Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

Research Article

Hepatocyte Growth Factor Activator Inhibitor Type 1 Regulates Epithelial to Mesenchymal Transition through Membrane-Bound Serine Proteinases

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Abstract

Hepatocyte growth factor activator inhibitor-1 (HAI-1), encoded by the *serine protease inhibitor Kunitz type 1* (*SPINT1*) gene, is a membrane-associated proteinase inhibitor that potently inhibits a variety of serine proteinases, including those that are membrane bound. Although HAI-1/SPINT1 is widely expressed by epithelial cells and cancer cells, its functional role is still unclear, particularly in cancer. Here, we show that stable knockdown of HAI-1/SPINT1 in the human pancreatic cancer cell line SUIT-2 induces an elongated spindle-like morphology associated with accelerated invasion, thereby mimicking an epithelial to mesenchymal transition (EMT). We found that HAI-1/SPINT1 knockdown significantly reduced the expression of E-cadherin and was accompanied by up-regulation of Smad-interacting protein 1 (SIP1), an E-cadherin transcriptional repressor. In addition, matrix metalloproteinase-9 (MMP-9) was up-regulated. Similar results were obtained in the HLC-1 lung carcinoma cell line. Moreover, a metastatic variant of SUIT-2 (S2-CP8) that showed loss of E-cadherin expression also showed a significantly reduced level of HAI-1/SPINT1. Engineered overexpression of HAI-1/SPINT1 in S2-CP8 resulted in reversion of E-cadherin expression and SIP1 down-regulation, which accompanied reestablishment of epithelial morphology in culture. The EMT caused by HAI-1/SPINT1 knockdown seemed to be mediated, at least partly, by membrane-bound serine proteinases, matriptase/ST14 and TMPRSS4, as knockdown of matriptase/ST14 or TMPRSS4 in HAI-1/SPINT1 knockdown SUIT-2 cells and HLC-1 cells resulted in reversion of SIP1 and/or MMP-9 expression levels. We suggest that interactions between HAI-1/SPINT1 and membrane-bound serine proteinases contribute to transcriptional and functional changes involved in EMT in certain carcinoma cells. [Cancer Res 2009;69(5):1828–35]

Introduction

Hepatocyte growth factor activator inhibitor-1 (HAI-1) is encoded by the *serine protease inhibitor Kunitz type 1* (*SPINT1*) gene. It is a membrane-associated Kunitz-type serine proteinase inhibitor expressed on the basolateral surface of various epithelial tissues, such as the gastrointestinal tract, breast, prostate, lung, and skin (1). It is also strongly expressed by placental cytotrophoblasts (2). HAI-1/SPINT1 is composed of an extracellular domain containing an NH_2-terminal Kunitz domain (KD1), a low-density lipoprotein receptor (LDLR)–like domain and a COOH-terminal Kunitz domain (KD2), followed by a transmembrane region and a short cytoplasmic domain (3, 4). To date, only a few examples of membrane-associated serine proteinase inhibitors have been reported (4, 5), and HAI-1/SPINT1 seems to have *in vivo* roles that are both unique and important (6). Previous studies showed that HAI-1/SPINT1 potently inhibits the action of a variety of trypsin-like serine proteinases that may be involved in carcinogenesis, invasion, and metastasis. These proteinases include hepatocyte growth factor activator (HGFA), matriptase/ST14, hepsin/TMPRSS1, and prostasin/PRSS8 (4, 7–10). Among them, matriptase/ST14 and hepsin/TMPRSS1 belong to the type II transmembrane serine protease superfamily, whereas prostasin/PRSS8 is a glycosyl-phosphatidylinositol–anchored protein (9, 11).

These membrane-bound proteases are likely to have important roles in cellular homeostasis and their dysregulated activities and expression have been implicated in tumor development and progression. To date, >20 membrane-bound serine proteinases have been reported (11). Based on the unique localization of HAI-1/SPINT1 on the cell surface, we have hypothesized that HAI-1/SPINT1 regulates multiple membrane-anchored serine proteinases and may have diverse functions in epithelial cells depending on the type of membrane-anchored serine proteinase coexpressed by the cell. To date, several studies have suggested a possible role of HAI-1/SPINT1 in the invasion of carcinoma cells. In breast cancer, inactivation of HAI-1/SPINT1 and its homologous protein HAI-2 significantly increased hepatocyte growth factor (HGF)–mediated breast cancer cell migration and invasion (12). Prostate cancer cells, after loss of HAI-1/SPINT1, showed an increased invasiveness and cellular motility *in vitro* (13). Engineered overexpression of HAI-1 in glioblastoma cells reduced invasiveness *in vitro* (14). However, the precise mechanisms regulating cellular invasiveness by HAI-1/SPINT1 in tumor cells have not yet been elucidated.

In carcinomas, a shift of their epithelial features toward a mesenchymal phenotype may occur during progression. This process, referred to as the epithelial to mesenchymal transition (EMT), provides mechanisms for epithelial cells to overcome the physical constraints imposed on them by intercellular junctions. Therefore, EMT enhances the motility of carcinoma cells that is often considered to be a prerequisite for tumor invasion (15, 16). E-cadherin is the best characterized molecular marker expressed by epithelial cells, and it is localized at the adherens junctions of the basolateral surface. Loss of E-cadherin expression and/or function is a well-recognized marker of EMT and promotes cell invasiveness (15, 16). In addition to the down-regulation of epithelial proteins, EMT up-regulates the expression of mesenchymal

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-3728

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

proteins such as vimentin and smooth muscle actin, which are associated with the invasive cellular phenotype, pseudopod formation, and cytoskeletal remodeling (15–17). In the process of EMT, E-cadherin can be silenced by different mechanisms, such as promoter hypermethylation or transcriptional repression (16). The primary transcriptional repressors of E-cadherin are zinc finger transcription factors, including Snail (Snai1), Slug (Snai2), smad-interacting protein 1 (SIP1, also known as ZEB2), ZEB1, and a basic helix loop helix transcription factor, Twist, which is also implicated in this process (16). The activation of these E-cadherin repressors may be induced by signaling mediated by growth factors/cytokines, such as HGF, insulin-like growth factor, epidermal growth factor, fibroblast growth factor, and transforming growth factor β (TGF-β) via their specific receptors (15–17). Pericellular activity of matrix metalloproteinase (MMP) is also implicated in the EMT phenomenon (18, 19). In addition to MMP, the membrane-bound serine proteinase TMPRSS4 was recently reported to initiate EMT processes (20).

In this study, we examined the functional role of HAI-1/SPINT1 in the human pancreatic cancer cell line SUIT-2, its metastatic sublines, and the HLC-1 lung cancer cell line. We found that knockdown of HAI-1/SPINT1 promoted EMT via reduced E-cadherin expression. This function of HAI-1/SPINT1 to regulate E-cadherin expression may be mediated by interaction between HAI-1/SPINT1 and membrane-bound serine proteinases.

