Achaete-Scute Complex Homologue 1 Regulates Tumor-Initiating Capacity in Human Small Cell Lung Cancer

Tianyun Jiang,1 Brendan J. Collins,2 Ning Jin,1 David N. Watkins,1 Malcolm V. Brock,1,2 William Matsui,1 Barry D. Nelkin,1 and Douglas W. Ball1,3

Departments of ‘Oncology, ’Surgery, and ‘Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

Abstract

The basic helix-loop-helix transcription factor achaete-scute complex homologue 1 (ASCL1) is essential for the development of normal lung neuroendocrine cells as well as other endocrine and neural tissues. Small cell lung cancer (SCLC) and non-SCLC with neuroendocrine features express ASCL1, where the factor may play a role in the virulence and primitive neuroendocrine phenotype of these tumors. In this study, RNA interference knockdown of ASCL1 in cultured SCLC resulted in inhibition of soft agar clonogenic capacity and induction of apoptosis. cDNA microarray analyses bolstered by expression studies, flow cytometry, and chromatin immunoprecipitation identified two candidate stem cell marker genes, CD133 and aldehyde dehydrogenase 1A1 (ALDH1A1), to be directly regulated by ASCL1 in SCLC. In SCLC direct xenograft tumors, we detected a relatively abundant CD133high-ALDHhigh-ASCL1high subpopulation with markedly enhanced tumorigenicity compared with cells with weak CD133 expression. Tumorigenicity in the CD133high population depended on continued ASCL1 expression. Whereas CD133high cells readily reconstituted the range of CD133 expression seen in the original xenograft tumor, CD133low cells could not. Our findings suggest that a broad range of SCLC cells has tumorigenic capacity rather than a small discrete population. Intrinsic tumor cell heterogeneity, including variation in key regulatory factors such as ASCL1, can modulate tumorigenicity in SCLC. [Cancer Res 2009;69(3):845–54]

Introduction

Despite the success of antismoking campaigns and extensive efforts to develop new therapeutic approaches, lung cancer remains the leading cause of cancer death in the United States, with small cell lung cancer (SCLC) accounting for ~13% of lung cancer cases (1). Up to 90% of SCLC tumors exhibit characteristic molecular abnormalities, including mutation of Rb and p53, and allelic losses on chromosome 3p (2). A striking feature of SCLC development remain largely unknown.

To further investigate the role of ASCL1 in SCLC, we used an RNA interference approach in SCLC cultures and subsequently in direct tumor xenografts. ASCL1 down-regulation in cultured SCLC resulted in inhibition of soft agar clonogenic growth and induction of apoptosis. The progenitor/stem cell markers CD133 and aldehyde dehydrogenase 1A1 (ALDH1A1) were first identified as potential targets for ASCL1 transcriptional up-regulation in SCLC by cDNA microarray, and then confirmed by RNA and protein expression analysis, flow cytometry, and chromatin immunoprecipitation (ChIP). To better capture the diversity of tumor-initiating capacity cells in SCLC tumors, we used a set of SCLC tumors implanted directly as s.c. xenografts in nu/nu mice. Using these xenograft tumors, we isolated a CD133high-ALDHhigh-ASCL1high subpopulation that was much more tumorigenic than CD133low-ALDHlow-ASCL1low cells from the same tumor. Knockdown of ASCL1 impaired the tumor-initiating capacity of CD133high-ALDHhigh-ASCL1high subpopulation. Therefore, high ASCL1 expression correlates with high tumorigenicity among SCLC subpopulations. ASCL1 seems to play a critical role in maintaining the ability to initiate new tumors.

Materials and Methods

Cell culture and transfection. Classic SCLC cell lines NCI-H1618, H345, H209, and H889; variant SCLC lines NCI-H82, H417, and H2106; and NSCLC...
lines NCi-H1299 and H460 (all obtained from the American Type Culture Collection) were maintained in RPMI 1640 supplemented with 5% fetal bovine serum (FBS). NCi-H1618 cells were transfected with either 25 nmol/L of nontargeting control (Dharmacon) or one of three ASCL1-targeting small interfering RNAs (siRNA) using Lipofectamine 2000 (Invitrogen). The ASCL1-targeting sequences were 5′-ACCGCGTCAAGTCG for 21 d. Viable colonies were visualized by flow cytometry. An aliquot from each sample was transferred to a tube containing DEAB, an ALDH inhibitor, to define background fluorescence. After incubation, the cells were washed twice with the Aldefluor assay buffer and analyzed using a FACSCalibur flow cytometer.

