Cooperativity of E-cadherin and Smad4 Loss to Promote Diffuse-Type Gastric Adenocarcinoma and Metastasis

Jun Won Park1,2, Seok Hoon Jang1, Dong Min Park1, Na Jung Lim1, Chuxia Deng3, Dae Yong Kim2, Jeffrey E. Green4, and Hark Kyun Kim1

Abstract
Loss of E-cadherin (CDH1), Smad4, and p53 has been shown to play an integral role in gastric, intestinal, and breast cancer formation. Compound conditional knockout mice for Smad4, p53, and E-cadherin were generated to define and compare the roles of these genes in gastric, intestinal, and breast cancer development by crossing with Pdx-1-Cre, Villin-Cre, and MMTV-Cre transgenic mice. Interestingly, gastric adenocarcinoma was significantly more frequent in Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice than in Pdx-1-Cre; Smad4F/F; Cdh1F/− mice, demonstrating that Cdh1 heterozygosity accelerates the development and progression of gastric adenocarcinoma, in combination with loss of Smad4 and p53. Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice developed gastric adenocarcinomas without E-cadherin expression. However, intestinal and mammary adenocarcinomas with the same genetic background retained E-cadherin expression and were phenotypically similar to mice with both wild-type Cdh1 alleles. Lung metastases were identified in Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice, but not in the other genotypes. Nuclear β-catenin accumulation was identified at the invasive tumor front of gastric adenocarcinomas arising in Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice. This phenotype was less prominent in mice with intact E-cadherin or Smad4, indicating that the inhibition of β-catenin signaling by E-cadherin or Smad4 downregulates signaling pathways involved in metastases in Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice. Knockdown of β-catenin significantly inhibited the migratory activity of Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ cell lines. Thus, loss of E-cadherin and Smad4 cooperates with p53 loss to promote the development and metastatic progression of gastric adenocarcinomas, with similarities to human gastric adenocarcinoma.

Implications: This study demonstrates that inhibition of β-catenin is a converging node for the antimetastatic signaling pathways driven by E-cadherin and Smad4 in Pdx-1-Cre; Smad4F/F; Trp53F/F; Cdh1F/+ mice, providing novel insights into mechanisms for gastric cancer metastasis. Mol Cancer Res; 12(8): 1–12. ©2014 AACR.

Introduction
Gastric cancer is the second most common cause of cancer-related death worldwide (1). p53, Smad4, and E-cadherin are frequently inactivated in human gastric cancer. TP53 mutations are reported in 0% to 21% of diffuse- and 36% to 43% of intestinal-type gastric cancers, respectively (2). Smad4, a co-Smad involved in both branches of the TGF-β/BMP signaling system (3), is inactivated in 40% of human gastric cancers by loss of heterozygosity (LOH), promoter hypermethylation, and somatic mutation (4). The CDH1 gene, coding for E-cadherin, is frequently inactivated in familial and sporadic diffuse-type gastric cancers (5). Germline CDH1 mutations are associated with a 80% lifetime risk of diffuse-type gastric cancer, and somatic inactivating E-cadherin mutations have been reported in 33% to 50% of sporadic diffuse-type gastric cancers (5). Promoter hypermethylation of CDH1, identified in up to 80% of patients with diffuse-type gastric cancers, is the most common second hit in the inactivation of wild-type CDH1 allele, but mechanisms leading to the inactivation of the wild-type CDH1 allele remain largely unknown (6, 7). A better understanding of the stepwise inactivation of E-cadherin would provide an opportunity for therapeutic intervention.

Valuable biologic insights into these gastric tumor suppressors have been obtained through studies of genetically engineered mouse models. Knockout of Smad4 in the germline of mice results in embryonic lethality (8), whereas mice heterozygous for mutant Smad4 in the germline develop in situ gastric carcinomas after 18 months of age.
It still remains to be elucidated how the loss of Smad4 in gastric epithelium, alone and in combination with other tumor suppressors, promotes the progression of gastric cancers. Although it was recently reported that Atp4b-driven p53 and E-cadherin knockout mice develop diffuse-type gastric cancers (10), the impact of E-cadherin heterozygosity on the development and progression of gastric cancer has not been previously evaluated. On the basis of human mutation profiles and prior studies, we assessed the role of one allelic loss of E-cadherin, alone or in combination with the loss of Smad4 and p53, on the development, progression, and metastasis of diffuse-type gastric adenocarcinomas in mice. In addition, we compared this gastric cancer model with intestinal and mammary cancer models of the same genetic background.

