Zic1 suppresses gastric cancer metastasis by regulating Wnt/β-catenin signaling and epithelial-mesenchymal transition

Qiwei Ge1,2 | Yingying Hu2,3 | Jiamin He2,3 | Fei Chen1,2 | Lunpo Wu1,2 | Xintao Tu4 | Yadong Qi2,3 | Zizhen Zhang1,2 | Meng Xue1,2 | Shujie Chen2,3 | Jing Zhong1,2 | Liangjing Wang1,2

Abstract
Gastric cancer (GC) patients with metastasis had limited treatment options and dismal outcome. We have previously reported the aberrant expression of Zic family member 1 (Zic1) in GC. However, the functional roles and underlying mechanism of Zic1 in GC metastasis remain unknown. Here, we demonstrate that lower expression of Zic1 was correlated with more lymph node metastasis and poor outcome of GC patients. Ectopic expression of Zic1 suppressed both lung metastasis and peritoneal tumor dissemination of GC in mice. The metastatic suppressing ability of Zic1 was mediated by regulating the process of cell invasion, adhesion and epithelial-mesenchymal transition (EMT). Mechanistically, Zic1 could downregulate Wnt targets including c-Myc and Cyclin D1 by inhibiting LEF transcriptional activity in GC cells. Notably, Zic1 was inversely related to the expression of Cyclin D1 in GC tissues tested. In addition, Zic1 could physically interact with β-catenin/transcription factor 4 (TCF4) and disrupt their complex formation, while not affecting β-catenin nuclear localization. Collectively, our study indicated that Zic1 suppressed GC metastasis through attenuating Wnt/β-catenin signaling and the EMT process. Our work may provide novel therapeutic strategies for the metastasis of GC.

KEYWORDS
EMT, gastric cancer, metastasis, Wnt/β-catenin, Zic1

Abbreviations: EMT, epithelial-mesenchymal transition; GC, gastric cancer; IHC, immunohistochemistry; qRT-PCR, quantitative real-time PCR; TCF4, transcription factor 4; Zic1, Zic family member 1.

Qiwei Ge, Yingying Hu, and Jiamin He contributed equally to this study.
Despite marked improvements in the diagnosis and treatments of gastric cancer (GC), GC remains one of the most common cancers and the third leading cause of cancer-related mortality globally.1,2 Over the past decades, the 5-year survival rate for early stage GC patients has been improved. However, patients at a late stage still have a very dismal outcome due to the rapid metastasis.3 Treatment options for patients with advanced tumors were extremely limited. A comprehensive understanding of the key molecular events in tumor metastasis is lacking.4 Thus, uncovering new therapeutic targets may help to improve the overall survival of GC patients.

As the first reported gene in the Zic family, Zic family member 1 (Zic1) is located on chromosome 3q25.1, which encodes a zinc finger transcription factor,5 and also serves as transcriptional cofactors.6,7 Zic1 plays important roles in early embryogenesis and organogenesis, particularly in the central nervous system.5,8 Accumulating evidence revealed that Zic1 could exhibit antitumorigenic effect in numerous epithelial cancers including endometrial cancer,9 colon cancer,10 thyroid cancer,11 and breast cancer.12 However, Zic1 could function as an oncogene in tumors originated from stroma, such as liposarcoma13 and pleural mesothelioma.14 We have previously identified that Zic1 was downregulated through promoter DNA methylation and acted as a tumor suppressor by regulating MAPK signaling and modulating cell-cycle distributions in GC.15,16 Detection of Zic1 promoter hypermethylation in plasma DNA can be a new strategy for the early diagnosis of GC.17

Zic1 has been shown to be involved in several key signaling pathways during early embryogenesis, including Wnt/β-catenin, TGF-β and Shh signaling.18 Other Zic family proteins, such as Zic219 and Zic520 have been shown to suppress β-catenin-induced axis duplication in Xenopus embryos. Zic5 played an oncogenic role in hepatocellular carcinoma through activating Wnt/β-catenin.21 Particularly, Zic1 is coexpressed with β-catenin in the myofibroblast of Dupuytren disease.22 Wnt/β-catenin signaling is closely associated with colorectal cancer and indisputably linked to many other cancers including GC.23,24 The Wnt/β-catenin signaling pathway is implicated in the maintenance of stem cell properties and serves as an important regulator of epithelial-mesenchymal transition (EMT) in cancer cells as well.25 Nevertheless, whether Zic1 regulates Wnt signaling in human cancers remains largely unknown.

