Forkhead Box F1 Regulates Tumor-Promoting Properties of Cancer-Associated Fibroblasts in Lung Cancer

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Abstract

Cancer-associated fibroblasts (CAF) attract increasing attention as potential cancer drug targets due to their ability to stimulate, for example, tumor cell growth, invasion, angiogenesis, and metastasis. However, the molecular mechanisms causing the tumor-promoting properties of CAFs remain poorly understood. Forkhead box F1 (FoxF1) is a mesenchymal target of hedgehog signaling, known to regulate mesenchymo-epithelial interactions during lung development. Studies with FoxF1 gain- and loss-of-function fibroblasts revealed that FoxF1 regulates the contractility of fibroblasts, their production of hepatocyte growth factor and fibroblast growth factor-2, and their stimulation of lung cancer cell migration. FoxF1 status of fibroblasts was also shown to control the ability of fibroblasts to stimulate xenografted tumor growth. FoxF1 was expressed in CAFs of human lung cancer and associated with activation of hedgehog signaling. These observations suggest that hedgehog-dependent FoxF1 is a clinically relevant lung CAF-inducing factor, and support experimentally the general concept that CAF properties can be induced by activation of developmentally important transcription factors. Cancer Res; 70(7): 2644–54. ©2010 AACR

Introduction

The tumor stroma provides a microenvironment that is critical for cancer cell growth, invasion, and metastatic progression. Cancer-associated fibroblasts (CAF) are a major constituent of the tumor stroma (1–4). CAFs commonly display myofibroblastic characteristics, including a prominent contractile ability and expression of α smooth muscle actin (αSMA). CAFs play key roles in promoting cancer progression via direct cell-cell interaction, through soluble factors such as growth factors and chemokines, or by modification of extracellular matrix components. They also participate in the modulation of inflammatory response or tumor angiogenesis. Direct targeting of CAFs, and targeting of CAF-derived factors, is attracting increasing attention as a novel strategy for cancer treatment (5, 6). It is becoming recognized that CAFs are heterogeneous and are likely to be composed of subsets, still remaining to be better defined (7–9).

The regulatory mechanisms that induce and control the CAF properties still remain largely unknown. Among the paracrine factors that act on fibroblasts, members of the transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) families have been implied as major regulators of CAFs. TGF-β can directly induce transdifferentiation of fibroblasts into myofibroblasts and is crucial for remodeling of extracellular matrices (10–12). PDGF is a potent mitogen and chemotactant of fibroblasts (13, 14). A recent study also identified induction of osteopontin as an important mechanism whereby fibroblasts stimulated by PDGF promote cancer growth (15). CXCL14 is another, recently identified, stimulator of CAF activity (16). In spite of these progresses, it is noteworthy that key transcription factors regulating CAFs have not been described.

Forkhead box F1 (FoxF1) is a transcription factor critically involved in mesenchymal-epithelial interaction of lung and gut morphogenesis. FoxF1 is expressed in descendants of splanchic mesoderm (i.e., mesenchymal cells of organs derived from primitive gut, such as lungs and intestine). Foxf1-null mutants show early embryonic lethality, and haploinsufficiency causes multiple defects in trachea, esophagus, lungs, and gallbladder (17–19). Moreover, experiments in liver and lung injury models have shown the importance of FoxF1 in tissue repair and homeostasis (20, 21). The importance of FoxF1 in human lung development is also highlighted...
by the recent findings that inactivating mutations of FoxF1 cause congenital malformations of the lungs (22).

It has been shown that hedgehog signaling can induce FoxF1 expression during lung organogenesis, gut development, and vasculogenesis (19, 23, 24). Recent biochemical analyses have also confirmed FoxF1 as a direct target of hedgehog signaling (25). Based on the recent discovery of hedgehog as an important paracrine signal for activation of tumor stroma (26–28), this study tested the hypothesis that FoxF1 plays a key role in tumor stromal cells, especially in fibroblasts. Because FoxF1 is abundantly expressed in the mesenchyme of lungs (29), the analyses focused on the potential role of FoxF1 in CAFs of lung cancer.

