

p37 from *Mycoplasma hyorhinis* promotes cancer cell invasiveness and metastasis through activation of MMP-2 and followed by phosphorylation of EGFR

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Abstract

High *Mycoplasma* infection in gastric cancer tissues suggests a possible association between *Mycoplasma* infection and tumorigenesis. By using human gastric cancer cells AGS and mouse melanoma cells B16F10 stably expressing p37, the major immunogen of *Mycoplasma hyorhinis*, we found that p37 enhanced cell motility, migration, and invasion *in vitro*. With experimental metastasis model in C57BL/6 mice, p37 adenovirus-infected B16F10 cells formed more metastasis lesions in the lung. Furthermore, p37 promoted the phosphorylation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase and the activity of matrix metalloproteinase-2 (MMP-2). Inhibitor of MMPs significantly blocked p37-induced EGFR but has little effect on extracellular signal-regulated kinase phosphorylation, whereas the p37-induced MMP-2 activation was only partially suppressed by inhibitor of MEK1/2 or by inhibitor of EGFR. However, all these inhibitors significantly reduced the p37-induced invasiveness of AGS cells. These results suggest that p37 may stimulate invasion by increasing the activity of MMP-2, thereby inducing EGFR phosphorylation and contributing to tumor metastasis on *M. hyorhinis* infection. p37 and its regulated molecules could be the potential targets for cancer therapy. [Mol Cancer Ther 2008;7(3):530–7]

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Introduction

Mycoplasmas are a heterogeneous group of the smallest organisms capable of self-replication. It can cause a wide variety of diseases in animals (1–5). However, investigating the possible association between *Mycoplasma* infection and carcinogenesis did not become more active until Tsai et al. reported that continuous infection of *Mycoplasma penetrans* or *Mycoplasma fermentans* could lead to multiple stages of malignant transformation in murine embryonic C3H cells, accompanied by abnormal karyotypes and some oncogenic up-regulation (6, 7).

p37 is a lipoprotein of *Mycoplasma hyorhinis* located on the outside of the cell membrane. It is thought to be part of a high-affinity transport system for *M. hyorhinis* and no homologous genes had been found in mammalian genomes (8, 9). Also, there are several lines of evidence indicating the association between p37 or *M. hyorhinis* and cancer (10–12). Recently, Ketcham et al. showed that recombinant p37 enhanced the invasiveness of prostate carcinoma cells and melanoma cells in a dose-dependent manner *in vitro* (13). Both our and other's works indicated p37 could induce expression and secretion of tumor necrosis factor- α (TNF- α) from human peripheral blood mononuclear cells (14–16). Our previous study also showed that p37 inhibited adhesion of human gastric cancer cells AGS (17). Thus, *M. hyorhinis* infection may facilitate tumor invasiveness via p37. However, the precise mechanisms responsible for it are not well understood. In this study, we established AGS cells and B16F10 cells stably expressing p37. *In vitro* experiments revealed that p37 promoted cell motility, migration, and invasion. p37 also enhanced the lung metastasis in C57BL/6 mice after i.v. injection of p37 adenovirus-infected B16F10 cells. Additionally, p37 promoted the activation of matrix metalloproteinase-2 (MMP-2) along with phosphorylation of epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK) 1/2 in a dose-dependent manner. Inhibitor of MMPs (GM6001) significantly blocked p37-induced EGFR phosphorylation, whereas inhibitor of EGFR (AG1478) has little effect on p37-induced MMP-2 activation. Moreover, inhibitors of EGFR, MEK1/2, and MMPs blocked the p37-induced invasion in AGS cells. Therefore, p37 may stimulate invasion, thereby inducing EGFR phosphorylation and contributing to tumor metastasis on *M. hyorhinis* infection.

Materials and Methods

Cell Culture and Other Reagents

Flp-In-T-REx-293 cell line was purchased from Invitrogen. Human gastric cancer cell line AGS and mouse

melanoma cell line B16F10 were from American Type Culture Collection. For maintenance, Flp-In-T-REx-293 cells were cultured in DMEM supplemented with 10% FCS. AGS cells were cultured in F-12 nutrient medium plus 10% FCS. B16F10 cells were cultured in RPMI 1640 plus 10% FCS. All culture media were purchased from Invitrogen. PD4, a mouse monoclonal antibody against p37, was generated in our laboratory (18). Antibodies to EGFR, pEGFR, ERK1/2, pERK1/2, MMP-2, and horseradish peroxidase-conjugated goat anti-mouse or rabbit antibodies were purchased from Santa Cruz. Transwell chambers (24-well) with 8.0- μ m pore membranes were purchased from Corning. Growth factor-reduced Matrigel was purchased from Becton Dickinson. Inhibitors of EGFR (AG1478), MMPs (GM6001), and MEK1/2 (U0126) were purchased from Sigma-Aldrich.