In this study, we found that the lower expression of Zic1 was associated with more lymph node metastasis as well as a poor prognosis of GC patients. Zic1 was shown to suppress the mobility of GC cells both in vitro and in vivo through modulating the EMT process. Zic1 could disrupt the interaction of β-catenin and transcription factor 4 (TCF4), hence inhibiting Wnt/β-catenin signaling. Thus, our study demonstrated the prognostic relevance of Zic1 in GC and may shed light on novel targeted therapy for metastatic GC patients.

2 | METHODS

2.1 | Cell lines and cell culture

Gastric cancer cell lines (AGS, BGC-823, and SGC-7901) were purchased from Riken Gene Bank (Tsukuba, Japan) and American Type Culture Collection (ATCC, MA, USA), and cultured with RPMI1640 medium and F12K medium (Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing, China). Human embryonic kidney epithelial cell line 293T (HEK293T) was purchased from Invitrogen. Cells were kept at 37°C in a 5% CO2-containing atmosphere.

2.2 | Plasmids and cell transfection

The full-length Zic1 open reading frame was cloned into mammalian expression vector pcDNA3.1 as previously described15 and then subcloned into the pFLAG-CMV4 vector with an N-terminal Flag. The pGL3 LEF-Luc reporter was kindly provided by Prof. Tianhua Zhou (Zhejiang University, China). Cells were transfected with FuGENE HD (Promega, Madison, WI) in accordance with the manufacturer’s protocol.

To generate cell lines with stably overexpression of Zic1, BGC-823, and SGC-7901 cells were transduced with CMV-GFP lentivirus encoding Zic1 with puromycin selection cassette. The expression level of Zic1 was confirmed by quantitative real-time PCR (qRT-PCR) and Western blot, and the expression of GFP was confirmed by the inverted fluorescence microscope.

2.3 | RNA extraction and quantitative real-time PCR

Total RNAs were extracted according to the protocol of TRIzol reagent (Invitrogen, Carlsbad, USA). Complementary DNA (cDNA) was synthesized using RT reagent kit with gDNA Eraser (TaKaRa, Otsu, Japan). SYBR Green Master Mix Kit (TaKaRa, Otsu, Japan). SYBR Green Master Mix Kit was used to perform qRT-PCR on a LightCycler 480 Instrument (Roche, Penzberg, Germany). U6 and β-actin were used as endogenous controls. Primers used for PCR reactions are listed in the Supporting Informations.

2.4 | Western blot

Protein samples were loaded on SDS-PAGE gels and transferred to PVDF membranes (Millipore, Bedford, USA). The following antibodies were used for Western blot: Zic1 from Abcam (1:1000, Shanghai, China); E-cadherin, N-cadherin, ZO-1, MMP2, Vimentin, β-catenin, c-Myc, Cyclin D1, TCF4, Histone H2A, GAPDH, Vinculin, and β-actin from Cell Signaling Technology (1:1000, Shanghai, China). An
HRP-linked Goat anti-Rabbit IgG (1:3000, Baoke, China) was used as the secondary antibody. Light-chain specific Mouse anti-Rabbit IgG (1:1000, CST, Shanghai, China) was used as the secondary antibody in Co-IP assay. The blots were visualized by chemiluminescence using the Amersham Imager 600 System (GE Healthcare Life Sciences, Pittsburgh, USA).

2.5 | Cell migration, invasion, and adhesion assays

In vitro cell migration and invasion were measured by Transwell chambers (Corning Inc, Corning, USA) and the chambers were coated with Matrigel (BD Biosciences, Franklin Lakes, USA), respectively. Briefly, cells transfected with indicated plasmids were cultured in serum-free medium for 24 hours and then 1 x 10^5 cells were plated to the upper chamber in 250 μL medium with 1% fetal bovine serum (FBS), while the lower chamber was filled with 600 μL medium with 15% FBS. After 24 hours incubation, cells on the outer surface of the insert bottom were fixed, stained with DAPI and then counted under an optical microscope in five predetermined fields. Cells invading through membrane were incubated with 0.1% crystal violet. Then crystal violet was washed out from the cells with 10% acetic acid and quantified on a microplate reader.