Materials and Methods

Cell lines. The human pancreatic adenocarcinoma cell line SUIT-2 (21) and its metastatic sublines S2-VP10 and S2-CP8 (22) were kindly provided by Dr. Takeshi Iwamura (Junwakai Memorial Hospital, Miyazaki, Japan). The human lung adenocarcinoma cell line HLC-1 and the colon adenocarcinoma line WiDr were also used (7). All cells except for HLC-1 were cultured in DMEM containing 10% fetal bovine serum (FBS). HLC-1 was cultured in Ham's F12, 10% FBS. S2-CP8 and S2-VP10 were established by Cutis-Pulmonary metastasis-culture (8 times) and Vein-Pulmonary metastasis-culture (10 times), via injection of parental SUIT-2 cells i.v. and s.c. into nude mice, respectively (22).

Reverse transcription-PCR and real-time reverse transcription-PCR. Total RNAs were extracted with the Trizol reagent (Life Technologies), followed by DNase I (Roche Applied Science) treatment and phenol-chloroform-isoamylalcohol extraction. For reverse transcription-PCR (RT-PCR), 3 μg of total RNA were reverse transcribed with a mixture of oligo(dT) and random primer and processed for each PCR reaction as described previously (23). The PCR products were analyzed by 1.5% agarose gel electrophoresis. For quantitative real-time RT-PCR using cyber green, PCR was performed in a LightCycler (Roche Applied Science). For internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin mRNAs were also measured in RT-PCR and real-time RT-PCR, respectively. The following primers were used for both conventional and real-time RT-PCR: HAI-1/SPINT1 forward, 5′-CAGCAGTGCCTCGAGTCTTGTC and reverse, 5′-GATGGCTACCACCACCACAATG (144 bp); E-cadherin forward, 5′-ACACCATCCTCAGCCAAGATCC and reverse, 5′-GTGGTGGGATTGAAGATCGGAG (232 bp); matriptase/ST14 forward, 5′-CTACATCGATGACAGAGGATTC and reverse, 5′-GTCGAAGGTGAAGTCATTGAAG (157 bp); TMPRSS4 forward, 5′-ACCGATGTGTTCAACTGGAAG and reverse, 5′-CATCCAATGATCCAGAGTGG (237 bp); MMP-9 forward, 5′-CACAACATCACCTATTGGATCC and reverse, 5′-TGGGTGTAGAGTCTCTCGCT (482 bp); SIP1 forward, 5′-GGGAGAATTGCTTGATGGAGC and reverse, 5′-TCTCGCCCGAGTGAAGCCTT (272 bp); Snail forward, 5′-CACTATGCCGCGCTCTTTCC and reverse, 5′-GTCGTAGGGCTGCTGGAAG (112 bp); Slug forward, 5′-GCCTCCAAA AAGCCAAACTACAG and reverse, 5′-ACAGTGATGGGGCTGTATGC (147 bp); Twist forward, 5′-ACAAGCTGAGCAAGATTCAGACC and reverse, 5′-TCCAGACCGAGAAGGCGTAG (150 bp); vimentin forward, 5′-AGGAAATGGCTCGTCACCTTCGTGAATA and reverse, 5′-AGGAGTTCGGTT GTTAAGAACTAGAGC (440 bp); GAPDH forward, 5′-GTGAAGGTCGGAGTCAACG and reverse, 5′-GGTGAAGACGCCAGTGGACTC (300 bp); β-actin forward, 5′-ATTGCCGACAGGATGCAG A and reverse, 5′-GAGTACTTGCGCTCAGGAGGA (89 bp).

Knockdown of HAI-1/SPINT1. The knockdown vector was constructed by using a short hairpin RNA (shRNA) expression retroviral vector pSINsi-hU6 (TAKARA Bio) as described previously (23). The target sequence of *HAI-1/SPINT1* gene was 5′-GGGCAGGCATAGACTTGAAGG and scramble is 5′-GGGACGGAGATTTCACGAGGA. For infection of retroviral vectors, Amphopack-293 packaging cells cultured in six-well plates were incubated with 1 μg of recombinant retroviral vector and 3 μL of TransFectin (Bio-Rad) for 12 h. After 48 h of cultivation, the supernatant containing the retroviral particles was collected, filtered, and used to infect target cells. The infected cells were subcultured at an appropriate density in fresh DMEM containing 0.5 mg/mL G418 (Nacalai Tesque), and the G418-resistant cell pools were established.

HAI-1/SPINT1 expression vector. Construction of human HAI-1/SPINT1 expression plasmid vector (pCI-HAI-1) was reported previously (7). The plasmid was linealized by *Xmn*I and transfected into the cultured S2-CP8 cells using Lipofectamine 2000 reagent (Life Technologies). After transfection, the cells were cultured in the presence of G418. The G418-resistant colonies were selected and screened for the expression of HAI-1/SPINT1. Mock-transfected clones were used as controls.

Small interfering RNA. To knock down the *matriptase/ST14* and *TMPRSS4* genes in HAI-1 knockdown SUIT-2 cells, small interfering RNAs (siRNA) were used. The sequence of matriptase/ST14 siRNA (Qiagen) was 5′-CGUCGUCACUUGUACACCAdTdT-3′ and TMPRSS4 Stealth RNAi (Invitrogen) was 5′-CAGCCUAUCUCAACUGGAUCUACAA-3′. Transfection was performed using Lipofectamine 2000 reagent. Forty-eight hours after the transfection, SUIT-2 HAI-1 knockdown cells were harvested and total mRNAs were extracted to examine the expression levels of matriptase/ST14, TMPRSS4, and EMT-related transcripts. Scrambled siRNAs were transfected as controls.

***In vitro* motility and invasion assays.** For the evaluation of *in vitro* motility of SUIT-2, a monolayer wounding (scratch) assay was performed. Cells were allowed to form a monolayer on a culture dish, and a wound was made by scratching the monolayer with a pipette tip. After the scratched cells were removed, the cells were cultivated for 12 h. The cellular motility was also estimated by using Chemotaxicells (polycarbonate filter, pore size 8 μm; Kurabo) coated with type IV collagen (3.6 μg per filter). Cells (1×10^5) in 100 μL of DMEM, 0.1% bovine serum albumin (BSA) were placed in the upper compartment and incubated for 24 h. After incubation, the cells on the upper surface of the filter were wiped off with a cotton swab. The cells on the lower surface were stained with hematoxylin and counted in 10 randomly selected fields (200-fold original magnification). For the evaluation of invasive capability, Matrigel invasion assay was performed using Chemotaxicells coated with 25 μg per filter of Matrigel (Life Technologies). Cells (1×10^5) in DMEM, 0.1% BSA were placed in the upper compartment and incubated for 72 h. As a chemoattractant, 1% FBS was added into the lower compartment. Invaded cells were counted as described above.

