Extracellular Signal–Regulated Kinase Signaling Pathway Regulates Breast Cancer Cell Migration by Maintaining slug Expression

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Abstract

Cell migration is a critical step in cancer cell invasion. Recent studies have implicated the importance of the extracellular signal–regulated kinase (ERK) signaling pathway in cancer cell migration. However, the mechanism associated with ERK-regulated cell migration is poorly understood. Using a panel of breast cancer cell lines, we detected an excellent correlation between ERK activity and cell migration. Interestingly, we noticed that a 48-hour treatment with U0126 [specific mitogen-activated protein/ERK kinase (MEK)-1/2 inhibitor] was needed to significantly inhibit breast cancer cell migration, whereas this inhibitor blocked ERK activity within 1 hour. This observation suggests that ERK-dependent gene expression, rather than direct ERK signaling, is essential for cell migration. With further study, we found that ERK activity promoted the expression of the activator protein-1 (AP1) components Fra-1 and c-Jun, both of which were necessary for cell migration. Combination of U0126 treatment and Fra-1/c-Jun knockdown did not yield further reduction in cell migration than either alone, indicating that ERKs and Fra-1/c-Jun act by the same mechanism to facilitate cell migration. In an attempt to investigate the role of Fra-1/c-Jun in cell migration, we found that the ERK-Fra-1/c-Jun axis regulated slug expression in an AP1-dependent manner. Moreover, the occurrence of U0126-induced migratory inhibition coincided with slug reduction, and silencing slug expression abrogated breast cancer cell migration. These results suggest an association between ERK-regulated cell migration and slug expression. Indeed, cell migration was not significantly inhibited by U0126 treatment or Fra-1/c-Jun silencing in cells expressing slug transgene. Our study suggests that the ERK pathway regulates breast cancer cell migration by maintaining slug expression. [Cancer Res 2009;69(24):9228–35]

Introduction

Invasion plays a critical role in tumor metastasis and is a multiple step process (1). During invasion, cells first interact with the surrounding extracellular matrix (ECM), subsequently degrade or remodel the surrounding ECM, and eventually migrate through the dissolved ECM to reach adjacent tissues (1). Among all these steps, cell migration is one of the most critical rate-limiting steps and is a complex process that requires the coordinate assembly and disassembly of focal adhesion complexes (2). Major effort has been exerted on defining the mechanisms associated with focal adhesion complex turnover and identifying molecules essential to this cellular event. However, the knowledge on the signaling pathways relevant to cell migration process is still lacking.

There are at least three families of mitogen-activated protein kinases (MAPK), namely, extracellular signal–regulated kinases (ERK), c-Jun NH_2-terminal kinases (JNK), and p38 MAPKs. In addition to their well-characterized role in cell proliferation/differentiation and cell survival/apoptosis, the MAPK signaling pathways also actively participate in cell migration. The lines of evidence connecting JNK to cell migration are (*a*) that inhibiting JNK activity or ablating JNK impairs cell migration of diverse cell types including fibroblasts, endothelial, and Schwann cells (3–5), and (*b*) that JNKs phosphorylate adhesion paxillin and non–JNK-phosphorylable paxillin inhibits cell migration (6). The lines of evidence linking p38 MAPK to cell migration are (*a*) that p38 inhibitor blocks integrin-mediated breast cancer cell migration (7) and (*b*) that caldesmon is a potent p38 substrate and p38-mediated caldesmon phosphorylation is required for smooth muscle cell migration (8). The use of chemical and genetic inhibitors has also implicated the role of the ERK signaling pathway in the cell migration (9–11). However, it is poorly understood how the ERK signaling pathway is involved in cell migration.

The activator protein (AP1) transcription factors are the dimers of the Fos (c-Fos, FosB, Fra-1, and Fra-2) and Jun (c-Jun, JunB, and JunD) families (12, 13), and activation of AP1 is essential for cancer initiation by Ras (14) and ETV-NTRK3 fusion oncogene (15). Among AP1 factors, Fra-1 is frequently elevated in metastatic breast, glioma, and thyroid cancers (16–18). Fra-1 may play a role in cell migration because ectopically expressing Fra-1 enhances cell migration and blocking Fra-1 reduces cell migration (19, 20). Because Fra-1 is expressed in an ERK-dependent manner (21–23), it is very likely that the ERK pathway regulates cell migration through Fra-1. This possibility is supported by a report showing that Fra-1 acts downstream of ERK to facilitate cell migration (24).

Slug is a member of the *snail* family and is upregulated in metastatic breast cancer and mesothelioma (25, 26). Recent lines of evidence strongly suggest that slug acts as a potent inducer of cell movement (27). For example, skin explants from slug-null mice have retarded migration rates of keratinocytes (28). Forced expression of slug in corneal explants resulted in higher rates of corneal epithelial cell migration (29). Because slug is capable of regulating integrin expression (30), it may affect cell migration by modulating cell-substratum interaction.

