Insulinoma-Associated Protein 1 Is a Crucial Regulator of Neuroendocrine Differentiation in Lung Cancer

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Insulinoma-associated protein 1 (INSM1) is expressed exclusively in embryonic developing neuroendocrine (NE) tissues. INSM1 gene expression is specific for small-cell lung cancer (SCLC), along with achaete-scute homolog-like 1 (ASCL1) and several NE molecules, such as chromogranin A, synaptophysin, and neural cell adhesion molecule 1. However, the underlying biological role of INSM1 in lung cancer remains largely unknown. We first showed that surgically resected SCLC samples specifically expressed INSM1. Forced expression of the INSM1 gene in adenocarcinoma cell lines (H358 and H1975) induced the expression of ASCL1, brain-2 (BRN2), chromogranin A, synaptophysin, and neural cell adhesion molecule 1; in contrast, knockdown of the INSM1 gene by siRNA in SCLC (H69 and H889) decreased their expression. However, forced/knockdown expression of ASCL1 and BRN2 did not affect INSM1 expression. A chromatin immunoprecipitation study revealed that INSM1 bound to the promoter region of the ASCL1 gene. A xenotransplantation assay using tet-on INSM1 gene-transfected adenocarcinoma cell lines demonstrated that INSM1 induced NE differentiation and growth inhibition. Furthermore, we found that INSM1 was not expressed in non-small-cell lung cancer and some SCLC cell lines expressing Notch1-Hes1. By forced/knockdown expression of Notch1 or Hes1 genes, we revealed that Notch1-Hes1 signaling suppressed INSM1, as well as ASCL1 and BRN2. INSM1, expressed exclusively in SCLC, is a crucial regulator of NE differentiation in SCLCs, and is regulated by the Notch1-Hes1 signaling pathway. (Am J Pathol 2015, 185: 3164–3177; http://dx.doi.org/10.1016/j.ajpath.2015.08.018)

Lung cancer is the leading cause of cancer-related mortality worldwide. Despite developments in the molecular analysis and understanding of lung cancer, the survival rate has not changed significantly in more than three decades.¹ Small-cell lung cancer (SCLC) accounts for 10% to 15% of all lung cancer and is genetically considered to be one of the most aggressive malignant tumors. Despite a high rate of response to first-line treatment, cases inevitably relapse because of the acquisition of multidrug resistance, resulting in a 5-year survival rate of 5% to 10%.² In recent years, focus has been on personalized treatment, as represented by epidermal growth factor receptor (EGFR)—tyrosine kinase inhibitor and anaplastic lymphoma kinase inhibitor for non-SCLC (NSCLC).³,⁴ In contrast, novel target molecules of therapies for SCLC have not yet been developed. Thus, the identification of target molecules is highly anticipated.

A striking feature of SCLC is the expression of poorly differentiated neuroendocrine (NE) phenotypes; the development of this feature is thought to be correlated with tumor malignancy, which includes rapid growth, a high metastatic rate at an early stage, and the acquisition of multidrug resistance.⁵—⁷

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Achaete-scute homolog-like 1 (ASCL1), a proneural basic helix-loop-helix transcription factor, and brain-2 (BRN2), a POU domain transcription factor, are reported to be expressed at high frequency in SCLC. ASCL1 is required to establish the lineage of pulmonary NE cells and is involved in the survival, growth, and acquisition of chemoresistance of SCLC. As a therapeutic target of SCLC, Osada et al reported that knockdown of ASCL1 induces growth inhibition and apoptosis in SCLC cell lines. These findings suggest that ASCL1 could play a pivotal role in the tumorigenesis of SCLC. BRN2 is a developmental neural cell-specific transcription factor that could play a role in maintaining the NE phenotype of SCLC with ASCL1. In addition, NE molecules [chromogranin A (CGA), synaptophysin (SYP), and neural cell adhesion molecule (NCAM)] are used as important complementary tools for the routine diagnosis of NE differentiation of lung cancer. These NE markers are useful for the clinical diagnosis of SCLC and have been reported as predictors of prognosis in SCLC patients. Although the Notch1-Hes1 signaling pathway is reported as an inhibitor of ASCL1 and NE molecules, it is largely unclear how the expression of ASCL1, BRN2, and NE molecules (CGA, SYP, and NCAM) is regulated.

