ZEB2–Sp1 cooperation induces invasion by upregulating cadherin-11 and integrin α5 expression

Eun-Hee Nam1,4, Yunhee Lee1,2,*, Xue-Feng Zhao3, Young-Kyu Park3, Jung Weon Lee4 and Semi Kim1,2,†

1Immunotherapy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejon 305-806, Korea, 2Department of Chemistry, Korea Advanced Institute of Science and Technology, Daejon 305-701, Korea, 3Department of Surgery, Chonnam National University Medical School, Hwasun 519-809, Korea and 4Department of Pharmacy, College of Pharmacy, Seoul National University, Seoul 151-742, Korea

*To whom correspondence should be addressed. Tel: +82-42-860-4228; Fax: +82-42-860-4149; E-mail: semikim@kribb.re.kr

Abbreviations: ATF, activating transcription factor; EMT, epithelial–mesenchymal transition; HEK293E, human embryonic kidney 293E; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA; SBD, Smad-binding domain; SD, standard deviation; TGF, transforming growth factor.

†These authors contributed equally to this work.

Introduction

The invasive nature of tumor cells is a major prerequisite to cancer metastasis. Invasion is enhanced by tumor cell activation of epithelial–mesenchymal transition (EMT) (1–4). EMT is characterized by loss of cell polarity and cell–cell interactions, modulation of cell–matrix adhesion, enhanced proteolytic activity (such as extracellular matrix degradation), reorganization of the cytoskeleton and acquisition of cell motility (2). During EMT, epithelial cells gradually lose their epithelial morphology, including E-cadherin-mediated cell–cell adhesion, and concomitantly acquire mesenchymal markers, such as vimentin, alpha smooth muscle actin and integrin α5 (2,5).

During EMT and tumor progression, cadherin switch occurs. This is a process whereby expression of epithelial E-cadherin is switched to expression of a mesenchymal cadherin, such as N-cadherin or cadherin-11 (6,7). Cadherin-11 (also called OB-cadherin) was initially identified in mouse osteoblasts (8) and is expressed in multiple cancers, including breast, prostate, colon and stomach (9–12). Cadherin-11 expression in aggressive breast and prostate cancer cells is associated with increased invasiveness and poor prognosis (11,12). However, the mechanisms underlying cadherin-11 expression and its role in colon or gastric cancer cells remain unknown.

Downregulation of E-cadherin is a well-known hallmark of EMT and correlates positively with tumor cell invasion and metastasis (2). Downregulation of E-cadherin is usually mediated by E-cadherin transcriptional repressors, including the ZEB family (ZEB1 and ZEB2) and the Snail superfamily of zinc finger factors (Snail and Slug), through interaction with proximal E-box elements in the E-cadherin promoter. ZEB2, initially characterized as a Snad-interacting protein (13), represses E-cadherin transcription by binding to bipartite E-box elements, and deletion of the Smad-binding domain (SBD) in ZEB2 reduces ZEB2 repression of the E-cadherin promoter in NMuMG-derived NM Ne cells (14). Recent studies show that E-cadherin repressors may directly or indirectly induce expression of certain genes (15–18) although the mechanisms by which this occurs during EMT are not fully understood.

Our laboratory investigates the roles of ZEB2 in the induction of mesenchymal genes during cancer cell EMT. We recently reported that ZEB2 directly upregulates transcription of integrin α5 and vimentin by cooperating with the transcription factor Sp1 (19). Sp1 acts as a basal/ubiquitous transcription factor, and Sp1 sites (GC boxes and related motifs) are constitutive promoter elements (20). However, recent studies report that Sp1 can either enhance or repress the promoter activity of genes involved in cell cycle progression, differentiation and oncogenesis, and dysregulation of Sp1 has been observed in many cancers (21). Furthermore, Sp1 has been reported to be involved in the transcriptional regulation of EMT and invasion (22). Therefore, Sp1 is of particular interest in this context, given its potential onco-genic functions.

In this study, we investigated the precise mechanism/pathway by which ZEB2–Sp1 cooperation contributes to invasion and EMT events. We found that ZEB2 activates the cadherin-11 promoter in an Sp1-dependent, but Smad- and E-box-independent, manner and suppresses E-cadherin transcription in an Sp1-independent manner, leading to cadherin switch. Furthermore, Sp1 is required for ZEB2-induced tumor cell invasion, mainly through induction of cadherin-11 and integrin α5, suggesting a potential role of the ZEB2/Sp1/cadherin-11–integrin α5 axis in induction of invasion. Double immunofluorescence analysis of tissue samples demonstrated nuclear colocalization of ZEB2 and Sp1 in tumor cells in human colorectal cancer tissues, providing further evidence of a cooperative role of ZEB2 and Sp1.

Materials and methods

Cell lines

Human embryonic kidney 293E (HEK293E) cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37°C/5% CO2. Human colon cancer (SW480 and LS174T), gastric cancer (AGS (American Type Culture Collection) and SNU-638), and liver cancer (SNU-398; Korean Cell Line Bank, Seoul, Korea) cells were maintained in Roswell Park Memorial Institute 1640 with 10% fetal bovine serum.