**Chromatin immunoprecipitation.** ChiP was performed using an EZ-ChiP kit (Millipore) on cells treated 72 h in the presence of control or ASCL1 siRNA. Cross-linking was performed with a 10-min incubation with 1% formaldehyde. After lysis, sonication yielded an average fragment size of 500 bp. Solubilized chromatin was precleared with salmon sperm DNA/ protein A agarose and immunoprecipitated with normal mouse IgG or Msi1 antibody (BD Pharmingen). PCR was performed using primers directed at conserved regions containing the ASCL1-binding E-box motif G/C-A-G/G-C/T-G-G/G (18) identified in the proximal promoter regions of the prominin 1 (PROM1)/CD133, ALDH1A1, and delta-like 3 (DLL3) genes: CD133, TGGGATTTACGCACTCAGGG (forward) and ACCTGGA-CGACATCCAGG (reverse); ALDH1A1, AGCCTGGTCTGGAAGACCC (forward) and TTACAAAGCGAATCTGGTG (reverse); and DLL3, GTCCTCTGATGGAAGCAGG (forward) and AGAGGCTCGGCTTCAAG-GAG (reverse). Human NIS (sodium iodide symporter) promoter region primers were used as a negative control: NIS, CTAGGCTTGGAGCAGG-GAGTC (forward) and CCTGGCTTGTGCCTGTCCT (reverse). Input used represents a 10-fold dilution of unprecipitated genomic DNA.

**Isolation of direct tumor xenograft cells.** Lxx3 and LX36 direct xenograft lines were derived from bronchoscopic biopsy specimens of treatment-naive SCLC patients, as previously described (19), expanded briefly in nu/nu mice, and stored in liquid nitrogen. To isolate xenograft tumor cells, careful dissection and then mechanical disaggregation were used to avoid the possible damage of cell surface markers by enzymatic digestion and to minimize contamination by stromal cells. Tumor tissues were put into 100-mm sterile Petri dishes with ice-cold serum-free medium, finely minced, triturated, and filtered. The cells were washed with serum-free medium and then treated with erythrocyte lysis buffer. For CD133 fluorescence-activated cell sorting (FACS) of live cells, cells were incubated with anti-CD133/1-PE at 4°C for 20 min. Sorting was performed using a FACS Vantage cell sorter (BD Immunocytometry Systems).

**Immunohistochemistry for cells in nude mice.** Animal studies were approved by the Johns Hopkins Animal Care and Use Committee. Viable cells were counted with trypan blue dye exclusion, resuspended in sterile Matrigel, and injected s.c. into the flanks of 4- to 6-wk-old female athymic nu/nu mice (Harlan Laboratories) using a 25-gauge needle. Tumor growth was assessed by caliper measurement. Animals were sacrificed at 32 d after implantation or when the tumor volume reached 0.5 cm³. Some of the tumor tissues were fixed in formaldehyde and stained with H&E for histologic analysis.

**Results**

ASCL1 maintains growth, apoptosis resistance, and soft agar cloning capacity in cultured SCLC. To study the role of ASCL1 in SCLC growth and tumorigenesis, we used RNA interference to repress endogenous ASCL1 expression, first in cultured SCLC cell lines and subsequently in SCLC direct xenograft tumors. To minimize the chance of off-target effects induced by the siRNA, we separately transfected samples of the SCLC cell line NCi-H1618 with three individual siRNA duplexes targeting ASCL1. As shown in Fig. 1A, each of the siRNA duplexes reduced endogenous ASCL1 expression both at the mRNA and the protein level. We compared the proliferation rate of ASCL1 knockdown cells with that of control cells using an MITT assay. To maintain effective knockdown throughout the experiment, we repeated the siRNA transfection at...
days 3 and 6. All three siRNA duplexes moderately inhibited proliferation (Fig. 1B). Cell cycle analysis with Hoechst 33258 showed that the sub-G1-G2 population increased from ~2% in control siRNA-treated cells to 5.5%, 7.6%, and 12.6% in ASCL1 siRNA-treated cells (Fig. 1C). Cell surface Annexin V reactivity increased from 7.9% in control cells to 17.7%, 19%, and 27.5% in SCLC cells 72 hours after ASCL1 siRNA treatment, confirming increased apoptosis (data not shown). The S-phase fraction decreased very modestly from 21.5% to 16.6% to 20%, with no significant changes in the distribution of G1 or G2-M. Repeat cell cycle analysis after BrdUrd incorporation similarly showed unchanged G1 and G2-M populations and minimal reduction in S phase (28.5% to 22.8–28%). These data indicate that knockdown of ASCL1 in cultured SCLC can cause induction of apoptotic cell death and modest repression of cell proliferation, largely consistent with earlier findings of Osada and colleagues (16).