Insulinoma-associated protein 1 (INSM1) is a zinc-finger transcriptional factor originally isolated from a human insulinoma subtraction library. It has been reported that INSM1 plays an important role in the development of pancreatic and intestinal NE cells, adrenal medulla cells, and basal neuronal progenitor cells in the neocortex, and in the neurogenesis of the embryonic olfactory epithelium. INSM1 mRNA is abundantly expressed in fetal developing neuronal and NE tissue, but is significantly reduced or restricted in adult tissues. The expression of INSM1 occurs in NE tumors, including insulinoma, pituitary tumor, pheochromocytoma, medullary thyroid carcinoma, medulloblastoma, neuroblastoma, and retinoblastoma. Several microarray gene expression profiling experiments of SCLC cell lines also reported INSM1 as a highly specific marker. In addition, a Northern blotting study revealed that 30 of 31 SCLC cell lines and four NSCLC cell lines with the NE phenotype expressed INSM1 mRNA, and they showed a high concordance with other NE markers, such as CGA and L-dopa decarboxylase. Thus, INSM1 was identified as a highly specific NE marker and as a prominent differential marker for SCLC, along with ASCL1, a critical transcriptional factor for NE tumor of lung cancer.

As a target of treatment, several groups reported the efficacy of INSM1 promoter-driven suicide gene therapy in SCLC cell lines. However, the underlying biological functions of INSM1 in lung cancer remain largely unclear.

To extend previous findings of the significance of INSM1 in lung cancer, we performed the following experimental studies. First, we examined INSM1 protein expression in 160 surgically resected cases of lung cancers [27 SCLCs, 86 adenocarcinomas (ADCs), and 47 squamous cell carcinomas (SCCs)] using immunohistochemical (IHC) staining, and in various human lung cancer cell lines using Western blot (WB) analysis to confirm the INSM1 protein expression pattern in lung cancer specimens and cell lines. Next, we used RNA interference and plasmid DNA transfection technology on lung cancer cell lines and subsequently added xenotransplantation experiments to elucidate the biological effects of INSM1 in lung cancer cell lines. Moreover, we examined mutual interactions among INSM1 and two representative NE transcription factors (ASCL1 and BRN2), and confirmed the regulatory effects of Notch1-Hes1 signaling on INSM1 expression to determine upstream and downstream regulators of INSM1. Our findings demonstrate that INSM1 is exclusively expressed in SCLC cases, regulates ASCL1, BRN2, and NE molecules (CGA, SYP, and NCAM) in SCLC cell lines, and is inhibited by the Notch1-Hes1 signaling pathway. In addition, we revealed the effect of INSM1 on the cell growth in lung cancer cell lines. Knockdown of INSM1 activates the apoptotic pathway and has an inhibitory effect on cell growth in SCLC; in contrast, forced expression of INSM1 inhibits the cell growth in NSCLC cell lines.

Materials and Methods

Cell Lines

Seven SCLC cell lines (H69, H889, SBC1, H69AR, H1688, SBC3, and SBC5), three ADC cell lines (A549, H358, and H1975), and three SCC cell lines (H226, H2170, and HCC15) were used in this study. H69, H889, H69AR, H1688, A549, H358, H1975, H226, and H2170 were purchased from ATCC (Manassas, VA), and SBC1, SBC3, and SBC5 were purchased from Japan Collection of Research Bioresources Cell Bank (Osaka, Japan). HCC15 was generously donated by Dr. Adi F. Gazdar (University of Texas Southwestern Medical Center, Dallas, TX).

Tissue Samples

Tissue samples of SCLCs (n = 27), ADCs (n = 86), and SCCs (n = 47), resected at the Department of Thoracic Surgery of Kumamoto University Hospital (Kumamoto, Japan) and the Department of Thoracic Surgery of National Minami-Kyushu Hospital (Kagoshima, Japan), were obtained from 160 patients for the following studies. A histological diagnosis of the samples was made according to the criteria of the World Health Organization. Additional sections were used for IHC staining. The study followed the guidelines of the Ethics Committee of Kumamoto University and National Minami-Kyushu Hospital.

WB Analysis

Cells were prepared for WB analysis, as previously described. A list of the primary antibodies used is shown in Table 1. The membrane was then washed and incubated with the respective secondary antibodies conjugated with peroxidase (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 1 hour, and
the immune complex was visualized with the electrochemiluminescence system (Santa Cruz Biotechnology, Santa Cruz, CA).

**IFA Data**

The cells were treated as previously described. The slides were heated using an autoclave in 0.01 mol/L citrate buffer (pH 7.0) (for CGA, SYP, and NCAM) for antigen retrieval, but this was not necessary to perform INSM1 immunofluorescence analysis (IFA) staining. A list of the primary antibodies used is provided in Table 1. Cells were incubated with the appropriate secondary antibodies (Alexa Fluor; Molecular Probes, Eugene, OR) and examined using a fluorescent microscope (Olympus, Tokyo, Japan). The specificity of immunolabeling of each antibody was tested by using normal mouse IgG (Santa Cruz Biotechnology) and normal rabbit IgG (Santa Cruz Biotechnology), and no staining was observed.

