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Neuroendocrine Cancer-Specific Up-Regulating Mechanism of Insulin-Like Growth Factor Binding Protein-2 in Small Cell Lung Cancer

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Small cell lung cancer (SCLC) exhibits insulin-like growth factor-dependent growth. SCLC is the most aggressive among known *in vivo* lung cancers, whereas *in vitro* growth of SCLC is paradoxically slow as compared with that of non-SCLC (NSCLC). In this study, we demonstrate that SCLC cells overexpress insulin-like growth factor binding protein (IGFBP)-2 via NeuroD, a neuroendocrine cell-specific transcription factor. Chromatin immunoprecipitation, electrophoretic mobility shift, and IGFBP-2 promoter assays all revealed that NeuroD binds to the E-box in the 5'-untranslated region of IGFBP-2. A NeuroD transgene in both airway epithelial and NSCLC cells up-regulated the transcription of IGFBP-2 and retarded cell growth. Recombinant IGFBP-2 repressed the growth of both airway epithelial and NSCLC cells in a dose-dependent manner. A NeuroD-specific small interfering RNA repressed IGFBP-2 expression in SCLC, and neutralization of IGFBP-2 and an IGFBP-2-specific small interfering RNA increased SCLC cell growth. Pathological samples of SCLC also expressed IGFBP-2 abundantly, as compared with NSCLC, and showed only rare (8%) IGFBP-2 promoter methylation, whereas

the IGFBP-2 promoter was methylated in 71% of adenocarcinomas and 29% of squamous cell carcinomas. These findings suggest that 1) SCLC has an IGFBP-2 overexpression mechanism distinct from NSCLC, 2) secreted IGFBP-2 contributes to the slow growth of SCLC *in vitro*, and 3) the epigenetic alterations in the IGFBP-2 promoter contribute to the striking differences in IGFBP-2 expression between SCLC and NSCLC *in vivo*. (Am J Pathol 2009, 175:976–987; DOI: 10.2353/ajpath.2009.081004)

Small cell lung cancer (SCLC) is distinct from non-SCLC (NSCLC) in terms of cell differentiation, disease progression, radiosensitivity, chemosensitivity, and patient prognosis.¹ Clinically, SCLC is one of the most aggressive malignant human neoplasms, due to its rapid growth and high metastatic potential. However, SCLC cells characteristically grow extremely slowly *in vitro*.² *In vitro* cell growth of SCLC cells is dependent on insulin-like growth factor (IGF), and SCLC cells use IGFs and gastrin-releasing peptide as autocrine signals *in vitro* and *in vivo*.^{3–5} Thus, IGF signaling is considered quite important for SCLC cell proliferation and may underlie the discrepancy between SCLC cell growth *in vitro* and *in vivo*.

SCLC and NSCLC cells produce IGF-binding proteins (IGFBPs).^{6–8} IGFBP-2 is one member of the IGFBP family of proteins. When secreted, IGFBP-2 binds to IGF-1/-2 and interferes with IGF receptors binding, which results in

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an insufficient response to IGF stimulation.^{9,10} Expression of early growth response-1 (*Egr-1*) is closely associated with IGFBP-2 induction, and heavy methylation of CpG islands in the *IGFBP-2* promoter causes *IGFBP-2* silencing in anaplastic NSCLC cell lines.¹¹

SCLC cells express various neuroendocrine cell-specific molecules, such as neural cell adhesion molecule (CD56), chromogranins, and synaptophysin.¹ NeuroD and achaete-scute homolog-like 1 (*ASCL1*), which are neuroendocrine cell-specific transcription factors, are also expressed in SCLC.^{12–15} NeuroD and *ASCL1* are members of a family of basic helix-loop-helix (bHLH) transcription factors associated with neurogenic cell fate/differentiation and play concomitant roles in neural, endocrine, and neuroendocrine development.¹⁶ *ASCL1* is expressed in neuronal precursor cells and NeuroD is important in hippocampal, cerebellar, retinal, and inner ear development, as well as maturation of pancreatic islet cells and differentiation of enteroendocrine cells in the small intestine.^{17–25} In the lungs, NeuroD deficiency increases neuroepithelial bodies and decreases the number of solitary pulmonary neuroendocrine cells, whereas *ASCL1* deficiency decreases neuroepithelial bodies.^{26,27} *ASCL1* is highly expressed in SCLC and has, therefore, been used as a diagnostic marker for neuroendocrine tumors.^{12,14} With the exception of their roles in development, however, the molecular mechanisms underlying the distinctive features of neuroendocrine cancers expressing NeuroD and *ASCL1* remain largely unresolved.

In the present study, we investigated IGFBP-2 expression in SCLC and unexpectedly observed that IGFBP-2 is more abundant in SCLC cell lines than in NSCLC cell lines. We speculated the existence of a SCLC-specific activating mechanism of *IGFBP-2* and tested whether binding of neuroendocrine cell-specific transcription factors could explain the overexpression of IGFBP-2. We also conducted immunohistochemistry and examined methylation status of the *IGFBP-2* promoter to verify overexpression of IGFBP-2 in pathological samples of SCLC.

Materials and Methods

Cells and Cell Culture

Seven human SCLC cell lines (TKB12, TKB15, TKB16, TKB17, Lu134A, Lu139, and Lu140), six NSCLC cell lines (adenocarcinoma lines TKB6 and TKB14, squamous cell carcinoma lines TKB4 and TKB20, and large cell carcinoma lines TKB1 and TKB5), and two SV40-immortalized peripheral lung epithelial cell lines (HPL1D and HPL1A) were used in this study.^{11,28,29} SCLC cells were cultivated in RPMI 1640 medium and NSCLC and lung epithelial cells were cultivated in Dulbecco's modified Eagle's medium. Both media were supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in 5% CO₂, and semiconfluent cells were used for the following experiments. For population doubling analysis,

2.0×10^5 cells were seeded onto 100-mm culture dishes and grown to semiconfluency. Cells were counted, and 2.0×10^5 cells were again seeded onto 100-mm dishes. Several passages were repeated in the same manner. The sum of population doublings (PDLs) at each point was calculated using the formula: $\Sigma PDL_n = \log_2 (\text{count}_n / 2.0 \times 10^5) + \Sigma PDL_{n-1}$. To measure secreted IGFBP-2 in culture medium, 1.0×10^6 cells were seeded onto 100-mm dishes and cultivated for 48 hours. Recovered media were centrifuged at $15,000 \times g$ for 5 minutes and used for enzyme-linked immunosorbent assay as described below.