Immunoblot analysis, immunohistochemistry, and immunofluorescence. To detect HAI-1/SPINT1 and E-cadherin proteins, cellular proteins were extracted at 80% confluency and fractionated into membrane and nuclear fractions using the ProteoExtract kit (Merck). Then, equal amounts of proteins were electrophoresed by standard SDS-PAGE under reducing conditions, transferred onto Immobilon membrane (Millipore), and the target proteins were detected by immunoblot as described previously (7, 23). Immunohistochemistry of formalin-fixed, paraffin-embedded tissue specimens was performed as described previously (1). For immunocytochemistry, cultured cells on glass slides were fixed with 4% paraformaldehyde in PBS for 30 min. After washing with PBS, the cells were incubated in 0.5% Triton X-100 in PBS for 10 min, washed with PBS, and incubated in blocking solution (1% BSA, 10% goat serum in PBS) for 30 min. Afterward, cells were incubated for 1 h with primary antibody. After washing with PBS, cells were incubated for 30 min with Alexa Fluor 555–conjugated goat

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

anti-mouse IgG (Invitrogen) at a dilution of 1:200 in PBS. Then, the cells were washed with PBS, counterstained with 4,′6-diamino-2-phenylindole (Sigma), and investigated with laser scanning confocal microscopy (TCS-SP2, Leica Microsystems). The primary antibodies used were as follows: anti-human HAI-1/SPINT1 mouse monoclonal antibody (1N7; ref. 1), anti-human E-cadherin monoclonal antibody (TAKARA Bio), and anti–β-actin monoclonal antibody (AC-74, Sigma).

Subcutaneous injection of tumor cells in nude mice. All of the animal work was carried out under protocols approved by University of Miyazaki Animal Research Committee, in accordance with international guiding principles for biomedical research involving animals. Cells (5×10^6/0.2 mL DMEM) were s.c. injected at the abdominal flank of 6-wk-old male nude mice (BALB/cAJc1-nu). The tumor volume was estimated by the formula $V = L \times W \times W/2$ [V, volume (mm^3); L, length (mm); W, width (mm)]. All mice were observed everyday and sacrificed at 5 wk postimplantation. Lungs were excised and fixed in 4% paraformaldehyde for 24 h. The numbers of metastatic lesion were counted in the largest cross-sectional specimens of both lungs of each mouse.

Statistics. Comparison between two unpaired groups was done with Mann-Whitney U test using Statview 5.0 program (Brainpower, Inc.). Significance was set at $P < 0.05$.

Results

The membrane-form of HAI-1/SPINT1 and E-cadherin show similar expression patterns in SUIT-2 cells *in vivo*. Initially, we examined the expression of HAI-1/SPINT1 in SUIT-2 cells using xenotransplanted tumor tissue from nude mice. As reported previously (21), SUIT-2 seemed to consist of heterogeneous subpopulations of tumor cells with different levels of differentiation, forming a mixture of differentiated tubular adenocarcinoma and poorly differentiated adenocarcinoma tissues when injected s.c., and the differentiated portion was predominant (Fig. 1*A*). They were also heterogeneous in their expression of HAI-1/SPINT1. Around 60% to 80% of the tumor area was strongly positive for HAI-1/SPINT1, showing apparent membrane surface localization. However, the expression of the membrane form of HAI-1/SPINT1 was significantly attenuated in less differentiated cells (Fig. 1*A*). Interestingly, this portion also showed reduced expression of membrane-associated E-cadherin, suggesting partial EMT (Fig. 1*A*).

Knockdown of HAI-1/SPINT1 expression results in loss of epithelial morphology in SUIT-2 cells *in vitro*. Next, we asked whether decreased HAI-1/SPINT1 expression had a causal relationship with SUIT-2 cell EMT. Preliminary RT-PCR analyses revealed that cultured SUIT-2 cells expressed HAI-1/SPINT1 mRNA and its presumed target proteinases, matriptase/ST14 and prostasin/PRSS8, but not hepsin/TMPRSS1 (Supplementary Fig. S1*A*). SUIT-2 cells also expressed TMPRSS4, another membrane-associated serine proteinase with a presumptive role in progression of pancreatic cancer (24). HGFA was expressed at a

