FRA-1 Proto-Oncogene Induces Lung Epithelial Cell Invasion and Anchorage-Independent Growth In vitro, but Is Insufficient to Promote Tumor Growth In vivo

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Abstract

FRA-1 forms activator protein-1 complexes in association with members of the JUN family and drives gene transcription. FRA-1 has been implicated in the development of airway squamous metaplasia and is frequently overexpressed in squamous cell carcinomas of the esophagus and stomach. We and others have shown a high level of persistent induction of FRA-1 by lung carcinogens, such as cigarette smoke and asbestos, in pulmonary epithelial cells. However, the exact roles of FRA-1 in regulating lung epithelial cell growth and invasion are poorly understood. To examine this aspect, we have stably overexpressed FRA-1 in human type-II-like alveolar malignant cell line (A549) and a nonmalignant bronchial epithelial cell line (BEAS-2B). FRA-1 greatly enhanced the rate of proliferation, motility, and invasion of A549 and BEAS-2B cells. In athymic nude mice, FRA-1, but not the control vector, rapidly enhanced tumor formation and metastasis by A549 cells. In contrast, FRA-1 failed to promote tumor formation by BEAS-2B. We suggest that FRA-1 can promote motility, invasion, and anchorage-independent growth of lung epithelial cells in vitro, but is insufficient for tumor formation. [Cancer Res 2007;67(13):6204–11]

Introduction

Lung cancer is the leading cause of cancer deaths in the United States and throughout the world. Pulmonary epithelial cells, the progenitor cell types that are implicated in the development of lung cancer, are the primary targets of notorious carcinogens such as tobacco smoke (1). As is commonly held for other neoplasms, the progression of lung cancer involves several genetic and molecular changes at the cellular level that begin as epithelial cell hyperplasia, evolve to dysplasia, and then to a life-threatening metaplasia (2, 3). Some of these molecular changes include a silencing of tumor suppressor genes, dysregulation of proto-oncogene expression and/or activation, and an up-regulation of genes that promote cell growth and transformation, leading to tumor development (2). In the case of advanced lung tumors, motility and invasion impart a metastatic character to the mutated cells, which results in the spread of the tumor to other organs (3, 4). However, the exact molecular mechanisms controlling these processes are incompletely understood.

The transcription factors JUN (c-JUN, JUN-B, JUN-D) and FOS (c-FOS, FOS-B, FRA-1, and FRA-2) constitute a group of “early-response proto-oncogenes” that are capable of forming activator protein-1 (AP-1) heterodimers in a signal-dependent manner. These molecules regulate the expression of genes involved in various biological processes, including cell proliferation, differentiation, transformation, inflammation, and pulmonary defense (5). Abnormal expression and/or activation of AP-1 proteins by various toxicants and mitogens contribute to the development of various diseases, including cancer (5). Other studies have shown a protracted induction of FRA-1 by asbestos, a potent lung carcinogen that causes mesothelioma. A prominent role for FRA-1 in asbestos-induced malignant transformation of rat pleural mesothelial cells has been shown (6). In particular, silencing of FRA-1 expression reverses the malignant phenotype of mesothelioma (7). We have recently shown that FRA-1 up-regulates the expression of genes involved in airway squamous metaplasia (8). The expression of FRA-1 is persistently induced in bronchial epithelial cells by lung carcinogens such as tobacco smoke (9), tumor-promoting phorbol esters, and mitogens (10, 11). Consistent with these observations, overexpression of FRA-1 has been noted in squamous cell carcinomas of the head and neck (12), esophagus, and stomach (13). The transition from the small cell to the non–small cell lung cancer (NSCLC) phenotype induced by the H-ras/c-myc oncogenes is accompanied by specific induction of FRA-1, suggesting a prominent role for this transcription factor in maintaining NSCLCs in a differentiated state (14).

Because the disruption of FRA-1 results is embryonic lethality (15), we have used a human type II alveolar cell line (A549) and a nontumorigenic bronchial epithelial cell line (BEAS-2B) to determine its role in lung cancer development. Here, we report that ectopic expression of FRA-1 in both cell lines enhanced cell motility, invasion, and anchorage-independent growth. However, FRA-1 alone was insufficient for oncogenic transformation of BEAS-2B cells.

