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Fibroblast Growth Factor (FGF) and FGF Receptor-Mediated Autocrine Signaling in Non–Small-Cell Lung Cancer Cells

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ABSTRACT

Despite widespread expression of epidermal growth factor (EGF) receptors (EGFRs) and EGF family ligands in non–small-cell lung cancer (NSCLC), EGFR-specific tyrosine kinase inhibitors (TKIs) such as gefitinib exhibit limited activity in this cancer. We propose that autocrine growth signaling pathways distinct from EGFR are active in NSCLC cells. To this end, gene expression profiling revealed frequent coexpression of specific fibroblast growth factors (FGFs) and FGF receptors (FGFRs) in NSCLC cell lines. It is noteworthy that FGF2 and FGF9 as well as FGFR1 IIIc and/or FGFR2 IIIc mRNA and protein are frequently coexpressed in NSCLC cell lines, especially those that are insensitive to gefitinib. Specific silencing of FGF2 reduced anchorage-independent growth of two independent NSCLC cell lines that secrete FGF2 and coexpress FGFR1 IIIc and/or FGFR2 IIIc. Moreover, a TKI [(±)-1-(anti-3-hydroxy-cyclopentyl)-3-(4-methoxy-phenyl)-7-phenylamino-3,4-dihydro-1*H*-pyrimido-[4,5-*d*]pyrimidin-2-one (RO4383596)] that targets FGFRs inhibited basal FRS2 and extracellular signal-regulated kinase phosphorylation, two measures of FGFR activity, as well as proliferation and anchorage-independent growth of NSCLC cell lines that coexpress FGF2 or FGF9 and FGFRs. By contrast, RO4383596 influenced neither signal transduction nor growth of NSCLC cell lines lacking FGF2, FGF9, FGFR1, or FGFR2 expression. Thus, FGF2, FGF9 and their respective high-affinity FGFRs comprise a growth factor autocrine loop that is active in a subset of gefitinib-insensitive NSCLC cell lines.

Autocrine growth factor production by cancer cells provides self-sufficiency in growth signals, one of the six hallmarks of cancer (Hanahan and Weinberg, 2000). Based on the frequent expression of EGFR in NSCLC (Hirsch et al., 2003) as well as the widespread coexpression with various EGF family ligands (Rusch et al., 1997), the EGFR is an attractive candidate for a receptor tyrosine kinase mediating autocrine growth in NSCLC. In this context, the EGFR inhibitors gefitinib and erlotinib were deployed in a series of clinical trials but yielded response rates of only 10 to 20% (Dancey, 2004; Hirsch and Bunn, 2005). Subsequent molecular analysis of the responsive lung tumors revealed a significant enrichment for gain-of-function EGFR mutations (Lynch et al., 2004; Han et al., 2005). The general insensitivity to EGFR-specific TKIs is also reflected in cultured NSCLC cell lines (Coldren et al., 2006; Helfrich et al., 2006). Thus, despite broad expression of EGFR in NSCLC, only a subset responds to EGFR inhibitors. Whereas rare mutations in EGFR that render the tyrosine kinase resistant to gefitinib and erlotinib have been identified (Pao et al., 2005), the limited response of NSCLC to

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ABBREVIATIONS: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; TKI, tyrosine kinase inhibitor; NSCLC, non–small-cell lung cancer; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; Ig, immunoglobulin; HITES, RPMI-1640 containing hydrocortisone/insulin/transferrin/estradiol/Na_3SeO_3/bovine serum albumin; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; RO4383596; (±)-1-(anti-3-hydroxy-cyclopentyl)-3-(4-methoxy-phenyl)-7-phenylamino-3,4-dihydro-1*H*-pyrimido[4,5-*d*]pyrimidin-2-one; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated kinase; PAGE, polyacrylamide gel electrophoresis; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; FRS2, FGF receptor substrate 2.

EGFR inhibitors may reflect the activity of additional receptor tyrosine kinase systems distinct from EGFR (Morgillo and Lee, 2005; Rikova et al., 2007). Candidate receptor tyrosine kinase systems include cMet (Engelman et al., 2007; Lutterbach et al., 2007), Axl (Wimmel et al., 2001; Shieh et al., 2005), and IGF-1 receptor (Morgillo and Lee, 2005) as well as novel tyrosine kinases such as Alk and Ros (Rikova et al., 2007). Moreover, a recent study by Stommel et al. (2007) in glioblastoma cell lines provides strong evidence for the coactivation of multiple receptor tyrosine kinases in cancer cells and the requirement for simultaneous blockade to achieve significant growth inhibition.

We examined archived gene expression array data obtained from a panel of NSCLC cell lines (Coldren et al., 2006) and noted frequent coexpression of distinct FGFs and FGFRs, suggesting that an FGFR-dependent autocrine signaling pathway may operate in a significant fraction of NSCLC. FGFs have been discovered from nematodes to man with 22 distinct FGFs presently identified in mammals (for review, see Eswarakumar et al., 2005; Grose and Dickson, 2005; Mohammadi et al., 2005). FGFs stimulate diverse responses in development and tissue maintenance by binding to and activating a family of four receptor tyrosine kinases designated FGFR1 to FGFR4. The extracellular domain of FGFRs contains two or three immunoglobulin-like (Ig-like) loops where the two membrane-proximal loops encode the FGF binding site. Of particular importance to FGF binding specificity is the third Ig loop, the N-terminal half of which is encoded by an invariant IIIa exon with alternative usage of IIIb or IIIc exons for the C-terminal half (Eswarakumar et al., 2005; Mohammadi et al., 2005). As a general rule, FGFRs encoding exon IIIb (FGFR IIIb) are expressed on epithelial cells, whereas the FGFRs encoding exon IIIc (FGFR IIIc) are expressed on mesenchymal cells (Mohammadi et al., 2005). By contrast, the ligands for FGFR IIIb are often expressed in mesenchymal cells, whereas ligands for FGFR IIIc are expressed in epithelial cells. This establishes a paracrine mechanism of signaling between epithelia and mesenchyme that is critical to normal development and tissue homeostasis. In this regard, FGF7 and FGF10 expressed in lung mesenchymal compartments exhibit high affinity for FGFR2 IIIb expressed on lung epithelial cells, providing key signals required for epithelial-mesenchymal interactions that are pivotal to embryonic lung development. By contrast, FGF2 and FGF9 exhibit negligible binding to FGFR2 IIIb but high-affinity binding to FGFR1 IIIc and FGFR2 IIIc.

The literature provides ample precedent for the involvement of distinct FGFs and FGFRs in cancers of epithelial origin (for review, see Grose and Dickson, 2005), including prostate, thyroid, skin, head and neck, and urinary bladder. In many instances, tumorigenesis is associated with amplified FGFR expression, although somatic mutations within the receptor coding sequences that confer gain of function have also been identified. In addition to FGFR mutations, inappropriate expression of FGF ligands presents an alternative mechanism by which FGFRs could be activated and could participate in oncogenesis. Our present study demonstrates coexpression of FGF2 or FGF9 as well as FGFR1 and/or FGFR2 in multiple NSCLC cell lines. Moreover, studies employing molecular silencing of FGF2 and a pharmacological FGFR inhibitor reveal a functional role for an FGFR autocrine signaling pathway in NSCLC cell lines, especially those that are insensitive to EGFR-specific TKIs.

Materials and Methods

Cell Culture. The NSCLC cell lines employed in this study were submitted to DNA profiling analysis by the University of Colorado Cancer Center to verify their authenticity. All cell lines were routinely cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37°C in a humidified 5% CO_2 incubator. Where indicated, the cells were switched to HITES medium (RPMI-1640 containing 10 nM hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 10 nM estradiol, 30 nM Na_3SeO_3 and 1% bovine serum albumin) to limit mitogenic inputs from serum components.

Quantitative Real-Time PCR Assay. Total RNA (3 μg) was reverse-transcribed in a volume of 20 μl using random hexamers and Moloney murine leukemia virus reverse transcriptase. Aliquots (1 μl) of 10-fold diluted reverse transcription reactions were subjected to PCR in 25-μl reactions with SYBR green Jumpstart Taq Readymix (Sigma, St. Louis, MO) and the primers listed in Table 1 using an I Cycler (Bio-Rad Laboratories, Hercules, CA). The real-time PCR amplification products from initial experiments were resolved by electrophoresis on 5% polyacrylamide gels to verify that the primer pairs amplified a single product of the predicted size. GAPDH mRNA levels were measured by quantitative RT-PCR in replicate samples as a housekeeping gene for normalization of the different mRNA expression, and the data are presented as "Relative Expression."