Western Blot Analysis

Whole-cell lysates (50 μ g protein/lane) were separated by 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher & Schell, Keene, NH). Membranes were blocked for 1 hour at room temperature with 1% skim milk in PBS containing 0.1% (v/v) Tween 20 (PBS-T) and then incubated with diluted goat polyclonal anti-IGFBP-2 (C-18, Santa Cruz Biotechnology, Santa Cruz, CA),¹¹ goat polyclonal anti-NeuroD (N-19, Santa Cruz Biotechnology), or mouse monoclonal anti-*ASCL1* (BD Biosciences Pharmingen, San Jose, CA)²⁸ for 1 hour at room temperature. After washing three times for 10 minutes with PBS-T at room temperature, membranes were incubated at room temperature for 30 minutes with a diluted peroxidase-labeled secondary antibody against goat (Santa Cruz Biotechnology) or mouse (Amersham Biosciences, Buckinghamshire, UK). The membranes were then washed three times for 10 minutes with PBS-T at room temperature, and immunopositive signals were visualized using an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences). Mouse monoclonal anti- β -actin (Sigma-Aldrich, St. Louis, MO) was used for the internal control.

Reverse Transcription-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), and cDNA synthesis and subsequent PCR reactions were performed using an RNA-LAPCR kit (Takara, Shiga, Japan) according to manufacturers' instructions. β -Actin served as the internal control. The number of PCR cycles was set at 35 for *egr-1*, NeuroD, *ASCL1*, IGF-1, and IGF-2; at 25 or 35 for IGFBP-2; and at 22 for β -actin. The forward and reverse primer sequences for *egr-1*, NeuroD, *ASCL1*, IGF-1, IGF-2, IGFBP-2, and β -actin are listed in Table 1. Densitometry scanning was conducted using NIH *Image* software (Rockville, MD).

IGFBP-2 Promoter Assay

Luciferase vectors (pGL4.10/*uc2*, Promega, Madison, WI) with various lengths of *IGFBP-2* promoter fragments inserted were used: pGL4.10BP2-522 (full-length *IGFBP-2* promoter inserted), -444, -314, -206, -134, and

Table 1. PCR Primers Used in this Study*

Target	Primer sequences [†]
<i>NeuroD</i>	F 5'-CCCCAGGGTTATGAGACTAT-3' R 5'-ACTTTGATCCCCGTGTTTCTTC-3'
<i>ASCL1</i>	F 5'-CAGCGCCCAAGCAAGTCAAGC-3' R 5'-GGCCATGGAGTTCAAGTCGTT-3'
<i>IGFBP-2</i>	F 5'-TTGCAGACAATGGCGATGACC-3' R 5'-GGGATGTGCAGGGAGTAGAGG-3'
<i>egr-1</i>	F 5'-GCAGCAGCAGCACCTTCAACC-3' R 5'-TTTCCCCTTTCCCTTTAGCAA-3'
<i>IGF-1</i>	F 5'-TCTTCAGTTCGTGTGTGGAGACAG-3' R 5'-CTCCGGAAGCAGCACTCATC-3'
<i>IGF-2</i>	F 5'-TCGTGCTGCATTGCTGCTTAC-3' R 5'-AGGTACAGCTGCGGAAACA-3'
<i>b-actin</i>	F 5'-GCGGGAATCGTGCGTGACAT-3' R 5'-GTGGACTTGGGAGAGGACTGG-3'
Ep site-mutated <i>IGFBP-2</i> promoter	F 5'-CCGTGCGAGTTCCCGCCCGCCGCTCGCTCGCTCG-3' R 5'-CGAGCGAGCGAGCGGGCGGGCGGGGAACTCGCACGG-3'
Ed site-mutated <i>IGFBP-2</i> promoter	F 5'-CCAAGCGAGTTCTCAGTGGTGGGTGATACCCAGGA-3' R 5'-TCCTGGGGTATCACCACCAGTGAAGCTCGCTTGG-3'
Ep-containing proximal area to <i>IGFBP-2</i> coding region (for ChIP)	F 5'-TCGCGAACTGAACTGAGAGCA-3' R 5'-CTGGCGGTGCGGCAGC-3'
Methylated <i>IGFBP-2</i> promoter (for MSP)	F 5'-TTTTAGAGTTCGTAGTTAACGCG-3' R 5'-ACCGAATCCTAAAAAACCGAC-3'
Unmethylated <i>IGFBP-2</i> promoter (for MSP)	F 5'-TTTTAGAGTTTGTAGTTAATGTGG-3' R 5'-CAACCAAATCCTAAAAACCAAC-3'

*F and R, forward and reverse primer sequences; Ep and Ed, E-box located proximal and distal to the *IGFBP-2* coding region; ChIP, chromatin immunoprecipitation; MSP, methylation-specific PCR.

[†]Mutated sequences are underlined.

–31.¹¹ For mutational analysis, full-length *IGFBP-2* promoter sequences with a distal (Ed) or proximal (Ep) E-box mutation located at –464 to –459 or + 55 to + 60 (each number indicates the 5' nucleotide position from the transcription start site [+1]), respectively, were constructed using the QuickChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA), specific mutagenic primer sets (Table 1), and a full-length *IGFBP-2* promoter-containing pT7 cloning vector, as described.¹¹ Cultured SCLC or HPL1A cells were transiently transfected using FuGENE6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer's instructions. Cells (1.0×10^5) were seeded in six-well plates and incubated for 24 hours. Cells were then co-transfected with 1 μ g/well of reporter vector (*IGFBP-2* promoter-containing pGL4.10/*uc2*) and 50 ng/well of control vector (pGL4.74TK, Promega). Twenty-four hours after transfection, cells were carefully washed with PBS, and luciferase activity was measured using a luminometer (Turner Biosystems, Sunnyvale, CA) and a Dual-Luciferase Reporter Assay System (Promega) according to manufacturers' instructions. Transfections were performed in triplicate, and each sample was measured three times.

