, 59). LPA regulates a myriad of cell signaling pathways to produce numerous effects, including cell proliferation, calcium homeostasis, cytoskeletal reorganization, cell adhesion, migration, and ion transport regulation (60). LPA promotes EOC metastasis through multiple mechanisms, including the stimulation of cyclooxygenase-2 expression (61, 62), promotion of proangiogenic factor production (vascular endothelial growth factor, IL-6, and IL-8) by EOC cells (63-66), enhancement of ovarian cancer cell chemoresistance (67), and activation of various invasion-associated proteases (50, 68-70). Symowicz et al. (61) reported that cyclooxygenase-2 was a mediator of LPA-induced aggressive behavior and MMP-2 secretion/activation. Our previous results showed that the LPA-induced expression of IL-8 is associated with increased expression of MMP-1 and MMP-7 (43), suggesting that protease expression/activation is one of the critical steps in facilitating LPA-induced EOC invasion and migration because many of the above-mentioned pathways lead to increased secretion or activation of MMPs. The first step to transduce cell surface signaling (LPA stimulation) is through

FIGURE 2. Overexpression of MMP-7 in DOV13 cells enhances invasiveness. A. Four MMP-7-overexpressing DOV13 clones, M7-GFP1, M7-GFP2, M7-GFP3, and M7-GFP6, which were selected by cell culture medium containing 800 μg/mL geneticin, showed positive GFP expression as viewed under fluorescence microscope. Magnification, ×100. Stably transfected vector control (>100) and the antisense MMP-7-expressing clone M7As-GFP3 (>200) positively expressed GFP. B. In vitro invasion assay of vector- and MMP-7-transfected DOV13 clones. The number of cells invaded through Matrigel-coated filter membrane in vector- or MMP-7-transfected DOV13 clones was counted as described in Materials and Methods. Cell invasiveness was expressed as the ratio of invaded cells in MMP-7-transfected DOV13 clones (M7-GFP1, M7-GFP2, M7-GFP3, and M7-GFP6) to that in vector-transfected control clone. C. Conditioned media from one invasion assay were collected and run on a 12% SDS-PAGE containing 0.1% gelatin to show the secretion and activation of MMP-7 in the MMP-7-overexpressing clones and vector control.
induction and activation of LPA receptors. Four mammalian cell surface LPA receptors have been identified thus far, with three of them belonging to the endothelial differentiation gene family of G protein–coupled receptors (LPA1, LPA2, and LPA3) and the fourth one (LPA4/GPR23/P2Y9) more closely related to the purinergic G protein–coupled receptor family (51). Disrupting LPA function by preventing LPA-LPA receptor interaction significantly reduces tumor progression and metastasis in experimental animals (71, 72). The signaling pathway that is activated by each individual LPA receptor remains unclear, although more studies that used specific LPA receptor siRNA or small molecular agonists and antagonists to determine the role of each receptor have been reported (46, 66, 73-75). In the EOC DOV13 cells, our preliminary results show that LPA1 is predominantly expressed, whereas LPA3 and LPA4 are the major receptors induced by the LPA treatment. LPA1 was previously shown to be the main regulator of cellular motility (51); however, no induction of LPA1 expression in DOV13 cells was seen by LPA treatment. More experiments are needed to evaluate the role of each LPA receptor in the regulation of DOV13 invasion and MMP secretion/activation. As LPA receptors also mediate multiple physiologic functions of LPA, knocking down LPA-regulated downstream proteases may represent a novel and more specific strategy for EOC therapy.