2.6 | Dual-luciferase reporter assay

BGC-823, AGS and HEK293T cells transiently expressing pFlag-CMV4-Zic1 or pFlag-CMV4-Vector were seeded in 12-well plates (2.5 x 10^4 cells per well) and transfected with LEF-Luc (200 ng) and pRL-TK vectors (10 ng, Promega) for 24 hours. The luciferase activities were measured using dual luciferase reporter system (Promega). Firefly luciferase activities were normalized to Renilla luciferase activities.

2.7 | Immunofluorescence assay

Cells grown on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked with 1% BSA. Then cells were incubated with the primary antibody (β-catenin, Cell signaling, 1:200) at 4°C overnight. After three washes using PBS-T, cells were incubated with AF488-conjugated secondary antibody (Thermo Scientific, Waltham, MA). The nucleus was counterstained with DAPI. Images were captured using fluorescence microscope MODEL BX51TRF (Olympus Corporation, Tokyo, Japan).

2.8 | Coimmunoprecipitation assay

BGC-823 or HEK293T cells were transfected with indicated plasmids. Nucleoprotein Extraction Kit (Sangon Biotech, Shanghai, China) was used for nuclear protein isolation according to the manufacturer's instructions. The lysates were mixed with protein A/G magnetic beads (Thermo Scientific) and then incubated with anti-Flag or specific antibody at 4°C overnight. Rabbit normal IgG was used as a negative control. The magnetic beads were collected by magnetic separator and washed with IP buffer three times to remove all the non-specific binding proteins. The bounded proteins were eluted with the elution buffer for western blot analysis.

2.9 | In vivo mouse models

For the lung metastasis model, 5 x 10^6 BGC-823 cells were intravenously injected through the tail vein of each 4-week-old female BALB/c nude mouse. For the peritoneal implantation metastasis model, 3 x 10^6 BGC-823 cells were injected into abdominal cavity of nude mice. All mice for metastasis assays were sacrificed at day 30 after injection. For tumor xenograft model, 1 x 10^7 SGC-7901 cells were subcutaneously injected into 4-week-old female BALB/c nude mice. All experimental procedures were approved by the Animal Ethics Committee of School of Medicine Zhejiang University.

2.10 | Clinical samples

Ninety patients with complete clinicopathologic characteristics and follow-up data who underwent surgery at the Second Affiliated Hospital, School of Medicine Zhejiang University and histologically diagnosed with GC were enrolled. Histological cancer types were evaluated by two independent pathologists in accordance with the TNM staging guide (2016) released by The American Joint Committee on cancer (AJCC). Tissue microarrays were made by paraffin-embedded consecutive sections. In addition, 50 surgically resected GC and matched adjacent non-cancerous specimens were obtained from Sir Run Run Shaw Hospital, School of Medicine Zhejiang University. Specimens were immediately frozen in liquid nitrogen and stored in −80°C. All patients were provided with informed written consents for obtaining study specimens. Experiments were approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine Zhejiang University.
2.11 | Immunohistochemistry

Tissue arrays were subjected to immunohistochemical staining for the expression of Zic1 and Cyclin D1, with the same primary antibodies as those used in western blot analysis, at a dilution of 1:100 in PBS. A pathologist and an investigator independently evaluated the scores of Zic1 and Cyclin D1 expression with an immunoreactivity score (IRS), which was combined by a score of the percentage of positive cells (1, 0%~25%; 2, 25%~50%; 3, 50%~75%; and 4, 75%~100%) and a score of the staining intensity (1, no staining of cells; 2, mild staining; 3, moderate staining; and 4, marked staining). We used an average score of stain in the cytoplasm and nucleus when different intensities were detected. The total score ranged from 0 to 16.