In this study, we observed an excellent correlation between ERK activity and cell migration among breast cancer cell lines.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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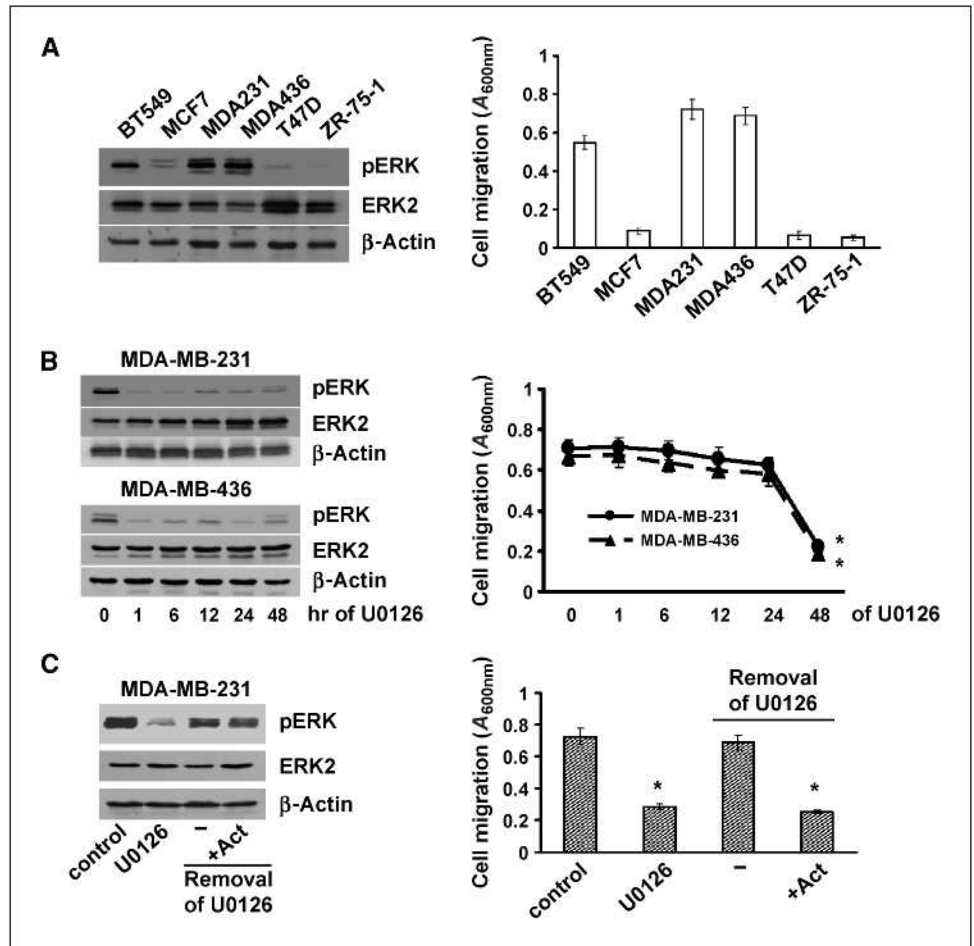

Figure 1. ERK-dependent gene expression is required for breast cancer cell migration. *A,* portions of overnight cultures of BT549, MCF7, MDA-MB-231, MDA-MB-436, T47D, and ZR-75-1 cells were lysed, and lysates were subjected to immunoblotting to detect phosphor-ERK (*pERK*), ERK2, and β-actin with the respective antibodies. The remaining portions were analyzed for cell migration using transwells as described in Materials and Methods. *Columns,* mean; *bars,* SE. *B,* MDA-MB-231 and MDA-MB-436 cells were treated with 5 μmol/L U0126 for various times. Portions of the cell cultures were analyzed for the levels of phosphor-ERK, ERK2, and β-actin and the remaining portions analyzed for cell migration. *Points,* mean; *bars,* SE. *, $P < 0.005$, versus 0 h. *C,* MDA-MB-231 cells were treated with 5 μmol/L U0126 for 2 d, washed with serum-free medium three times to remove U0126, and then incubated in the absence or presence of 2 μg/mL actinomycin for 4 h. A portion of the cell culture was analyzed for phosphor-ERK, ERK2, and β-actin levels and the remaining portion assayed for cell migration. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control.

However, our results indicate that ERK-dependent gene expression, rather than direct ERK signaling, is involved in cell migration. To determine the potential involvement of AP1 dimer in ERK-regulated cell migration, we showed that Fra-1 and c-Jun expression is dependent on ERK activity. Although prolonged treatment with U0126 [a MAP/ERK kinase (MEK)-1/2 inhibitor], Fra-1, and c-Jun short hairpin RNAs (shRNA) was capable of inhibiting MDA-MB-231 and MDA-MB-436 cell migration, combined treatment of all three did not show additive effect on the reduction in cell migration. This observation supports the notion that ERK and AP1 work in the same signaling axis to facilitate cell migration. To define the role of Fra-1/c-Jun in cell migration, we performed a microarray analysis with total RNA isolated from Fra-1/c-Jun knockdown MDA-MB-231 cells and identified slug as a Fra-1/c-Jun–regulated gene. Silencing slug expression diminished MDA-MB-231 and MDA-MB-436 cell migration, and forced slug expression effectively reversed the inhibition of cell migration caused by prolonged U0126 treatment or Fra-1/c-Jun shRNA. Our results suggest that the ERK-Fra-1/c-Jun axis regulates breast cancer cell migration by maintaining slug expression.

Materials and Methods

Immunoblotting. Immunoblotting was done as previously described (31). To determine the effect of U0126 on ERK phosphorylation, Fra-1, c-Jun, Fra-2, or slug, cells were treated with 5 μmol/L U0126 for various times followed by immunoblotting. To determine shRNA effect, cells were infected with lentiviral vectors containing shRNAs for 4 d and then lysed for immunoblotting. The antibodies used in immunoblotting were all obtained from Santa Cruz Biotechnology.

Cell migration assay. Cell migration was assayed by transwell and wound healing assays as described previously (31). For transwell assay, cells were detached with 10 mmol/L EDTA–containing PBS, then resuspended in serum-free medium and allowed to migrate for 4 h. To determine the effect of U0126 on cell migration, cells were treated with 5 μmol/L U0126 for various times in medium containing 10% FCS. To determine the effect of Fra-1, c-Jun, or slug knockdown, cells were infected with lentiviral vector containing luciferase (control), Fra-1, c-Jun, or slug shRNA for 4 d before the analysis of cell migration. To determine the effect of murine slug expression on U0126-mediated inhibition of cell migration, cells were infected with lentiviral vector encoding murine slug cDNA for 2 d and then treated with 5 μmol/L U0126 for another 2 d, followed by cell migration assay. To determine the effect of murine slug expression on Fra-1/c-Jun shRNA–mediated inhibition of cell migration, cells were first infected with lentiviral vectors containing Fra-1 and c-Jun shRNAs for 2 d and then again infected with lentiviral vector encoding murine slug cDNA for 2 d before assaying cell migration.

Microarray analysis. Total RNA was isolated from control (luciferase shRNA) or Fra-1/c-Jun-knockdown MDA-MB-231 cells using Trizol (Invitrogen). The microarray was done using Illumina whole genome BeadArray technology. The microarray data were initially processed using the lumi package in R (32). The data were then transformed using the variance stabilizing transformation and robust spline normalization (33). The processed data were analyzed using the LIMMA package in R (34).