Transfection with expression vectors and small interfering RNA

Cells were transfected with a vector expressing ZEB2 (pCS3 SIP1; a kind gift from Dr D. Huylebroeck, University of Leuven, Belgium) using Lipofectamine 2000 or electroporation (Invitrogen, Carlsbad, CA, USA).
CA). Cells were transfected with small interfering RNA (siRNA) specific to ZEB2, cadherin-11, Smad2, Smad3 (Dharmacon, Lafayette, CO), integrin α5 (5′-GGACCAAGGCCAGAACGCTT-3′) or Sp1 (5′-GGUGAGCUCAGAUUGUUUGATT-3′) by Lipofectamine 2000 for 48 h.

The mutant ZEB2 lacking SBD (residues from 437 to 487) (ZEB2ASBD1) (13) was generated from pCS3 SIP1. N-terminal and C-terminal segment-encoding parts of the DNA were amplified by PCR with primer sets (5′-AGATCTGAACTTCAAGGTGCAGC-3′ and 5′-TGTGATAACCCATTGAGCAGAGCAG-3′) for N-terminal segment and 5′-GAGTCCAAATGGTTATCACAGGAAGCCATG-3′ and 5′-TCTAGGACAGTGGAAGGGCTG-3′ for C-terminal segment), hybridized and then extended using Pfu polymerase. The resulting amplified DNA was digested with BglII/XbaI and then cloned into the pCS3 SIP1.

Where indicated, cells were transfected with siRNA for 24 h and further transfected with plasmids. Cells were transfected with ZEB2-expressing vector for 24 h and then treated with mithramycin A (50 nM, Sigma, St Louis, MO) or 0.2% dimethyl sulfoxide for 24 h. Cells were transfected with siRNA for 42 h and then treated with MG132 (2 μM, Sigma) or 0.1% dimethyl sulfoxide for 6 h.

**Immunoblot analysis**

Whole cell lysates were prepared using radioimmunoprecipitation assay buffer as described (23) and analyzed using the following primary antibodies: anti-β-actin, anti-α-tubulin, anti-Sp1 (PEP2) and anti-glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA); anti-myc (Upstate Biotechnology); anti-E-cadherin and anti-integrin α5 (BD Biosciences, San Jose, CA); anti-vimentin (Sigma); anti-phospho-c-Jun N-terminal kinase (JNK; T183/Y185), anti-JNK, anti-phospho-activating transcription factor (ATF-2) (T71), anti-AFP-2, anti-Smad2 and anti-Smad3 (Cell Signaling, Danvers, MA); anti-ZEB2 (6E5; Active Motif, Tokyo, Japan); and anti-cadherin-11 (Invitrogen).

**Reverse transcription–polymerase chain reaction from cell lines**

Total RNA was isolated using TRIzol (Invitrogen), and complementary DNA was synthesized using reverse transcriptase (Bioneer, Daejon, Korea). Real-time quantitative PCR reactions were performed using SYBR Green (PKT, Seoul, Korea) with ZEB2-specific primers (5′-TTTCCGGAGAATTCGTGA-3′ and 5′-CATCCATGACATCAGCTC-3′), cadherin-11-specific primers (5′-CCAACAGCCCCGATAAGGTAT-3′ and 5′-TTGATTTCTGCTGCAAA GAC-3′) and glyceraldehyde 3-phosphate dehydrogenase-specific primers (5′-CATGACCAACAGCTCATGCTTCC-3′ and 5′-AAGGCCATGCCCAGTCTTCTC-3′), with an annealing temperature of 58°C.

**Construction of the cadherin-11 promoter**

The human cadherin-11 promoter region (−1350 to +137) was obtained by PCR with primer set (5′-CGCCGACGTAGTTTCCGGGCTTGCAT-3′ and 5′-CCGCTCGAGCAGGCCCCTTGGACG-3′) using genomic DNA from SW480 cells. The PCR product was digested with XhoI and then cloned into the pcDNA3 basic vector (Promega, Southampton, UK) to generate pCad11-1350luc construct.

**Deletion and mutagenesis of the cadherin-11 promoter**

Deletion constructs of the human cadherin-11 promoter were generated by PCR using the following forward primers: 5′-GAATCTCAGGGGTGGCGGTTCCATC-3′ and 5′-CCGCTCTGAGGCTCCGTCC-3′ and reverse primers 5′-GGACCAAGGCCAGAACGCTT-3′ or Sp1 (5′-GGUGAGCUCAGAUUGUUUGATT-3′) by using the pcDNA3 basic vector. The mutant cadherin-11 promoter reporter construct (Sp1 site mutations; −1111 to +100 and −100 to +96) was generated from the pCad11-150luc construct by customer service (Bioneer, Daejon, Korea).