**Materials and Methods**

**Reagents and antibodies.** Anti-α-tubulin, β-actin, FLAG, and αSMA antibodies were from Sigma-Aldrich. Anti–PDGF receptor (PDGFR) α, PDGFRβ, and phosphotyrosine (phos-Tyr) antibodies were from Cell Signaling. Anti-CD31 α-receptor (PDGFR) and α Calbiochem and used at the concentration of 3 μg/mL of antibody was from BD Pharmingen. TGF-β1 and PDGF-AA were used at the concentration of 1 and 100 ng/mL, respectively. LY364947 (TGF-β type 1 receptor inhibitor) was from Calbiochem and used at the concentration of 3 μmol/L.

**Cell culture.** NIH3T3, C3H10T1/2, A549, IMR-90, and 293T cells and primary murine lung fibroblasts (MLF) were cultured in DMEM. MLFs were isolated from the explant outgrowth of lungs derived from wild-type or Foxf1 heterozygous mice. MLFs were immortalized by retroviral transduction of SV40 large T antigen. To obtain the conditioned medium of NIH3T3 and IMR-90 cells, serum-free Opti-MEM (Life Technologies) and DMEM with 0.5% fetal bovine serum (FBS) supplemented with 20 μg/mL heparin (Sigma-Aldrich) were used, respectively.

**Cloning of the human FoxF1 cDNA.** The open reading frame of FoxF1, derived from the cDNAs of human lung fibroblasts, was amplified by PCR using high-fidelity Taq polymerase (LA-Taq, TaKaRa). The purified PCR fragment of FoxF1, derived from the cDNAs of human lung fibroblasts, was first cloned into pCR2.1 vector with TA Cloning kit (Invitrogen) and subcloned into the retroviral expression vector MSCV-ires-neo. Correctness of the final insert was confirmed by DNA sequencing.

**Retrovirus infection.** Transient transfection into 293T cells was performed using PolyFect reagent (Qiagen). 293T cells were cotransfected with the expression vector and ecotropic helper plasmid to produce culture supernatant containing retroviruses. Target cells were cultured for 48 h in the virus-containing conditioned medium in the presence of 10 μg/mL polybrene before drug selection with 2 μg/mL G418 (Sigma-Aldrich).

**Fluorescence microscopy.** Cells were fixed in 1:1 acetone-methanol solution and incubated with primary antibody diluted with Blocking One solution (Nacalai Tesque) for 1 h. The cells were then incubated with secondary antibody for 1 h and stained with TOTO-3 (Invitrogen Molecular Probes).

**Immunoblot analysis.** The detailed procedures were described previously (16). Glycoproteins were bound to lectin-agarose (Sigma-Aldrich) and used for the detection of phosphotyrosine, PDGFRα, and PDGFRβ.

**RNA isolation and reverse transcription-PCR.** To obtain clinical cases for mRNA expression analysis, immunohistochemistry for FoxF1 was performed on tissue microarrays representing 190 non–small cell lung cancer cases, with available corresponding fresh-frozen tissue samples. Sixteen samples were selected based on stromal FoxF1 expression (7 positive and 9 negative cases). Total RNA (RNA integrity number values > 7.5) was isolated from 5 to 10 frozen sections (10 μm) using the RNeasy Mini kit (Qiagen). The procedures of quantitative reverse transcription-PCR (RT-PCR) analysis were described previously (16). Expression level was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primer sequences are shown in Supplementary Table S2.

**RNA interference.** Lipofectamine 2000 reagent (Invitrogen) was used for small interfering RNA (siRNA) transfection into IMR-90 cells, and the final concentration of siRNA was 100 nmol/L. Human FoxF1 siRNA (Stealth RNAi HSS142031) and negative control siRNA were purchased from Invitrogen.