To study the effect of long-term knockdown of ASCL1 on SCLC, we developed lentiviruses expressing shRNAs directed against two different target sequences of ASCL1 using pL3.7, which also expresses green fluorescent protein (GFP) as a marker for infection efficiency. Luciferase shRNA in the same vector was used as a negative control. Fluorescence microscopy indicated that ~80% of NCI-H1618 cells were infected (data not shown). ASCL1 knockdown was effective, as shown in Fig. 1D. Either of the ASCL1-targeting shRNA lentiviruses caused ~80% reduction in soft agar cloning efficiency.

**Genes induced by ASCL1 in SCLC include CD133 and ALDH1A1.** To identify possible regulatory targets for ASCL1 in SCLC, we compared mRNA expression profiles between control and ASCL1 siRNA-treated cells using Affymetrix U133 Plus 2.0 arrays. Table 1 shows genes selected according to a threshold of a 2-fold reduction by all the three individual siRNAs, following 72 or 120 hours of treatment. Genes regulated to that extent by fewer than three of the effective siRNAs were excluded as possible off-target effects. For the purpose of this study, we focused on genes whose expression decreased with ASCL1 knockdown based on the

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Knockdown of ASCL1 results in induction of apoptosis and reduced clonogenicity in cultured SCLC. A, NCI-H1618 cells were transfected with a control siRNA (Dharmacon) or one of three individual siRNA duplexes against ASCL1. Quantitative RT-PCR and immunoblotting performed 48 h after transfection showed effective knockdown of ASCL1. B, NCI-H1618 cells were treated with ASCL1 siRNA thrice at days 0, 3, and 6 and grown in complete medium. Reduced proliferation is shown by MTT assay. Points, mean (n = 3); bars, SD. C, cell cycle analysis with Hoechst 33258 was performed 48 h after treatment with siRNA. The percentages of sub-G1 and S phase are indicated below. D, NCI-H1618 cells infected with lentivirus expressing shRNA against luciferase or ASCL1 were seeded in 0.4% soft agar at a density of 20,000 per well and grown for 21 d. Viable colonies were visualized and counted after crystal violet staining. Columns, mean (n = 3); bars, SD. Immunoblotting of cell lysates collected before seeding showed efficient knockdown of ASCL1 expression.
primary role of this transcription factor as a transcriptional activator. The regulation of expression of these genes by ASCL1 was validated by real-time reverse transcription-PCR (RT-PCR; Table 1). One of these genes is DLL3, encoding a Notch ligand, which has been identified as an ASCL1 transcriptional target in mammalian nervous system development (4, 20). To further understand the mechanisms by which ASCL1 regulates the expression of these genes, we compared the rate of decay of ASCL1 and candidate target gene mRNAs following siRNA treatment. Figure 2A shows that expression of PROM1/CD133, MMP10, and DLL3 mRNA decreased by about 40% to 70% at 24 hours, ~8 hours after effective suppression of ASCL1 protein by siRNA treatment. These findings suggest that these genes could be either direct or rapidly activated indirect targets of ASCL1.

To understand ASCL1 regulation of the PROM1/CD133 gene, we first analyzed expression levels of the four adjacent PROM1/CD133 alternative initiating exons (21). Alternate exon 1A was most abundantly expressed by quantitative RT-PCR in NCI-H1618 cells; expression of this exon decreased by approximately 5- to 7-fold following ASCL1 knockdown (data not shown). A candidate ASCL1-binding E-box motif (4) was identified 104 bp 5' of exon 1A. ChIP analysis (Fig. 2B) confirmed association of endogenous ASCL1 protein to the CD133 promoter region that was eliminated by ASCL1 siRNA. Comparable specific ASCL1 association with the ALDH1A1 proximal promoter region was also detected (Fig. 2B, middle). A DLL3 promoter region, orthologous to a mouse DLL3 region reported to bind ASCL1 (20), also showed specific association with ASCL1 in human SCLC cells.