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared using a CellLytic NuCLEAR Extraction kit (Sigma, St. Louis, MO). Cells were lysed with lysis buffer (10 mmol/L Tris-HCl pH 7.5, 2 mmol/L MgCl₂, 3 mmol/L CaCl₂, 300 mmol/L sucrose) containing 0.1% IGEPAL CA-630, 1 mmol/L dithiothreitol, and a protease inhibitor cocktail, and nuclear proteins

were extracted with an extraction buffer (20 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl₂, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 25% [v/v] glycerol) containing 1 mmol/L dithiothreitol and a protease inhibitor cocktail. The nuclear extracts were quickly frozen and preserved at –80°C for further use. For the binding reaction with the Ep region, 5 μ g nuclear extract was mixed with 5 pmol digoxigenin-labeled double-stranded probe in a final volume of 20 μ l containing 20 mmol/L Tris-HCl (pH 7.9), 50 mmol/L NaCl, 1 mmol/L EDTA, 5% glycerol, 1 μ g poly-dIdC, and 1 mmol/L dithiothreitol. The sense sequences of the wild-type Ep and mutant Ep (mEp) sites were 5'-GG-GCCGTGCCACCTGCCCGCCCGC-3' and 5'-GGGCGTG-GCGAGTTCCCCGCCCGC-3', respectively. Mutations are underlined. For supershift experiments, antibodies against NeuroD (N-19, Santa Cruz Biotechnology) or ASCL1 (C-16, Santa Cruz Biotechnology)²⁸ were added to the mixture and left 30 minutes at 4°C before electrophoresis in 5% acrylamide/bisacrylamide (29:1) gels with recirculating 0.25× Tris-Borate-EDTA buffer (2 hours, 200 V) at 4°C. 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 (ChIP) was performed using the ChIP kit (Upstate, Temecula, CA) according to manufacturer's instructions with some modifications. Briefly, 1.0×10^6 TKB15 cells at 80% confluence were

cross-linked by adding formaldehyde to the tissue-culture medium at a final concentration of 1% (v/v). After fixation for 10 minutes, scraped cells were washed twice with cold PBS containing protease inhibitors and were collected by centrifugation at $700 \times g$ at 4°C. Fixed cells were resuspended in SDS lysis buffer containing protease inhibitors, and lysed cells were sonicated on ice to an average DNA length of 200 to 500 bp. Samples were centrifuged at $13,300 \times g$ at 4°C for 10 minutes to remove insoluble material. The chromatin solution was pre-cleaned with the addition of Protein G beads for 2 hours at 4°C, and then incubated with 10 μ g of the following antibodies: goat anti-NeuroD (N-19, Santa Cruz Biotechnology), goat anti-ASCL1 (C-16, Santa Cruz Biotechnology), and non-immunized goat IgG (Santa Cruz Biotechnology). The antibody/chromatin mixtures were precipitated with Protein G beads, and the beads were sequentially washed with ChIP wash buffer. Complexes were eluted twice with 200 μ l elution buffer for each ChIP reaction. Cross-linking was reversed by adding 4 μ l of 5 M/L NaCl and 1 μ l RNase, and samples were incubated at 65°C overnight. Protein was removed from DNA by adding proteinase K at 37°C for 2 hours, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. The forward and reverse PCR primers used to detect target sequences containing the Ep site are described in Table 1.

RNA Interference

Double-stranded small interfering RNAs (siRNAs) for NeuroD (25-mer) were designed by RNAi Designer (Invitrogen) and synthesized using Stealth RNAi (Invitrogen). TKB15 cells (1.0×10^5) were seeded in six-well plates, and 500 pmol of siRNA duplex was transfected using X-tremeGENE siRNA transfection reagent (Roche Diagnostics) according to manufacturer's instructions. The siRNA sequences for NeuroD and scrambled control were 5'-UCGAAGGACAUAAUUAUUGUCUAUGG-3' and 5'-UAUCAUUGAUGUAUCGGAACUGAAG-3', respectively. After siRNA-treated TKB15 cells were cultivated for 24 hours, they were harvested, and total RNA samples were prepared to conduct semiquantitative RT-PCR for NeuroD, IGFBP-2, and β -actin (internal control). To force constitutive expression of siRNA for IGFBP-2 in SCLC cells, a retrovirus-based siRNA expression vector (pSIN-siDKI, Takara, Japan), which can simultaneously express two kinds of siRNA to enhance knockdown effectiveness, was used as described below.

Construction of Retroviral Vectors and Transfection

cDNA encoding NeuroD was generated by reverse transcriptase (RT)-PCR using the specific primer set described above. We used a retrovirus-based Tet-On Advanced Inducible Gene Expression System (pTet-On Advanced and pRevTRE, Clontech, Mountain View, CA), because stably NeuroD-expressing clones could not be established using retrovirus. A pTet-On Advanced

Table 2. Oligonucleotide Sequences for Construction of pSINsiDKI-IGFBP-2 or pSINsiDKI-Negative Control Vector*

siIGFBP-2-1 (for pSINsiDKI-IGFBP-2)
F 5'-GATCCGCAGTGCAGATGTCTCTGATAGTGCTC-CTGGTTGTCAGAGACATCTTGCCTGCTTTTAT-3'
R 5'-CGATAAAAAAGCAGTGCAGATGTCTCTGACAA-CCAGGAGCACTATCAGAGACATCTTGCCTGCG-3'
siIGFBP-2-2 (for pSINsiDKI-IGFBP-2)
F 5'-CTAGAGTGCATCTCTTCTCAATCACAGGGAAG-CGAGTCTGATTGT AGAAGAGATGACACTTTTTCTGCA-3'
R 5'-GGAAAAAAGTGTCTATCTCTTCTACAATCAGACTCG-CTTCCCTGTGATTGTAGAAGAGATGACACT-3'
Scrambled control-1 (for pSINsiDKI-negative control)
F 5'-GATCCGTCTTAATCGCGTATAAGGCTAGTGCTCC-TGTTGGCCTTATACGCGATTAAGCTTTTAT-3'
R 5'-CGATAAAAAAGTCTTAATCGCGTATAAGGCCAAC-CAGGAGCACTAGCCTTATACGCGATTAAGACG-3'
Scrambled control-2 (for pSINsiDKI-negative control)
F 5'-CTAGAGTCTTAATCGCGTATAAGGCCACAGGG-AAGCGAGTCTGGCCTTATACGCGATTAAGACTTTTTCTGCA-3'
R 5'-GGAAAAAAGTCTTAATCGCGTATAAGGCCAGACT-CGCTTCCCTGTGGCCTTATACGCGATTAAGACT-3'

*F and R, forward and reverse primer sequences.

vector (Clontech), which expresses reverse tetracycline-controlled transactivator protein, was initially transfected into HPL1A or TKB5 cells using Lipofectamine 2000 (Invitrogen). After neomycin (1 mg/ml) selection for 3 weeks, stable transfectants were cloned. Next, the NeuroD or ASCL1²⁸ cDNA-inserted pRevTRE vector was transfected into HEK293T cells using Lipofectamine 2000. After 24 hours of transfection, conditioned media were recovered as virus solution. The desired genes were introduced by incubating HPL1A or TKB5 cells with viral solution containing 10 μ g/ml polybren (Sigma) for 24 hours. Cells were selected with hygromycin (500 μ g/ml) for 3 weeks.