**Promoter reporter assay**

Cells were transfected with Lipofectamine 2000. For transfection, 2 × 105 cells were seeded on 6-well plates. After 24 h incubation, 2 μg of reporter plasmid DNA and 1.8 μg of ZEB2 expression vector were cotransfected. At 48 h posttransfection, firefly luciferase activity was measured with a dual-luciferase reporter assay system (Promega). Transfection efficiency was normalized by measuring Renilla luciferase activity, encoded by the cotransfected Renilla luciferase vector (pRL-TK). The E-cadherin promoter (−308 to +41) construct was kindly provided by Dr G. Bex (VIB-Ghent University, Belgium) (14), and the vimentin promoter (−411 to +60) construct was kindly provided by Dr S. Rittling (The Forsyth Institute, MA) (24). The integrin α5 promoter (−908 to +241) construct was described previously (19).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation (ChIP) assays were performed according to the instructions of the ChIP Assay Kit from Upstate Biotechnology (Lake Placid, NY). Briefly, the equivalent of 2 × 106 SNU-638 cells was used per ChIP reaction using rabbit anti-ZEB2 (H260, Santa Cruz Biotechnology) or rabbit anti-Sp1 (PEP2, Santa Cruz Biotechnology). As a control antibody, normal rabbit IgG was used. Immunoprecipitated and input DNA were analyzed by PCR with cadherin-11 promoter-specific primers (5′-AGGCTTGGGCTTCTCAG-3′ and 5′-GCTATGGCAGAATGAGGA-3′ for −191 to −62 and 5′-AGCTTGGCGTCCATC-3′ and 5′-CCCTACGACTGCTC-3′ for −112 to −100). ChIP assay using SNU-398 cells transfected with siRNA for 48 h was also performed in a similar manner.

**Invades and cell migration assays**

Invades assays were performed as described (25). Cells were plated in serum-free medium on Transwell inserts (Corning, NY) coated with 25 μg of Matrigel (BD Biosciences) for invasion assays. After incubation for 48 h at 37°C/5% CO2, the inserts were fixed with 3.7% paraformaldehyde/phosphate-buffered saline for 20 min and then stained with crystal violet. The number of cells that had invaded was counted in five representative (×200) fields per insert. Cell migration assays were performed in a similar manner but without the Matrigel coating.

**Cell proliferation and viability assays**

Cell proliferation and viability were determined by colorimetric water-soluble tetrazolium assay (Ta KaBio, Otsu, Shiga, Japan) and cell-staining analysis with calcein AM (Invitrogen) and propidium iodide (Sigma). Specifically, SW480 cells transfected with siRNA for 48 h were seeded into 96-well plates at 3000 cells/well and incubated for 48 h. Cells were then incubated with 1 μg/ml of calcein AM for 4 h. Cell survival was measured at 440 nm using a spectrophotometric microplate reader (Molecular Devices, Sunnyvale, CA). In parallel, cells were incubated in 2 μM calcein AM and 1 μg/ml propidium iodide in phosphate-buffered saline at room temperature for 30 min in the dark. Stained cells were visualized using a fluorescence microscope (Leica DM IL LED; Leica, Wetzlar, Germany).

**Tissue samples**

Nine colorectal adenocarcinoma tissue samples (3 cases stage II, and 6 cases stage III) were provided by the Chonnam National University Hwasun Hospital, a member of the National Biobank of Korea, which is supported by the Ministry of Health, Welfare and Family Affairs. All samples derived from the National Biobank of Korea were obtained with informed consent under institutional review board-approved protocols. Tumor tissues were harvested from colon (n = 5) and rectum (n = 4). Within 30 min following surgical bowel resection, tissue specimens were dissected from the tumor and immediately frozen in optimal cutting temperature compound (Sakura, Tokyo, Japan). The tissue specimens were dissected from the edge of the tumors to ensure that they contained the invasive part of the tumors and their paired normal mucosa control tissues. Context of tissue samples was confirmed by hematoxylin and eosin stain.

**Tissue immunofluorescent stain**

Optimal cutting temperature compound-embedded 6 μm thick frozen tissue sections were processed for immunofluorescence analysis as per standard protocol. Sections were immersed in 10% goat serum in phosphate-buffered saline for 20 min to block non-specific binding and then incubated with rabbit anti-Sp1 (1:50 dilution; Santa Cruz Biotechnology) and mouse anti-ZEB2 (1:100 dilution; Active Motif) antibodies overnight at 4°C. Sp1 and ZEB2 were detected using Alexa Fluor® 594 goat anti-mouse IgG (1:2000 dilution; Molecular Probes) secondary antibodies, respectively. Sections were counterstained with 4,6-diamidino-2-phenylindole (Invitrogen) to visualize cell nuclei. Cystained sections were analyzed under the OLYMPUS FX10-ASW confocal laser scanning biological microscope system.

**Statistical analysis**

Statistical analyses were performed using a Student’s t-test, where P < 0.05 was considered statistically significant.