Table 1. Selected ASCL1-regulated genes identified by Affymetrix U133 microarray and validated by quantitative RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change</th>
<th>Pathway involved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affymetrix chip</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>ASCL1</td>
<td>6.5</td>
<td>8.6–20</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>8.6</td>
<td>10–22</td>
</tr>
<tr>
<td>PROM1/CD133</td>
<td>2.0</td>
<td>3.3–10</td>
</tr>
<tr>
<td>DLL3</td>
<td>2.5</td>
<td>1.8–2</td>
</tr>
<tr>
<td>MMP10</td>
<td>3.1</td>
<td>3–12</td>
</tr>
<tr>
<td>LIFR</td>
<td>3.0</td>
<td>5–7.1</td>
</tr>
<tr>
<td>RAB3B</td>
<td>2.5</td>
<td>8–3–20</td>
</tr>
<tr>
<td>PTPT4A3</td>
<td>2.3</td>
<td>2.5–3.6</td>
</tr>
<tr>
<td>SNF1L2</td>
<td>2.2</td>
<td>2.6–5.6</td>
</tr>
<tr>
<td>EPHA4</td>
<td>2.2</td>
<td>2.6–5.6</td>
</tr>
<tr>
<td>SCN3A</td>
<td>2.3</td>
<td>5–5.3</td>
</tr>
</tbody>
</table>

The effect of ASCL1 knockdown on CD133 and ALDH1A1 mRNA expression raised the possibility that ASCL1 could regulate the tumor-initiating capacity of SCLC tumor cells. CD133 and ALDH1A1 have been characterized as progenitor/stem cell markers in normal development, and they are also expressed in tumor-initiating populations in several different tumor types (22–26). To explore this potentially important function of ASCL1, we first confirmed significant regulation of these markers at the protein level. CD133 protein levels significantly decreased by immunoblot following ASCL1 siRNA treatment in cultured NCI-H1618 and NCI-H209 cells (Supplementary Fig. S1). By FACS analysis, an impressive fraction of control NCI-H1618 cells, 65%, was positive for CD133, normalized to an IgG control (Fig. 2C). This fraction dropped to 36% after 3 days of treatment with ASCL1 siRNA. It is noteworthy that the distribution of CD133 followed a Gaussian rather than a bimodal pattern, indicating a continuous range of CD133 expression rather than discrete populations of positive and negative cells. Treatment with ASCL1 siRNA resulted in skewing of the CD133 distribution leftwards (Fig. 2C). Aldefluor assays were performed to measure the activity of ALDH, which converts substrate BAAA to the fluorescent product BAA. The distribution of Aldefluor activity was also continuous; knockdown of ASCL1 caused its leftward movement with a dramatic decrease of Aldefluor bright cells from 58.9% to 21.4% after 5 days of ASCL1 siRNA treatment (Fig. 2C).

Table 1. Selected ASCL1-regulated genes identified by Affymetrix U133 microarray and validated by quantitative RT-PCR

Table 1. Selected ASCL1-regulated genes identified by Affymetrix U133 microarray and validated by quantitative RT-PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Fold change</th>
<th>Pathway involved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Affymetrix chip</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>ASCL1</td>
<td>6.5</td>
<td>8.6–20</td>
</tr>
<tr>
<td>ALDH1A1</td>
<td>8.6</td>
<td>10–22</td>
</tr>
<tr>
<td>PROM1/CD133</td>
<td>2.0</td>
<td>3.3–10</td>
</tr>
<tr>
<td>DLL3</td>
<td>2.5</td>
<td>1.8–2</td>
</tr>
<tr>
<td>MMP10</td>
<td>3.1</td>
<td>3–12</td>
</tr>
<tr>
<td>LIFR</td>
<td>3.0</td>
<td>5–7.1</td>
</tr>
<tr>
<td>RAB3B</td>
<td>2.5</td>
<td>8–3–20</td>
</tr>
<tr>
<td>PTPT4A3</td>
<td>2.3</td>
<td>2.5–3.6</td>
</tr>
<tr>
<td>SNF1L2</td>
<td>2.2</td>
<td>2.6–5.6</td>
</tr>
<tr>
<td>EPHA4</td>
<td>2.2</td>
<td>2.6–5.6</td>
</tr>
<tr>
<td>SCN3A</td>
<td>2.3</td>
<td>5–5.3</td>
</tr>
</tbody>
</table>