**IHC Staining**

Formalin-fixed, paraffin-embedded specimens were cut into sections (4 μm thick) and mounted onto MAS-GP—coated slides (Matsunami Glass Ind, Osaka, Japan). After being deparaffinized and rehydrated, the sections were heated using an autoclave in 0.01 mol/L citrate buffer (pH 7.0) for antigen retrieval. The sections were incubated with 0.3% H2O2 in absolute methanol for 20 minutes to block endogenous peroxidase activity. Then, the sections were heated using an autoclave in 0.01 mol/L citrate buffer (pH 7.0) (for CGA, SYP, and NCAM) for antigen retrieval, but this was not necessary to perform INSM1 immunofluorescence analysis (IFA). A list of the primary antibodies used in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP). We were incubated with the primary antibodies shown in Table 1 at 4°C overnight. This was followed by sequential 1-hour incubations with the secondary antibodies (En Vision+ System-HRP-Labeled Polymer; Dako) and negative control (for ChIP).
antibody was tested by using normal mouse IgG (Santa Cruz Biotechnology) and normal rabbit IgG (Santa Cruz Biotechnology), and no staining was observed. In addition, we confirmed that as positive IHC controls for INSM1, pancreatic islets and insulinoma were positive for INSM1.

Transfection with siRNA

siRNAs for INSM1, ASCL1, BRN2, Notch1, Hes1 mRNA, and negative control (scrambled random siRNA) were purchased from Santa Cruz Biotechnology and transfected into cells at a concentration of 20 nmol/L by using an electroporator (NEPA21 pulse generator; Nepa Gene, Chiba, Japan), as described in the manufacturer’s instructions. The cells were harvested at 48 hours after transfection.

Cell Counting Assay

H69 and H889 cells that were transfected with siRNA as a negative control, INSM1, and BRN2 were seeded at equivalent densities (2.5 × 10^5 cells/mL) in 6-well plates. At 24-hour intervals, cells were trypsinized and then counted using a TC20 Automated Cell Counter (BioRad Laboratories, Richmond, CA). The experiments were repeated separately in triplicate to confirm reproducibility.

Plasmid Construction and Transfection

**INSM1-Expressing Vector**

To construct pTRE3G-ZsGreen1-INSM1, INSM1 cDNA was obtained from the pCMV6-XL5-INSM1 vector (OriGene, Rockville, MD) and inserted into the Mlu1 and BamHI sites of the pTRE3G-ZsGreen1-mock vector (Clontech, Mountain View, CA). The tet-on INSM1-transfected H358 and H1975 cells were established by using this vector and the tet-on system, as described below.

**Notch1-Expressing Vector**

Human Notch1 intracellular domain cDNA, a fragment (5262 to 7665) of the human Notch1 gene, was PCR amplified using cDNA from H358 cells and cloned into the Mlu1 and BamHI sites of the pTRE3G-ZsGreen1-mock vector (Clontech). The tet-on Notch1-transfected H69 and H889 cells were established by using this vector and the tet-on system, as described below.

**Hes1-Expressing Vector**

To construct pTRE3G-ZsGreen1-Hes1, a fragment (1 to 843) of the human Hes1 gene was PCR amplified using cDNA from H358 cells and cloned into the Mlu1 and BamHI sites of the pTRE3G-ZsGreen1-mock vector (Clontech). The tet-on Hes1-transfected H69 and H889 cells were obtained by using this vector and the tet-on system, as described below.
**ASCL1-Expressing Vector**

Human ASCL1 cDNA was obtained from the pOTB7-ASCL1 vector (RIKEN BRC through the National Bio-Resource Project of MEXT, Tsukuba, Japan) and inserted into the EcoRI and XbaI sites of the pCMV6-XL5-mock vector. The ASCL1-transfected H358 and H1975 cells were obtained by transfection with this vector using an electroporator (Nepa Gene), as described in the manufacturer’s instructions.

**BRN2-Expressing Vector**

To construct pCMV6-XL5-BRN2, a BRN2 expression vector, a fragment (from 1 to 1332) of the human BRN2 was PCR amplified using cDNA from H69 cells, and cloned into the EcoRI and XbaI sites of the pCMV6-XL5-mock vector. The BRN2-transfected H358 and H1975 cells were obtained by transfection with this vector using an electroporator (Nepa Gene), as described in the manufacturer’s instructions.

