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Oncogenic KRAS-induced interleukin-8 overexpression promotes cell growth and migration and contributes to aggressive phenotypes of non-small cell lung cancer

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Abstract

The CXC chemokine interleukin-8 (IL-8) is an angiogenic growth factor that is overexpressed in various cancers, including non-small cell lung cancer (NSCLC). Previously, IL-8 was shown as a transcriptional target of RAS signaling, raising the possibility of its role in oncogenic KRAS-driven NSCLC. Using microarray analysis, we identified *IL-8* as the most downregulated gene by shRNA-mediated KRAS knockdown in NCI-H1792 NSCLC cells where IL-8 is overexpressed. NSCLC cell lines harboring *KRAS* or *EGFR* mutations overexpressed IL-8, while IL-8 levels were more prominent in *KRAS* mutants compared to *EGFR* mutants. IL-8 expression was downregulated by shRNA-mediated KRAS knockdown in *KRAS* mutants or by treatment with EGFR tyrosine kinase inhibitors and EGFR siRNAs in *EGFR* mutants. In our analysis of the relationship of IL-8 expression with clinical parameters and mutation status of *KRAS* or *EGFR* in 89 NSCLC surgical specimens, IL-8 expression was shown to be significantly higher in NSCLCs of males, smokers, and elderly patients and those with pleural involvement and *KRAS* mutated adenocarcinomas. In *KRAS* mutant cells, the MEK inhibitor markedly decreased IL-8 expression, while the p38 inhibitor increased IL-8 expression. Attenuation of IL-8 function by siRNAs or a

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neutralizing antibody inhibited cell proliferation and migration of *KRAS* mutant/IL-8 overexpressing NSCLC cells. These results indicate that activating mutations of *KRAS* or *EGFR* upregulate IL-8 expression in NSCLC; IL-8 is highly expressed in NSCLCs from males, smokers, elderly patients, NSCLCs with pleural involvement, and *KRAS*-mutated adenocarcinomas; and IL-8 plays a role in cell growth and migration in oncogenic KRAS-driven NSCLC.

Keywords

non-small cell lung cancer; KRAS; interleukin-8; molecular target

KRAS encodes a small GTP-binding protein that is involved in many cellular processes including proliferation, differentiation, and apoptosis.[1] Wild-type KRAS has intrinsic GTPase activity, which catalyzes the hydrolysis of bound GTP to GDP and thereby inactivates the RAS growth-promoting signal, whereas mutant KRAS is locked into the GTP-bound state, leading to constitutive RAS signaling. Activating *KRAS* mutations play essential roles in malignant transformation in various human cancers including non-small cell lung cancer (NSCLC).[1] *KRAS* mutations are found in ~ 25% of NSCLC but almost never in small cell lung cancer (SCLC)[2,3] and are associated with poor prognosis of NSCLC patients.[4] To improve survival for patients with NSCLC, there is an urgent need to develop therapeutic modalities for NSCLC harboring *KRAS* mutations. Therapeutic approaches targeting oncogenic Ras including farnesyl transferase inhibitors have failed in the treatment of NSCLC[5]; moreover, *KRAS* mutations are associated with resistance to EGFR tyrosine kinase inhibitors (EGFR-TKIs) for NSCLC.[6,7] Thus, no effective treatment strategies have been established for *KRAS* mutant NSCLC.

A functional relationship between inflammation and cancer has been suggested for a long time.[8] The CXC chemokine interleukin-8 (IL-8), which was originally identified as a neutrophil chemoattractant with inflammatory activity,[9] is an important proinflammatory mediator relevant to cancer development.[10] Increasing evidence suggests an important role for IL-8 in tumor progression and metastasis by promoting cell proliferation and angiogenesis in NSCLC.[11–17] Furthermore, previous studies have reported that elevated IL-8 expression is an unfavorable prognostic factor in NSCLC.[16,18,19] In a previous study, IL-8 was shown to be a transcriptional target of RAS signaling,[20] raising the possibility of its role in oncogenic KRAS-driven NSCLC.

In a recent study, we performed a microarray analysis to compare gene expression profiling of mutant KRAS-disrupted NSCLC clones to those of the mutant KRAS expressing clones.[21] Consequently, we identified *IL-8* as the most down-regulated gene (−17.4 fold-change) by mutant KRAS knockdown in NCI-H1792 NSCLC cell line harboring a heterozygous *KRAS* mutation. In this study, we confirmed that prior to KRAS knockdown, H1792 cells overexpressed IL-8 at both the mRNA and the protein levels and that short hairpin RNA (shRNA)-mediated KRAS knockdown downregulated IL-8 expression. These results led us to examine IL-8 expression in a panel of lung cancer cell lines and clinically annotated surgical resection specimens and to analyze the relationship of IL-8 expression with clinicopathological parameters and *KRAS* mutation status. We also assessed whether attenuation of IL-8 function inhibited *in vitro* cell growth and migration of *KRAS* mutant/IL-8 overexpressing NSCLC cells. Here, we describe the positive association between IL-8 expression, *KRAS* mutations and certain clinicopathological features and therapeutic significance of IL-8 expression in *KRAS* mutated NSCLC.

Material and Methods

Cell lines and culture conditions

Twenty-two small cell lung cancer (SCLC) cell lines (NCI-H187, -H209, -H345, -H378, -H524, -H526, -H740, -H865, -H889, -H1045, -H1092, -H1184, -H1238, -H1339, -H1607, -H1618, -H1672, -H1963, -H2141, -H2171, -H2227, and HCC33), 10 NSCLC cell lines harboring *KRAS* mutations (NCI-H23, -H157, -H358, -H441, -H460, -H1264, -H1792, -H2009, -H2122, and HCC4017), 10 NSCLC cell lines harboring *EGFR* mutations (NCI-H820, -H1650, -H3255, -H1975, HCC827, HCC2279, HCC2935, HCC4006, HCCC4011, and PC9), 10 NSCLC cell lines with wild-type *KRAS*/*EGFR* (NCI-H322, -H520, -H661, -H838, -H1299, -H1395, -H1437, -H2077, -H2126, and HCC95), and immortalized human bronchial epithelial cell lines (HBEC3 and HBEC4, established as described[22]), were obtained from the Hamon Center collection (University of Texas Southwestern Medical Center). BEAS-2B (ATCC), HBEC3, and HBEC4 cell lines were used as noncancerous controls. Cancer cells were cultured with RPMI 1640 medium supplemented with 5% fetal bovine serum. The immortalized human bronchial epithelial cell lines were cultured with Keratinocyte-SFM (Invitrogen, Carlsbad, CA) medium with 50 μg/ml bovine pituitary extract (Invitrogen) and 5 ng/ml EGF (Invitrogen). All of the cell lines have been DNA fingerprinted for provenance using the PowerPlex 1.2 kit (Promega, Madison, WI) and confirmed to be the same as the DNA fingerprint library maintained either by ATCC or by the Minna/Gazdar lab (which is the primary source of the lines). The lines were also tested to be free of mycoplasma by e-Myco kit (Boca Scientific, Boca Raton, FL).