We further tested 10 SCLC lines (7 classic and 3 variant) for the concordance of ASCL1, CD133, and ALDH. The seven classic SCLC lines all expressed abundant ASCL1, six of seven had abundant CD133 expression by FACS, and six of seven had significant ALDH activity. In contrast, the variant SCLC lines, which lack significant neuroendocrine marker expression, also lacked detectable ASCL1 and ALDH activity, and one of three expressed CD133 (Supplementary Table S1). In two NSCLC cell lines that lack ASCL1 expression (NCI-H1299 and NCI-H460), only a small subpopulation (under 5%) expressed CD133 and had ALDH activity (Supplementary Fig. S2). Therefore, ASCL1 and CD133 expression, and ALDH activity seem to be generally concordant across a broad range of lung cancer cell lines.

ASCL1 is coregulated with CD133 and ALDH1A1 in SCLC tumor subpopulations. To better model the range and diversity of tumor-initiating capacities in native human SCLC tumors, we performed further studies using direct SCLC tumor xenografts developed by two of the coauthors (D.N.W. and M.V.B.). These direct tumor xenografts were generated by collecting tumor cells from bronchoscopic biopsies of extensive-stage SCLC patients and directly implanting them s.c. in athymic nude mice. These xenograft lines, LX33 and LX36, have been frozen and expanded briefly in mice. LX33 and LX36 xenograft tumors exhibited typical SCLC morphology by H&E staining, expressed classic neuroendocrine markers and ASCL1, and lacked detectable Rb protein by
immunoblot, similar to many classic SCLC lines and tumors (Fig. 3A; data not shown). We detected abundant CD133-positive and Aldefluor bright cells in both of these SCLC xenografts (Fig. 3B; data not shown). In addition, similar to the SCLC cell lines, the distribution of CD133 followed a Gaussian rather than a bimodal distribution. We selected the highest and the lowest 5% of cells by FACS and defined them CD133^{high} and CD133^{low} cells, respectively. The purity, as assessed by CD133 antibody against a second epitope, was ~90% for CD133^{high} and 98% for CD133^{low} cells, indicating considerable enrichment of both subpopulations (Fig. 3B, bottom). The fraction of human cells was estimated at ~93% using FACS for human-specific CD59 for both CD133^{high} and CD133^{low} subpopulations (Supplementary Fig. S3). To test for contamination by mouse endothelial cells and progenitors that could be reactive for CD133, we performed control FACS using anti-mouse CD31. Both CD133^{high} and CD133^{low} subpopulations had <3% CD31-reactive cells (data not shown).

Because both CD133 and ALDH1A1 are apparent targets of ASCL1, we compared mRNA expression levels of ASCL1, ALDH1A1, and CD133 in CD133^{high} and CD133^{low} subpopulations to determine whether expression of these genes normally cosegregates in SCLC. Quantitative real-time PCR of two different direct xenograft lines revealed that CD133^{high} cells expressed much higher levels of ASCL1 and ALDH1A1 than did CD133^{low} cells (Fig. 3C). Immunoblotting also confirmed the coexpression of CD133 and ASCL1. These data indicate that ASCL1 is jointly regulated with CD133 and ALDH1A1 in these SCLC xenograft tumor subpopulations.