Cell Proliferation and Anchorage-Independent Growth Assays. To measure the effect of FGFR inhibition on cell proliferation, NSCLC cells were seeded in 96-well plates at 1000 cells/well; RO4383596 was added the next day at concentrations ranging from 0 to 3 μM and cultured for 5 additional days. Viable cells were subsequently estimated with a modified MTT assay as described previously (Carmichael et al, 1988). For measurement of anchorage-independent cell growth, 20,000 cells were suspended in 0.75 ml of HITES containing 5% fetal bovine serum and 0.4% Difco agar noble (BD Biosciences, Sparks, MD) and overlaid on base layers containing 0.75 ml of HITES containing 5% fetal bovine serum and 0.5% agar noble in 12-well plates. In experiments testing the effect of RO4383596 on anchorage-independent growth, the drug was added to the base layers at 2-fold concentrations such that diffusion into the top layers yielded the desired final drug concentrations. The plates were incubated in a 37°C CO_2 incubator for 21 days and viable colonies were stained for 24 h with nitro blue tetrazolium. After digital photography, the colonies were quantified using the Meta-

TABLE 1
Sequences of primers for quantitative RT-PCR

Gene	Forward Primer	Reverse Primer
FGF2	CGGCTGTACTGCAAAAACGG	GATGTGAGGGTCGCTCTTCTCC
FGF7	GATCCTGCCAACTTTGCTCTACAG	TGCTCTGGAGTCATGTCATTGC
FGF9	GAAAGACCACAGCCGATTTG	TTCATCCCGAGGTAGAGTCC
FGFR1	TAATGGACTCTGTGGTGCCCTC	ATGTGTGGTTGATGCTGCCG
FGFR2	CGCTGGTGAGGATAACAACACG	TGGAAGTTCATACTCGGAGACCC
FLK1	TCTCTCTGCCTACCTCACCTGTTTC	CTGACTGATTCCTGCTGTGTTGTC
FLT1	TTCCTCTGAAATGGATGGCTCC	AGCAATACTCCGTAAGACCACACG
GAPDH	GCCAAATATGATGACATCAAGAAGG	GGTGTCGCTGTTGAAGTCAGAG

Morph imaging software program (Molecular Devices, Sunnyvale, CA).

FGF2 ELISA. NSCLC cell lines seeded in 12-well plates were cultured in 1 ml of HITES medium for three days. Thereafter, the medium was collected and assayed for FGF2 using a Quantikine human FGF basic assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The cell monolayers were lysed in 250 μl of mitogen-activated protein kinase lysis buffer (0.5% Triton X-100, 50 mM β-glycerophosphate, pH 7.2, 0.1 mM Na_3VO_4, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.3 M NaCl, 2 μg/ml leupeptin, and 4 μg/ml aprotinin) and cell protein was measured by the Bradford assay. Secreted FGF2 measured by ELISA was normalized to cellular protein and presented as picograms of FGF2 per milligram of cell protein.

FGF2 Silencing with shRNAs. Two distinct shRNAs (clone ID TRCN0000003329 targeting FGF2 sequences 799–819 and clone ID TRCN0000003332 targeting FGF2 sequences 756–776) encoded in the pLKO.1 lentiviral vector were obtained from Open Biosystems (Huntsville, AL). The pLKO.1 constructs encoding the FGF2 shRNAs as well as pLKO.1 encoding a control shRNA targeting GFP were packaged into lentiviruses and used to transduce H226 and H1703 cells according to the manufacturer's protocol. Stable transfectants were selected with 1 μg/ml puromycin and drug-resistant cells were pooled and screened for FGF2 secretion by ELISA. The cultures were submitted to anchorage-independent growth assays within three passages after the initial puromycin selection.

Immunoblot Analyses. For analysis of phospho-ERK and phospho-FRS2, growth factor or drug-treated NSCLC cells were rinsed once with phosphate-buffered saline, lysed in mitogen-activated protein kinase lysis buffer, and centrifuged (5 min at 13,000 rpm). The particulate fractions were discarded and the soluble extracts were mixed with SDS sample buffer and submitted to SDS-PAGE. After electrophoretic transfer onto nitrocellulose, the filters were blocked in 3% bovine serum albumin (Cohn Fraction V; MP Biomedicals, Inc., Irvine, CA) in Tris-buffered saline with 0.1% Tween 20 and then incubated with rabbit polyclonal anti-phospho-ERK or rabbit polyclonal phospho-FRS2-Y196 antibodies (Cell Signaling Technology, Inc., Danvers, MA) for 16 h at 4°C. The filters were washed thoroughly in Tris-buffered saline with 0.1% Tween 20, then incubated with alkaline phosphatase-coupled goat anti-rabbit antibodies and developed with LumiPhos reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. The filters were subsequently stripped and re-probed for total ERK1 and ERK2 using a mixture of rabbit polyclonal anti-ERK1 and ERK2 antibodies or FRS2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Because ERK2 was more abundant relative to ERK1 in the NSCLC cell lines, the respective phospho-ERK2 and total ERK2 bands were submitted to densitometry analysis and the data are presented as the ratio of phospho-ERK2 to total ERK2.

For immunoblot analysis of FGFR1, FGFR2, EGFR, and the α-subunit of NaK-ATPase, NSCLC cells were collected in phosphate-buffered saline, centrifuged (5 min, 1000*g*) and suspended in hypotonic lysis buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 4 μg/ml PMSF, 2 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM dithiothreitol). The cells were homogenized by six to eight passes through a 26-gauge syringe needle, and the homogenate was submitted to centrifugation at 1000*g* for 5 min to pellet nuclei and unbroken cells. The resulting supernatants were centrifuged (10,000*g* for 10 min) to collect the membrane fragments that were subsequently resuspended in hypotonic lysis buffer. Aliquots of the membrane preparations containing 100 μg of protein were submitted to SDS-PAGE and immunoblotted for FGFR1, FGFR2, and NaK-ATPase α-subunit with antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). EGFR was detected with a rabbit polyclonal antibody from Cell Signaling Technology, Inc.

Results

Specific FGFs and FGFRs are Coexpressed in NSCLC Cell Lines. When a data set from the recent gene expression profiling of a panel of NSCLC cell lines (Coldren et al., 2006) was queried for the coexpression of known receptor tyrosine kinases and their respective polypeptide growth factors, frequent expression of FGF2, FGF9, FGFR1, and FGFR2 was observed (data not shown). The platelet-derived growth factor and VEGF family of growth factors were also predicted to be widely expressed in NSCLC cell lines, but the expression values

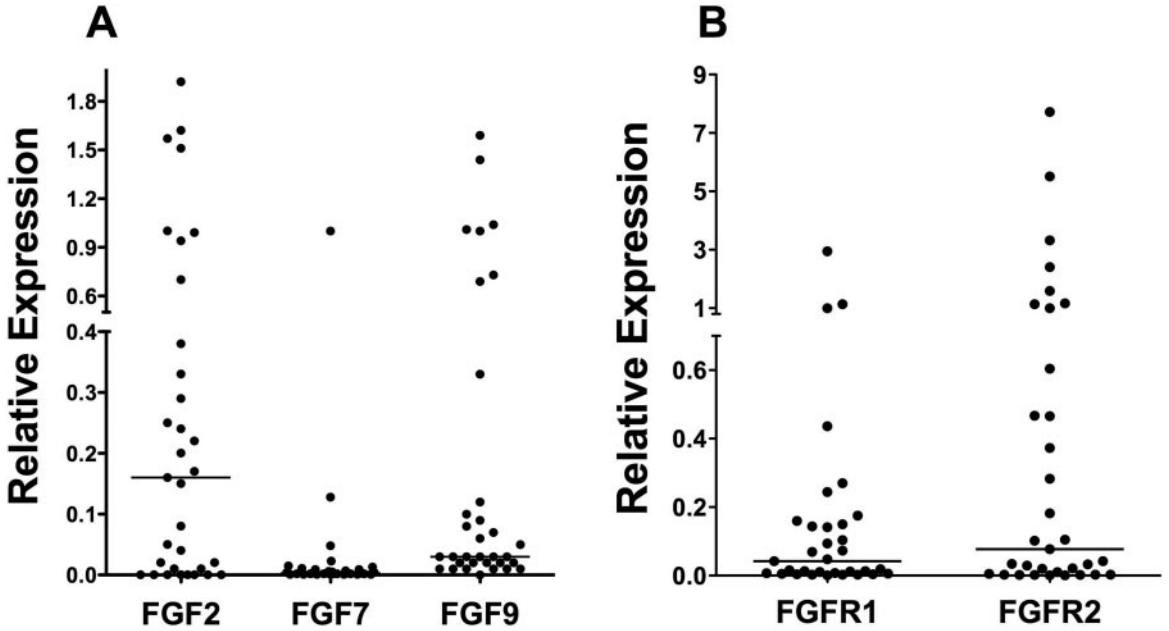


Fig. 1. Expression of FGFs and FGFRs in NSCLC cell lines. Total RNA prepared from a panel of 33 NSCLC cell lines was reverse-transcribed and submitted to quantitative RT-PCR with primers (see Table 1) specific for FGF2, FGF7, or FGF9 (A) or FGFR1 or FGFR2 (B). The values are normalized to GAPDH mRNA measured in replicate samples and presented as relative expression. The horizontal bars denote the median expression among the 33 NSCLC cell lines.

for PDGFRs (PDGFRα, PDGFRβ) and VEGFRs (FLT1, FLK1) were uniformly low or absent with the exception of H1703 cells, which express PDGFRα (Rikova et al., 2007). Quantitative RT-PCR analyses of 33 NSCLC cell lines confirmed the gene expression profiling data and revealed a non-Gaussian distribution of FGF2 and FGF9 mRNA expression (Fig. 1A). By contrast, only one NSCLC cell line expressed FGF7 at a level different from the low to undetectable expression observed in the other NSCLC cell lines. Moreover, FGFR1 and FGFR2 mRNAs were also widely expressed in a non-Gaussian manner among the 33 NSCLC cell lines (Fig. 1B). By contrast, FGFR3 and FGFR4 mRNAs were more restricted (not shown) and did not correlate with FGF2 or FGF9 expression or response to an FGFR TKI described below. Based on these findings, we highlighted FGF2, FGF9, FGFR1, and FGFR2 as putative components of an autocrine signaling pathway in NSCLC cell lines for further exploration.