Expression and Purification of p37

After transfecting p37 into the Flp-In-T-REx-293 cells, we established a stable cell line expressing p37 (19). To purify p37 protein from the culture medium, we collected supernatant from the stable 293 cells. Purified anti-p37 monoclonal antibody PD4 was conjugated to Sepharose-4B beads (Sigma). After centrifugation and filtration, the supernatant was loaded onto the PD4-Sepharose-4B column at 4°C. The column was washed with PBS and the p37 protein was eluted with elution buffer (0.2 mol/L Gly-HCl, pH 2.8). The eluted sample was then neutralized with 1 mol/L Tris (pH 8.8) and dialysed against PBS at 4°C. The purified p37 protein was quantified at OD280 nm, aliquoted, and stored at -80°C. Following this protocol (20), 1 L supernatant yielded ~2 mg p37 protein.

Cell Mobility Assay

AGS cell mobility was assessed using a wound healing assay (21). The cells were seeded into six-well tissue culture dishes and cultured until confluent to get cell monolayers, which were then carefully wounded using sterile pipette tips and any cellular debris was removed by washing with PBS. The wounded monolayers were then incubated in medium with varying concentration of p37. Photos were captured at 0, 12, 24, and 36 h after wounding.

Cell Migration and Invasion Assay

Cell migration assay was carried out by using tissue culture-treated 6.5-mm Transwell chamber with 8.0- μ m pore membranes. According to the previous method (22), the bottom chamber was filled with 800 μ L medium containing 10% FCS and 2% bovine serum albumin as chemoattractant. Cells resuspended in serum-free medium, containing p37 with inhibitors or DMSO, were carefully transferred onto the top chamber of each Transwell apparatus at a density of 10^6 cells/mL (100 μ L/chamber). Cells were allowed to migrate for 24 h at 37°C. After removing the cells that remained in the top chamber, the top surface of each membrane was cleared of cells with a cotton swab. Cells that had penetrated to the bottom side of the membrane were then fixed in methanol, stained using hematoxylin, and counted in nine randomly selected microscopic fields ($\times 200$) per well.

The invasion assay was similar to the migration procedure described above, except that the upper side of the membranes was coated with a uniform thickness of 100 μ g Matrigel for 60 min at room temperature before experiment.

Animal Assay

All the animal experiments were approved by the Medical Ethics Committee of Beijing Institute for Cancer Research. Female C57BL/6 mice (Vital River Laboratories) were 8 to 10 weeks old, weighed 18 to 20 g, and maintained in a germ-free environment in the animal facility. To investigate the role of p37 in metastasis *in vivo*, we used the adenovirus expressing p37 to infect mouse melanoma cell line B16F10 and injected the infected cells into the tail vein of C57BL/6 mice (2.5×10^5 cells per recipient). After 25 days, the mice were sacrificed and the quantity of metastasis lesions on the surface of lung was counted.

Gelatin Zymography

AGS cells in subconfluent culture (~70-80% cell density of confluent culture) were washed and refreshed with serum-free F-12 and then incubated with or without p37 protein for 24 h. In some experiments, cells were preincubated for 1 h with inhibitors or DMSO before incubation with p37. The enzymatic activity of electrophoretically separated gelatinolytic enzymes in the conditioned medium of AGS cells was determined by gelatin zymography as described previously (23). Zones of gelatinolytic activity were detected as clear bands against a blue background. Densitometric analysis was done using Scion Image program.

Western Blot Analysis

To assess the effect of p37 on EGFR and ERK activation, AGS cells were starved in serum-free medium for 24 h and then stimulated with different concentration of p37 protein for 24 h. In parallel, cells were preincubated with GM6001 (5 μ mol/L) or DMSO for 1 h before adding the p37 protein. Cells were lysed in lysis buffer as described previously (17). Equal aliquot of the total protein was separated by 12% SDS-PAGE and blotted to nitrocellulose membrane then probed with anti-EGFR, anti-pEGFR, anti-ERK1/2, or anti-pERK1/2.

Statistical Analyses

The data were analyzed by ANOVA. The statistical analysis was done using SPSS 11.0 software (SPSS) and $P < 0.05$ was considered significantly.

