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Growth Regulation via Insulin-Like Growth Factor Binding Protein-4 and -2 in Association with Mutant K-ras in Lung Epithelia

Hanako Sato,* Takuya Yazawa,*
Takehisa Suzuki,* Hiroaki Shimoyamada,*
Koji Okudela,[†] Masaichi Ikeda,* Kenji Hamada,[‡]
Hisafumi Yamada-Okabe,[‡] Masayuki Yao,[§]
Yoshinobu Kubota,[§] Takashi Takahashi,[¶]
Hiroshi Kamma,^{||} and Hitoshi Kitamura*

From the Departments of Pathobiology* and Urology,[§] Yokohama City University Graduate School of Medicine, Kanagawa; the Department of Pathology 1,[†] Hamamatsu Medical University, Shizuoka; the Pharmaceutical Research Department 4,[‡] Kamakura Research Laboratories, Chugai Pharmaceutical Company Limited, Kanagawa; the Department of Molecular Oncology,[¶] Nagoya University Graduate School of Medicine, Nagoya; and the Department of Pathology,^{||} Kyorin University School of Medicine, Tokyo, Japan

Gain-of-function point mutations in *K-ras* affect early events in pulmonary bronchioloalveolar carcinoma. We investigated altered mRNA expression on K-Ras activation in human peripheral lung epithelial cells (HPL1A) using oligonucleotide microarrays. Mutated K-Ras stably expressed in HPL1A accelerated cell growth and induced the expression of insulin-like growth factor (IGF)-binding protein (IGFBP)-4 and IGFBP-2, which modulate cell growth via IGF. Other lung epithelial cell lines (NHBE and HPL1D) revealed the same phenomena as HPL1A by mutated *K-ras* transgene. Lung cancer cell growth was also accelerated by mutated *K-ras* gene transduction, whereas IGFBP-4/2 induction was weaker compared with mutated K-Ras-expressing lung epithelial cells. To understand the differences in IGFBP-4/2 inducibility via K-Ras-activated signaling between nonneoplastic lung epithelia and lung carcinoma, we addressed the mechanisms of IGFBP-4/2 transcriptional activation. Our results revealed that Egr-1, which is induced on activation of Ras-mitogen-activated protein kinase signaling, is crucial for transactivation of IGFBP-4/2. Furthermore, *IGFBP-4* and *IGFBP-2* promoters were often hypermethylated in lung carcinoma, yielding low basal expression/weak induction of IGFBP-4/2.

These findings suggest that continuous K-Ras activation accelerates cell growth and evokes a feedback system through IGFBP-4/2 to prevent excessive growth. Moreover, this growth regulation is disrupted in lung cancers because of promoter hypermethylation of *IGFBP-4/2* genes. (Am J Pathol 2006, 169:1550–1566; DOI: 10.2353/ajpath.2006.051068)

Lung cancer is a leading cause of cancer-related death in many countries. Among the various histological types of lung cancer, adenocarcinomas are significantly more prevalent than squamous cell carcinoma.^{1,2} Adenocarcinomas arising in the peripheral lung exhibit so-called bronchioloalveolar (alveolar-lining) growth that is distinguishable from other forms of lung cancer, and bronchioloalveolar carcinoma is classified as a subtype of pulmonary adenocarcinomas.^{2–6}

The *ras* genes, *H-ras*, *K-ras*, and *N-ras*, encode 21-kd GTP-binding proteins that localize to the inner plasma membrane, transduce extracellular signals to various effector proteins, and activate various signal transduction systems such as those controlling cell proliferation or transformation. Among the three *ras* genes, alleles carrying mutant *K-ras* occur in ~30% of human adenocarcinomas, and most of these affected alleles involve a point mutation in codon 12 (Gly→Val). Mutated *K-ras* is oncogenic, a consequence of constitutive activation attributable to reduced intrinsic GTPase activity, resulting in excessive activation of its downstream effectors.⁷ Phosphatidylinositol 3 kinase (PI3K) and Raf are among the best-studied downstream effectors of K-Ras.⁸ PI3K phosphorylates membrane phosphatidylinositides to recruit and activate many factors that contain a pleckstrin homology domain, such as Akt and PDK, which transmit signals mediating

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Address reprint requests to Takuya Yazawa, M.D., Ph.D., Department of Pathobiology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-0004, Japan. E-mail: tk yazawa@med.yokohama-cu.ac.jp.

cell survival, cell cycle progression, and glucose metabolism.⁹ We recently reported that *K-ras* gene activation in human airway epithelial cells and lung adenocarcinoma cells enhances cell motility via Akt activation.¹⁰ On the other hand, Raf activates mitogen-activated protein kinase (MAPK) via phosphorylation of MAPKK (MAPK kinase, MEK).¹¹ Activated MAPK binds to other kinases, translocates to the nucleus, and interacts with other transcription factors to regulate the expression of genes that facilitate cell cycle progression and inhibit apoptotic cell death.^{11–14}

Atypical adenomatous hyperplasia is classified as a precancerous lesion, according to the World Health Organization.^{15–19} Like adenocarcinomas, atypical adenomatous hyperplasia cells often carry a mutated *K-ras* gene, and thus *K-ras* gene mutations are considered to be involved in early-stage tumorigenesis of lung adenocarcinoma.^{20–24} However, it is unclear which kinds of genes are up-regulated in lung airway epithelial cells in response to the continuous activation of the mutated *K-ras* gene.

Here, we show that *K-ras* gene activation in lung airway epithelia, including lung cancer cells, not only accelerates cell growth but also induces two kinds of growth-modulating factors, IGFBP-4 and IGFBP-2, through the MEK-MAPK-Egr-1 pathway. Furthermore, IGFBP-4 and IGFBP-2 expression/induction levels are substantially lower in lung cancer cells because of hypermethylation of *IGFBP-4* and *IGFBP-2* gene promoters. These results suggest that induction of IGFBP-4 and IGFBP-2 in lung airway epithelia is one of the feedback mechanisms controlling excessive growth via *K-ras* gene activation and that neo-plastic airway epithelia might gradually lose these growth-modulating systems as tumor aggressiveness increases.

Materials and Methods

Cells and Cell Culture

Human peripheral lung epithelial cell lines (HPL1A and HPL1D; donated from Aichi Cancer Center Research Institute, Nagoya, Japan), a human bronchial epithelial cell line (NHBE, which was immortalized by simian virus 40), and non-small-cell lung cancer cell lines (A549, H820, TKB6, TKB14, TKB1, and TKB5) were used in this study.^{10,25,26} Cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin and were maintained at 37°C in 5% CO₂. Subconfluent cells were used for the following experiments.

To examine cell growth rate, cells were seeded at 5 × 10⁴ cells/60-mm dish in 3 ml of medium and cultivated for up to 96 hours. After washing with phosphate-buffered saline (PBS) (pH 7.4), the cells were harvested by trypsinization and counted. Each examination was performed in triplicate. Statistical analysis was performed with the paired *t*-test, and differences between values were considered statistically significant at *P* < 0.05.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), and cDNA synthesis and subsequent PCR reactions were performed using an RNA-PCR kit (Takara, Shiga, Japan) according to the manufacturer's instructions. The alteration of mRNA levels was investigated by semiquantitative RT-PCR. β -Actin served as an internal control. The cycle number of PCR was set in the range that the PCR product was exponentially increasing and was determined as 25 cycles for IGFBP-4 and IGFBP-2, 30 cycles for *egr-1*, and 22 cycles for β -actin. Densitometry scanning was conducted using National Institutes of Health (Rockville, MD) image computer software. The forward and reverse primer sequences specific for IGFBP-4, IGFBP-2, *egr-1*, and β -actin are detailed in Table 1.

Construction of Expression Vectors and Transfection

We used PCR to amplify cDNA from a wild-type *K-ras*-expressing cancer cell line or a mutated *K-ras* cDNA-inserted plasmid vector (pSW11–1 containing a *K-ras* gene with a point mutation, Gly→Val, in codon 12; Riken Gene Bank, Wako, Japan) and *K-ras*-specific primers described in Table 1. The PCR products were cloned using the pT7Blue vector (Novagen, Madison, WI) and sequenced. The inserts were excised with *Hind*III and *Bam*HI and then reinserted into the expression vector pcDNA3.1 (Invitrogen, San Diego, CA). The mutated *K-ras*-inserted, wild-type *K-ras*-inserted, or empty vector was transfected into HPL1A, HPL1D, NHBE, and TKB5 using a lipofection method (Superfect; Qiagen, Hilden, Germany). After selection for 3 weeks, the stable transfectants of mutated *K-ras*, wild-type *K-ras*, and empty vector were cloned and named SW, K, and C, respectively. To construct IGFBP-4 or IGFBP-2 expression vectors, cDNAs were obtained by RT-PCR using total RNA of HPL1A-SW and specific primer sets (Table 1) and were inserted into pcDNA3.1. Transfections using TKB5 cells and subsequent selection were performed as above, and the established clones were named TKB5-BP4 and TKB5-BP2, respectively.

