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Aberrant upregulation of *ASCL2* by promoter demethylation promotes the growth and resistance to 5-fluorouracil of gastric cancer cells

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Achaete scute-like 2 (*ASCL2*), a basic helix-loop-helix transcription factor, plays an essential role in the maintenance of adult intestinal stem cells. However, the function of *ASCL2* in gastric cancer (GC) is poorly understood. Therefore, we investigated the roles and regulatory transcription mechanisms of *ASCL2* in GC. Gene expression and methylation data analysis showed that *ASCL2* was upregulated and hypomethylated in GC tissues. Using real-time RT-PCR and pyrosequencing analysis, we confirmed that *ASCL2* was overexpressed and hypomethylated in GC tissues compared to adjacent normal tissues. We then investigated the mechanisms underlying the aberrant expression of *ASCL2* in GC and found that treatment with a methylation inhibitor induced *ASCL2* expression in GC cell lines. MBD-sequencing assay also revealed hypermethylation of the promoter region of *ASCL2* in GC cell lines, which barely expressed the *ASCL2* gene. Furthermore, *ASCL2* expression levels were inversely correlated with GC patient survival. Ectopic overexpression of *ASCL2* showed that *ASCL2* increased cell growth and promoted resistance to 5-fluorouracil in GC cells. These results suggest that *ASCL2* might play an important role in gastric tumor growth and chemoresistance, and could be a useful prognostic marker for GC patients. (*Cancer Sci* 2013; 104: 391–397)

Gastric cancer (GC) is the second most common cause of cancer-related death in the world. The incidence rate of GC is highest in East Asia, Eastern Europe and parts of Latin America.[1] However, the precise mechanisms underlying gastric carcinogenesis are not yet fully understood.[2] One of the most important hallmarks of tumor development and progression is DNA methylation.[3] While the role of hypermethylation in the silencing of tumor suppressor genes is well-documented,[4] recent works have also shown that hypomethylation contributes to tumor progression in numerous types of human cancer, including GC.[5,6] DNA methylation is also a dynamic epigenetic indicator that undergoes extensive changes during differentiation of self-renewing stem cells and is responsible for the regulation of expression of some stem cell markers, such as *CD133*,[7] *OCT4* and *NANOG*.[8]

The achaete scute-like 2 (*ASCL2*) gene is a member of the basic helix-loop-helix (*bHLH*) family of transcription factors, which initiates transcription by binding to the E-box.[9] It is involved in the determination of neuronal precursors in both the peripheral and central nervous systems.[10] It is also a cancer stem cell (CSC) marker,[11] and some reports have revealed that *ASCL2* promotes cell growth and migration in colon cancer.[12,13] The CSC is a fundamental concept in tumor biology and presents a new cellular target for anticancer drug development. Currently, methods to eradicate CSC include blocking self-renewal signaling, inhibiting the survival of those cells, and targeting CSC surface markers.[14] Lung tumor $CD133^+$ cells are highly tumorigenic, endowed with stem-like features and are spared by cisplatin treatment.[15] Ectopic expression of *OCT4* and *NANOG* in lung cancer cells increases the $CD133^+$ subpopulation and sphere formation and enhances drug resistance.[16] Therefore, targeting transcription factors related to CSC might be an effective method for increasing the sensitivity to anticancer drugs.

In this study, we examine the transcriptional regulatory mechanisms of *ASCL2* and its role in GC. Using the GENT database and other sources to mine gene methylation data, we identified *ASCL2* as both overexpressed and hypomethylated in GC patients. Finally, we explored the effects of *ASCL2* overexpression on GC cell growth and resistance to anticancer drugs.

Materials and Methods

Cell lines and tissue samples. Gastric cancer cell lines were cultured in complete RPMI 1640 medium. All cell lines were obtained from the Korean Cell Line Bank (http://cellbank.snu.ac.kr/index.htm), and all complete media contained 10% FBS (HyClone, Thermo Scientific, Rockford, IL, USA), 100 U/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine and 0.5 mM HEPES. A total of 114 tumors were collected from Chungnam National University Hospital. All samples were obtained with informed consent, and the Internal Review Board at Chungnam National University Hospital approved their use.

Real-time RT-PCR and western blots. Real-time RT-PCR and western blots were carried out as described previously (for details see Supporting Information).[6]

***ASCL2* stable cell line.** Human *ASCL2* cDNA was amplified from the cDNA of GC tissue using the following primers: 5′-ATA AGC TTA TGG ACG GCG GCA CAC TGC-3′ (sense) and 5′- ATC TCG AGT CAG TAG CCC CCT AAC CAG-3′ (antisense). This PCR product was then subcloned into the *Hind*III/*Xho*I sites of the pcDNA3.1(+) vector (Invitrogen), and the *ASCL2* sequence was verified by sequencing. *ASCL2* expression plasmids were transfected into MKN-1 cells using Lipofectamine Plus (Invitrogen) according to the manufacturer's protocol. Transfected cells were cultured for 2 days before selection in complete media with 500 μg/mL of G418. *ASCL2* mRNA and protein levels

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were assessed by RT-PCR and western blot analysis, respectively.

MBD-sequencing. Genomic DNA (5 μg) was fragmented at 44 psi for 1 min with a nebulizer (Illumina, San Diego, CA, USA) and then subjected methylated DNA enrichment using the MethylMiner methylated DNA enrichment kit (Invitrogen).