**Migration assay.** Cell migration assay was performed using cell culture inserts with 8-μm pore size (Corning). The upper surface of the chamber was coated with 50 μg/mL of type I collagen (BD Biosciences) diluted with 0.02 N acetic acid. The conditioned medium was put in the well under the chamber, and A549 cells lentivirally transduced with green fluorescent protein (GFP; 6 × 105 per well) were seeded in the upper side of the chamber. After 24 h, cells on the lower surface of the chamber were trypsinized, resuspended in PBS, and measured for GFP fluorescence. The experiments were performed at least thrice.

**Collagen gel contraction assay.** Type I collagen [rat tail tendon collagen (RTTC)] was purchased from BD Biosciences. Collagen gels were prepared by mixing RTTC, distilled water, 4× DMEM, 10× gel contraction buffer (200 mmol/L HEPES, 2.2% NaHCO3, 0.05 N NaOH), FBS, and cells. The final concentrations were 1.5 mg/mL RTTC, 1× DMEM, 1× buffer, and 6 × 105 cells/mL. The mixture (500 μL) was cast into each well of a 24-well culture plate. The solution was then allowed to polymerize at 37°C for 30 min. After polymerization, the gels were gently released from the plate and transferred into 60-mm tissue culture dishes. The surface area of the gels was measured as the relative value compared with that of the 6-cm dish. These values were normalized to that of the control gel at 0 h.

**Animal experiments.** Severe combined immunodeficient (SCID) mice, 5 to 8 wk old, were from the Microbiology, Tumor and Cell Biology animal unit, Karolinska Institutet. The mixture of 2 × 106 NIH3T3 cells or MLFs, with or without 4 × 105 A549 cells, was resuspended in 100 μL PBS and injected s.c. into mice. Tumor volume was approximated as 4/3 π d1d2d3/2 (a, b, and c: three different axes). The animal experiments were conducted in accordance with national guidelines and approved by the Stockholm North Ethical Committee on Animal Experiments.
**Immunohistochemistry for CD31.** The sections from fresh-frozen tumor samples were briefly fixed with 3.7% formalin and methanol, blocked in 20% goat serum, and incubated with rat anti-mouse CD31 (1:200 dilution), biotinylated goat anti-rat antibody (Vector Laboratories), and streptavidin-Cy3 (Sigma-Aldrich). The slides were mounted with 4′,6-diamidino-2-phenylindole (DAPI)–containing Vectashield mounting medium (Vector Laboratories). Vascular density was analyzed in tumors derived from A549 cells coinjected with GFP- or FoxF1-expressing NIH3T3 cells.
FoxF1 were put on ice and treated with or without PDGF-AA for 1 h. Cell lysates were subjected to immunoblotting for PDGFRα.

**Foxf1 knockout mice.** Foxf1 knockout mice with C57/BL6 background were generated by Mahlapuu and colleagues (30), and the detailed procedures of gene targeting and genotyping were described previously.

**Lung tumor tissue microarrays.** The analyzed tissue microarrays were composed of 247 cases, surgically treated for stage I to IIIA lung cancer or carcinoma, at the University Hospital of Lund, between 1981 and 1984 ($n = 119$) and 1995 and 1997 ($n = 128$). None of the patients had received any systematic neoadjuvant or adjuvant therapy. There were 154 (62.3%) men and 93 (37.7%) women. After histopathologic evaluation on H&E-stained slides, tissue microarrays were constructed as described previously (31). After a median follow-up of 4,569 y, 53 (21.5%) patients were alive and 194 (78.5%) were dead.

**Immunohistochemistry for FoxF1 on tissue microarrays.** The antibody for FoxF1 was from the Human Proteome Resource (Human Protein Atlas, Sweden) and was used with 1:100 dilution (32). To retrieve the antigen, slides were boiled in a microwave oven at 650 W in Target retrieval buffer, high pH (DAKO). The detailed procedures of immunohistochemistry were described previously (33). By light microscopy, the tissue sections were evaluated for the nuclear staining of FoxF1 in CAFs, which were morphologically identified as stromal spindle cells. We excluded the cases where fibroblasts could not be morphologically identified. The positivity of FoxF1 in CAFs was scored as positive or negative. All the samples were anonymized and independently scored by two evaluators. In case of disagreement, the samples were reexamined and a consensus was reached by the evaluators. Kaplan-Meier analysis and log-rank test were used to illustrate differences in recurrence-free and overall survival according to FoxF1 expression in CAFs.