Analysis of slug promoter activity. The construction of wild-type and AP1-mutant slug promoter reporter plasmids and luciferase assay were

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described in Supplementary Data. The luciferase was analyzed using Duel Luciferase System (Promega) according to the manufacturer's protocol. To determine the effect of U0126 on slug or Rous sarcoma virus (RSV) promoter activities, cells were transfected with the reporter gene constructs for 1 d, and 5 μmol/L U0126 was then added to the cells for another 2 d before luciferase activity assay. To determine the effect of silencing Fra-1/c-Jun on promoter activity, the reporter gene construct was transfected into Fra-1/c-Jun shRNA–expressing cells for 2 d followed by assaying of luciferase activity.

***In vitro* invasion assay, tumor outgrowth, and lung metastasis.** *In vitro* cell invasion was assayed using Matrigel invasion chamber (Cellbio Labs) according to the manufacturer's protocol. Control or slug-knockdown MDA-MB-231 cells were added into Matrigel-coated chamber and allowed to invade for 24 h.

In vivo tumor development was done by s.c. injecting control or slug-knockdown MDA-MB-231 cells (3×10^6 per mouse) at the 4th mammary fat pad area of female athymic nude mice (6–7 wk of age, Harlan Laboratories). Xenografts were measured using a caliper and tumor volume (V) was calculated by the equation $V = (L \times W^2) \times 0.5$, where L is the length and W is the width of a xenograft. To determine metastasis to the lung, mice were sacrificed at 8 wk after injection and lungs removed for formalin fixation. The sections were cut from the fixed lung tissue and subjected to H&E staining. The metastatic lesions were visualized under a microscope.

Statistical analysis. Statistical analyses of cell migration and Matrigel invasion were done by Student's t test using Microsoft Excel software. Comparison between animals receiving control and those receiving slug knockdown cells was made by the Student-Newman-Keuls test using the SPSS software program (SPSS, Inc.). $P < 0.05$ was considered statistically significant.

Results

ERK-dependent gene expression, rather than direct ERK signaling, is required for cell migration. To investigate the potential role of the ERK signaling pathway in cell migration, we examined both ERK activity and cell migration in six breast cancer cell lines. Immunoblotting with anti–phosphor-ERK(Thr[202]/Tyr[204]) monoclonal antibody showed that ERK phosphorylation (ERK activity) was high in BT549, MDA-MB-231, and MDA-MB-436 cell lines, but low in MCF7, T47D, and ZR-75-1 cell lines (Fig. 1*A*). Transwell migration assay showed that lines with high ERK activity were migratory, whereas lines with low ERK activity were poorly

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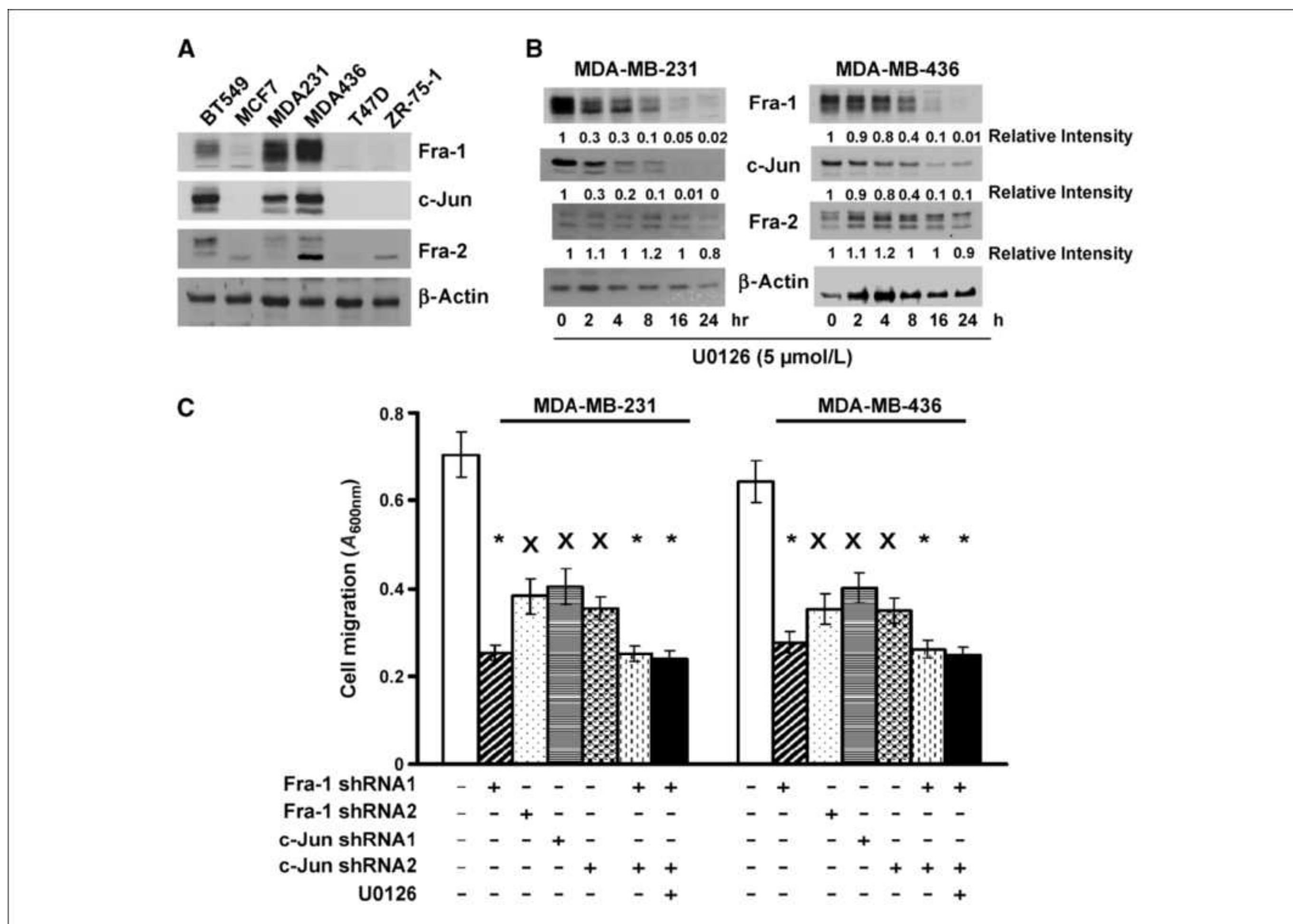