**Tet-On System**

The Tet-On 3G Inducible Expression System with ZsGreen1 (Clontech) was used to establish the tet-on cell lines, according to the manufacturer’s instructions, to avoid difficulty in obtaining stable cell lines because of unexpected growth-inhibitory effects caused by the transfected genes. In brief, clones expressing tet-on 3G transactivator protein were obtained by the transfection of H69, H889, H358, and H1975 with the pCMV-Tet3G vector (Clontech). Selected positive clones were then cotransfected with pTRE3G-Zsgreen1-INSM1 (for H358 and H1975), pTRE3G-Zsgreen1-Notch1 (for H69 and H889), pTRE3G-Zsgreen1-Hes1 (for H69 and H889), and Linear Puromycin Marker (Clontech). Transfected cells were selected with 5 μg/mL puromycin (Clontech). The resistant clones were expanded and examined for the induction of each protein and ZsGreen1 on the addition of 0.5 μg/mL doxycycline (Dox) by WB analysis, quantitative RT-PCR, and IFA. Cells were transfected by using a NEPA21 pulse generator (Nepa Gene), as described in the manufacturer’s instructions.

**Quantitative RT-PCR**

The Fast Pure RNA Kit (Takara Bio, Shiga, Japan) was used to isolate total RNA from each cell line. cDNA was produced using a ReverTra Ace qPCR RT-Kit (Toyobo, Osaka, Japan), according to the manufacturer’s instructions. cDNA was subjected to quantitative SYBR Green real-time PCR by using SYBR Premix Ex Taq II (Takara Bio). A list of the specific primers is provided in Table 2. Real-time quantitative PCR was performed with a Thermal Cycle Dice Real-Time System (Takara Bio) by using 40 cycles of a three-stage program with the following conditions: 2 seconds at 95°C, 10 seconds at 60°C, and 15 seconds at 72°C, as recommended by the manufacturer. The products were quantified during the initial exponential phase of amplification above the baseline. Data were obtained from triplicate reactions. The means and SDs of the copy number were normalized to the value for glyceraldehyde-3-phosphate dehydrogenase mRNA.

**ChIP Assay**

A PCR primer was designed to cover the promoter region on ASCL1, and another PCR primer for a non-promoter region on ASCL1 was designed as a negative control. The arrangements of the primers are shown in Table 2. Chromatin was immunoprecipitated using anti-INSM1 and normal rabbit IgG (Santa Cruz Biotechnology). Approximately 2 × 10⁶ cells (H69, H889, H358, and H1975) were used per chromatin immunoprecipitation (ChIP) reaction. In brief, cells were harvested, fixed in 1% formaldehyde, and sonicated to produce chromatin.
fragments 300 to 500 bp in length. The precipitated DNA was extracted using phenol-chloroform and then purified. The fold enrichment was calculated as the difference between each sample and the negative control using quantitative RT-PCR analysis, as described above. Individual assays were repeated three times to confirm the reproducibility of the experiment.

**Tumor Xenograft Growth in Vivo and Histopathological Evaluation**

The tet-on H358 and H1975, which express INSM1 in the presence of Dox, were established by using a Tet-On 3G Inducible Expression System with ZsGreen1 (Clontech). A total of $2 \times 10^5$ cells were injected s.c. into the back of 10 mice [Rag$^{-/-}$:Jak$3^{-/-}$ mice; a generous gift from Prof. Seiji Okada (Kumamoto University)]. After tumor formation was confirmed after 3 weeks, the mice were divided into two groups, in which the average tumor sizes were similar (approximately 1000 mm$^3$). One group was given drinking water supplemented with 4 mg/L Dox, and the other group was given normal water. Five weeks after the first injection, the tumors were removed and measured. The samples were fixed with 10% formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin, and additional sections were IHC stained, as described above. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

**Statistical Analysis**

All data were obtained from independent experiments, and are expressed as the means ± SD of triplicate determinations. The differences in the mean values between the two groups were statistically analyzed using t-test. SPSS version 18 (SPSS Inc., Chicago, IL) was used for statistical analyses. $P < 0.05$ was considered significant.

**Results**

**INSM1 Is Expressed in SCLC Tissue Sections and SCLC Cell Lines**

Previous studies revealed that INSM1 expression is specific for SCLC and NSCLC with NE phenotypes. However, to
our knowledge, no study has analyzed the expression of INSM1 protein. To examine the INSM1 protein expression pattern, we stained surgically resected lung cancer tissues (27 SCLCs, 86 ADCs, and 47 SCCs) immunohistologically for INSM1 (Figure 1A) and performed WB analysis in 13 lung cancer cell lines (seven SCLCs, three ADCs, and three SCCs) (Figure 1B). By IHC, INSM1 was expressed exclusively in all pure SCLC specimens and the SCLC component in combined SCLC specimens (Table 3). In addition, WB analysis revealed that INSM1 protein was expressed in four of seven SCLC cell lines, but was not expressed in NSCLC cell lines (Figure 1B).