Briefly, methylated DNA was precipitated from the fragmented genomic DNA (1 μg) via binding to the methyl-CpG-binding domain of human MBD2 protein coupled to magnetic Dynabeads. The methylated fragments were then eluted with 2 M NaCl elution buffer (Invitrogen) and purified with the Qiagen (Valencia, CA, USA) MinElute PCR Purification Kit. Purified methylated DNA was then processed to generate a library for sequencing with an Illumina Genome Analyzer. These sequencing libraries were generated using the ChIP-Seq Sample prep kit (Illumina). Finally, the resulting sequences were mapped to the human genome using ELAND from the Genome Analyzer data analysis pipeline. The sequencing data were visualized as a custom track in the University of California, Santa Cruz Genome Browser. The peaks are in regions that show high density of methylated cytosines, and troughs are in regions with low density of methylated cytosines.

Pyrosequencing analysis. The promoter region of *ASCL2* (−795 to −667 upstream of transcription start site) was amplified using primers designed by PSQ Assay Design (Biotage AB, Kungsgatan, Sweden). Pyrosequencing was performed as described previously.[6] The primer sequences are shown in Table S1.

5-aza-2′-deoxycitidine (5-Aza-dC) treatment. For 5-aza-dC treatment, cells were seeded at a density of 1×10^6 cells /100 mm dish, then treated 24 h later with 2 μM of 5-Aza-dC (Sigma-Aldrich, Taufkirchen, Germany). Incubation with 5-Aza-dC continued for 4 days with media changes at 2-day intervals.

Cell proliferation and anchorage-dependent colony formation. The cell proliferation assay was performed using a Cell-Counting Kit-8 (Dojindo, Kumamoto, Japan). Cells were harvested and plated in 96-well plates and maintained at 37°C in a humidified incubator. At the indicated time points, we added CCK-8 solution into the wells, incubated the solution, and then measured the absorbance at 450 nm to determine the number of vital cells in each well. To examine the long-term effects, we seeded the cells in a 6-well plate using complete medium and cultured them for 14 days at 37°C. After the indicated time period, the cells were washed with PBS, fixed in 70% ethanol, and finally stained using crystal violet.

Caspase-3/7 assay. MKN-1 cells were seeded in 96-well plates and treated with 5-FU (10 μg/mL). After 48 h of treatment, the cells were washed once with 1× PBS, trypsinized, washed again with 1× PBS, and then resuspended in PBS. Cells were mixed with caspase-Glo-3/7 reagent (Promega, Madison, WI, USA) and incubated for 1 h at room temperature with agitation. The luciferase activity was measured using a luminometer and then normalized according to protein concentration.

Bisulfite sequencing. Genomic DNA (1 μg) was modified by sodium bisulfite using the EZ DNA Methylation kit (ZYMO Research, Irvine, CA, USA) according to the manufacturer's instructions. Bisulfite-modified DNA was amplified using a primer set targeted to the region of interest. Next, these PCR products were cloned using the pGEM-T Easy Vector (Promega). Clones were randomly chosen for sequencing.

Immunohistochemistry. Immunostainings of ASCL2 were performed on mixed human gastric tissues ($n = 10$) containing normal and tumor regions using the labeled streptavidin-biotin method after microwave antigen retrieval. Briefly, the formalin-fixed, paraffin-embedded 4 μm-thick sections were dewaxed in xylene, rehydrated through graded alcohol, and placed in an endogenous peroxide block for 15 min. Sections were then washed in water before antigen retrieval, placed in a citrate buffer, and microwaved for 10 min. Diluted anti-ASCL2 monoclonal antibody (1:100) was then applied, and antibody binding was detected using an avidin–biotin–peroxidase complex kit (Vextor Laboratories, Burlingame, CA, USA) using diaminobenzidine tetrahydrochloride solution (Biogenex, San Ramon, CA, USA).

Statistical analysis. Statistical analyses of group differences were performed using Student's *t*-test. A *P*-value <0.05 was considered significant. The following parameters were obtained from the medical records of the 114 patients studied: age, gender, histology, lymph node metastasis, tumor stage and *Helicobacter pylori* infection. Statistical analyses of correlation between expression and methylation were performed using the R statistical programming language (http://cran.r-project.org/).

Results

Negative correlation between methylation and gene expression of *ASCL2* in gastric camcer tissues. We first investigated the expression level of *ASCL2* in GC using the GENT database,[17] and observed *ASCL2* upregulation in tumor tissues compared to normal samples (Fig. S1A). *ASCL2* promoter is known to be a target of CpG methylation.[12] To inspect the methylation status of *ASCL2*, we collected human tissue methylation datasets from public resources, and created a methylation database named MENT (submitted; currently available at http://mgrc.kribb.re.kr:8080/MENT/). *ASCL2* methylation was inversely correlated with *ASCL2* expression in gastric normal or tumor tissues, suggesting a negative correlation between the expression and methylation of *ASCL2* (Fig. S1B). To confirm this hypothesis, we performed quantitative RT-PCR and pyrosequencing using GC and adjacent normal tissues for expression and methylation, respectively. As expected, *ASCL2* was both upregulated and hypomethylated in tumor tissues compared with the controls (Fig. 1A,B). A majority of patients (66 of 90) showed higher expression levels of *ASCL2* mRNA in tumor tissues than in adjacent normal samples. In contrast, a majority of patients (69 of 90) showed a lower methylation status for *ASCL2* in tumor tissues than in normal tissues. Immunostaining also showed that ASCL2 was expressed in intestinal metaplasia and tumor tissue, but barely in normal regions (Fig. 1C). We assessed whether methylation of the *ASCL2* CpG sites was negatively correlated with *ASCL2* expression and found a significant relationship (Fig. 1D). This suggests that increases in *ASCL2* expression in tumor tissues might be associated with DNA hypomethylation.