**Materials and Methods**

**Mice**

Mouse studies were conducted with the approval of the Animal Care and Use Committees of National Cancer Center of Korea and the National Cancer Institute, Bethesda, MD. *Pdx-1-Cre* mice (B6.FVB-Tg(Ip1-Cre)1Tuv), originally generated by Dr. Lowy (11), were provided through the Mouse Models of Human Cancers Consortium (MMHCC) repository at the NCI Frederick Cancer Research Center, Frederick, MD. B6.Cg-Tg(Vil-Cre)20Sy, FVB/N-Tg (MMTV-Cre)7Mul, and FVB.B129-Tg(129Pdx1-Cre)8Mklm (12) mice were also provided by the MMHCC. B6.129-Cdh1tm2Kem/J mice, which have *loxP* sites flanking exons 6 to 10, were purchased from The Jackson Laboratory (13). Conditional Smad4 knockout mice (*Smad4fl/fl*) on the Black Swiss, B6, and 129 backgrounds were previously described (14).

Compound conditional knockouts of Smad4, p53, and E-cadherin were bred with *Pdx-1-Cre* mice, *Villin-Cre* mice, and *MMTV-Cre* mice, to perform targeted deletion for these genes in gastric, intestinal, and mammary epithelium, respectively. *Pdx-1-Cre* mice express Cre in mucosal epithelial cells of the gastric antrum and duodenum as well as the pancreatic islet cells (15). *Villin-Cre* mice expressed Cre in progenitor cells of the intestinal epithelial mucosa (16) and of the antrum (17). *MMTV-Cre* mice expressed Cre in mammary epithelial cells and striated ductal cells of the salivary gland (18). The strain background for crosses was controlled to avoid confounding variables in comparing tumor-free survival across the genotypes. Offspring mice were genotyped using polymerase chain reaction (PCR) assays for tail DNA. Mice positive for *Pdx-1-Cre* or *Villin-Cre* mice and *MMTV-Cre* mice, to perform targeted deletion for these genes in gastric, intestinal, and mammary epithelium, respectively. *Pdx-1-Cre* mice express Cre in mucosal epithelial cells of the gastric antrum and duodenum as well as the pancreatic islet cells (15). *Villin-Cre* mice expressed Cre in progenitor cells of the intestinal epithelial mucosa (16) and of the antrum (17). *MMTV-Cre* mice expressed Cre in mammary epithelial cells and striated ductal cells of the salivary gland (18). The strain background for crosses was controlled to avoid confounding variables in comparing tumor-free survival across the genotypes. Offspring mice were genotyped using polymerase chain reaction (PCR) assays for tail DNA. Mice positive for *Pdx-1-Cre* or *Villin-Cre* mice and *MMTV-Cre* mice, to perform targeted deletion for these genes in gastric, intestinal, and mammary epithelium, respectively.

**β-Catenin and the migration assays**

For the measurement of β-catenin mRNA expression and activity, we performed quantitative real-time PCR and Dual-Luciferase Reporter Assay (Promega). Primary cultured cells (1.5 × 10^5^ cells per well) were treated in quadruplicate for 16 hours in 12 wells with RPMI-1640 media containing 0.5% FBS with 100 ng/mL of BMP2 (R&D Systems; 355-Bm), 400 ng/mL of noggin (R&D Systems; 6057-NG), 5 ng/mL of TGFβ1 (gift from Dr. Lalage Wakefield, NCI, Bethesda, MD), or Wnt3a conditioned media produced by L Wnt-3A cells (gift from Dr. Tae-Aug Kim, NCI, Bethesda, MD). For migration assays, primary cultured cells (2.5 × 10^5^ cells per well) were plated on 24-well inserts with an 8-μm pore size (353097, BD Biosciences) in serum-free RPMI-1640 media. Media containing 10% FBS were added to the 24-well lower insert chambers (354578, BD Biosciences). After 24 hours, cells that migrated to the lower insert chamber were counted at three high-power fields (×200). Student *t* tests were used for statistical analyses.

**Results**

**Gastric tumors formed in Pdx-1-Cre;Smad4<sup>fl/fl</sup>,Trp53<sup>fl/fl</sup>, Cdh1<sup>fl/fl+</sup> mice recapitulate human diffuse-type gastric adenocarcinomas**

Twenty-one of 25 *Pdx-1-Cre;Smad4<sup>fl/fl</sup>,Trp53<sup>fl/fl</sup>, Cdh1<sup>fl/fl+</sup> mice (84%) developed spontaneous tumors in the glandular stomach (Fig. 1A and S1). Of 4 *Pdx-1-Cre;Smad4<sup>fl/fl</sup>,Trp53<sup>fl/fl</sup>, Cdh1<sup>fl/fl+</sup> mice without gastric adenocarcinomas, 2 mice were killed by scheduled sacrifice at 6 months, and the other 2 mice died of duodenal cancer obstruction at 7.4 and 8.2 months, respectively. The most common cause of death in *Pdx-1-Cre;Smad4<sup>fl/fl</sup>,Trp53<sup>fl/fl</sup>, Cdh1<sup>fl/fl+</sup> mice was carcinomatous lesions were identified in a given organ, it was censored for the development of carcinoma on the day of necropsy (19). *MMTV-Cre*–positive female mice were euthanized when mammary tumors reached 2 cm in diameter. Liver, spleen, and lung were harvested at necropsy to assess for metastases.