Tumor Specimens of NSCLC Patients

Tumor specimens were obtained from 89 patients (45 men and 44 women) with primary NSCLC cancer who underwent surgery between July 2003 and May 2008 at the Gunma University School of Medicine Hospital (Gunma, Japan). Of 89 patients, 48 were smokers and 41 were never smokers. Tumors were histologically classified as adenocarcinomas (N = 77) or squamous cell carcinomas (N = 12), according to the criteria of the World Health Organization. We classified the postsurgical pathological stage as stage I in 57 tumors (IA, 38; IB, 19), stage II in 11 tumors (IIA, 6; IIB, 5), and stage III/IV in 21 tumors (IIIA, 16; IIIB, 4; IV, 1), according to the tumor-node-metastasis classification. Noncancerous lung specimens (N = 5) obtained from five patients were used as normal controls for tumor specimens. Mutations in *KRAS* at codon 12 or *EGFR* in exons 19 and 21 were analyzed using the Smart Amplification Process version 2 assay (DNAFORM, Kanagawa, Japan) followed by direct sequencing to confirm the presence of these mutations.[23] The study protocol was approved by the institutional review board of Gunma University Graduate School of Medicine. The specimens were immediately frozen after collection and stored at −80°C until mRNA extraction was performed.

Use of retroviral shRNA vectors and synthetic small interfering RNA (siRNA)

To achieve mutant-specific KRAS knockdown, the pRS-KRAS-C12 retroviral vector producing shRNA against the *KRAS* G12C mutation (GGT to TGT) was used.[21] Cells were infected with retroviral vectors by previously described methods.[24] siRNAs targeting against IL-8 or EGFR were obtained from the siGENOME library (Dharmacon, Lafayette, CO). A siRNA against Tax (the human leukemia virus gene) was used as a nontargeting control.[25] One day before transfection, 1×10^5 cells were plated on each well of six well plates. Cells were transfected with 30 nM siRNAs using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's protocol. After 72 hr, cells were harvested to verify the silencing effects on the expression of the target genes.

Quantitative real-time RT-PCR

mRNA expressions of *IL-8*, *CXCR1*, *CXCR2,* and *EGFR* were analyzed by quantitative real-time RT-PCR.[26] Briefly, total RNA was extracted using the RNeasy mini kit (Qiagen), and cDNA was synthesized using 2 μg of total RNA with the SuperScript II First-Strand Synthesis using the oligo (dT) primer system (Invitrogen) according to the manufacturer's protocol. Primers and probes for *IL-8*, *CXCR1*, *CXCR2*, and *EGFR* were purchased from Applied Biosystems (assay ID: Hs00174103_m1, Hs00174146_m1, Hs00174304_m1, and Hs01076078_m1, respectively). For the quantitative analysis, the *TBP* gene was used as an internal reference gene to normalize input cDNA. We then standardized the expression levels of the *IL-8*, *CXCR1*, *CXCR2,* and *EGFR* genes by dividing the value for a tumor cell line by the mean of values obtained in noncancerous bronchial epithelial cell lines ($N = 3$) for the analysis of cell lines (the mean of the three non-cancerous lines = 1) or by dividing the value for a tumor specimen by the mean of values obtained in normal lung tissues ($N = 5$) for the analysis of tumor specimens (the mean of the five normal lung tissues = 1). PCR was performed using the Gene Amp 7700 Sequence Detection System and software (Applied Biosystems). The comparative Ct method was used to compute relative expression values.

Western blot analysis

Western blotting was performed using whole cell lysates, separated on SDS/polyacrylamide gel, and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Keene, NH).[25] The membranes were incubated with mouse monoclonal anti-KRAS (Santa Cruz, Santa Cruz, CA), mouse monoclonal anti-α-tubulin (Calbiochem, San Diego, CA), rabbit polyclonal anti-extracellular signal-regulated kinase (ERK) 1/2 (Cell Signaling), and rabbit polyclonal anti-phospho-ERK1/2 antibodies (Cell Signaling). The membranes were developed with peroxidase-labeled anti-mouse or anti-rabbit IgG (Amersham Pharmacia, Piscataway, NJ) by Super Signal chemiluminescence substrate (Pierce, Rockford, IL).

Enzyme-linked immunosorbent assay

The concentration of IL-8 in the cultured medium was determined by enzyme-linked immunosorbent assay (ELISA) using the human IL-8 duo set kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

MTT and IL-8 neutralization assays

Cell viability was measured using the 3-(4,5 dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) Cell Growth Assay Kit (Chemicon International, Temecula, CA).[27] Briefly, 48 hr after siRNA transfection, trypan blue-negative viable cells (2,000 cells for H157, H460 and H1792; 4,000 cells for H2122) were re-plated and cultured in 96-well plates in replicates of eight. After 72 hr, the MTT assay was performed as previously described.[27] For the IL-8 neutralizing assay, cells were treated with the anti-human IL-8 antibody (R&D Systems) or the IgG1 isotype control antibody (R&D Systems), and the MTT assay was performed 72 hr later.

Colony formation assay

In-vitro growth characteristics were tested by a colony formation assay.[25] Briefly, after 48 hr of siRNA transfection, cells were harvested, and 500 trypan blue-negative viable cells were replated in each well of six-well plates. The cells were cultured in RPMI 1640 supplemented with 5% serum, and surviving colonies were counted 10 days later after staining with methylene blue.

Migration assay

Cell migration was measured in a modified Boyden chamber.[27] Briefly, polycarbonate filters with 8 μm pores were coated with 100 μg/ml of collagen in 0.5 M acetic acid for 16 hr. The coated filter was then placed on a 12-blind-well chemotaxis chamber, and 10^5 cells in 100 μl per well were loaded into the upper wells. The cells were incubated for 15 min before loading. After incubation at 37°C in 5% CO_2 for 4 hr, the filter was disassembled. The upper side of the filter was then scraped free of cells. The cells on the lower side of the filter were fixed with methanol and stained with a Diff-Quick staining kit (International Reagent, Kobe, Japan). The number of cells that migrated to the lower side of the filter was counted. The number of migrated cells in each experiment was adjusted by the cell viability assay (trypan-blue assay) to correct for anti-proliferation effects of the IL-8 neutralizing antibody (corrected migrated cell number = counted migrated cell number/percentage of viable cells).[28]

Statistical analysis

Statistical analyses were performed using version 5.0 of GraphPad Prism for Windows (GraphPad Software, San Diego, CA). $p < 0.05$ was considered significant, and $p \geq 0.05$ but $p < 0.1$ was considered borderline significant.