To conduct a retrovirus-based siRNA experiment targeted to IGFBP-2, two kinds of synthesized double-stranded oligonucleotide inserts, which are specific for production of IGFBP-2 siRNAs, were ligated with a promoter cassette and linearized pSINsiDKI vector (Takara). Sequences of the IGFBP-2 siRNA insert and negative control siRNA insert are shown in Table 2. These siRNA-producing vectors were transfected into HEK293T cells with Lipofectamine 2000, and the desired genes were then introduced by incubating TKB15 cells with the viral solution containing 10 μ g/ml polybren (Sigma) for 24 hours. Cells were selected with neomycin (1 mg/ml) for 3 weeks and then used for experiments.

Recombinant IGFBP-2 Supplementation

To avoid other growth factors besides IGFs as far as possible, the concentration of heat-inactivated FCS supplemented into Dulbecco's modified Eagle's medium was reduced to 1%. Cultured HPL1A or TKB5 cells (1.0×10^4 cells/well) were seeded in 96-well plates in 100 μ l medium supplemented with various amount of recombinant IGFBP-2 (rIGFBP-2, R&D Systems, Minneapolis, MN). Final concentrations of rIGFBP-2 were 0, 100, 250, 500, 750, and 1000 ng/ml. Cells were cultivated for 72 hours,

and cell growth was evaluated using an MTT assay system (Seikagaku Corp., Tokyo, Japan) and a microplate reader (Bio-Rad Laboratories, Hercules, CA) according to manufacturers' instructions. Absorbance was measured at a wavelength of 450 nm. Experiments were performed in triplicate, and each sample was measured three times.

IGFBP-2 Neutralization

To avoid other growth factors besides IGFs as far as possible, the concentration of heat-inactivated FCS supplemented into RPMI 1640 medium was reduced to 1%. Cultured SCLC cells (1.0×10^4 cells/well) were seeded in 96-well plates in 50 μ l medium. After 24 hours of culture, 50 μ l medium supplemented with goat anti-IGFBP-2 IgG or non-immunized goat IgG were added (final concentration, 50 μ g/ml; R&D Systems) and cells were cultivated for 72 hours. Cell growth was evaluated using an MTT assay system (Seikagaku Corp.) and a microplate reader (Bio-Rad Laboratories) according to manufacturers' instructions. Absorbance was measured at a wavelength of 450 nm. Experiments were performed in triplicate, and each sample was measured three times.

Immunohistochemistry and Methylation-Specific PCR

The institutional ethical boards of both Yokohama City University and Kanagawa Cardiovascular and Respiratory Disease Center Hospital approved examination of a series of 77 surgically resected primary lung cancer tissues. Histologically diagnosed 12 small cell carcinomas, 48 adenocarcinomas, and 17 squamous cell carcinomas were used for immunohistochemistry and methylation-specific PCR.¹ All SCLC patients were limited disease,³⁰ and all NSCLC patients were pathological stage I.³¹ IGFBP-2 immunohistochemistry was conducted using formalin-fixed, paraffin-embedded tissue sections. Sections were stained with H&E or immunostained using a goat polyclonal anti-IGFBP-2 (C-18, Santa Cruz Biotechnology)¹¹ and an EnVision system (DAKO, Carpinteria, CA) after microwave treatment to retrieve antigenicity of tissue sections. Negative control immunostaining was performed with non-immunized goat IgG (Santa Cruz Biotechnology) in place of the primary antibody. Immunostaining was detected with diaminobenzidine (Nichirei, Tokyo, Japan), and nuclei were counterstained with hematoxylin. Expression of IGFBP-2 in lung cancer cells was subdivided into four degrees: negative (0), positive in less than 25% of cancer cells (1+), positive in 25 to 50% of cancer cells (2+), and positive in more than 50% of cancer cells (3+).

Cancer cell nuclei were microdissected from formalin-fixed, paraffin-embedded lung cancer tissue sections using a laser capture microdissection system (PALM-MBIII, Carl Zeiss, Munich, Germany). To reverse the covalent links between protein and DNA, the nuclei were incubated in 300 mmol/L NaCl at 65°C for 12 hours. After ethanol precipitation, the samples were digested with

proteinase K (Wako, Tokyo, Japan) at 37°C overnight and the DNA was purified with phenol/chloroform, precipitated with ethanol, and then treated with sodium bisulfite using a MethylEasy DNA bisulfite modification kit (Human Genetic Signatures, Macquarie Park, Australia) according to manufacturer's instructions. Genomic DNA (1 μ g/20 μ l) was denatured by NaOH (final concentration, 0.273 M/L) for 15 minutes at 37°C and 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, and resuspended in reagent 3. The primers for methylation-specific PCR were constructed using promoter sequence data and primer design software. Since Egr-1 plays an important role in up-regulating IGFBP-2 expression, and Egr-1-binding sites within 130 bp of the transcription start site are important for IGFBP-2 expression,¹¹ we designed methylated and unmethylated IGFBP-2 promoter-specific primer sets that amplify the region containing Egr-1-binding sites (Table 1).

Enzyme-Linked Immunosorbent Assay

Cells (1.0×10^6) were cultivated for 48 hours, and secreted IGFBP-2 in the media was measured. Media were quickly frozen and preserved -80°C for further use. Secreted IGFBP-2 was measured using a DuoSet ELISA Development System (DY674, R&D Systems) and a microplate reader (Bio-Rad Laboratories) according to manufacturers' instructions. Absorbance was measured at a wavelength of 450 nm. The amount of secreted IGFBP-2 in the culture media was corrected for the number of cells harvested.

Statistical Analysis

All data are presented as mean $\pm$ SD. The unpaired Student's *t*-test was used for analyses. Two-tailed significance was set at $P < 0.05$.