**Results**

**FoxF1 modulates fibroblast morphology and expression of CAF markers.** To establish FoxF1 gain-of-function models, NIH3T3 and C3H10T1/2 murine fibroblasts were retrovirally transduced with control GFP or FLAG-tagged FoxF1. Cell populations were used for further experiments to avoid effects possibly caused by clonal variations. Ectopic FoxF1 was logically identified as stromal spindle cells. We excluded the nuclear staining of FoxF1 in CAFs, which were morphologically identified as stromal spindle cells. We excluded the cases where fibroblasts could not be morphologically identified. The positivity of FoxF1 in CAFs was scored as positive or negative. All the samples were anonymized and independently scored by two evaluators. In case of disagreement, the samples were reexamined and a consensus was reached by the evaluators. Kaplan-Meier analysis and log-rank test were used to illustrate differences in recurrence-free and overall survival according to FoxF1 expression in CAFs.

About the efficacy of transduction, we noted GFP fluorescence in 98% of the cells in the GFP-transduced cell population as confirmed by fluorescence-activated cell sorting analysis (data not shown). Ectopic FoxF1 induced expression of integrin β3, which has been identified as a FoxF1 target (34), confirming that FLAG-tagged FoxF1 is functionally active (Supplementary Fig. S1B).

FoxF1 expression conferred morphologic changes, including a spindle-like shape, similar to the cells stimulated by TGF-β1, whereas control cells displayed a round or cuboidal appearance (Fig. 1A). Cells were also immunostained for αSMA, an established marker of activated fibroblasts, which is also commonly expressed in CAFs. Interestingly, forced expression of FoxF1 resulted in enhanced staining of αSMA in these fibroblasts (Fig. 1B, left). This effect was also confirmed by immunoblotting (Fig. 1B, right).

To generate a FoxF1 loss-of-function model, IMR-90 human lung fibroblasts, expressing endogenous FoxF1, were used for silencing experiments. IMR-90 cells were transiently transfected with siRNA duplex specific to human FoxF1 (si FoxF1) or with control siRNA duplex (si NTC; Fig. 1C). FoxF1 was knocked down to 32% of the basal level as confirmed by quantitative RT-PCR (Supplementary Fig. S1C), and the effect was supported by downregulation of integrin β3 (Supplementary Fig. S1D). Silencing of FoxF1 was also confirmed by FoxF1 immunoblotting (Fig. 1D, left).

IMR-90 cells treated with FoxF1 siRNA showed a more elongated appearance at confluency compared with the control cells (Fig. 1C). Whereas control fibroblasts treated with TGF-β1 showed larger cell size, cell morphologic change was marginal even after TGF-β1 stimulation in the cells transfected with FoxF1 siRNA (Fig. 1C). Despite the differences in cell morphology, silencing of FoxF1 did not influence overall expression of αSMA in a bulk population as determined by immunoblot analysis (data not shown).

It has been reported that PDGFRα is expressed in CAFs of a subset of lung cancers (33). FoxF1 knockdown resulted in decreased PDGFRα expression (Fig. 1D) and attenuation of its phosphorylation following PDGFR-α treatment (Fig. 1D, right), whereas PDGFRβ expression was not affected.

The observed morphologic changes and FoxF1-dependent expression of PDGFRα are thus compatible with FoxF1 being involved in induction of lung CAF properties.

**FoxF1 controls the expression of hepatocyte growth factor and fibroblast growth factor-2.** Hepatocyte growth factor (HGF) and fibroblast growth factor-2 (FGF-2) are well-established CAF-derived stimulators of tumor growth that act, predominantly, on malignant and endothelial cells, respectively (35, 36). The expression of HGF and FGF-2 was confirmed by quantitative RT-PCR (Supplementary Fig. S1C), confirming that FLAG-tagged FoxF1 is functionally active (Supplementary Fig. S1B).