Results

p37 Promotes Cell Motility, Migration, and Invasion *In vitro*

The effect of p37 on the motility capability of AGS cells was analyzed using a wound healing assay. Recombinant p37 protein was affinity purified and verified by SDS-PAGE staining and Western blot (Supplementary Fig. S1).¹ Confluent monolayers of AGS cells were scratch wounded

¹ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

with sterile pipette tips and incubated for a further 36 h in the absence or the presence of p37 protein. We observed that cell flattening and spreading along the edges of the wound was enhanced in the presence of p37 (AGS + p37) or stably transfected with p37 (AGS-P) compared with the respective controls (Fig. 1). Similarly, the migration ability of AGS cell was promoted when transfected with p37 or treated with p37 protein ($\geq 20 \mu\text{g}/\text{mL}$; Fig. 2A). It was shown as dose-dependent manner (data not shown).

A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the confines of the primary tumor environment (24). To examine the effect of p37 on cell invasion, a modified Transwell chamber assay was carried out to determine the ability of AGS cells to invade through biological matrices *in vitro*. As shown in Fig. 2A, when transfected with p37 gene or treated with p37 protein, the cells capable of invading through the filter coated with Matrigel were increased by $\sim 127.16\%$ ($P < 0.001$) and 155.81% ($P < 0.001$) compared with the control, respectively. Additionally, the recombinant adenovirus expressing p37 were established. We then used the uninfected B16F10 cells (B16), cells infected with p37 adenovirus (Ad-p37), or control adenovirus (Ad-c) to perform the Transwell experiments, and similar results were obtained (Fig. 2B).

p37 Stimulates Tumor Metastasis in C57BL/6

Now that p37 promoted cell invasion and migration *in vitro*, we wonder whether p37 plays a role in tumor metastasis. However, we found that AGS cells were difficult to form tumor in mice. Therefore, we chose the

p37 adenovirus-infected B16F10 cells for cancer metastasis assay *in vivo*. Twenty-five days after the tail vein injection, the lung metastasis lesions of C57BL/6 mice were counted. In this experiment, we found that the p37 group had much more metastasis than control groups (Fig. 3; $P < 0.001$).

p37 Up-Regulates the Activity but not the Expression of MMP-2

Tumor cell invasion through matrix and tissue barriers requires the combined effects of increased cell motility and regulated proteolytic degradation of matrix. Elevated levels of the MMPs in tumor tissue have been generally correlated with cancer cell invasion and metastasis. As shown in Fig. 4A, p37 had no effect on the protein level of 72-kDa form of MMP-2 (proMMP-2) in AGS cells, but the level of proMMP-2 in the medium was significantly up-regulated after transfection with p37 gene or treatment with p37 protein in the zymography assay, indicating that p37 promotes proMMP-2 secreting from the AGS cells. As p37 enhanced cell motility, migration, and invasion *in vitro* and stimulated metastasis *in vivo*, we propose the possibility of indirect way(s) by which p37 up-regulate the activity of MMP-2.

p37-Induced MMP-2 Activation Cannot Be Blocked by EGFR Inhibitor

To further understand the mechanism of p37-induced MMP-2 activation, we next used MEK1/2 and EGFR inhibitor to examine the roles of mitogen-activated protein kinases. As shown in Fig. 4B, the p37-induced MMP-2 activation was only partially inhibited by MEK1/2 inhibitor (U0126), whereas the EGFR inhibitor (AG1478) had no effect. As a control, the MMP-2 activity was strongly blocked by MMP inhibitor (GM6001).