Four of seven SCLC cell lines showed the same expression patterns of INSM1, ASCL1, and CGA. Although their expression patterns were not similar, BRN2 and other NE marker molecules (CGA, SYP, and NCAM) were exclusively expressed in SCLC cell lines.

In contrast, by IHC, Notch1 was expressed in all of the NSCLC specimens and the NSCLC component of combined SCLC specimens (Table 3 and Supplemental Figure S1). WB analysis of lung cancer cell lines revealed that Notch1 was strongly expressed in NSCLC cell lines and in SCLC cell lines that did not have INSM1 and ASCL1 expression. This implies that Notch1 might have a biological function that opposes those of INSM1 and ASCL1. Although Hes1 was reported to be a key effector of the NE network in the Notch1 pathway, Hes1 expression was not always consistent with the Notch1 expression pattern, suggesting that various pathways could exist. We summarize the results of IHC staining of INSM1, CGA, SYP, NCAM, and Notch1 in Table 3.

**INSM1 Is a Crucial Regulator of ASCL1, BRN2, and NE Molecules in Lung Cancer Cells**

To investigate the biological effect of INSM1 in lung cancer cell lines, we first conducted INSM1 knockdown experiments using RNA interference in H69 and H889 cells as representatives of SCLC cells that express INSM1 (Figure 1B and Figure 2A). Knockdown of INSM1 expression in H69 and H889 cells resulted in significant reduction of the protein expression of ASCL1, BRN2, and NE molecules (CGA, SYP, and NCAM) (Figure 2A).
Next, to confirm that INSM1 actually affects ASCL1, BRN2, and NE molecules, we performed INSM1 transfection experiments and obtained stable cell lines that express it only in the presence of Dox using the tet-on inducible expression system applied to H358 and H1975 cells, which are representatives of NSCLC cell lines without the expression of INSM1 and other NE molecules. The expression of ASCL1 and NE molecules was induced by the forced expression of INSM1 (Figure 2, B and C). Although WB analysis did not detect the induction of BRN2 protein, an increase of the BRN2 mRNA expression level was detected by quantitative RT-PCR (Figure 2D). These results suggested that INSM1 is strongly involved in ASCL1 expression, so we performed a ChIP assay to find out whether ASCL1 is a direct target of INSM1 or not. The results demonstrated that INSM1 binds to the promoter region of ASCL1 directly (Figure 2E). In addition, the induction of the NE molecule proteins was confirmed by IFA analysis in vitro (Figure 3) and IHC staining in the tumor tissue sections formed from xenotransplanted cells (Figure 4A). Although the forced expression of INSM1 induced NE differentiation in lung cancer cell lines, the histological pattern did not change to NE morphology (Figure 4A).

The Effect of INSM1 on Proliferation and Apoptosis in Lung Cancer Cell Lines

The sizes of xenotransplanted tumors from tet-on INSM1-transfected H1975 cells with Dox (Dox⁺) were significantly smaller than those from tet-on INSM1-transfected H1975 cells without Dox (Dox⁻) (Figure 4B). Moreover, WB analysis showed that the forced expression of INSM1 reduced...
phospho-histone H3, Bcl-2, caspase-3, and cyclin D1, but increased cleaved caspase 3 (Figure 4C). In addition, a significant difference of proliferative activity was observed between the Dox− and Dox+ tumors by counting Ki-67−labeled cells (Dox− versus Dox+, 77.6 ± 6.2 versus 33.2 ± 9.2; N = 5; P < 0.01) (Figure 4D). These findings suggest that the forced expression of INSM1 in NSCLC cell lines suppresses cell proliferation activity and induces apoptotic activity. On the other hand, in the SCLC cell lines, knockdown of INSM1 expression decreased Bcl-2, but increased phospho-histone H3 and cleaved caspase 3. These findings suggest that knockdown of INSM1 expression facilitated the apoptotic pathway, but increased cell proliferative activity (Figure 4E). In the present study, the facilitated apoptotic activation seemed to be greater than the proliferative activity. The results of the cell counting assay could be explained by these results (Figure 4F).