A panel of NSCLC cell lines that had previously been characterized for sensitivity to the EGFR inhibitor gefitinib (Coldren et al., 2006; Helfrich et al., 2006), was assayed for FGF2 and FGF9 mRNA using quantitative RT-PCR with primers listed in Table 1. NSCLC cell lines H226, H520, H661, H1299, H1703, and Colo699 are insensitive to gefitinib ($IC_{50} > 4$ μM), whereas H322c, H358, H1648, HCC827, and HCC4006 are sensitive to gefitinib ($IC_{50} < 0.5$ μM). Among the latter, HCC827 and HCC4006 bear EGFR exon 19 deletions conferring gain of function. As shown in Fig. 2A, high FGF2 and FGF9 mRNA expression largely segregated with the gefitinib-insensitive NSCLC cell lines, although HCC827 and HCC4006 expressed detectable FGF2 mRNA. The mean relative FGF2 mRNA expression in the group composed of H226, H520, H661, H1299, H1703, and Colo699 cells (0.88 ± 0.29) was statistically greater ($p < 0.02$) than for H322c, H358, H1648, HCC827, and HCC4006 cells (0.092 ± 0.057). Moreover, analysis of conditioned medium from the NSCLC cell lines by ELISA revealed that FGF2 protein was readily secreted into the medium by NSCLC cells that expressed significant FGF2 mRNA (Fig. 2B).

Similar analysis of FGFR1 and FGFR2 mRNA levels in the NSCLC cell lines revealed exclusive segregation of FGFR1 mRNA in the cell lines previously defined as being gefitinib-insensitive (Fig. 3A). The mean FGFR1 mRNA relative expression in the group composed of H226, H520, H661, H1299, H1703, and Colo699 cells (0.96 ± 0.43) was statistically greater ($p < 0.005$) than the mean expression for H322c, H358, H1648, HCC827, and HCC4006 cells (0.006 ± 0.002). By contrast, the expression of FGFR2 mRNA was not different between the two groups of cells (Fig. 3A). FGFR1 protein as assessed by immunoblot analysis coincided closely with FGFR1 mRNA measured by quantitative RT-PCR (Fig. 3B). Although FGFR2 protein was readily detected in H1648 and Colo699 cells that express high levels of FGFR2 mRNA (Fig. 3A), a significant level of FGFR2 protein was also detected in H226, H520, and H661 cells, which expressed very low levels of FGFR2 mRNA. Thus, FGF2 or FGF9 and FGFR1 and/or FGFR2 are coexpressed in H226, H520, H661,

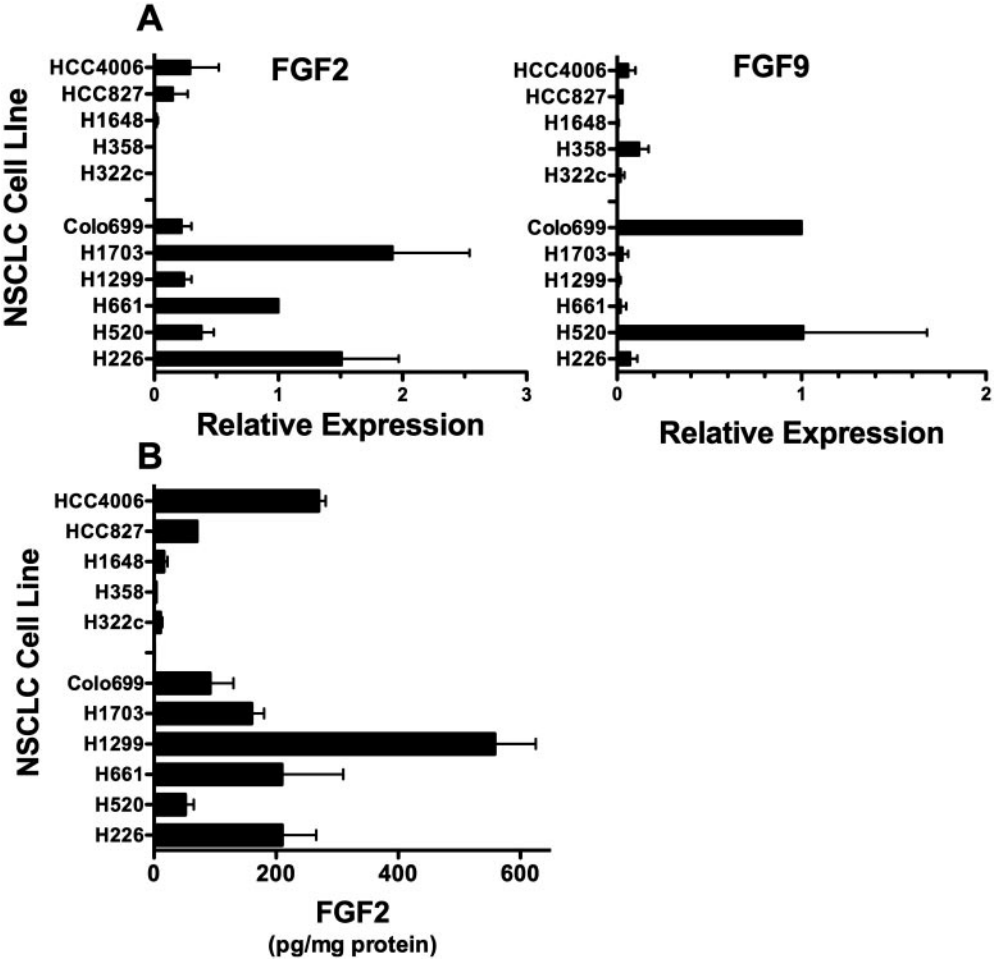


Fig. 2. Expression of FGF2, FGF7 and FGF9 mRNA and FGF2 secretion by NSCLC cell lines. A, quantitative RT-PCR assay for FGF2 and FGF9 mRNAs was performed on the indicated NSCLC cell lines and normalized for GAPDH mRNA levels. B, NSCLC cell lines were cultured for 2 days in serum-free HITES medium. FGF2 secreted into the conditioned medium was measured by ELISA (R&D Systems) and normalized to protein measured in the attached cells.

H1299, H1703, and Colo699 cells. By contrast, HCC827 and HCC4006 express FGF2, but no detectable expression of FGFR1 or FGFR2 mRNA or protein.

FGFR1 and FGFR2 mRNAs are alternatively spliced within the third extracellular, membrane-proximal immunoglobulin-like loop to generate the respective FGFR IIIb and FGFR IIIc proteins, which display distinct FGF family member binding properties (Eswarakumar et al., 2005; Grose and Dickson, 2005; Mohammadi et al., 2005). FGFR1 IIIb, FGFR1 IIIc, and FGFR2 IIIc bind FGF2 and FGF9 with high affinity, whereas FGFR2 IIIb binds FGF7 and FGF10. We employed published (Murgue et al., 1994; Kwabi-Addo et al., 2001) PCR strategies to amplify the regions of the FGFR1 and FGFR2 cDNAs flanking the third extracellular immunoglobulin loops and the resulting cDNA products were digested with restriction enzymes that discriminate between the FGFR IIIb and FGFR IIIc sequences. As shown in Fig. 4, the FGFR1 PCR products amplified from cDNA prepared from Colo699, H520, H1703, H1299, and H226 cells were completely digested with AflII, which cuts within the FGFR1 IIIc sequence, but not Bsm1, which cuts the product arising from the FGFR1 IIIb mRNA. Analysis of FGFR1 mRNA from H661 cells also revealed only FGFR1 IIIc (not shown). Thus, FGFR1 IIIc is the sole FGFR1 isoform expressed in NSCLC cells. Similar analysis of FGFR2 PCR products revealed expression of FGFR2 IIIc (cut by HincII) in Colo699 and H226 cells (Fig. 4) as well as H520 and H661 (not shown). By contrast, H322c, H358, H1648, HCC827 (Fig. 4), and HCC4006 (not shown) exclusively expressed the FGFR2 IIIb splice product that is digested by Ava I (Fig. 4). Therefore, expression of FGF2 and/or FGF9 in H226, H520, H661, H1299, H1703, and Colo699 cells coincides with expression of one or more of their high-affinity receptors, FGFR1 IIIc and/or FGFR2 IIIc. Although FGF2 mRNA and protein are expressed in HCC827 and HCC4006 cells, neither FGFR1 IIIc nor FGFR2 IIIc were expressed in these cell lines.