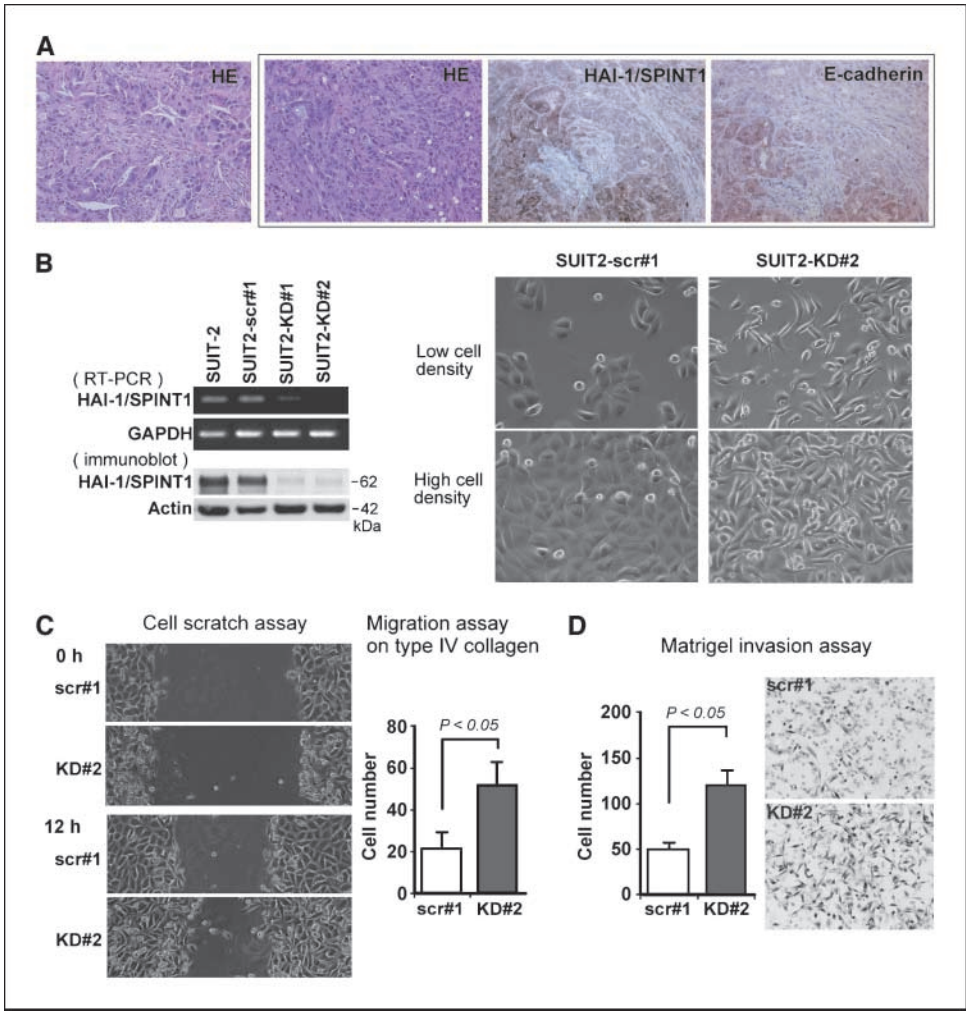

Figure 1. Reduced HAI-1/SPINT1 expression is accompanied by a less differentiated, spindle cell morphology in SUIT-2 cells. *A,* histology of xenografted SUIT-2 cells in nude mice. H&E (*HE*)–stained specimens of well to moderately differentiated portion (*left*) and moderately to poorly differentiated portion (*right*) are shown, and the serial sections of the latter portion were immunostained for HAI-1/SPINT1 and E-cadherin. HAI-1/SPINT1 and E-cadherin showed similar expression patterns in the tumor tissue (original magnification, ×200). *B,* stable knockdown of HAI-1/SPINT1 in SUIT-2 induces a spindle-like morphology. RT-PCR analysis and immunoblot analysis indicated that both HAI-1/SPINT1 mRNA and protein were significantly decreased in two independent SUIT-2 cell pools (HAI-1 *KD#1* and *KD#2*) infected with HAI-1 shRNA retroviral vector compared with parental and scrambled control SUIT-2 cells. Under phase-contrast microscopy, cultured control SUIT-2 showed cobblestone-like epithelial morphology (*SUIT2-scr#1*). Knockdown of HAI-1/SPINT1 induced spindle morphology, with a tendency for piling up (*SUIT2-KD#2*). *C,* cell scratch assay (*left*) and random migration assay on type IV collagen (*right graph*). The confluent monolayer was wounded by scratching with a pipette tip. At 0 and 12 h after scratching, photographs of the wound in the monolayer were taken. *Columns,* means of triplicate experiments; *bars,* SD. *D,* effects of HAI-1 knockdown on invasiveness of SUIT-2 cells through Matrigel. *Columns,* mean of triplicate experiments; *bars,* SD.

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

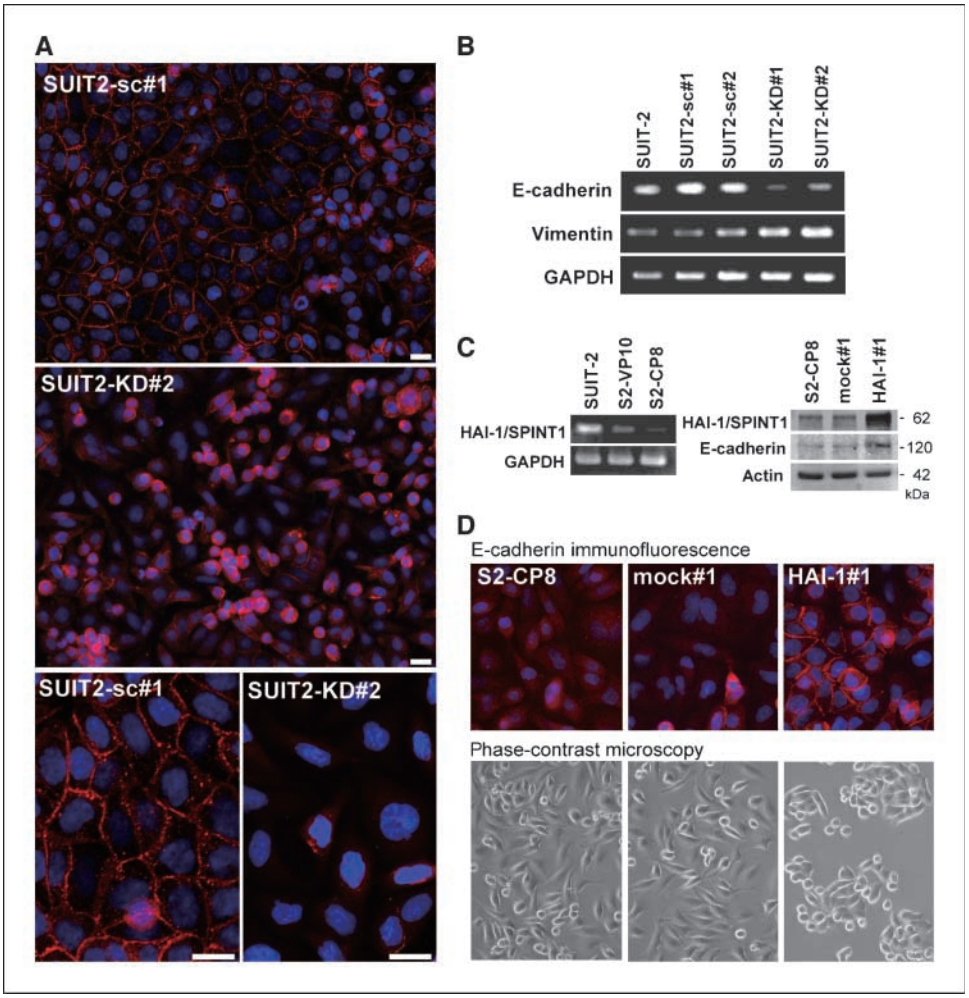

Figure 2. Effects of HAI-1/SPINT1 on E-cadherin expression. *A,* E-cadherin immunofluorescence (*red*) analysis of cultured scrambled control (*SUIT2-sc#1*) and HAI-1/SPINT1 knockdown (*SUIT2-KD#2*) cells. Nuclei were stained with 4′,6-diamidino-2-phenylindole (*blue*). Membrane-associated E-cadherin protein was significantly decreased in knockdown cells. *Bar,* 20 μm. *B,* RT-PCR analysis of E-cadherin and vimentin mRNA in SUIT-2, two scrambled control cell pools (*SUIT2-sc#1* and *SUIT2-sc#2*), and two HAI-1/SPINT1 knockdown cell pools (*SUIT2-KD#1* and *SUIT2-KD#2*). E-cadherin mRNA levels were decreased and vimentin mRNA levels were increased in knockdown cells. *C,* RT-PCR analysis of HAI-1/SPINT1 mRNA in SUIT-2 and two metastatic sublines of SUIT-2 (*S2-CP8* and *S2-VP10*), and immunoblot analyses of HAI-1/SPINT1 and E-cadherin in S2-CP8, mock-transfected control S2-CP8 (*mock#1*) and S2-CP8 overexpressing HAI-1/SPINT1 (*HAI-1#1*). The same amount of protein (whole extracts) was applied to each lane and β-actin was used as control for the extract quantity and quality. *D,* effects of HAI-1/SPINT1 overexpression on E-cadherin expression and culture morphology of S2-CP8 cells. Immunofluorescence analysis of E-cadherin and culture morphology of S2-CP8, mock-transfected control (*mock#1*), and HAI-1/SPINT1–transfected (*HAI-1#1*) cells. Engineered overexpression of HAI-1/SPINT1 up-regulated E-cadherin expression and induced epithelial culture morphology in S2-CP8 cells.

very low level. This cell line also expressed c-Met receptor tyrosine kinase (HGF receptor), but not HGF (Supplementary Fig. S1*A*).