Figure 2. The ERK-Fra-1/c-Jun axis is involved in cell migration. *A,* BT549, MCF7, MDA-MB-231, MDA-MB-436, T47D, and ZR-75-1 cells were lysed, and lysates were subjected to immunoblotting to detect Fra-1, c-Jun, Fra-2, and β-actin with the respective antibodies. *B,* MDA-MB-231 and MDA-MB-436 cells were treated with 5 μmol/L U0126 for various times and then lysed for immunoblotting to detect Fra-1, c-Jun, Fra-2, and β-actin. *C,* MDA-MB-231 and MDA-MB-436 cells were infected either with control lentiviral vector (luciferase shRNA) or with vector containing Fra-1, c-Jun shRNA, or both. Some Fra-1/c-Jun shRNA–expressing cells were further treated with 5 μmol/L U0126 for 2 d. Cells were detached with 10 mmol/L EDTA and then assayed for cell migration as described in Materials and Methods. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control; ×, $P < 0.01$, versus control.

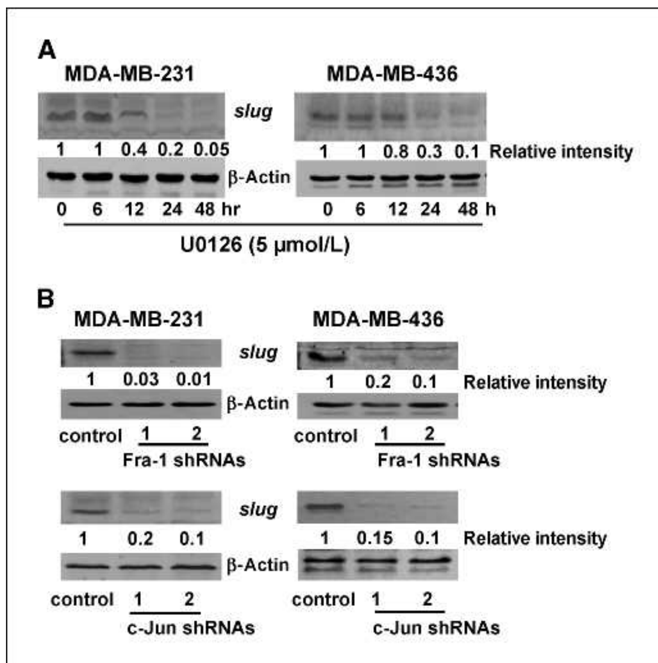

Figure 3. The ERK-Fra-1/c-Jun axis regulates slug expression. *A,* MDA-MB-231 and MDA-MB-436 cells were treated with 5 μmol/L U0126 for various times and then lysed for immunoblotting to detect slug and β-actin with the respective antibodies. *B,* MDA-MB-231 cells were infected with lentiviral vector containing luciferase (control), Fra-1, or c-Jun shRNA for 4 d and then lysed for immunoblotting to detect slug and β-actin.

migratory (Fig. 1*A*). These results show an excellent correlation between ERK activity and cell migration.

We next investigated the importance of ERK activity in cell migration by treating MDA-MB-231 and MDA-MB-436 cells with 5 μmol/L U0126 (a specific MEK1/2 inhibitor) for various times. One-hour U0126 treatment was enough to block more than 90% of ERK phosphorylation (Fig. 1*B*); however, cell migration was only substantially inhibited after 2 days of U0126 treatment (Fig. 1*B*). The time disparity between the rapid inhibition of ERK phosphorylation (<1 hour) and delayed onset of cell migration inhibition (~2 days) strongly suggests that the ERK does not participate in cell migration through direct signaling.

The ERK signaling pathway regulates the expression of various genes (35). We thus tested whether ERK-dependent gene expression was required for breast cancer cell migration. MDA-MB-231 cells were first treated with U0126 for 2 days and U0126 was then removed by several washes. Cells were cultured in 10% FCS–containing medium with or without 2 μg/mL actinomycin (RNA synthesis inhibitor) for 4 hours followed by the analyses of ERK phosphorylation and cell migration. ERK activity was completely restored 4 hours after removal of U0126, and actinomycin had no effect on ERK reactivation (Fig. 1*C*). Removal of U0126 restored cell migration (Fig. 1*C*); however, the presence of actinomycin prevented cell migration (Fig. 1*C*). As actinomycin prevents *de novo* gene expression, these results suggest that ERK-regulated gene expression is required for cell migration.

Fra-1/c-Jun and ERKs regulate breast cancer cell migration through the same mechanism. Fra-1 is regulated in an ERK-dependent manner (21–23) and has been indicated to be important for cell migration (19, 20). To investigate whether Fra-1 and other AP1 components were functionally linked to ERK in the regulation of cell migration, we first examined the levels of Fra-1, Fra-2, and c-Jun in breast cancer cell lines. Fra-1 and c-Jun levels were high in BT549, MDA-MB-231 and MDA-MB-436 lines (Fig. 2*A*), and these lines also displayed high ERK activity and were migratory (Fig. 1*A*). In contrast, Fra-1 and c-Jun levels were low in MCF7, T47D, and ZR-75-1 lines (Fig. 2*A*), and these lines also exhibited low ERK activity and were poorly migratory (Fig. 1*A*). Fra-2 was seen in all but the T47D line, and its levels were not correlated to ERK activity (Fig. 2*A*). This observation shows an excellent correlation between the levels of Fra-1/c-Jun and ERK activity/cell migration in breast cancer cells.