To determine whether the coexpression of FGFs and FGFRs in NSCLC cell lines is also observed in primary NSCLC tumors, the expression of FGF2, FGF9, FGFR1, and FGFR2 mRNAs was measured by quantitative RT-PCR in total RNA prepared from nine primary human NSCLC tumor samples obtained through the University of Colorado Lung SPORE. The findings in Table 2 show expression values for FGF2 and FGF9 in which six of the nine tumors examined expressed FGF2 or FGF9 at levels greater than median values (0.13 and 0.03 for FGF2 and FGF9, respectively). In addition, FGFR1 mRNA was expressed at levels similar to those detected in NSCLC cell lines and was

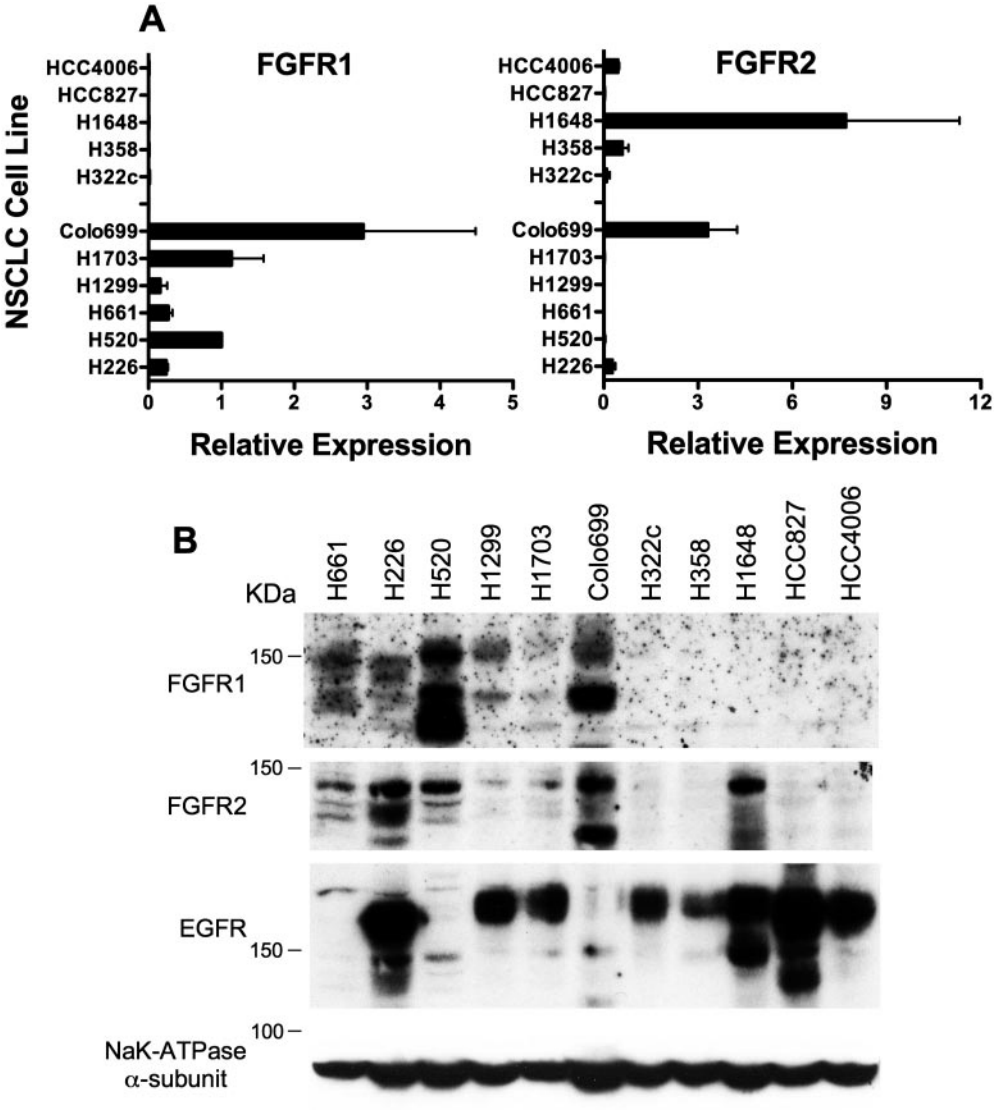
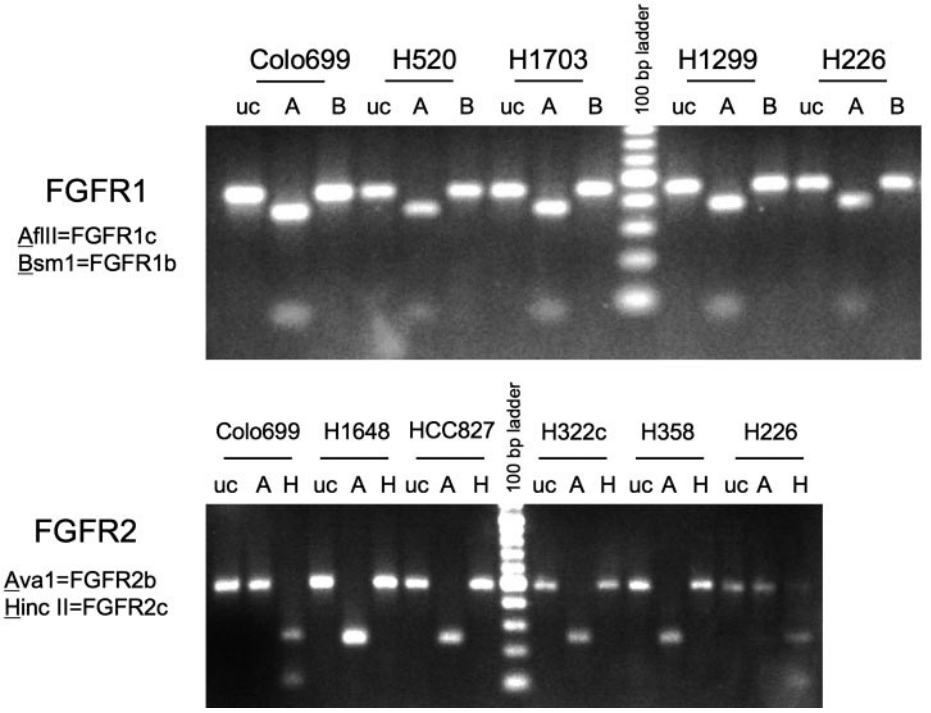


Fig. 3. FGFR1 and FGFR2 protein expression in NSCLC cell lines. A, quantitative RT-PCR assay for FGFR1 and FGFR2 mRNAs was performed on the indicated NSCLC cell lines and normalized for GAPDH mRNA levels. B, membrane preparations from the indicated NSCLC cell lines were resolved by SDS-PAGE and immunoblotted for FGFR1 and FGFR2. The filters were stripped and reprobed for EGFR or the α-subunit of NaK-ATPase as a loading control.

exclusively spliced to yield FGFR1 IIIc (not shown). Likewise, FGFR2 was expressed at levels greater than the median level in four of the nine tumors and analysis of the IIIb/IIIc status of FGFR2 revealed FGFR2 IIIc expression, alone or in combination with FGFR2 IIIb, in four of the nine tumors. Thus, these data indicate that FGF2 and FGF9 are frequently coexpressed with FGFR1 IIIc and/or FGFR2 IIIc in primary NSCLC tumors.

We tested whether NSCLC cell lines expressing FGFR1 IIIc or FGFR2 IIIc responded to exogenous FGF2 as predicted by their FGFR isoform status. The NSCLC cell lines were incubated with EGF or FGF2 for 15 min and cell extracts were submitted to immunoblot analysis of phospho-ERK as a measure of receptor activation. As shown in Fig. 5, FGF2-treatment of NSCLC cell lines expressing FGFR1 IIIc and/or FGFR2 IIIc (H226, H520, H661, H1299, H1703, Colo699) stimulated ERK phosphorylation. H520, H661 and Colo699 cells failed to respond to EGF, consistent with little or no expression of EGFR in these lines (see Fig. 3B). By contrast, H322c, H358, H1648, HCC827, and HCC4006 responded to EGF, but not exogenous FGF2, with increased phospho-ERK. The NSCLC cell lines exhibit the FGF2-stimulated phospho-ERK responses predicted by the FGFR isoform expression status.