By using a retroviral vector expressing HAI-1/SPINT1 shRNA, we successfully knocked down *HAI-1/SPINT1* gene expression. We observed a significant reduction in the level of HAI-1/SPINT1 mRNA in two independent HAI-1/SPINT1 knockdown cell pools (*SUIT2-KD#1* and *SUIT2-KD#2*) compared with parental cells and control cell pools treated with scrambled shRNA (*SUIT2-scr#1* and *SUIT2-scr#2*; Fig. 1*B*). HAI-1/SPINT1 protein levels were also reduced significantly (Fig. 1*B*). Scrambled shRNA cells did not alter their expression of HAI-1/SPINT1. Interestingly, knockdown of HAI-1/SPINT1 significantly altered culture morphology. Although parental SUIT-2 cells and control scrambled shRNA-expressing cells grew in monolayer cultures as epithelial clusters, HAI-1/SPINT1 knockdown cells showed loss of cell-cell contracts, spindle fibroblastic morphology, and cell scattering (Fig. 1*B*). Moreover, the HAI-1/SPINT1 knockdown resulted in increased migration in cell scratch assay and also in a migration assay on type IV collagen (Fig. 1*C*). Invasiveness as measured in Matrigel was also significantly enhanced *in vitro* ($P < 0.05$; Fig. 1*D*). On the other hand, *in vitro* cellular growth rate was modestly suppressed by HAI-1/SPINT1 knockdown (Supplementary Fig. S1*B*).

HAI-1/SPINT1 regulates E-cadherin expression of SUIT-2 and its metastatic variant. Together, the acquisition of a fibroblastoid morphology and the enhanced invasiveness of HAI-1/SPINT1 knockdown SUIT-2 cells suggested that these cells had undergone an EMT *in vitro*. To better understand this change, we assessed the expression of E-cadherin on the cell surface. Following shRNA-mediated loss of HAI-1/SPINT1, the cell surface E-cadherin proteins were markedly decreased (Fig. 2*A*). Moreover, the level of E-cadherin mRNA was also down-regulated, accompanying enhanced expression of vimentin mRNA (Fig. 2*B*), suggesting that the knockdown of HAI-1/SPINT1 somehow influenced intracellular signaling that regulated *E-cadherin* gene transcription.

Next, we examined the expression of HAI-1/SPINT1 and E-cadherin in S2-VP10 and S2-CP8, metastatic sublines of SUIT-2 (22). RT-PCR analysis showed that the expression level of HAI-1/SPINT1 was low in S2-VP10 compared with that of its parent (SUIT-2) and was almost undetectable in S2-CP8 (Fig. 2*C*). As the expression level of E-cadherin was correlated with that of HAI-1/SPINT1 in these cell lines and was significantly lower in S2-CP8, we then tested the effect of forced expression of HAI-1/SPINT1 on S2-CP8 to confirm the relationship between HAI-1/SPINT1 and E-cadherin. Overexpression of HAI-1/SPINT1 in S2-CP8 cells increased E-cadherin protein levels (Fig. 2*C*) and restored both membrane-associated E-cadherin and epithelial morphology *in vitro* (Fig. 2*D*). We concluded that HAI-1/SPINT1 regulated E-cadherin expression and EMT of the SUIT-2 cell line and its metastatic derivatives.

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

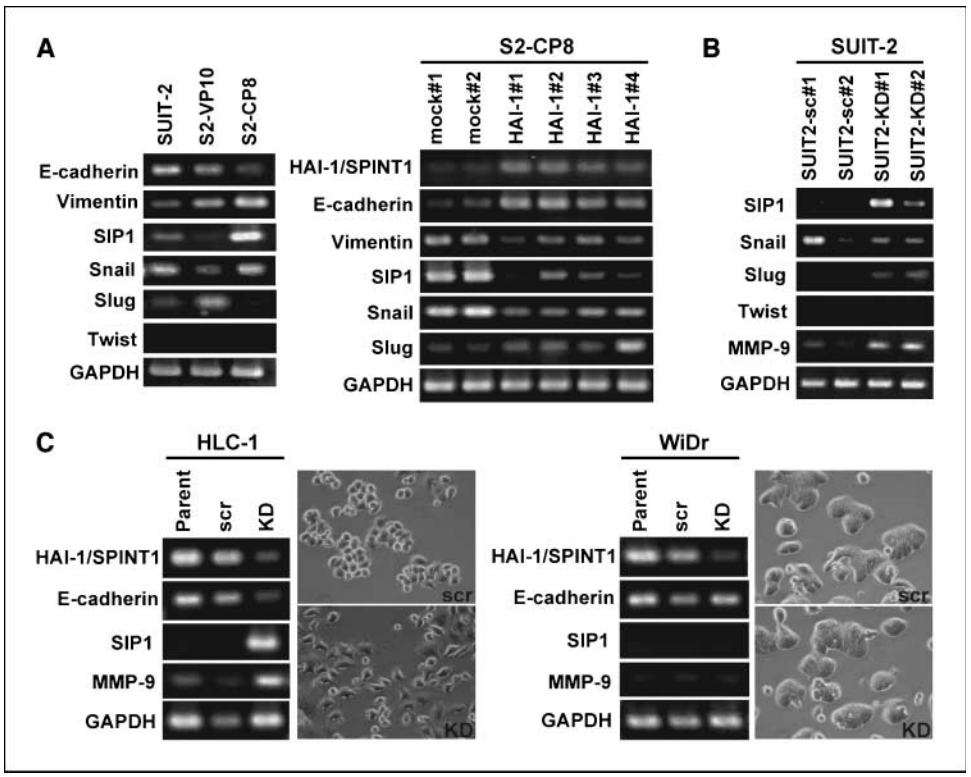

Figure 3. HAI-1/SPINT1 modulates E-cadherin transcriptional repressors and MMP-9. *A,* RT-PCR analysis of transcriptional repressors of E-cadherin in SUIT-2, S2-VP10, and S2-CP8, and effects of HAI-1/SPINT1 overexpression. Four independent overexpression clones (*HAI-1#1, HAI-1#2, HAI-1#3*, and *HAI-1#4*) and two mock-transfected control clones were examined. *B,* RT-PCR analysis of transcriptional repressors of E-cadherin in scrambled control cells (*SUIT2-sc#1* and *SUIT2-sc#2*) and HAI-1/SPINT1 knockdown SUIT-2 cells (*SUIT2-KD#1* and *SUIT2-KD#2*). *C,* RT-PCR analysis of HAI-1– and EMT-related transcripts in the HLC-1 human lung carcinoma cell line and the WiDr human colon carcinoma cell line and effects of HAI-1/SPINT1 knockdown. Nontreated cells (*parent*), scrambled shRNA-treated cells (*scr*), and HAI-1/SPINT1 shRNA–treated cells were examined. Culture morphologies of the cells are also shown. Knockdown of HAI-1/SPINT1 resulted in EMT in HLC-1 cells but not in WiDr cells.