**ASCL1 is critical for enhanced tumor-initiating capacity in the CD133^{high} SCLC subpopulation.** CD133 has been identified as a marker for tumor cells with high tumor-initiating capacity in brain, colon, prostate, and pancreatic cancer (24, 27–29). We evaluated the tumor-initiating capacity of CD133^{high} and CD133^{low} fractions in SCLC direct xenograft tumors by s.c. injection into athymic nu/nu mice. Comparable viability, by trypan blue dye...
exclusion, was observed in CD133\textsuperscript{high} and CD133\textsuperscript{low} cells following isolation (79% and 74%) and equal numbers of viable cells were injected. As shown in Fig. 4A, as few as 1,000 CD133\textsuperscript{high} cells reliably generated s.c. tumors after 4 weeks. In contrast, although 100,000 and 25,000 CD133\textsuperscript{low} cells showed similar tumor formation capacity as CD133\textsuperscript{high} cells, 5,000 and 1,000 CD133\textsuperscript{low} cells were much less efficient in initiating tumors. Moreover, the tumors generated by CD133\textsuperscript{low} cells were significantly smaller than those generated by the CD133\textsuperscript{high} xenograft fraction. Tumor growth curves showed that it took longer for CD133\textsuperscript{low} cells to form palpable tumors (Fig. 5D). The tumors generated by transplantation of CD133\textsuperscript{high} cells showed a similar distribution of CD133 expression compared with parental tumors derived from unsorted cells (Fig. 4B), suggesting that CD133\textsuperscript{high} cells are able to give rise to CD133\textsuperscript{low} cells and reconstitute the whole tumor population. In contrast, tumors arising from CD133\textsuperscript{low} cells had a consistently smaller fraction of CD133\textsuperscript{high} cells than did unselected parental tumors. These data indicate that cells with tumor-initiating capacities are enriched in the CD133\textsuperscript{high} subpopulation. Low CD133 expression did not exclude tumor-initiating cells, but this capacity was expressed at a lower rate, and CD133\textsuperscript{low} cells were unable to fully and rapidly reconstitute cells with high CD133 expression.

To study whether ASCL1 plays a critical role in tumorigenicity of CD133\textsuperscript{high} cells, we infected the freshly isolated CD133\textsuperscript{high} cells with ASCL1-expressing vector, and tumor formation was observed (Fig. 3A). H&E staining of direct xenograft tumor LX33 and LX36 showed typical SCLC morphology. B, freshly isolated cells from direct xenograft tumors were labeled with CD133 antibody conjugated with PE. Top, the highest and the lowest 5% of cells were selected by FACS; bottom, CD133\textsuperscript{high} and CD133\textsuperscript{low} fractions were reanalyzed with CD133-APC targeted against a second CD133 epitope. C, quantitative RT-PCR and immunoblotting show coexpression of ASCL1, CD133, and ALDH1A1 in CD133\textsuperscript{high} cells derived from LX36 (left) and LX33 (right) direct xenograft tumors (black columns, CD133\textsuperscript{high} cells; white columns, normalized to CD133\textsuperscript{low}). Representative data from two independent experiments are shown.

![Figure 3](image-url)
with ASCL1 shRNA or a control luciferase shRNA lentivirus. We observed effective knockdown of ASCL1 protein in CD133\textsuperscript{high} cells. As shown in Fig. 5A, ASCL1 knockdown resulted in ASCL1 levels comparable with those seen in CD133\textsuperscript{low} cells from the same tumor. Whereas eight of eight mice injected with 5,000 CD133\textsuperscript{high} cells infected with control virus developed tumors, only four of eight mice injected with CD133\textsuperscript{high} cells infected with ASCL1 shRNA lentivirus developed tumors. Three of eight mice injected with 5,000 CD133\textsuperscript{low} cells developed tumors (Fig. 5B). Similar proportions were observed when 1,000 cells were used. Tumor growth curves showed that the tumors derived from CD133\textsuperscript{high} cells grew much faster than those from CD133\textsuperscript{low} cells of the same direct xenograft line (Fig. 5C and D). Treatment of CD133\textsuperscript{high} cells with ASCL1 shRNA lentivirus dramatically decreased the tumor proliferation rate compared with control CD133\textsuperscript{high} cells. Thus, the CD133\textsuperscript{low} fraction showed much lower tumor-initiating capacity, and ASCL1 knockdown effectively reduced the tumorigenicity of the CD133\textsuperscript{high} fraction to the level seen in CD133\textsuperscript{low} cells. Similar results were obtained in a second xenograft tumor line LX33 (Supplementary Fig. S4).

In summary, these data suggest that a naturally existing SCLC tumor subpopulation jointly enriched for CD133, ASCL1, and ALDH has an enhanced tumor-initiating capacity, which is at least partially dependent on ASCL1 regulation.