Materials and Methods

Cell cultures. A549, a human NSCLC cell line that exhibits type II-like alveolar epithelial characteristics, and BEAS-2B, a human normal bronchial epithelial cell line immortalized by SV40 T antigen, were obtained from the American Type Culture Collection and cultured in RPMI and DMEM, respectively, supplemented with 5% fetal bovine serum (FBS), unless otherwise indicated. Cells were transfected with a mouse FRA-1 wild-type cDNA (16), p53 cDNA bearing mutation in Arg175 to histidine (R175H, hereafter noted as p53R mutant; ref. 17), or K-ras cDNA (Val12; ref. 18)
cloned into the pCMV-neo mammalian expression vectors. Stable cell lines expressing FRA-1, p53R, mutant, or K-ras mutant were selected in the presence of 600 μg/mL G418. Several independent colonies stably expressing the FRA-1 (hereafter referred to as A549-F1 or BEAS-F1 cells), p53R mutant (BEAS-p53R), or K-ras (BEAS-Kras) were pooled, and FRA-1 mRNA expression was analyzed by reverse transcription-PCR (RT-PCR) using gene-specific primers (see below). Protein expression was confirmed by Western blot analysis using an antibody specific for human and mouse FRA-1 (Santa Cruz Biotechnology). As a control group, cells stably transfected with an empty vector, pCMV were generated and are referred to as A549-C or BEAS-C.

**Gene expression and reporter analysis.** Total RNA was isolated from stable transfectants, and PCR was done using forward (F) and reverse (R) primer sequences for mouse FRA-1 (F, 5′-GGCTTCGTA-GATGCGACGAG-3′ and R, 5′-GGCCATTCGTA-CAAGGCGGAGA-3′), α-actin (F, 5′-GAGAAATCTGGCACCACC-3′ and R, 5′-TACCCTCTGTA-GTGTCGAC-3′) amplification was used as a reference. The RT-PCR assays were carried out in duplicate and confirmed in two independent experiments. For Western analysis, a comparable amount of whole cell lysate (40 μg) was separated, and membranes were probed with FRA-1 (SC-183) and FRA-2 (SC-604X; Santa Cruz Biotechnology, Inc.) and α-actin antibodies (Cell Signaling Technology, Inc.).

A reporter bearing seven copies of the consensus AP-1 binding site of TPA response element (TRE, 5′-TGACTAA-3′) upstream of Luc (referred to as TRE-Luc) was obtained from Stratagene and transfected along with a reference plasmid pHK.

**Electrophoretic mobility shift assays.** Nuclear extracts from A549-C and A549-F1 cells were prepared, and electrophoretic mobility shift assays (EMSA) were done using double-stranded 32P-labeled TRE (5′-GCGTTGAT-GCTCAGGCGGAGG-3′) and OCT-1 (5′-TGCGAGGCAATCATCAGAAGA; Promega Corp.) probes as described previously (8). Nuclear extracts (2–3 μg) were incubated with the binding buffer on ice for 10 min before the addition of 32P-labeled double-stranded DNA probes. To determine the presence of a specific protein in the complex, nuclear extracts were incubated with unlabeled TRE, anti-FRA-1 antibodies (1–2 μg) or non-immune immunoglobulin G (IgG) for 1 h before the addition of the labeled probe.

**Cell proliferation analysis.** Cells were seeded onto 96-well plates at a density of 5 × 10^3 or 2 × 10^4 cells per well in 100 μL medium containing 5% FBS. After 24 h incubation, cells were maintained in culture medium with or without serum. After 48 h, cell proliferation was assessed with the CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega).

**Cell motility assays.** Cells were plated at equal density in six-well plates and grown to confluence. Wounds were then generated with a sterile pipette, cells were rinsed twice with PBS, and fresh culture medium in 0.5% agarose was added on top, and the medium was changed every 4 days.

**Matrigel invasion assays.** For invasion assays, modified Boyden chamber Transwell polycarbonate filters (6.5 mm in diameter, 8 μm pore size; Costar) were coated with 100 μL of Matrigel (Sigma) at a 1:2 dilution in serum-free RPMI 1640 and air-dried for 24 h. Cells were placed in serum-free medium for 24 h before experiments. Cells (1 × 10^4) were layered in the upper compartment of Transwell inserts. Medium containing 10% FBS was used in the bottom wells. Cells were allowed to incubate for 48 h, and invaded cells adherent to the undersurface of the insert were fixed and stained, and readings were taken at OD_{595} using appropriate blank controls.