Gene Chip Analysis

Cell lines HPL1A-SW, HPL1A-K, and HPL1A-C were used for gene chip analyses. cDNAs were synthesized using the Reverse Superscript Choice System (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. Purified total RNA (5 µg) was hybridized with an oligo-dT primer containing the T7 promoter sequence and then incubated with 200 U of Super Script II reverse transcriptase at 42°C for 1 hour. The cDNA was extracted with phenol/chloroform with Phase Lock Gel (Eppendorf-5 Prime, Inc., Boulder, CO) and concentrated by ethanol precipitation. The cRNA was also synthesized using the MEGAscript T7 kit (Ambion, Austin, TX) at 37°C for 6 hours. Mononucleotides and short oligonucleotides

Table 1. PCR Primers Used in This Study

Target	Forward primer sequence
IGFBP-4 cDNA	5'-TTCATCCCCATCCCCAACTGC-3'
IGFBP-2 cDNA	5'-TTGCAGACAATGGCGATGACC-3'
egr-1 cDNA	5'-GCAGCAGCAGCACCTTCAACC-3'
β -Actin cDNA	5'-GCGGGAAATCGTGCGTGACAT-3'
Wild-type K-ras cDNA	5'-GACGGCCGAAGCTTGCTGAAAATGACTGAATATAAACTTG-3'
Mutated K-ras cDNA	5'-GACGGCCGAAGCTTGCTGAAAATGACTGAATATAAACTTG-3'
IGFBP-4 cDNA containing full coding region	5'-GCGGTATCCTGCCCCCTCTGC-3'
IGFBP-2 cDNA containing full coding region	5'-GGGCGAGGGAGGAGGAAGAAG-3'
<i>IGFBP-4</i> promoter	
–1298 to +297 (for Luc)*	5'-CACGCCCCGGCTAACTT-3'
–855 to +297 (for Luc)	5'-TTCAACGCAAACTT-3'
–185 to +297 (for Luc)	5'-ACTGCGCCGCTTCCTTCTTCG-3
–125 to +297 (for Luc)	5'-CGACTCAGGACAGCGCC-3'
+11 to +297 (for Luc)	5'-GCAGCCGCTCAGCCCC-3'
–565 to –174 (for ChIP) [†]	5'-TGCGGGGGCGGGAGAGGT-3'
–317 to +131 (for ChIP)	5'-GGCGGGGCGGGACGAGAC-3'
–695 to –43 (for BS) [‡]	5'-TGAATGGGTTTGAGAGGTAAATATT-3'
Egr-1-binding site-mutated <i>IGFBP-4</i> promoter	
Site 1 mutant (for Luc)	5'-CAAATGCGCCCTGGGGAGATTGCTAGGTCGGGAGAGGTTGCAAGGGGCAAGTG-3'
Site 2 mutant (for Luc)	5'-GAGACTGAGGCCGCTTGGTAGGGCGGGACGAGACTC-3'
Site 3 mutant (for Luc)	5'-GAAAAGGACTTTTCAGATGCTACGGCGGCTACGGCGGCGACTCAGGAC-3'
Site 4 mutant (for Luc)	5'-GACTCAGGACAGCGCCCTATCCCCCTAACGGCCGCTC-3'
Site 5 mutant (for Luc)	5'-CTCTCCCCCTCGCCCTACCTAGCTCCCCCACCTCTGGGAAG-3'
<i>IGFBP-2</i> promoter	
–522 to +114 (for Luc)	5'-TCTAGACGGGTCTGAAACTCC-3'
–444 to +114 (for Luc)	5'-CCCCAGGATGGAAGG-3'
–314 to +114 (for Luc)	5'-TGAGCCGACTGAAATCTACTT-3'
–206 to +114 (for Luc)	5'-TCGCGAACTGAACTGAGAGCA-3'
–134 to +114 (for Luc)	5'-CAGAAGAGTGCGGAGGGGACGG-3'
–31 to +114 (for Luc)	5'-GCCCGCAGCCAACGC-3'
–202 to +113 (for ChIP)	5'-CAGAAGAGTGCGGAGGGGACGG-3'
–413 to +201 (for BS)	5'-GTGATATTTTAGGATGGAAGGAGTT-3'
Egr-1-binding site-mutated <i>IGFBP-2</i> promoter	
Site 1 mutant (for Luc)	5'-CACTTGCCGGCGCGAGTAAGTGTGCGGGGGGAAG-3'
Site 2 mutant (for Luc)	5'-GAGGGGAGAAGGCATAGGGCGGGGAGAAG-3'
Site 3 mutant (for Luc)	5'-CTTTAGGACCCGGCTGCTACGGCGAGGGAGGAG-3'
<i>Egr-1</i> promoter	
–600 to –363 (for BS)	5'-GTTTGGGTTTTTTTAGTTTAGTTTA-3'
–374 to –75 (for BS)	5'-TTGGGTAGTATTTTATTGGAGTGG-3'

*Luciferase assay; [†]chromatin immunoprecipitation assay; [‡]bisulfite sequencing.
 Mutated sequences are underlined.

(table continues)

were removed by column chromatography (CHROMA SPIN+STE-100 column; Takara), and the cRNA in the eluates was sedimented by ethanol. Gene expression analyses were conducted using high-density oligonucleotide microarrays (HuGeneFL array, HuU95; Affymetrix, Santa Clara, CA) according to the manufacturer's instructions.^{27,28} For hybridization with oligonucleotides on the chips, the cRNA was fragmented at 95°C for 35 minutes, and hybridization was performed at 45°C for 12 hours. After washing the chips, the chips were incubated with a biotinylated antibody against streptavidin and stained with streptavidin R-phycoerythrin to increase the hybridization signal, as described in the manufacturer's instructions. Each pixel level was collected with a laser scanner (Affymetrix), and the expression levels were analyzed using Affymetrix Microarray Suite version 4.0 software. Clustering analyses were conducted using the EISEN cluster program.²⁹

Western Blot Analysis

Total cell lysates (50 μ g protein per lane) were separated by 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were blocked for 1 hour at room temperature with 1% skim milk in Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBS-T) and then incubated with diluted mouse monoclonal anti-p44/42 MAPK, mouse monoclonal anti-phosphorylated MAPK (each from Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-IGFBP-4, or goat polyclonal anti-IGFBP-2 antibody (each from Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hour. After washing three times for 10 minutes with TBS-T at room temperature, membranes were incubated at room temperature for 30 minutes with a

Table 1. *Continued*

Reverse primer sequence	Size (bp)
5'-CTGCTACCCACGCTTCCTTA-3'	767
5'-GGGATGTGCAGGGAGTAGAGG-3'	340
5'-TTTCCCTTTCCCTTTAGCAA-3'	1405
5'-GTGGACTTGGGAGAGGACTGG-3'	863
5'-CAGGATCCTCATTACATAATTACACACTTTG-3'	599
5'-CAGGATCCTCATTACATAATTACACACTTTG-3'	599
5'-CACACCCATGCTCACAAACAC-3'	969
5'-AGCAAGAAGGAGCAGGTGTGG-3'	1305
5'-GACCGCCCCGAGGACTGG-3'	1595
5'-GACCGCCCCGAGGACTGG-3'	1152
5'-GACCGCCCCGAGGACTGG-3'	482
5'-GACCGCCCCGAGGACTGG-3'	422
5'-GACCGCCCCGAGGACTGG-3'	287
5'-AAGCGGCGCAGTTTGAA-3'	392
5'-AGCGGGGAAGTTAGCAG-3'	448
5'-CCTTCCCAAAAATAAAAAACC-3'	653
5'-CACTTGCCCCTTGCAACCTCTCCCGACCTAGCAATCTCCCAGGGCGCATTG-3'	5857
5'-GAGTCTCGTCCCGCCCTACCAAGGCGGCC TCAGTCTC-3'	5857
5'-GTCCTGAGTCGCGCCCGTAGCCGCCGTAGCATCTGAAAGTCCTTTTC-3'	5857
5'-GAGGCGGCCGTTAGGGGATAGGGCGCTGTCCTGAGTC-3'	5857
5'-CTTCCAGAGGTGGGGGAGCTAGGTAGGGC GAGGGGGAGAG-3'	5857
5'-CTGGCGGTCGGCAGC-3'	636
5'-CTGGCGGTCGGCAGC-3'	588
5'-CTGGCGGTCGGCAGC-3'	428
5'-CTGGCGGTCGGCAGC-3'	320
5'-CTGGCGGTCGGCAGC-3'	248
5'-CTGGCGGTCGGCAGC-3'	145
5'-CTGGCGGTCGGCAGC-3'	315
5'-CCCAATAACAACAACAAC-3'	649
5'-CTTCCCCCGACACTTACTCGCGCCGGCAAGTG-3'	4898
5'-CTTCTCCCGCCCTATGCCTTCTCCCTC-3'	4898
5'-CTCCTCCCTCGCCGTAGCAGCCGGTCTAAAG-3'	4898
5'-AATACTACCCAAATAAAAAATTATTCC-3'	238
5'-CATATATAAAAAACAAAAACCCTAATATA-3'	300

diluted peroxidase-labeled secondary antibody against mouse (Amersham Biosciences, Buckinghamshire, UK), rabbit (Amersham Biosciences), or goat (Santa Cruz Biotechnology). The membranes were then washed three times for 10 minutes with TBS-T at room temperature, and immunopositive signals were visualized using an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences). Mouse anti- β -actin antibody (Sigma, St. Louis, MO) was used for the internal control.