Results

SCLC Cell Lines Overexpress IGFBP-2

IGFBP-2 plays a role as growth modulator in lung epithelial cells, and the growth-modulating system in anaplastic NSCLC cell lines is often disrupted by hypermethylation of the IGFBP-2 promoter.¹¹ We sought to expand on these observations by assessing IGFBP-2 expression in SCLC cells by Western blot analysis, and we obtained a surprising result (Figure 1A). Some NSCLC cell lines expressed a modest amount of IGFBP-2, whereas all of the SCLC cell lines examined expressed IGFBP-2 much more abundantly than the NSCLC cells. Because we had found previously that Egr-1 plays an important role in up-regulating IGFBP-2 expression in lung epithelium and NSCLC cells,¹¹ we examined whether SCLC cells showed sustained and abundant expression of *egr-1* under maintenance culture conditions (medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin) by semiquantitative RT-

Figure 1. Expression of IGFBP-2 and egr-1 in lung cancer cell lines. **A:** Western blot analysis of IGFBP-2 in two airway epithelial cell lines, seven SCLC cell lines, and six NSCLC cell lines. All SCLC cell lines examined overexpressed IGFBP-2. β -Actin (Act-B) served as an internal control. Sm, small cell carcinoma; Ad, adenocarcinoma; Sq, squamous cell carcinoma; La, large cell carcinoma. **B:** Semiquantitative RT-PCR analysis of egr-1 in the same panel of cell lines. After the RT reaction, 35 or 22 cycles of PCR were conducted for egr-1 and β -actin (Act-B), respectively. All cell lines examined expressed egr-1, and the expression levels varied in each SCLC or NSCLC cell line. Amplified RT-PCR product sizes for egr-1 and β -actin are 1405 and 863 bp, respectively.

PCR (Figure 1B). Although expression levels of egr-1 varied between individual SCLC and NSCLC cell lines, Egr-1 expression was not much greater in SCLC cells than NSCLC cells. This result suggested that there is another mechanism operating in SCLC cells to up-regulate IGFBP-2 expression.

NeuroD Binds to the E-box Located at the 5'-Untranslated Region and Stimulates Basal Transcription of IGFBP-2 Gene

We next tested the activity of the IGFBP-2 promoter by conducting IGFBP-2 promoter assays using the SCLC cell lines. The data were compared with results from HPL1A lung epithelial cells to identify SCLC-specific up-regulation mechanisms. Binding sites for Egr-1 are located at -98 to +5 of the IGFBP-2 promoter (numbers indicate the 5' nucleotide position from the transcription start site [+1]). In HPL1A, promoter activity for the pGL4.10BP2-444 and the pGL4.10BP2-314 constructs declined in comparison with the full-length IGFBP-2 promoter, although there was no loss of promoter activity for constructs pGL4.10BP2-206 and pGL4.10BP2-134 (Figure 2A), as described.¹¹ In contrast, the IGFBP-2 promoter activity of SCLC cell lines was not significantly affected by shortening the promoter length, strongly suggesting that a SCLC-specific IGFBP-2 transactivation

mechanism exists in a site proximal to the coding region of IGFBP-2.

We used computer software (MatInspector, Genomatix, Muenchen, Germany) to search the IGFBP-2 promoter for putative neuroendocrine cell-specific transcription factor binding sites, focusing on bHLH transcription factors. As shown by RT-PCR, the seven SCLC cell lines expressed NeuroD and ASCL1, whereas airway epithelial cell lines and NSCLC cell lines did not (Figure 2B). Because two E-boxes are located distal (Ed) and proximal (Ep) to the IGFBP-2 coding region and the Ep site is a putative NeuroD-binding site, we constructed luciferase vectors with mutated IGFBP-2 promoters and conducted luciferase assays to examine whether these cis-acting elements, Ep and Ed, stimulate IGFBP-2 promoter activity. Replacing the wild-type Ep site with mutant Ep (pGL4.10BP2-mEp) significantly reduced IGFBP-2 promoter activity, whereas replacing wild-type Ed site with mutant Ed (pGL4.10BP2-mEd) did not affect activity (Figure 2C). To examine which factor(s) bind to the Ep site under *in vitro* conditions, we conducted an electrophoretic mobility shift assay using an Ep-specific oligonucleotide probe and nuclear extracts from TKB12 (SCLC), TKB15 (SCLC), and TKB5 (NSCLC) cell lines. SCLC-specific signals were identified (Figure 2D, left panel), and the signals supershifted in response to supplementation with anti-NeuroD, but not with anti-ASCL1 (Figure 2D, right panel). We also performed a ChIP-based PCR analysis using nuclear lysate from TKB15 and a primer set that amplifies the Ep-containing region; this analysis confirmed that NeuroD binds to the Ep site (Figure 2E).

To examine whether and how NeuroD activates IGFBP-2, we established NeuroD-inducible transfectants from HPL1A and TKB5 cells (HPL1A-pRevNeuroD and TKB5-pRevNeuroD, respectively) and conducted a comparative study with empty vector-transfected or stably ASCL1-expressing cells (HPL1A-pRevEmpty, TKB5-pRevEmpty, HPL1A-pRevASCL1, and TKB5-pRevASCL1, respectively). NeuroD or ASCL1 protein was promptly induced in HPL1A-pRevNeuroD/TKB5-pRevNeuroD or HPL1A-pRevASCL1/TKB5-pRevASCL1 cells by supplementation with 1 μ g/ml doxycycline for 24 hours (Figure 2F). IGFBP-2 mRNA and protein increased in HPL1A-pRevNeuroD/TKB5-pRevNeuroD cells in response to NeuroD induction, but not in HPL1A-pRevASCL1/TKB5-pRevASCL1 cells in response to ASCL1 induction (Figure 2F). However, the level of IGFBP-2 induction in TKB5-pRevNeuroD cells was much weaker than that in HPL1A-pRevNeuroD cells (Figure 2F). Differences in IGFBP-2 inducibility between HPL1A and TKB5 cells were likely caused by the methylation status of the IGFBP-2 promoter; the IGFBP-2 promoter of TKB5 is heavily methylated,¹¹ whereas that of HPL1A is hardly methylated (data not shown). We also performed a siRNA experiment for NeuroD using TKB15. siRNA targeted to NeuroD promptly repressed NeuroD and IGFBP-2 mRNA in the TKB15 cell line (Figure 2G). These findings suggest that NeuroD stimulates basal transcription of IGFBP-2 in SCLC cells.