Results

IL-8 overexpression is induced by activating mutations of KRAS or EGFR in NSCLC cells

To assess the association between IL-8 expression and *KRAS* mutations in lung cancer, we first examined *IL-8* mRNA expression in a panel of lung cancer cell lines by quantitative RT-PCR analysis. *IL-8* mRNA levels were significantly higher in NSCLCs harboring *KRAS* mutation or *EGFR* mutations than those with wild-type *KRAS*/*EGFR* (Fig. 1*a*). In contrast, IL-8 expression was low or undetectable in SCLCs, in agreement with a previous study.[29] We next examined IL-8 protein levels in cultured NSCLC cell lines. IL-8 protein levels were significantly higher in *KRAS* mutants or *EGFR* mutants than those with wild-type *KRAS*/*EGFR* (Fig. 1*b*). Quantitatively, differences in IL-8 levels were more prominent in *KRAS* mutants than *EGFR* mutants compared to NSCLCs with wild-type *KRAS*/*EGFR*. Notably, we were able to confirm the IL-8 overexpression in NCI-H1792 cells with a baseline protein level of 5,662 pg/ml, and microarray analysis revealed transcriptional downregulation of IL-8 expression (fold-change was −17.4) by shRNA-mediated KRAS knockdown.[21] We also examined the expression of IL-8 receptors, CXCR1 and CXCR2, to determine whether these receptors are also differentially expressed in NSCLC cell lines according to the mutation status of *EGFR* and *KRAS*. There were no significant differences in the *CXCR1* and *CXCR2* mRNA expression levels between NSCLCs with or without these mutations (Figs. 1*c* and 1*d*).

NCI-H2122 NSCLC cells exhibited the highest level of IL-8 protein among all lines (19,477 pg/ml) and had the same type of *KRAS* mutation at codon 12 (G12C) as H1792 cells. Therefore, we established KRAS-disrupted H2122 clones and KRAS-disrupted H1792 clones using the pRS-KRAS-C12 vector (Fig. 2*a*) and assessed whether shRNA mediated KRAS knockdown downregulates IL-8 expression in H1792 and H2122 cell lines. In both lines, mutant KRAS knockdown resulted in significant reduction of IL-8 expression at both mRNA and protein levels (Figs. 2*b* and 2*c*).

We also assessed whether attenuation of oncogenic EGFR activity by siRNA-mediated EGFR knockdown or by treatment with EGFR-TKIs (gefitinib and erlotinib) downregulates IL-8 expression in HCC827 cells, which exhibit the highest basal IL-8 protein levels (7,791 pg/ml) among all *EGFR* mutant NSCLCs. The treatments with EGFR-TKIs or EGFR siR-

NAs resulted in significant reduction of IL-8 expression at both mRNA and protein levels (Figs. 2*d*–2*h*), suggesting that activating *EGFR* mutations transcriptionally upregulate IL-8 expression.

Association of IL-8 expression with clinicopathological parameters and mutation status of KRAS or EGFR in NSCLC

To evaluate the association between IL-8 expression and clinicopathological parameters of NSCLC, *IL-8* mRNA expression was examined in surgical specimens of 89 NSCLC patients by quantitative RT-PCR. IL-8 expression was significantly higher in the specimens from males, smokers and elderly patients (Fig. 3*a*), although IL-8 expression was elevated in subsets of patients from each clinicopathological group. In contrast, there were no significant differences in IL-8 expression levels according to histological subtypes and pathological stages (data not shown). IL-8 expression was significantly higher in NSCLC specimens with pleural involvement and was higher in those with lymphatic permeation or vascular involvement, both with borderline significance (Fig. 3*b*). We also evaluated the association between IL-8 expression and mutation status of *KRAS* or *EGFR* in a subgroup of lung adenocarcinomas to avoid histological bias since mutations of *KRAS* or *EGFR* are observed in adenocarcinomas but rarely in squamous cell lung carcinomas.[30,31] In fact, all *KRAS* and *EGFR* mutations were found in adenocarcinomas but not in squamous cell carcinomas in our study. IL-8 expression was significantly higher in lung adenocarcinomas with *KRAS* mutations than those with wild-type *KRAS* (Fig. 3*c*), whereas we did not observe higher IL-8 expression in adenocarcinomas with *EGFR* mutations than those with wild-type *EGFR* (data not shown). Furthermore, we divided the adenocarcinoma population into four groups according to *KRAS* mutation status and smoking history and then compared the IL-8 expression levels to avoid smoking-related bias since the possible association between *KRAS* mutations and smoking status in lung adenocarcinoma has been indicated.[32] In the adenocarcinoma population, there was a significant difference in the IL-8 expression levels among these four groups (Fig. 3*d*). Thus, IL-8 appears to be preferentially overexpressed in lung adenocarcinomas with *KRAS* mutations compared to those with *EGFR* mutations. In addition, several lung adenocarcinomas with wild-type *KRAS/EGFR* had elevated levels of IL-8 expression, possibly through other mechanisms.

To evaluate the prognostic significance of IL-8 expression and *KRAS* mutation status in NSCLC, we divided patients with NSCLC into four groups according to the IL-8 expression level and *KRAS* mutation status and compared disease-free survival among these groups. There was a tendency of poor survival in patients with NSCLC with IL-8 high/*KRAS* mutant versus IL-8 low/*KRAS* mutant (Fig. 3*e*), although the patient numbers are too small to draw any firm conclusions. Overall, IL-8 overexpression appears to be associated with unfavorable outcome for patients with NSCLC harboring *KRAS* mutations.

Effects of the MEK and p38 inhibitors on IL-8 expression in NSCLC cells with mutations of KRAS or EGFR

A previous study demonstrated that IL-8 is transcriptionally upregulated through the ERK-MAPK pathway activation.[20] In the current study, we found that KRAS knockdown was accompanied with ERK dephosphorylation in H1792 and H2122 cells (Fig. 4*a*), suggesting that oncogenic KRAS induces ERK activation in *KRAS* mutant NSCLC cells. To further assess whether IL-8 upregulation is involved in ERK-MAPK pathway activation, we examined the effect of the MEK inhibitor U0126 on IL-8 expression in *KRAS* mutant NSCLC cell lines H1792, H157, H460, and H2122, in which IL-8 was most highly expressed at basal levels of 5,662, 9,772, 12,663, and 19,477 pg/ml, respectively. In all cases, U0126 markedly downregulated IL-8 expression at both mRNA and protein levels (Figs. 4*b* and 4*c*). These lines were also treated with the p38 inhibitor SB202190 because

p38 was shown as an upstream mediator of IL-8 transcription.[33–35] Unexpectedly, the p38 inhibitor upregulated IL-8 expression in three NSCLC lines (H460, H157, and H2122) while having no effect on H1792 cells (Figs. 4*b* and 4*c*). Furthermore, in studies of H2122 cells with shRNA-mediated KRAS knockdown, we found that U0126 still was able to downregulate IL-8 mRNA and protein expression, while KRAS knockdown blocked SB202190-mediated IL-8 upregulation (Figs. 4*d* and 4*e*). We also examined the effect of U0126 and SB202190 on IL-8 expression in the EGFR mutant HCC827 cells. Unlike the case of KRAS mutant NSCLC cells, both inhibitors significantly reduced IL-8 expression at mRNA and protein levels in HCC827 cells (Figs. 4*f* and 4*g*).