Supplementation Experiments with MEK Inhibitor, Recombinant IGF-1 (rIGF-1), rIGFBP-4, and rIGFBP-2

HPL1A, HPL1D, NHBE, and TKB5 cells (1×10^5) were seeded in 100-mm dishes and cultured for 24 hours.

After rinsing with PBS three times, cultures were incubated in 10 ml of medium with or without 20 μ M MEK inhibitor (U0126; Promega, Madison, WI) for up to 24 hours. For recombinant IGF-1 (rIGF-1), rIGFBP-4, and rIGFBP-2 supplementation experiments, HPL1A, HPL1D, NHBE, and TKB5 cells were seeded in six-well plates at 5×10^4 cells/well in 3 ml of medium containing 600 μ g of rIGFBP-4 (Genzyme-Techne Corp., Minneapolis, MN) or rIGFBP-2 (Genzyme-Techne). After 30 minutes, 75 ng of rIGF-1 (Genzyme-Techne) was added to the medium, and the cells were cultured for 48 hours. For rIGF-1 supplementation experiments using TKB5 derivatives, TKB5-C, TKB5-BP4 (stably IGFBP-4-transfected TKB5 clone), and TKB5-BP2 (stably IGFBP-2-transfected clone), 5×10^5 cells were seeded in 100-mm dishes in 10 ml of medium containing 0 to 50 ng/ml rIGF-1 and incubated for 48 hours.

IGFBP-4 and IGFBP-2 Neutralization Experiment

HPL1A-SW, HPL1D-SW, or NHBE-SW cells were seeded in 96-well plates at 1×10^4 cells/well in 50 μ l of medium. After culture for 24 hours, 50 μ l of medium supplemented with goat anti-IGFBP-4, goat anti-IGFBP-2, or nonimmunized goat antibody (final concentration, 50 μ g/ml) (R&D Systems, Minneapolis, MN) was added and cultivated for up to 48 hours. Cell growth was evaluated using a MTT assay system (Seikagaku Corp., Tokyo, Japan) and a microplate reader (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm.

IGFBP-4 and IGFBP-2 Promoter Assay

The *IGFBP-4* and *IGFBP-2* promoters are located in regions 1298 bp and 522 bp (EMBL accession no. Y12508 and AY398667) from the transcription start site, respectively.^{30,31} Various lengths of *IGFBP-4* and *IGFBP-2* promoter fragment-inserted constructs, pGL4.10IGFBP4-1298, -855, -185, -125, and +11 and pGL4.10IGFBP2-522, -444, -314, -206, -134, and -31 [each number indicates the 5' nucleotide position of insert from the transcription start site (+1)] were generated by PCR using specific primer sets (Table 1). Each amplified promoter DNA fragment was subcloned into the pT7Blue cloning vector (Novagen) and inserted into the pGL4.10 *luc2* luciferase plasmid (Promega). For construction of pGL4.10IGFBP4-522, the distal region of the inserted promoter DNA fragment of pGL4.10IGFBP4-855 was cut out with *KpnI* and *HpaI* and religated using a *KpnI*-*HpaI* linker.

For mutational analysis, full-length *IGFBP-4* or *IGFBP-2* promoter sequences with mutation in each Egr-1-binding site were constructed using a quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA), specific mutagenic primer sets (Table 1), and a full-length *IGFBP-4* or *IGFBP-2* promoter-inserted pT7 cloning vector, according to the manufacturer's instructions. In brief, PCR using a normal *IGFBP-4* or *IGFBP-2* promoter sequence-inserted pT7 cloning vector and a respective mutagenic primer set specific for each Egr-1-binding site was performed, and the parental DNA template in the reactant was digested with *DpnI* (New England BioLabs, Beverly, MA). The nicked plasmids that were generated by PCR and had mutated *IGFBP-4/2* promoter sequences in each Egr-1-binding site were repaired in XL1-Blue cells (Stratagene). Then, the mutated *IGFBP-4/2* promoter sequences were inserted into the pGL4.10 *luc2* luciferase plasmid (Promega).

HPL1A-SW cells were transiently transfected using the FuGENE6 transfection reagent according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). Cells were seeded at 50% confluency in six-well plates. After 24 hours, cells were co-transfected with 1 μ g/well of reporter plasmid vector (promoter DNA-inserted pGL4.10 *luc2*) and 50 ng/well of control vector (pGL4.7TK) (Promega). At 24 hours after transfection,

cells were washed three times with PBS, and the lysates were subjected to the luciferase assay. Luciferase activity was measured according to the manufacturer's instructions using a luminometer (Turner Biosystems, Sunnyvale, CA). Transfections were performed in quadruplicate, and experiments were performed at least three times.

Electrophoretic Mobility Shift Assay (EMSA)

For binding reaction with the putative Egr-1-binding sequence in the *IGFBP-4* and *IGFBP-2* promoters, 2 μ l of recombinant human Egr-1 protein (Alexis Biochemicals, Lausen, Switzerland) was mixed with 1 pmol of digoxigenin-labeled double-stranded IGFBP-4 or IGFBP-2 promoter-specific DNA probes with or without methylated CpGs (Table 2) containing 20 mmol/L Tris-HCl, pH 7.9, 50 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 5% glycerol, 1 μ g of poly dIdC, and 5 mmol/L dithiothreitol. The mixture was incubated 30 minutes at 20°C. Unlabeled DNA probe containing consensus Egr-1-binding sequence (5'-GCGGGGCG-3') or nonspecific sequence was used for the competitor (Table 2). Probes with mutation in the Egr-1-binding sites (Table 2) were also used to examine whether Egr-1 could bind to the mutated sequences used in the mutational promoter assay. These oligonucleotide probes were purchased from Greiner Japan (Atsugi, Japan). For supershift experiments, a rabbit IgG antibody against Egr-1 (Santa Cruz Biotechnology) or a nonimmunized rabbit IgG antibody (Santa Cruz Biotechnology) was added to the mixture and left 30 minutes at 4°C before gel electrophoresis in 5% acrylamide/bisacrylamide (29:1) gels with 0.25 $\times$ TBE buffer for 3 hours at 150 V and 4°C with recirculating buffer. The protein-probe complexes were contact-blotted and fixed with UV for 5 minutes on positively charged nylon membranes (Perkin-Elmer, Wellesley, MA), and the digoxigenin-labeled probes were detected with a chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed essentially as described.³² Cultivated HPL1A-SW cells (1×10^7) with or without 20 μ mol/L U0126 treatment for 4 hours were exposed to formaldehyde (final concentration, 1%) added directly to the tissue culture medium for 10 minutes. Cells were pelleted, washed in PBS three times, and lysed in lysis buffer (5 mmol/L PIPES, pH 8.0, 85 mmol/L KCl, and 0.5% Nonidet P-40 containing protease inhibitors). Nuclei were pelleted and lysed in 500 μ l of nuclei lysis buffer (50 mmol/L Tris-HCl, pH 8.1, 10 mmol/L ethylenediaminetetraacetic acid, and 1% SDS) containing protease inhibitors, and sonicated using a microtip until the average DNA fragment was ~500 bp. The samples were centrifuged at 16,000 $\times g$ for 5 minutes, and the 50 μ l of supernatant were diluted 1:10 with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L ethylenediaminetetraacetic

Table 2. Probes for Electrophoretic Mobility Shift Assay (Sense Sequences)