HAI-1/SPINT1 modulates the expression level of E-cadherin repressor SIP1. HAI-1/SPINT1 influenced the levels of both E-cadherin protein at the cell surface and E-cadherin mRNA. Therefore, we addressed whether E-cadherin repressor was involved in HAI-1/SPINT1–mediated E-cadherin expression. Initially, we examined the expression of E-cadherin repressors SIP1, Snail, Slug, and Twist in SUIT-2, S2-VP10, and S2-CP8 cells and found that SIP1 was significantly up-regulated in S2-CP8 (Fig. 3*A*). The expression level of Snail was not detectably altered in metastatic variants compared with parental SUIT-2 cells. On the other hand, S2-VP10 showed modestly increased expression of Slug and did not show SIP1 up-regulation. Twist was not expressed in these cells. We then examined the effects of overexpression and knockdown of HAI-1/Spint1 on the expression of these E-cadherin repressors in S2-CP8 and SUIT-2 cells, respectively. Notably, HAI-1/SPINT1 overexpression induced significant down-regulation of SIP1 in S2-CP8 (Fig. 3*A*), and, consequently, knockdown of HAI-1/SPINT1 resulted in significant up-regulation of SIP1 expression in SUIT-2 (Fig. 3*B*). Snail was also down-regulated in HAI-1/SPINT1-expressing S2-CP8 cells. The modulation of Slug expression by HAI-1/SPINT1 showed conflicting results between overexpression and knockdown studies (Fig. 3*A* and *B*). Twist was not induced by the knockdown of HAI-1/SPINT1. In addition, we found that MMP-9 mRNA was apparently increased in HAI-1/SPINT1 knockdown SUIT-2 cells compared with scrambled control (Fig. 3*B*), although MMP-9 was not up-regulated in the metastatic sublines (data not shown).

We next assessed whether HAI-1/SPINT1–mediated alteration of E-cadherin expression was SUIT-2 specific. For this purpose, we transfected an HAI-1/SPINT1 shRNA retroviral vector into HLC-1 and WiDr cell lines, both of which expressed high levels of HAI-1/SPINT1 (7). As shown in Fig. 3*C*, knockdown of HAI-1/SPINT1 resulted in a significant reduction of E-cadherin expression with morphologic changes, suggesting EMT in HLC-1 cells but not in WiDr cells. Similar to SUIT-2 cells, both SIP1 and MMP-9 were up-regulated in response to HAI-1/SPINT1 knockdown in HLC-1 cells. On the other hand, neither SIP1 expression nor MMP-9 expression was induced in WiDr cells by the knockdown of HAI-1/SPINT1. Therefore, HAI-1/SPINT1 regulates E-cadherin expression and EMT in some but not all carcinoma cells.

HAI-1/SPINT1 regulates EMT via interactions with serine proteinases. We proposed that the EMT-inducing effects of HAI-1/SPINT1 knockdown were attributable to the resulting excess of proteinase activity. To test this hypothesis, we examined the effects of serine proteinase inhibitors on E-cadherin expression in HAI-1/SPINT1 knockdown SUIT-2 cells. As shown in Fig. 4*A*, serine proteinase inhibitors, aprotinin and nafamostat mesylate, partially restored the expression of E-cadherin in HAI-1/SPINT1 knockdown SUIT-2 cells. These results suggested that the deregulated activity of serine proteinase in the absence of HAI-1/SPINT1 may be responsible for the observed EMT phenomenon. On the other hand, the metalloproteinase inhibitor GM6001 did not show such an effect (Fig. 4*A*). Because HAI-1/SPINT1 is localized on the plasma membrane and the expression level of HGF activator was very low in SUIT-2 cells, it is reasonable to postulate that membrane-bound serine proteinase may be responsible for the HAI-1/SPINT1 knockdown-induced EMT. To clarify this possibility, we assessed whether silencing of *matriptase/ST14* or *TMPRSS4* could reverse EMT. Matriptase/ST14 is a cognate proteinase of HAI-1/SPINT1 in various epithelial tissues (10) and TMPRSS4 was initially identified as a protein overexpressed in pancreatic carcinoma cells, including SUIT-2 (24), and is involved in EMT by the colon cancer cell line SW480 (20). Interestingly, knockdown of TMPRSS4 reduced the SIP1 level in HAI-1/SPINT1 knockdown SUIT-2 cells, accompanying recovery of E-cadherin expression (Fig. 4*B*). On the other hand, matriptase/ST14 knockdown did not

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

down-regulate SIP1, but down-regulated MMP-9 expression with modest reversion of E-cadherin expression (Fig. 4*B*). Partial reversion of the epithelial morphology was also observed in HAI-1/SPINT1 and TMPRSS4 double knockdown SUTI-2 cells (Fig. 4*C*). Similar experiments were also performed in HLC-1 cells. In contrast to SUIT-2, silencing of the *matriptase/ST14* gene resulted in down-regulation of both SIP1 and MMP-9, with partial recovery of epithelial morphology in HAI-1/SPINT1 knockdown HLC-1 cells (Fig. 4*D*). TMPRSS4 was also expressed in HAI-1/SPINT1 knockdown HLC-1 cells, and knockdown of TMPRSS4 resulted in similar but less evident effects on HLC-1 cells compared with SUIT2-KD#2 cells (Fig. 4*D*).

Effect of HAI-1/SPINT1 on metastasis *in vivo*. Finally, we examined the effect of HAI-1/SPINT1 on the metastatic capabilities of SUIT-2 and S2-CP8 cells *in vivo* using a nude mouse model. After s.c. inoculation of HAI-1/SPINT1 knockdown SUIT-2 or control (scrambled shRNA) SUIT-2 cells, we found that tumor growth rate of the knockdown cells was lower than that of control SUIT-2 cells (Supplementary Fig. S2), showing a similar tendency to that observed in the *in vitro* culture study. Five weeks after xenotransplantation, the mice were sacrificed and the number of pulmonary metastases was counted. The subcutaneous tumor of HAI-1/SPINT1 knockdown SUIT-2 tended to show less differentiated histology compared with scrambled control (Supplementary Fig. S3), confirming the *in vitro* findings. Contrary to our expectation, the incidence of pulmonary metastasis was not increased in HAI-1/SPINT1 knockdown cells (3 of 10) compared with control cells (2 of 10). However, the number of metastatic lesions in each metastatic case was increased in mice receiving HAI-1/SPINT1 knockdown cells (Supplementary Table S1), although the primary tumors were smaller than those of control cells (mean tumor volume ± SD at autopsy: 1,522 ± 574 mm^3 and