Recent studies have identified MMP-7 as a promising new therapeutic target in cancers (76), where elimination of MMP-7 is associated with low invasiveness and slow tumor growth in breast cancer (77, 78). In colon and rectal cancer, MMP-7 expression is a promising biomarker predicting nodal metastasis (79) and MMP-7 also has an important role in human rectal carcinoma progression (80). MMP-7-deficient mice showed reduced prostate tumor–induced osteolysis and receptor activator of nuclear factor-κB ligand processing (81). MMP-7 is also involved in cell dissociation and the subsequent invasion of pancreatic cancer cells (82). Fas down-regulation and a consequential increased resistance to Fas ligand–triggered apoptosis resulted from up-regulated MMP-7 in colorectal cancer cells could be a key mechanism for their escape from immune surveillance (83). However, few studies other than the first report by Tanimoto et al. (32) have investigated the role of MMP-7 in EOC. The significant difference of MMP-7 genotype and allelotype distribution in ovarian cancer patients from that in healthy controls found by Li et al. (84) suggests a possible association between the MMP-7 A/G with susceptibility to EOC. We have previously shown that MMP-7 is overexpressed of cellular motility (51); however, no induction of LPA1 expression in DOV13 cells was seen by LPA treatment. More experiments are needed to evaluate the role of each LPA receptor in the regulation of DOV13 invasion and MMP secretion/activation. As LPA receptors also mediate multiple physiologic functions of LPA, knocking down LPA-regulated downstream proteases may represent a novel and more specific strategy for EOC therapy.

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![FIGURE 3.](image)

**MMP-7 expression is correlated to DOV13 invasiveness.**

A. The relative expression of MMP-7 in sense-transfected (p47-M7-6) and antisense-transfected (p47-M7As-3) DOV13 cells to that in vector control was analyzed by real-time PCR in triplicate as described in Materials and Methods. ***, P < 0.01 versus vector control.** B. Representative image from 1.5% agarose gel electrophoresis of MMP-7 regular PCR products in vector control (V), clone P47-M7-6 (M7), and clone P47-M7As-3 (As). C. Matrigel invasion assay of vector-transfected, MMP-7 sense-transfected, and MMP-7 antisense-transfected DOV13 clones. The relative cell invasion was expressed as percentage of cells invaded in vector control. *, P < 0.05 versus vector control; **, P < 0.01 versus vector control.

![FIGURE 4.](image)

**LPA stimulates the secretion and activation of MMP-7 in DOV13 conditioned medium.** Subconfluent DOV13 cells were starved in serum-free medium for 24 hours and then treated with LPA (10-80 μmol/L) for another 24 hours in serum-free medium. Conditioned medium was then collected and centrifuged. The supernatant of each sample was used for the following analyses. A. ELISA. Total MMP-7 protein in supernatants of conditioned medium from each treatment was quantified by Quantikine MMP-7 ELISA kit as described in Materials and Methods. *, P < 0.05 versus control; **, P < 0.01 versus control (n = 3). B. Gelatin zymography.
in the invasive front of EOC and promotes invasion. MMP-7 secretion is increased by vascular endothelial growth factor and IL-8, which are mediators of LPA-induced EOC cell motility and invasion (17, 44, 51), suggesting a role of MMP-7 in the LPA-induced EOC metastasis. In the present study, we clarified the role of MMP-7 in EOC by sense and antisense MMP-7 gene transfection. We showed that MMP-7 expression was closely correlated to EOC invasiveness and LPA induced the secretion/activation of MMP-7 in EOC cells. We then examined the effect of MMP-7 knocking down on the LPA-induced invasion using MMP-7 antisense and siRNA transfection. Our results showed that MMP-7 down-regulation by MMP-7 full-length antisense cDNA transfection significantly decreased LPA-induced DOV13 invasion. However, in addition to the inhibition on MMP-7 expression, MMP-7 antisense transfection seems to have an inhibitory effect on MMP-2 activity (Fig. 5C). It remains unclear whether this inhibitory effect is nonspecific or is due to the subsequent effect of MMP-7 regulation on MMP-2 activity. Because siRNA has recently been exploited to knock down individual genes with high specificity (85, 86), we adopted this technology to resolve the specificity problems encountered with antisense transfection. With observed 30% to 70% knocking down of MMP-7 transcription, siRNA significantly reduced LPA-induced invasion and partially reversed LPA-induced MMP-7 activation in the EOC cell lines DOV13 and R182 (Figs. 6 and 7) without significantly affecting MMP-2 activation (Fig. 6E) and expression (data not shown). More efficient silencing of MMP-7 may be achieved by construction of a siRNA-expressing plasmid that can be stably transfected. More cell lines are also being tested to evaluate the effect of MMP-7 silencing on EOC metastasis.