<i>IGFBP-4</i> promoter	
Egr-1-binding site 1	5'-GGGAGATTGCGGGGGCGGGAGAGGT-3'
Egr-1-binding site 1, mutated	5'-GGGAGATTGCTAGGTCGGGAGAGGT-3'
Egr-1-binding site 1, methylated	5'-GGGAGATTGC ^m GGGGGC ^m GGGAGAGGT-3'
Egr-1-binding site 2	5'-GCCGCCTTGGCGGGGCGGGACGAGA-3'
Egr-1-binding site 2, mutated	5'-GCCGCCTTGGTAGGGGCGGGACGAGA-3'
Egr-1-binding site 2, methylated	5'-GCC ^m GCCTTGGC ^m GGGGC ^m GGGAC ^m GAGA-3'
Egr-1-binding site 3	5'-AGATGCGGCGGCGGCGGCGGCGGCG-3'
Egr-1-binding site 3, mutated	5'-AGATGCTACGGCGGCTACGGCGGCG-3'
Egr-1-binding site 3, methylated	5'-AGATGC ^m GGC ^m GGC ^m GGC ^m GGC ^m GGC ^m GGC ^m G-3'
Egr-1-binding site 4	5'-GGACAGCGCCCCCTCCCCTAACGGC-3'
Egr-1-binding site 4, mutated	5'-GGACAGCGCCCTATCCCCTAACGGC-3'
Egr-1-binding site 4, methylated	5'-GGACAGC ^m GCCCCCTCCCCTAAC ^m GGC-3'
Egr-1-binding site 5	5'-CCCTCGCCCGCCCCGGCTCCCCAC-3'
Egr-1-binding site 5, mutated	5'-CCCTCGCCCTACCTAGCTCCCCAC-3'
Egr-1-binding site 5, methylated	5'-CCCTC ^m GCCC ^m GCCC ^m GGCTCCCCAC-3'
<i>IGFBP-2</i> promoter	
Egr-1-binding site 1	5'-CTTGCCGGCGCGAGGGAGTGTCGGGG-3'
Egr-1-binding site 1, mutated	5'-CTTGCCGGCGCGAGTAAGTGTCGGGG-3'
Egr-1-binding site 1, methylated	5'-CTTGCC ^m GGC ^m GC ^m GAGGGAGTGTC ^m GGGG-3'
Egr-1-binding site 2	5'-GGAGAAGGCAGGGGGCGGGGAGAAG-3'
Egr-1-binding site 2, mutated	5'-GGAGAAGGCATAGGGGCGGGGAGAAG-3'
Egr-1-binding site 2, methylated	5'-GGAGAAGGCAGGGGGC ^m GGGGAGAAG-3'
Egr-1-binding site 3	5'-ACCCGGCTGCGGCGGCGAGGGAGGA-3'
Egr-1-binding site 3, mutated	5'-ACCCGGCTGCTACGGCGAGGGAGGA-3'
Egr-1-binding site 3, methylated	5'-ACCC ^m GGCTGC ^m GGC ^m GGC ^m GAGGGAGGA-3'
Competitor for Egr-1	5'-GCTCGCGGGGGCGATCGAAT-3'
Nonspecific probe	5'-AGCGAGCTTGCCTTTCTGTCC-3'

C^m, methylated cytosine.

Mutated sequences are underlined.

acid, 16.7 mmol/L Tris-HCl, pH 8.1, and 167 mmol/L NaCl) containing protease inhibitors, 50 μ g/ml yeast tRNA, and 20 μ g/ml sonicated salmon sperm DNA. Immunoprecipitations were performed at 4°C for 2 hours using 550 μ l of diluted supernatant and protein G-Sepharose beads (Pharmacia) conjugated with or without a rabbit IgG antibody against Egr-1 (Santa Cruz Biotechnology). Protein G-Sepharose beads bound with a non-immunized rabbit IgG (Santa Cruz Biotechnology) were used as the negative control. PCR was conducted using LATaq polymerase (Takara), purified DNA fragments in the immunoprecipitates, and the specific primer sets for amplifying *IGFBP-4* or *IGFBP-2* promoter DNA including Egr-1-binding sites (Table 1).

RNA Interference Experiment

Double-stranded small interfering RNAs (25-mer) were designed by RNAi Designer (Invitrogen) and synthesized using Stealth RNAi (Invitrogen). HPL1A-SW cells (1×10^5) were seeded in six-well plates, and 500 pmol of siRNA duplex was transfected using the X-tremeGENE siRNA transfection reagent (Roche Diagnostics, Penzberg, Germany) and Opti-MEM-1 (Invitrogen) according to the manufacturer's instructions. The siRNA sequence for *egr-1* and scrambled control was 5'-AGCAAUUUC-AAUUGUCCUGGGAGA-3' and 5'-AGCGGAUUUAACU-UUACUGGUCAGA-3', respectively. After the siRNA-treated HPL1A-SW cells were cultured for 14 hours, they were harvested and total RNA was extracted.

Bisulfite Sequencing

DNA was extracted from TKB1 and TKB5 cells and then treated with sodium bisulfite using a MethylEasy DNA bisulfite modification kit (Human Genetic Signatures, Macquarie Park, Australia) according to the manufacturer's instructions. In brief, genomic DNA (3 μ g) in a volume of 20 μ l was denatured by NaOH (final concentration, 0.273 mol/L) for 15 minutes at 37°C. The denatured DNA was then treated with 220 μ l of combined reagent 1 and reagent 2 for 16 hours at 55°C. The bisulfite-modified DNA was purified, precipitated, resuspended in reagent 3, and used for PCR. The primers for bisulfite sequencing were constructed using promoter sequence data and primer design software. The primer sequences are shown in Table 1. The PCR products were purified, ligated into the pT7Blue vector (Novagen), and sequenced. At least 10 separate clones were chosen for sequence analysis.

Recombinant IGF-1 Supplementation Experiment on 5-Azacytidine (5-AzaC)-Treated Cells

TKB1 and TKB5 cells were cultured with medium supplemented with a final concentration of 10 μ mol/L 5-azaC for 48 hours, after which 50 μ mol/L rIGF-1 (final concentration) was added and cultured for up to 24 hours. Total RNA was extracted from the harvested cells, and the

Figure 1. A: Expression of phosphorylated MAPK (P-MAPK) and MAPK in stable transfectants, HPL1A-C (empty vector-transfected HPL1A), HPL1A-K (wild-type *K-ras* vector-transfected HPL1A), and HPL1A-SW (mutated *K-ras* vector-transfected HPL1A). The protein extracts from the HPL1A-C, -K, and -SW cells were resolved by SDS-PAGE and blotted with antibodies against phosphorylated MAPK and MAPK. β -Actin (Act-B) served as an internal control. **B:** Growth of HPL1A-C, -K, and -SW cells. HPL1A-SW, HPL1A-K versus HPL1A-C: * $P < 0.05$; HPL1A-SW versus HPL1A-K: † $P < 0.05$. Bar, +SD. **C:** Up-regulation of IGFBP-4 and IGFBP-2 through the transfection of wild-type or mutated *K-ras* gene. Left: Result of a gene chip analysis on IGFBP-4 and IGFBP-2 expression in HPL1A-C, -K, and -SW cells. Relative expression levels of IGFBP-4 and IGFBP-2 mRNAs are shown. Right: Expression of IGFBP-4 and IGFBP-2 proteins in HPL1A-C, -K, and -SW cells. The protein extracts from the HPL1A-C, -K, and -SW cells were resolved by SDS-PAGE and blotted with antibodies against IGFBP-4 and IGFBP-2. β -Actin (Act-B) served as an internal control.

improvement of induction of IGFBP-4, IGFBP-2, and *egr-1* was examined by RT-PCR.

IGFBP-4, IGFBP-2, Phosphorylated MAPK, and *Egr-1* Immunohistochemistry

IGFBP-4, IGFBP-2, phosphorylated MAPK, and *Egr-1* immunohistochemistry were conducted on 33 surgically resected pulmonary adenocarcinomas. Seven of the thirty-three tumors had a mutated *K-ras* gene. This study was approved by the Ethical Committee of Yokohama City University School of Medicine. Formalin-fixed, paraffin-embedded serial sections were dewaxed, and a microwave treatment was conducted to retrieve antigenicity of tissue sections. Sections were immunostained using rabbit anti-IGFBP-4 (Santa Cruz Biochemistry), goat anti-IGFBP-2 (Santa Cruz Biotechnology), mouse monoclonal anti-phosphorylated MAPK (Cell Signaling Technology), and rabbit polyclonal anti-*Egr-1* (Santa Cruz Biotechnology) antibodies and a tyramide-coupled signal amplification procedure using a CSA kit (DAKO, Carpinteria,

CA). Immunostaining was detected with diaminobenzidine (Nichirei, Tokyo, Japan), and nuclei were stained with hematoxylin.