IL-8 siRNAs or an IL-8 neutralizing antibody abolishes in vitro cell growth and migration of KRAS mutant NSCLC cells

To assess whether attenuation of IL-8 function leads to growth inhibition of *KRAS* mutant NSCLC cells, we examined the effect of siRNA mediated IL-8 knockdown on *in vitro* cell growth of H1792 cells. IL-8 siRNAs silenced IL-8 expression compared to the untreated H1792 cells (Fig. 5*a*). Under this condition, IL-8 siRNAs (but not mock siRNA) significantly inhibited cell proliferation (Fig. 5*b*) and colony formation (Fig. 5*c*) in H1792 cells, suggesting that siRNA-mediated IL-8 knockdown abolishes *in vitro* cell growth of *KRAS* mutant NSCLC cells.

To confirm that the growth-inhibitory effect of IL-8 attenuation is not specific for H1792 but is commonly observed in other *KRAS* mutant/IL-8 overexpressing NSCLC cells, we examined the effect of an IL-8 neutralizing antibody on *in vitro* cell growth in four NSCLC lines with *KRAS* mutations and IL-8 overexpression (H1792, H157, H460, and H2122). In all cases, IL-8 neutralization resulted in a modest but significant inhibition of cell growth (Fig. 5*d*). In addition, we confirmed that the growth-inhibitory effects of IL-8 neutralization were similar to that of U0126 (Fig. 5*d*), which markedly downregulated IL-8 expression in these lines (Figs. 4*b* and 4*c*). We also assessed the effect of IL-8 neutralization on cell migration of H460 and H157 cell lines, which were assessable by the migration assay. IL-8 neutralization resulted in a significant reduction of migrated cells compared to those with the control antibody (Fig. 5*e*). Overall, these results suggest that attenuation of IL-8 function abolishes *in vitro* cell growth and migration of *KRAS* mutant NSCLC cells.

Discussion

KRAS and *EGFR* mutations are currently recognized as important molecular abnormalities in NSCLC because of their clinical implications in EGFR-TKI therapy. *EGFR* mutations are associated with a favorable response to EGFR-TKIs,[36–39] while *KRAS* mutations are associated with resistance to EGFR-TKIs.[6,40] Although several studies have reported IL-8 overexpression in NSCLC,[12,16,17,19,29] the association between IL-8 expression and mutations of *KRAS* or *EGFR* remains unknown. In this study, using a panel of lung cancer cell lines and NSCLC specimens, we found that IL-8 was abundantly expressed in NSCLCs with *KRAS* mutations and a subset of NSCLCs with *EGFR* mutations. IL-8 overexpression was downregulated by shRNA-mediated KRAS knockdown in *KRAS* mutant NSCLC cells, consistent with the previous findings that ectopic expression of mutant KRAS resulted in IL-8 upregulation in human cells.[20,41] Our findings demonstrate that activating *KRAS* mutations induce IL-8 overexpression in NSCLC, highlighting important roles of IL-8 in the development of NSCLC with *KRAS* mutations. Furthermore, we found for the first time that a subset of *EGFR* mutant NSCLC cell lines and tumor specimens (which all occurred in never smokers) showed elevated IL-8 expression and EGFR-TKIs or EGFR siRNAs downregulated IL-8 expression in *EGFR* mutant cells, suggesting that activating *EGFR* mutation also induces IL-8 overexpression in NSCLC. In contrast, we could not observe significantly higher IL-8 expression in *EGFR* mutant tumor specimens compared to those

with wild-type *EGFR*. Thus, activating *EGFR* mutations are unlikely to be common mechanisms of IL-8 overexpression in NSCLC.

IL-8 expression analysis in NSCLC tumor specimens revealed that some NSCLCs with wild-type *KRAS*/*EGFR* also overexpressed IL-8, suggesting that there are other mechanisms of IL-8 upregulation. Previous studies have reported that cigarette smoking leads to IL-8 upregulation by inducing IL-8 release from bronchial epithelial cells[42–44] and that IL-8 is differentially expressed in bronchial epithelial cells in smokers having lung cancer compared to smokers without lung cancer.[45] In our study, IL-8 expression was significantly higher in NSCLC tumors from smokers than in those from nonsmokers. Of note, in a subgroup of NSCLCs with wild-type *KRAS*/*EGFR*, IL-8 expression was significantly higher in the tumors derived from smokers ($p = 0.0143$), whereas in a subgroup of *KRAS* mutant NSCLCs, no significant difference was observed between smokers and nonsmokers. Therefore, it is possible that IL-8 plays a role in tobacco-related carcinogenesis of NSCLC, which may partially explain the mechanisms of KRAS/EGFR independent IL-8 overexpression.

The ERK-MAPK pathway plays a central role in oncogenic KRAS-driven malignant phenotypes of NSCLC.[2,21] We found that shRNA-mediated KRAS knockdown was accompanied by ERK dephosphorylation and that the MEK inhibitor completely blocked IL-8 expression in *KRAS* mutant NSCLC cells. These results are in agreement with a previous study showing that IL-8 is transcriptionally upregulated through the ERK-MAPK pathway activation.[20] Our findings that MEK inhibitor-mediated IL-8 downregulation was unaffected by KRAS knockdown suggest that oncogenic KRAS-induced IL-8 overexpression is highly responsible for ERK-MAPK pathway activation in NSCLC. Interestingly, treatment with the p38 inhibitor resulted in IL-8 upregulation, which was inhibited by KRAS knockdown. This finding suggests that the negative feedback from p38 to ERK is present in NSCLC as observed in other types of cancers[46] and that oncogenic KRAS upregulates this negative feedback.

We found that both of the U0126 and p38 inhibitors downregulated IL-8 expression in EGFR mutant HCC827 cells. A recent study indicated that EGFR related IL-8 production from NSCLC cells is stimulated through the ERK activation.[47] Since HCC827 cells highly express phosphorylated-ERK and EGFR-TKIs are able to inhibit the ERK phosphorylation in this cell line,[48,49] it is likely that oncogenic *EGFR* mutations upregulate IL-8 expression in NSCLC cells, at least in part, through the ERK-MAPK pathway activation. In addition, unlike the cases of KRAS mutant NSCLC cells, the p38-MAPK pathway activation may also be one of the mechanisms of IL-8 upregulation in EGFR mutant NSCLC cells.