In summary, the present study is the first to show a close correlation of MMP-7 expression with EOC invasiveness and we are the first to report that MMP-7 plays a critical role in the LPA-induced EOC invasion. We report a new mechanism by which LPA stimulates EOC invasion and that LPA induces EOC invasion through the secretion/activation of MMP-7. The mechanism is a supplement of the previous known induction of proMMP-2 activation by LPA. Because previous studies found that MMP-7 could activate proMMP-2 and proMMP-9 in EOC and promote invasion, the effect of LPA on MMP activation may be initiated by MMP-7 secretion/activation. Although more studies are required to elucidate the MMP activation cascade, our results suggest that MMP-7 is to be considered as a new target for developing effective therapeutics to inhibit EOC metastasis.

Materials and Methods

Materials

Synthetic lysophospholipids (LPA 18:1) were products of Avanti Polar Lipids, Inc. (Alabaster, AL). Protein concentrations were determined using the Bio-Rad DC kit (Bio-Rad Laboratories, Inc., Hercules, CA) with bovine serum albumin as a standard. Matrigel, invasion chambers, and 24-well plates were from Becton Dickinson (Bedford, MA); quenched fluorogenic DQ-gelatin was obtained from Molecular Probes (Eugene, OR); the DNA-free and RETROscript RT kits were

FIGURE 5. MMP-7 antisense transfection reduces LPA-induced increase of DOV13 invasion and gelatinolytic activity. A. Matrigel invasion assay of vector control, P47-M7-GFP6, and P47-M7As-GFP (P47-M7As-3) treated with vehicle (0) or LPA (20 μmol/L). Vector-transfected DOV13 cells, clone P47-M7-GFP6, and P47-M7As-GFP were starved for 24 hours and then trypsinized and resuspended at a concentration of 3 x 10^5/mL. LPA at 20 μmol/L or vehicle was added to the cells in triplicate. Cells (0.5 mL) were then coated on the upper well of Boyden chamber and incubated at 37°C for 24 hours. The number of cells migrated through the Matrigel was counted as described in Materials and Methods. The invasiveness was expressed as the ratio of invaded cells in LPA-treated group to that in nontreated control. B. Typical image of stained invading cells on the bottom side of Matrigel-coated filter membrane. C. Gelatinolytic activity assay. The gelatinolytic activity of MMPs was evaluated by degrading DQ-gelatin as described in Materials and Methods. ns, non-significant. **, P < 0.01 versus nontreated control (n = 3).
from Ambion (Austin, TX); Taq-Pro complete was from Denville (Metuchen, NJ); SYBR Green I Supermix was from Bio-Rad Laboratories; PCR primers were from Integrated DNA Technologies (Coralville, IA); and Tris base, EDTA, and gelatin were purchased from Sigma (St. Louis, MO). All other chemicals were of analytic grade.

**ELISA and Gelatin Zymography**

Total MMP-7 concentration in DOV13 conditioned medium was determined by Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN) as described previously (44). All assays were done in triplicate. For zymography, conditioned medium was resolved under nonreducing conditions on SDS-PAGE gels.