Results

Altered Cell Growth and Gene Expression Caused by Mutated *K-ras*

The success of stable transfection of mutated or wild-type *K-ras* gene (genomic integration) into the cell line HPL1A was confirmed by Southern blotting (data not shown). It was evident that the transfected genes were active because the level of phosphorylated (activated) MAPK increased substantially in HPL1A-SW (stably transfected with mutated *K-ras* gene) and increased modestly in HPL1A-K (stably transfected with wild-type *K-ras* gene) compared with HPL1A-C (stably transfected with empty vector) (Figure 1A). Stable expression of mutated *K-Ras* significantly accelerated cell growth of

Figure 2. **A:** Growth of HPL1D-C, -K, -SW; NHBE-C, -K, -SW; and TKB5-C, -K, and -SW cells. SW or K versus empty vector-transfected (C): * $P < 0.05$; SW versus K: † $P < 0.05$. Bar, +SD. **B:** Expression of phosphorylated MAPK (P-MAPK) and MAPK (top) and IGFBP-4/IGFBP-2 mRNAs (bottom) in each transfectant. The protein extract from each transfectant was resolved by SDS-PAGE and blotted with antibodies against phosphorylated MAPK and MAPK. The number of PCR cycles for IGFBP-4 and IGFBP-2 after the reverse transcription reaction are indicated. β -Actin protein or mRNA (Act-B) served as an internal control for each cell line. **C:** Alteration of P-MAPK and MAPK protein expression in HPL1A-SW (left) and of IGFBP-4 and IGFBP-2 mRNA expression in HPL1A-SW, HPL1D-SW, NHBE-SW, and TKB5-SW (right). Representative data in response to a MEK inhibitor (U0126) are shown at the right (U0126 treatment for 12 hours). The relative expression level of IGFBP-4 or IGFBP-2 mRNA (ratio) was calculated as follows: (density of IGFBP-4 or IGFBP-2/density of β -actin)/(density of IGFBP-4 or IGFBP-2 (U0126(-))/density of β -actin (U0126(-))) $\times 100$. The number of PCR cycles for IGFBP-4 and IGFBP-2 after the reverse transcription reaction is indicated below. β -Actin protein or mRNA (Act-B) served as an internal control in each cell line. **D:** Alteration of cell growth by supplementation with rIGF-1, rIGFBP-4, or rIGFBP-2 in HPL1A, HPL1D, NHBE, and TKB5 cells. Cells with no treatment served as controls. *Statistically significant difference from rIGF-1 ($P < 0.05$). Bar, +SD. **E:** Results of IGFBP-4 or IGFBP-2 neutralization experiment using lung epithelial cell lines transfected with mutated *K-ras* gene, HPL1A-SW, HPL1D-SW, and NHBE-SW. Cell proliferation was evaluated by MTT assay. Absorbance at wavelength 450 nm of each sample was measured. *Statistically significant difference from goat nonimmunized antibody ($P < 0.05$). Bar, $\pm$ SD. **F:** Expression of IGFBP-4 and IGFBP-2 proteins in TKB5-BP4 (stable IGFBP-4 expression vector transfectant) or TKB5-BP2 (stable IGFBP-2 expression vector transfectant). Empty vector-transfected TKB5 (TKB5-C) served as a control. The protein extracts from the TKB5-C, TKB5-BP4, and TKB5-BP2 cells were resolved by SDS-PAGE and blotted with antibodies against IGFBP-4 and -2. β -Actin (Act-B) served as an internal control. **G:** Cell growth of TKB5-C, TKB5-BP4, and TKB5-BP2. Empty vector-transfected TKB5 (TKB5-C) served as a control. *Statistically significant difference from TKB5-C ($P < 0.05$). Bar, $\pm$ SD. **H:** Alteration of cell growth by supplementation with rIGF-1 in TKB5-BP4 or TKB5-BP2. Cells were cultivated in the culture medium containing 0 to 50 ng/ml rIGF-1 and incubated for 48 hours. Empty vector-transfected TKB5 (TKB5-C) served as a control. *Statistically significant difference from TKB5-C ($P < 0.05$). Bar, $\pm$ SD.

HPL1A ($P < 0.05$, HPL1A-SW versus HPL1A-C and HPL1A-K), and wild-type *K-ras* transgene also modestly accelerated cell growth ($P < 0.05$, HPL1A-K versus HPL1A-C) (Figure 1B).

To investigate changes in gene expression caused by mutated *K-ras* in HPL1A cells, we performed a gene chip analysis (HuU95, Affymetrix) using the three stable transfectants, HPL1A-SW, -K, and -C. We focused on genes involved in growth regulation and consequently found that HPL1A-SW cells (carrying mutated *K-ras*) had elevated expression of two kinds of IGFBP mRNAs, encoding IGFBP-4 and IGFBP-2, both of which have potent growth-inhibitory function (Figure 1C, left). The up-regulation of IGFBP-4 and IGFBP-2 expression was also confirmed at the protein level (Figure 1C, right).

To examine whether IGFBP-4 and IGFBP-2 are up-regulated in the other lung airway cell lines and lung cancer cells, we tried to establish clones that stably expressed the mutated or wild-type *K-ras* gene using NHBE cells (a bronchial epithelial cell line), HPL1D (a peripheral airway epithelial cell line), and TKB5 (a lung cancer cell line), and we obtained NHBE-SW, -K, -C, HPL1D-SW, -K, -C, and TKB5-SW, -K, and -C clones, respectively. Mutated *K-Ras* expression significantly accelerated cell growth of NHBE, HPL1D, and TKB5 (NHBE-SW, HPL1D-SW, and TKB5-SW cells) (Figure 2A), and the expression of IGFBP-4 and IGFBP-2 was up-regulated (Figure 2B). However, the basal and inducible expression levels of both IGFBP-4 and IGFBP-2 in TKB5 were extremely low compared with lung airway epithelial cell lines, because the specific products were barely detectable in the RT-PCR reactions in which an additional 10 cycles of PCR was done (Figure 2B).

We next examined whether continuous *K-Ras* activation is actually linked to the up-regulation of IGFBP-4 and IGFBP-2 expression. In response to a MEK inhibitor, U0126, the level of phosphorylated (activated) MAPK protein immediately decreased, followed by gradually decreased expression of IGFBP-4 and IGFBP-2 mRNAs from 2 hours after U0126 treatment (Figure 2C). These findings suggested that IGFBP-4 and IGFBP-2 expression is closely associated with the activation of the *K-Ras*-MEK-MAPK pathway.

Previous reports have indicated that IGFBP-4 and IGFBP-2 inhibit cell growth by binding to IGF-1 and IGF-2.^{33,34} Thus, we used recombinant IGF-1 (rIGF-1), rIGFBP4, and rIGFBP-2 to test the effect of IGFBP-4 and IGFBP-2 on cell growth of lung airway epithelial cells and lung cancer cells. In all cells examined, rIGF-1-mediated growth acceleration was promptly reduced by addition of rIGFBP-4 and rIGFBP-2 to culture media (Figure 2D). Since it was confirmed that abundant IGFBP-4 and IGFBP-2 was secreted in the culture media of mutated *K-ras* gene-transfected lung airway epithelial cells (data not shown), we conducted IGFBP-4/IGFBP-2 neutralization experiments using HPL1A-SW, HPL1D-SW, and NHBE-SW cells and antibodies against IGFBP-4 and IGFBP-2. As shown in Figure 2E, supplementation with anti-IGFBP-4 and IGFBP-2 antibodies significantly raised cell growth rate of examined cell lines. These findings indicated the existence of IGF in the culture media, and

Figure 3. Expression of P-MAPK, MAPK, IGFBP-4, and IGFBP-2 in cultured lung cancer cells. A549 and H820 lung adenocarcinoma cell lines possess mutated *K-ras* gene, whereas TKB6 and TKB14 adenocarcinoma cells and TKB1 and TKB5 large cell carcinoma cells do not. The protein extracts from the each cell line were resolved by SDS-PAGE and blotted with antibodies against phosphorylated MAPK, MAPK, IGFBP-4, or IGFBP-2. Ad, adenocarcinoma; La, large cell carcinoma. β -Actin (Act-B) served as an internal control.

we confirmed by RT-PCR that HPL1A-SW, HPL1D-SW, and NHBE-SW cells express IGF-1/2 and IGF receptor mRNAs (data not shown).

We next established clones of TKB5 lung cancer cells, TKB5-BP4 and TKB5-BP2, that stably expressed IGFBP-4 or IGFBP-2, respectively. We could not, however, establish stable transfectants of the NHBE, HPL1A, and HPL1D epithelial cell lines. It was confirmed that TKB5-BP4 or TKB5-BP2 expressed high levels of IGFBP-4 or IGFBP-2, respectively (Figure 2F), that these proteins were secreted into the culture media (data not shown), and that cell growth of these transfectants was significantly retarded in comparison with the empty vector-transfected clone (TKB5-C) (Figure 2G); moreover, rIGF-1-mediated growth acceleration was significantly diminished in TKB5-BP4 and TKB5-BP2 cells (Figure 2H).

These data suggested that IGFBP-4 and IGFBP-2 act as growth-modulating factors for lung airway epithelial cells and lung cancer cells and that *K-Ras* activation-mediated signaling therefore simultaneously stimulates cell growth and the growth-modulating system via IGFBP-4 and IGFBP-2. We reasoned that these seemingly paradoxical phenomena reflect an inherent feedback mechanism that prevents excessive growth caused by extrinsic growth signals.