We next investigated whether ERK activity was required for Fra-1/c-Jun expression by treating MDA-MB-231 and MDA-MB-436 cells with 5 μmol/L U0126 for various times. Immunoblotting with the respective antibodies showed that the levels of Fra-1 and c-Jun were greatly decreased at 8 hours and nearly abolished at 24 hours (Fig. 2*B*), suggesting that ERK activity is required for high levels of Fra-1 and c-Jun in migratory breast cancer cells. However, U0126 did not significantly alter Fra-2 expression in both lines (Fig. 2*B*). This corroborates the observation that there is no clear correlation between Fra-2 and ERK activity in breast cancer cells (Fig. 2*A*).

To determine the importance of Fra-1/c-Jun in breast cancer cell migration, we designed two shRNAs each for Fra-1 and c-Jun and expressed them in MDA-MB-231 and MDA-MB-436 cells. Although all shRNAs effectively suppressed their target gene expression (Supplementary Fig. S1), they did not affect the status of ERK phosphorylation (Supplementary Fig. S1), which agrees that ERK acts upstream to regulate Fra-1/c-Jun expression. Transwell migration assay showed that silencing either Fra-1 or c-Jun reduced cell migration by more than 60% (Fig. 2*C*), but Fra-1 and c-Jun shRNAs together did not lead to greater inhibition of cell migration than did either shRNA alone (Fig. 2*C*). Similar results were also obtained with wound healing assay (Supplementary Fig. S2). These results indicate that Fra-1 and c-Jun are involved in cell migration through the same mechanism. To rule out the potential off-target effect of shRNAs, we expressed murine Fra-1 and c-Jun in Fra-1/c-Jun-knockdown MDA-MB-231 cells and found that their expression was able to rescue cell migration to the control level (Supplementary Fig. S3). In a parallel experiment, we examined the combinational effect of inhibiting ERK activity and silencing Fra-1/c-Jun on cell migration. MDA-MB-231 and MDA-MB-436 cells expressing both Fra-1 shRNA1 and c-Jun shRNA2 were treated with U0126 for 2 days followed by the analysis of cell migration. Additional U0126 treatment exhibited only slightly greater inhibitory effect on cell migration than Fra-1/c-Jun knockdown alone (Fig. 2*C*; Supplementary Fig. S2). Because the expression of Fra-1 and c-Jun is ERK dependent (Fig. 2*B*), these results suggest that the ERK signaling pathway may indirectly participate in cell migration by promoting Fra-1/c-Jun expression.

The ERK-Fra-1/c-Jun axis regulates slug expression. U0126 abolished Fra-1/c-Jun expression by more than 90% in less than 1 day (Fig. 2*B*) but did not significantly inhibit cell migration until 2 days of treatment (Fig. 1*B*). As Fra-1 and c-Jun are components of the AP1 dimer, we reasoned that a particular AP1-regulated gene might be essential for cell migration. To search for such gene, we performed a microarray analysis with total RNA collected from control and Fra-1/c-Jun-knockdown MDA-MB-231 cells. Using more than 2-fold difference as the cut-off standard, we detected that the levels of 15 genes were elevated and those of 43 genes decreased in Fra-1/c-Jun-knockdown

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cells in comparison with the control (Supplementary Table S1). Among these genes, the expression of slug was decreased more than 3-fold (Supplementary Table S1).

Slug has been previously described as an inducer of cell movement (27); we thus hypothesized that Fra-1/c-Jun facilitated cell migration by regulating slug expression. To test this hypothesis, we treated MDA-MB-231 and MDA-MB-436 cells with U0126 for various times followed by immunoblotting to detect slug. Slug expression was significantly inhibited following 2 days of U0126 treatment in both lines (Fig. 3*A*) and this time point coincided with U0126-mediated inhibition of cell migration (Fig. 1*B*). In parallel, we analyzed the levels of slug in Fra-1- or c-Jun-knockdown cells and found that silencing either Fra-1 or c-Jun was able to down-regulate slug expression (Fig. 3*B*).

To further investigate the mechanism associated with ERK/Fra-1/c-Jun–regulated slug expression, we constructed a slug promoter reporter plasmid by linking the slug promoter to a luciferase gene and transfected this plasmid into MCF7, MDA-MB-231, MDA-MB-436, and ZR-75-1 cells. Assaying luciferase activity showed that slug promoter activity was much greater in MDA-MB-231 and MDA-MB-436 cells (displaying high ERK activity and Fra-1/c-Jun expression) than in MCF7 and ZR-75-1 cells (displaying low ERK activity and Fra-1/c-Jun expression; Fig. 4*A*). In the subsequent experiments, we transfected MDA-MB-231 and MDA-MB-436 cells with the slug or RSV promoter reporter plasmid for 1 day followed by 2-day U0126 treatment. U0126 inhibited more than 70% of slug promoter activity in comparison with the untreated cells in both lines, but reduced RSV promoter activity by only less than 20% (Supplementary Fig. S4*A*). Similarly, forced expression of dominant-negative MEK1 led to a 60% to 70% reduction in slug promoter activity but only a marginal decrease in RSV promoter activity (Fig. 4*B*; Supplementary Fig. S4*A*). These results suggest that the activity of ERK is specifically required for slug gene transcription.

To determine the importance of Fra-1/c-Jun in slug gene transcription, the slug or RSV promoter construct was transfected into Fra-1- or c-Jun-knockdown MDA-MB-231 and MDA-MB-436 cells. Silencing Fra-1 or c-Jun alone abolished 60% to 70% of slug promoter activity in comparison with the control, and silencing them