Despite many studies indicating an essential role for, and therapeutic significance of, oncogenic KRAS in NSCLC, no effective strategies for the treatment of NSCLC harboring *KRAS* mutations have been established. Recently, the VEGF monoclonal antibody bevacizumab has been approved and several other antiangiogenic agents are being tested for treatment of NSCLC patients[50]; thus, tumor-related angiogenesis has become an attractive therapeutic target for NSCLC. Given the fact that IL-8 functions as an angiogenic factor and IL-8 neutralization suppresses *in vivo* tumor growth and angiogenesis of NSCLC,[14,15] our findings of oncogenic KRAS-induced IL-8 overexpression raise the possibility of anti-IL-8 therapy for *KRAS* mutant NSCLC. Also, we found that IL-8 siRNAs or the IL-8 neutralizing antibody inhibited cell proliferation and migration of KRAS mutant NSCLC cells, consistent with previous studies demonstrating that IL-8 neutralization inhibited cell proliferation and migration in NSCLC cell lines including H460 cells.[12,14] These findings strongly suggest that IL-8 could be a therapeutic target for *KRAS* mutant NSCLC.

In previous studies, IL-8 expression was associated with angiogenesis, lymph node metastasis, and unfavorable outcome in patients with NSCLC.[16–19] We found that IL-8 was overexpressed in NSCLC tumors with pleural involvement, lymphatic permeation and vascular invasion, suggesting that IL-8 overexpression potentially contributes to the aggressive phenotypes of NSCLC. Furthermore, in *KRAS* mutant NSCLC patients, disease-free survival of patients with higher IL-8 expression tended to be shorter compared to those with lower IL-8 expression. Because patient numbers were small in this study, further investigation with a larger number of patients with NSCLC will likely elucidate the prognostic significance of IL-8 expression in *KRAS* mutant NSCLC.

In conclusion, the present study demonstrates the positive association between *KRAS* mutations, IL-8 overexpression, and certain clinicopathological features in NSCLC. Further *in vivo* studies will be needed to evaluate the effectiveness of an anti-IL-8 treatment strategy for KRAS mutant NSCLC. The findings of IL-8 overexpression in a subset of NSCLCs with *EGFR* mutations or wild-type KRAS/EGFR also suggest that IL-8 could be upregulated in some of these lung cancers as well; thus, mechanisms of IL-8 upregulation irrelevant to *KRAS* mutations should be elucidated.

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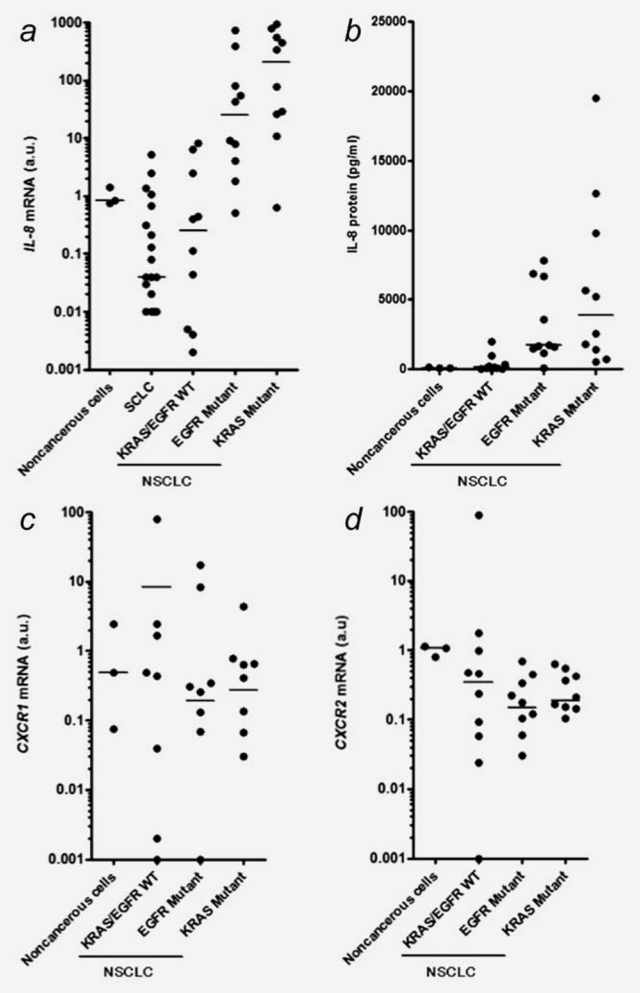

Figure 1.

(*a*) Expression of *IL-8* mRNA in human bronchial epithelial cell lines (noncancerous cells; $N = 3$), SCLC cell lines ($N = 22$), NSCLC cell lines with wild-type *KRAS*/*EGFR* (KRAS/EGFR WT; $N = 10$), NSCLC cell lines harboring *EGFR* mutations (EGFR mutant; $N = 10$) or *KRAS* mutations (KRAS mutant; $N = 10$). IL-8 levels were significantly higher in KRAS mutant ($p < 0.01$) or EGFR mutant ($p < 0.05$) than in KRAS/EGFR WT. (*b*) IL-8 protein concentration in cultured lung cancer cell lines as determined by ELISA assay. Twenty-four hours after plating 10^6 cells on 100 mm dishes, culture medium was replaced with 10 ml of RPMI 1640 medium with 5% serum. After cells were cultured for additional 48 hr, medium was collected, and an ELISA assay was performed. IL-8 levels were significantly higher in EGFR Mutant ($p < 0.05$) or KRAS Mutant ($p < 0.01$) NSCLCs than in KRAS/EGFR WT. Expressions of (*c*) *CXCR1* and (*d*) *CXCR2* mRNA in the noncancerous and NSCLC cell lines. The arbitrary units (a.u.) of the expressions of *IL-8*, *CXCR1*, and *CXCR2* were calculated as described in Materials and Methods. Points represent the mean IL-8 levels from three independent experiments. Lines represent the median levels in each group. Differences were analyzed by Kruskal–Wallis test with Dunn's multiple comparisons.