2.12 | Statistical analysis

All experiments were performed at least three times. All data are presented as the Mean ± SD. Statistical analyses were performed in IBM SPSS Statistics (Version 22.0, IBM, Armonk, NY, USA) and a P value of less than .05 was considered statistically significant. The Mann–Whitney U-test was used to analyze the nonparametric data in clinical samples. χ² test was used to assess the association of Zic1 expression with the clinicopathological parameters. Kaplan–Meier plots were used for overall survival rates, then compared with the log-rank test. Spearman’s rank correlation test was used to analyze the correlation between expression level of different proteins in clinical samples. Significant differences between groups were determined using the Student’s t test.

3 | RESULTS

3.1 | Zic1 is downregulated in GC and correlated with poor prognosis

We first assessed the expression of Zic1 in 50 primary GC tissues as well as their matched adjacent noncancerous tissues (Cohort 1) and showed that the expression of Zic1 was downregulated in the GC tissues at both mRNA level (P < .001, Figure 1A) and protein level (Figure 1B). Further immunohistochemistry (IHC) staining illustrated a lower expression of Zic1 in GC tissues in another independent cohort with 90 GC patients (Cohort 2). Zic1 is widely expressed in normal gastric epithelial tissues, and is mainly expressed in the nucleus of...
surface mucous foveolar cells. Quantitative analysis indicated that Zic1 was significantly lower expressed in GC tissues comparing to the noncancerous tissues \( (P < .001, \text{Figure 1C,D}) \).

We then evaluated the association between the expression of Zic1 and several clinicopathological parameters as well as the patient prognosis. Patients with lymph node metastasis showed a significantly lower expression of Zic1 in the cancer tissues comparing to those patients without metastasis (Figures 1E and S1). Downregulation of Zic1 was also observed in GC patients with late TNM stage \( (P = .011, \text{Table 1}) \). Kaplan–Meier analysis showed that patients with lower expression level of Zic1 in GC tissues had a poor prognosis \( (P = .0092, \text{Figure 1F}) \). Collectively, these results indicated that Zic1 was downregulated in GC and correlated with poor prognosis in the patients.

### 3.2 Zic1 inhibits GC cells invasion and epithelial mesenchymal transition

To investigate the mechanism underlying Zic1 mediated metastasis, we applied in vitro studies to explore the functional roles of Zic1. As Zic1 is silenced in most GC cell lines,\(^{15}\) we ectopically expressed Zic1 in GC cells (BGC-823 and AGS) with pFlag-CMV4-Zic1 plasmid, and overexpression of Zic1 markedly inhibited cell migration and cell invasion in BGC-823 and AGS cells (Figure 2A,B), but promoted cell adhesion in both parental cells (Figure 2C).

Epithelial mesenchymal transition (EMT) has been recognized as a key step in the progression of cancer metastasis.\(^{26}\) Thus, we hypothesized that EMT might be involved in the process of GC metastasis. The epithelial markers, E-cadherin and ZO-1, were upregulated in both cell lines upon ectopic expression of Zic1, whereas the expression of mesenchymal markers, including N-cadherin, Vimentin, and MMP2 were reduced (Figure 2D). These effects were further confirmed by qRT-PCR analysis of EMT markers (Figure 2E), suggesting that Zic1 could suppress GC cells metastatic ability by regulating the process of cell invasion, adhesion, and EMT.

### 3.3 Zic1 suppresses GC lung and peritoneal metastasis in mice

We next investigated whether Zic1 could play a role in GC metastasis in vivo. BGC-823 cells transduced with lentivirus carrying either GFP or Zic1-GFP were injected into abdominal cavity of nude mice. Mice received cells with Zic1 overexpression exhibited less peritoneal metastasis than the control group (Figure 3A,B). We also measured the effect of Zic1 on lung metastasis by intravenous injection of these GFP-positive cells to