Effects of ASCL1 and BRN2 in Lung Cancer Cells

We also conducted knockdown of ASCL1 and BRN2 expression in H69 and H889 cells, and transient transfection of ASCL1 and BRN2 in H358 and H1975 cells, to confirm the effects of ASCL1 and BRN2 in lung cancer cells. Knockdown or forced expression of ASCL1 and BRN2 did not affect INSM1 expression at all, suggesting that INSM1 is not regulated by ASCL1 and BRN2 (Figures 5 and 6). CGA and SYP protein expression was significantly suppressed by the knockdown of ASCL1 and BRN2 in H69 and H889 cells, but NCAM expression was slightly decreased, suggesting the existence of various pathways (Figure 5A and Figure 6A). NE molecules (CGA, SYP, and NCAM) were up-regulated in ASCL1-transfected H358 and H1975 cells at the protein level (Figure 5B). Meanwhile, the protein expression of NE molecules was not detected, but their mRNA was up-regulated in BRN2-transfected H358 and H1975 cells (Figure 6, B and C). These findings indicate that ASCL1 is a more crucial regulator for NE molecules than BRN2. The impact of ASCL1 on BRN2 expression was heterogeneous. BRN2 protein expression was significantly decreased in the H69 cells transfected with small interfering (si)ASCL1 (Figure 5A), and ASCL1 protein expression was slightly decreased in H69 cells transfected with siBRN2 (Figure 6A), suggesting that there is a mutual interaction between these two molecules in H69 cells. On the other hand, there seems to be no interaction of ASCL1 and BRN2 in H889 cells (Figure 5A and Figure 6A). In NSCLC cell lines, ASCL1 mRNA expression was increased in the BRN2-transfected H358 cells, but was not affected in the BRN2-transfected H1975 cells (Figure 6C). Meanwhile, BRN2 mRNA expression level was increased in ASCL1-transfected H1975 cells, but was not affected in ASCL1-transfected H358 cells (Figure 5C). Therefore, the transcriptional program driven by ASCL1 is heterogeneous, as previously reported.33 The present results demonstrate that ASCL1 seems to have a stronger effect on the regulation of NE differentiation than BRN2, even though BRN2 also regulates the NE molecules’ mRNA; furthermore, INSM1 should be considered as an upstream regulator for ASCL1, BRN2, and NE molecules in lung cancer cells. It has been shown that the knockdown of ASCL1 induces growth inhibition and apoptosis in SCLC cell lines17; however, the significance of BRN2 in SCLC on cell growth has not been clarified. In SCLC cell lines (H69 and H889), knockdown of BRN2 had no effects on cell proliferation and apoptosis in H69; in contrast, the
apoptotic pathway was facilitated and cell growth was suppressed in H889 (Supplemental Figure S2).

Effect of Notch1-Hes1 Signaling on INSM1, ASCL1, and BRN2

The protein expression of INSM1 and Notch1 showed the opposite patterns from each other, suggesting that the Notch1 signaling pathway could regulate INSM1 expression and ASCL1 (Figure 1B). Notch1-Hes1 signaling was reported to work as an inhibitor of ASCL1 and NE features.11,17 To elucidate whether Notch1-Hes1 signaling can regulate the expression of INSM1, we conducted Notch1 or Hes1 knockdown experiments using RNA interference in SCLC cell lines expressing Notch1 and Hes1 (H69AR and SBC3), and forced expression experiments in SCLC cell lines without the expression of Notch1 or Hes1 (H69 and H889) (Figure 1B). INSM1 was not induced in both H69AR and SBC3 cell lines transfected with siNotch1 (Figure 7A), but was expressed in only the H69AR cells transfected with siHes1 (Figure 7C). Forced expression of Notch1 or Hes1 significantly suppressed INSM1 expression, ASCL1, and BRN2 (Figure 7, B and D). We previously reported that forced expression of Notch1 resulted in the inhibition of SCLC growth.17 In the present study, we confirmed the effects of Notch1 and Hes1 on SCLC cell growth in supplemental experiments (Supplemental Figure S3). Forced expression of Notch1 or Hes1 in SCLC cell lines (H69 and H889) decreased phospho-histone H3 and Bcl-2, but increased cleaved caspase 3, suggesting that the proliferation was suppressed and the apoptotic pathway was facilitated, similar to the findings in a previous report.17 These findings demonstrated that Notch1-Hes1 signaling suppresses INSM1, ASCL1, and BRN2 in lung cancer cell lines, and forced expression of Notch1-Hes1 signaling inhibits cell growth in SCLC cell lines.

Figure 6  Brain-2 (BRN2) does not affect insulinoma-associated protein 1 (INSM1) expression, but is involved in the expression of neuroendocrine (NE) molecules and achaete-scute homolog-like 1 (ASCL1) in the lung cancer cell lines. BRN2 was knocked down or transiently expressed in lung cancer cell lines. All analyses were performed in triplicate. β-Actin was used as an internal control in Western blot (WB), whereas glyceraldehyde-3-phosphate dehydrogenase was also used in quantitative RT-PCR (RT-qPCR) analysis. A: The NE marker molecules [chromogranin A (CGA), synaptophysin (SYP), and neural cell adhesion molecule (NCAM)] decrease by knockdown of the BRN2 gene in the small-cell lung carcinoma (SCLC) cell lines. ASCL1 expression is slightly decreased only in H69 treated with siBRN2. INSM1 does not change at all. B: WB analyses reveal that the forced expression of the BRN2 gene does not affect the expression of other NE molecules in the adenocarcinoma (ADC) cell lines. C: RT-qPCR analysis reveals that BRN2 does not affect the expression of INSM1 mRNA in both cell lines. The expression of ASCL1, CGA, and NCAM mRNAs increases in H358 transfected with BRN2; on the other hand, the expression of CGA, SYP, and NCAM mRNA increases in H1975 transfected with BRN2. Data are given as means ± SD (C). **P < 0.01, ***P < 0.001. Si, small interfering.
Discussion