We then examined the relationship between *ASCL2* expression and survival of GC patients. We first divided the patients into two groups: a group with low *ASCL2* expression and the other group with high *ASCL2* expression. No significant difference was observed in the overall survival rate between the two groups. Then, we divided the patients into two groups based on tumor stages: early-stage (I + II) and late-stage (III + IV), and assessed survival differences within the sub-groups. Kaplan–Meier survival analysis showed that significant differences in survival existed among late-stage patients according to *ASCL2* expression levels, but not in early-stage patients (Fig. 1E). We also examined the relationship between *ASCL2* methylation and survival of GC patients. However, there was no difference in the survival rate between the two groups (data not shown). We analyzed the correlation between the expression and DNA methylation of *ASCL2* and clinicopathological parameters in GC patients. Neither expression nor methylation of *ASCL2* were associated other parameters (Tables S2 and S3).

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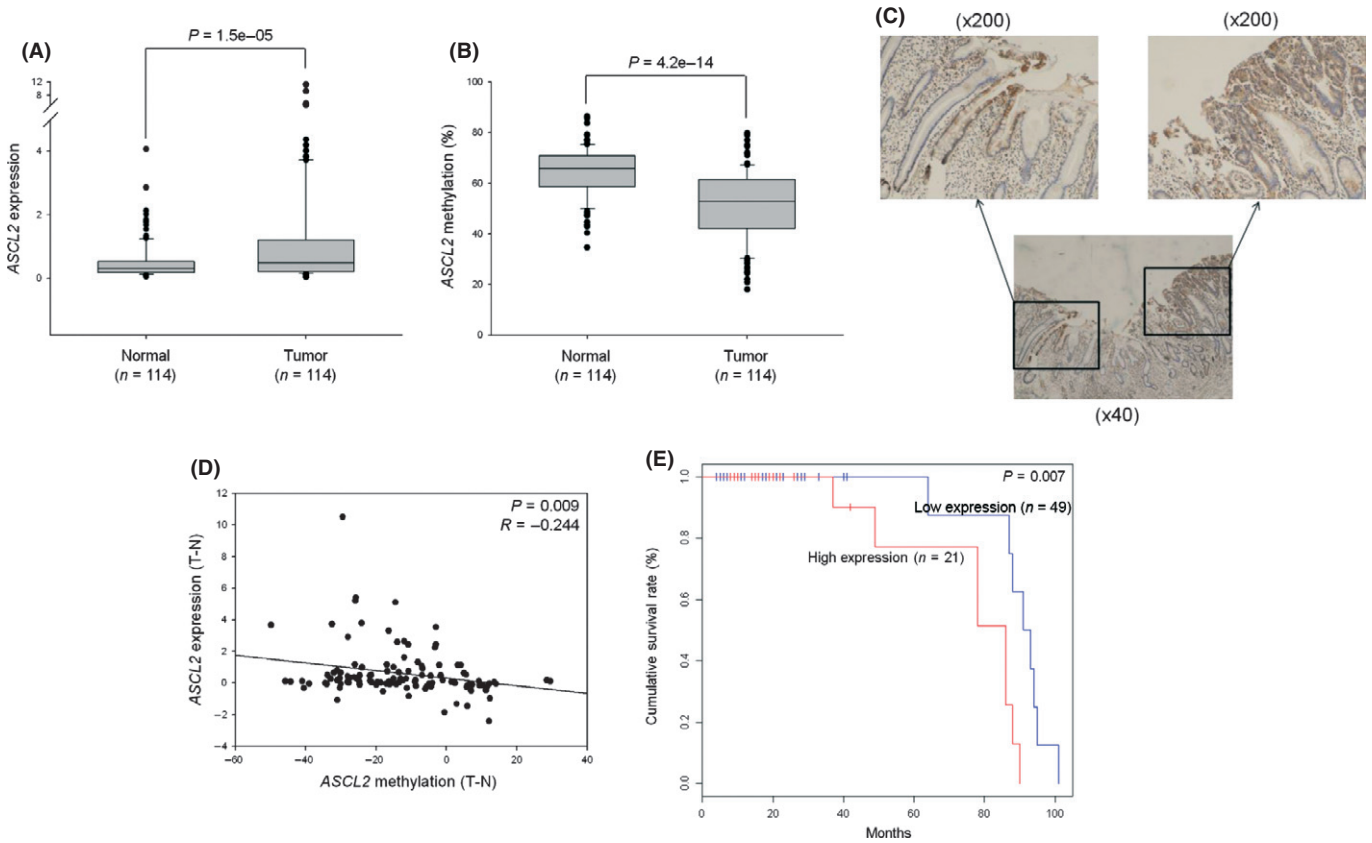

Fig. 1. Correlation between expression level and methylation status of *ASCL2* in gastric cancer (GC) tissues. (A) Quantitative RT-PCR for *ASCL2* in 114 paired human tumor and adjacent normal tissues. (B) *ASCL2* methylation for 114 paired GC and adjacent normal tissues. Pyrosequencing analysis was performed at the three CpG sites of *ASCL2* promoter. The boxes are bounded above and below by the 25th and 75th percentiles. The lines in the boxes indicate the median values. (C) ASCL2 immunostaining of gastric tissues. Sections shown are representative of 10 mixed human gastric tissues containing normal and tumor regions. (D) Correlation between the methylation and expression of *ASCL2*. The *x*-axis and *y*-axis are the difference in methylation and expression, respectively, between paired tumor and normal tissues (tumor minus normal). (E) A Kaplan–Meier plot of survival for patient groups with high and low levels of *ASCL2* mRNA expression in late-stage (III + IV) GC. Comparison of the two groups was done by log rank test.