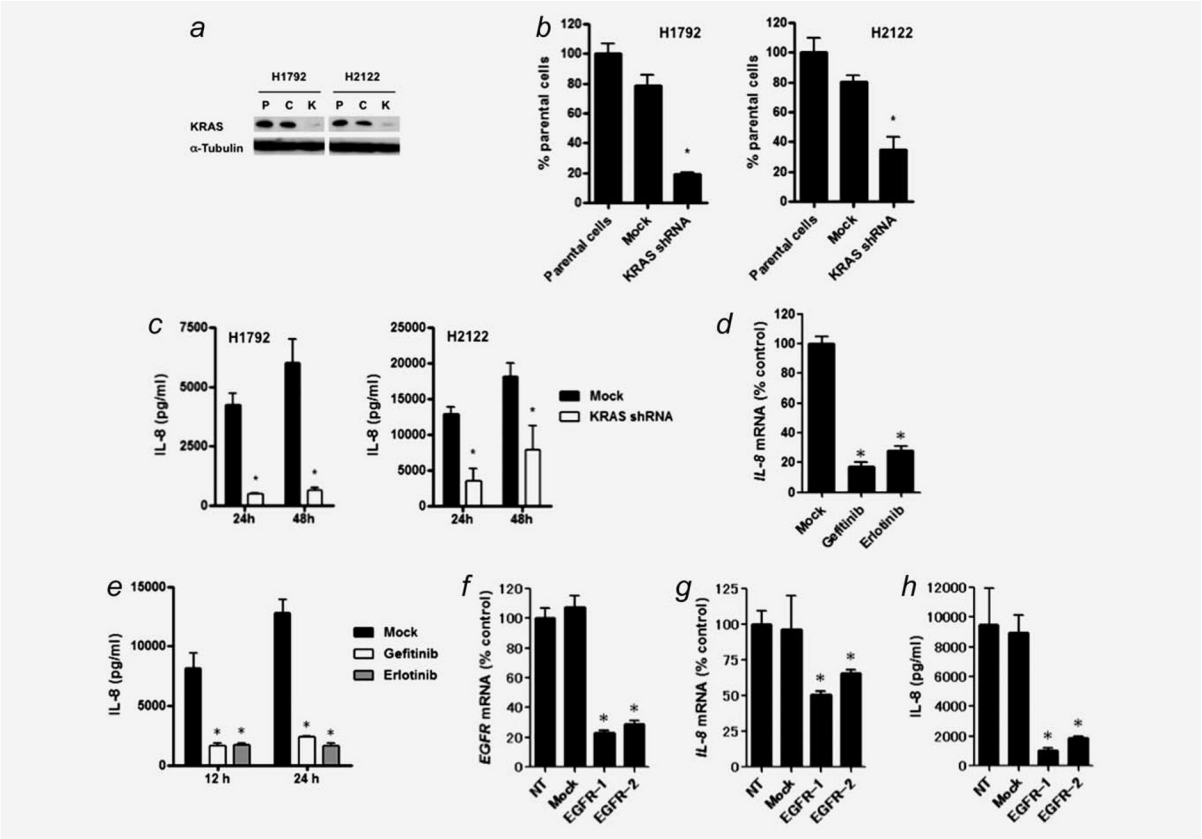

Figure 2.

(*a*) Stable knockdown of mutant KRAS expression by retroviral-mediated shRNA in H1792 and H2122 cells, both of which harbor the *KRAS* G12C mutation (GGT to TGT) in codon 12. P: parental cells, C: pRS (non-targeting shRNA) vector-infected cells, K: pRS-KRAS-C12-infected cells. Thirty micrograms of whole cell lysate was loaded per lane, and western blots were performed using α-Tubulin expression levels as a loading control. (*b*) shRNA-mediated KRAS knockdown reduces *IL-8* mRNA expression in H1792 and H2122 cells as determined by quantitative real-time RT-PCR. *$p < 0.01$ for comparison between KRAS knockdown cells and the parental cells by Kruskal–Wallis test with Dunn's multiple comparisons. Mean levels of the parental cells are set at 100%. (*c*) shRNA-mediated KRAS knockdown reduced IL-8 protein levels in cultured medium of H1792 and H2122 cells as determined by ELISA assay. *$p < 0.001$ for comparisons between KRAS knockdown cells and the parental cells by ANOVA with Bonferroni multiple comparisons. Column represents mean ± SD from four independent experiments. (*d*) Treatment with gefitinib or erlotinib transcription ally down-regulated IL-8 expression in HCC827 NSCLC cells harboring *EGFR* mutations. After treatment with gefitinib (1 μM) or erlotinib (1 μM) for 24 hr, cells were harvested for quantitative real-time RT-PCR analysis. Columns represent means ± SD from four independent experiments. *$p < 0.001$ for comparison of Mock treatment (DMSO alone) by ANOVA with Bonferroni multiple comparisons. (*e*) Treatment with gefitinib or erlotinib reduced IL-8 protein levels in cultured medium of HCC827 cells. After treatment with gefitinib (1 μM) or erlotinib (1 μM) for 12 or 24 hr, cultured medium was collected, and IL-8 concentration was determined by ELISA assay. Columns represent means ± SD from three independent experiments. *$p < 0.001$ for comparison of Mock treatment (DMSO alone) by ANOVA with Bonferroni multiple comparisons. (*f*) siRNA-mediated EGFR knockdown in HCC827 cells as evaluated by quantitative RT-PCR. NT: treatment with

medium alone; Mock: treatment with Tax siRNA. Two siRNAs targeting different sites of *EGFR* mRNA (EGFR-1 and EGFR-2) were used. Columns represent the mean ± SD from four independent experiments. $*p < 0.0001$ for comparisons between NT and each siRNA treatment by ANOVA with Bonferroni multiple comparisons. shRNA-mediated EGFR knockdown reduced IL-8 expression at (*g*) mRNA and (*h*) protein levels in HCC827 cells as evaluated by quantitative real-time RT-PCR and ELISA assays. Twenty-four after siRNA transfection, the medium was replaced by fresh medium. After 48 hr, cells and supernatants were harvested for the assays. $*p < 0.0001$ for comparisons between NT and each siRNA treatment by ANOVA with Bonferroni multiple comparisons. Mean levels of NT are set at 100% for Fig. 2F and 2G.

