

# LncRNA *TRERNA1* Function as an Enhancer of *SNAI1* Promotes Gastric Cancer Metastasis by Regulating Epithelial-Mesenchymal Transition

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**Long noncoding RNA (lncRNA) has been implicated in cancer, but little is known about the role of lncRNAs as regulators of tumor metastasis. In the present study, we demonstrate that lncRNA *TRERNA1* acts like an enhancer of *SNAI1* to promote cell invasion and migration and to contribute to metastasis of gastric cancer (GC). *TRERNA1* is significantly unregulated in GCs and GC cell lines. Increased *TRERNA1* is positively correlated with lymph node metastasis of GCs. RNA immunoprecipitation (RIP) and chromatin immunoprecipitation (ChIP) assays revealed that *TRERNA1* functions as a scaffold to recruit *EZH2* to epigenetically silence epithelial-mesenchymal transition marker *CDH1* by H3K27me3 of its promoter region. *TRERNA1* knockdown markedly reduced GC cell migration, invasion, tumorigenicity, and metastasis. Depletion of *TRERNA1* reduced cell metastasis of GCs in vivo. Taken together, our findings indicated that *TRERNA1* serves as a critical effector in GC progression by regulating *CDH1* at the transcription level. It is implied that *TRERNA1/CDH1* is a new potential target for GC therapy.**

## INTRODUCTION

Gastric cancer (GC) is one of the most common human cancers and the second leading cause of cancer-related mortality worldwide despite the greater progress in the diagnosis and treatment of human malignancy.<sup>1,2</sup> Metastasis is a major cause of death that greatly hinders treatment success, despite rapid advances in medical technology of GC.<sup>3</sup> Therefore, identifying pivotal genes and understanding their molecular mechanisms is a key to cancer prognosis and therapeutics for GC. Since the early 2000s, the majority of studies have focused on protein-coding genes, and a vast class of genes, including *AP-1*, *NF-κB*, *SNAI1*, *Sp1*, *EGR*, *CREB*, and *ATF*, is identified that was involved in the progression and metastasis of GC.<sup>4–11</sup> But so far, the molecular mechanism underlying GC development and progression remains unclear.

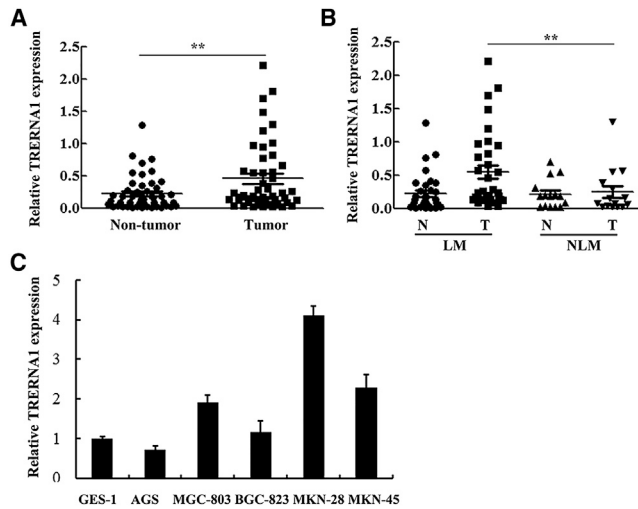
With the progress of DNA sequencing and analytical techniques, researchers have found that 98.5% of DNA sequences do not code for

proteins and have postulated that 80% of genome sequences are transcribed into primary transcripts and have biochemical functions.<sup>12</sup> To date, the crucial roles of these noncoding RNAs have been shown in various biological regulatory processes, especially in cancer. Long noncoding RNAs (lncRNAs) are defined as transcripts that contain more than 200 nucleotides that do not display the typical properties of well-characterized protein-coding RNAs that act as decoys and guides to facilitate both proximal and distal macromolecular interactions,<sup>13–16</sup> and accumulating evidence also suggests that lncRNAs play roles in regulating a wide range of cellular processes, including apoptosis, proliferation, cell migration, and invasion through affecting various aspects of protein, DNA, and RNA expression and interactions.<sup>17–21</sup> Recent evidence has demonstrated that the aberrant upregulated expression of lncRNAs, such as *TGUI*, *SNHG5*, *H19*, *HOXA11-AS*, and *UCAL*, is involved in cell invasion and metastasis of GC.<sup>22–25</sup> Therefore, it is very important for clinical diagnosis and therapy of GC to explore the biological function of more lncRNAs.

lncRNA *TRERNA1* was identified and located close to *SNAI1*, which is an epithelial-mesenchymal transition (EMT) master regulator transcription factor. A retrospective analysis shows that *TRERNA1* is overexpressed in lymph node metastatic human breast cancer samples and suggests that *TRERNA1* plays an important role in cell invasion and metastasis.<sup>26,27</sup> In the present study, we found the expression of lncRNA *TRERNA1* was significantly upregulated in tumor tissues compared with that in normal tissues in GC. Enhanced expression of *TRERNA1* was correlated with lymph node metastasis in GC. Our data showed that *TRERNA1* contributed to GC cell invasion and metastasis both in vitro and in vivo. Further studies have shown

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**Figure 1. LncRNA *TRERNA1* Is Upregulated in GC Tissues and Cells**