5-Aza-dCs restores the expression of *ASCL2* gene in gastric cancer cell lines. To investigate whether 5-Aza-dCs, an inhibitor of the DNMT enzymes, influences *ASCL2* expression, we treated two cell lines (SNU-601 and MKN-1) with 5-Aza-dCs. The expression of *ASCL2* in SNU-601 and MKN-1 cells was augmented by 5-Aza-dC treatment (Fig. 2A). When we analyzed the methylation status on the *ASCL2* promoter by pyrosequencing, both cell lines exhibited strong demethylation after 5-aza-dC treatment (Fig. 2B), suggesting that DNA methylation is one of the mechanisms responsible for the expression of the *ASCL2* gene. We then performed an MBD-sequencing assay using the SNU-601 and SNU-620 cell lines to examine the methylation status broadly across the *ASCL2* promoter. Notably, SNU-620 expressed very little *ASCL2*, but the *ASCL2* expression was partially restored by 5-Aza-dCs treatment (data not shown). These results indicated that the promoter region of *ASCL2* in those two *ASCL2* silent cell lines was strongly hypermethylated, which was confirmed by bisulfite sequencing (Fig. 2C,D).

***ASCL2* overexpression promotes the growth of gastric cancer cells.** Although aberrant upregulation of *ASCL2* was found in tumor tissues, we could not detect ASCL2 protein expression in GC cell lines. Thus, we investigated the effects of *ASCL2* via overexpression in GC cells. Recently, it was reported that an *ASCL2* blockade in colon cancer cell lines inhibits cell proliferation.[13] Therefore, we examined the proliferative role of *ASCL2* in GC using *ASCL2*-overexpressing cell lines. MKN-1 cells were transfected with empty vector (Vector) or *ASCL2*-expressing vector (*ASCL2*) and selected with G418. The expression of *ASCL2* in mixed clones after selection was estimated by RT-PCR and western blot analysis (Fig. 3A). We first examined whether *ASCL2* overexpression affected cell growth in MKN-1 cells. The results showed a significant difference in growth between *ASCL2*-overexpressing and control cells (Fig. 3B). In the colony-forming assay, *ASCL2* overexpressing cells also established more and larger colonies than control cells (Fig. 3C). We also performed a migration assay, but found no difference in migration between *ASCL2* and control-transfected cell lines (data not shown).

***ASCL2* promotes resistance to 5-fluorouracil in gastric cancer cells.** To further examine the functional role of *ASCL2*, cells were treated with 5-fluorouracil (5-FU), one of the most effective chemotherapeutic drugs in the GC treatment. Our results showed different susceptibility to the anti-proliferative effect of 5-FU. *ASCL2* overexpressing cells exhibited better overall survival compared with control cells in a dose-dependent manner (Fig. 4A). Our data also revealed that cell growth differences were augmented in a time-dependent manner (Fig. 4B,C). To assess the effect of 5-FU on apoptosis, caspase activity was also examined. Levels of caspase-3/7 activity were increased in control cells compared to the *ASCL2* overexpressing cells after 5-FU treatment (Fig. 4D).

***ASCL2* upregulates *LGR5* expression at transcriptional level.** We investigated whether *ASCL2* plays a role in regulating the