**Immunohistochemistry**

Immunohistochemistry analyses were performed on 5-μm, formalin-fixed, paraffin-embedded slides from tumors arising in the mutant mice. The following antibodies were used: rabbit polyclonal anti-E-cadherin antibody (1:200; Cell Signaling Technology; #3195), rabbit polyclonal anti-Ki-67 antibody (1:200; Abcam; ab15580), mouse monoclonal anti-β-catenin antibody (1:200; BD Transduction Laboratories; 610154), rabbit monoclonal anti-vimentin antibody (1:100; Cell Signaling Technology; #5741), and rabbit monoclonal anti-MMP7 antibody (1:100; Cell Signaling Technology; #3801). The invasive front of the tumor was defined as the microscopic interface between normal tissue of the host mouse and the tumor mass invading the submucosa or deeper regions of the stomach (20). Differences in the percentage of positive immunostaining between the various genotypes were evaluated using the Student *t* test.
duodenal obstruction, followed by gastric outlet obstruction.

Histologically, tumors arising in the glandular stomach of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice resembled human diffuse-type gastric adenocarcinomas based on the histopathologic findings, and were invasive into the muscle layers and regional lymph nodes (Fig. 1B).

Duodenal adenocarcinomas and forestomach squamous cell carcinomas were also identified in 36% and 24% of these mice, respectively (Supplementary Table S1). In addition, 2 mice were noted to have adenocarcinomas in the pancreas (8%), which was interpreted as invasion of primary duodenal or gastric adenocarcinomas. This pattern of tumor distribution is consistent with the known tissue specificity of the Pdx-1 promoter (15).

Because gastric adenocarcinoma was the most common type of carcinoma in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice, we focused our analysis on adenocarcinomas arising in the glandular stomach. DNA microarray analyses and immunohistochemistry of four gastric cancers from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice compared with normal stomach epithelium revealed that these tumors were positive for mucin 6 and TFF2, and negative for mucin 5a, pepsinogen C, somatostatin, and gastric intrinsic factors, suggesting the deep antral gland origin of these tumors (ref. 21; Supplementary Fig. S3).

E-cadherin loss is required for the development of diffuse-type gastric adenocarcinoma

Scheduled sacrifices performed at 4 and 5 months revealed no gastric premalignant lesions such as atrophic gastritis, metaplasia, or dysplasia, in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice. In 2 of 4 Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice screened at 6 months of age, intramucosal adenocarcinomas with signet ring cell feature were observed (Fig. 1C). Interestingly, E-cadherin immunostaining was lost in adenocarcinoma cells by 6 months of age, suggesting that E-cadherin loss is an early event required for the diffuse-type gastric carcinogenesis in mice.

To further evaluate the role of E-cadherin in constraining diffuse-type gastric carcinogenesis, a cohort (n = 25) of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice was used in this study.
1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice was compared with a cohort (n = 28) of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice. The median gastric adenocarcinoma-free survival of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ was 8.0 months [95% confidence intervals (CI), 7.5–8.4], whereas only one gastric adenocarcinoma was identified by a scheduled sacrifice at 9 month of age, in the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mouse cohort (log-rank P < 0.001 and stratified log-rank P < 0.001; Fig. 1D). Whereas E-cadherin expression was retained in a gastric adenocarcinoma arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ (Fig. 1C), all of the gastric adenocarcinomas arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice were negative for E-cadherin immunostaining, and all of the gastric adenocarcinomas analyzed from this group were also found to express low levels of Cdh1 mRNA compared with normal tissue (Fig. 2A and B).

LOH at the Cdh1 locus was identified in two of 17 Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ gastric adenocarcinomas tested (11.8%; Fig. 3A). Next, we investigated epigenetic changes leading to the loss of E-cadherin, but found no evidence for Cdh1 promoter hypermethylation (Fig. 3B and C) or deacetylation (Fig. 3D). MicroRNA microarray analyses revealed no difference in expression level of microRNAs targeting Cdh1, such as miR-9, between Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ gastric adenocarcinomas and normal tissue (data not shown).