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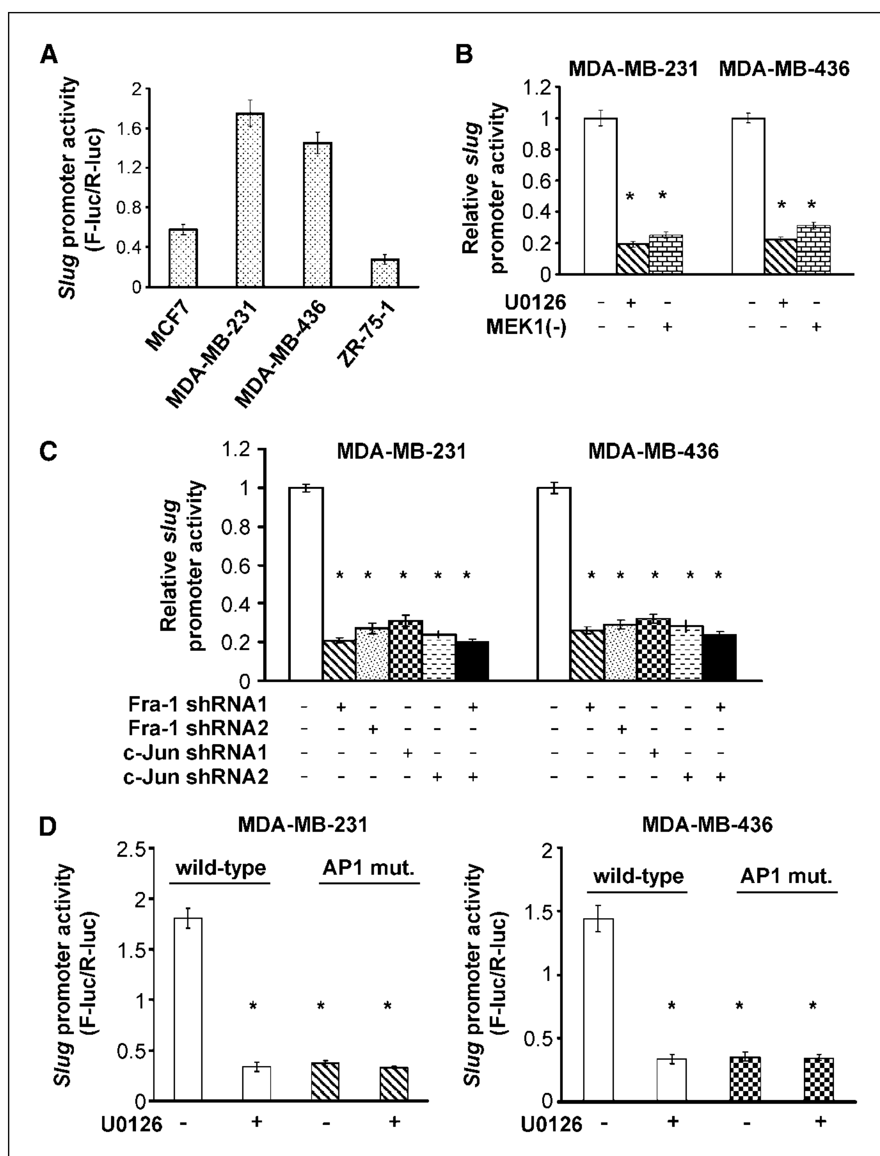

Figure 4. Slug is expressed in an AP1-dependent manner. *A,* MCF7, MDA-MB-231, MDA-MB-436, and ZR-75-1 cells were transfected with the slug promoter luciferase reporter plasmid for 2 d and then lysed; cell lysates were measured for luciferase activity as described in Materials and Methods. *Columns,* mean; *bars,* SE. *B,* MDA-MB-231 and MDA-MB-436 cells were transfected with the slug promoter plasmid for 1 d, and 5 μmol/L U0126 was then added to the cells for another 2 d. In a parallel experiment, the slug promoter plasmid was cotransfected into the cells with dominant-negative MEK1 expression vector [MEK1(−)] for 3 d. Cells were lysed, and cell lysates analyzed for luciferase activity. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control. *C,* the slug promoter plasmid was transfected into MDA-MB-231 and MDA-MB-436 cells that expressed Fra-1 or c-Jun shRNA or both for 2 d. Cells were lysed, and cell lysates analyzed for luciferase activity. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control. *D,* MDA-MB-231 and MDA-MB-436 cells were transfected with the slug promoter plasmid or plasmid containing slug promoter with mutation in AP1 consensus site for 1 d, and 5 μmol/L U0126 was added to the cells for another 2 d. Cells were lysed, and cell lysates measured for luciferase activity. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus cells with wild-type promoter (no U0126).