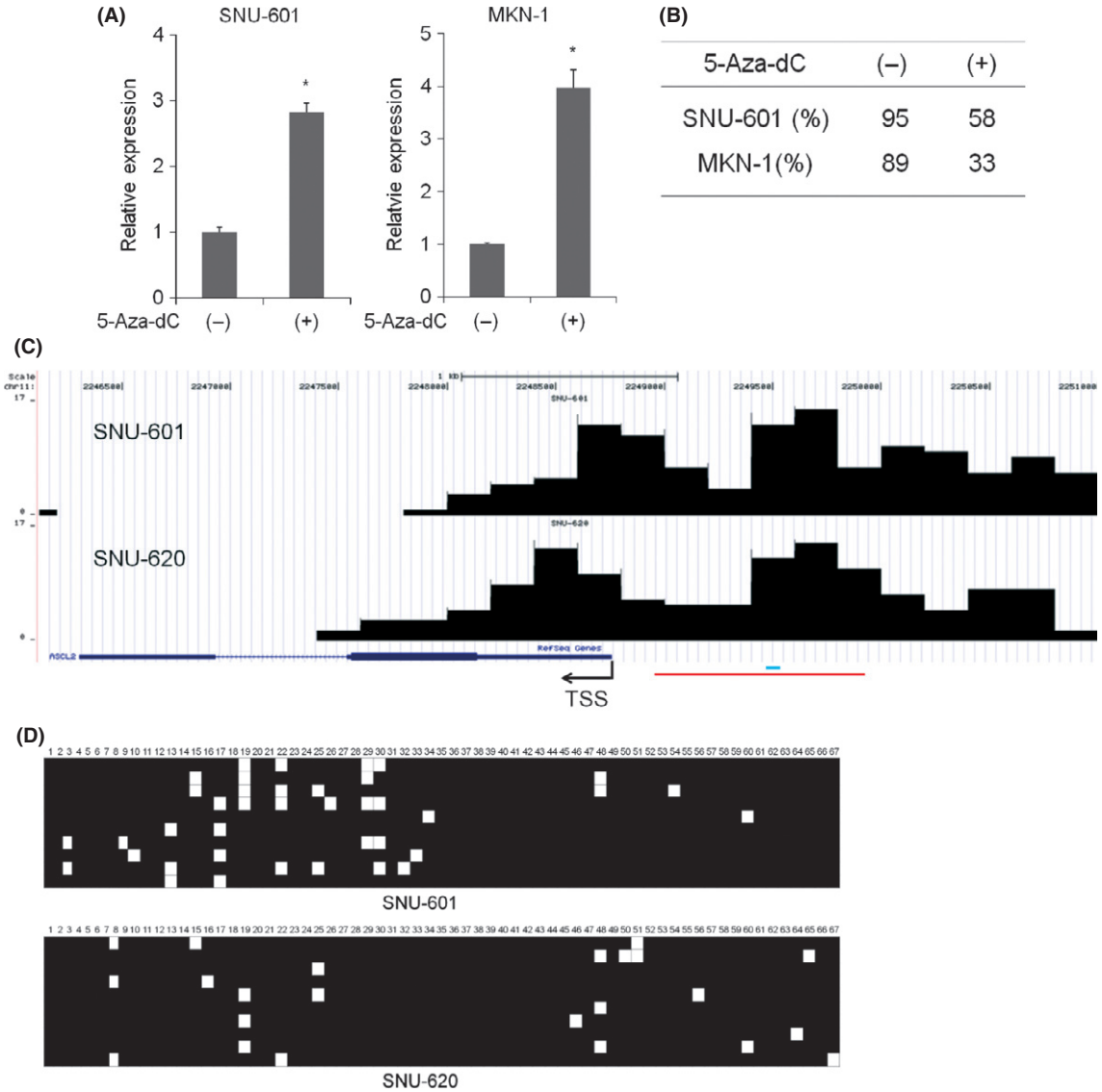

Fig. 2. *ASCL2* upregulation by 5-Aza-dCs treatment in gastric cancer (GC) cells. (A) Cells were treated for 4 days with 2 μM of 5-Aza-dC, as described in Materials and Methods. Quantitative RT-PCR was performed using specific primers for the *ASCL2* gene. (B) Pyrosequencing analysis in SNU-601 and MKN-1 cells after 5-Aza-dC treatment. (C) MBD-sequencing analysis of cell lines SNU-601 and SNU-620. Black bars represent the methylation status. Red and blue lines indicate the bisulfite sequencing and pyrosequencing region, respectively. (D) *ASCL2* methylation status was analyzed by bisulfite sequencing in GC cell lines. A total of 67 CG sites exist in the bisulfite sequencing region. Squares represent CpG dinucleotides either unmethylated (open) or methylated (closed).

expression of stemness-related genes in MKN-1 cells, as *ASCL2* is known to bind to promoters from this group.[11,13] The expressions of a few selected stemness-related genes were analyzed by real time PCR in *ASCL2*-overexpressing and control cells. Among the tested genes, only *LGR5* was significantly upregulated by *ASCL2* overexpression (Fig. 5A). Transient transfection experiments also showed the induction of *LGR5* by *ASCL2* overexpression (Fig. 5B). We then investigated the correlation between *ASCL2* and *LGR5* expression using the GENT database. We observed significant positive correlations between *ASCL2* and *LGR5* expression in normal and tumor tissues (Fig. 5C,D). The expression of *LGR5* in tumors was also higher than that in normal tissues (Fig. 5E). These data suggest that *ASCL2* might be an important factor that regulates the expression of *LGR5*. *ASCL2* binds approximately 500 bp upstream from the *LGR5* gene transcription start site (TSS) in intestinal cells.[11] Hence, we constructed an *LGR5* reporter gene containing three E-box sites, covering approximately 1 kb upstream from the *LGR5* gene TSS. However, *ASCL2* did not affect the reporter gene activity (data not shown), suggesting that there may be another binding site that is important for *LGR5* expression.

Discussion

ASCL2 is a transcription factor essential for the differentiation of the nervous system.[18] Although a few reports have revealed that *ASCL2* affects cell growth and migration in colon cancer,[12,13] little is known on the biological roles of *ASCL2* and its signaling pathway. Furthermore, little is known about the molecular mechanisms regulating *ASCL2* expression in cancer. DNA methylation is an important mechanism for regulating gene expression during cancer progression. Many tumor suppressor genes and oncogenes are regulated in cancers via

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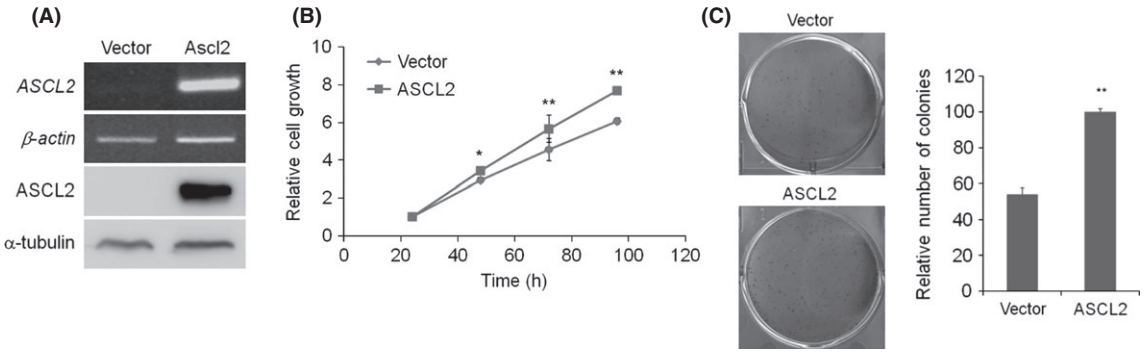