(A) Relative expression of lncRNA *TRERNA1* in GC tissues compared with the corresponding non-tumor tissues ( $n = 48$ ). (B) Patients with LM showed high levels of lncRNA *TRERNA1* compared with NLM patients. (C) qRT-PCR analysis of relative expression of lncRNA *TRERNA1* in GC cells. lncRNA *TRERNA1* expression levels were normalized to  $\beta$ -actin. Data are means  $\pm$  SD. \*\* $p < 0.01$ . LM, lymph node metastasis; N, non-tumor tissues; NLM, no lymph node metastasis; T, tumor tissues.

that *TRERNA1* not just inhibits the expression of *CDH1* by enhancing *SNAI1* expression, but also epigenetically silenced *CDH1* by recruiting *EZH2* to carry out histone methylation of its promoter region. These studies will help to clarify the role of *TRERNA1* in GC progression and as a potential therapeutic target.

## RESULTS

### Increased Expression Level of lncRNA *TRERNA1* Is Correlated with Lymph Node Metastasis in GCs

To explore the potential relationship between the expression pattern of *TRERNA1* with GC carcinogenesis and progression, we examined the expression levels of lncRNA in 48 GC tissues with paired non-tumor tissue as control. As revealed by qRT-PCR analysis, the expression level of lncRNA *TRERNA1* was remarkably higher in tumor tissues compared with adjacent non-tumor tissues (Figure 1A). After evaluating the correlation between *TRERNA1* and the clinicopathological characteristics, we found a significant relationship between *TRERNA1* expression level and lymph node metastasis in GC specimens (Figure 1B), although no significant correlation was observed among *TRERNA1* to GC patients' age, gender, TNM staging, and the differentiation degree of tumors (Table 1). In six GC cell lines, compared with the immortalized normal gastric epithelial cell line GES-1, there is upregulated expression of *TRERNA1* in four cell lines, including KMN-28, MKN-45, BGC-823, and MGC-803, and downregulated expression of *TRERNA1* in the AGS cell (Figure 1C). These results indicated the potential role of *TRERNA1* during GC carcinogenesis, particularly in the metastasis process.

**Table 1. Clinicopathological Correlation of *TRERNA1* Expression in GC Cases**

Feature	Expression of <i>TRERNA1</i>		p Value
	T > N	T $\leq$ N	
Gender			0.809
Male	21	12	
Female	9	6	
Age			0.582
$\geq 60$	23	15	
<60	7	3	
Histologic Grade			0.825
Poor	13	7	
Moderate	6	5	
High	11	6	
Lymph Node Metastasis			0.034 <sup>a</sup>
Yes	21	7	
No	9	11	
TNM Staging			0.878
Stage I/II	19	11	
Stage III/IV	11	7	
Lauren's Classification			0.414
Diffuse type	18	9	
Intestinal type	12	9	

N, non-tumor tissues; T, tumor tissues.

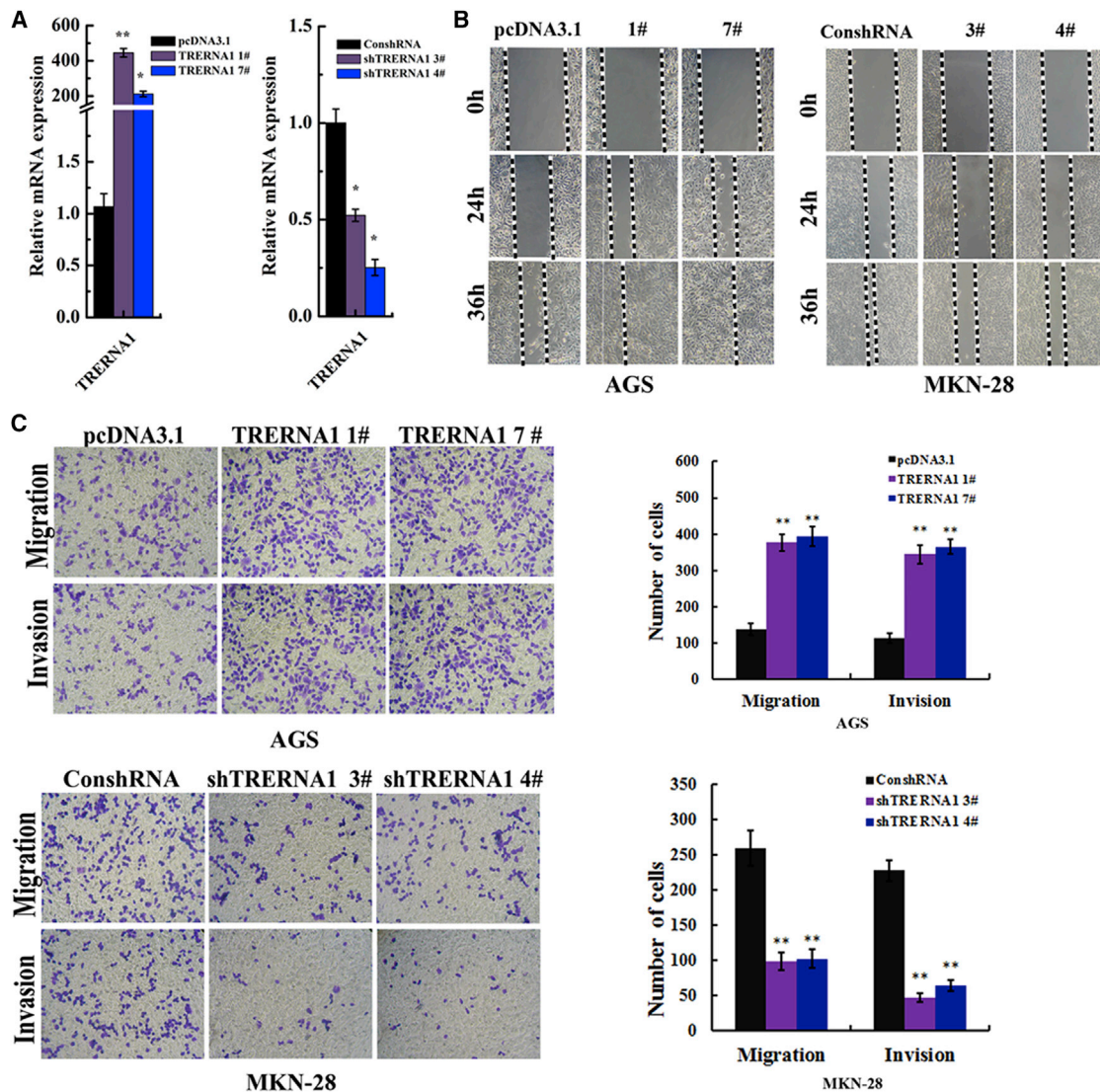
<sup>a</sup> $\chi^2$  test, \* $p < 0.05$ .