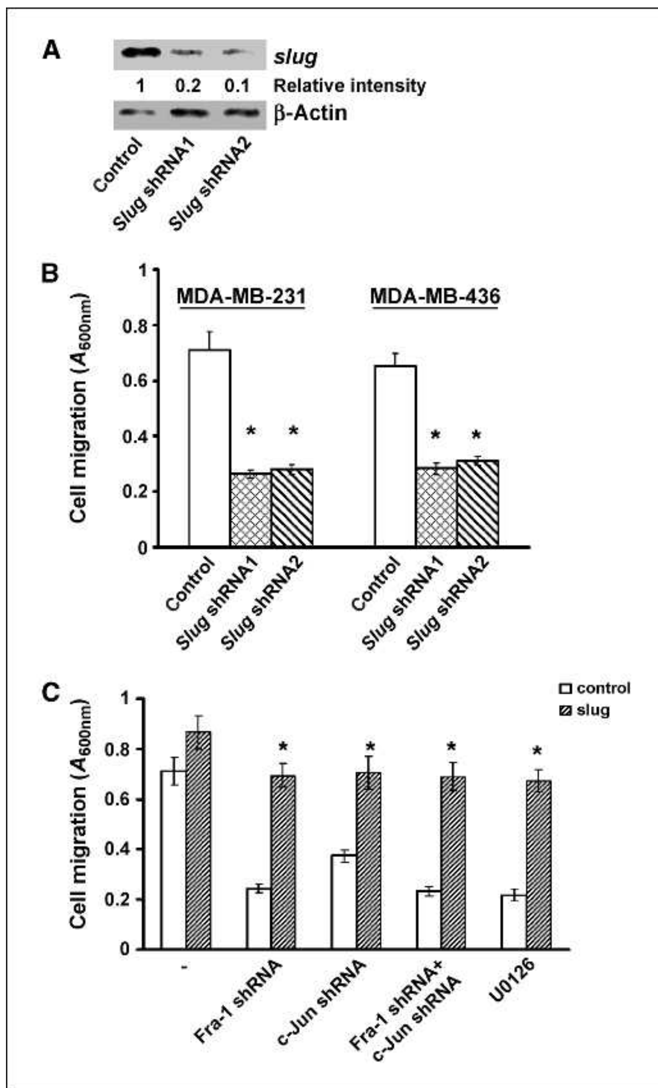

Figure 5. Slug is essential for ERK-Fra-1/c-Jun axis–regulated cell migration. *A,* MDA-MB-231 cells were infected with lentiviral vector containing luciferase shRNA (control) or slug shRNA for 4 d and then lysed for immunoblotting to detect slug and β-actin with the respective antibodies. *B,* MDA-MB-231 and MDA-MB-436 cells were infected with control (luciferase shRNA) or slug shRNA–containing lentiviral vector for 4 d, then detached with 10 mmol/L EDTA and assayed for cell migration as described in Materials and Methods. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control. *C,* MDA-MB-231 cells were infected with empty lentiviral vector (control) or vector encoding murine slug cDNA for 4 d. Control cells or cells with stable murine slug cDNA expression were further infected with lentiviral vector containing luciferase shRNA, Fra-1 shRNA, c-Jun shRNA, or both Fra-1 and c-Jun shRNAs for 4 d. In a parallel experiment, control and murine slug cDNA–expressing cells were treated with 5 μmol/L U0126 for 2 d. Cells were detached and analyzed for cell migration. *Columns,* mean; *bars,* SE. *, $P < 0.005$, versus control.

simultaneously did not further diminish slug promoter activity (Fig. 4*C*). In contrast, Fra-1 or c-Jun shRNA had little effect on RSV promoter activity (Supplementary Fig. S4*B*). These results indicate that Fra-1 and c-Jun work by the same mechanism to regulate slug transcription. In parallel, we overexpressed Fra-1 and c-Jun in MCF7 and ZR-75-1 cells and subsequently measured slug promoter activity. Forced Fra-1/c-Jun expression led to a 15% to 20% increase in slug promoter activity (Supplementary Fig. S4*C*). Interestingly, expressing Fra-1/c-Jun together with constitutively active MEK1 greatly enhanced slug promoter activity (Supplementary Fig. S4*C*). As the ERK signaling pathway is known to affect Fra-1/c-Jun phosphorylation status, these results suggest that in addition to the presence of Fra-1/c-Jun, ERK-mediated Fra-1/c-Jun phosphorylation may also be important for slug expression.

In further studies, we performed mutagenesis at the potential AP1 consensus sequence (GTGACTTCA→GTAGATTCA) in the slug promoter and determined how this mutation affected slug promoter activity in MDA-MB-231 and MDA-MB-436 cells. The mutation in AP1 site resulted in a >70% reduction in slug promoter activity in both lines (Fig. 4*D*) and additional treatment of U0126 did not further reduce the activity of the slug mutant promoter (Fig. 4*D*). These results suggest that the ERK-Fra-1/c-Jun axis regulates slug expression through the AP1 site in the slug promoter.

Slug expression is required for cell migration. To determine the role of slug in cell migration, we designed two slug shRNAs and found that both slug shRNAs were capable of effectively suppressing slug expression (Fig. 5*A*). Transwell migration assay showed that silencing slug led to MDA-MB-231 and MDA-MB-436 cells migrating at about 40% of the rate observed with control cells (Fig. 5*B*). Similar results were also obtained with wound healing assay (Supplementary Fig. S5). As forced murine slug expression completely reversed the inhibition of cell migration caused by slug shRNA (Supplementary Fig. S5), these results show that the presence of slug is essential for cell migration.

We next expressed murine slug transgene in Fra-1/c-Jun-knockdown MDA-MB-231 cells and then analyzed the migration of these cells. Whereas forced slug expression moderately enhanced the migration of parental cells, it elevated migration of Fra-1/c-Jun-knockdown cells to those seen in the control cells (Fig. 5*C*). Similarly, forced slug expression also largely rescued U0126-mediated inhibition of cell migration (Fig. 5*C*). These results suggest that the ERK-Fra-1/c-Jun axis regulates cell migration by maintaining slug expression.

Slug expression is required for *in vitro* cell invasion and *in vivo* metastasis. To investigate the role of slug in cell invasion, we examined how silencing slug affected the *in vitro* invasiveness of MDA-MB-231 cells by performing Matrigel invasion assay. MDA-MB-231 cells exhibited robust invasion, but the expression of slug shRNAs blocked more than 80% of their abilities to invade Matrigel (Fig. 6*A*). Similar results were also obtained with MDA-MB-436 cells (data not shown). Subsequently, we examined the importance of slug in *in vivo* tumor outgrowth and metastasis by s.c. injecting control or slug-knockdown MDA-MB-231 cells in the mammary fat pad of nude mice. We detected palpable tumors within 7 days in both groups, and daily monitoring up to 8 weeks revealed no statistically significant difference in tumor outgrowth between them (Supplementary Fig. S6). When lung sections from euthanized mice were analyzed by H&E staining, we observed significant metastatic lesions in the lungs of animals receiving control cells (5.3 ± 2.4 lesions per section; Fig. 6*B*). In contrast, metastatic lesion was not detected from any animal receiving slug-knockdown cells (Fig. 6*B*). Taken together, these results suggest that slug is needed for both invasion and metastasis but not for tumor outgrowth.

Discussion

The enzymatic ERK activity is increased in invasive breast carcinomas as compared with normal tissues or benign lesions (36, 37). As invasive cancer cells are migratory, it is reasonable to postulate that the enzymatic ERK activity could be involved in cancer