![Graph A](image1)

**FIGURE 6.** MMP-7 siRNA transfection suppresses LPA-induced DOV13 invasion. **A.** Real-time reverse transcription-PCR results show the average knocking down of MMP-7 mRNA by siMMP-7-09 and siMMP-7-31 from three independent siRNA transfection experiments. **B.** The invasiveness of siRNA MMP-7-09-, MMP-7-31-, and MMP-7-20-transfected DOV13 cells was evaluated with the presence of LPA at 10 μmol/L or vehicle control. The ratio of cells invaded by LPA treatment to nontreated control was plotted as relative invasion. **C.** Gelatin zymography (12% SDS-PAGE) analysis of conditioned medium collected from the above invasion assay showing active MMP-7. **D.** ELISA shows the decrease of total MMP-7 secreted in siMMP-7-transfected DOV13 cells compared with NC-siRNA-transfected DOV13 cells. **E.** The effect of siRNA MMP-7-31 and MMP-7-20 transfection on DOV13 invasion was evaluated with the presence of 20 μmol/L LPA or vehicle control. **F.** Gelatin zymography (12% gel) analysis of conditioned medium collected from the above invasion assay showing MMP-2 and active MMP-7.

### Table 1. siRNA Duplex Sequences Used in This Work

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<th>Name</th>
<th>Target exon</th>
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<td>CAUAUGUUCEUGAAUGCCT</td>
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containing 0.1 mg/mL gelatin. Gel was rinsed with 2.5% Triton X-100 followed by incubation in developing buffer and finally stained with Coomassie blue as reported previously (17). Areas where have gelatinolytic activity were visualized as transparent bands.

**Generation of Plasmid Constructs**

For sense MMP-7-expressing plasmid (pEXP47-M7-GFP), the full-length human MMP-7 cDNA (MGC-3913, American Type Culture Collection mammalian gene collection, Manassas, VA) was subcloned into the mammalian expression vectors pcDNA-DEST47 (Invitrogen, Carlsbad, CA) using Invitrogen Mammalian Expression System with Gateway Technology as reported previously (35). For antisense MMP-7, the coding region of MMP-7 was subcloned into pcDNA3.1-NT-GFP-TOPO vector (Invitrogen) from the pOTB7-MMP-7 plasmid by PCR using the forward primer 5'-GCCAGTAACTATAAGGTAG-3' and the reverse primer 5'-GTTCTGCCTGAAGTTTAAATTCTTTCTTGAATTAC-3'. The orientation of the MMP-7 insert in the vector was verified by sequencing of the insert, and the antisense direction construct (pEXP-M7As-GFP) was used for MMP-7 knockdown experiments (Fig. 1). pcDNA-DEST47-transfected or pcDNA3.1-NT-GFP-TOPO-transfected DOV13 cells were used as vector control.

**Cell Culture and Establishment of Stable Cell Lines**

The ovarian carcinoma cell line DOV13 was provided by Dr. R. Bast, Jr. (M. D. Anderson Cancer Center, Houston, TX), and R182 was provided by Dr. G. Mor (Yale University, New Haven, CT) and cultured as reported previously (5, 44). Experiments were done with cells in the logarithmic phase of growth. DOV13 cells were transfected with expression vectors coding for GFP alone (vector control), GFP fused with MMP-7 (pEXP47-M7-GFP), or GFP fused with antisense MMP-7 (pEXP-M7As-GFP) using the Fugene 6 transfection reagent (Roche, Indianapolis, IN) following the protocol offered by the manufacturer as reported previously (35). For stable transfection, the transfected cells were selected with geneticin (G418; 800 μg/mL) 24 hours after transfection. Stably transfected clones were isolated using cloning cylinders and maintained in normal culture medium containing 600 μg/mL geneticin. Clones were screened and selected for MMP-7 expression and invasiveness analysis.

**siRNA Transfection**

siRNA duplexes were predesigned to human MMP-7 by Ambion. The sequences of siRNA used in this study were shown in Table 1. DOV13 and R182 cells were transfected, and the transfection conditions were optimized according to the instructions of the Silencer siRNA Transfection II kit (Ambion). Briefly, cells that reached 80% confluence were trypsinized, centrifuged, and resuspended at a concentration of 1 × 10^5/mL. siPORT NeoFX (5 μL) was diluted into 95 μL Opti-MEM I, vortexed well, and incubated at room temperature for 10 minutes. The required amount (10 nmol/L) of siRNA was diluted into a final volume of 100 μL in Opti-MEM I and gently mixed. The siPORT NeoFX/Opti-MEM mixture was then added to the siRNA solution, mixed well, and incubated at room temperature for 10 minutes and then added to six-well plate, with the addition of 2.3 mL cells to the plate. The plate was then incubated at 37°C in the presence of 5% CO2 until further analysis.