**Three-dimensional collagen gel migration/invasion assays.** Three-dimensional collagen gel matrices were prepared using rat tail collagen type I solution (Upstate Biotechnology) mixed in a ratio of 1:1 with 2× RPMI 1640 containing 5% FBS. To a 24-well plate, 400 μL of collagen solution was added and allowed to polymerize at 37°C. Above this collagen lattice, cells (5 × 10^3 per well) were suspended in RPMI containing 5% FBS. The cells were allowed to attach for 1 h at 37°C. After the incubation, excess medium was removed, and an upper collagen cast (300 μL) was layered above the cells and allowed to polymerize at 37°C for 30 min. RPMI containing 5% FBS was added on top, and the medium was changed every 4 days.

**Soft agar assays.** Viable cells (5 × 10^3) were mixed in the RPMI medium in 0.5% agar, plated on the top of 1% agar base, and covered with RPMI containing 5% FBS. Cultures were maintained at 37°C in the incubator for 15 days. The medium was changed every 3 days, and the number of anchorage-independent colonies was counted in five random fields.

**S.c. and pulmonary xenografts.** Viable cells (2 × 10^6 cells) were injected s.c. into right and left flank of 5-week-old athymic nude (NCr nu/nu, Taconic) mice (two mice per group). Tumor volume was assessed, as detailed elsewhere (19), every 2 days for 2 weeks, and photographs were taken. For metastasis experiments, 5 × 10^5 cells were injected into the tail vein of athymic nude mice (four mice per group) on day 0. On day 14, mice were sacrificed, and India ink was injected into the trachea. Lungs were preserved in Fekete’s solution, images were captured, and visible tumor nodules were counted as described previously (19).

**RNA interference.** To silence the FRA-1 expression, cells were transfected with SMART pool small interfering RNA specific for mouse FRA-1 (Dharmacon Inc.) or the control scrambled siRNA using TransIT-TKO transfection reagent (Mirus). After 48 h incubation, cells were harvested, counted, and seeded for cell invasion assay, Western blotting, and RNA analyses.

**Statistics.** Data presented are the means ± SD of n independent assays as noted in the figure legends. Statistical significance was determined using the Student’s t test. Significance was set at P ≤ 0.05.

## Results

Stable expression of FRA-1 in human lung alveolar epithelial carcinoma cells. As discussed above, FRA-1 is transiently induced in most cells, but its expression is persistent in malignant tumors. To mimic the persistent expression of FRA-1 and to define its role in lung cancer cell growth, motility and invasion, we stably transfected the human adenocarcinoma cell line A549 with a FRA1-expression vector (16). We have previously shown that the endogenous FRA-1 expression is low in A549 cells but is strongly induced by mitogens, oxidants, cytokines, and carcinogens (9–11, 20). Stable cell clones overexpressing FRA-1 (referred to as A549-F1 cells) or a control empty vector (termed A549-C) were isolated following selection with G418, pooled, and used for subsequent gene expression and functional studies. The overexpression of FRA-1 was confirmed by RT-PCR and Western blot analyses (Fig. 1A). The A549-C cells showed little or no expression of FRA-1 (top). Overexpression of FRA-1 did not alter the expression of another FOS protein, FRA-2 (bottom).

The biological activity of ectopically expressed FRA-1 was assessed by EMSA using a 32P-labeled consensus AP-1 binding site. The formation of a constitutive AP-1 complex was noted in lanes 1 and 2. Formation of this AP-1 complex could be competed out by an unlabeled TRE oligo, confirming its specific DNA binding property (lane 6). The difference in AP-1 complex...
Equal amounts of whole-cell lysates (40 transfectants for gene expression analyses and nuclear extracts for DNA binding assays. RT-PCR was done using the FRA-1 or β-actin gene-specific primers (top). Equal amounts of whole-cell lysates (40 μg) were subjected to Western blot analysis with anti–FRA-1, FRA-2, and β-actin antibodies (bottom). B, nuclear extracts (3 μg) were incubated with 32P–end-labeled consensus TRE (top) or OCT1 (bottom) oligo probes (lanes 1–4). In competition assays, nuclear extracts were incubated without (lane 5) or with unlabeled TRE oligo. C, in supershift analysis, nuclear extracts were incubated with 1–2 μg of non-immune IgG (lanes 1–2) or anti–FRA-1 antibody (lanes 3–4) for 1 h on ice before the addition of the labeled TRE. EMSA gel exposed to a shorter time (top) and a longer time (bottom). Arrow, position of AP-1 complex; ↑, position of supershifted (SS) complex with FRA-1 antibody. A representative autoradiogram from two independent experiments is shown. D, cells were transfected with 100 ng of the TRE reporter construct along with 1 ng of pRL-TK plasmid. After 24 h incubation, the cells were harvested, and luciferase activity was analyzed. The fold induction was calculated, with the reporter values for the A549-C cells being set to 1.0. Data are means ± SD of triplicate from a representative experiment. Each experiment was repeated at least twice.