FGF2 Silencing Inhibits Anchorage-Independent Growth of H226 and H1703 Cells. Combined, the findings in Figs. 2 to 5 demonstrate coexpression of FGF2 or FGF9 and functional FGFR1 or FGFR2 in H226, H520, H661, H1299, H1703, and Colo699 cells. To directly test the role of



Cell Line	FGFR1 isoform	FGFR2 isoform
H226	FGFR1c	FGFR2c
Colo699	FGFR1c	FGFR2c
H520	FGFR1c	FGFR2c
H661	FGFR1c	FGFR2c
H1299	FGFR1c	-
H1703	FGFR1c	-
H322c	-	FGFR2b
H358	-	FGFR2b
H1648	-	FGFR2b
HCC827	-	FGFR2b
HCC4006	-	FGFR2b

Fig. 4. Expression of FGFR1 and FGFR2 IIIb and IIIc splice variants in NSCLC cells. Total RNA from the indicated NSCLC cell lines was reverse-transcribed and submitted to PCR with published primers (Kwabi-Addo et al., 2001) flanking the cDNA sequences encoding the alternatively spliced third Ig-like extracellular domains from FGFR1 or FGFR2. The amplified FGFR1 PCR products were incubated with AflII (A) that digests the FGFR1 IIIc isoform or Bsm1 (B) that digests the FGFR1 IIIb isoform or incubated with neither enzyme (uc). Similar analysis was performed on the FGFR2 PCR products where Ava 1 (A) cuts the FGFR2 IIIb isoform and Hinc II (H) cuts the FGFR2 IIIc isoform; uc is uncut product. The reactions were submitted to electrophoresis on 1.5% agarose gels and stained with ethidium bromide.

TABLE 2

Expression of FGF and FGFR mRNAs in primary NSCLC tumors

Primary lung tumor total RNA obtained through the Tissue Procurement Core of the UCHSC Lung SPORE was reverse-transcribed and submitted to quantitative RT-PCR with primers (see Table 1) specific for FGF2, FGF9, FGFR1, or FGFR2. The values are normalized to GAPDH mRNA and presented as mean relative expression and S.E.M. The status of the FGFR IIIb/IIIc splice variants for FGFR1 and FGFR2 was determined as described in the legend to Fig. 4. FGFR1 IIIc was the sole FGFR1 isoform detected in the primary tumor mRNA samples.

Tumor ID	Relative Expression				
	FGF2	FGF9	FGFR1	FGFR2	FGFR2 isoform
11898	0.06 ± 0.001	0.04 ± 0.018	0.12 ± 0.027	0.29 ± 0.037	FGFR2b
18187	1.12 ± 0.24	0.00 ± 0.000	0.32 ± 0.073	0.56 ± 0.038	FGFR2c
16219	0.14 ± 0.000	0.46 ± 0.089	0.39 ± 0.115	0.07 ± 0.014	
21023	1.00 ± 0.000	0.00 ± 0.001	0.49 ± 0.051	1.00 ± 0.000	FGFR2c
16895	0.13 ± 0.010	0.13 ± 0.055	0.18 ± 0.035	0.04 ± 0.001	
21236	0.06 ± 0.025	1.00 ± 0.000	0.31 ± 0.045	0.51 ± 0.127	FGFR2b and c
21211	0.05 ± 0.002	0.01 ± 0.003	1.00 ± 0.000	0.03 ± 0.010	
21460	0.04 ± 0.027	0.01 ± 0.005	0.10 ± 0.040	0.03 ± 0.004	
21450	0.16 ± 0.018	0.03 ± 0.011	0.40 ± 0.030	0.46 ± 0.088	FGFR2b and c

FGF2 as an autocrine growth factor in H226 and H1703 cells, endogenous FGF2 mRNA was silenced by stable expression of shRNA constructs (see *Materials and Methods*). Compared with a control shRNA targeting GFP, the FGF2-specific shRNAs (3329 and 3332) mediated a significant reduction in FGF2 mRNA (not shown) and FGF2 secretion (Fig. 6A). In addition, silencing of FGF2 in H226 and H1703 cells decreased basal phospho-ERK levels relative to cells transduced with the GFP control (Fig. 6B), indicating decreased proximal activity of FGFRs. It is noteworthy that the decrease in pERK levels in H1703 cells expressing the FGF2 shRNA was not as extensive as observed in H226 cells, possibly resulting from the ongoing activity of the activated PDGFRα present in H1703 cells (Rikova et al., 2007). Finally, anchorage-independent growth of both H226 and H1703 cells transduced with the FGF2 shRNAs were inhibited compared with transfectants expressing the control GFP shRNA (Fig. 6C). In H226 cells, the reduction in soft agar growth by the two distinct shRNAs was consistent with the degree of reduction in FGF2 expression. These data provide molecular evidence for the role of FGF2 as an autocrine factor in H226 and H1703 cells.

A TKI Selective for FGFR, VEGFRs, and PDGFRs Inhibits ERK Activation and Transformed Growth of FGF and FGFR-Expressing NSCLC Cell Lines. RO4383596 is a small-molecule inhibitor of the related family of FGFRs, VEGFRs, and PDGFRs (McDermott et al., 2005). Published studies demonstrated that RO4383596 directly inhibits VEGFR, PDGFR, and FGFRs with an IC_{50} ~30 to 40 nM, blocks autophosphorylation of VEGFR stimulated by exogenous VEGF, and inhibits growth factor-stimulation of effector signal pathways including ERK1/2 and Akt (McDermott et al., 2005). It is noteworthy that RO4383596 inhibited both VEGF and FGF2-stimulated proliferation of human umbilical cord endothelial cells (McDermott et al., 2005). The Affymetrix data set (Coldren et al., 2006) reveals little or no expression of PDGFRα and PDGFRβ mRNA in NSCLC cell lines, except for H1703 cells, which express PDGFRα (not shown). In addition, mRNA levels for the VEGFRs FLT1 and FLK1 were less than 2 and 0.4%, respectively, of the levels measured in human microvascular endothelial cells as assessed by quantitative RT-PCR (data not shown). Thus, RO4383596 was employed as a TKI to test the requirement of the putative FGF-FGFR autocrine pathway

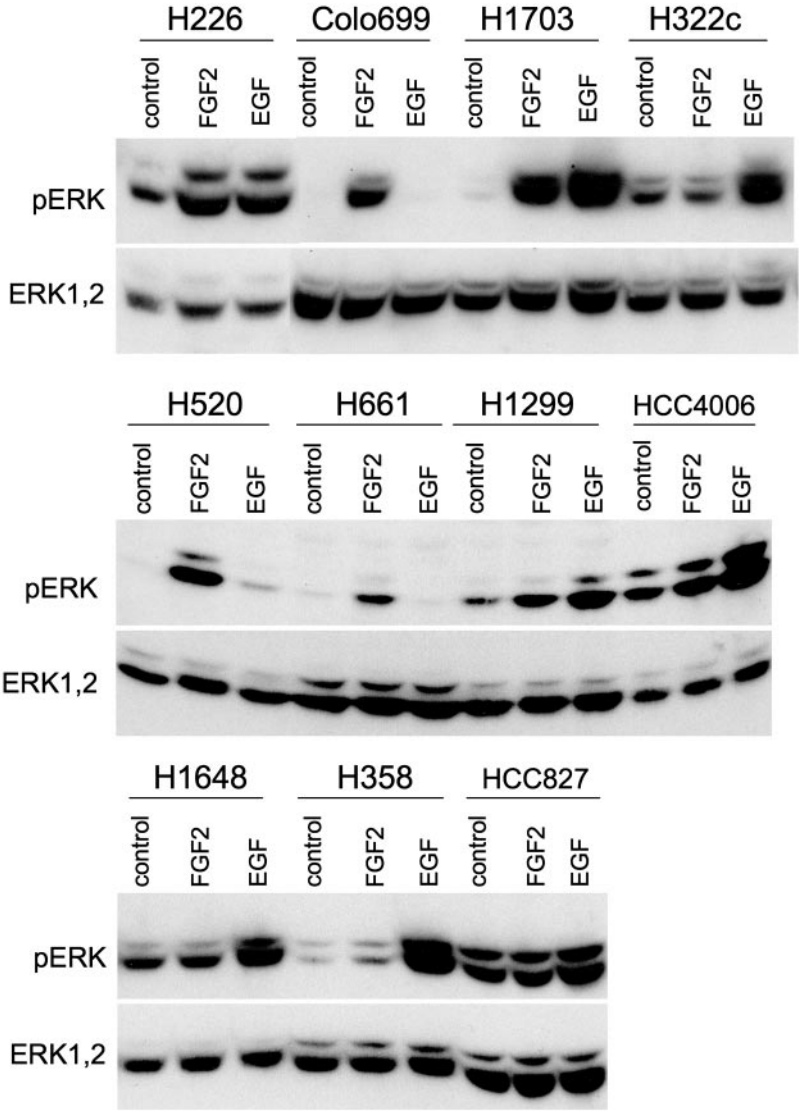


Fig. 5. ERK pathway activation by exogenous FGF2 or EGF. The indicated NSCLC cell lines were incubated for 2 h in serum-free HITES medium and then for another 15 min with or without 10 ng/ml EGF or 10 ng/ml FGF2. Cell extracts were prepared, resolved by SDS-PAGE, and immunoblotted for phospho-ERK1/ERK2 as a measure of EGFR and FGFR activation. The filters were stripped and re-probed with total ERK antibodies to verify equal sample loading.

for transformed growth of NSCLC cells with the caveat that H1703 cells express an additional target, PDGFRα, for this drug.