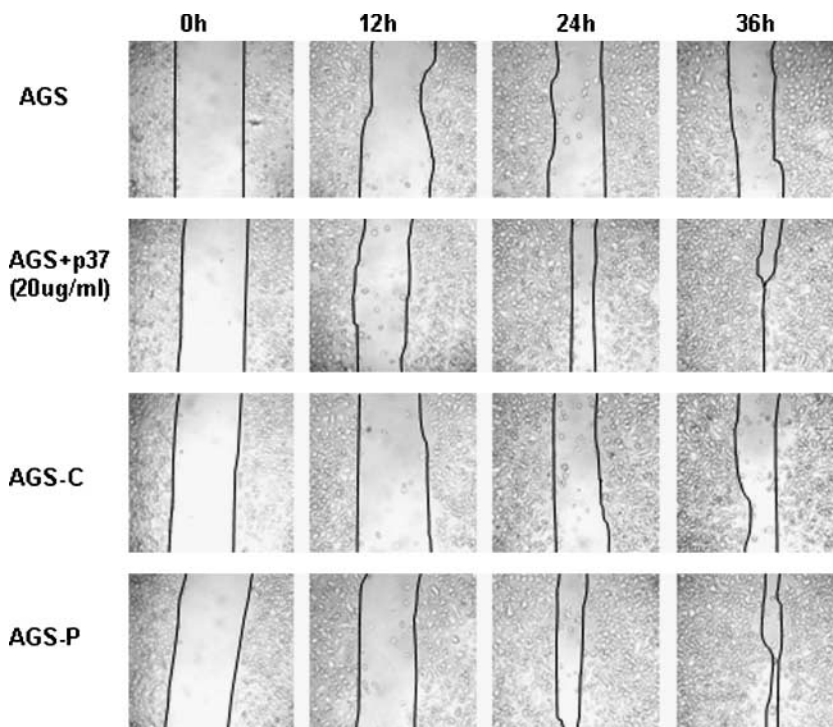
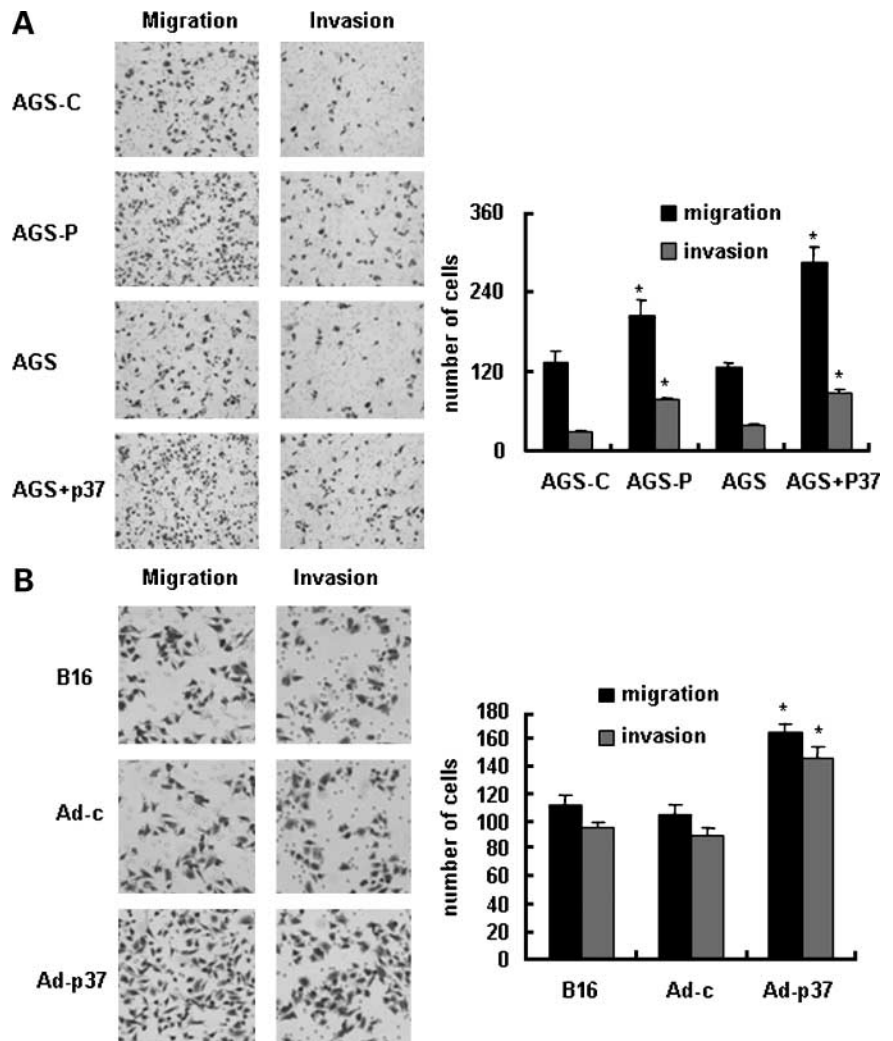


Figure 1. p37 enhances the wound healing ability of AGS cells. AGS cells were seeded into six-well tissue culture dishes and cultured until confluent to get cell monolayers, then were carefully wounded using sterile pipette tips, and any cellular debris was removed by washing with PBS. The wounded monolayers were incubated in medium with indicated concentration of p37. Photos were taken at 0, 12, 24, and 36 h after wounding.

Figure 2. p37 promotes migration and invasion of different cells *in vitro*. **A**, migration and invasion of AGS cells transfected with p37 (AGS-P) or control (AGS-C) treated by p37 protein (20 μ g/mL; AGS + p37) or bovine serum albumin (AGS). **B**, migration and invasion of uninfected B16F10 cells (B16), cells infected with p37 adenovirus (Ad-p37), or control adenovirus (Ad-c). Three independent experiments were done. Average of nine microscopic fields for each experimental condition; bars, SE. *, $P < 0.001$.



p37 Promotes Phosphorylation of EGFR and ERK1/2, Which Could Be Significantly Blocked by MMP Inhibitor

To study the role of EGFR in p37-induced invasion, the total or phosphorylated EGFR and ERK1/2 were assessed. As shown in Fig. 5A, p37 enhanced phosphorylation of EGFR at a concentration of 20 μ g/mL and ERK1/2 at 50 μ g/mL, respectively. To examine whether the effect of p37 on EGFR phosphorylation was downstream of the activity of MMPs, which could promote the cleavage and secretion of EGF-like ligands into the culture medium (25), the cells were preincubated with an inhibitor of MMPs (GM6001) for 1 h before treatment with p37. We noticed that p37-induced EGFR phosphorylation was strongly suppressed by GM6001 (Fig. 5B).