| Clinicopathological characters | Zic1 expression | \( \chi^2 \) | \( P \) value\(^a\) |
|-------------------------------|----------------|-----------|----------------|
| Age (year)                    |                |           |                |
| 60                            | 23             | 17        | 0.0574         | .811 |
| \( \geq 60 \)                 | 30             | 20        |                | .       |
| Gender                        |                |           |                |
| Male                          | 38             | 22        | 1.4686         | .226 |
| Female                        | 15             | 15        |                | .       |
| Tumor size (cm)               |                |           |                |
| <5.0                          | 40             | 21        | 3.4943         | .062 |
| \( \geq 5.0 \)                | 13             | 16        |                | .       |
| Invasive depth                |                |           |                |
| T1-T2                         | 21             | 11        | 0.9307         | .335 |
| T3-T4                         | 32             | 26        |                | .       |
| Lymph node metastasis         |                |           |                |
| N = 0                         | 26             | 9         | 5.6081         | .017* |
| N = 1-3                       | 27             | 28        |                | .       |
| TNM stage                     |                |           |                |
| I-II                          | 33             | 13        | 6.4177         | .011* |
| III-IV                        | 20             | 24        |                | .       |

\(^{a}\)Chi-square test.

\( * \) \( P < .05 \).
the nude mice. Fewer numbers of lung metastatic nodules were observed in the Zic1 overexpression group demonstrated by the lower green fluorescence in the lung as well as fewer lung nodules indicated by H&E staining (Figure 3C,D). Additionally, overexpression of Zic1 in tumor cells significantly prolonged survival of the mice ($P < .05$, Figure 3E). Since EMT process is critical in lung metastasis, we measured the expression of several key EMT markers in the metastatic tissues.

**FIGURE 2** Zic1 regulates GC cell migration, invasion and adhesion. A, Cell migration was assessed by transwell assays. Cells which migrated to the bottom of the membrane were stained with DAPI. The mean number of visible migratory cells was counted in five random high-power fields. Scale bar, 100 μm. B, Cell invasion was assessed by transwell invasion assay. Invaded cells at the bottom of the membrane were stained with crystal violet, washed with 10% acetic acid and the amount of crystal violet was detected on a microplate reader (540 nm). Scale bar, 100 μm. C, Cell adhesion was quantified by cell adhesion assay. Cells adhered to the plates were stained with crystal violet. The crystal violet was dissolved in 10% acetic acid and the absorbance was measured at 540 nm. Scale bar, 100 μm. D-E, Western blot analysis (D) and qRT-PCR (E) were performed to measure the expression level of EMT markers after ectopic expression of Zic1 in GC cell lines. *$P < .05$, **$P < .01$, ***$P < .001$
We found that the epithelial marker E-cadherin was upregulated in tumors, while the mesenchymal markers N-cadherin and Vimentin were downregulated in Zic1 overexpression group (Figure 3F). We also examined the role of Zic1 in tumor growth in vivo. Nude mice subcutaneously transplanted with SGC-7901 cells expressing either empty vector or Zic1 vector (Figure S2A,B) developed solid tumors in 20 days. There was no significant difference of tumor weight between two groups (Figure S2C,D), indicating that ectopic expression of Zic1 may have no effect on tumor growth in a mouse xenograft model. Taken together, these results showed that Zic1 could suppress the EMT process and GC metastasis in vivo.

3.4 | Zic1 regulates Wnt/β-catenin signaling pathway in GC

Previous reports have showed that Zic family proteins regulate Wnt/β-catenin signaling during embryogenesis.\(^6,19\) In this regard, we proposed that Zic1 might act on the Wnt/β-catenin pathway to suppress tumor metastasis. Indeed, overexpression of Zic1 significantly reduced the expression level of Wnt/β-catenin downstream genes, including Cyclin D1, c-Myc, and Axin2 (Figure 4A). Interestingly, we found that ectopically expression of Zic1 downregulated Cyclin D1 and c-Myc with or without LiCl (20 mM) stimulation, which could stabilize β-catenin through GSK-3β (Figure 4B). Consistently, we observed that Cyclin D1 and c-Myc were downregulated in lung metastasis tissues from nude mice in Zic1 overexpression group in vivo (Figure 4C). To investigate whether Wnt/β-catenin signaling pathway was inhibited by Zic1, we performed dual-luciferase reporter assays with LEF reporter. Overexpression of Zic1 drastically inhibited the LEF luciferase activity in all cell lines tested (BGC-823, AGS and HEK293T cells), indicating that Wnt/β-catenin signaling was attenuated by Zic1 (Figure 4D).