Figure 1. Ectopic FRA-1 expression in human lung adenocarcinoma cells. A, total RNA and whole cell lysates were prepared from stable A549-C and A549-F1 transfectants for gene expression analyses and nuclear extracts for DNA binding assays. RT-PCR was done using the FRA-1 or β-actin gene-specific primers (top). Equal amounts of whole-cell lysates (40 μg) were subjected to Western blot analysis with anti–FRA-1, FRA-2, and β-actin antibodies (bottom). B, nuclear extracts (3 μg) were incubated with 32P–end-labeled consensus TRE (top) or OCT1 (bottom) oligo probes (lanes 1–4). In competition assays, nuclear extracts were incubated without (lane 5) or with unlabeled TRE oligo. C, in supershift analysis, nuclear extracts were incubated with 1–2 μg of non-immune IgG (lanes 1–2) or anti–FRA-1 antibody (lanes 3–4) for 1 h on ice before the addition of the labeled TRE. EMSA gel exposed to a shorter time (top) and a longer time (bottom). Arrow, position of AP-1 complex; ↑, position of supershifted (SS) complex with FRA-1 antibody. A representative autoradiogram from two independent experiments is shown. D, cells were transfected with 100 ng of the TRE reporter construct along with 1 ng of pRL-TK plasmid. After 24 h incubation, the cells were harvested, and luciferase activity was analyzed. The fold induction was calculated, with the reporter values for the A549-C cells being set to 1.0. Data are means ± SD of triplicate from a representative experiment. Each experiment was repeated at least twice.

Overexpressed FRA-1 increases lung cancer cell proliferation and motility. We next examined the effects of ectopically expressed FRA-1 on the morphologic features and proliferation of A549 cells. In contrast to the control cell line A549-C, which seemed to contain compact epithelial cells with a cobblestone-like appearance, the A549-F1 cells assumed a fibroblastoid shape with long extended processes (Fig. 2A). Given the importance of cell proliferation and survival in the regulation of the cancer cell progression and metastasis, we first compared the proliferation rates of A549-F1 cells and A549-C under normal conditions and conditions of growth factor deprivation. An equal number of viable cells were plated at two different densities and cultured in serum-free medium, and the number of viable cells was measured after 48 h incubation. Under these conditions, the cell proliferation rate of A549-F1 was 9-fold greater than that of the A549-C cells (Fig. 2B). No significant differences in proliferation rate were observed when these cells were cultured in medium containing normal levels of serum (10%; Fig. 2C). These differences indicate that FRA-1 imparts neoplastic properties to the cells under conditions of growth factor deprivation. To assess the effect of FRA-1 overexpression on cell migration/motility, we used "scratch wound-healing" assays. The cell motility following wound generation was greater in A549-F1 cells at all time points (Fig. 2D). Closure of the wound was almost complete in A549-F1 cells within 40 h of incubation, but not in the case of similarly treated A549-C1 cells. These results indicate that FRA-1 overexpression can lead to potentiation of lung cancer cell growth and migration.

Overexpression of FRA-1 enhances cell invasion. The invasion of cancer cells through the basement membrane is a key event during metastasis. Therefore, we used two complementary approaches to assess the effect of FRA-1 overexpression on the ability of A549 cells to invade the basement membrane. In the first approach, cells were plated on the two-chamber Transwell inserts, and the cellular migration to the lower surface of the inserts was determined after 48 h. As shown in Fig. 3A, ectopically expressed FRA-1 greatly increased the cell migration rate. A 12-fold greater number of A549-F1 cells (filled column) than A549-C cells (open column) was found in the bottom wells.
In a second approach, the invasive potential of both the A549-F1 and A549-C cells was analyzed using modified Boyden chambers (Fig. 3C). Cells were placed on the Matrigel-coated two-chamber Transwell inserts, and after 48 h of incubation, the invaded cells were fixed and stained with crystal violet. The number of cells that crossed the Matrigel barrier to the lower surface of the inserts was about 2-fold higher in the case of A549-F1 cells (Fig. 3C, filled column) than A549-C cells (Fig. 3C, open column). These results clearly indicate that FRA-1 overexpression markedly enhances the invasive potential of lung adenocarcinoma cells.