Inhibitors of EGFR, MEK1/2, and MMPs Suppress p37-Induced Invasion of AGS Cells

Our results revealed that p37 enhanced MMP-2 activation and subsequently induced EGFR phosphorylation, so we next sought to examine whether the inhibitors of EGFR, MEK1/2, or MMPs could suppress the p37-induced inva-

sion. As expected, all of these inhibitors blocked p37-induced invasiveness in AGS cells (Fig. 6A). This result suggested all these molecules are related to p37-promoted AGS cell invasiveness.

Discussion

The importance of human-associated mycoplasmas first became apparent in the 1960s on the characterization of the novel human respiratory pathogen *Mycoplasma pneumoniae* (3–5). It was reported that infection of *Mycoplasma* caused chromosomal aberrations (3). Other research in the 1980s reported that an arthropod spiroplasma could rapidly transform mouse and monkey cells (6). In the 1990s, there were discrete reports that *Mycoplasma* could transform some human cells (6, 7).

PD4, a specific monoclonal antibody that recognizes the unique *M. hyorhinae*-specific protein p37, was used to detect *Mycoplasma* infection in paraffin-embedded carcinoma tissues (18). The results indicated that positive rate of

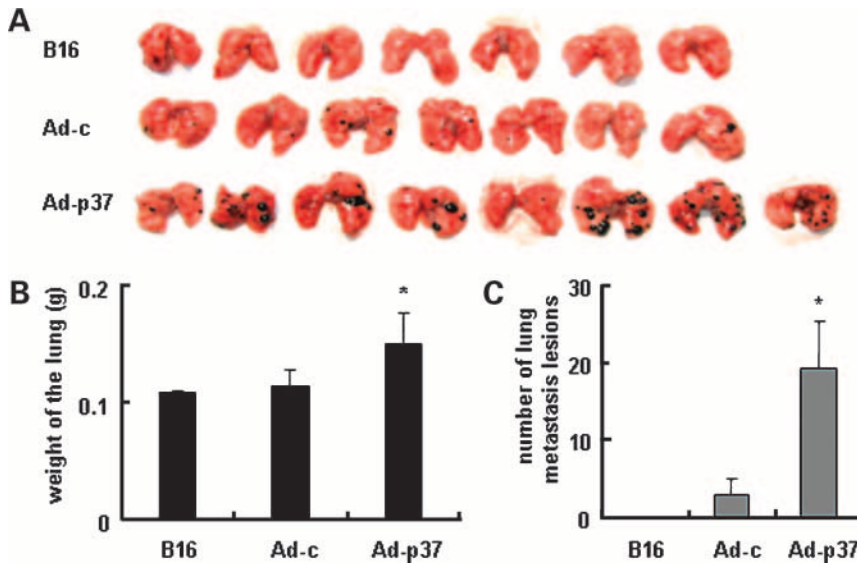


Figure 3. p37 stimulates tumor metastasis in C57BL/6 mice. **A**, metastasis lesions of mice lungs after tail vein injection with B16F10 cells. **B**, weight of mice lungs. **C**, number of metastasis lesions. *, $P < 0.001$. *B16*, uninfected B16F10 cells; *Ad-c*, B16F10 cells infected by control adenovirus; *Ad-p37*, B16F10 cells infected by p37 adenovirus.

infection in gastric carcinoma, esophageal cancer, colon carcinoma, and lung cancer was ~50% but was <25% in other gastric diseases, such as chronic superficial gastritis, gastric ulcer, or intestinal metaplasia (18).

Cancer progression involves a sequential series of critical genetic and molecular alterations inducing the deregulation of cell proliferation, adhesion, migration, and invasion and leading to the lethality associated with metastatic spread of malignant tumors. *M. hyorhinis* and p37 have long been associated with increased invasiveness and tumor metastasis (7–13, 26, 27). One aspect is the elevated tumor invasion, as suggested by a leukocyte adherence inhibition response, correlated with the presence of *M. hyorhinis* in patients with lung cancer, colon cancer, and breast cancer (26). Additionally, two independent groups showed that surface expression of p37 in FS9 mouse fibrosarcoma cells was associated with a highly invasive phenotype as measured in the *in vitro* invasion assay (8, 10, 27). Antibody against p37 inhibited the invasive

potential of infected FS9 cells in the *in vitro* assay (10, 26). Using Abercrombie’s confronted explants assay, it was shown that antibodies against p37 reverted malignant cells to a normal phenotype (11). Moreover, p37 antibodies were reported to reduce the lung metastasis of colon cancer in the nude mice model (12).