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Figure 1. FoxF1 regulates morphologic changes of fibroblasts and expression of αSMA and PDGFRα. A, NIH3T3 cells transduced with GFP or FoxF1 were cultured in the presence or absence of TGF-β1 for 48 h and analyzed by phase-contrast microscopy. Scale bar, 100 μm. B, left, NIH3T3 and C3H10T1/2 cells transduced with GFP or FoxF1 were immunostained for αSMA (red) and analyzed by fluorescence microscopy. Nuclei were visualized with TOTO-3 (blue). Scale bar, 100 μm. Right, immunoblotting for αSMA and FLAG in NIH3T3 and C3H10T1/2 cells transduced with GFP or FLAG-tagged FoxF1. α-Tubulin was used as loading control. C, IMR-90 cells transfected with si NTC (control siRNA) or si FoxF1 (siRNA for FoxF1) were cultured in the presence or absence of TGF-β1 and analyzed by phase-contrast microscopy. Scale bar, 100 μm. D, left, IMR-90 cells transfected with si NTC or si FoxF1 were put on ice and treated with or without PDGF-AA for 1 h. Cell lysates were subjected to immunoblotting for PDGFRα, PDGFRβ, and FoxF1. β-Actin was used as loading control. Right, immunoblotting for phosphotyrosine (P-Tyr), PDGFRα, and PDGFRβ in the lectin-agarose fractions.
characterized in the FoxF1 loss- and gain-of-function models. These analyses revealed strong upregulation of HGF and FGF-2 in FoxF1-transduced cells (Fig. 2A). Furthermore, downregulation of these factors was observed in the cells where FoxF1 was silenced (Fig. 2B). Consistently, the concentration of HGF in the conditioned medium of FoxF1 siRNA–transfected cells was decreased compared with the control (data not shown).

**Figure 2.** FoxF1 regulates HGF and FGF-2 expression. A and B, quantitative RT-PCR for HGF and FGF-2 in NIH3T3 or C3H10T1/2 cells transduced with GFP or FoxF1 (A) and in IMR-90 cells transfected with si NTC or si FoxF1 (B). C, quantitative RT-PCR for FoxF1 and HGF in MLFs derived from wild-type (+/+) or Foxf1 heterozygous (+/−) mice. Bars, SD.
Figure 3. FoxF1 expression controls the ability of fibroblasts to stimulate lung cancer cell migration and collagen gel contraction. A, A549 cell migration assays using the conditioned medium from NIH3T3 cells transduced with GFP or FoxF1 (GFP-CM or FoxF1-CM; left) or IMR-90 cells transfected with si NTC or si FoxF1 (si NTC-CM or si FoxF1-CM; right). Bars, SD. *P* value was calculated by Student’s *t* test. B and C, collagen gels containing NIH3T3 cells transduced with GFP or FoxF1 cultured in 2% FBS-containing medium (B), or wild-type or Foxf1 heterozygous MLFs cultured in 10% FBS-containing medium (C), were analyzed. Figures show representative pictures taken 24 h (B, bottom) and 8 h (C, bottom) after seeding, and results from quantitation of the surface area of collagen gels at the indicated time points (B and C, top). Triplicate study. Bars, SD. The circle with dashed lines corresponds to the diameter of the control pellet of GFP-transduced NIH3T3 cells or wild-type MLFs. D, collagen gels containing NIH3T3 cells transduced with GFP or FoxF1 were cultured in 10% FBS-containing medium in the presence of control DMSO or LY364947 for 72 h. Left, quantitation of the surface area of collagen gels at the indicated time points; right, photographs of collagen gels after 24 h. The result is presented in the same manner as in B.
For further loss-of-function experiments, primary MLFs from Foxf1 heterozygous mice were used, which were isolated from genotyped mice (Supplementary Fig. S2A). Morphologic difference was not obviously observed between wild-type and heterozygous MLFs (Supplementary Fig. S2B). FoxF1 expression in heterozygous MLFs was reduced to approximately the half seen in wild-type MLFs (Fig. 2C, left). Similar to the effect of FoxF1 knockdown in human lung fibroblasts, the expression of HGF was decreased in heterozygous MLFs (Fig. 2C, right).