In contrast with the differences in the incidences of gastric adenocarcinomas between the genotypes, duodenal adenocarcinoma-free survival was similar between Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ and Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice (9.0 vs. 9.1 months; log-rank P = 0.15; Fig. 2C). E-cadherin was retained in duodenal adenocarcinomas arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice, and, therefore, was relatively overexpressed compared with gastric adenocarcinomas from the same mice (Fig. 2A and B). Next, we evaluated intestinal and mammary adenocarcinomas in mice created through the loss of the same set of genes using Villin-Cre and MMTV-Cre transgenes, respectively, to compare the tumor-promoting effects of E-cadherin heterozygosity across the different target tissues. Intestinal adenocarcinoma-free survival was similar between Villin-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ and

Figure 2. A, E-cadherin immunostaining of gastric adenocarcinomas in the top (#6, #9, and #16) arising in three representative Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice, duodenal adenocarcinoma (DU) from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice, colorectal adenocarcinoma (CO) from Villin-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice, and mammary adenocarcinoma (MA) from MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice. B, RNA expression levels from real-time RT-PCR and DNA microarray analysis (in linear scales) for Cdh1 of tumors relative to normal stomach of Cre-negative mice (WT). Error bars, SDs. C, i, duodenal adenocarcinoma-free survival of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice compared with Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice. ii, colorectal adenocarcinoma-free survival of Villin-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice compared with Villin-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice. iii, mammary adenocarcinoma-free survival of MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice compared with MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1F/þ mice.
Villin-Cre;Smad4F/F;Trp53F/F;Cdh1+/− mice (5.2 vs. 5.4 months; log-rank \( P = 0.27 \); Fig. 2C). No distant metastases were observed in either genotype, except for one Villin-Cre;Smad4F/F;Trp53F/F;Cdh1+/− mouse developing skin metastasis. E-cadherin was retained in intestinal adenocarcinomas formed in Villin-Cre;Smad4F/F;Trp53F/F;Cdh1+/− mice (Fig. 2A).

MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1+/− and MMTV-Cre;Smad4+/−;Trp53F/F;Cdh1+/− mice were also not different in the mammary carcinoma-free survival (10.4 vs. 12.1 months; log-rank \( P = 0.73 \)) and lung metastasis [33.3% (3 of 9) vs. 35.7% (5 of 14), respectively; \( P \) for \( \chi^2 \) test = 0.91; Fig. 2C]. Histologically, MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1+/− tumors were invasive ductal carcinomas with a squamous component (Supplementary Fig. S4). Mammary adenocarcinomas arising in MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1+/− mice also retained E-cadherin (Fig. 2A), perhaps accounting for why lobular carcinomas were not observed in this model as has been reported in K14cre;Trp53F/F;Cdh1F/F (22). These results clearly demonstrate that E-cadherin loss is important for the development of gastric adenocarcinomas, but not for the development of intestinal or mammary adenocarcinomas.

It was surprising to us that the Cdh1 promoter was not significantly methylated in gastric adenocarcinomas formed in our Cdh1 heterozygous mice. In human diffuse-type gastric cancers, the CDH1 promoter hypermethylation has...
been identified in up to 80% of patients. To gain further insight into the clinical relevance of these mouse tumor data, we examined the E-cadherin mutation profiles of 13 young (<40 years old) Korean patients with gastric cancer (Supplementary Table S3). Three of 13 (23.1%) patients demonstrated very low levels of E-cadherin immunostaining and mRNA expression (Fig. 3E and F), and 1 of them harbored a germline missense CDH1 mutation [c.1018A>G (p. T340A)] that was previously reported in a hereditary diffuse gastric cancer kindred (23). Promoter hypermethylation of the CDH1 gene was not identified in any of the three E-cadherin-negative patients (Fig. 3G). Thus, transcriptional repression of CDH1 was possibly due to epigenetic changes other than promoter methylation or other regulatory factors that may be mechanisms for E-cadherin inactivation in humans as in our mutant mice.