**Results**

ZEB2 induces cadherin-11 expression by activating the cadherin-11 promoter

Previously we showed that integrin α5 is transcriptionally upregulated by ZEB2 through cooperation with Sp1. In an attempt to further identify the subset of genes upregulated by ZEB2 in cancer cells and to
explore the functional significance of Sp1 in ZEB2-induced invasion, we first explored whether ZEB2 is involved in cadherin switch during cancer cell EMT. We examined whether ZEB2 upregulates cadherin-11 and N-cadherin expression. Transient transfection of SW480 cells, which normally express little to no endogenous ZEB2, with a ZEB2 expression plasmid upregulated cadherin-11 expression at both the messenger RNA and protein levels (Figure 1A and B), whereas N-cadherin expression was not induced by ZEB2 (data not shown). Knockdown of ZEB2 by siRNA in SNU-638 and SNU-398 cells, which express endogenous ZEB2, significantly reduced cadherin-11 messenger RNA levels (Figure 1C).

To explore whether ZEB2 activates the cadherin-11 promoter, cells were transiently transfected with the ZEB2 expression vector and a reporter plasmid driven by the cadherin-11 promoter (+1350/+137). ZEB2 induced 3.2-, 5.5- and 1.5-fold increases in cadherin-11 promoter activity in SW480, AGS and HEK293E cells, respectively (Figure 1D). Furthermore, suppression of ZEB2 by siRNA reduced cadherin-11 promoter activity by 45% in SNU-398 cells (Figure 1E).

To delineate the region(s) of the cadherin-11 promoter responsible for induction by ZEB2, deletion mutants of the promoter were generated (Figure 2A). By successive 5′-deletion from position −1350 to −80 in the promoter region, we identified the region within the cadherin-11 promoter (−150/−80) that is transactivated by ZEB2 (Figure 2B). This region contains the consensus binding site for Sp1 but does not contain E-box elements or Smad-binding elements (Figure 2A).

We analyzed the functional involvement of the Sp1 site in the −150/−80 region using reporter assays. Mutation of the proximal Sp1 site (−111/−107 and −100/−96) resulted in a substantial decrease in ZEB2-mediated activation of the cadherin-11 promoter in SW480 and AGS cells (Figure 2C), indicating functional significance of the proximal Sp1 site for the effects of ZEB2, although the extent of ZEB2-mediated gene expression that is dependent on Sp1 may vary depending on cell type or context. Notably, mutation of the proximal Sp1 site reduced the basal cadherin-11 promoter activity modestly or not at all, whereas elimination of the region containing the Sp1 site, −150 to −80, resulted in a substantial decrease in both ZEB2-induced and basal cadherin-11 promoter activity, suggesting the possible involvement of this region in basal cadherin-11 promoter activity.

The binding of endogenous ZEB2 and Sp1 to the cadherin-11 promoter was examined by ChIP with SNU-638 cells, which express endogenous ZEB2. Chromatin fragments containing the cadherin-11 promoter (−191/−62) were specifically pulled down by anti-ZEB2 and anti-Sp1 antibodies, but an irrelevant region (−1122/−1007) was not efficiently pulled down (Figure 2D). Furthermore, suppression of ZEB2 by siRNA substantially reduced immunoprecipitation of chromatin fragments containing the cadherin-11 promoter (−191/−62), using anti-Sp1 and anti-ZEB2 in SNU-398 cells (Figure 2E).

Consistent with the data shown in Figure 2A–C, these observations indicate that the −191/−62 region is probably involved in the interaction of ZEB2 and Sp1 with the cadherin-11 promoter.

Sp1, but not Smad, is required for ZEB2 activation of the cadherin-11 promoter

To investigate whether Sp1 is required for ZEB2-induced cadherin-11 promoter activation, Sp1 was suppressed by siRNA or treatment with mithramycin A, an Sp1-DNA-binding inhibitor, in SW480 and AGS cells. Real-time qPCR analysis showed that ZEB2-mediated transcription of cadherin-11 was abolished in both cell types following pharmacological inhibition of Sp1 with mithramycin A (Figure 3A). Immunoblot analysis also showed that Sp1-binding activity is required for ZEB2-induced cadherin-11 expression in SW480 and AGS cells (Figure 3B). Sp1 depletion by siRNA also significantly reduced the induction of cadherin-11 by ZEB2 in SW480 and AGS cells (Figure 3C; see below). Taken together, these observations indicate that ZEB2 activates the cadherin-11 promoter through cooperation with Sp1, as has been shown for the integrin α5 promoter (19).

ZEB2 was originally identified as a Smad-interacting protein, and amino acid residues from 437 to 487 were identified as the SBD (13). ZEB2 appeared to downregulate E-cadherin transcription through E-box elements in a Smad-dependent manner in NMuMG-derived NMe cells (13,14). Therefore, we explored whether Smad is involved in ZEB2-induced cadherin-11 transcription. We generated mutant ZEB2 lacking the SBD (ZEB2ΔSBD) (13) and confirmed that it was expressed at a level similar to that of wild-type ZEB2 (Figure 3D). Reporter assays demonstrated that the mutant ZEB2 activated the cadherin-11 promoter to an extent similar to wild-type ZEB2 in SW480 (Figure 3E) and AGS cells (Supplementary Figure S1A is available at Carcinogenesis Online). This is consistent with the observation that the region of the cadherin-11 promoter transactivated by ZEB2 (−150/−80) does not contain Smad-binding elements (Figure 2A and B). Furthermore, integrin α5 and vimentin promoters were also activated by mutant ZEB2 in SW480 (Figure 3E) and AGS cells (Supplementary Figure S1B is available at Carcinogenesis Online), indicating that ZEB2 upregulates mesenchymal gene expression in a Smad-independent, but Sp1-dependent, manner.