### *TRERNA1* Promotes GC Cell Migration, Invasion, and Metastasis In Vitro

To assess the potential role of *TRERNA1* in GC carcinogenesis, we obtained stable monoclonal cells to test and compare the cell phenotype after *TRERNA1* knockdown and overexpression in MKN-28 and AGS cells (Figure 2A). Wound healing assay showed that AGS cells that stably enforced *TRERNA1* displayed a notably faster recovery compared with control cells; conversely, MKN-28 cells that knocked down *TRERNA1* showed a slower recovery compared with control cells (Figure 2B). Similarly, in transwell migration assay and matrigel invasion assay, enforced *TRERNA1* expression level increased the ability of cell migration and invasion in AGS cells ( $p < 0.05$ ; Figure 2C); however, suppressed *TRERNA1* expression level decreased the ability of cell migration and invasion in MKN-28 cells ( $p < 0.05$ ; Figure 2C). These results implied an important role of *TRERNA1* in cell migration and invasion in GCs.

### Depletion of *TRERNA1* Reduced Cell Metastasis of GCs In Vivo

To validate the important role of *TRERNA1* in cell metastasis of GCs in vivo, MKN-28 cells that are transfected with *TRERNA1*-short hairpin RNA (shRNA) or negative control (NC)-shRNA were inoculated into nude mice by tail vein injection. After injection for 45 days, the treated nude mice were sacrificed to dissect lungs for analysis. As shown in Figure 3, inhibition of *TRERNA1* by shRNA effectively



**Figure 2. *TRERNA1* Regulates Gastric Cancer Cell Migration and Invasion In Vitro**

(A) Overexpression and knockdown efficiency of *TRERNA1* after transfection of the overexpression and shRNA construct in AGS and MKN-28 cells. (B) Wound healing assay showed that the enforced expression of *TRERNA1* displayed a notably faster recovery compared with control cells in AGS cells (left); conversely, there was a slower recovery in *TRERNA1* knockdown clones compared with control in MKN-28 cells (right). (C) *TRERNA1* modulated GC cell migration and invasion in vitro by transwell migration and invasion assays in AGS and MKN-28 cells. \**p* < 0.05; \*\**p* < 0.01.

suppressed tumor cells metastasis compared with the control. These data further demonstrated the important role of *TRERNA1* in GC tumor cell metastasis both in vitro and in vivo.

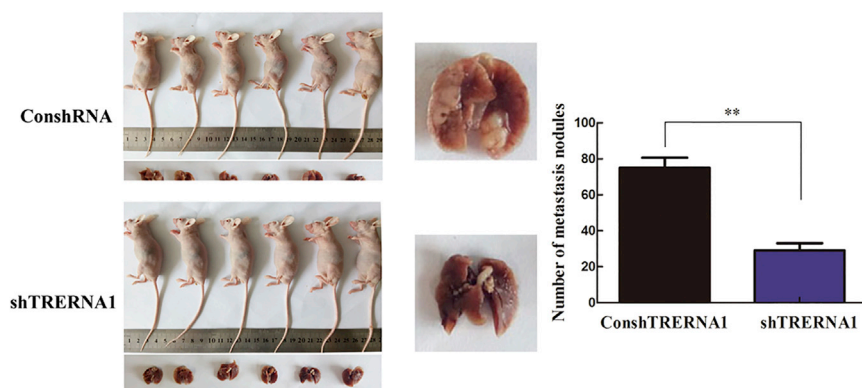
***TRERNA1* Regulated Nearby Gene *SNAI1* Expression by Acting Like an Enhancer**

Accumulating evidence shows that lncRNA participates in multiple cell processes by regulating gene expression in different levels. In order to explore the function and mechanism of *TRERNA1* on cell migration and invasion, we investigated the subcellular location of *TRERNA1* in GC cells. We fractionated GC cells into cytoplasmic

and nuclear fractions, and separation was confirmed by the presence of *GAPDH* mRNA in the cytoplasm fraction and nuclear *U6* mRNA predominantly in the nuclear fraction (Figure 4A, right). qRT-PCR results showed that *TRERNA1* distributes in both the cytoplasm (AGS: 58.7% ± 3.78%, MKN-28: 65.6% ± 4.65%) and the nucleus of GC cells (AGS: 41.3% ± 2.54%, MKN-28: 36.4% ± 3.725%) (Figure 4A, left). The data indicate that *TRERNA1* may play a regulatory role in both the cytoplasm and nucleus in GCs.