The ERK mitogen-activated protein kinases represent a conserved signal pathway activated distal to diverse growth factor receptors as well as oncogenes such as Ras and Src. By contrast, the FGF receptor substrate 2 (FRS2) docking protein serves as a proximal component of FGFR and Trk-mediated signal pathways (Eswarakumar et al., 2005). Treatment of H226 cells with FGF2 increased both ERK and FRS2-Y196 phosphorylation (Fig. 7), and both activities were inhibited in a dose-dependent manner by RO4383596. EGF treatment elicited a small effect on FRS2 phosphorylation and strongly increased ERK phosphorylation. Neither response was inhibited by RO4383596, indicating that neither the EGFR nor conserved signaling components that participate in growth factor-stimulated ERK phosphorylation are targets for RO4383596.

When the basal phosphorylation states of ERK1 and ERK2 are used as a measure of growth factor and oncogene signaling in NSCLC cell lines cultured in serum-free HITES medium without exogenous growth factors, RO4383596 elicited a dose-dependent inhibition of phospho-ERK in H226, H520, H661, H1299, H1703, and Colo699 cells (Fig. 8, A and B). By contrast, little or no influence of RO4383596 on pERK levels in H322c, H358, H1648, HCC827, and HCC4006 cells was

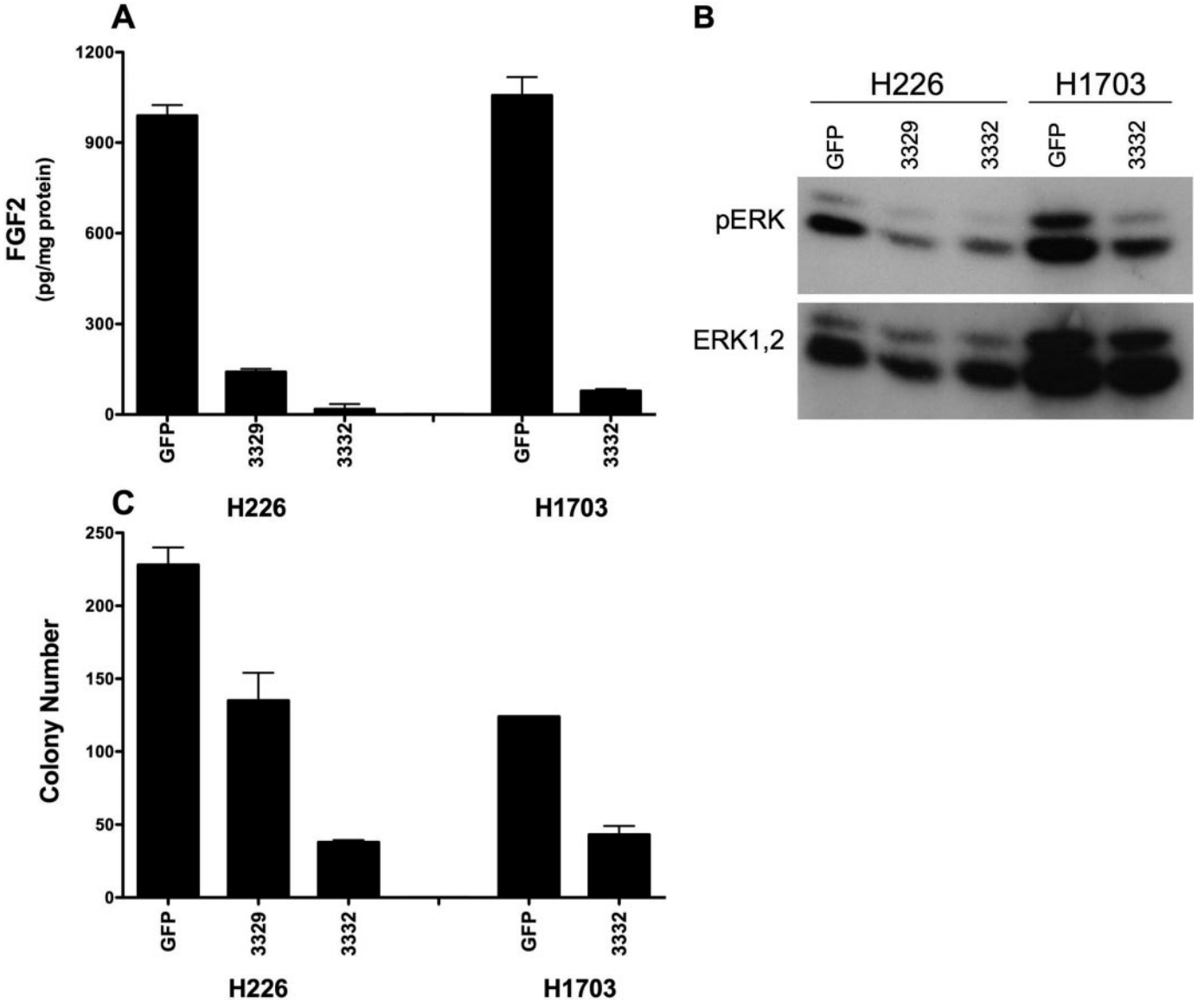


Fig. 6. FGF2 silencing inhibits ERK activity and anchorage-independent growth of H226 and H1703 cells. A, the FGF2 shRNA-encoding lentiviral vectors (TRCN0000003329 or TRCN0000003332) or the pLKO.1 vector encoding a shRNA targeting GFP as a control were packaged into lentiviruses, transduced into H226 or H1703 cells, and stable transfectants were selected with puromycin. Pooled puromycin-resistant cultures were seeded in 12-well plates and aliquots of media conditioned by the cells for three days were assayed for FGF2 by ELISA. The data are presented as picograms of FGF2 per milligram of cellular protein (mean ± S.E.M.), where GFP indicates cells transduced with the control GFP shRNA and 3329 and 3332 denote cultures transduced with the TRCN0000003329 or TRCN0000003332 constructs, respectively. B, basal ERK phosphorylation in the H226 and H1703 transfectants was measured by immunoblot analysis as described under *Materials and Methods*. The pERK levels relative to total ERK levels in H226–3329, H226–3332, and H1703–3332 were 24, 9 and 16%, respectively, of the levels observed in the appropriate H226-GFP and H1703-GFP controls. C, the H226 and H1703 transfectants expressing the GFP or FGF2 shRNAs were assayed for anchorage-independent growth as described under *Materials and Methods*.

observed (Fig. 8, A and B). Similar treatment of the different NSCLC cell lines with gefitinib revealed no inhibition of pERK levels in H226, H520, H661, H1299, H1703, or Colo699 cells, but strong inhibition in H322c, H358, H1648, HCC827, and HCC4006 cells (Fig. 8, A and B), confirming their previously described sensitivity to EGFR-specific TKIs (Coldren et al., 2006). As a proximal measure of FGFR signaling status in the NSCLC cell lines, the phosphorylation status of FRS2 was monitored in several NSCLC cell lines treated with or without RO4383596. As shown in Fig. 8C, the intensity and mobility of pFRS2 was markedly altered upon RO4383596-treatment in H226, H520, and Colo699 cells, but not in H322c, H358, and H1648, indicating that basal FRS2 phosphorylation is regulated by FGFRs in the former cell lines. Combined, these findings indicate that an RO4383596-sensitive FGFR-mediated autocrine signaling pathway contributes to the phosphorylation and activity of FRS2 and ERK in H226, H520, H661, H1299, H1703, and Colo699 cells, whereas, as predicted, an EGFR-dependent pathway is dominant for maintaining the phosphorylation of ERK1 and ERK2 in H322c, H358, H1648, HCC827, and HCC4006 cells.