The above results show that ectopic FRA-1 promotes malignant potential of A549 cells. To show that this is a true FRA-1 effect, we assessed the effect of FRA-1 siRNA on the invasive potential of both the A549-F1 and A549-C cells using modified Boyden chambers. As anticipated, the silencing of FRA-1 expression (bar 3) significantly reduced the invasive potential of A549-F1 cells (see Supplementary Fig. S1), confirming that ectopic FRA-1 is responsible for inducing greater level of invasive potential in A549-F1 cells.

**FRA-1 promotes anchorage-independent growth.** We next examined the behavior of the stable transfectants in a three-dimensional Matrigel invasion assay, a system that closely resembles physiologic growth conditions. Cells were cultured in a three-dimensional Matrigel system and observed by phase-contrast microscopy for 2 weeks. As compared with the control A549-C cells, the A549-F1 cells formed a higher number of invasive colonies, which were enlarged and aberrantly shaped (Fig. 4A). A549-F1 cells more effectively invaded the surrounding basement membrane matrix; this migration resulted in the formation of a number of colonies. Similarly, soft agar tumor assays (Fig. 4B) revealed that A549-F1 cells had an enhanced ability to form colonies under nonadherent conditions when compared with the A549-C cells. As seen under high magnification (Fig. 4B, right), the size and density of the anchorage-independent colonies of A549-F1 cells were generally much larger than those of the parental cells. This difference could be directly attributed to the increased rate of cell proliferation caused by FRA-1 overexpression. The number of colonies generated was five times higher in the case of the A549-F1 cells.

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**Figure 2.** Effect of FRA-1 overexpression on A549 cell proliferation and motility. **A,** cells were fixed in methanol, stained with toluidine blue, and examined by phase-contrast microscopy. Images in (i) and (ii) are at ×20 magnification, whereas (iii) and (iv) were taken at ×40 magnification. **B,** cells were seeded in a 96-well plate and maintained on 1% serum. After 48 h incubation, cell numbers were quantified using Cell Titer Glo reagent. Columns, means of six separate wells (n = 6) from two independent experiments; bars, SD. **C,** cells were cultured as in (B) but maintained on 10% serum instead of 1%. **D,** Ectopic FRA-1 enhances cell motility in wound-healing assays. A uniform scratch was made in each confluent monolayer culture, and the extent of closure was monitored under phase-contrast microscopy at the indicated time points and photographed. Representative images of two independent experiments done in duplicate are shown.
cells (Fig. 4C, filled column) than the A549-C cells (open column). Thus, overexpression of FRA-1 increased the oncogenic potential of A549 cells, as reflected by a high colony-forming efficiency and anchorage-independent growth rate.

**Ectopic FRA-1 promotes on tumor growth and lung metastasis potential of A549 in vivo.** To determine their relative oncogenic abilities A549-C and A549-FRA-1 cells were injected (s.c.) into athymic nude mice, and tumor formation was monitored for 17 days. A549-F1 cells expressing FRA-1 formed large tumors (Fig. 5A) and grew robustly (Fig. 5B) as compared with A549-C. To assess whether FRA-1 overexpression enhances metastatic potential, A549-C and A549-F1 cells were injected into the tail vein of nude mice, and tumor formation was monitored for 60 days. The data showed that A549-F1 cells formed larger tumors and more distant metastases than A549-C cells (Fig. 5C).

**Figure 3.** FRA-1 overexpression enhances the invasive potential of A549. A, equal numbers of viable stable cell transfectants were seeded on the top of the Transwell inserts and incubated for 48 h in serum-containing medium. The cells that migrated through the filters were stained with 0.01% crystal violet and examined under a light microscope. Arrows, cells that migrated to the lower chamber of the Transwell insert. The stained filters were suspended in acetic acid and methanol (1:1), and the absorbance was read at OD_{595}. Columns, means from three independent experiments done in triplicate; bars, SD. C, cell invasion assays were done using modified Boyden chambers. The cells that migrated through the Matrigel-coated filters after 48 h were stained with 0.01% crystal violet and quantified at OD_{595}. Data are means of three separate experiments done in duplicate.