*TRERNA1* is located at chromosome 20q13.13. In 150 kb neighbor regions to *TRERNA1* there are five protein-coding genes: *SNAI1*,





**Figure 3. *TRERNA1* Stable Knockdown in MKN-28 Cells Significantly Reduced the Number of Visible Nodules on the Lung Surface after Tail Vein Injection in Nude Mice**

Lungs from each group are shown (left). The middle panel is representative of pulmonary nodules, and the right panel is a statistical analysis of the number of nodules from two groups. Bar graph represents the number of visible nodules on the lung surface. Error bars represent mean  $\pm$  SD.  $p < 0.05$ ,  $**p < 0.01$ .

*UBE2V1*, *TMEM189*, *CEBPB*, and *RNF114* (Figure 4B, upper). We assay these gene expression levels in knockdown or overexpression of *TRERNA1* by qRT-PCR (primers are listed in Table S1). Depletion of *TRERNA1* led to decreased expression of their neighboring protein-coding gene *SNAI1*, but did not affect the other protein-coding genes surrounding *TRERNA1* in mRNA level (Figure 4B, middle). Conversely, overexpression of *TRERNA1* increased the expression of *SNAI1* (Figure 4B, lower), but did not affect the other protein-coding genes surrounding *TRERNA1*. The same results observed in western blot analysis demonstrated that *TRERNA1* was able to regulate the expression of *SNAI1* protein levels in *TRERNA1* knockdown and overexpression of GC cell clones (Figure 4C). These results implied that *TRERNA1* functions as an enhancer in regulating the protein-coding gene *SNAI1* expression in *cis*.

#### ***TRERNA1* Enhances *SNAI1* and Modulates *CDH1* Expression in GC Cells**

*SNAI1* is a member of the snail zinc-finger family, which has been shown to function as the regulators of cell migration, adhesion, and EMT in cancer progression.<sup>28–30</sup> Previous studies have shown that *SNAI1* binds to E-boxes of *CDH1* promoter to repress its transcription.<sup>31</sup> Downregulated *CDH1* is one of the hallmarks of EMT in tumor. To investigate whether *SNAI1* is a repressor of *CDH1* in GC cells, we detected the *SNAI1* expression level either in RNA interference or in enforced expression. The results showed that *SNAI1* overexpression significantly reduced the expression of *CDH1* in AGS cell lines (Figure 5A); on the contrary, downregulation of *SNAI1* contributes to increased expression of the *CDH1* in MKN-28 cell lines (Figure 5B).

Then, we evaluated the effects of *TRERNA1* on gene transcripts and protein levels of EMT-related genes, which were suspected to initiate the invasion process of many tumor cells by qRT-PCR and western blot in GC cells. Enforced expression of *TRERNA1* in AGS cells significantly suppressed expression of *CDH1*; conversely, knockdown of *TRERNA1* in MKN-28 cells that increased the expression of *CDH1* was determined by qRT-PCR (Figure 6A). Similar results were observed that *TRERNA1* modulates the expression of *CDH1* proteins after knockdown or overexpression of *TRERNA1* in GC cells (Figure 6B). These data clearly provide insights into the mechanisms

that *TRERNA1* upregulated *SNAI1* to suppress *CDH1* expression and contributed to GC cell invasion and metastasis.