Analysis of the influence of RO4383596 on proliferation and anchorage-independent growth of the NSCLC cell lines revealed dose-dependent inhibition of proliferation (Fig. 9A) and/or anchorage-independent growth (Fig. 9B) of H226, H520, H661, H1299, H1703, and Colo699 cells by RO4383596. By contrast, proliferation or anchorage-independent growth of gefitinib-sensitive H322c, H358, H1648, HCC827, and HCC4006 cells was not significantly influenced by RO4383596. Thus, RO4383596 inhibits ERK pathway activation and proliferation/anchorage-independent growth in a set of gefitinib-insensitive NSCLC cell lines that coexpress FGF2 or FGF9 and FGFR1 IIIc or FGFR2 IIIc and supports the involvement of FGF and FGFR autocrine signaling as a dominant pathway mediating transformed growth in a subset of NSCLC cells. It is not possible to assign an exclusive autocrine role for FGF2 and FGFR1 in H1703 cells from their sensitivity to RO4383596. As previously mentioned, these cells also express activated PDGFRα (Rikova et al., 2007) and RO4383596 is an effective inhibitor of this receptor tyrosine kinase as well (McDermott et al., 2005). Based on the ability of FGF2 silencing to reduce ERK activity and anchorage-independent growth, it is likely that both FGFR1 and PDGFRα contribute to transformed growth in H1703 cells.

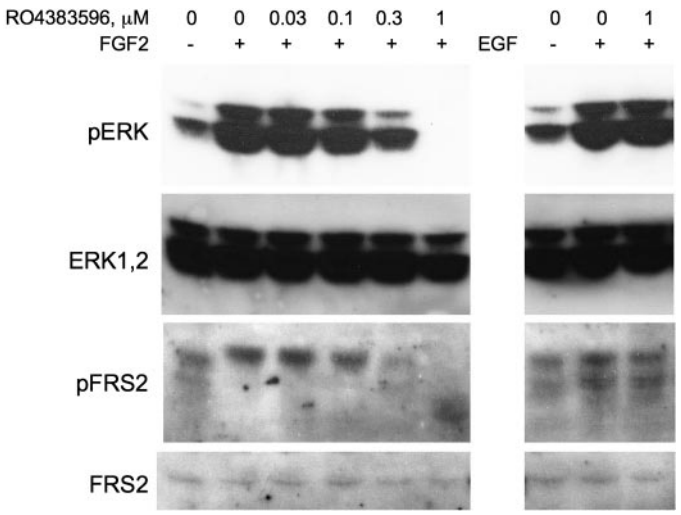


Fig. 7. RO4383596 inhibits FGF2-stimulated, but not EGF-stimulated, ERK and FRS2 phosphorylation in H226 NSCLC cells. H226 cells in 35-mm wells were incubated for 1 h in HITES medium in the presence of the indicated concentrations of RO4383596; DMSO was added as a diluent control. Subsequently, FGF2 or EGF was added at 10 ng/ml for another 15 min, and cell extracts were prepared and immunoblotted for phospho-ERK and phospho-FRS2-Y196 as described under *Materials and Methods*. The filters were stripped and reprobed with antibodies to total ERK or FRS2 to verify equal protein loading. Note that the mobility of pFRS2 is decreased after FGF2 treatment, consistent with increased phosphorylation of the protein on additional sites. In addition, the mobility of pFRS2 detected in cells treated with FGF2 and 1 μM RO4383596 was greatly increased, suggesting that FRS2 is dephosphorylated after TKI treatment.

Discussion

Previously published studies have demonstrated expression of FGF2 and FGFRs in human lung cancers and in NSCLC cell lines (Berger et al., 1999; Chandler et al., 1999; Kuhn et al., 2004). In addition, rare somatic mutations in FGFR1 that may confer gain of function have been identified (Zhao et al., 2005) and amplification of the FGFR1 gene has been detected in human NSCLC, albeit at a very low frequency (Davies et al., 2005). Our results support these previous studies and also provide molecular evidence for an active FGF autocrine signaling pathway in a subset of NSCLC cell lines. Our demonstration of an active FGF-FGFR autocrine loop in NSCLC cell lines also provides a mechanism for the observed insensitivity of some NSCLC tumors and cell lines to EGFR-specific TKIs. Our data suggest that gefitinib-insensitive NSCLC cell lines employ alternative receptor tyrosine kinases, such as the FGFR, to establish self-sufficiency in growth. Previous studies have shown that gefitinib-sensitive NSCLC tumors and cell lines are enriched for the adenocarcinoma and bronchoalveolar carcinoma histological type (Miller et al., 2004). By contrast, among the NSCLC cell lines in which we demonstrated FGFR-dependent autocrine signaling, H226, H520, and H1703 are derived from squamous cell carcinomas, and H661 and H1299 are derived from large cell carcinomas. Thus, FGFR inhibitors may better target the squamous cell and large cell histologies of NSCLC cell lines that frequently exhibit a high degree of insensitivity to EGFR TKIs.

A recent study (Rikova et al., 2007) employed a phosphotyrosine proteomic approach to survey for tyrosine kinases that are active in lung cancer cell lines and primary tumors. In contrast to our present findings, only three of 41 NSCLC cell lines showed evidence for activated FGFR1, suggesting that the FGF/FGFR pathway is not a major receptor pathway active in NSCLC cell lines. It is noteworthy that the NSCLC cell line panel employed in the aforementioned study was composed of ~75% adenocarcinomas, whereas we find that squamous cell and large cell carcinomas are enriched in the FGF and FGFR-expressing NSCLC cell lines. In addition, each experimental approach (proteomics, genomics, etc.) will have unique biases, and tyrosine-phosphorylated peptides derived from FGFR1 or FGFR2 may not be efficiently detected by the experimental approaches employed. Alternatively, the selected phosphopeptides from FGFR1 or FGFR2 may not serve as sensitive indicators of FGFR activity in cell lines or tumors.

A conclusion from our experiments is that FGFR1 IIIc and/or FGFR2 IIIc and their respective ligands, FGF2 and FGF9, undergo coselection as components of an autocrine