**Discussion**

The bHLH transcription factor ASCL1/Mash1 was initially discovered as an early regulator of mammalian neural development, promoting lineage commitment of neural and oligodendroglial progenitors in the central nervous system (CNS), as well as sympathoadrenal and enteric precursors in the peripheral nervous system (3, 7, 8). In addition to its role in neural development, ASCL1 is essential for the appearance of lung neuroendocrine cells as well as several other neuroendocrine tissues, including calcitonin-producing thyroid C cells and adrenomedullary chromaffin cells (10, 30). Several lines of evidence suggested that ASCL1 could promote neuroendocrine tumorigenesis in the lung, including a transgenic overexpression model and cell culture–based RNA interference models (12, 14). In this study, we show that knockdown of ASCL1 induces apoptosis and moderate growth inhibition in cultured SCLC cells, consistent with a previous report (16). We show that repression of ASCL1 causes a more dramatic reduction of soft agar clonogenicity and tumorigenicity in nude mice. Furthermore, variations in ASCL1 expression levels within SCLC tumors positive) and hard to infect (GFP negative) cells (data not shown).
seem to correlate with the tumor-initiating capacity of these subpopulations.

Identification of transcriptional targets of ASCL1 in SCLC that are relevant to the initiation of new tumors has been inconclusive, to date. By cDNA microarray analysis following ASCL1 knockdown, we identified two previously identified target genes of ASCL1, DLL3 and RAB3B, involved in Notch signaling in the developing nervous system and neurotransmitter vesicle trafficking, respectively (4, 31, 32). We confirmed that ASCL1 associates with human DLL3 gene promoter, similar to findings in the developing mouse CNS. Interestingly, we have not observed down-regulation of any classic neuroendocrine differentiation markers, such as chromogranins, synaptophysin, and SCG2. Possible explanations include persistence of sufficient threshold levels of ASCL1, redundant transcriptional activators, or, less likely, prolonged stability of these neuroendocrine mRNAs. The ASCL1-regulated genes in our study have been implicated in several critical signaling pathways in tumorigenesis, cell proliferation, and tumor metastasis (Table 1), indicating that besides an effect on neuroendocrine differentiation, ASCL1 may exert a more profound influence on classic SCLC.

CD133, ALDH1A1, leukemia inhibitory factor receptor (LIFR), and EPHA4 are putative stem cell markers or involved in regulating stem/progenitor cell function or differentiation. We showed association of endogenous ASCL1 with the CD133 and ALDH1A1 promoter regions. CD133 and ALDH were previously identified as markers for high tumor-initiating capacity in glioblastoma multiforme as well as colon, breast, and pancreatic cancer (27, 28, 33, 34). Whether SCLC tumors have discrete populations with enhanced tumor-initiating capacity was not previously known. Thus, we investigated the possibility of using CD133 and ALDH1A1 as markers for tumor-initiating capacity in SCLC. Because established SCLC cell lines could lose their heterogeneity of tumor initiation capacity after extensive passaging in serum-containing medium, we used a direct xenograft model, in which the cancer cells collected from SCLC patients were injected s.c. and expanded briefly in mice. Presumably, these low-passage direct xenograft tumor cells have undergone some selective pressure for the ability to form s.c. tumors rather than to grow in suspension culture. This model is of particular interest for studying a characteristic feature of tumor-initiating cells—the ability to generate non-tumorigenic end cells. We found that CD133high cells were highly tumorigenic, with only 1,000 CD133high cells forming aggressive tumors in a short period of time. The similarity of CD133 distribution of the tumors generated by CD133high and unsorted parental cells suggested that CD133high cells are able to give rise to a CD133low subpopulation. On the other hand, significantly more CD133low cells were required to initiate tumors and CD133low cells may not be able to effectively reconstitute the whole tumor population, including CD133high cells. Interestingly, CD133 expression in secondary tumors from CD133low cells was somewhat more abundant than the original CD133low subfraction (compare Figs. 3B and 4B). It is unknown whether this increase represents proliferation of remaining CD133high cells, conversion of CD133low cells, or a combination of both processes.

The cosegregated expression of CD133, ALDH1A1, and ASCL1 in a cellular subpopulation with high tumorigenicity seems broadly consistent with the cancer stem cell hypothesis. However, we could not completely exclude tumorigenic cells by selecting CD133low cells. Furthermore, we observed Gaussian rather than discrete bimodal distributions of CD133 expression and ALDH activity.