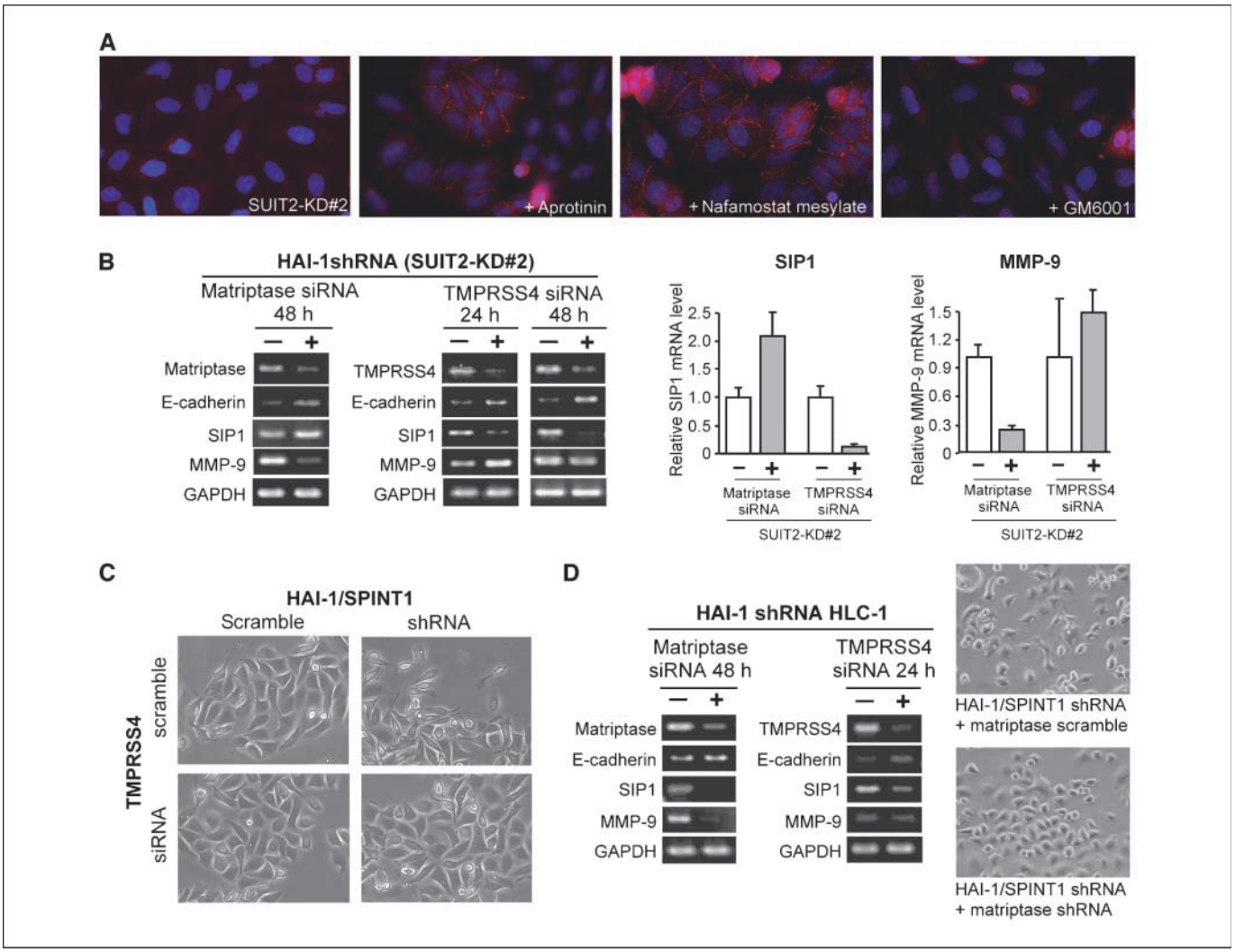

Figure 4. Possible involvement of serine proteinase activity in HAI-1/SPINT1 knockdown–induced EMT. *A,* HAI-1/SPINT1 knockdown cells (*SUIT2-KD#2*) were treated with serine protease inhibitors (200 μg/mL of aprotinin or 50 μmol/L of nafamostate mesylate) or MMP inhibitor (GM6001, 25 μmol/L) in DMEM, 2% FBS for 72 h. The expression of E-cadherin was examined by immunofluorescence staining using anti–E-cadherin antibody. Serine proteinase inhibitors, but not MMP inhibitor, restored E-cadherin expression. *B,* HAI-1 knockdown SUIT-2 cells (*SUIT2-KD#2*) were transiently transfected with siRNA to knockdown serine proteinase matriptase/ST14 or TMPRSS4. Control cells were treated with scrambled siRNA. Twenty-four and/or 48 h later, the expression levels of EMT-related transcripts (E-cadherin, SIP1, Snail, and MMP9) were analyzed by RT-PCR (*left*). The real-time RT-PCR data at 48 h ($n = 3$) are also shown (*bottom graph*; *columns,* mean; *bars,* SD). *C,* culture morphologies at 48 h posttransfection of TMPRSS4 siRNA, showing partial reversion of epithelial morphology of SUIT2-KD#2 cells. *D,* effect of *TMPRSS4* gene silencing (24 h after siRNA transfection) and *matriptase/ST14* gene silencing (48 h after siRNA transfection) on the expression of EMT-related molecules. Scrambled sequences were used as controls. The morphology of HAI-1/SPINT1 knockdown HLC-1 cells (48 h treatment) is also shown.

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

$2,565 \pm 1086$ mm^3 for HAI-1/SPINT1 knockdown cells and control cells, respectively, $P < 0.05$). The data suggested a trend in which the sizes of metastatic lesions in the knockdown groups were smaller than those in the control groups (Supplementary Fig. S3; Supplementary Table S1). Only 19% of the pulmonary metastases were composed of >10 tumor cells in nude mice transplanted with HAI-1/SPINT1 knockdown SUIT-2, whereas 67% of the lesions were composed of >10 tumor cells in control SUIT-2–transplanted mice. S2-CP8 cells showed increased incidence of lung metastasis compared with SUIT-2 cells. Forced expression of HAI-1/SPINT1 in S2-CP8 did not alter the incidence of lung metastasis. However, the number of metastatic lesions in lungs of each metastatic case was decreased by the expression of HAI-1/SPINT1 (Supplementary Table S1).

Discussion

Many molecular mechanisms that contribute to EMT have been subjected to analysis (15–17, 19). These mechanisms usually involve growth factors/cytokines, their cognate receptors, and downstream signaling molecules. The resultant signaling cascade induces E-cadherin transcription repressors such as Snail, Slug, SIP1, and Twist, eventually resulting in a loss of epithelial morphology. Proteinase activities, particularly MMPs, have been implicated in EMT by activating growth factors and their receptors and cleaving cell-cell and cell–extracellular matrix (ECM) adhesion molecules (18, 19). Recently, in addition to MMPs, the membrane-bound serine proteinase TMPRSS4 was reported to initiate EMT (20). In this study, we show for the first time that the membrane-associated serine proteinase inhibitor HAI-1/SPINT1 has a significant regulatory role in EMT of certain carcinoma cells. In this capacity, HAI-1/SPINT1 seems to interact with membrane-bound serine proteinases.