**Smad4 cooperates with E-cadherin in constraining the development of gastric adenocarcinoma by inhibiting cell-cycle progression**

No neoplastic lesions were found at the time of necropsy of Pdx-1-Cre;Trp53F/F;Cdh1F/F mice (Fig. 4A). These results suggest that loss of Smad4 is required for the development of gastric adenocarcinoma in Pdx-1-Cre;Trp53F/F;Cdh1F/F mice (log-rank \( P = 0.005 \) and stratified log-rank \( P = 0.009 \); Fig. 4A). Because all of the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice analyzed were found to develop E-cadherin-negative gastric adenocarcinomas, tumors from those mice were compared with tumors from Pdx-1-Cre;Trp53F/F;Cdh1F/F mice. Six mice in the Pdx-1-Cre;Trp53F/F;Cdh1F/F cohort (\( n = 15 \)) developed gastric adenocarcinomas with a median tumor-free survival of 9.4 months for this group (Fig. 4A), but did not develop distant metastases. This phenotype is consistent with that of the previously reported Atp4b-Cre;Trp53F/F;Cdh1F/F mice (10). Gastric adenocarcinoma-free survival was significantly longer in Pdx-1-Cre;Trp53F/F;Cdh1F/F mice than in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice (median, 9.4 vs. 8.0 months; log-rank \( P = 0.007 \) and stratified log-rank \( P = 0.007 \)), demonstrating the role of Smad4 in constraining gastric cancer progression (Fig. 4A). Gastric adenocarcinomas formed in Pdx-1-Cre;Trp53F/F;Cdh1F/F mice were less invasive than those arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice (Fig. 4B). Gastric adenocarcinomas formed in Pdx-1-Cre;Trp53F/F;Cdh1F/F mice exhibited lower Ki-67 positivity than Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice (median, 42.8% vs. 71.7%, respectively; \( P < 0.001 \)), suggesting that Smad4 constrains tumor progression through the inhibition of the cell cycle (Fig. 4C and D).

**Loss of E-cadherin and Smad4 cooperates to promote lung metastasis through the accumulation of nuclear \( \beta \)-catenin**

Importantly, three of 21 Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice with gastric adenocarcinomas (14.2%) developed lung metastases. The metastatic lesions had similar...
cytologic features to the primary gastric tumors, including the lack of E-cadherin immunostaining (Fig. 5A). Prompted by the Gene Set Enrichment Analysis (GSEA) showing the enrichment of \( \text{Lef1} \) target genes (involved in the Wnt signaling pathway) in the gastric cancers arising in \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice (Supplementary Table S2), we sought to evaluate β-catenin (a regulator of Wnt signaling) by immunostaining the mutant mouse tumors. Nuclear β-catenin accumulation was identified at the invasive front of gastric adenocarcinomas from \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice, suggesting the association of nuclear β-catenin accumulation with tumor progression (\( P \) for \( t \) test between the invasive front and tumor center < 0.001; Supplementary Fig. S5; ref. 24). β-Catenin target genes, such as MMP7 and vimentin, were focally overexpressed among nuclear β-catenin–positive carcinoma cells at the invasive front, suggesting an epithelial-to-mesenchymal transition (EMT; Supplementary Fig. S5 and 5C). Ki-67 immunostaining was decreased at the invasive front of these mice, compared with the tumor center (Supplementary Fig. S5).

A gastric carcinoma from a \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mouse did not demonstrate nuclear β-catenin staining (Fig. 6A). Duodenal carcinomas from \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice (n = 4), with intact E-cadherin expression, also demonstrated significantly lower nuclear β-catenin positivity at the invasive front than gastric carcinomas from mice with the same genotype (n = 14; median, 8.5% vs. 29.3%; \( P \) for \( t \) test < 0.001; Fig. 6B). Invasive fronts of these tumors with E-cadherin expression did not express vimentin (data not shown). Thus, complete loss of E-cadherin may be a prerequisite for nuclear β-catenin accumulation across the different epithelial tumor types. The \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mouse with a gastric adenocarcinoma had no distant metastases (log-rank \( P \) value for metastasis-free survival between \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) and \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice.

Figure 5. A, hematoxylin and eosin (H&E; ii and iii) and E-cadherin immunostaining (iv) of lung metastases of gastric carcinomas arising in three \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice (#3, #14, and #22). Corresponding primary tumors are shown in A, i. B, metastasis-free survival of \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) compared with \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice. C, metastasis-free survival of \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) compared with \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) and \( \text{Pdx-1-Cre;Smad4}^{\text{F/F}};\text{Trp53}^{\text{F/F}};\text{Cdhl}^{\text{F/F}}} \) mice.
Given the low nuclear β-catenin positivity of the duodenal adenocarcinomas from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice that did not metastasize (Fig. 6B), these results suggest that the metastatic propensity of Pdx-1-Cre;Trp53F/F;Cdh1F/+ mice may be attributable, at least in part, to the activation of the β-catenin signaling pathway following E-cadherin loss.