Lower expression of β-catenin has been shown to inhibit canonical Wnt pathway. However, overexpression of Zic1 did not affect the overall expression (Figures 4E and S3A) or nuclear localization of β-catenin (Figure 4F). Immunofluorescence assays showed similar β-catenin nuclear localization in both control and Zic1 overexpression cells in two different cell lines (BGC-823, Figure 4G and AGS cells, Figure S3B). Collectively, these data implicated that Zic1 could inhibit Wnt/β-catenin signaling pathway without affecting the nuclear localization of β-catenin.

3.5 | Zic1 interacts with β-catenin and TCF4

It is well established that nuclear β-catenin associates with TCF4 to activate target gene transcription.\(^27\) Since β-catenin level in the nucleus was unaffected by Zic1, we hypothesized that Zic1 may directly inhibit the function of β-catenin/TCF4 complex in GC cells. First, we examined whether Zic1
could interact with the nuclear components of β-catenin. Ectopically expressed Zic1 could interact with β-catenin and TCF4 in both BGC-823 and HEK293T cells (Figure 5A). Furthermore, endogenous β-catenin and TCF4 could be co-immunoprecipitated with Zic1 in HEK293T cells (Figure 5B,C). To examine the simultaneous presence of Zic1, TCF4,
2169

and β-catenin within the same ternary complex, a two-step coimmunoprecipitation assay was performed. We observed that in the eluates immunoprecipitated by anti-Flag-Zic1, β-catenin could also be coimmunoprecipitated with Zic1 and TCF4 (Figure 5D). Taken together, these data illustrated that Zic1, TCF4, and β-catenin might form a ternary complex.

Next, we asked how this interaction affected the function of β-catenin. Reciprocal Co-IP assays using endogenous proteins revealed that Zic1 could disrupt the interaction between β-catenin and TCF4 (Figure 5E,F). Collectively, Zic1 plays an essential role in the inhibition of Wnt signaling through disrupting the formation of β-catenin/TCF4 complex.

3.6 | Zic1 is inversely correlated with Cyclin D1 in GC

To further investigate the relationship between Zic1 and Wnt target gene in GC, we assessed the expression level of Zic1 and Cyclin D1 in GC and noncancerous tissues. Cyclin D1 was significantly upregulated in GC tissue as compared to noncancerous tissues (Figure 6A). The expression level of Zic1 and Cyclin D1 is negatively correlated by IHC staining in GC tissues (Spearman’s correlation coefficient $R = -0.223$, Figure 6B,C).

4 | DISCUSSION

For patients with advanced tumors, prognostic biomarkers are required for a better risk assessment. In this regard, we analyzed human GC specimens from two independent cohorts and revealed that the lower expression of Zic1 was associated with more lymph node metastasis and poor outcome of patients. We have previously found that Zic1 inhibited cell proliferation through p-Akt and p-Erk1/2 inactivation.10 Zic1 could suppress thyroid cancer cell migration and invasion in vitro by modulating PI3K/Akt and MAPK pathways.
and enhancing FOXO3a transcriptional activity. These findings suggest that Zic1 exhibit antitumorigenic effect via regulating numerous downstream targets.

In our current study, overexpression of Zic1 suppressed both lung and peritoneal tumor metastasis of GC in nude mice. Mechanically, we found that overexpression of Zic1 upregulated E-cadherin and ZO-1 as well as downregulating N-cadherin and Vimentin (Figures 2D and 3F), indicating that Zic1 modulates the process of EMT. EMT is an important biological process which polarized epithelial cells undergo multiple cellular changes to acquire a mesenchymal cell phenotype with enhanced migratory capacity and invasiveness. In epithelial carcinomas, EMT drives tumor cells to leave the primary tumor and invade to the adjacent tissue as well as the blood vessels, setting the stage for metastatic dissemination. Furthermore, it is well known that Wnt/β-catenin signaling has a major impact on EMT. Nuclear β-catenin binds to members of the TCF/LEF family of transcription factors including Snail1/2 and Twist to promote EMT.