**RNA Preparation and Quantitative Reverse Transcription-PCR**

Total RNA was extracted from stably transfected DOV13 clones or 48 hours after transfection of DOV13 cells with MMP-7 siRNA using the GenElute Mammalian Total RNA kit (Sigma). After DNase I treatment using DNA-free kit (Ambion), 1 μg total RNA was reverse transcribed to first-strand cDNA using RETROscript kit according to the protocol provided by the kit. Reverse-transcribed products were then amplified using iQ SYBR Green Supermix for real-time PCR. The MMP-7, LPA1, LPA2, LPA3, LPA4, and glyceraldehyde-3-phosphate dehydrogenase primers were as described in previous studies (17, 35, 44, 87). PCR products were analyzed electrophoretically

**FIGURE 7.** MMP-7 siRNA transfection decreases the secretion of total MMP-7 and suppresses the LPA-induced invasion in R182 cells. A. Matrigel invasion assay of MMP-7 siRNA-transfected R182 cells with or without the treatment of 10 μmol/L LPA. Cell invasion was expressed as fold difference of invading cells in LPA-treated R182 cells to that in the nontreated control. **, P < 0.01 versus nontreated control; ***, P < 0.001. ns, not significant (P > 0.05). B. Total MMP-7 ELISA. The total MMP-7 secreted in the MMP-7 siRNA-transfected R182 conditioned medium was expressed as percentage of total MMP-7 concentration in NC-siRNA-transfected cells. *, P < 0.05 versus NC-siRNA.
by 1.5% agarose gels in the presence of 0.5 μg/mL ethidium bromide, and DNA was visualized under UV light. Real-time PCRs were carried out on iCycler (Bio-Rad) as reported previously (35).

Matrigel Invasion Assay

Analysis of in vitro invasion was done as described previously (17, 35, 44). Briefly, stably or transiently transfected DOV13 cells (1.5 × 10^5 per well) were seeded in the upper compartment of Matrigel-coated inserts (11 μg/filter, 8-μm pore size) after 24 hours of serum starvation and incubated at 37°C in a humidified atmosphere. After 24 hours, noninvaded cells were removed from the upper surface of the filters with a cotton swab followed by fixation and staining with the Diff-Quick staining kit (Fisher Scientific, Morris Plains, NJ). All experiments were completed in triplicate, and each clone was tested at least in two independent assays. Cells fixed on the lower face of filters were counted and quantified as described previously (17, 35, 44).

Gelatinolytic Activity Assay by Quenched Fluorogenic DQ-Gelatin

Vector and antisense MMP-7-transfected cells were plated onto 12-well plates at a density of 4 × 10^5 per well and incubated overnight in a humidified atmosphere at 37°C with 5% CO2. Cells were treated with LPA for 24 hours followed by overnight starvation in serum-free medium, then conditioned medium was collected and centrifuged, and 100 μL supernatant from each sample was incubated with quenched fluorogenic DQ-gelatin in reaction buffer for 24 hours as described previously (17). The fluorescence was measured at 485/515 nm using the fluorescence microplate reader (Wallac Victor 1420 Multilabel Counter, Perkin-Elmer Life and Analytical Sciences, Torrance, CA). The fluorescence intensity of untreated control (gelatinolytic activity) was designated as 1, and the gelatinolytic activity of LPA-treated DOV13 clones is expressed as the ratio of their measured fluorescence to that in untreated control.

Statistical Analysis

Gelatinase activity and cell invasion assays were done in triplicate in three separate experiments. Data analysis was done using one-way ANOVA and unpaired t test (GraphPad Prism, GraphPad Software, Inc., San Diego, CA). A P < 0.05 was considered statistically significant.

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References

in vivo colon carcinoma cell lines affects tumorigenicity.


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