IGFBP-4 and IGFBP-2 Expression in Cultured Lung Cancer Cells

To examine IGFBP-4 and IGFBP-2 expression in cancer cells, we conducted IGFBP-4 and IGFBP-2 Western blot analysis on six cultured human lung cancer cell lines. As shown in Figure 3, A549 and H820 adenocarcinoma cells, which have mutated *K-ras* gene, expressed high levels of IGFBP-4 and IGFBP-2. TKB6 and TKB14 adenocarcinoma cells had relatively modest IGFBP-2 ex-

pression and a barely detectable level of IGFBP-4; IGFBP-4/IGFBP-2 levels in TKB1 and TKB5 large cell carcinoma cells, both are derived from the most dedifferentiated form of lung cancer, were extremely low despite of the fact that MAPK was adequately phosphorylated/activated. As shown in Figure 2B, TKB5 cells had quite poor inducibility of IGFBP-4 and IGFBP-2 in comparison with airway epithelial cells, even though the *K-ras* gene was continuously and highly activated. These data strongly suggested that dedifferentiated lung cancer cells are somehow deficient in the signaling pathway between K-Ras activation and IGFBP-4/IGFBP-2 activation, resulting in a disruption of feedback mechanisms against growth acceleration via extracellular growth stimulation.

IGFBP-4 and IGFBP-2 Promoter Activation through Egr-1

To understand how *IGFBP-4* and *IGFBP-2* gene expression is regulated and which kinds of abnormalities cause deficient expression of IGFBP-4 and IGFBP-2 in dedifferentiated lung cancer cells, we further investigated the function of *IGFBP-4* and *IGFBP-2* promoters and the relevant transcription factors. We made constructs carrying a luciferase reporter gene and various lengths of *IGFBP-4* or *IGFBP-2* promoter DNA and transfected them into HPL1A-SW cells. As shown in Figure 4A, as the promoter fragment lengths were shortened, IGFBP-4 promoter activity gradually declined from the pGL4.10IGFBP4-522; IGFBP-2 promoter activity of pGL4.10IGFBP2-444, which had a 78-bp deletion of the distal region, declined up to 35% compared with the vector encoding the full promoter. The pGL4.10IGFBP2-421 and pGL4.10IGFBP2-314 also revealed low luciferase activity. However, the activity gradually strengthened in going from the pGL4.10IGFBP2-206 to the pGL4.10IGFBP2-134 constructs. These results suggested that transacting factor-binding sites are scattered across a 750-bp region from the transcription start site of the *IGFBP-4* promoter, that repressive element is located in the middle region in the *IGFBP-2* promoter, and that the downstream region of the *IGFBP-2* promoter is crucial for *IGFBP-2* gene activation.

Next, we searched for putative transcription factor-binding sites in the *IGFBP-4* and *IGFBP-2* promoter sequences using computer software. Based on the search data and results from the IGFBP-4 and IGFBP-2 promoter assays, we focused on the transcription factor, Egr-1, which is one of the immediate early response genes that is activated via the MEK/ERK-MAPK signaling pathway in response to growth factors such as IGF, HGF, and EGF.^{35–38} The gene chip analysis demonstrated consistent results that *egr-1* mRNA in HPL1A-SW cells increased 2.8-fold and 2.5-fold in comparison with HPL1A-C and HPL1A-K, respectively, and continuous up-regulation of *egr-1* mRNA was confirmed in mutated *K-ras* gene-transfected lung epithelial cells (Figure 4B). The Egr family consensus-binding sequence, 5'-GCGGGGGCG-3', and several putative Egr-1-binding sites were identified in the regions that are crucial for

IGFBP-4 and *IGFBP-2* gene activation (Figure 4A). To obtain direct evidence, we conducted EMSAs using recombinant Egr-1 proteins and oligonucleotide probes containing Egr-1-binding consensus motif or putative Egr-1-binding sites predicted by computer analysis (Table 2), and it was confirmed that IGFBP-4 and IGFBP-2 promoters possess five and three Egr-1-binding sites, respectively (Figure 4C). To examine whether Egr-1 actually binds to the *IGFBP-4* and *IGFBP-2* genes, we performed a chromatin immunoprecipitation-based PCR analysis using HPL1A-SW cells with or without U0126 treatment. As shown in Figure 4D, two primer sets specific for *IGFBP-4* promoter amplified 392-bp and 448-bp DNA fragments, which contained Egr-1-binding site 1 to site 3 and site 2 to site 5 sequences, respectively, and the primer set specific for *IGFBP-2* promoter amplified 315-bp DNA fragments containing Egr-1-binding site 1 to site 3 sequences. It was also evident that Egr-1 binding to the *IGFBP-4* or *IGFBP-2* promoter was decreased by the U0126 treatment (Figure 4D). The reduction of *egr-1* mRNA expression after treatment with U0126 was paralleled by a reduction in IGFBP-4 and IGFBP-2 (Figure 4E). Furthermore, a small interfering RNA (siRNA) targeted to *egr-1* promptly repressed IGFBP-4 and IGFBP-2 mRNA expression (Figure 4F).

To examine in detail the functionality of the Egr-1 binding sites, we conducted mutational analysis using *IGFBP-4/2* promoter sequences with mutation in each Egr-1 binding site. We confirmed that Egr-1 did not bind to the synthesized mutated sequences of Egr-1-binding sites (Table 2 and Figure 4G, top). The mutational analysis using mutated *IGFBP-4/2* promoter-inserted luciferase vectors revealed that the mean promoter activity of *IGFBP-4* declined to 73.8% (mutation in Egr-1-binding site 1), 66.3% (mutation in site 2), 77.8% (mutation in site 3), 53.7% (mutation in site 4), and 52.3% (mutation in site 5) and that the mean promoter activity of *IGFBP-2* declined to 77.0% (mutation in Egr-1-binding site 1), 21.5% (mutation in site 2), and 57.8% (mutation in site 3), compared with the vector encoding the respective normal promoter (Figure 4G, bottom). These results confirmed that Egr-1 induction through the activation of the Ras-MEK-MAPK signaling pathway is involved in IGFBP-4 and IGFBP-2 expression in lung airway epithelial cells.

Hypermethylation of IGFBP-4 and IGFBP-2 Promoters in Lung Cancer Cells

The levels of basal and induced expression of IGFBP-4 and IGFBP-2 were quite low in TKB1 and TKB5 large cell carcinoma cells despite significant activation of the MEK-MAPK signaling pathway (Figures 2B and 3). Because IGFBP-4, IGFBP-2, and *egr-1* promoters contain CpG islands (Figure 5A), we next investigated whether the methylation status of *IGFBP-4*, *IGFBP-2*, and *egr-1* promoters correlated with deficient expression/induction of IGFBP-4 and IGFBP-2 in lung cancer cells. PCR using bisulfite-treated DNA was performed and the PCR products were cloned and sequenced. As shown in Figure 5A, TKB1 and TKB5 cells revealed severe methylation in and

A

B

C

D

E

F

G

around the Egr-1-binding sites of the *IGFBP-4* promoter. The Egr-1-binding sites in the *IGFBP-2* promoter of TKB1 and TKB5 cells were also considerably methylated, whereas no methylation was detected in the examined regions of the *egr-1* promoter. To confirm decreased Egr-1 binding affinity to the Egr-1-binding sites containing methylated CpGs, we conducted an EMSA using Egr-1-binding site-specific probes with methylation of CpG dinucleotides and compared the binding affinity between unmethylated and methylated probes. As shown in Figure 5B, the signal densities of Egr-1-probe complexes were decreased by CpG methylation. Furthermore, we tested whether IGFBP-4 and IGFBP-2 expression in TKB1 and TKB5 cells could be recovered by demethylating the genomic DNA using 5-azaC. The induction of *egr-1* was complete within 1 hour after rIGF-1 supplementation (data not shown), and IGFBP-4 and IGFBP-2 induction was apparently improved by treatment with 5-azaC (Figure 5C).

IGFBP-4, IGFBP-2, Phosphorylated MAPK, and Egr-1 Expression in Lung Adenocarcinoma Tissue

Finally, we performed immunohistochemistry for IGFBP-4, IGFBP-2, phosphorylated MAPK, and Egr-1 on surgically resected pulmonary adenocarcinoma tissues and compared their expression between adenocarcinoma cells and nonneoplastic airway epithelial cells. The results are summarized in Table 3 and representative microphotographs are shown in Figure 6, A–D. In cases in which IGFBP-4 and IGFBP-2 staining was positive in adenocarcinoma cells, we noted that the nonneoplastic airway epithelium involved in or adjacent to cancerous tissues often expressed high levels of IGFBP-4 and IGFBP-2 (Figure 6, A and B; Table 3). The signal of phosphorylated MAPK and Egr-1 was localized in the nuclei of the adenocarcinoma cells and nonneoplastic airway epithelial cells (Figure 6, C and D). Adenocarcinoma cells carrying a mutated *K-ras* gene intensely expressed IGFBP-4 and IGFBP-2, whereas ~88 and 39% of cases revealed

focal weak or no expression of IGFBP-4 and IGFBP-2 in adenocarcinoma cells without *K-ras* gene mutation, respectively (Table 3). These immunohistochemical findings were completely consistent with *in vitro* data concerning phosphorylated MAPK, Egr-1, IGFBP-4, and IGFBP-2 expression in lung airway epithelial cells and lung cancer cells.