We have detected the p37 protein in the medium of MGC-803 and AGS cells infected with *M. hyorhinis* (data not shown). Recent reports revealed that purified recombinant p37 bound to cell surfaces and stimulated the invasiveness of human cell lines (13). Our previous work revealed that p37 inhibited adhesion of human gastric cancer cells AGS (17). The primary cause of malignancy is the loss of normal control of cell motility and growth. In our previous work, p37 did not show significant effect on the growth of AGS cells (data not shown); thus, p37 may not be involved in the *Mycoplasma* metabolic machinery that adds additional stress to the host cells. At the same time, we found that the S phase of AGS cells stably

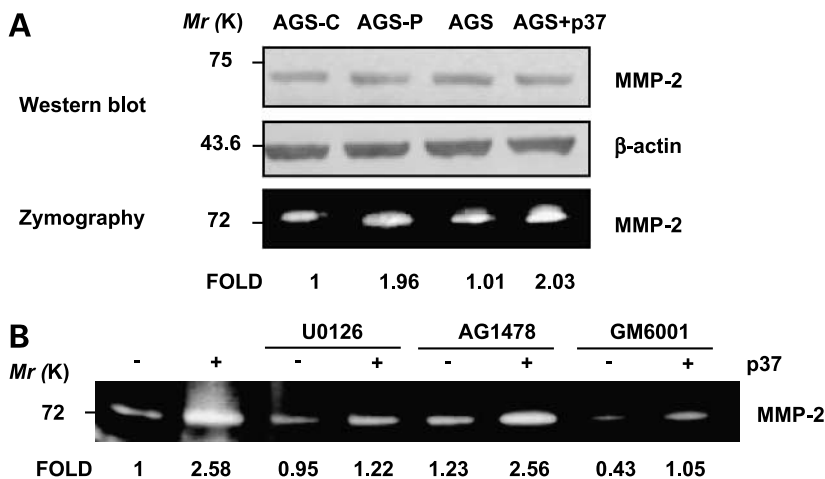


Figure 4. Effects of p37 and different inhibitors on MMP-2 activity. **A**, p37 up-regulates the expression of proMMP-2 in medium but not in AGS cells. AGS were either transfected with p37 or treated with p37 protein (20 μg/mL). Cell lysates were subjected to Western blot analysis and the media were subjected to gelatin zymography. Densitometric analysis was done using Scion Image program and fold of activation was shown. **B**, gelatin zymography of conditioned medium of AGS cells after treated with U0126 (10 μmol/L), AG1478 (20 μmol/L), or GM6001 (5 μmol/L), in the presence or absence of p37 protein (20 μg/mL).