We observed that E-cadherin expression was reduced by the mutant ZEB2 lacking SBD to an extent similar to wild-type ZEB2 in SW480 and HEK293E cells (Figure 3F). In addition, E-cadherin reduction by ZEB2 was not substantially recovered by knockdown of Smad2 or Smad3 in SW480 and HEK293E cells (Supplementary Figure S2 is available at Carcinogenesis Online), indicating that Smad(s) may be involved in ZEB2-mediated downregulation of E-cadherin in a cell- or context-dependent manner. On the other hand, E-cadherin reduction by ZEB2 in SW480 and HEK293E cells was not substantially affected by siRNA-mediated Sp1 depletion (Figure 3G) although the E-cadherin promoter contains a GC-rich box. Taken together, these results indicate that ZEB2 upregulates mesenchymal gene expression in a Smad- and E-box-independent, but Sp1-dependent, manner, whereas ZEB2 downregulates E-cadherin expression in an E-box-dependent and Sp1-independent manner.

ZEB2 upregulates Sp1 through enhancing Sp1 protein stability

Previously we observed that ZEB2 enhanced Sp1 expression in SW480 and HEK293E cells (19); however, the underlying mechanism remains unknown. Therefore, we explored the mechanism by which ZEB2 enhances Sp1 expression. Based on the observation that ZEB2 physically binds to Sp1 (19), we hypothesized that ZEB2-mediated Sp1 upregulation may be due, in part, to enhanced protein stability. Therefore, we investigated the effects of the proteasome inhibitor MG132 on ZEB2 depletion-mediated Sp1 downregulation. SNU-398 liver cancer cells, which express a high level of endogenous ZEB2, were transiently transfected with ZEB2-specific siRNA for 42 h and treated with MG132 for 6 h before lysis. siRNA-mediated suppression of ZEB2 reduced the level of Sp1, and this effect was substantially reversed by MG132 treatment (Figure 4A). However, MG132 did not modulate glyceraldehyde 3-phosphate dehydrogenase expression, indicating that ZEB2 suppression selectively induced proteasome-dependent degradation of Sp1 in SNU-398 cells. Consistent with the level of Sp1, MG132 reversed the effects of ZEB2 depletion on the expression of integrin α5 and the phosphorylation of JNK and ATF-2 (Figure 4A; see below). Notably, MG132 appeared to increase the basal level of ATF-2 to some extent. This observation suggests that ZEB2 modulates Sp1 level, at least in part, in a proteasome-dependent manner.

Sp1 is required for ZEB2-induced cancer cell invasion mainly through upregulation of cadherin-11 and integrin α5

We explored whether Sp1 is required for ZEB2-induced EMT and invasion. Consistent with our previous findings (19), transient transfection of SW480 colorectal cancer cells with a ZEB2 expression plasmid significantly induced cell invasion in a reconstituted basement membrane (Matrigel) and cell migration, compared with transfection with empty vector (Figure 4B). siRNA-mediated depletion of Sp1 resulted in significant suppression of basal invasiveness (Figure 4B,
ZEB2–Sp1 cooperation induces invasion via cadherin-11 and integrin α5

lane 1 versus lane 3) but did not substantially affect cell proliferation or viability (Supplementary Figure S3 is available at Carcinogenesis Online). Furthermore, suppression of Sp1 expression dramatically reduced ZEB2-mediated invasion and cell migration (Figure 4B, lane 2 versus lane 4). Immunoblot analysis confirmed that induction of cadherin-11, vimentin (Figure 4C) and integrin α5 (Figure 4C) (19)