Because no distant metastases were identified in mice with intact Smad4 (Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F+) mice that did not metastasize (Fig. 6B), these results suggest that the metastatic propensity of Pdx-1-Cre;Trp53F/F;Cdh1F/F mice may be attributable, at least in part, to the activation of the β-catenin signaling pathway following E-cadherin loss.

Figure 6. A, representative immunohistochemistry findings for E-cadherin and β-catenin at the invasive front of tumors across genotypes. B, percentage of nuclear β-catenin–positive cells at the invasive front of gastric adenocarcinomas from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ cohort (n = 14) and Pdx-1-Cre;Trp53F/F;Cdh1F/+ cohort (n = 4) and duodenal carcinomas from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice (n = 4). Box, interquartile range with median. C, vimentin and MMP7 immunohistochemistry at the invasive front of gastric adenocarcinomas between Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ and Pdx-1-Cre;Trp53F/F;Cdh1F/+ mice. D, percentage of vimentin- and MMP7-positive cells in gastric adenocarcinomas arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice (n = 10) and Pdx-1-Cre;Trp53F/F;Cdh1F/+ mice (n = 5).

Cdh1F/+ = 0.013; Fig. 5B). Given the low nuclear β-catenin positivity of the duodenal adenocarcinomas from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice that did not metastasize (Fig. 6B), these results suggest that the metastatic propensity of Pdx-1-Cre;Trp53F/F;Cdh1F/F mice may be attributable, at least in part, to the activation of the β-catenin signaling pathway following E-cadherin loss.

Because no distant metastases were identified in mice with intact Smad4 (Pdx-1-Cre;Trp53F/F;Cdh1F+) and Pdx-1-Cre;Trp53F/F;Cdh1F/F;Trp53F/F;Cdh1F/+ mice, we investigated possible mechanisms for the role of Smad4 in suppressing metastasis. Gastric adenocarcinomas arising in Pdx-1-Cre;Trp53F/F;Cdh1F/+ (n = 4) demonstrated nuclear β-catenin accumulation at the invasive front, but not as frequently as gastric adenocarcinomas from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice (n = 14), consistent with a prior report (ref. 10; P for t test = 0.047; Fig. 6B). Gastric cancers formed in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice demonstrated higher expression of vimentin and MMP7 at the invasive front than those arising from Pdx-1-Cre;Trp53F/F;Cdh1F/+ mice (t test P values for the positivity, 0.021 and 0.037, for vimentin and MMP7, respectively; Fig. 6C and D). MMP8 expression also tended to be higher in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice (Supplementary Fig. S6).

To further evaluate the effect of Smad4 loss on β-catenin (Ctnnb1) activity, we established primary cultured cell lines from gastric adenocarcinomas arising from Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ mice. Consistent with our in vivo results, Ctnnb1 mRNA expression was higher in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ cells than in Pdx-1-Cre;Trp53F/F;Cdh1F/+ cells (P < 0.001; Fig. 7A). Similar results were obtained when Smad4 was overexpressed in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ cells. Ctnnb1 mRNA was
significantly low in the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ cells stably expressing Smad4 similar to the endogenous level (designated as Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+–Smad4), compared with vector-only transfected Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/+ cells (P = 0.006; Fig. 7B). We wished to examine how Smad4 repressed β-catenin expression by determining whether repression was induced through the TGFβ or BMP signaling pathways, as Smad4 can mediate both pathways. Among BMP family members, BMP2 was selected for subsequent functional experiments based on its putative role as a gastric tumor suppressor (25–27) and the activity in our cell lines. Our results demonstrated that BMP2 significantly suppressed β-catenin expression when Smad4 was expressed, whereas TGFβ did not have these effects, and that BMP2 modestly augmented Smad4-induced Ctnnb1 downregulation (Fig. 7A and B). BMP2-induced repression of β-catenin expression was reversed by treatment with noggin, a BMP antagonist (Fig. 7A and B). Concordantly, Tcf/Lef reporter activity was lower in Smad4-expressing cell lines than in Smad4-null cells, and BMP2 treatment suppressed Tcf/Lef reporter activity in Smad4-expressing cells (Fig. 7C and D). The specificities of the responses to BMP2 and TGFβ in these experiments are depicted in Supplementary Fig. S7. These findings suggest that canonical BMP signaling through Smad4 mediates transcriptional repression of
Ctnnb1, and that Ctnnb1 is overexpressed with the loss of Smad4. We determined that Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cells demonstrated higher migratory activity than Pdx-1-Cre;Trp53–/–;Cdh1–/– cells using the Boyden chamber assay (P < 0.001; Fig. 7E and Supplementary Fig. S8). This was consistent with our in vivo results demonstrating increased metastatic propensity of the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice compared with Pdx-1-Cre;Trp53F/F;Cdh1F/F mice. To determine whether the increased migration was due to increased β-catenin expression, we performed β-catenin knockdown experiments on two independent Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cell lines. Knockdown of β-catenin, using two different short hairpin RNAs, significantly inhibited migratory activity of both Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cell lines (P < 0.001; Fig. 7F), whereas it did not affect the monolayer growth rate of these cells (Supplementary Fig. S9). In addition, knockdown of β-catenin in the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cell lines led to a mesenchymal-to-epithelial morphologic switch (Fig. 7G), with downregulation of EMT-activating transcription factors including Twist1, but with unchanged, almost undetectable level of Cdh1 expression (Fig. 7H and Supplementary Fig. S10). These results collectively suggest that the loss of E-cadherin and Smad4 expression cooperate to promote lung metastasis partly through the β-catenin activation.