These experiments reveal that the expression of HGF and FGF-2 is regulated by FoxF1 and thus establish that FoxF1 controls the production by fibroblasts of tumor-promoting paracrine factors.

**FoxF1 controls the ability of fibroblasts to stimulate lung cancer cell migration.** Next, we explored the role of FoxF1 in controlling fibroblast-stimulated cancer cell migration. The conditioned medium derived from FoxF1-transduced fibroblasts was more potent, compared with

![Figure 4.](image)

**Figure 4.** FoxF1 status determines the ability of fibroblasts to promote xenografted tumor growth and angiogenesis. A and B, A549 cells were s.c. injected into SCID mice, with or without NIH3T3 cells transduced with GFP or FoxF1 (NIH-GFP or NIH-FoxF1; A), or wild-type or Foxf1 heterozygous MLFs (B). Tumor volume (A, left, and B) or tumor weight (A, right) was measured at the time of excision (25 d in A and 37 d in B). Bars, SD. C and D, vascular density was analyzed in tumors derived from A549 cells coinjected with GFP- or FoxF1-expressing NIH3T3 cells. C, the blood vessels were immunostained for CD31 and nuclei were visualized with DAPI (blue). Scale bar, 200 μm. D, vascular density was quantified by measuring the percentage of the area positively stained for CD31 and DAPI. Bars, SD. n = 8 (A, B, and D). P values were calculated by Student’s t test.
that from control fibroblasts, in stimulating migration of A549 lung cancer cells (Fig. 3A, left). Similar experiments were performed using conditioned medium from control-transfected or FoxF1 siRNA–transfected IMR-90 cells. Consistently, the conditioned medium derived from IMR-90 cells transfected with FoxF1 siRNA was less potent in stimulating migration of A549 cells (Fig. 3A, right).

**FoxF1 enhances the contractile ability of fibroblasts.** CAFs and other activated fibroblasts are characterized by enhanced contractile property, which can be induced by TGF-β and PDGF (37). Tissue contraction mediated by CAFs is considered as one of the causes of increased interstitial fluid pressure, which hampers drug delivery to cancer tissues (38). To investigate the role of FoxF1 in the contractile ability of fibroblasts, collagen gel contraction assays were performed.

FoxF1-expressing fibroblasts clearly enhanced collagen gel contraction compared with the control fibroblasts (Fig. 3B). Consistently, Foxf1 heterozygous MLFs were less potent in inducing collagen gel contraction compared with wild-type MLFs (Fig. 3C).

To investigate the TGF-β dependency of the FoxF1-induced enhanced gel contraction, experiments were performed in the presence or absence of TGF-β type I receptor inhibitor, LY364947. The increased gel contraction ability of FoxF1-transduced cells was also observed in the presence of TGF-β signaling inhibition, suggesting that FoxF1 controls gel contraction through mechanisms that are largely independent of TGF-β signaling (Fig. 3D).

The experiments presented in Fig. 3 thus establish that FoxF1 status of fibroblasts controls functional activities in vitro, such as stimulation of cancer cell migration and gel contraction, associated with CAFs.

**Figure 5.** FoxF1 expression in human lung cancer and its association with survival. A, representative photographs of immunohistochemistry for FoxF1 in three histologic subtypes of lung cancer. Scale bar, 100 μm. B, Kaplan-Meier analysis of lung tumor cases divided into positive or negative according to FoxF1 expression in CAFs. Left, overall population; middle, female population; right, population of large cell lung cancer. P value was calculated by log-rank test. n.s., not significant. C, quantitative RT-PCR for FoxF1, αSMA, PDGFRα, HGF, and FGF-2 was performed in triplicate for each sample (n = 16) to analyze associations with FoxF1 expression. Expression levels were normalized to that of HPRT, and correlation coefficients (r) were calculated. To test statistical significance, a t value was calculated for each data set. The significance was shown as P < 0.01 (t > 2.977) or P < 0.05 (t > 2.145).
**FoxF1 status of fibroblasts determines their ability to support subcutaneous tumor growth.** To address the *in vivo* effects of FoxF1, coinjection of fibroblasts together with A549 lung cancer cells was performed.