The present study is the first to confirm that INSM1 is expressed exclusively in SCLC specimens using IHC staining and also the first to elucidate the significance of INSM1 in the NE differentiation of lung cancer cells. We herein revealed that INSM1 regulates ASCL1, BRN2, and NE molecules (CGA, SYP, and NCAM), and has a role in proliferation and apoptosis. SCLC is an aggressive and highly metastatic tumor with an extremely poor prognosis. Although usually responsive to chemotherapy, recurrent tumors almost always develop in a chemoresistance type.1,2 Therefore, an emphasis on elucidating the critical molecules that correlate with malignant biological behavior and on exploring therapeutic targets must be focused on in SCLC.

INSM1 is a zinc finger transcription factor originally isolated from a human insulinoma subtraction library.20 INSM1 expression is specific in NE tumors, including SCLC, pituitary tumor, pheochromocytoma, medullary thyroid carcinoma, neuroblastoma, and retinoblastoma.20,27,29 INSM1 is abundantly expressed in SCLC, but it is not detected after birth in normal adult lung tissue, implying the INSM1 expression could contribute to the SCLC characteristics. Although INSM1 was identified as a highly specific marker of SCLC, the molecular mechanism remained to be elucidated.9,10

Several lines of evidence have suggested that ASCL1 could promote NE tumorigenesis and be involved in the survival, growth, and acquisition of chemoresistance of SCLC; in addition, ASCL1 is reported as a therapeutic target of SCLC cell lines.12,13 ASCL1 is essential for the
development of normal lung NE cells and other endocrine and neural tissues. Recently, INSM1 was reported as one of the gene targets in ASCL1-driving pathways. The present results demonstrated that the proteins of ASCL1 and NE molecules (CGA, SYP, and NCAM) and BRN2 mRNA expression levels were induced by forced expression of the INSM1 in NSCLC cell lines. In addition, the protein expression of ASCL1, BRN2, and NE molecules was significantly suppressed by knockdown of the INSM1 expression. We also confirmed that INSM1 binds to the promoter region of ASCL1 by using ChIP assay analysis. Thus, we concluded that INSM1 is an upstream regulator of ASCL1, and assumed that INSM1 function is exerted in the presence of ASCL1, because we observed INSM1 expression in the fetal lung of ASCL1-deficient mice (T.L., unpublished observation), in which no pulmonary NE cells are present. We also concluded that INSM1 is a regulator of the expression of BRN2 and NE molecules in lung cancer cell lines. Taking these findings together, INSM1 in SCLC is extremely important in various biological features.

NE molecules (CGA, SYP, and NCAM) are used as important complementary tools for the diagnosis of NE tumor of lung cancer. Several reports have suggested that the expression of these NE molecules contributes to SCLC malignancy. To extend previous findings of the expression of NE marker molecules in SCLC, we also investigated the effects of ASCL1 and BRN2 on the NE molecules’ pathway. The present study showed that ASCL1 was involved in the protein expression of NE molecules. We could not detect the expression of the NE molecules by the forced expression of BRN2, but their mRNA levels were increased. BRN2 is a developmental neural cell—specific transcription factor that plays some role in maintaining the NE phenotype of SCLC with ASCL1. A few reports have indicated that BRN2 is a higher-level regulator of ASCL1. By forced knockdown experiments of BRN2, we found that the relationship between ASCL1 and BRN2 is heterogeneous. A mutual interaction was observed in H69 cells, but they were independent in H889 cells in regulating NE differentiation. Although the BRN2 mRNA expression level was increased by the forced expression of ASCL1 in H1975, the level of ASCL1 mRNA was increased by the forced expression of BRN2. These findings suggested that the mutual relationship between ASCL1 and BRN2 is complex. In addition, only INSM1 can regulate all of ASCL1, BRN2, and NE molecules constantly. As described, the present studies investigated the roles of three transcriptional factors in NE differentiation, and it is expected that these would contribute significantly to future SCLC research.