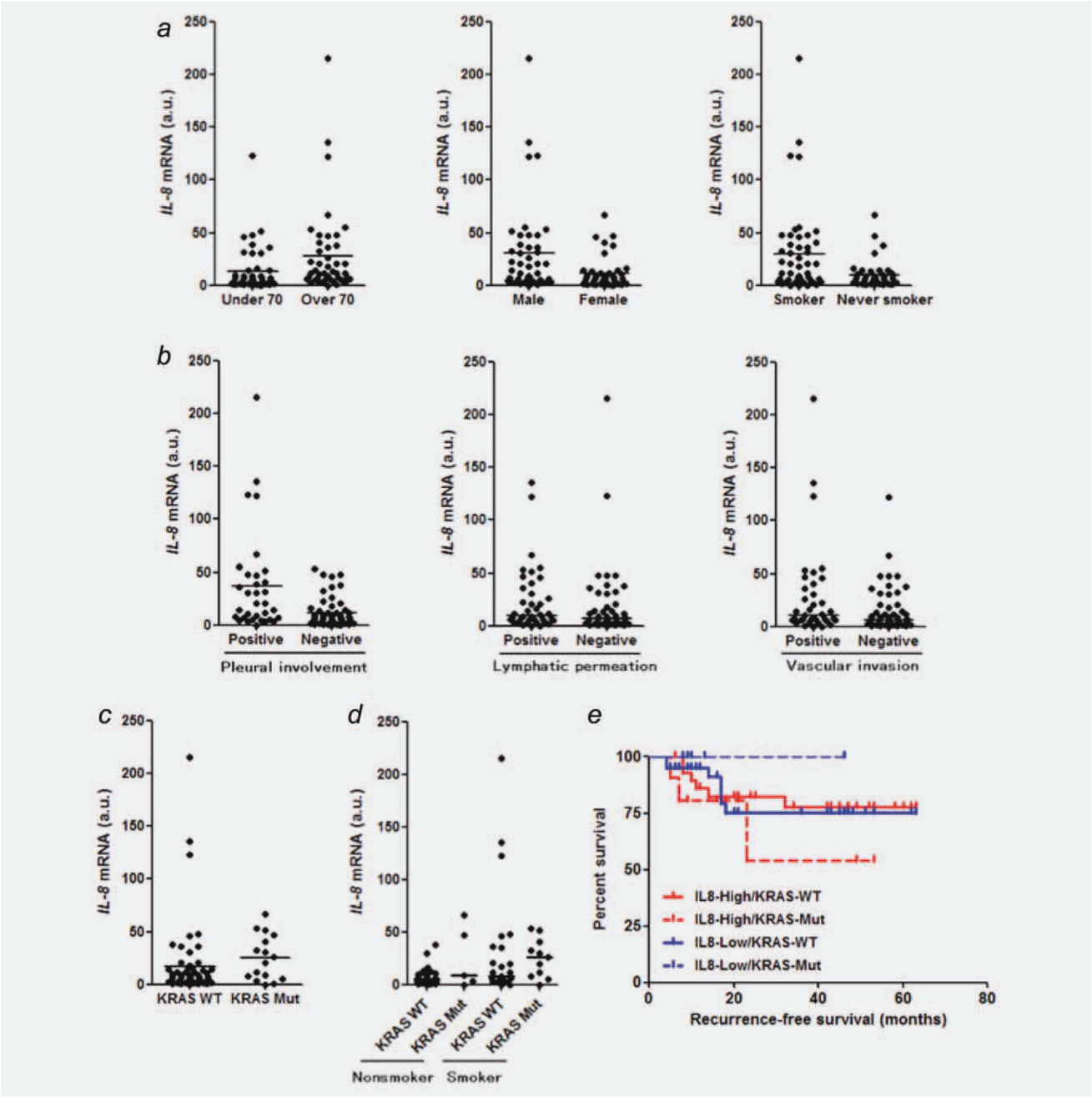

Figure 3.

(*a*) Comparisons of *IL-8* mRNA expression levels between patients with NSCLC under the age of 70 and those over 70 years old ($p = 0.0016$), males and females ($p = 0.0056$), smokers and nonsmokers ($p = 0.007$); (*b*) NSCLC specimens with or without pleural involvement ($p = 0.0003$), NSCLC specimens with or without lymphatic permeation ($p = 0.0871$), NSCLC specimens with or without vascular invasion ($p = 0.054$); (*c*) adenocarcinoma specimens with or without *KRAS* mutations ($p = 0.0355$). IL-8 expression levels in NSCLC surgical specimens were normalized to the mean (= 1 a.u.) of values obtained from five different noncancerous lung tissues. Points represent the mean IL-8 levels obtained from three independent experiments. Lines represent median IL-8 levels in each group. Differences

were analyzed by Mann–Whitney test. (*d*) Comparison of *IL-8* mRNA expression among the four groups of KRAS wild-type/nonsmoker, KRAS mutant/nonsmoker, KRAS wild-type/smoker and KRAS mutant/smoker (the median levels of these groups were 5.9, 9.2, 8.7, and 26.3, respectively; $p = 0.0279$ by Kruskal–Wallis test). (*e*) Kaplan–Meier analysis of disease-free survival (month) for NSCLC patients who were divided according to the IL-8 expression levels and *KRAS* mutation status. KRAS-WT: *KRAS* wild-type; KRAS-Mut: *KRAS* mutation; IL8-High: ≤7.5 a.u.; IL8-Low: >7.5 a.u. Note that the median IL-8 level of all NSCLC tumor specimens was 7.5 a.u.

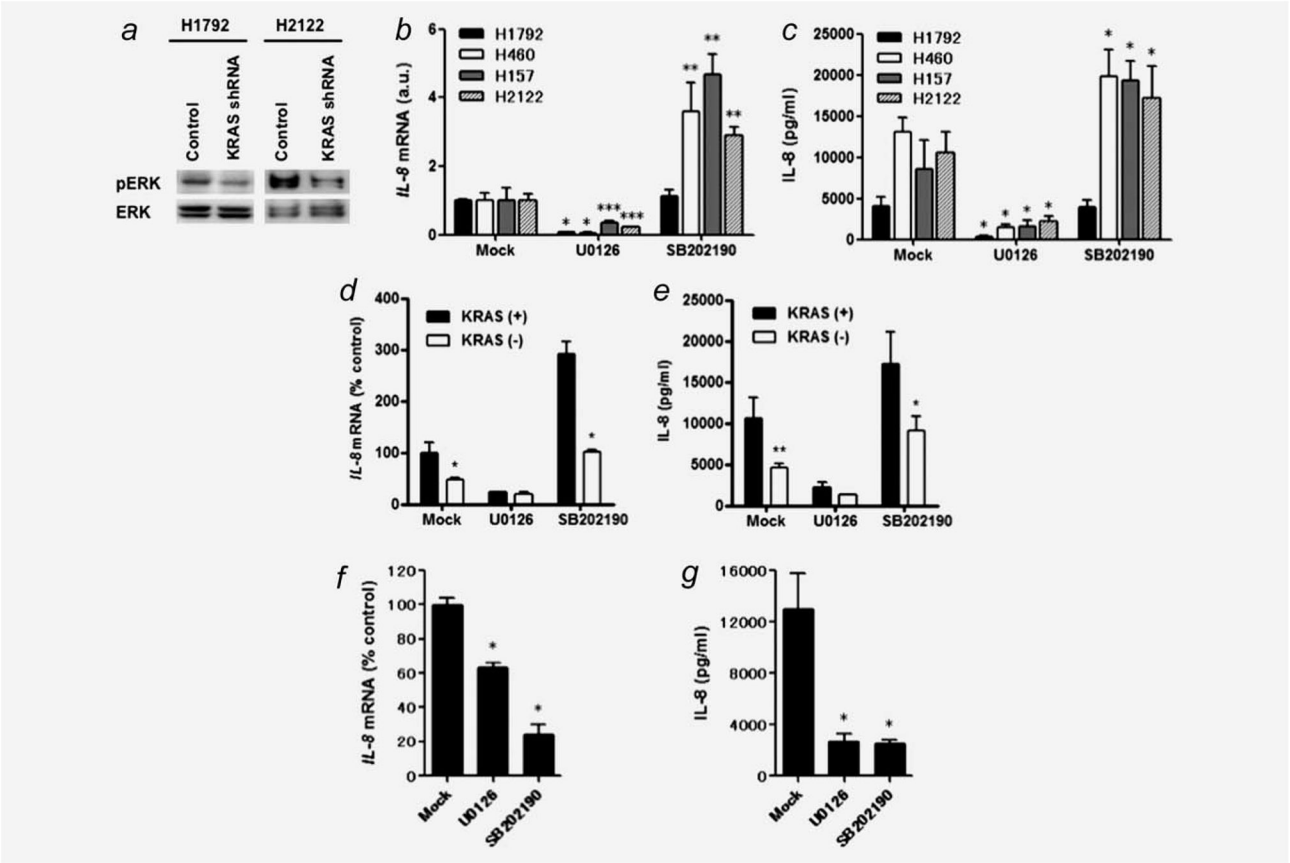

Figure 4.