FoxF1-expressing NIH3T3 cells were more potent in enhancing subcutaneous tumor growth compared with GFP-expressing control fibroblasts (Fig. 4A). All the xenografted tumors derived from A549 cells coinjected with GFP- or FoxF1-expressing NIH3T3 cells were composed of A549 cell-derived cancer tissues with gland-like structures and surrounding stromal tissues. Histologic appearances and epithelial-stromal ratios were not different between these two groups, suggesting that the increase in tumor volume is caused by enhanced proliferation of A549 cancer cells as well as corresponding stromal cells (data not shown). In agreement with these findings, coinjection of wild-type MLFs with A549 cells resulted in enhanced tumor growth compared with what was observed following coinjection with Foxf1 heterozygous MLFs (Fig. 4B).

To further explore the tumor-promoting effect of FoxF1, tumor angiogenesis was analyzed in tumors derived from coinjection of A549 cells and either control GFP-transduced or FoxF1-transduced NIH3T3 cells. Vascular density, as determined by CD31-positive area, was significantly higher in the tumors derived from the mixture of FoxF1-expressing fibroblasts and A549 cells (Fig. 4C and D).

Taken together, these findings thus show that FoxF1 status of fibroblasts determines their ability to support subcutaneous tumor growth, and also suggest that enhancement of tumor angiogenesis is involved in the protumorigenic effect of FoxF1-expressing fibroblasts.

**FoxF1 expression in human lung cancer and its association with survival.** To evaluate the clinical significance of these model-derived findings, FoxF1 status was determined by immunohistochemistry in human lung tumor samples. For the validation of antibody, we confirmed that FoxF1 was specifically detected in paraformaldehyde-fixed and paraffin-embedded FoxF1-transfected cells (Supplementary Fig. S3A).

Distinct staining of FoxF1 in the nucleus was observed in a subset of lung tumor tissues and was restricted to tumor stromal cells (Fig. 5A). We focused on the FoxF1 positivity in fibroblasts (CAFs), which were morphologically identified as stromal spindle cells. Of 247 cases, positive nuclear staining of FoxF1 in CAFs was found in 110 (44.5%) cases. In a part of these cases, stromal cells adjacent to the tumor cells showed stronger staining compared with those further apart from the tumor cells (Supplementary Fig. S3B). FoxF1 expression in CAFs was observed in a variety of histologic subtypes of lung cancer and was not significantly associated with any particular subtype (Supplementary Table S1). Although FoxF1 positivity in CAFs did not show significant association with survival in the overall population (Fig. 5B, left), it predicted poor prognosis when restricted to the female population (Fig. 5B, middle) as well as the patients of large cell lung cancer (Fig. 5B, right).

To further substantiate the *in vitro* studies showing that FoxF1 controls the expression of αSMA, PDGFRα, HGF, and FGF-2 (Figs. 1 and 2), the associations between FoxF1 expression and these genes were investigated in clinical samples. Interestingly, quantitative RT-PCR analyses, performed on tumor-derived RNA, revealed significant correlation between FoxF1 and all these genes (Fig. 5C).

These results thus provide strong support for the clinical relevance of the experimental results obtained from the tissue culture and animal studies described above.

**FoxF1 expression is strongly associated with activation of hedgehog signaling.** Studies on the roles of FoxF1 during organogenesis have identified hedgehog signaling as a potent upstream regulator of FoxF1 expression (19, 25). Potential associations between FoxF1 expression and activation of hedgehog signaling in clinical samples were therefore analyzed to obtain preliminary information on the mechanism(s) governing the differential FoxF1 expression in the stroma of human lung cancer.

Expression of Gli1, patched1 (PTCH1), and patched2 (PTCH2) was used to monitor hedgehog signaling activation (39). The expression levels of these three genes were all strongly correlated with the expression of FoxF1 (Fig. 6).