In EMT, cells switch from the polarized, epithelial phenotype to a highly motile mesenchymal phenotype. Data implicate EMT in the progression of primary tumors toward metastasis. Our study showed that HAI-1/SPINT1 knockdown up-regulated SIP1 and MMP-9 mRNA levels, both of which have been implicated in EMT (17, 19). We also showed that HAI-1/SPINT1 knockdown down-regulated E-cadherin expression, which led to loss of epithelial morphology and enhanced invasiveness *in vitro*. E-cadherin expression by SUIT-2 and HLC-1 cells undergoing HAI-1/SPINT1 knockdown was partially rescued by silencing of *matriptase/ST14* and/or *TMPRSS4*. Thus, EMT observed after knockdown of HAI-1/SPINT1 was likely caused by deregulated activities of matriptase/ST14 and TMPRSS4. Consistent with these observations, Carney and colleagues (25) have shown that deletion of the *HAI-1/SPINT1* gene abrogates the epithelial properties of keratinocytes in zebrafish, and this phenotype was rescued by inactivation of the *matriptase/ST14* gene. In mouse models, loss of HAI-1/SPINT1 disrupted the epithelial integrity of a population of matriptase/ST14–expressing chorionic trophoblasts and reduced levels of E-cadherin, perturbing their normal differentiation, which, in turn, led to failure to form the placental labyrinth (6, 26, 27). The important contribution of HAI-1/SPINT1 to the maintenance of cell surface E-cadherin and epithelial morphology was also confirmed in the experiments using S2-CP8, a metastatic variant of SUIT-2 (22). S2-CP8 showed loss of E-cadherin expression, enhanced SIP1 expression and a significantly decreased level of HAI-1/SPINT1 compared with parental SUIT-2 cells. Forced expression of HAI-1/SPINT1 in S2-CP8 significantly restored E-cadherin expression, which seemed to be mediated by the down-regulation of SIP1. On the other hand, MMP-9 level was not increased in S2-CP8. This may be due to low expression level of matriptase/ST14 in S2-CP8 compared with SUIT-2 (data not shown). Although *HAI-1/SPINT1* silencing caused E-cadherin loss–induced EMT, it was not sufficient to enhance metastasis in nude mice. However, it may increase the numbers of metastatic foci as long as the host permits the establishment of metastasis.

HAI-1/SPINT1 is expressed in a variety of epithelial cells and also in cancer cells, and is implicated in inhibitory activity toward HGFA and membrane-bound trypsin-like serine proteases, such as matriptase/ST14, hepsin/TMPRSS1, and prostasin/PRSS8 (4, 7–10). It also efficiently inhibits trypsin and human kallikrein 1–like peptidases (4, 28). Therefore, the role of HAI-1/SPINT1 might depend on the combination of HAI-1/SPINT1–sensitive proteinases present in the microenvironment of the cells. Among these proteinases, matriptase/ST14 is expressed on the cell surface concomitantly with HAI-1/SPINT1 in many epithelial tissues (10, 29, 30). Recent studies have indicated that HAI-1/SPINT1 is not only an inhibitor of matriptase/ST14 but may also act as a molecular chaperon that is required to localize matriptase/ST14 to the proper cell surface destination (10, 31). The tight regulation of matriptase/ST14 activity by HAI-1/SPINT1 is critical in the development and functions of certain tissues (6, 27, 32). Matriptase/ST14 degrades ECM proteins (10, 30). It also activates HGF, urokinase-type plasminogen activator, and protease-activated receptor-2 (30). Moreover, matriptase/ST14 may act upstream of other membrane-associated serine proteinases such as prostasin/PRSS8 (33). Therefore, deregulated matriptase/ST14 activity and abnormal subcellular localization, which may be caused by the absence of HAI-1/SPINT1, might result in multiple phenotypic alterations. Indeed, transgenic expression of matriptase/ST14 results in carcinogenesis in mice in a HAI-1/SPINT1–inhibitable manner (10, 34), and the ratio of matriptase to HAI-1/SPINT1 seems to be a biomarker of prostatic cancer progression (35). On the other hand, little is known regarding the functions of TMPRSS4 *in vivo*. Although a trypsin-like activity of TMPRSS4 has been shown by a study using synthetic substrates (20), its physiologic substrate is not known and the sensitivity of TMPRSS4 activity to HAI-1/SPINT1 remains to be determined. TMPRSS4, which was initially termed TMPRSS3, was identified as a protein up-regulated in pancreatic cancer (24). It is also up-regulated in gastric, colon, and thyroid cancers (24, 36), suggesting that TMPRSS4 may have a role in tumor progression. In fact, recent study has revealed that overexpression of TMPRSS4 in colon cancer cells enhances invasiveness and induces EMT (20), findings that are in accord with our present observations. Interestingly, signaling mediated by matriptase/ST14 and TMPRSS4 seems to be distinct from each other in SUIT-2 cells. The silencing of *matriptase/ST14* in HAI-1/SPINT1 knockdown SUIT-2 cells resulted in reversion of MMP-9 level, whereas the silencing of *TMPRSS4* resulted in reversion of SIP1 level.

An important question unanswered in this study is the mechanism by which interactions between HAI-1/SPINT1 and serine proteinases alter the signaling that induces EMT. It is possible that in the absence of HAI-1/SPINT1, membrane-associated serine proteinase(s) enhance the activity of EMT-inducing growth factor/cytokine(s). Although the expression of HGFA is very low in SUIT-2, matriptase/ST14 can activate HGF (4, 10, 30), and HGF is a well-known inducer of EMT via its receptor c-Met. However, SUIT-2 cells did not express endogenous HGF.

Published OnlineFirst February 17, 2009; DOI: 10.1158/0008-5472.CAN-08-3728

TGF-β is also an important inducer of EMT and certain membrane-bound serine proteinases may facilitate TGF-β signaling by activating the latent form of TGF-β, similar to serine proteinases plasmin and thrombin (37). In this study, we observed the up-regulation of SIP1 in cells undergoing EMT, which supports this hypothesis, as SIP1 mediates TGF-β–induced E-cadherin down-regulation (17). Further biochemical experiments using specific inhibitors of TGF-β signaling molecules will be required to clarify this possibility. At present, we do not know whether TMPRSS4 activates latent TGF-β. Another possible mechanism is degradation of cell surface E-cadherin by deregulated serine proteinase activities on the cell surface. However, soluble E-cadherin was not detectably increased in supernatants of HAI-1/SPINT1 knockdown cells (data not shown). Thus, proteolytic cleavage of cell surface E-cadherin may not be responsible for EMT observed in this study. An alternative possibility is that membrane-bound serine proteinases and HAI-1/SPINT1 transduced signals via their intracytoplasmic domains. This possibility is attractive but highly speculative at present.

In summary, the results of this study suggest that HAI-1/SPINT1 acts as a novel regulator of E-cadherin and that its inactivation plays a role in inducing EMT of carcinoma cells. Further studies regarding interactions between HAI-1/SPINT1 and membrane-associated serine proteinases and subsequent alteration of intracellular signaling will broaden our understanding of epithelial integrity and EMT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 9/26/2008; revised 11/24/2008; accepted 12/4/2008; published OnlineFirst 02/17/2009.

Grant support: Ministry of Education, Science, Sports, Culture, and Technology of Japan grants-in aid 20390114 (H. Kataoka) and 20790307 (T. Fukushima).

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Cancer Res 2009;69:1828-1835. Published OnlineFirst February 17, 2009.