Fig. 3. The effect of *ASCL2* on cell growth. (A) RT-PCR (top) and western blot (bottom) analysis of MKN-1 cells stably expressing *ASCL2*. (B) Proliferation was measured at 24, 48, 72 and 96 h. The data are presented as the means ± SD of triplicate experiments. (C) Anchorage-dependent colony formation assay in a monolayer culture. Columns, results of triplicate experiments; error bars, SD. **P* < 0.05, ***P* < 0.01.

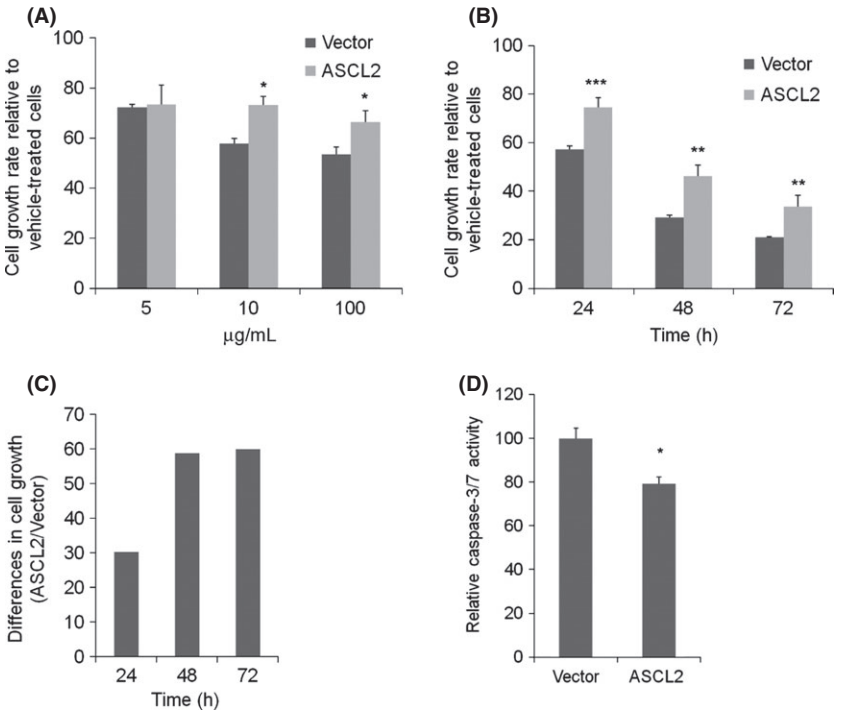

Fig. 4. The effect of *ASCL2* on resistance to 5-fluorouracil. (A) MKN-1 cells were seeded in 96-well plates, and the cell growth of MKN-1 cells were analyzed by CCK-8 reagent after 24 h exposure to the indicated doses of 5-fluorouracil. (B) Cell growth of MKN-1 cells were measured at 24, 48 and 72 h after exposure to 5-fluorouracil (10 μg/mL). (C) Differences (ASCL2 divided by Vector) in cell growth in panel B. (D) A plot of caspase-3/7 activity normalized to protein concentration in MKN-1 cells after 48 h exposure to 5-fluorouracil (10 μg/mL). Columns, results of triplicate experiments; error bars, SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

epigenetic mechanisms.[4,6] There are some clues as to the involvement of epigenetics in *ASCL2* expression at transcriptional levels.[12,19] However, those reports do not fully reveal the mechanism of *ASCL2* expression. Here, we showed that promoter methylation might be an important mechanism for the increased expression of *ASCL2* in tumor tissues. We also found that the *ASCL2* promoter region was extensively methylated in GC cell lines, suggesting that this pattern may maintain the expression status of *ASCL2*.

Cancer stem cells are a very small population of cancer cells in tumor tissues. Primarily, they function as stem cells, with self-renewal and differentiation potential. CSC play decisive roles in malignancy, metastasis and recurrence.[20] Therefore, the development of CSC-targeted therapies is critical to improving the survival and overall quality of life in cancer patients. Four transcription factors, *OCT4*, *SOX2*, *KLF4* and *c-MYC*, can induce pluripotent stem cells through overexpression, although *KLF4* and *c-MYC* may be replaced by *Lin28* and *Nanog*.[21,22] These transcription factors are known to be involved in tumor progression. For example, double knockdown of *OCT4* and *NANOG* reverses epithelial-mesenchymal transition and blocks the tumorigenic and metastatic ability of lung adenocarcinoma.[16] *SOX2* is also expressed in early stage breast tumors; *SOX2* knockdown prevents sphere formation and delays tumor generation.[23] Reduced expression of *ASCL2* leads to arrest at the G2/M cell cycle checkpoint. Lowered levels of *ASCL2* can also lead to colon cancer tumor growth arrest, both *in vitro* and *in vivo*.[12,13] Here, we have also