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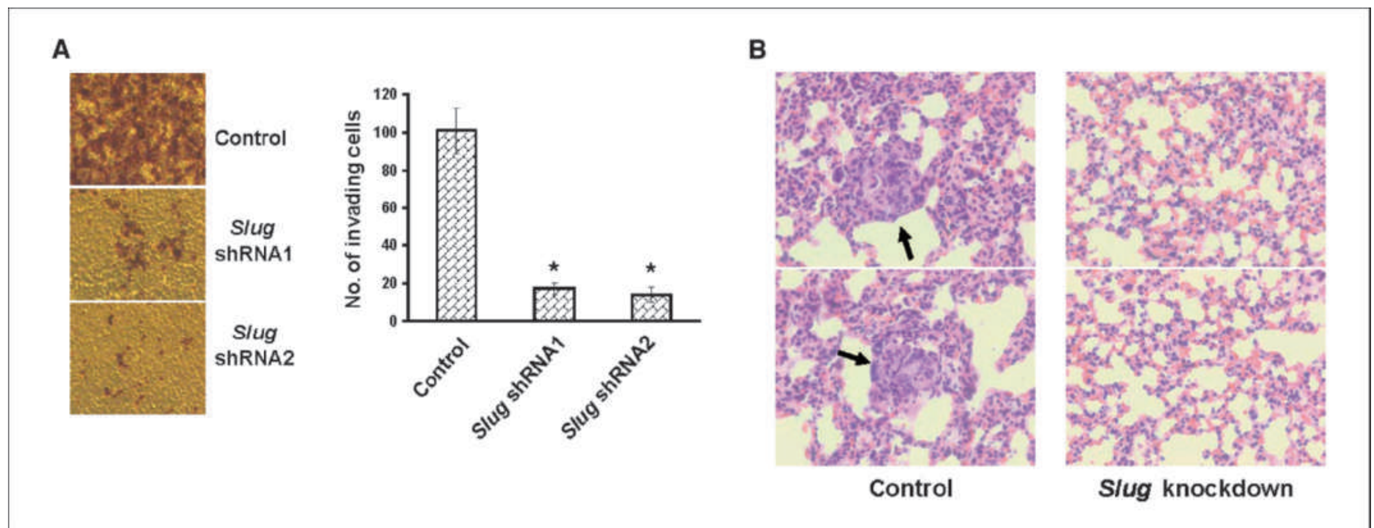

Figure 6. Slug is essential for *in vitro* invasion and lung metastasis. *A,* control or slug-knockdown MDA-MB-231 cells were added into invasion chambers and allowed to invade for 24 h. The cells on the undersurface of chambers were stained and visualized under a microscope. *Columns,* mean; *bars,* SE. *, $P < 0.001$, versus control. *B,* the lungs from mice receiving either control or slug-knockdown MDA-MB-231 cells for 8 wk were fixed and sectioned. The sections were subjected to H&E staining and visualized under a microscope. *Arrows,* metastatic lesions. Magnification, ×40.

cell migration. This notion is supported by our observation that the levels of ERK activity correlated extremely well with the migratory status of breast cancer cells (Fig. 1*A*). Recent studies have implicated the involvement of the ERK pathway in cell migration (9). However, the mechanism associated with ERK-regulated cell migration is not clear. We showed that a 48-hour U0126 treatment was required to significantly inhibit MDA-MB-231 and MDA-MB-436 cell migration, whereas ERK activity was diminished within 1 hour of U0126 treatment (Fig. 1*B*). This observation suggests that direct ERK signaling is unlikely to be responsible for breast cancer cell migration. Using actinomycin to block *de novo* RNA synthesis, we found that ERK-dependent gene expression is necessary for MDA-MB-231 cell migration (Fig. 1*C*). In search for the genes essential for breast cancer cell migration, we turned our attention to the components of the AP1 complex because the expression of the AP1 component Fra-1 is regulated by the ERK signaling pathway (21) and Fra-1 acts as a downstream effector of ERK to facilitate colon carcinoma cell migration (24). In our studies, we detected high levels of Fra-1/c-Jun only in migratory breast cancer cell lines (Fig. 2*A*) and the expression of Fra-1/c-Jun was diminished by 16-hour U0126 treatment (Fig. 2*A*). To determine the importance of Fra-1/c-Jun in cell migration, we found that silencing either Fra-1 or c-Jun significantly decreased breast cancer cell migration (Fig. 2*C*). Importantly, combination of Fra-1/c-Jun knockdown and U0126 treatment did not yield any further inhibition of cell migration than either Fra-1/c-Jun knockdown or U0126 treatment alone (Fig. 2*C*), strongly suggesting that ERK and Fra-1/c-Jun act through the same mechanism to facilitate cell migration. Our findings confirmed the importance of Fra-1 in cell migration as reported by previous studies (19, 20, 24) and also showed the necessity of c-Jun for cell migration (Fig. 2*C*). As Fra-1 is known to interact with c-Jun to form an AP1 heterodimer to promote gene expression, the requirement of both Fra-1 and c-Jun suggests that Fra-1/c-Jun AP1 dimer–driven gene expression is essential for breast cancer cell migration. In fact, we noticed that U0126-induced Fra-1/c-Jun downregulation occurred at least 1 day earlier than U0126-induced inhibition of cell migration (16 versus 48 hours). This observation also supports the notion that Fra-1/c-Jun regulates cell migration by promoting a particular gene expression in an AP1-dependent manner.

Recent studies have implicated that slug acts as an inducer of cell movement (27). In our studies, we showed that the expression of slug is regulated by the ERK-Fra-1/c-Jun signaling axis through the AP1 consensus sequence in the slug promoter (Figs. 3 and 4). Moreover, we found that U0126-induced inhibition of cell migration coincided with U0126-induced reduction in slug expression (Figs. 1 and 3) and that silencing slug expression abrogated breast cancer cell migration (Fig. 5*A* and *B*), cell invasion, and spontaneous metastasis (Fig. 6*A* and *B*). More importantly, the ability of U0126 or Fra-1/c-Jun shRNAs to inhibit cell migration is lost in cells expressing slug transgenes (Fig. 5*C*). Our results strongly support the role of slug in cell migration. Recent studies have reported that slug and its related protein, *snail*, can regulate integrin expression (30, 38). Because integrins are essential for cell-matrix substratum interaction and cell migration, we reason that the ERK-Fra-1/c-Jun/slug axis affects breast cancer cell migration through regulation of the integrins. This possibility is buttressed by a previous study in which activator protein (AP1) was shown to regulate integrin expression through the ERK pathway (39).

In conclusion, our data show that the ERK signaling pathway facilitates breast cancer cell migration by regulating slug expression in an AP1-dependent mechanism. This knowledge suggests that anti–breast cancer approaches may be developed by targeting the ERK-AP1-slug axis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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