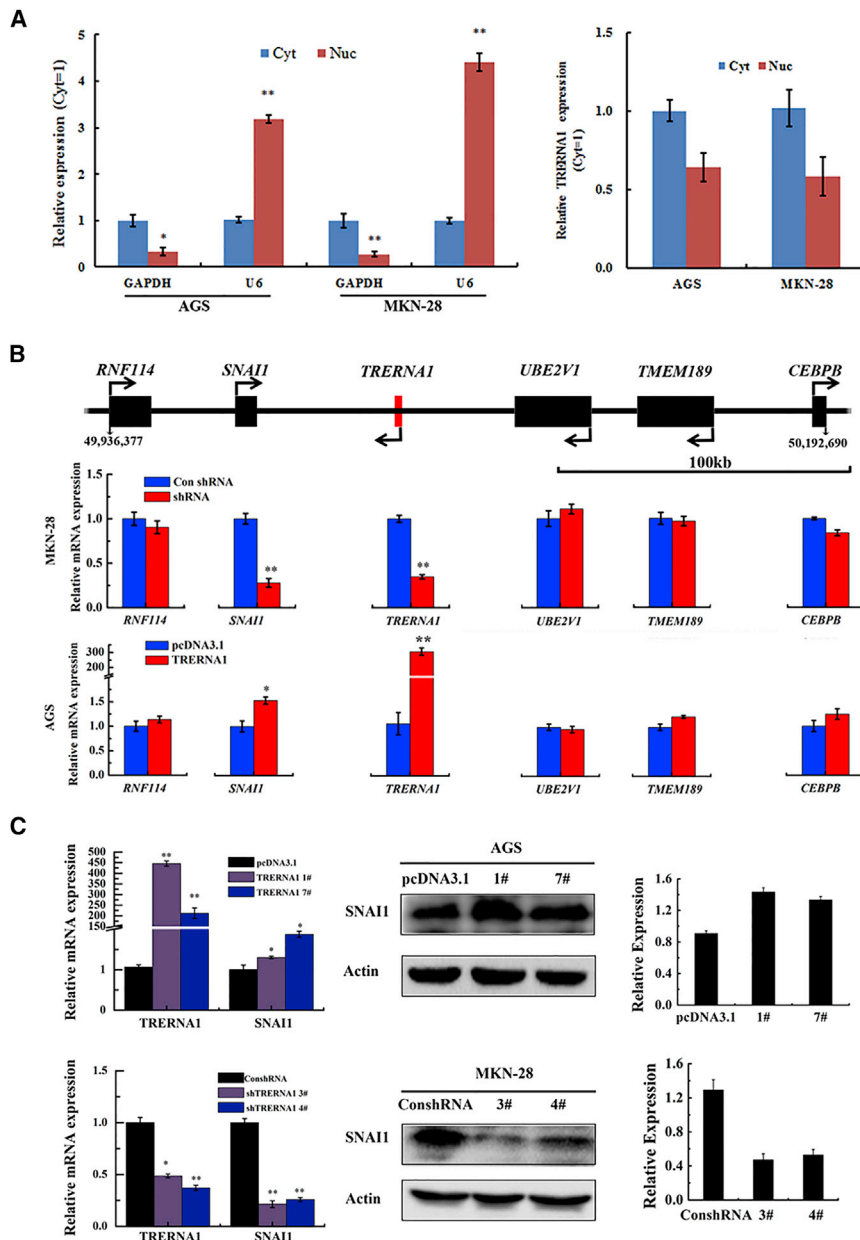
#### **lncRNA *TRERNA1* Altered the H3K27me3 Level of *CDH1* Gene by Binding to EZH2**

It has been found that the expression of *CDH1* is not only regulated by the transcription factor *SNAI1*, but also by epigenetic modification, such as microRNA (miRNA), DNA methylation, and histone modification.<sup>32–36</sup> It is well known that lncRNA-mediated epigenetic gene silencing by PRC2 appears to be an evolutionarily conserved mechanism between plants and animals,<sup>37</sup> we first detected whether *TRERNA1* combined with EZH2 using RNA binding protein immunoprecipitation technology (RIP), and we observed a strong binding of *TRERNA1* to EZH2 (Figure 7A). Next, we conducted chromatin immunoprecipitation (ChIP) assays to map histone modifications in the proximal region of the *CDH1* promoter in MKN-28 cells. We found that knockdown of *TRERNA1* decreased the H3K27me3 levels of *CDH1* promoter compared with the control (Figure 7B). These results demonstrated that *TRERNA1* epigenetically silenced *CDH1* expression by physical association with EZH2 in GC cells.

#### **DISCUSSION**

GC is one of the leading causes of cancer death worldwide, especially in China.<sup>38,39</sup> Despite the fact that strategies in the treatment of advanced and metastatic cancer have improved dramatically in recent years, metastasis leads to most cancer deaths and remains one of the most enigmatic aspects of GC.<sup>2,40,41</sup> Therefore, understanding the dynamic interactions of these pathways will help us to identify promising molecular targets for cancer therapy and key obstacles to their clinical treatment.<sup>42</sup>

lncRNAs have been shown to be involved in cellular processes, including apoptosis, proliferation, cell migration, and invasion.<sup>19,21,43</sup> Evidence has also indicated that lncRNAs act as crucial determinants of GC metastasis.<sup>22,44,45</sup> Identification of lncRNAs involved in cancer progression will further improve our understanding in cancer. lncRNAs can exert their regulatory function through a variety of mechanisms, including chromatin remodeling, RNA processing, localization, translation, and mRNA stability and even as a competing endogenous RNA.<sup>46–48</sup>



**Figure 4. *TRERNA1* Regulates *SNIA1* Function Like an Enhancer**

(A) The relative distribution of *TRERNA1* in the cytoplasm and nucleus as identified using qRT-PCR; GAPDH was used as a cytosolic marker, and U6 was used as a nuclear marker in AGS and MKN-28 cells. (B) *TRERNA1* is a regulator of *SNIA1* expression. The relative position of the adjacent gene to the *TRERNA1* (upper) and the effects on mRNA levels for the surrounding genes after knockdown or enforced expression of *TRERNA1* in MKN-28 and AGS cells as determined by qRT-PCR (middle and lower panels) are shown. Scale bar, 100 kb. (C) Western blot assays detected the expression of *SNIA1* after the transfection of the overexpression and shRNA *TRERNA1* plasmid in GC cell lines. \**p* < 0.05, \*\**p* < 0.01. Cyt, cytoplasm; Nuc, nucleus.

icantly upregulated in GC tissues and correlated with lymph node metastasis in GC. In vitro and in vivo, the data showed that *TRERNA1* exerts an oncogenic function in GC cell invasion and metastasis. Further molecular mechanism study indicated that *TRERNA1* modulates *CDH1* expression by enhancing *SNIA1* expression. To our surprise, we found that *TRERNA1* epigenetically regulates the expression of the *CDH1* gene by recruiting to *EZH2*. These results suggested that lncRNA *TRERNA1* regulates *CDH1* expression and contributes to EMT by various molecular mechanisms. Our studies not only help to clarify the role of *TRERNA1* in GC progression and metastasis, but also as a potential therapeutic target. Importantly, our data implied that *TRERNA1* can regulate tumor-related gene expression by multiple mechanisms in diverse cancers.