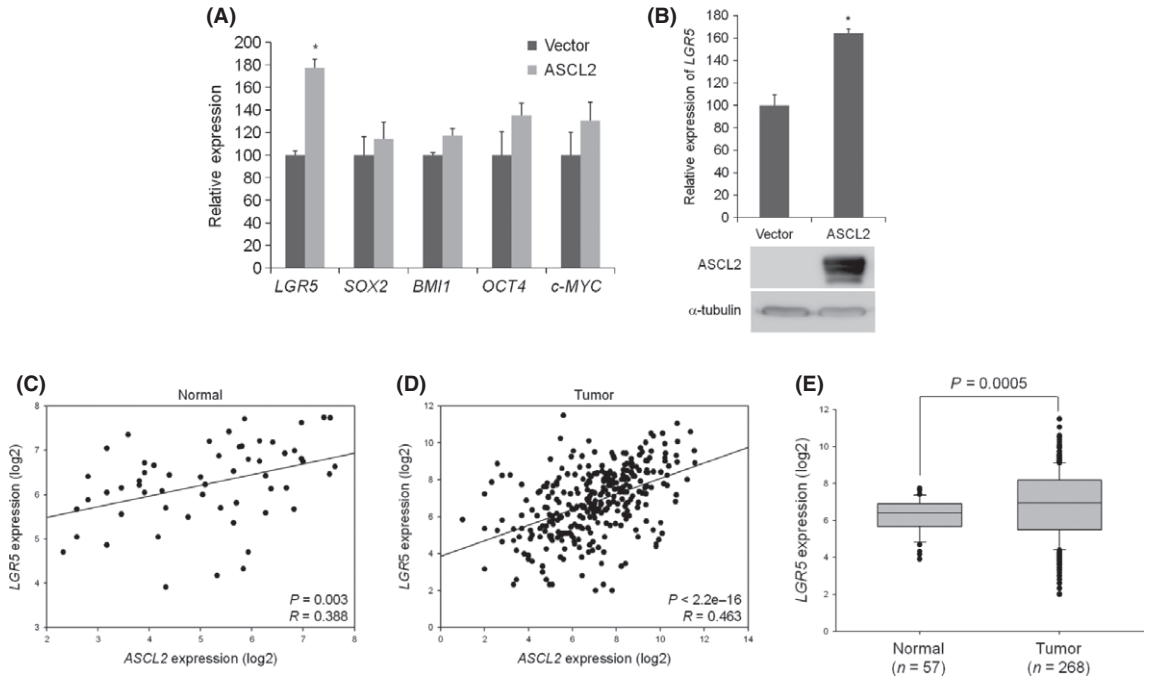

Fig. 5. *ASCL2* upregulates *LGR5* expression at transcriptional level in MKN-1 cells. (A) Quantitative RT-PCR analysis of expression of stemness-related genes in MKN-1 cells stably expressing *ASCL2*. (B) Quantitative RT-PCR (top) and western blot (bottom) analysis of *LGR5* and *ASCL2* expression in MKN-1 cells transfected with *ASCL2*, respectively. (C,D) The comparison of the expression levels of *ASCL2* and *LGR5* in gastric normal and tumor tissues, respectively, using GENT database. (E) The comparison of the expression levels of *LGR5* in gastric normal and tumor tissues. Columns, results of triplicate experiments; error bars, SD. **P* < 0.05.

shown that *ASCL2* overexpression results in increased growth of GC cells.

Cancer stem cells are relatively resistant to commonly used cancer therapies, such as radiation and chemotherapy. Indeed, some groups have demonstrated that CSC or cells expressing stem cell markers exhibit resistance to cancer therapies. Our work has revealed that *ASCL2* can induce resistance to 5-FU in GC cells. Wnt signaling promotes chemoresistance in multiple myeloma and hepatocellular carcinoma cells.[24,25] Breast cancer cells expressing CSC markers are also resistant to radiation, and ectopic expression of Wnt or β-catenin augments this resistance.[26,27] Recently, a report has noted that the upregulation of Wnt6 using chemotherapeutics enhances the resistance of GC cells to anthracycline drugs.[28] *ASCL2* is a target of the Wnt pathway, and expression of *ASCL2* mRNA is reduced in colon cancer cells transfected with β-catenin siRNA,[12] suggesting that *ASCL2* may control one of the important components in Wnt-mediated chemoresistance. *LGR5* is a member of the canonical Wnt pathway cascade and regulates cell proliferation and differentiation. *LGR5* also has prognostic and tumor biological significance in GC[29,30] and is associated with increased tumor recurrence.[31] Although we have not identified promoter elements in *LGR5* regulated by *ASCL2*, we suggest that *LGR5* might be one of the *ASCL2* target genes and that cooperation between *ASCL2* and *LGR5* could be an important component of cell growth and chemoresistance in GC.

In conclusion, we demonstrated that upregulation of *ASCL2* expression in GC tissues was accomplished by epigenetic mechanisms. Moreover, high *ASCL2* expression was associated with poor prognosis in GC patients. We also explored the effects of ASCL2 overexpression in GC and revealed that overexpression of *ASCL2* promoted the growth of GC cells as well as significant resistance to 5-FU, indicating that *ASCL2* is a potential prognostic marker in GC. Although more detailed studies need to be performed to identify the underlying mechanism of *ASCL2* effects on GC, the results presented here support the notion that *ASCL2* targeting may elevate the efficacy of current GC therapies.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; **80**: 827–41.
2 Yuasa Y. Control of gut differentiation and intestinal-type gastric carcinogenesis. *Nat Rev Cancer* 2003; **3**: 592–600.
3 Feinberg AP, Tycko B. The history of cancer epigenetics. *Nat Rev Cancer* 2004; **4**: 143–53.
4 Baylin SB. DNA methylation and gene silencing in cancer. *Nat Clin Pract Oncol* 2005; **2**(Suppl. 1): S4–11.
5 Ehrlich M. DNA methylation in cancer: too much, but also too little. *Oncogene* 2002; **21**: 5400–13.