Our study has revealed, for the first time, the effect of Zic1 on Wnt/β-catenin signaling in GC. Zic1 inhibited the luciferase activity of LEF reporter and downregulated Wnt signaling targets including c-Myc and Cyclin D1. Importantly, Zic1 could interact with β-catenin/TCF4 complex and disrupt the interaction of β-catenin and TCF4 without affecting the normal nuclear localization of β-catenin. The expression and activity of β-catenin are regulated on multiple layers, including protein stability, nuclear accumulation, and transcriptional activity. Mutations in APC or CTNNB1 could stabilize β-catenin, which leads to aberrant activation of the Wnt/β-catenin signaling, thus play important roles in the progression of various cancers, especially in colorectal cancer. However, mutations in APC or CTNNB1 are rarely detected in GC, indicating that other mechanisms could be attributable to the activation of Wnt signaling in gastric carcinogenesis. Zic1 is silenced by promoter DNA hypermethylation in GC, which implies that epigenetic alternations could also contribute to the aberrant activation of Wnt signaling. Second, the key molecular events in the Wnt signaling are the β-catenin nuclear accumulation and the transcriptional activity of β-catenin/TCF4 complex. With regard to β-catenin nuclear accumulation, previous studies have reported that some proteins, such as FoxM1, BCL9, and Tw1 could regulate the shuttling of β-catenin between the cytoplasm and the nucleus. However, our results showed that Zic1 might not...
affect the nuclear accumulation of β-catenin. At last, we showed that Zic1 could function as a transcriptional cofactor to regulate the Wnt/β-catenin signaling in GC. It was reported that cofactors including CBP/p300,33 FOXP3,34 and ICAT,35 could interact with β-catenin in the nucleus to regulate the transcriptional activity. As a transcription factor, Zic proteins could bind to Gli-binding DNA sequences in a sequence-specific manner. Furthermore, Zic proteins could physically interact with Gli proteins via zinc-finger domains to act as transcriptional cofactors and modulate the hedgehog signaling pathway.36 There are three reported domains in Zic1 protein named as N-terminally located ZOC domain, zinc finger N-flanking conserved region (ZF-NC), and zinc finger domain.6 In the current study, we found that Zic1 could interact with β-catenin/TCF4 complex using Co-IP assays. Whether Zic1 has a direct interaction with TCF4 or β-catenin, and which domain or sequence participates in the above interaction, remain to be further studied.

Zic family proteins had been reported to suppress Wnt/β-catenin signaling in Xenopus gastrula embryos, and Zic3 could physically interact with TCF1.20 In human tumors, activation of the Wnt signaling has been linked tightly to colon cancer which harbors frequent activating mutations within Wnt pathway.37 Zic1 and Zic2 had been shown to inhibit the Wnt/β-catenin signaling in SW480 colon cancer cells.38 A recent study revealed that Zic5 could suppress the transcription of GLUT1 in a β-catenin/TCF4-dependent manner to downregulate glucose uptake in HCT116 colon cancer cells, while Zic2 played a completely opposite role.39 However, Zic1 and Zic4 were highly expressed through promoter DNA demethylation in desmoid tumor,40 which is another type of tumor with irregular activation of the Wnt signaling.41 These studies indicate that the function of Zic family differs greatly among tumor histological types. The collaborative or competitive actions of Zic proteins in tumor progression are worth further detailed investigation.

In conclusion, the results presented here, strongly suggest that Zic family proteins play essential roles in tumor progression. We show that Zic1 is downregulated in human GC and functions as a tumor suppressor by inhibiting tumor metastasis. Mechanistically, Zic1 could negatively regulate Wnt signaling by attenuating the function of β-catenin/TCF4 complex (Figure 6D). Our findings uncover a novel mechanism for aberrant activation of the Wnt/β-catenin signaling in GC, suggesting that Zic1 may serve as a potential therapeutic target for the treatment of GC.