Discussion

K-Ras is activated on activation of receptor tyrosine kinase signaling, resulting in the activation of the MEK-MAPK signaling pathway. It is generally accepted that the activation of the MEK-MAPK signaling pathway regulates cell cycle progression and modulates the activity of apoptotic factors.³⁹ In this study, we investigated the alteration of gene expression caused by transfection with a mutated *K-ras* gene in lung airway epithelial cells, and we found that two kinds of growth-modulating molecules, IGFBP-4 and IGFBP-2, are up-regulated via the activation of the K-Ras-MEK-MAPK-Egr-1 pathway in growth-accelerated lung airway epithelial cells. Egr-1 is an immediately responsive zinc finger transcription factor^{40,41} and is rapidly and transiently expressed on various stimuli such as growth factors and acute physical injury via the MEK/ERK-MAPK signaling pathway.^{35–38,42} Dai and colleagues³⁰ reported the functional promoter for human *IGFBP-4* gene and referred to the possible contribution of Egr-1 to IGFBP-4 expression on the basis of existence of consensus Egr-1-binding sequence in the promoter. We demonstrated here that Egr-1 is continuously up-regulated in lung airway epithelial cells transfected with a mutated *K-ras* gene (Figure 4B) and that Egr-1 actually binds to the IGFBP-4 and IGFBP-2 promoters and induces IGFBP-4/2 expression (Figure 4, C–G). Cancer cells produce various kinds of growth factors/cytokines and thus undergo accelerated growth via autocrine and paracrine mechanisms.^{43,44} Our study demonstrates that IGFBP-4, IGFBP-2, phosphorylated MAPK, and Egr-1 are often intensely expressed *in vivo* in nonneoplastic airway epithelial cells localized in and around cancerous lesions

Figure 4. A: Luciferase assay using various lengths of *IGFBP-4* (top) or *IGFBP-2* (bottom) promoter sequence-inserted pGL4.10 luciferase vector. Egr-1-binding sites (open circles), TATA boxes (closed boxes), and luciferase cDNAs (open boxes) are indicated. Transfections were performed in quadruplicate, and experiments were performed at least three times. The results are shown as mean $\pm$ SD. **B:** Expression of *egr-1* mRNA in HPL1A-C, -K, and -SW cells. Semiquantitative RT-PCR using specific primers for *egr-1* cDNA was conducted. β -Actin (Act-B) served as an internal control. **C:** Top, EMSA using recombinant Egr-1 protein (Egr-1) and putative Egr-1-binding site-specific double-stranded probes. The sequence of putative Egr-1-binding sites and competitors are described in Table 2. Competition assay was done in the presence of 100-fold molar excess of unlabeled double-stranded oligonucleotides possessing consensus Egr-1-binding motif (Cons) or nonspecific nucleotide sequences (NS). Bottom, supershift assay conducted using rabbit anti-Egr-1 IgG (Egr-1) or nonimmunized rabbit IgG (NI) antibody. **Arrow**, specific signal; **arrowhead**, supershifted signal. **D:** Ch-IP-based PCR analysis using HPL1A-SW cells with or without U0126 treatment for 4 hours. Formaldehyde-treated cells were sonicated, and DNA fragments conjugated with nuclear proteins were immunoprecipitated with or without rabbit anti-Egr-1 IgG antibody (Egr-1 IgG and no Ab, respectively), or with nonimmunized rabbit IgG antibody (rab IgG). Two primer sets specific for *IGFBP-4* promoter were prepared to amplify 392-bp and 448-bp DNA fragments containing Egr-1-binding site 1 to site 3 and site 2 to site 5 sequences, respectively. The primer set specific for *IGFBP-2* promoter was prepared to amplify 315-bp DNA fragments containing Egr-1-binding site 1 to site 3 sequences. Input, input DNA; M, ladder marker. **E:** Alteration of *egr-1*, IGFBP-4, and IGFBP-2 mRNA expression after treatment with the MEK inhibitor U0126. The relative expression level of IGFBP-4 or IGFBP-2 mRNA was calculated as follows: (density of IGFBP-4 or IGFBP-2)/(density of β -actin) $\times$ 100. NT, no treatment with U0126. β -Actin (Act-B) served as an internal control. M, ladder marker. **F:** Forced repression of *egr-1* and after alteration of IGFBP-4/2 expression in HPL1A-SW cells. The *egr-1*-specific or scrambled control siRNA was treated on HPL1A-SW cells for 14 hours. β -Actin served as an internal control. Relative expression level of IGFBP-4 or IGFBP-2 mRNA (ratio) was calculated as follows: (density of IGFBP-4 or IGFBP-2)/(density of β -actin) $\times$ 100. NT, no treatment with U0126. **G:** Top, EMSA using recombinant Egr-1 protein (Egr-1) and Egr-1-binding site-specific double-stranded probes or respective mutant probes. The sequences of wild-type or mutated Egr-1-binding sites are described in Table 2. **Arrow**, specific signal. Bottom, luciferase assay using wild-type or mutated *IGFBP-4* or *IGFBP-2* promoter sequence-inserted pGL4.10 luciferase vector. Mutated sequences of Egr-1-binding sites are described in Table 1. Wild-type Egr-1-binding sites (open circles), mutated Egr-1-binding sites (closed circles), TATA boxes (closed boxes), and luciferase cDNAs (open boxes) are indicated. Transfections were performed in quadruplicate, and experiments were performed at least three times. The results are shown as mean $\pm$ SD.

(Figure 5—continues)

Figure 5. A: Structures of *IGFBP-4*, *IGFBP-2*, and *egr-1* promoters and results of bisulfite sequencing using specific primer sets described in Table 1. Bars with bilateral open boxes and nucleotide number indicate sequencing regions with respective forward and reverse primers and their position relative to the transcription start site. Methylated or unmethylated CpG sites in the *IGFBP-4*, *IGFBP-2*, and *egr-1* promoters are indicated by closed or open circles, respectively. Egr-1-binding sites (site 1 to site 5 of *IGFBP-4* promoter and site 1 to site 3 of *IGFBP-2* promoter) are indicated by open boxes. **B:** EMSA using recombinant Egr-1 protein and Egr-1-binding site-specific probes with or without methylated cytosine. The sequences of used probes are described in Table 2. **Arrow**, specific signal. UM, oligonucleotide probe with unmethylated CpGs; M, oligonucleotide probe with methylated CpGs. **C:** Restoration of rIGF-1-induced IGFBP-4 and IGFBP-2 expression via 5-azaC treatment in TKB1 and TKB5. TKB1 and TKB5 cells were cultured with a 10 μ mol/L 5-azaC for 48 hours. Then, rIGF-1 (final concentration, 50 μ mol/L) was added and cells were further cultivated for up to 24 hours. Representative data (6 hours after supplementation with rIGF-1) are shown. β -Actin (Act-B) served as an internal control.

(Figure 6 and Table 3). Minuto and colleagues⁴⁵ reported that the concentration of IGF-1 is elevated in lung cancerous tissue. Our RT-PCR analyses provide evidence that lung cancer cells often express IGF-1/2 mRNA and IGF receptors (data not shown). Narasaraaju and colleagues⁴⁶ reported the IGF and IGF receptor expression in rat airway epithelial cells during lung injury and recovery. We confirmed that mutated *K-ras* gene-transfected lung airway epithelial cells produce IGFs and express IGF receptors (data not shown) and that cell growth is actually accelerated by neutralization using antibodies against IGFBP-4 and IGFBP-2 (Figure 2E). Therefore, IGFBP-4 and IGFBP-2 expression in the *in vivo* airway epithelial cells localized in and adjacent to cancerous lesions might result from locally acting growth factors/cytokines secreted from lung cancer cells and themselves. In this study, we conducted reporter gene assays using various lengths of *IGFBP-4* and *IGFBP-2* promoter elements. It has been shown that estrogen receptor-Sp1 complexes⁴⁷ or Sp3 (Sp3-binding site: -861 nucleotides to -856 nucleotides from the transcription start site)⁴⁸ regulates the promoter activity and thus the expression of human IGFBP-4. Our search for putative transcription factor-binding sites yielded two putative sites for thyroid transcription factor 1 (TTF-1), which is specifically expressed in lung airway epithelial cells, in the distal region of the human *IGFBP-4* promoter (-1059 nucleotides to -1045 nucleotides, and -922 nucleotides to -908 nucleotides from the transcription start site). However, both Sp3 and TTF-1 are not considered to play fundamental roles in *IGFBP-4* gene induction via continuous *K-ras* gene activation in lung airway epithelial cells, because the luciferase activity of the vector lacking the Sp3- and TTF-1-binding sites (pGL4.10IGFBP4-855) did not differ significantly from that of pGL4.10IGFBP4-1298 (full-length *IGFBP-4* promoter). Sp1 binds to GC-elements

and has consensus DNA-binding motif (GGGCGG). The motif was overlapped in our examined Egr-1-binding sites 1 and 2 of *IGFBP-4* promoter (Table 2), and we confirmed that Sp1 actually binds to these sites (data not shown). Although the results of the gene chip analysis showed no considerable up-regulation of Sp1 expression through the wild-type or mutated *K-ras* gene transfection (HPL1A-C/HPL1A-K = 1:1.1 and HPL1A-K/HPL1A-SW = 1:1.2, respectively), Sp1 could participate in basal expression of IGFBP-4.