(*a*) shRNA-mediated KRAS knockdown reduces phosphorylated ERK levels in H1792 and H2122 cells. Thirty microgram of whole cell lysate was loaded per lane, and western blots were performed using total ERK expression levels as a loading control. (*b*) Effects of U0126 and SB202190 on *IL-8* mRNA expression in KRAS mutant/IL-8 overexpressing NSCLC cell lines. Twenty-four hour after 5×10^5 cells were plated in each well of 6-well plates, cultured medium was replaced with 2 ml of the growth medium with U0126 (10 μM) or SB202190 (10 μM). After additional culture for 24 hr, cells were harvested for real-time RT-PCR analysis. Columns represent means ± SD from four independent experiments. *$p <$ 0.01, **$p < 0.001$, ***$p < 0.05$ for comparisons of the treatment with DMSO alone (Mock) to the treatment with U0126 or SB202190 on each cell line by ANOVA with multiple comparisons. (*c*) Effects of U0126 or SB202190 on IL-8 protein expression from KRAS mutant/IL-8 overexpressing NSCLC cells. After treatments with U0126 or SB202190 for 24 h, cultured medium was collected, and IL-8 concentration was determined by ELISA. Columns represent means ± SD from four independent experiments. *$p < 0.001$ for comparisons of the treatment with DMSO alone (Mock) to the treatment with U0126 or SB202190 on each cell line by ANOVA with Bonferroni multiple comparisons. Effects of KRAS knockdown on IL-8 expression at (*d*) mRNA and (*e*) protein levels in H2122 cells treated with U0126 or SB202190. IL-8 levels were determined at 24 hr posttreatment. Columns represent means ± SD from four independent experiments. *$p < 0.001$, **$p < 0.01$ for comparison between mutant KRAS-disrupted cells (white square) and mutant KRAS-expressing (pRS vector-infected) cells (black square) on each treatment by ANOVA with Bonferroni multiple comparisons. Effects of U0126 and SB202190 on *IL-8* expression at (*f*) mRNA and (*g*) protein levels in HCC827 cells. The experimental procedures and conditions

were the same as Figures 4*b* and 4*c*. *$p < 0.001$ for comparisons of the treatment with DMSO alone (Mock) to the treatment with U0126 or SB202190 by ANOVA with Bonferroni multiple comparisons.

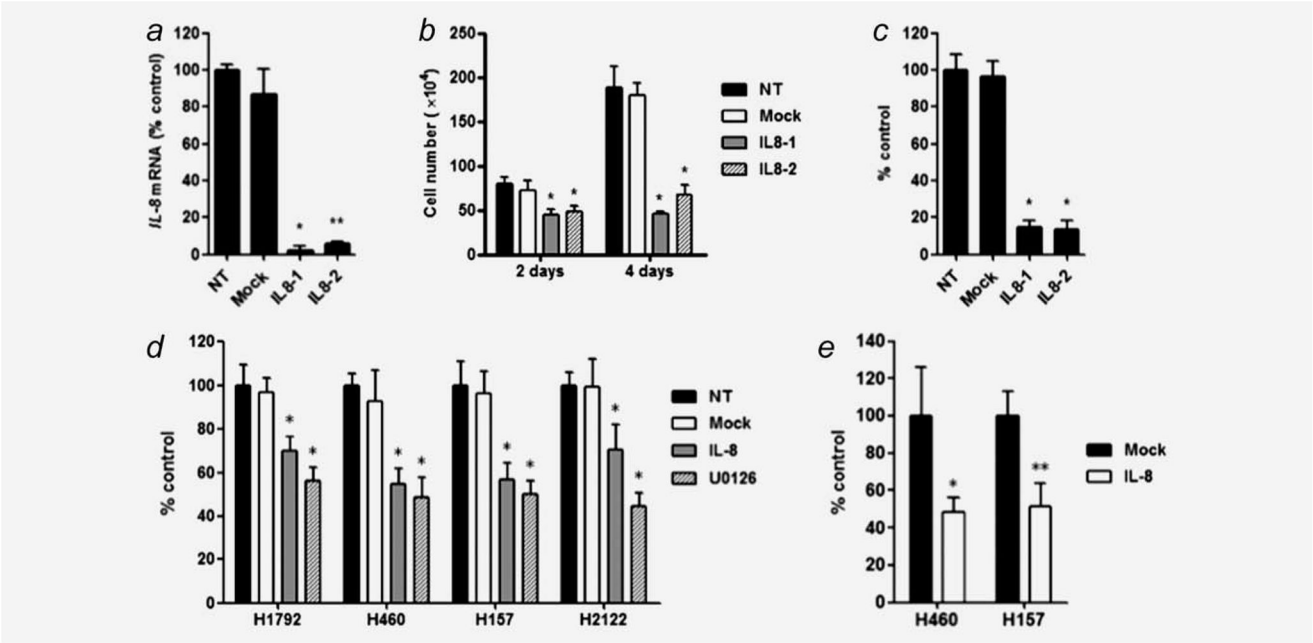

Figure 5.

(*a*) siRNA mediated knockdown of IL-8 expression in H1792 cells as evaluated by quantitative RT-PCR. NT: treatment with medium alone; Mock: treatment with Tax siRNA as a negative control. Two siRNAs targeting different sites of *IL-8* mRNA (IL8-1 and IL8-2) were used to knockdown IL-8. Columns represent the mean ± SD from four independent experiments. *$p < 0.001$, **$p < 0.01$ for comparisons between NT and each siRNA treatment by Kruskal–Wallis test with Dunn's multiple comparisons. siRNA-mediated IL-8 knockdown inhibited cell proliferation and colony formation as evaluated by (*b*) cell growth analysis using trypan-blue staining and (*c*) colony formation assay in H1792 cells. Columns represent the mean ± SD from six independent experiments for cell growth analysis and three independent experiments for colony formation assay. *$p < 0.001$ for comparison between NT and each siRNA treatment by ANOVA with Bonferroni multiple comparisons. (*d*) IL-8 neutralization mediated by an IL-8 antibody inhibits cell proliferation of KRAS mutant/IL-8 overexpressing NSCLC cell lines as evaluated by MTT assay. Cells were treated with medium alone (NT), IgG1 control antibody (Mock; 10 μg/ml), IL-8 antibody (IL-8; 10 μg/ml) and U0126 (10 μM) for 72 hr. *$p < 0.001$ for comparison between NT and each treatment by ANOVA with Bonferroni multiple comparisons. Columns represent the mean ± SD from replicates of eight from two independent experiments (*e*) IL-8 neutralization inhibits cell migration of H460 and H157 cells. *$p < 0.01$; **$p < 0.05$ for comparison between Mock and IL-8 antibody treatments by unpaired *t*-test. Columns represent means ± SD from three independent experiments.