Fig. 1. ZEB2 induces cadherin-11 expression. (A and B) SW480 cells were transfected with the ZEB2 expression vector for 48 h. (A) Transfected cells were lysed and used for immunoblotting. Anti-myc and anti-ZEB2 (6E5; active motif) were used to detect exogenous myc-tagged ZEB2 and total ZEB2, respectively. glyceraldehyde 3-phosphate dehydrogenase and α-tubulin were used as internal controls. (B) Real-time qPCR analysis of cadherin-11 messenger RNA level. (C) SNU-638 and SNU-398 cells were transfected with ZEB2-specific siRNA or negative-control siRNA for 48 h. Transfected cells were lysed and used for real-time qPCR analysis of cadherin-11 and ZEB2 messenger RNA levels. Suppression of ZEB2 by siRNA was confirmed by immunoblotting using anti-ZEB2 (6E5). (D) Cells were cotransfected with the ZEB2 expression vector and the cadherin-11 promoter construct in the pGL3 vector for 48 h. Overexpression of ZEB2 was confirmed by immunoblotting using anti-ZEB2 (6E5). Firefly luciferase activity, representing cadherin-11 promoter activity, was measured and normalized to Renilla luciferase activity to determine transfection efficiency. (E) Cells were transfected with siRNA for 24 h and further transfected with the cadherin-11 promoter construct for 24 h. Suppression of ZEB2 by siRNA was confirmed by immunoblotting using anti-ZEB2 (6E5). Reporter assays were performed as in (D). Values represent mean ± standard deviation (SD). *P < 0.05.
by ZEB2 was reduced by suppression of Sp1, whereas reduction of E-cadherin by ZEB2 was sustained (Figure 4C). Notably, suppression of Sp1 appeared to reduce the basal level of E-cadherin to some extent although the E-cadherin promoter activity was not substantially reduced by knockdown of Sp1 as shown in Figure 3G. ZEB2-induced phosphorylation of JNK and ATF-2 was also reduced by Sp1 knockdown (Figure 4C; see below). These observations suggest that ZEB2-induced invasion and expression of mesenchymal genes are dependent on Sp1.

It has been reported that cadherin-11 expression is associated with increased invasiveness and motility in breast and prostate cancer cells and glioma cells (11, 12, 26, 27). In contrast, proapoptotic, tumor suppressor activity of cadherin-11 has also been reported in common cancers (28). Thus, the role of cadherin-11 in cancer growth and...
progression remains controversial. In addition, the role of cadherin-11 in colorectal cancer cells is unknown. Therefore, we examined the effect of cadherin-11 suppression on colorectal cancer cell invasion and migration. siRNA-induced knockdown of cadherin-11 in SW480 cells significantly inhibited invasion and cell migration (Figure 5A). In addition, in agreement with our previous report showing that integrin α5 is required for invasion of SNU-638 gastric cancer cells and HEK293E cells (19), suppression of integrin α5 in SW480 cells also inhibited invasion and cell migration (Figure 5A). Immunoblot analysis showed that suppression of cadherin-11 reduced integrin α5 and vimentin expression (Figure 5B). Interestingly, depletion of integrin α5 also reduced cadherin-11 and vimentin expression (Figure 5B), indicating the presence of reciprocal regulation between cadherin-11 and integrin α5. Suppression of cadherin-11 or integrin α5 reduced phosphorylation of JNK and ATF-2 (Figure 5B). Furthermore, ZEB2-induced invasion and cell migration were significantly reduced by suppression of cadherin-11 or integrin α5 (Figure 5C). Immunoblot analysis showed that ZEB2 induced phosphorylation of JNK and ATF-2 and that these effects were reduced by knockdown of cadherin-11 or integrin α5 (Figure 5D). In addition, suppression of cadherin-11 and integrin α5 resulted in reduction of ZEB2-induced expression of integrin α5 and cadherin-11, respectively (Figure 5D), confirming the presence of bidirectional regulation between cadherin-11 and integrin α5. Taken together, these results suggest that the ZEB2/Sp1/cadherin-11–integrin α5 axis may contribute to invasiveness of cancer cells (Figure 5E).
In addition, the role of cadherin-11 on colorectal cancer cell invasion and migration was further examined using LS174T cells, which are known to be metastatic (29). Suppression of cadherin-11 in LS174T cells decreased invasion and cell migration (Supplementary Figure S4 is available at Carcinogenesis Online). ZEB2 moderately upregulated cadherin-11 expression,

Fig. 3. Sp1, but not Smad, is required for ZEB2 activation of the cadherin-11 promoter. (A and B) Effect of Sp1-DNA binding inhibitor (mithramycin A) on ZEB2-induced cadherin-11 expression in SW480 and AGS cells. Cells were transfected with the ZEB2 expression vector or empty vector for 24 h and then treated with mithramycin A (50 nM) or vehicle control (dimethyl sulfoxide, 0.2%) for 24 h. (A) Real-time qPCR analysis of cadherin-11 messenger RNA level. Values represent mean ± SD. *P < 0.05 compared with empty vector + dimethyl sulfoxide; §P < 0.05, compared with ZEB2 + dimethyl sulfoxide. (B) Immunoblot analysis of lysates of cells transfected with ZEB2 and treated with mithramycin A. (C) Effect of Sp1-specific siRNA on ZEB2-induced cadherin-11 expression. Cells were transfected with siRNA for 24 h and further transfected with ZEB2 expression vector or empty vector for 48 h. Cadherin-11 messenger RNA level was analyzed by real-time qPCR. Values represent mean ± SD. *P < 0.05 compared with empty vector + control siRNA; §P < 0.05, compared with ZEB2 + control siRNA. (D) SW480 cells were transfected with wild-type ZEB2 expression vector or SBD-deleted mutant ZEB2 (ZEB2ΔSBD51) for 48 h prior to lysis for immunoblotting. Anti-myc was used to detect wild-type and mutant myc-tagged ZEB2. (E) SW480 cells were cotransfected with the wild-type or mutant ZEB2 expression vector and cadherin-11 promoter (pcad11-1350luc), integrin α5 promoter (−908/+241) or vimentin promoter (−411/+60) construct for 48 h. Reporter assays were performed as in Figure 1D. (F) SW480 and HEK293E cells were cotransfected with the wild-type or mutant ZEB2 expression vector and E-cadherin promoter (−308/+41) construct for 48 h. Reporter assays were performed as in Figure 1D. Values represent mean ± SD. *P < 0.05. (G) Effect of Sp1-specific siRNA on ZEB2-suppressed E-cadherin promoter activity in SW480 and HEK293E cells. Reporter assays were performed as in Figure 1D. Values represent mean ± SD. *P < 0.05 compared with empty vector + control siRNA.
ZEB2–Sp1 cooperation induces invasion via cadherin-11 and integrin α5