**Figure 4.** FRA-1 increases the invasion and colony-forming ability of lung cancer cells. A, cells were grown on three-dimensional (3D) Matrigel for 15 d and photographed by phase-contrast microscopy. Magnification, ×20 (left). Right, magnified spheroids. B, soft agar assays were done as detailed in Materials and Methods. Phase-contrast microscopy of a representative experiment showing the increased number of colonies formed by the A549-F1 cells is shown. C, data represent the number of colonies formed from two independent experiments done in triplicate.
FRA-1 induces malignant phenotypes in normal bronchial epithelial cells in vitro, but is not sufficient for tumor formation in vivo. The above studies were done in A549 cells, which exhibit type-II alveolar cell differentiation-specific characteristics but possess malignant potential (ATCC). Thus, we examined the effects of FRA-1 in normal human bronchial epithelial cells, BEAS-2B. This cell line does not form tumors when injected into nude mice (ATCC). BEAS-2B cells stably expressing the empty pcMV vector (BEAS-C) or FRA-1 (BEAS-F1) were generated. To avoid a clonal bias, all experiments used pools of at least 10 stable colonies in each case. The expression of ectopic FRA-1 in BEAS-C (lane 1) and BEAS-F1 (lane 2) was confirmed by RT-PCR and Western blot analyses (Fig. 6A, left). In transient transfection assays, we observed significantly elevated AP-1 activity in BEAS-F1 cells (Fig. 6A, right, column 2) as compared with BEAS-C cells (column 1). We have used several different methods, including cell motility, proliferation, migration, matrigel-invasion, and anchorage-independent soft agar growth assays, and in vivo tumor growth as measures of their transformed phenotype. As shown in Fig. 6B, BEAS-F1 cells migrated into the injured area faster than BEAS-C cells were expressing pcMV. When cultured in serum-free conditions, the proliferation rate of BEAS-F1 was 6-fold greater than that of the BEAS-C cells (Fig. 6C, left). Transwell migration assays revealed a greater increase in cell migration rate in BEAS-F1 (6-fold) as compared with BEAS-C cells (Fig. 6C, right). Boyden chamber assays (Fig. 6D, left) showed that overexpression of FRA-1 greatly enhances the ability of BEAS-2B cells (filled column) to invade the basement membrane. The number of colonies obtained in soft agar growth assays were more than 3-fold greater in BEAS-F1 cells than BEAS-C cells (Fig. 6D, right).

On the basis of these in vitro characteristics, we measured invasive potential of BEAS-2B cells. We next examined if BEAS-2B cells overexpressing FRA-1 have capability to form tumors in vivo (Fig. 6D). As positive controls, we have used BEAS-2B cells overexpressing K-ras (BEAS-Kras) or p53 mutant (BEAS-p53R). Neither BEAS-C (data not shown) nor BEAS-F1 cells formed tumors following implantation into nude mice (see Fig. 6E and Supplementary Fig. S2). In contrast, BEAS-2B cells expressing K-ras (BEAS-Kras) or p53 mutant (BEAS-p53R) formed large tumors (Supplementary Fig. S2, arrows) and grew robustly (Fig. 6E). Thus, FRA1 alone seems to be insufficient for promoting tumor formation in vivo.

Discussion

The present study investigated the role of FRA-1 in regulating lung epithelial cell growth and invasion. The results of assays measuring three-dimensional Matrigel migration and invasion and soft agar growth revealed that FRA-1 overexpression greatly enhances both the growth and invasive potential of lung epithelial cells. We have previously shown that various agents that promote tumor cell growth and invasion persistently activate FRA-1 expression in pulmonary (type II and bronchial) epithelial cells (9–11, 20). Thus, the present findings are important in that the activation of this transcription factor by mitogens and carcinogens plays key roles in promoting cell growth, malignant transformation, and invasive potential of pulmonary epithelial cells. Our findings are consistent with recent experimental evidence obtained from animal models, in which aberrant activation and expression of JUN and FOS family members have been shown to play key roles in the development of tumorigenesis (5). The fact that FRA-1 also regulates asbestos-induced mesothelioma (6, 7) and is important for mammary (16, 21), colon (22), and brain (23) cancer cell motility and invasion further reinforces the significance of the role of FRA-1 in lung carcinogenesis and tumor progression.