Figure 2. NeuroD binds to the E-box located at the 5'-untranslated region of the *IGFBP-2* gene. **A:** Luciferase assay vectors containing various lengths of *IGFBP-2* promoter sequences were transiently co-transfected with a pGL4.71TK control vector into SCLC cells and HPL1A cells (used as representative non-neuroendocrine cells). After 24 hours, cells were harvested and the cell lysates were subjected to the promoter assay. Columns, mean of three experiments in triplicate; bars = SD. **B:** RT-PCR analysis of ASCL1 and NeuroD in a panel of cell lines. ASCL1 and NeuroD were expressed only in SCLC cell lines. Amplified RT-PCR product sizes for ASCL1 and NeuroD are 374 and 1306 bp, respectively. Act-B, β -actin. **C:** Alteration of *IGFBP-2* promoter activity in association with E-box (Ed or Ep) mutation. Promoter activity was reduced by Ep mutation. Statistically significant difference ($P < 0.05$) from BP2-522 or BP2-mEd; columns, mean of three experiments in triplicate; bars, SD; mEp, mutant Ep; mEd, mutant Ed. **D (Left):** Electrophoretic mobility shift assay using Ep site-specific double-stranded oligonucleotide probe and nuclear lysate from TKB12, TKB15, and TKB5. **Arrows** indicate SCLC-specific nuclear protein-probe complexes; NS, nonspecific signal. **D (Right):** Competition and supershift assays using Ep site-specific double-stranded oligonucleotide probe and nuclear lysate from TKB15. The competition assay was done in the presence of 100-fold molar excess of unlabeled double-stranded oligonucleotide possessing Ep or mutated Ep (mEp) sequence. The supershift assay was conducted using anti-ASCL1 or anti-NeuroD antibody. **Arrows** indicate SCLC-specific nuclear protein-probe complexes. **Astrisk** indicates a supershifted signal; NS, nonspecific signal. **E:** ChIP-based PCR analysis using nuclear lysate from TKB15 and a specific primer set to amplify the Ep-containing site. DNA fragments conjugated with nuclear proteins were immunoprecipitated with goat anti-NeuroD, goat anti-ASCL1, or non-immunized goat IgG antibody. Fragments (315 bp) containing Ep site were amplified from DNA obtained by ChIP using anti-NeuroD antibody. Input, input DNA. **F:** NeuroD stimulates basal transcription of *IGFBP-2*. *IGFBP-2* mRNA and protein expression in HPL1A and TKB5 were up-regulated through induction of ASCL1. Cells were treated with 1 μ M of doxycycline for 24 hours. Act-B, β -actin; NS, nonspecific signal. Amplified RT-PCR product for *IGFBP-2* is 340 bp. **G:** Alteration of *IGFBP-2* mRNA expression in TKB15 through forced repression of NeuroD using siRNA targeted to NeuroD. *IGFBP-2* expression in TKB15 was reduced by NeuroD repression. Act-B, β -actin.

IGFBP-2 Represses Cell Growth of Cultured SCLC Cells

Establishment of NeuroD-inducible transfectants allowed us to analyze the influence of NeuroD on cell growth. Cell growth of NeuroD-expressing transfectants (HPL1A-pRevNeuroD and TKB5-pRevNeuroD) was significantly retarded in comparison with pRevEmpty (control) or pRevASCL1 transfectants (Figure 3A). The *in vitro* growth of SCLC cell lines was very slow, with doubling times of 102.6, 104.9, 96.5, 108.3, 92.0, 74.7, and 96.0 hours, respectively, for TKB12, TKB15, TKB16, TKB17, Lu134A, Lu139, and Lu140 under maintenance culture conditions. *In vitro* SCLC cells secreted greater amounts of IGFBP-2 than NSCLC or airway epithelial cells (Figure 3B, top panel). Abundant IGFBP-2 was expressed in TKB15,

TKB17, and Lu140 (Figure 1A and Figure 3B, top panel), and these cell lines revealed relatively slow growth among SCLC cell lines. Furthermore, NeuroD-induced HPL1A and TKB5 cells secreted abundant IGFBP-2, as did cultivated SCLC cells (Figure 3B, bottom panel). Therefore, we hypothesized that IGFBP-2 overexpression in SCLC cells might be one cause of slow SCLC cell growth *in vitro*.

To define the contribution of the IGF system to lung cancer cell growth, we investigated IGF expression in lung cancer cell lines and conducted a recombinant IGFBP-2 supplementation experiment. As shown in Figure 3C, three of seven SCLC cell lines (TKB15, Lu134A, and Lu140) produced IGF-1. IGF-2 expression was found in all cell lines examined. Among SCLC cell lines, TKB12, TKB16, and Lu140 expressed modest amounts of IGF-2,

Figure 3. IGFBP-2 represses cell growth of cultured SCLC cells. **A:** Alteration of population doublings in HPL1A and TKB5 through forced expression of NeuroD or ASCL1. Each transfectant was cultivated with media supplemented with 1 μ g/ml doxycycline. NeuroD induction retarded cell growth of both HPL1A and TKB5, but ASCL1 did not. Statistically significant difference ($*P < 0.05$) from pRevEmpty or pRevASCL1. Data are shown as mean $\pm$ SD of three experiments in triplicates. Bars = SD. **B:** Enzyme-linked immunosorbent assay of IGFBP-2 in culture media from a panel of cell lines (**top**) and from ASCL1- or NeuroD-induced HPL1A/TKB5 cells (**bottom**). **Top:** 1×10^6 cells were seeded onto 100-mm culture dishes and cultivated for 48 hours, and secreted IGFBP-2 in the media was measured. The amounts of secreted IGFBP-2 were normalized to the number of harvested cells. Statistically significant difference ($*P < 0.05$) from each airway epithelial cell line or NSCLC cell line. **Bottom:** 1×10^6 transfectants were seeded onto 100-mm culture dishes and cultivated with media containing 1 μ g/ml doxycycline for 48 hours, and secreted IGFBP-2 in the media was measured. The amounts of secreted IGFBP-2 were compensated by harvested cell numbers. Statistically significant difference ($*P < 0.05$) from pRevEmpty or pRevASCL1. Columns, mean of three experiments in triplicate; bars, SD; Sm, small cell carcinoma; Ad, adenocarcinoma; Sq, squamous cell carcinoma; La, large cell carcinoma. **C:** RT-PCR analysis of IGF-1/-2 in a panel of cell lines. TKB15, Lu134A, and Lu140 expressed IGF-1, and all examined cell lines expressed IGF-2. Amplified RT-PCR product sizes for IGF-1 and IGF-2 are 114 and 173 bp, respectively. Act-B, β -actin. **D:** Recombinant IGFBP-2 supplementation experiment. 1.0×10^4 HPL1A or TKB5 cells were seeded in 96-well plates in 100 μ l of 1% FCS-containing medium supplemented with 0 to 1000 ng/ml (final concentration) of recombinant IGFBP-2. Cell growth was evaluated using an MTT assay system. *Statistically significant difference ($P < 0.05$) from non-immunized goat IgG (NI). BP2, IGFBP-2. **E:** Alteration of SCLC cell growth by neutralization of secreted IGFBP-2. 1.0×10^4 SCLC cells were seeded in 96-well plates in 50 μ l of 1% FCS-containing medium. After 24 hours of culture, 50 μ l of 1% FBS-containing medium supplemented with goat anti-IGFBP-2 IgG or non-immunized goat IgG (NI) antibody was added (final concentration, 50 μ g/ml), and cells were cultivated for 72 hours. Cell growth was evaluated using an MTT assay system. *Statistically significant difference ($P < 0.05$) from non-immunized goat IgG (NI). BP2, IGFBP-2. **F:** siRNA experiment using IGFBP-2-specific siRNA-producing retrovirus. **Top:** IGFBP-2 production in TKB15 cells was repressed by transfection with IGFBP-2-specific siRNA-producing retrovirus. siCTL, control siRNA; siIGFBP-2, IGFBP-2-specific siRNA. **Bottom:** Cell growth of TKB15 was accelerated by repressed IGFBP-2 production. Statistically significant difference ($*P < 0.05$) from control siRNA-producing retrovirus-transduced TKB15. Data are shown as mean $\pm$ SD of three experiments in triplicate; bars = SD.