With regard to the mechanisms governing *IGFBP-2* gene expression, our reporter gene assays indicated that distal region of the *IGFBP-2* promoter, as well as proximal region, is crucial for *IGFBP-2* gene activation (Figure 4A). Because the E-box is located in the distal region of the *IGFBP-2* promoter (-465 nucleotides to -460 nucleotides from the transcription start site) and the chromatin immunoprecipitation assays showed that USF-1 binds to the E-box (data not shown), USF-1 might be a putative important transcription factor for *IGFBP-2* gene activation. Previous reports found that the binding of Sp1 to the GC box in the proximal region of the rat *IGFBP-2* promoter is essential for promoter activity.⁴⁹ The human and rat *IGFBP-2* promoters are similar in terms of being TATA-less and having a potent Sp1-binding site in the proximal region.³¹ Although Sp1 expression was not up-regulated by continuous *K-ras* gene activation as mentioned above, we confirmed through the EMSA and chromatin immunoprecipitation assay that Sp1 actually binds to the *IGFBP-2* promoter (data not shown). Therefore, Sp1 might be an important transcription factor for basal IGFBP-2 expression. Cazals and colleagues⁵⁰ reported that NF-kappa B is involved in transcriptional activation of rat IGFBP-2 in oxidant-exposed lung alveolar epithelial cells. Our database search for putative transcription factor-binding sites in the human *IGFBP-2* promoter revealed one potent

Table 3. *In Vivo* IGFBP-4 and IGFBP-2 Expression in Nonneoplastic Airway Epithelial Cells and Adenocarcinoma Cells

Adenocarcinoma with K-ras mutation (7 patients)						Adenocarcinoma without K-ras mutation (26 patients)					
Cancer cell						Cancer cell					
	IGFBP-4	++	+	+/-	-		IGFBP-4	++	+	+/-	-
Nonneoplastic Epithelial cell*	++	6/7 (86%)	1/7 (14%)	0/7 (0%)	0/7 (0%)	Nonneoplastic Epithelial cell	++	1/26 (4%)	1/26 (4%)	1/26 (4%)	0/26 (0%)
	+	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		+	0/26 (0%)	1/26 (4%)	0/26 (0%)	2/26 (8%)
	+/-	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		+/-	0/26 (0%)	0/26 (0%)	2/26 (8%)	2/26 (8%)
	-	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		-	0/26 (0%)	0/26 (0%)	0/26 (0%)	16/26 (60%)
Cancer cell						Cancer cell					
	IGFBP-2	++	+	+/-	-		IGFBP-2	++	+	+/-	-
Nonneoplastic Epithelial cell	++	6/7 (86%)	1/7 (14%)	0/7 (0%)	0/7 (0%)	Nonneoplastic Epithelial cell	++	4/26 (15%)	4/26 (15%)	0/26 (0%)	0/26 (0%)
	+	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		+	0/26 (0%)	8/26 (31%)	5/26 (19%)	1/26 (4%)
	+/-	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		+/-	0/26 (0%)	0/26 (0%)	2/26 (8%)	0/26 (0%)
	-	0/7 (0%)	0/7 (0%)	0/7 (0%)	0/7 (0%)		-	0/26 (0%)	0/26 (0%)	0/26 (0%)	2/26 (8%)

++, Diffusely and intensely positive; +, focally and intensely positive; +/-, focally and weakly positive; -, negative; (%), frequency of positive cases.

*Nonneoplastic airway epithelial cell localized in or adjacent to adenocarcinoma lesion.

c-Rel/NF- κ B-binding site (-166 nucleotides to -156 nucleotides from the transcription start site), which is localized in the shortened region between pGL4.10IGFBP2-206 and pGL4.10IGFBP2-134. However, the luciferase activity of pGL4.10IGFBP2-134 was greater than that of pGL4.10IGFBP2-206; therefore, the NF- κ B site is not considered to be strongly involved in human *IGFBP-2* promoter activation in concert with continuous *K-ras* gene activation in lung airway epithelial cells.

It is widely accepted that cancer is a multistep process with a genetic basis and that *K-ras* gene mutations are involved in early-stage tumorigenesis of lung adenocarcinoma.^{20-24,51} Our previous studies and this current study demonstrated that continuous *K-ras* gene activa-

tion via mutated *K-ras* transgene actually up-regulates growth and motility of immortalized lung airway epithelial cells.¹⁵ We had obtained evidence that mutated *K-ras* transgene causes senescence to primarily cultured lung epithelial cells (unpublished observations). These findings and observations suggest that the event of *K-ras* gene mutation is enough for excessive growth of transformed lung airway epithelial cells to form early tumor lesions like atypical adenomatous hyperplasia. However, Yoshida and colleagues²⁴ described that atypical adenomatous hyperplasia with *EGFR* mutation could develop into invasive adenocarcinoma with fewer genetic alterations and consequently shorter time than that with *K-ras* mutation. We demonstrated in this study that *K-ras* gene

Figure 6. Immunohistochemistry using sister sections of a case of adenocarcinoma and antibodies against IGFBP-4 (A), IGFBP-2 (B), phosphorylated MAPK (C), and Egr-1 (D). IGFBP-4 (A) and IGFBP-2 (B) are expressed both in adenocarcinoma cells (left) and nonneoplastic airway epithelial cells (right). The signals of phosphorylated MAPK (C) and Egr-1 (D) are localized in the nuclei of the adenocarcinoma cells and nonneoplastic airway epithelial cells.

activation stimulates not only cell growth but also growth-modulating system via IGFBP-4 and IGFBP-2, simultaneously. These facts imply that *K-ras* mutation alone is insufficient, and accumulation of multiple genetic alterations including DNA hypermethylation is necessary for lung neoplastic cells to gain aggressiveness.

DNA hypermethylation is often seen in cancer cells and is important for gene silencing.⁵² The lung cancer cell lines we examined, TKB1 and TKB5, were deficient for induction of IGFBP-4 and IGFBP-2 despite the fact that MAPK was activated sufficiently for this purpose, even under the conditions of basal culture, transfection with mutated *K-ras*, or stimulation by IGF-1. Because *egr-1*, *IGFBP-4*, and *IGFBP-2* promoters contain CpG islands, we postulated that promoter methylation might be relevant to the promoter activity of these genes in TKB1 and TKB5 cells. Actually, the *IGFBP-4* promoter including *Egr-1*-binding sites was severely methylated, and the *Egr-1*-binding sites in *IGFBP-2* promoter were also highly methylated (Figure 5A), and methylation of *Egr-1*-binding sites reduced *Egr-1*-binding affinity (Figure 5B). However, CpG methylation was hardly detected in the *egr-1* promoter (Figure 5A). Although we are not able to properly explain the basis for the differences in methylation patterns between *IGFBP-4/2* and *egr-1* in this study, it is reasonable to propose that IGFBP-4/2-mediated growth regulatory systems become deficient as lung cancers gain aggressiveness through gene silencing by promoter hypermethylation. To our knowledge, this is the first report to describe the hypermethylation of *IGFBP-4* and *IGFBP-2* promoters in cancer cells. Further studies are necessary to understand the mechanistic basis of differences in methylation patterns between *IGFBP-4/2* and *egr-1* promoters.

In summary, we have demonstrated that continuous *K-ras* gene activation facilitated by transfection of a mutant *K-ras* in lung airway epithelial cells not only accelerates cell growth but also activates a growth regulatory system via induction of IGFBP-4 and IGFBP-2. Our data also show that *Egr-1* plays a key role in the induction of IGFBP-4 and IGFBP-2 and that the growth regulatory system becomes deficient because of hypermethylation of *IGFBP-4* and *IGFBP-2* promoters. These findings suggest that the growth-modulating systems via IGFBP-4 and IGFBP-2 are considered as one of the feedback mechanisms maintained in lung airway epithelia to prevent excessive growth on stimulation with extracellular growth factors and that the growth regulatory system becomes disrupted as lung cancers gain aggressiveness.

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