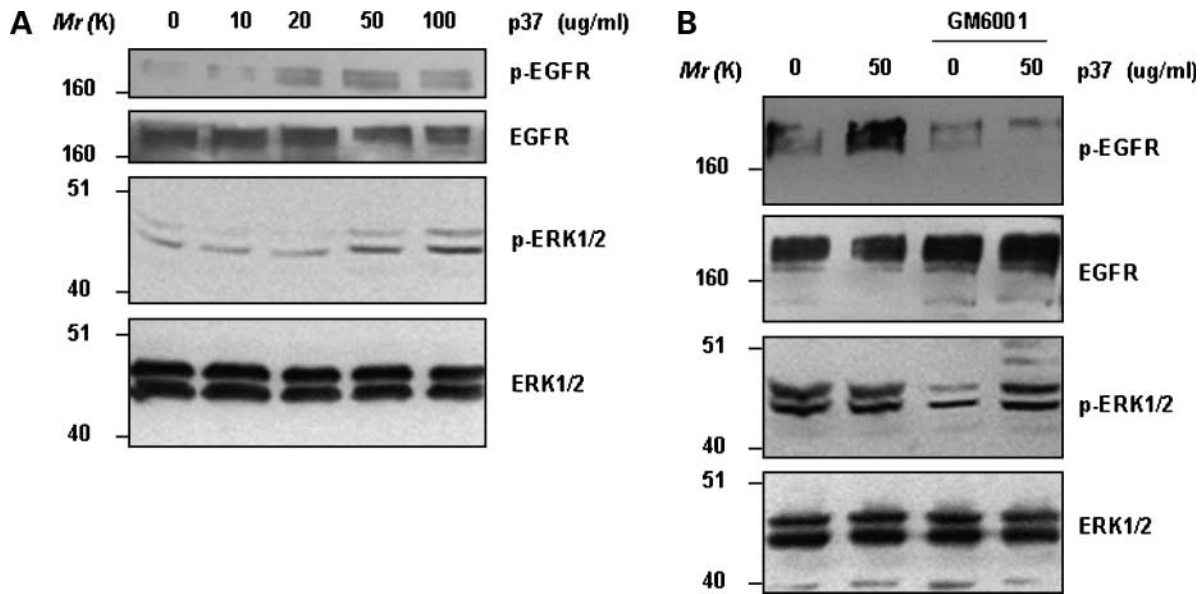


Figure 5. p37 stimulates EGFR and ERK1/2 phosphorylation in AGS cells. **A**, expression of pEGFR, total EGFR protein, pERK1/2, and total ERK1/2 protein in AGS cells treated with increasing concentrations of p37 protein. **B**, GM6001 inhibits the p37-induced pEGFR. AGS cells were preincubated with GM6001 (5 μmol/L) for 1 h and then stimulated with p37 (50 μg/mL) for 24 h. Cell lysates were subjected to Western blot with indicated antibodies.

transfected with p37 was longer than that of control cells in flow cytometry analysis. It remains a possibility that p37 may affect the growth or survival of AGS cells.

Metastasis is a multistep, multifactor process involving cancer cells, host cells, extracellular matrix, and many metastasis-associated molecules (24). We have reported that the AGS cells, after being transfected with the p37 gene, were smaller, more spherical, and easy to detach from each other. Their adhesion to matrix was also diminished

(17). In this study, we found that p37 also contributed to increase the cell motility. Furthermore, we found that the migration and invasion of AGS and B16F10 cells were promoted by p37, either being transfected with p37, or treated with p37 protein or infected with p37 adenovirus (Fig. 2). In the migration assay, the stimulation by p37 was dose dependent (data not shown). In addition, we have also established the experimental metastasis model in C57BL/6 by i.v. injection of p37 adenovirus-infected B16F10 cells.

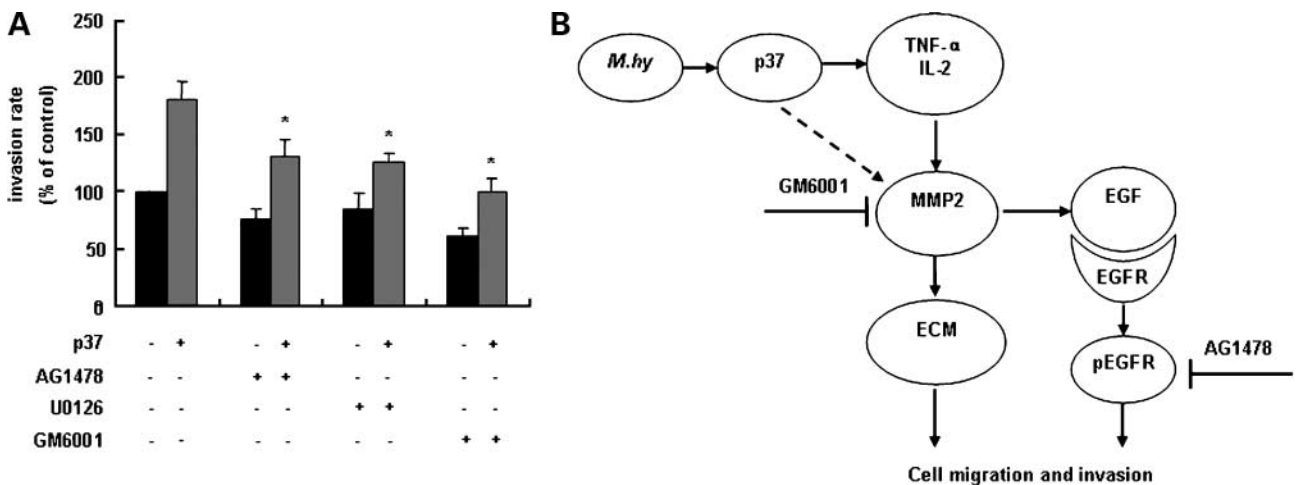


Figure 6. **A**, effects of p37 and inhibitors on the invasion ability of AGS cells. Cells were pretreated with AG1478 (20 μmol/L), U0126 (10 μmol/L), or GM6001 (5 μmol/L) followed by treatment with p37 protein (20 μg/mL). The cells invading through Matrigel were counted under microscope in nine random fields at ×200. Representative of three separate experiments. *, $P < 0.001$. **B**, proposed model for signal pathways involved in the p37-induced invasion and metastasis. During *M. hyorhinis* infection, p37 protein promotes cytokines (e.g., TNF-α and interleukin-2) secretion from human peripheral blood mononuclear cells. Either by TNF-α or by alternative pathways, p37 potentiates MMP-2 secretion and activation. The activated MMP-2 then degrades extracellular matrix (ECM) and induces EGFR phosphorylation, thereby contributing to cell migration and invasion.