Discussion

Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice described in this study are unique in several aspects. In contrast with previously reported spontaneous murine gastric tumors, which are of parietal cell or neuroendocrine lineage (10, 28–30), gastric adenocarcinomas formed in these mice are of the mucus-secreting gastric epithelial cell origin. In addition, tumor-suppressor genes most commonly inactivated in human gastric adenocarcinomas were targeted to be knocked out in the gastroduodenal epithelium of this model (2–5). While Smad4 is inactivated in 40% of human gastric cancers (4), functional roles of Smad4 in gastric cancer progression have not been fully evaluated using in vivo models. This study provides functional evidence for the role of Smad4 in suppressing gastric cancer progression. Although this study focused on gastric adenocarcinomas, it also demonstrates a role of Smad4 in suppressing duodenal carcinomas. Although Pdx-1-Cre;Trp53F/F;Cdh1F/F+ mice developed no duodenal carcinomas, the additional loss of Smad4 significantly promoted duodenal carcinomas (Supplementary Table S1), confirming that Smad4 loss in the intestinal epithelium promotes carcinogenesis (31). Possibly, had Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice not died of duodenal obstruction, they might have developed gastric cancers at later time points.

The specific role of E-cadherin loss in the development of diffuse-type gastric adenocarcinomas was clearly documented by this cross-tissue tumorigenesis study. These results validate and extend a prior study suggesting a role for E-cadherin in the development of gastric cancer (10, 32). In contrast with breast cancers arising in humans with germline E-cadherin mutation, mammary adenocarcinomas arising in MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice retained E-cadherin expression, and were not more aggressive than tumors arising in MMTV-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice. Thus, the selection pressure for E-cadherin loss may be relatively low in the context of the mouse mammary gland, compared with the stomach. This is consistent with the lower lifetime risk for breast cancer than for stomach cancer in germline E-cadherin mutation carriers (33). Studies are ongoing in our laboratory to identify gastric tissue-specific epigenetic events or signaling pathway activation leading to E-cadherin loss that may be promoted by loss of Smad4 or p53.

Notably, 14.3% of gastric adenocarcinomas arising in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice metastasized to the lung, which is a highly unique finding for a genetically engineered mouse model for gastric cancers. To date, only a few genetically engineered mouse models of gastric cancer have been reported, and none of them develop distant metastases (10, 28–30, 34). After Helicobacter inoculation, insulin-gastrin (INS-GAS) transgenic mice develop invasive gastric carcinomas with a frequency of 75% (28), but without distant metastases. Thirty percent of mice deficient in p52 trefoil factor develop multifocal intraepithelial or intramuscular carcinomas, but no distant metastases (29). Gp130F/F mice lacking in the SHP2-binding site on the IL6 family receptor gp130 develop gastric cancer with submucosal invasion only (30). The K19-C2mE mice expressing COX-2 and microsomal prostaglandin E synthase-1 in the stomach develop dysplastic tumors in the gastric epithelium (34). Atp4b-Cre;Cdh1F/F;Trp53F/F+ mice develop diffuse-type gastric cancers that frequently metastasize to lymph nodes but not to the distant visceral organs (10). Thus, gastric cancer metastasis may require a concerted action of key molecular events, including losses of E-cadherin and Smad4, as demonstrated in this study.