Table 3. IGFBP-2 Expression and Methylation Status of the *IGFBP-2* Promoter for Each Histologic Type

Histologic type	Methylation status	IGFBP-2 expression				Total
		0	1+	2+	3+	
Small cell carcinoma	Methylated	0	0	0	1	1 (8%)
	Unmethylated	0	0	0	11	11 (92%)
	Total	0 (0%)	0 (0%)	0 (0%)	12 (100%)	12 (100%)
Adenocarcinoma	Methylated	10	17	7	0	34 (71%)
	Unmethylated	0	1	8	5	14 (29%)
	Total	10 (21%)	18 (38%)	15 (31%)	5 (10%)	48 (100%)
Squamous cell carcinoma	Methylated	0	4	1	0	5 (29%)
	Unmethylated	0	1	4	7	12 (71%)
	Total	0 (0%)	5 (29%)	5 (29%)	7 (42%)	17 (100%)

as did non-neoplastic airway epithelial cell lines, squamous cell carcinoma cell lines, and large cell carcinoma cell lines. Also shown in Figure 3D, supplementation with recombinant IGFBP-2 significantly repressed the growth of HPL1A and TKB5 in a dose-dependent manner. The cell growth of TKB5, which did not express IGFBP-2 (Figure 1A), was repressed by less than 70% compared with control, and that of HPL1A, which expressed a modest amount of IGFBP-2 (Figure 1A), was also repressed to approximately 80%. Using a specific anti-IGFBP-2 on SCLC revealed that growth of all SCLC cell lines examined was accelerated by up to 10% to 20% by neutralization of IGFBP-2 (Figure 3E). Differences in growth acceleration among SCLC cell lines were considered to be associated with IGFBP-2 and IGF-1/-2 expression levels. To obtain more direct evidence, we constructed a retrovirus vector–based siRNA targeted to IGFBP-2 and established stably IGFBP-2-siRNA–expressing TKB15 cells. The IGFBP-2 expression level is too high to completely knock down IGFBP-2 expression; however, we succeeded in substantially reducing IGFBP-2 expression in TKB15 cells (Figure 3F, top panel), and confirmed that cell growth was accelerated by sustained inhibition of IGFBP-2 expression (Figure 3F, bottom panel). These results strongly suggest that the IGF system plays an important role in lung cancer cell growth, and that IGFBP-2 contributes to repression of SCLC cell growth *in vitro*.

IGFBP-2 Is Overexpressed and Its Promoter Is Rarely Methylated in SCLC in Vivo

To determine whether SCLC cells produce large amounts of IGFBP-2 *in vivo* as well as *in vitro*, we performed IGFBP-2 immunohistochemistry using 77 formalin-fixed, paraffin-embedded lung cancer tissue sections. The results are summarized in Table 3. All SCLCs examined were strongly positive for IGFBP-2 (Figure 4A), whereas the IGFBP-2 signal intensity in NSCLCs varied tumor by tumor, but was generally weaker than in SCLCs (Figure 4B, Table 3).

Because the *IGFBP-2* promoter has CpG islands and is often hypermethylated in lung cancer cell lines derived from anaplastic NSCLC,¹¹ we speculated that differences of IGFBP-2 expression among *in vivo* SCLC and NSCLC cells might be caused not only by NeuroD-associated induction, but also by *IGFBP-2* silencing via pro-

motor hypermethylation. Therefore, we conducted methylation-specific PCR using microdissected cancer cell nuclei from lung cancer tissues and examined whether the methylation status of the *IGFBP-2* promoter is associated with IGFBP-2 expression *in vivo* in lung cancer cells. There was a striking difference in the degree of methylation in the Egr-1-binding region of the *IGFBP-2* promoter between SCLCs and NSCLCs, though the Egr-1-binding region of the *IGFBP-2* promoter of the stage I NSCLCs tested was not yet fully methylated. Amplified products from methylated *IGFBP-2* promoter were detected in 71% of adenocarcinomas (34/48) and 29% of squamous cell carcinomas (5/17), but were detectable in only 8% of SCLCs (1/12; Table 3 and see Supplemental Figure S1 at <http://ajp.amjpathol.org>). Furthermore, most of the methylated NSCLC cases revealed low or deficient IGFBP-2 expression (Table 3). These findings suggest that differences in the methylation status of the *IGFBP-2* promoter contribute to differences in IGFBP-2 expression levels between SCLC and NSCLC, in addition to the neuroendocrine phenotype-linked up-regulating mechanism of IGFBP-2.