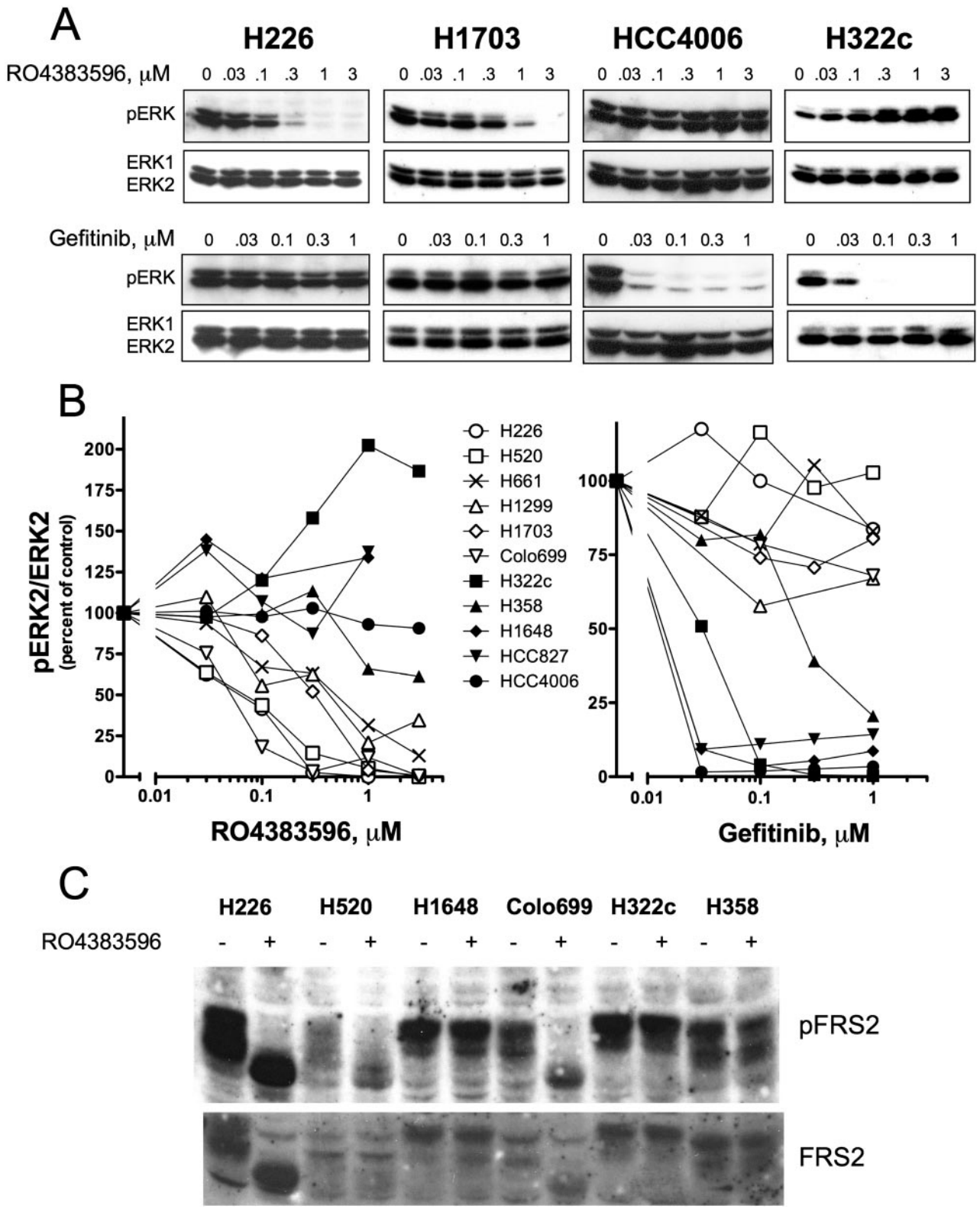


Fig. 8. RO4383596 inhibits basal ERK and FRS2 phosphorylation in NSCLC cell lines coexpressing FGFs and FGFRs. A, NSCLC cell lines were seeded in 12-well plates and cultured in full medium to 50 to 90% confluence. The growth medium was replaced with serum-free HITES medium, and after a 2-h incubation, RO4383596 or gefitinib was added at the indicated concentrations, and incubation was continued for another 2 h. Cell-free extracts were prepared and submitted to immunoblot analysis for pERK as described under *Materials and Methods*. The filters were stripped and reprobed for total ERK1 and ERK2 with rabbit polyclonal antibodies from Santa Cruz Biotechnology, Inc. Representative pERK and ERK1/ERK2 blots are shown for H226, H1703, H322c, and HCC4006 cells. B, immunoblot data from the entire panel of NSCLC cell lines was submitted to densitometry analysis and is presented as the ratio of pERK2 to total ERK2. C, the indicated NSCLC cell lines were incubated in HITES medium for 16 h and then for an additional 2 h with or without 1 μM RO4383596. Extracts were prepared and immunoblotted for pFRS2-Y196 as described under *Materials and Methods*. The filters were subsequently stripped and reprobed for total FRS2.

signaling pathway during initiation and progression of NSCLC. It is noteworthy that no NSCLC cell line expressing FGFR1 IIIc or FGFR2 IIIc was found that did not also express FGF2 or FGF9. However, HCC827 and HCC4006 expressed FGF2 but lacked detectable expression of FGFR1 IIIc or FGFR2 IIIc. As single markers, FGFR1 IIIc or FGFR2 IIIc may be a more reliable indicator of FGF and FGFR-dependent autocrine signaling. FGFR signaling is critical for lung development and tissue homeostasis, although FGFR2 IIIb, FGF7, and FGF10 are the key players in this regard (Eswarakumar et al., 2005). However, our studies did not detect coexpression of FGFR2 IIIb with FGF7 or FGF10 in human NSCLC cell lines. Rather, FGFR1 IIIc and FGFR2 IIIc are coexpressed with their high-affinity ligands FGF2 or FGF9. The literature indicates that FGF2 and FGFR1 seem to be most highly expressed in vascular compartments of the lung (Powell et al., 1998). Thus, ectopic expression of FGF2, FGF9, FGFR1 IIIc, and FGFR2 IIIc may be induced during lung tumorigenesis. In support of this possibility, enhanced bronchial expression of FGF2 and FGFR1 is observed in chronic obstructive pulmonary disease (Kranenburg et al., 2002, 2005), where the risks for chronic obstructive pulmonary disease and lung cancer aggregate (Schwartz and Ruckdeschel, 2006). An alternative mechanism for the observed coexpression of specific FGFs and FGFRs in NSCLC cell lines is that a specific lung epithelial cell type or progenitor may express FGF2 or FGF9 and FGFR1 IIIc and/or FGFR2 IIIc and serve as a precursor for the NSCLC cells that exhibit an FGFR autocrine signaling loop. In support, a recent study by Ince et al. revealed a higher degree of gene expression similarity between a given nontransformed mammary epithelial cell type and its transformed derivative than between different types of nontransformed mammary epithelial cell precursors (Ince et al., 2007), indicating that tumor cells retain a high degree of gene expression similarity with their nontransformed precursors.

In addition to autocrine signaling through EGFR and FGFR pathways in NSCLC, the literature documents that multiple receptor tyrosine kinases will participate in lung oncogenesis (Rikova et al., 2007) including cMet, which is amplified in some gefitinib-insensitive NSCLC (Engelman et al., 2007; Lutterbach et al., 2007). Inspection of the gene expression array dataset derived from the NSCLC cell lines (Coldren et al., 2006) also predicts the coexpression of the Axl family of receptor tyrosine kinases and their ligands, Gas6 and protein S (Hafizi and Dahlback, 2006), in a significant fraction of NSCLC cell lines. In fact, a potential role for an Axl/Gas6 autocrine loop in NSCLC has been previously invoked (Wimmel et al., 2001; Shieh et al., 2005). Finally, an IGF-1 receptor signaling system has been proposed as a transforming pathway in NSCLC (Morgillo and Lee, 2005). Our own preliminary studies reveal frequent overexpression of the IGF-1 receptor in NSCLC cell lines, although coexpression of the ligands IGF1 or IGF2 is less clear. However, it is important to consider paracrine involvement of growth factor receptors as well where the tumor microenvironment may provide growth factors that stimulate receptor tyrosine kinases expressed on lung cancer cells. Combined, our present study and the literature indicate that the EGFR does not function as a single dominant receptor tyrosine kinase in autocrine growth of NSCLC, but that multiple autocrine loops will participate. Thus, effective blockade of autocrine and paracrine signaling in primary NSCLC tumors will require precise identification of the active receptor tyrosine kinase pathways through appropriate biomarkers. The fact that growth factors such as FGF2 are secreted molecules appearing in extracellular fluids of patients with cancer (Nguyen et al., 1994) suggests that the autocrine factors themselves may serve as informative biomarkers in this regard.

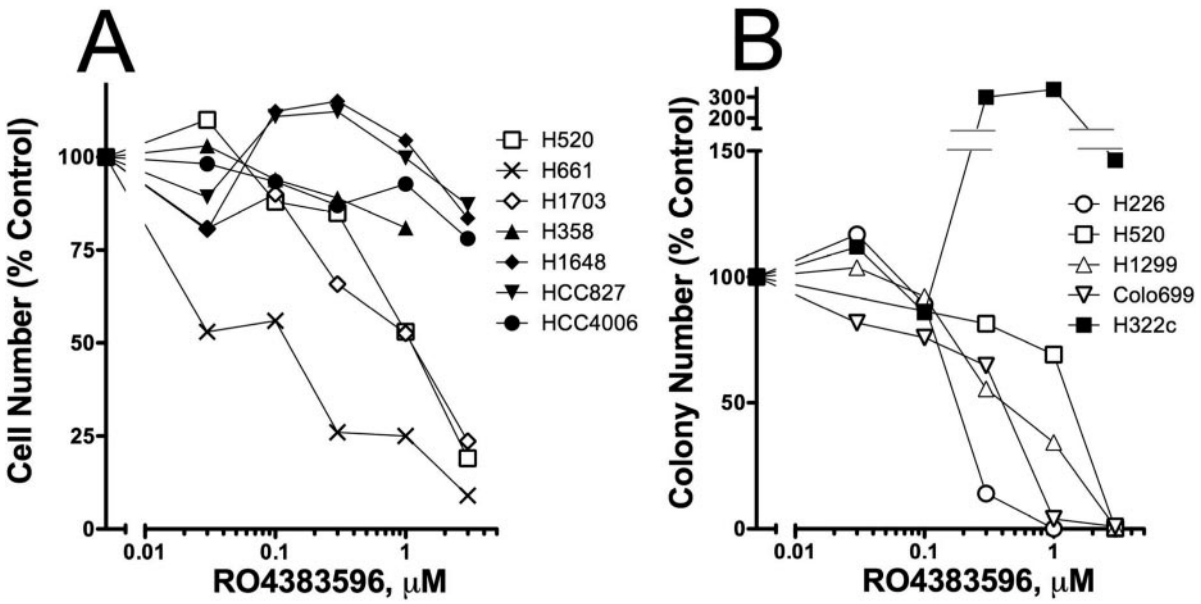


Fig. 9. Selective inhibition of NSCLC proliferation and anchorage-independent growth by RO4383596. A, the indicated NSCLC cell lines were seeded at 2000 cells/well in 96-well plates and subsequently incubated for 5 days with growth medium containing the indicated concentrations of RO4383596. Viable cells were measured with the MTT assay as described under *Materials and Methods*. B, anchorage-independent growth of the indicated NSCLC cell lines in the presence of RO4383596 was measured as described under *Materials and Methods*. The plates were incubated for three weeks, the colonies were stained with nitro blue tetrazolium, and quantified after digital photography and MetaMorph software analysis. The symbols in the figure are consistent with those used in Fig. 8.

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