We also examined a xenotransplanted tumor with H358-tet-on INSM1 and H1975-tet-on INSM1. Although before the experiments we had imagined that forced expression of INSM1 could induce NE morphology, there were no histological differences between the Dox− and Dox+ tumors. However, the induction of NE differentiation was confirmed by IHC staining in the tumor with the induction of INSM1 by Dox treatment. Presumably, INSM1 was not involved in lung cancer histology, but the contribution to NE differentiation was proved in vivo and in vitro.

Moreover, we found that INSM1 affected the cell proliferation and apoptosis of lung cancer cells. Little has been known about the effect of INSM1 on the proliferation of lung cancer cells. Xenotransplanted tumors with cells forced to express INSM1 were significantly smaller. In addition, we proved that the forced expression of INSM1 suppressed the cell proliferative activity and activated the apoptotic pathway in NSCLC cell lines. On the other hand, in SCLC cell lines, the knockdown of INSM1 expression resulted in the inhibition of cell growth and the activation of the apoptotic pathway. Therefore, we concluded that INSM1 could not only function as a regulator of the NE differentiation pathway, but also act as a regulator of cell growth. Although the effects of INSM1 on lung cancer cell lines varied depending on the cell type, knockdown of INSM1 expression suppressed NE differentiation and cell growth in SCLC, suggesting that INSM1 could be a target of SCLC treatment.

Several reports have indicated functional interactions between Notch1-Hes1 signaling and ASCL1, and we previously reported that Notch1 signaling suppresses ASCL1 and the NE molecules, and inhibits the cell growth of SCLC. However, to date, how INSM1 expression is regulated has remained unclear. Interestingly, in our WB study using lung cancer cell lines, those with Notch1 expression did not have INSM1 expression, and vice versa. In addition, all cases of surgically resected pure SCLC specimens and the SCLC component of combined SCLC specimens were negative for IHC staining for Notch1 (Supplemental Figure S1 and Table 3). Through the forced expression/knockdown of Notch1 or Hes1 experiments, we ascertained that Notch1-Hes1 signaling suppresses all of INSM1, ASCL1, and BRN2. Notably, Hes1, a key effector of the Notch signaling pathway, was considered as a stronger suppressor than Notch1, and different pathways from Notch1 could exist. This could explain why the protein expression of INSM1 was evident only by the knockdown of Hes1. In addition, we examined the IHC for Notch1 in the H358-tet-on INSM1 tumor xenograft model, but the expression of Notch1 did not differ between Dox− and Dox+ tumors (Supplemental Figure S4). These results
support the assertion that Notch1 is an upstream regulator of INSM1 (Figure 8).

A high incidence of mutations in TP53 and RB1 has been identified in small-cell carcinoma of the lung,34,35 and these bi-allelic TP53 and RB1 lesions seem to be fundamental genetic events in small-cell carcinogenesis. This was confirmed by a mouse experimental study, in which small-cell carcinomas were induced in the lungs of mice deficient in Tp53 and Rb1.36 Among these two genes, mutation or loss of RB1 seems to be more characteristic in the development of small-cell carcinoma because RB1 was detected as the most decreased protein in small-cell carcinoma compared with non—small-cell carcinoma,37 and because Rb1 knockout mice show the proliferation of NE cells in the developing lungs.38

In the past 10 years, many cases with transformation from NSCLC to SCLC have been reported in one of the resistance mechanisms against EGFR tyrosine kinase inhibitors, and these tumors were approved as small-cell carcinoma by histology and IHC for NE markers.39 Interestingly, according to Niederst et al,40 all of the cases of transformation from ADC to small-cell carcinoma had loss of RB1. In the present study, we analyzed NE differentiation from the viewpoints of transcriptional regulation and Notch signaling, but not from the viewpoint of genetic alterations, especially in RB1. Although it has been reported that shRNA-mediated depletion of RB1 in EGFR mutant cancer cells resistant to an EGFR tyrosine kinase inhibitor did not induce NE differentiation,40 there should be underlying mechanisms between RB1 mutations and NE differentiation that must be induced by the up-regulation of INSM1 and ASCL1 accompanying Notch1-Hes1 dysfunction.

In summary, SCLC exclusively overexpresses INSM1, and we demonstrated that INSM1 regulates the NE differentiation pathway represented by ASCL1, BRN2, and NE molecules (CGA, SYP, and NCAM) in lung cancer in vitro and in vivo (Figure 8). We also revealed the expression of INSM1 on the cell growth in lung cancer cells. Furthermore, we confirmed that the Notch1-Hes1 signaling pathway suppresses INSM1 expression. Further study of INSM1, considered as a crucial transcriptional factor involved in highly specific molecules in SCLC (ASCL1, BRN2, CGA, SYP, and NCAM), is needed. The present study promotes our understanding of the significance of INSM1 in SCLC, which will hopefully lead to the development of novel targeted therapies and an improved prognosis in SCLC.

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Supplemental Data

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References


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