These findings thus suggest that activation of hedgehog signaling is an important upstream regulator of the FoxF1 expression in human lung cancer.

**Discussion**

This study shows, through the systematic use of loss- and gain-of-function models, that FoxF1 is a regulator of key...
characteristics of lung CAFs. These include upregulation of αSMA and PDGFRα (Fig. 1), secretion of paracrine growth factors such as FGF-2 and HGF (Fig. 2), induction of contractile and migration-stimulatory properties (Fig. 3), and in vivo protumorigenic effects (Fig. 4).

It has previously been shown that inactivation of one allele each of FoxF1 and FoxF2 causes defects in gut development and increases stomatal Wnt5a expression, leading to epithelial hyperproliferation (24, 40). In the current study, we have revealed that FoxF1 regulates the expression of protumorigenic factors HGF and FGF-2. HGF has been well studied as a fibroblast-derived stimulator of lung cancer cells (35) and thus is a candidate regulator of FoxF1-mediated modulation of A549 cell migration. Furthermore, activation of PDGF signaling in fibroblasts leads to upregulation of molecules that promote cancer cell migration, which could be another possible mechanism.

The mechanisms of protumorigenic and proangiogenic effects of FoxF1-expressing fibroblasts in vivo remain to be elucidated. Alterations of the composition of the micro-environments in the xenografted tumors, which involves different types of host-derived stromal cells, might be involved. HGF and FGF-2 are candidate mediators of these effects.

Importantly, clinical relevance of these findings is supported by the FoxF1 expression in human lung CAFs (Fig. 5). Finally, the correlation between the expression of FoxF1 and markers for hedgehog signaling (Fig. 6) suggests hedgehog pathway as an important upstream regulator of FoxF1 expression.

An improved understanding of the molecular basis for the protumorigenic properties of CAFs is of obvious importance for understanding tumor growth and for the development of novel stroma-targeted therapies. About CAF recruitment and expansion, it is well established that members of PDGFR and TGF-β families play important roles (2, 14, 41). Recently, other classes of paracrine signaling molecules, such as chemokines and members of the hedgehog family, have also been shown to stimulate the tumor-promoting effects of fibroblasts (16, 28).

In parallel with these studies, it has been investigated whether genetic or epigenetic alterations underlie the CAF properties. Strong evidence showing consistent epigenetic alterations has been provided (42–44). About the presence of somatic mutations in CAFs, reports with conflicting results have been presented (45, 46). However, it is noteworthy that the information about key transcription factors governing the CAF properties is the most limited. The present description of FoxF1 as a potent inducer of the CAF properties thus presents one of the first studies that identify a transcription factor controlling functionally important CAF properties.

Our studies suggest hedgehog signaling as a key regulator of FoxF1 expression in the stroma of human lung cancer. Hedgehog signaling is receiving increasing attention as an important pathway for tumor stroma growth and function (47, 48). One key study emphasizing the importance of hedgehog signaling in tumor stroma showed therapeutic effects of hedgehog inhibitors in several tumor models, where the malignant cells were not sensitive to hedgehog inhibitors (28). The stroma-promoting action of hedgehog signaling is also supported by studies in genetic models, which have shown that ablation of the hedgehog receptor smoothened in epithelial cells did not affect pancreatic tumor growth (26). Furthermore, hedgehog inhibitors were also recently shown to promote tumor drug uptake in a genetic model of pancreatic cancer through mechanisms that involved remodeling of the fibroblastic stroma (27). Our findings provide additional support for an important role of stromal hedgehog signaling, including FoxF1 activation, also in lung cancer.

In conclusion, the study has identified FoxF1 as a previously unrecognized clinically relevant regulator of lung cancer fibroblasts. The findings provide novel mechanistic understanding of the molecular control of a key cell type of the lung cancer stroma and suggest general principles to be further evaluated in other types of cancer. The findings should thereby ultimately contribute to the development of novel CAF-targeting therapies for lung cancer and other tumor types.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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