The metastatic phenotype of our mutant mice provides a unique opportunity to dissect the roles of E-cadherin and Smad4 in gastric cancer progression, and in relation to EMT. Without exposure to carcinogens, the gastric epithelium of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice lost E-cadherin expression and developed adenocarcinomas that progressed through EMT, suggesting the importance of EMT in gastric cancer progression. Nuclear β-catenin accumulation, suggesting activation of the canonical Wnt/β-catenin pathway, was accompanied by EMT phenotype such as vimentin overexpression (24). Given that this phenotype was not observed in gastrointestinal tumors of our mutant mice that retained E-cadherin, the unique metastatic phenotype of Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F+ mice may be attributable in part to the activation of the β-catenin signaling pathway following E-cadherin loss (35). Our study also provides functional evidences for the causal role of β-catenin for the migration process related to metastasis of gastric cancer, consistent with data in the literature suggesting that β-catenin promotes the metastatic progression of breast and
colorectal cancers by transcriptional activation of vimentin, MMP7, fibronectin, and other prometastatic genes (35–38). The antimetastatic role of Smad4 in gastric cancer, which has not been previously demonstrated in vivo, is in line with previous data for colorectal and prostate cancers (39, 40). Notably, nuclear β-catenin accumulation at the invasive front was more prominent in our Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F mice than in Pdx-1-Cre;Trp53F/F;Cdh1F/F, which is partly consistent with a report by Shimada and colleagues that Atp6v0a-Cre;Cdh1F/F;Trp53F/F mice do not overexpress β-catenin (10). In addition, our Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cells demonstrated enhanced β-catenin activity and migratory activity compared with Pdx-1-Cre;Trp53F/F;Cdh1F/F cells. Thus, our study is the first to demonstrate the Smad4 loss-induced β-catenin activation in gastric cancer, and is consistent with a prior report that Smad4 mediates canonical BMP signaling by repressing the transcription of β-catenin in SW480 colon cancer cells (41). Therefore, inhibition of β-catenin may be a converging node for the antimetastatic signaling pathways driven by E-cadherin and Smad4 in Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F cells. Other possible mechanisms for the antimetastatic role of Smad4 may include suppression of proliferation, leading to proliferative dormancy (40).

Taken together, we conclude that loss of E-cadherin and Smad4 cooperate to promote the development and metastatic progression of p53-null diffuse-type gastric carcinoma. By performing gastro-duodenal epithelium-specific knock-out of one allele of Cdh1 and both alleles of p53 and Smad4, which are frequently inactivated in human gastric cancers, we have created a genetically engineered mouse model that develops distant metastasis. Gastric adenocarcinomas that formed in this genetic context, but not intestinal or mammary adenocarcinomas, lose E-cadherin expression and undergo EMT. These results closely recapitulate human diffuse-type gastric cancers, and sharpen our understanding of the interaction between E-cadherin and Smad4, two commonly inactivated tumor suppressors in gastric cancer. In addition, the Pdx-1-Cre;Smad4F/F;Trp53F/F;Cdh1F/F animal model will be extremely useful for preclinical studies, given its similarities with human diffuse-type gastric carcinoma and its metastatic propensity.

**Disclosure of Potential Conflicts of Interest**
No potential conflicts of interest were disclosed.

**Disclaimer**
The study sponsor has no roles in the study design in the collection, analysis, and interpretation of data.

**Authors’ Contributions**
Conception and design: J.W. Park, C. Deng, D.Y. Kim, J.E. Green, H.K. Kim
Development of methodology: J.W. Park, S.H. Jang, D.M. Park, C. Deng, J.E. Green, H.K. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.W. Park, D.M. Park, N.J. Lim, C. Deng, J.E. Green, H.K. Kim
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.W. Park, S.H. Jang, D.M. Park, N.J. Lim, C. Deng, D.Y. Kim, J.E. Green, H.K. Kim
Writing, review, and/or revision of the manuscript: J.W. Park, C. Deng, D.Y. Kim, J.E. Green, H.K. Kim
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.W. Park, C. Deng, J.E. Green, H.K. Kim
Study supervision: C. Deng, D.Y. Kim, J.E. Green, H.K. Kim
Other: Interpreting pathology, J.W. Park

**Grant Support**
This work was supported by the Proteogenomic Research Program through the National Research Foundation of Korea and the Converging Research Center Program (2013K0000429) funded by the Korean Ministry of Education, Science and Technology: by National Cancer Center Grant 1210051; and by the NIH intramural program, Center for Cancer Research, National Cancer Institute, Bethesda, MD.

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.

Received April 8, 2014; accepted April 10, 2014; published OnlineFirst April 30, 2014.

**References**
Cooperativity of E-cadherin and Smad4 Loss to Promote Diffuse-Type Gastric Adenocarcinoma and Metastasis

Jun Won Park, Seok Hoon Jang, Dong Min Park, et al.

Mol Cancer Res Published OnlineFirst April 30, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-14-0192-T

Supplementary Material
Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2014/04/30/1541-7786.MCR-14-0192-T.DC1

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.