Discussion

We have demonstrated here that SCLC cells overexpress IGFBP-2 via binding of NeuroD to the E-box located at the 5'-untranslated region of the *IGFBP-2* promoter. NeuroD is a neurogenic differentiation factor with a bHLH structure that plays important roles in developing neuronal/neuroendocrine and sensory organs such as the brain, retina of the eye, inner ear, pancreatic islets, enteroendocrine cells, and pulmonary neuroendocrine cells.^{17–27,32} In this study, we have demonstrated that NeuroD induces a molecule that is not directly related to neuronal/neuroendocrine differentiation.

IGFBP-2 is one of the most highly expressed IGFBPs in neuroblastomas, glial tumors, and prostate cancer.^{33–36} Elevated serum IGFBP-2 levels have been reported in neoplasms of several organs, including the central nervous system, prostate, ovaries, and colorectum.^{35,37–39} Neuroblastomas, medulloblastomas, and retinoblastomas, which are classified as primitive neuroectodermal tumors, express NeuroD.⁴⁰ We confirmed that cultured neuroblastoma cells express both NeuroD and abundant IGFBP-2 (data not shown). Recently, Cindolo et al re-

Figure 4. IGFBP-2 immunohistochemistry in lung cancers. **A:** Small cell carcinoma cells revealed strong positivity for anti-IGFBP-2 antibody. Signal was not seen in the negative control. **B:** Adenocarcinoma cells did not express IGFBP-2. H.E., hematoxylin and eosin stain; IGFBP-2, IGFBP-2 immunostaining; N.C., negative control. Scale bar = 100 μ m.

ported that cultured prostate cancer cells express NeuroD in response to cAMP treatment, and that NeuroD positivity in prostate cancer cells *in vivo* increases with cancer progression.³⁶ However, glial tumor cells do not express NeuroD,^{33,34} and there is no report that common ovarian or colon cancers express NeuroD. Therefore, our results strongly suggest that IGFBP-2 expression in neuroectodermal tumor cells and prostate cancer cells might be up-regulated by NeuroD, and that IGFBP-2 expression mechanisms in glial tumor, ovarian cancer, and colon cancer cells differ from those of primitive neuroectodermal and neuroendocrine tumors.

Cultured SCLC cells characteristically show slow growth in comparison with NSCLC cells.^{2,41,42} We have demonstrated here that NeuroD-expressing airway epithelial cells and NSCLC cells produce abundant IGFBP-2 and have retarded growth. Furthermore, cell growth of cultured SCLC cells is accelerated by antibody neutralization of IGFBP-2 and by forced repression of IGFBP-2 production by RNA interference. In a previous study, growth of cultured NSCLC cells was repressed by supplementation with recombinant IGFBP-2 in culture media and by IGFBP-2 transgene expression.¹¹ In the present study, it was shown that lung cancer cell lines including SCLC produce IGF-1/-2 and that recombinant IGFBP-2 repressed growth of cultured airway epithelial and NSCLC cells in a dose-dependent manner (Figure 3D). Taken together, these *in vitro* results suggest that the IGF system is important for lung cancer cell growth and

that secreted IGFBP-2 modulates growth by sequestering IGFs.

The decreased IGFBP-2 expression in NSCLC *in vivo* was likely due to *IGFBP-2* silencing via methylation of the CpG islands in the *IGFBP-2* promoter, since the *IGFBP-2* promoter of *in vivo* NSCLC cells, especially adenocarcinoma cells, was often methylated (Figure 4B, Table 3). These results are quite reasonable for a model in which secreted IGFBP-2 binds to IGF and inhibits its binding to IGF receptors, thereby acting as a growth modulator, and is consistent with the observed reduction of IGFBP-2 expression associated with cancer progression.¹¹ By contrast, the *IGFBP-2* promoter of *in vivo* SCLC was rarely methylated, and *in vivo* SCLC cells overexpressed IGFBP-2, as shown in Figure 4A and Table 3. Why, then, does *in vivo* SCLC overexpressing IGFBP-2 paradoxically reveal aggressive growth? From our previous study¹¹ and present *in vitro* investigation, it could be interpreted that overexpressed IGFBP-2 in SCLC functions as a brake against growth acceleration signals associated with oncogene activation, tumor suppressor gene inactivation, and the gastrin-releasing peptide autocrine system,^{5,43} but is not sufficient to modulate the excessive growth activity of *in vivo* SCLC. However, recent reports have shown that membrane-bound IGFBP-2 binds to the extracellular matrix and is associated with migration and invasion, whereas secreted or exogenous IGFBP-2 represses cell growth.^{44,45} Furthermore, Sethi et al have reported that extracellular matrix proteins protect SCLC

cells against apoptosis.⁴⁶ Most cultured *in vitro* SCLC cells characteristically grow floating in the culture medium or weakly attached to the culture dish,^{41,42} indicating that SCLC cells *in vitro* do not have sufficient contact with extracellular matrix, as compared with NSCLC cells. These reports and the characteristics of cultured SCLC suggest the possibility that SCLC acquires aggressiveness by interacting with the extracellular matrix, either directly or via membrane-bound IGFBP-2.

Another possible explanation for this paradox is that neuroendocrine cell-specific molecules participate in aggressiveness and poor prognosis of SCLC. ASCL1, a neuronal/neuroendocrine cell-specific bHLH transcription factor, is frequently overexpressed in SCLC cells^{12,13} and is recognized as a biomarker of SCLC.^{12,14,15,47} We previously showed that ASCL1 represses an immune-related molecule, class II transactivator (CIITA), by competitively binding to the E-box located in the *CIITA* promoter, and that CIITA repression causes severe deficiency of major histocompatibility complex expression, resulting in poor anti-cancer immunity against SCLC.^{11,28} Osada et al reported that ASCL1 functions as a dual transcription factor by activating neuroendocrine differentiation and repressing putative tumor suppressors.⁴⁸ These reports suggest that neuroendocrine cell-specific bHLH transcription factors may contribute to distinctive behaviors of neuroendocrine cancer cells.

In summary, SCLC fatefully overexpresses IGFBP-2 via a neuroendocrine differentiation-associated transcription factor, NeuroD, and the overexpressed IGFBP-2 contributes to the slow growth of SCLC *in vitro*. Further investigations into the function of IGFBP-2 *in vivo* and the downstream molecules regulated by neuroendocrine cell-specific transcription factors are needed to resolve the paradox that IGFBP-2-overexpressing SCLC is aggressive and associated with poor prognoses *in vivo*. A deep understanding of SCLC-specific behaviors by these further investigations is needed to promote development of effective therapeutic strategies against the poor prognosis of neuroendocrine cancers.

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