doi: 10.1111/cas.12076

6 Kwon OH, Park JL, Kim M *et al.* Aberrant upregulation of LAMB3 and LAMC2 by promoter demethylation in gastric cancer. *Biochem Biophys Res Commun* 2011; **406**: 539–45.
7 Tabu K, Sasai K, Kimura T *et al.* Promoter hypomethylation regulates CD133 expression in human gliomas. *Cell Res* 2008; **18**: 1037–46.
8 Deb-Rinker P, Ly D, Jezierski A, Sikorska M, Walker PR. Sequential DNA methylation of the Nanog and Oct-4 upstream regions in human NT2 cells during neuronal differentiation. *J Biol Chem* 2005; **280**: 6257–60.
9 Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol Cell Biol* 2000; **20**: 429–40.
10 Miyamoto T, Jinno Y, Sasaki T *et al.* Genomic cloning and localization to chromosome 11p15.5 of the human achaete-scute homolog 2 (ASCL2). *Cytogenet Cell Genet* 1996; **73**: 312–14.
11 van der Flier LG, van Gijn ME, Hatzis P *et al.* Transcription factor achaete scute-like 2 controls intestinal stem cell fate. *Cell* 2009; **136**: 903–12.
12 Jubb AM, Chalasani S, Frantz GD *et al.* Achaete-scute like 2 (ascl2) is a target of Wnt signalling and is upregulated in intestinal neoplasia. *Oncogene* 2006; **25**: 3445–57.
13 Zhu R, Yang Y, Tian Y *et al.* Ascl2 Knockdown results in tumor growth arrest by miRNA-302b-related inhibition of colon cancer progenitor cells. *PLoS ONE* 2012; **7**: e32170.
14 Zhou BB, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nat Rev Drug Discov* 2009; **8**: 806–23.
15 Bertolini G, Roz L, Perego P *et al.* Highly tumorigenic lung cancer CD133+ cells display stem-like features and are spared by cisplatin treatment. *Proc Natl Acad Sci U S A* 2009; **106**: 16281–6.
16 Chiou SH, Wang ML, Chou YT *et al.* Coexpression of Oct4 and Nanog enhances malignancy in lung adenocarcinoma by inducing cancer stem cell-like properties and epithelial-mesenchymal transdifferentiation. *Cancer Res* 2010; **70**: 10433–44.
17 Shin G, Kang TW, Yang S, Baek SJ, Jeong YS, Kim SY. GENT: gene expression database of normal and tumor tissues. *Cancer Inform* 2011; **10**: 149–57.
18 Hermansen SE, Kalstad T, How OJ, Myrmel T. Inflammation and reduced endothelial function in the course of severe acute heart failure. *Transl Res* 2011; **157**: 117–27.
19 de Sousa EMF, Colak S, Buikhuisen J *et al.* Methylation of cancer-stem-cell-associated Wnt target genes predicts poor prognosis in colorectal cancer patients. *Cell Stem Cell* 2011; **9**: 476–85.
20 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105–11.
21 Takahashi K, Tanabe K, Ohnuki M *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007; **131**: 861–72.
22 Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S. Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 2008; **322**: 949–53.
23 Leis O, Eguiara A, Lopez-Arribillaga E *et al.* Sox2 expression in breast tumours and activation in breast cancer stem cells. *Oncogene* 2012; **31**: 1354–65.
24 Noda T, Nagano H, Takemasa I *et al.* Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. *Br J Cancer* 2009; **100**: 1647–58.
25 Kobune M, Chiba H, Kato J *et al.* Wnt3/RhoA/ROCK signaling pathway is involved in adhesion-mediated drug resistance of multiple myeloma in an autocrine mechanism. *Mol Cancer Ther* 2007; **6**: 1774–84.
26 Woodward WA, Chen MS, Behbod F, Alfaro MP, Buchholz TA, Rosen JM. WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc Natl Acad Sci USA* 2007; **104**: 618–23.
27 Phillips TM, McBride WH, Pajonk F. The response of CD24(−/low)/CD44 + breast cancer-initiating cells to radiation. *J Natl Cancer Inst* 2006; **98**: 1777–85.
28 Yuan G, Regel I, Lian F *et al.* WNT6 is a novel target gene of caveolin-1 promoting chemoresistance to epirubicin in human gastric cancer cells. *Oncogene* 2013; **32**: 375–87.
29 Reya T, Clevers H. Wnt signalling in stem cells and cancer. *Nature* 2005; **434**: 843–50.
30 Simon E, Petke D, Boger C *et al.* The spatial distribution of LGR5+ cells correlates with gastric cancer progression. *PLoS ONE* 2012; **7**: e35486.
31 Gerger A, Zhang W, Yang D *et al.* Common cancer stem cell gene variants predict colon cancer recurrence. *Clin Cancer Res* 2011; **17**: 6934–43.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The expression and methylation level of *ASCL2* in gastric normal and cancer tissues.

Doc. S1. Material and methods details.

Table S1. Primer information.

Table S2. *ASCL2* expression in tumors with respect to clinicopathologic characteristics.

Table S3. *ASCL2* methylation in tumors with respect to clinicopathologic characteristics.