In the present study, tumor growth assays done with A549-F1 and A549-C cells have revealed that overexpression of FRA-1 significantly enhances the formation of tumor metastases (Fig. 5B). Lung tumor nodules formed by the A549-C cell may be attributed to either endogenous FRA-1 or other oncogenic mutations in the
parent cell line. To determine the role of endogenous FRA-1 in promoting metastasis, we attempted to ablate its expression using expression vectors coding for FRA-1–specific shRNAs. However, none of the shRNA expressing cell lines survived to form stable cell clones (data not shown), indicating an important role for FRA-1 in the survival of lung adenoma cells. Therefore, it was technically impossible to perform tumorigenic assays with A549 cells lacking endogenous FRA-1 expression. Interestingly, although overexpression of FRA-1 in nonmalignant human bronchial epithelial cells promoted mitogen-independent cell proliferation, migration and invasion, and anchorage-independent growth (Fig. 6), it failed to promote tumor growth in vivo (Fig. 6E), whereas BEAS-2B cells overexpressing p53 mutant or K-ras induced tumor growth. In contrast to our results, ectopic expression of Fra-1 in rat fibroblasts, Rat-1A, results in anchorage-independent growth in vitro and tumor development in athymic mice (24). This discrepancy may be due to differences in cell types, species origin, and genetic constitution of the cells used in these reports. Rat1A is an immortalized rat fibroblastic cell line (25), whereas BEAS-2B is a SV-40 immortalized human bronchial epithelial cell line (26, 27). Nonetheless, our current findings suggest that FRA-1 expression alone is not sufficient to drive tumor growth in vivo. In support of this notion, Jochum et al. (28) have shown that ubiquitous expression of FRA-1 enhanced osteosclerosis, but did not induce lung tumors. We also found that targeted expression of FRA-1 specifically in bronchial epithelial cells under the control of a Clara cell-specific promoter formed no detectable lung tumors in mice.

Previous studies have shown transformability and tumorigenic potential of BEAS-2B cells with certain oncogenes and carcinogens. For example, transfection of v-Ki-Ras (29), v-Ha-Ras (30), and a mutant form of p53 (31) or treatment with a tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (32)
induces neoplastic transformation of BEAS-2B cells and tumorigenicity in mice. It is unclear why FRA-1 when overexpressed in non-malignant BEAS-2B has failed to promote tumor growth in vivo in athymic nude mice, despite imparting malignant transformation-like characteristics in vitro. It is likely that tumor growth and metastasis require additional events such as mutational inactivation of either tumor suppressor(s) and/or activation of certain proto-oncogenes, in addition to FRA-1. For example, several studies have reported that A549 cells have mutations in the K-Ras oncogene. Such mutations have not been reported thus far in the case of BEAS-2B cell line. Mutations in K-Ras have been frequently noted in lung cancer tissues and cell lines (33), and overexpression of K-Ras oncogene in mice causes lung tumor development (34). FRA-1 is a critical downstream effector of Ras-extracellular signal-regulated kinase pathway. Thus, it is likely that FRA-1 in cooperation with an additional oncogene(s) may promote lung tumor growth in vivo. Importantly, FRA-1 cannot bind DNA on its own and requires another protein, such as Jun, to bind to the AP-1 sites. Recently, Maeno et al. (35) have shown that overexpression of c-Jun promotes cell viability and anchorage-independent growth of BEAS-2B cells. However, no experiments were done to evaluate the oncogenic ability of these cells in this study. Interestingly, similar to FRA-1, c-Jun expression using the ubiquitin promoter in transgenic mice caused no lung tumor formation (36). Thus, we propose that although FRA-1 or c-Jun expression augments malignant potential of transformed lung epithelial cells, their expression alone is not sufficient to promote lung tumor growth in vivo.

In summary, our findings suggest that the activation of FRA-1 by carcinogens and mitogens may be a critical determinant of tumor cell progression and invasion. Delineating the mechanisms by which FRA-1 promotes tumor growth and metastasis in malignant cells but not in nonmalignant cells in vivo may provide further insight into lung cancer development.

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References

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