The p37 adenovirus-infected B16F10 group showed much more lung metastasis lesions than control group. These suggested that p37 had direct or indirect function in metastasis.

After the *Helicobacter pylori* was reported to induce gastric cancer (28), infection or chronic inflammation has been associated with increased incidence of malignancy. Infiltration of innate immune cells, elevated activities of MMPs, and increased angiogenesis and vasculature density are a few examples of the similarities between chronic and tumor-associated inflammation (29). Because of the high potential to degrade tissues, MMPs are involved in a number of physiologic processes, such as embryogenesis and wound healing, as well as pathologic processes, such as tumor angiogenesis and metastasis (30). In addition, several MMPs, such as MMP-1, MMP-2, MMP-3, MMP-7, and MMP-11, directly modulate the activity of several growth factors, such as TNF- α , insulin-like growth factor-1, EGF, and fibroblast growth factors (31–35). It is reported that the production and activity of some MMPs are up-regulated in response to specific stimuli during tissue injury or inflammation when there is a need for tissue remodeling and degradation (36). We then hypothesized that the stimulatory effect of p37 or mycoplasmal infection on tumor invasion and migration is associated with MMPs. To test this, we employed the zymography assay. After being treated with p37 protein, level of proMMP-2 in the medium of AGS cells were up-regulated. Furthermore, we found that the p37-induced invasion was strongly blocked by the MMP inhibitor. These suggested that MMP-2 may be involved in p37-induced invasiveness.

We reported previously the down-regulation of ICAM-1 and integrin β_1 in AGS cells transfected with p37 gene (17). A collagen-binding integrin $\alpha_1\beta_1$ functions as a negative regulator of EGFR signaling through the activation of a protein tyrosine phosphatase (37). In this study, we found that p37 promoted phosphorylation of EGFR and ERK1/2 in a dose-dependent manner. Many growth factor receptors are substrates of MMPs. The fibroblast growth factor 1 is cleaved by MMP-2 (32), whereas two members of the EGFR family, HER2 and HER4, are substrates for unidentified MMPs (38, 39). We wonder if the effect of p37 on EGFR phosphorylation was secondary to the activity of MMPs, which could promote the cleavage and secretion of EGF-like ligands into the culture medium (40). In our present work, the p37-induced EGFR phosphorylation was greatly suppressed by MMP inhibitor GM6001, whereas the p37-induced MMP-2 activation was not blocked by EGFR inhibitor AG1478. These results suggested that MMP-2 may be the upstream molecule to activate EGFR in this event.

Biological factors lead to carcinogenesis mainly through two ways: promoting genomic instability and inducing some kinds of cytokine secretion. Although there were discrete reports that *Mycoplasma* could transform some cells (6, 7), there was no mycoplasmal genetic integration in host genome (41), which suggests that the latter way is more important. Some inflammation factors and growth factors, such as interleukin-1, TNF- α , and EGF, were reported to

affect the transcription and activity of MMPs (42). In this study, we revealed that the activity of MMP-2 was greatly induced by p37. MMP-2 is secreted into the tissue in a latent form, proMMP-2. The activation of this latent enzyme is controlled by cleavage of NH₂-terminal prodomain. This is coordinated by the membrane-type metalloproteinases in participation with the tissue inhibitor of metalloproteinases (43). Moreover, TNF- α was found to significantly induce membrane-type metalloproteinase expression and stimulate proMMP-2 activation through nuclear factor- κ B signal transduction pathway (44). We have reported that p37 induced secretion of TNF- α from human peripheral blood mononuclear cells (14). TNF- α may play similar role during p37-induced MMP-2 activation and cell invasiveness. In this scenario, on *M. hyorhinis* infection, p37 protein promotes cytokines (e.g. TNF- α and interleukin-2) secretion followed by up-regulated MMP-2 secretion and activation. The activated MMP-2 then degrades extracellular matrix and elicits signaling events through EGFR/MEK/ERK phosphorylation, thereby contributing to cell migration and invasion (Fig. 6B).

To our knowledge, this is the first report that p37 enhance tumor invasion and migration, which was mainly mediated by MMP-2 and the EGFR/MEK/ERK pathway. Our results also underlie the potential application of MMP-2 and/or EGFR inhibitor in the treatment of *M. hyorhinis*-induced diseases. However, the molecular mechanism of effect of p37 on tumor cells needs further exploration.

Acknowledgments

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