On the basis of the data from our study, we propose the following model for clarifying the molecular mechanism of *TRERNA1* involved in GC progression and metastasis. *TRERNA1* fraction

in the nucleus acts as an enhancer-like function to suppress *CDH1* expression by enhancing transcription of the *SNIA1* gene and epigenetically silencing the expression of *CDH1* by binding to PRC2.

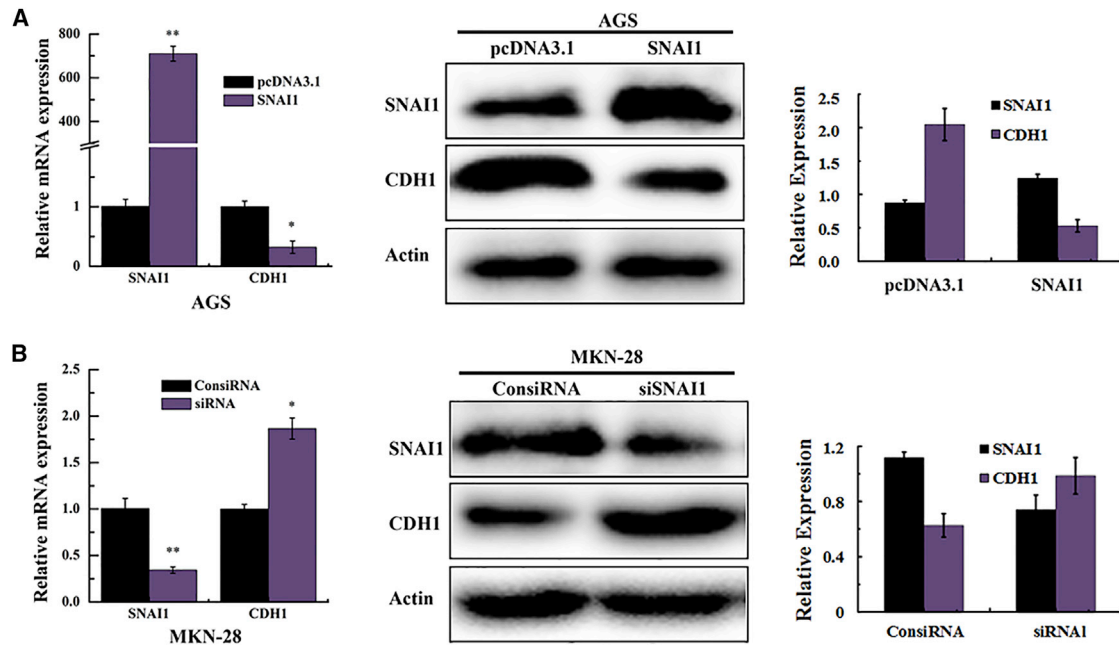
**MATERIALS AND METHODS**

**Collection of Tissue Specimens**

A total of 48 fresh GC tissues and paired adjacent non-tumor tissues were obtained from patients who had undergone surgical resection between 2010 and 2014 at the Third Affiliated Hospital of Harbin Medical University, China. Histopathological diagnosis was confirmed by an experienced pathologist. All of the tissue samples were washed with sterile PBS before being snap frozen in liquid

Despite the fact that growing evidence shows that aberrant lncRNA expression plays a key role in carcinogenesis and progression of cancer,<sup>49</sup> the underlying biological and molecular mechanisms of lncRNAs in diverse tumors are not yet fully elucidated.

*TRERNA1* was first reported by Ørom et al.,<sup>26</sup> who indicated that *TRERNA1* plays an important role in the activation of critical regulators of development and differentiation. Gumireddy et al.<sup>27</sup> showed that *TRERNA1* is upregulated in paired clinical breast cancer primary and lymph node metastasis samples, and that its expression stimulates tumor invasion in vitro and metastasis in vivo. Our results from investigation of GC showed that *TRERNA1* was signif-



**Figure 5. Transcription Factor *SNAI1* Represses the Expression of *CDH1***

(A and B) The expression of *CDH1* after transfection of the overexpression construct (A) and siRNA (B) of *SNAI1* in AGS and MKN-28 cells detected by qRT-PCR and western blot assays. \* $p < 0.05$ ; \*\* $p < 0.01$ .

nitrogen until total RNA was extracted. No patients had been treated with radiotherapy or chemotherapy before surgery. This study was approved by the Ethics Committee of Southeast University, and informed consent was obtained from each patient involved in the study.

#### Cell Culture

The GC cell lines (MKN-28, MKN-45, BGC-823, AGS, and MGC-803) and the human gastric epithelial cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Wisent), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) in an incubator humidified with 5%  $\text{CO}_2$  at 37°C.

#### RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen). For qRT-PCR, RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara). qRT-PCR analyses were performed using SYBR Green (Takara). The results were normalized to the expression of  $\beta$ -actin. The primers were listed in Table S1. All experiments were performed using the  $2^{-\Delta\Delta\text{Ct}}$  method, and each experiment was performed in triplicate.

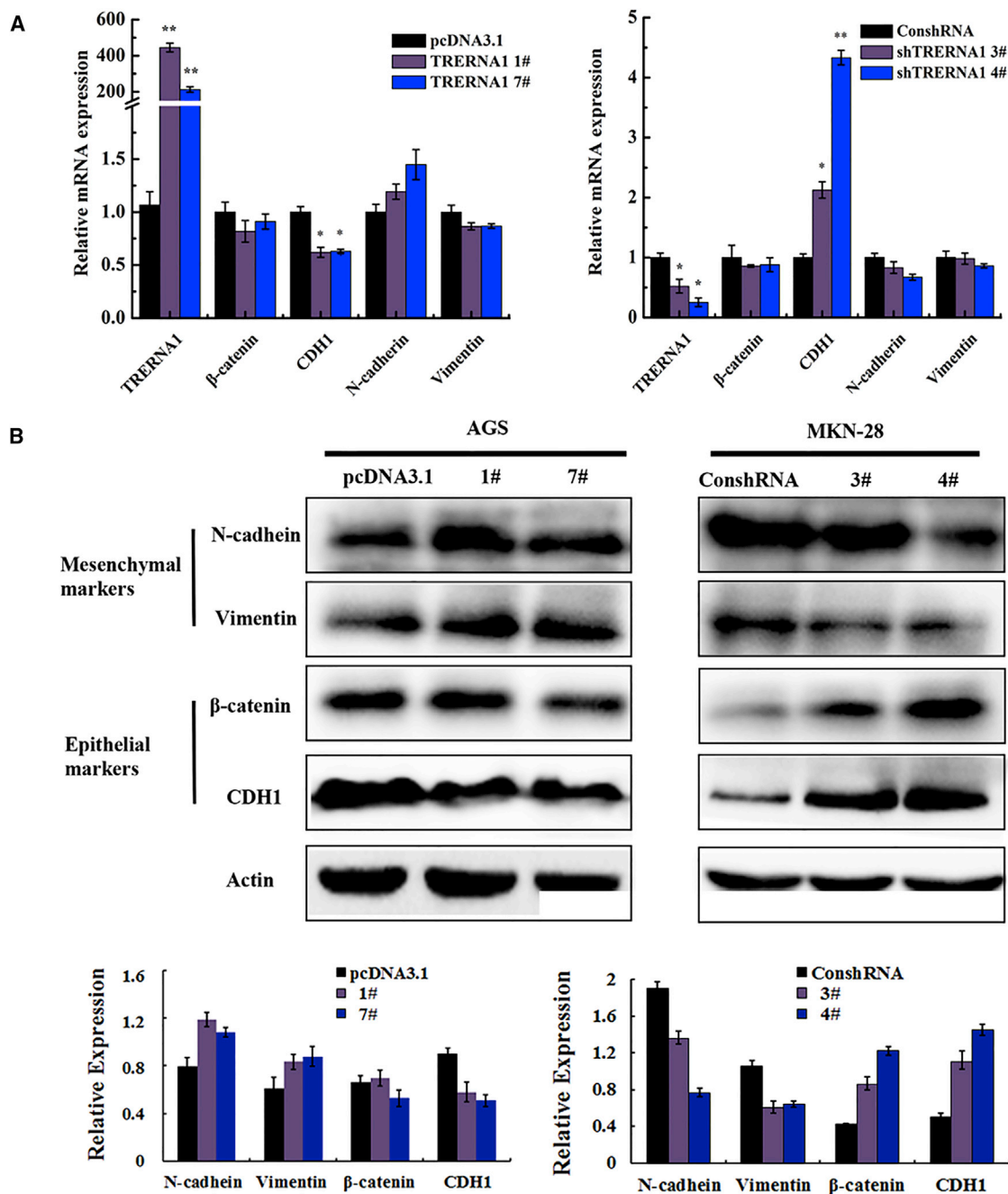
#### Plasmid Construction and Cell Transfection

The cDNA of lncRNA *TRERNA1* (NR\_051976.1) were synthesized by GENEWIZ and then cloned into pcDNA3.1. DNA segments of

*TRERNA1*-shRNA and small interfering RNA (siRNA) for *SNAI1* were synthesized by GENEWIZ and ligated into the BglII/Hind III sites of pSUPER-EGFP vector after annealing.<sup>50</sup> The cDNA of *SNAI1* was isolated by RT-PCR and then cloned into the Hind III/EcoRI sites of pcDNA3.1. The primers sequences for PCR amplification are as follows: forward 5'-CCCCAAGCTTATGCCGCGCTCTTTCCTCGTCAG-3' and reverse 5'-GGTGGAAATTCTCAGCGGGACATCCTGAGCAGC-3'. Transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). The stable cell clones were selected with G418.