Fig. 4. Sp1 is upregulated by ZEB2 through enhanced Sp1 protein stability and is required for ZEB2-induced EMT marker expression and invasion. (A) SNU-398 cells were transfected with siRNA for 42 h and then treated with MG132 (2 μM) or vehicle control (dimethyl sulfoxide, 0.1%) for 6 h before lysates preparation for immunoblot analysis. (B and C) SW480 cells were transfected with siRNA for 24 h and further transfected with the ZEB2 expression vector for 24 h. (B) Transfected cells were allowed to invade Matrigel (3 × 10⁴ cells) or migrate toward collagen type I (1 × 10⁴ cells) for 48 h. The number of cells that had invaded or migrated was counted in five representative high-power (×200) fields per Transwell insert and averages were obtained. Values represent mean ± SD. *P < 0.05 compared with empty vector + control siRNA; §P < 0.05 compared with ZEB2 + control siRNA. (C) Transfected cells were lysed and used for immunoblotting. Anti-myc was used to detect myc-tagged ZEB2.
and ZEB2-induced invasion and cell migration were significantly reduced by suppression of cadherin-11 (Supplementary Figure S4 is available at Carcinogenesis Online). These results suggest that cadherin-11 may be associated with increased invasiveness and motility in colorectal cancer cells.

Nuclear expression of ZEB2 correlates positively with Sp1 expression in human colorectal cancers

To obtain evidence of the cooperation of ZEB2 and Sp1 in human carcinomas, we analyzed a series of nine human colorectal cancers for coexpression of ZEB2 and Sp1. In all nine cases, Sp1 expression
ZEB2–Sp1 cooperation induces invasion via cadherin-11 and integrin α5.

Discussion

It is well known that ZEB2 acts as a transcriptional suppressor of E-cadherin through recognition of E-box elements in the E-cadherin promoter. Our study shows that ZEB2 acts as both a transcriptional repressor in an E-box-dependent, but Sp1-independent, manner and a transcriptional activator in an E-box-independent, but Sp1-dependent, manner. In our system, ZEB2 played both roles in a Smad(s)-independent manner. KLF4 recognizes and binds a GC-rich box in the proximal promoter of E-cadherin to induce E-cadherin expression (30). Our observation that Sp1, which may compete with KLF4 for GC box binding (20,31), is not involved in E-cadherin downregulation by ZEB2 suggests that ZEB2–Sp1 cooperation is specific for induction of mesenchymal genes. Previously, we found that ZEB2 directly upregulates integrin α5 (19). Here, we report that ZEB2 is a direct mediator of the switch from E-cadherin to cadherin-11, which results in a mesenchymal transition that allows for greater tumor cell invasiveness.

Taken together, these data show that ZEB2 contributes to EMT by coordinating changes in cell–cell interactions, such as cadherin switch, with changes in cell–matrix interactions through modulation of specific integrin(s) expression. Cadherins and integrins mediate cell–cell and cell–extracellular matrix interactions, respectively, and play important roles during cell proliferation, differentiation, survival, migration and gene expression (32,33). Crosstalk between integrins and cadherins has been suggested; e.g. integrins can cause downregulation of E-cadherin expression via integrin-linked kinase (34) and disrupt cadherin-mediated cell–cell adhesion via activation of RhoA and Rac1 (35). In addition, integrin β1 and β3 regulate the surface distribution and activity of N-cadherin in migrating neural crest cells (36). However, a relationship between integrin and cadherin-11 has not been established. Here, our results reveal that expression levels of cadherin-11 and integrin α5 are mutually dependent. In addition to their functions as adhesion molecules, cadherin-11 and integrin α5 appear to function interdependently, and possibly synergistically, to modulate activity of specific signaling pathways (such as JNK) associated with invasiveness. It is possible that integrin α5 and cadherin-11 physically interact on the cell surface to modulate cellular signaling events and invasion although this remains to be determined.

Furthermore, ZEB2 appears to play a significant role in regulating this positive relationship. These observations suggest that the ZEB2/Sp1–cadherin-11–integrin α5 axis contributes to invasion and accelerated aggressiveness of a malignancy (Figure 5E).