Figure 5. ASCL1 regulates tumor-initiating capacity. A, freshly isolated CD133high cells from direct xenograft line LX36 were infected with lentivirus expressing control luciferase shRNA or ASCL1 shRNA for 4 d. Immunoblotting shows effective knockdown of ASCL1. B, reduced percentage of tumor-bearing mice after ASCL1 knockdown compared with control shRNA. C, reduced mean tumor volume following ASCL1 knockdown. *, P < 0.05 versus CD133high tumor. **P < 0.01 versus CD133high tumor (two-tailed Student’s t test). Columns, mean; bars, SD. D, reduced mean tumor volume following ASCL1 knockdown at various time points after implantation.
In addition, the very high tumorigenicity of CD133<sup>high</sup> cells could be modulated by ASCL1 knockdown. On balance, our data favor a stochastic variation model of tumorigenicity in SCLC, with a large fraction of cells able to initiate a new tumor (35). However, because our studies use xenograft lines selected for s.c. tumor survival, we may not have characterized some cells present in the original cancer with low tumor-initiating potential.

Recently, Eramo and colleagues (36), focusing mainly on NSCLC, reported that a small fraction of CD133-positive cells in human lung cancer carry tumor-initiating capacity. In the present study, we further show that in SCLC, ASCL1 may actively maintain the high-tumor-initiating phenotype of a relatively abundant, rather than discrete, subpopulation of SCLC cells. The detailed mechanisms by which ASCL1 modulates tumor-initiating capacity remain unclear. We detected abundant expression of several additional stem cell–related genes such as *Masashi, Bmi1*, and *EZH2* (37, 38), which were equivalent in both CD133<sup>high</sup> and CD133<sup>low</sup> subpopulations and not regulated by ASCL1 in cultured SCLC. The brachonoleuvar progenitor marker CD34<sup>+</sup> was low in both populations (data not shown). We could not exclude the possibility that ASCL1 may function as a transcription repressor for some critical targets. Most recently, Osaka and colleagues (31) reported that ASCL1, when overexpressed in the adenocarcinoma line A549, repressed expression of DKK1, a negative regulator of Wnt/β-catenin pathway, potentially contributing to growth in that model system. We observed modest up-regulation of DKK3, but not DKK1, by ASCL1 siRNA in cultured SCLC cells (data not shown). Although an earlier study on cell lines and our own unpublished data did not favor a major role for the Wnt pathway in SCLC (40), we could not exclude the possibility that lower levels of Wnt pathway activity are significant.

A recent study systematically explored ASCL1 transcriptional targets in the normal developing brain (18). Among the 14 direct target genes identified in this study, including the transcription factors Insm1, Isl1, Lifx8, and Pou3f1, none satisfied our criteria for 2-fold regulation by all three target siRNAs in SCLC cells. To account for the surprising lack of overlap in transcriptional targets of ASCL1 between normal developing brain and SCLC, we hypothesize that there are numerous powerful differences in transcriptional context. Potentially important influences in SCLC include the absence of Rb, known to associate with over 200 proteins including many transcription factors, alterations in key histone modulators including polycomb group regulators, as well as promoter hypermethylation. Collectively, these genetic and epigenetic alterations may account for the apparent paradox of how a proneural transcription factor plays a key role in neuroendocrine tumor virulence.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 7/20/2008; revised 10/17/2008; accepted 10/20/2008.

**Grant support:** National Cancer Institute grant ROI CA070244 (D.W. Ball), Flight Attendant’s Medical Research Institute Clinician-Scientist Awards (D.W. Ball and D.N. Watkins), and a generous charitable donation (A.G. to D.W. Ball).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Vincent C. Daniel and Jared S. Hierman for providing valuable assistance in procuring the xenografts.

Microarray data were deposited online at http://www.ebi.ac.uk/microarray/, accession number E-MEXP-1857.

**References**


28. Miki J, Furusato B, Li H, et al. Identification of putative stem cell markers, CD133 and CAC84, in hTERT-immortalized primary nonmalignant and malig...


Achaete-Scute Complex Homologue 1 Regulates Tumor-Initiating Capacity in Human Small Cell Lung Cancer

Tianyun Jiang, Brendan J. Collins, Ning Jin, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-08-2762

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/01/26/0008-5472.CAN-08-2762.DC1

Cited articles
This article cites 40 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/3/845.full#ref-list-1

Citing articles
This article has been cited by 27 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/69/3/845.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.