#### Wound Healing, Migration, and Invasion Assays

Wound healing, migration, and invasion assays were performed as described previously.<sup>33</sup> Cells were cultured in standard conditions until 80%–90% confluence during a scratch wound was generated using a 200  $\mu\text{L}$  pipette tip, and the spread of the wound closure was observed under the microscope. For transwell migration assay, cells ( $4 \times 10^5$ ) were plated within the top chamber (Millipore) with the non-coated membrane. For invasion assays, matrigel (BD Biosciences) was polymerized in transwell inserts for 30 min at 37°C. In both assays, cells were plated within the top chamber in medium without serum; the lower chamber filled with 10% FBS was used as a chemoattractant. After 36 hr incubation, the cells that did not migrate or invade through the pores were gently removed with a cotton swab. All of the cells were stained with crystal violet and counted in five fields by inverted microscope. All experiments were repeated independently three times.



**Figure 6. TRERNA1 Regulates EMT Marker Expression in GC Cells**

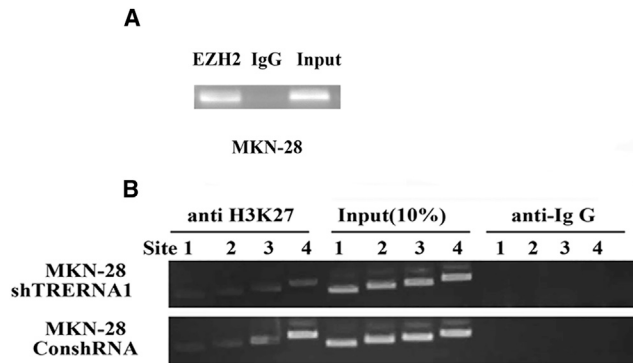
(A) qRT-PCR was used to measure expression levels of EMT-related markers (*CDH1*, *N-cadherin*, *Vimentin*, and  $\beta$ -*catenin*) in AGS and MKN-28 cells, which transfected with construct (pcDNA3.1+*TRERNA1*) or shRNA-*TRERNA1* and normalized to  $\beta$ -actin expression. (B) *TRERNA1* affects the expression of EMT-related genes in GC cells as determined by western blot analysis. \* $p < 0.05$ ; \*\* $p < 0.01$ .

### Xenograft Mouse Model

Four-week-old male nude mice, weighing 20 g, were housed in an air-conditioned room and maintained under pathogen-free (SPF) conditions. Ten nude mice were randomly divided into two groups, and MKN-28 cells stably transfected with sh-*TRERNA1* or empty

vector were injected into the tail veins of 10 mice ( $1 \times 10^6$  cells/mouse). After 45 days, the nude mice were sacrificed to dissect lungs for quantitative analysis of lung metastases. All animal studies were approved by the Animal Welfare and Research Ethics Committee at Southeast University, and all protocols were conducted strictly in





**Figure 7. *TRERNA1* Represses Transcription of *CDH1* by Recruiting PRC2 Subunits EZH2 to the *CDH1* Promoter**

(A) The *TRERNA1* directly interacts with PRC2 subunits EZH2 by RIP assay (RIP experiments using anti-EZH2 antibody and IgG as a negative control were performed in MKN-28 cells, and the coprecipitated RNA was subjected to PCR for *TRERNA1*). (B) ChIP-PCR of H3K27me3 enrichment of the promoter region of the *CDH1* after knockdown *TRERNA1* in MKN-28 cells.

accordance with the Guide for the Care and Use of Laboratory Animals.

#### Western Blot Assay and Antibodies

Cells protein lysates were separated by 10% SDS-PAGE, transferred to 0.22  $\mu$ m NC membranes (Sigma), and incubated with specific antibodies. Anti-CDH1 and *SNAI1* (Bioworld Technology), anti- $\beta$ -catenin, and *N-cadherin* (4061) (Cell Signaling Technology), *Vimentin* (sc-6260) (Santa Cruz Biotechnology), and anti- $\beta$ -actin (Sigma-Aldrich) were used as controls. Protein detection was performed with Super Signal Chemiluminescence Substrate (Pierce).

#### Subcellular Fractionation Location

Separation of the nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies) according to the manufacturer's instructions, and RNA was used for subsequent reverse transcription reaction and real-time PCRs (SYBR Premix Ex Taq; TaKaRa). The primer sequences are shown in Table S1.

#### Chromatin Immunoprecipitation Assays

ChIP assays were performed using the Chromatin Immunoprecipitation Kit according to the manufacturer's instructions (Millipore). Immunoprecipitations were performed using anti-H3K27me3 (Millipore) and/or normal mouse IgG used as the negative control. PCR was performed with the primers designed from the sequence of the *CDH1* promoter listed in Table S2, covering upstream of the transcription start site of the *CDH1* gene.

#### RIP

RIP experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. Antibody for RIP assays of *EZH2* was from Abcam (ab3748). The co-precipitated RNAs were detected by conventional RT-PCR.

#### Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software. The significance of differences between groups was estimated by Student's *t* tests and  $\chi^2$  tests as appropriate. Pearson's correlation coefficient was calculated using Prism5 software (GraphPad). A *p* value < 0.05 was considered statistically significant.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.omtn.2017.06.021>.

#### AUTHOR CONTRIBUTIONS

H.F. designed the experiments and modified the manuscript. H.W. conducted the experiments and wrote the paper. W.S., Y.H., Z.C., and P.G. performed the animal experiment and data collection. Statistical analysis was performed by X.L. and X.S. Y.Q. analyzed the data from qRT-PCR. K.Z. and M.Z. collected the clinical sample and analyzed the clinicopathological characteristics.

#### CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

#### ACKNOWLEDGMENTS

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