It has been reported that cadherin-11 is associated with increased invasiveness in breast and prostate cancer cells (11,26). In contrast, tumor suppressor activity of cadherin-11 has also been suggested (28). Cadherin-11 contributes to pulmonary fibrosis by mediating transforming growth factor (TGF)-β-induced EMT and is induced by TGF-β1 (37). However, mechanisms underlying cadherin-11 induction remain unknown, and the role of cadherin-11 in colon cancer cells is not fully understood. Our study shows that cadherin-11 is induced by ZEB2 through cooperation with Sp1, and that cadherin-11 is required for colon cancer cell migration and invasion.

We observed that suppression of Sp1 abolished ZEB2-induced cell invasion and migration, suggesting that ZEB2–Sp1 cooperation is critical for invasiveness. In addition, cadherin-11 and integrin α5 are, probably, primary modulators of ZEB2–Sp1 cooperation-induced invasion although we cannot completely rule out the possibility that other mesenchymal genes are involved in ZEB2-mediated invasion. On the other hand, it appeared that, in our system, E-cadherin downregulation is required, but not sufficient, for ZEB2-induced invasion because...
Fig. 6. Nuclear expression of ZEB2 correlates positively with Sp1 expression in human colorectal cancers. (A and B) Double immunofluorescence analysis of ZEB2 and Sp1 expression in sections of human colorectal carcinomas. (A) Representative images showing correlation of nuclear colocalization of ZEB2 and Sp1 in tumor cells in cancer tissues. (B) Representative images showing nuclear localization of Sp1 and cytoplasmic localization of ZEB2 in normal epithelial cells in normal tissues. Scale bars, 5 μm; TD, Transmitted light detection. (C) Quantification of the relative percentage of nuclear expression of ZEB2 in normal versus tumor cells. *P < 0.0001, compared with normal cells. One hundred cells per field, in three different fields of each tissue (normal versus tumor; n = 9) were quantified.
restoration of E-cadherin is not a prerequisite for suppression of invasion. Together, these observations suggest the functional significance of involvement of specific mesenchymal genes, such as cadherin-11 and integrin α5, in invasion induced by ZEB2 during tumor progression.

ZEB2 was originally identified as a Smad-interacting protein (13). Both ZEB1 and ZEB2 bind to Smads (38); whereas ZEB1 synergizes with Smads to induce expression of TGF-β target genes, such as 3TP, p21, p15 and c-Jun, ZEB2 represses those genes (38). Here, we show that ZEB2 upregulates mesenchymal gene expression in a Smad-independent, but Sp1-dependent, manner. It has been reported that Smad proteins and Sp1 cooperatively regulate expression of TGF-β target genes, including matrix metalloproteinase-11, cyclin D1 and Smad7, as well as TGF-β-mediated pancreatic cancer cell migration (39,40). Therefore, it is possible that ZEB2–Sp1 may cooperate with Smad(s) to induce expression of certain mesenchymal genes under particular conditions/contexts.

In addition, it remains unclear whether ZEB2 can cooperate with transcription factors other than Sp1 to induce EMT and invasion under certain conditions/contexts. For example, we cannot completely rule out the possibility that transcription factors other than Sp1, which can compete with Sp1 for GC box binding, may be involved in ZEB2-mediated mesenchymal gene expression. It is intriguing that ZEB2 expression was associated with the phosphorylation of ATF-2, probably through activation of the JNK-signaling pathway, in a cadherin-11–integrin α5-dependent manner. This raises the possibility that ZEB2 can also modulate the activity of other transcription factors, such as activator protein-1, in addition to Sp1, and implies that ZEB2 can confer not only invasiveness but also other features, such as stem cell-like properties and capacity for survival or proliferation, on cancer cells. It would be worth exploring whether ZEB2 may cooperate with and/or modulate the activity of other transcription factors in the context of tumor progression.

Immunofluorescence analysis of human colorectal cancer tissues demonstrated nuclear colocalization of ZEB2 and Sp1 in tumor cells. It is possible that ZEB2 translocates from cytoplasm to nucleus during tumorogenesis and/or tumor progression. Nuclear translocation of ZEB2 and maintenance of ZEB2 in nuclei may be related to the presence of Sp1; e.g. physical interaction of ZEB2 with Sp1 may positively modulate ZEB2 protein translocation and/or stability. We observed that ZEB2 modulates the level of Sp1, at least in part, in a proteasome-dependent manner (Figure 4A), which may result in enhanced Sp1 transcriptional activity (19). It would be worth exploring whether Sp1 can modulate ZEB2 translocation and stability, which would suggest the existence of a positive feedback loop between ZEB2 and Sp1 during EMT and metastasis.

In conclusion, cadherin-11 and integrin α5 are proposed to be leading factors in the association between ZEB2–Sp1 cooperation and induction of invasion, implying that these factors and ZEB2 itself are interesting potential targets for novel therapies against cancer metastasis.

Supplementary material
Supplementary Figures S1–S4 can be found at http://carcin.oxfordjournals.org/

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References


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