ACKNOWLEDGMENTS
We thank Prof. Tianhua Zhou (Zhejiang University, China) for providing relevant plasmids. We thank Dr Baizhou Li (Department of Pathology, Second Affiliated Hospital of Zhejiang University School of Medicine, China) for the help of interpreting immunohistochemistry images. We are grateful to Dr Yang Guo (Zhejiang University, China) for insightful discussions. This work was supported by National Key R&D Program of China (2016YFC1303200), the National Natural Science Foundation of China (81702308) and Natural Science Foundation of Zhejiang Province (LY18H160009, LQ17H160010).

COMPETING INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
L.J. Wang and J. Zhong conceived and designed the study. Q.W. Ge, Y.Y. Hu, J.M. He, F. Chen, L.P. Wu, Y.D. Qi, and Z.Z. Zhang performed experiment. Q.W. Ge, Y.Y. Hu, J.M. He, and L.J. Wang analyzed and interpreted the data. Q.W. Ge and X.T. Tu drafted the manuscript. L.J. Wang, J. Zhong, S.J. Chen, and M. Xue critically revised the manuscript. All authors read and approved the final manuscript.

REFERENCES
1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca-Cancer J Clin. 2018;68:394-424.
2. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136:E359-E386.
3. Thrumurthy SG, Chaudry MA, Chau I, Allum W. Does surgery have a role in managing incurable gastric cancer? Nat Rev Clin Oncol. 2015;12:676-682.
4. Lambert AW, Pattabiraman DR, Weinberg RA. Emerging biological principles of metastasis. Cell. 2017;168:670-691.
5. Aruga J, Minowa O, Yaginuma H, et al. Mouse Zic1 is involved in cerebellar development. J Neurosci. 1998;18:284-293.
6. Aruga J. The role of Zic genes in neural development. Mol Cell Neurosci. 2004;26:205-221.
7. Ali RG, Bellchambers HM, Arkell RM. Zinc fingers of the cerebellum (Zic): transcription factors and co-factors. Int J Biochem Cell Biol. 2012;44:2065-2068.
8. Milet C, Maczkowiak F, Roche DD, Monsoro-Burq AH. Pax3 and Zic1 drive induction and differentiation of multipotent, migratory, and functional neural crest in Xenopus embryos. Proc Natl Acad Sci USA. 2013;110:5528-5533.
9. Wong YF, Cheung TH, Lo KW, et al. Identification of molecular markers and signaling pathway in endometrial cancer in Hong Kong Chinese women by genome-wide gene expression profiling. Oncogene. 2007;26:1971-1982.
10. Gao L, Chen S, Zhong J, et al. ZIC1 is downregulated through promoter hypermethylation, and functions as a tumor suppressor gene in colorectal cancer. PLoS ONE. 2011;6:e16916.
11. Qiang W, Zhao Y, Yang Q, et al. ZIC1 is a putative tumor suppressor in thyroid cancer by modulating major signaling pathways and transcription factor FOXO3a. J Clin Endocrinol Metab. 2014;99:E1163-E1172.
12. Han W, Cao F, Gao XJ, et al. ZIC1 acts a tumor suppressor in breast cancer by targeting survivin. Int J Oncol. 2018;53:937-948.
13. Brill E, Gobble R, Angeles C, et al. ZIC1 overexpression is oncogenic in liposarcoma. Cancer Res. 2010;70:6891-6901.
14. Cheng YY, Kirschner MB, Cheng NC, et al. ZIC1 is silenced and has tumor suppressor function in malignant pleural mesothelioma. J Thorac Oncol. 2013;8:1317-1328.

15. Wang LJ, Jin HC, Wang X, et al. ZIC1 is downregulated through promoter hypermethylation in gastric cancer. Biochem Biophys Res Commun. 2009;379:959-963.

16. Zhong J, Chen S, Xue M, et al. ZIC1 modulates cell-cycle distributions and cell migration through regulation of sonic hedgehog, PI(3)K and MAPK signaling pathways in gastric cancer. BMC Cancer. 2012;12:290.

17. Chen X, Lin Z, Xue M, Si J, Chen S. Zic1 Promoter hypermethylation in plasma DNA Is a potential biomarker for gastric cancer and intraepithelial neoplasia. PLoS ONE. 2015;10:e0133906.

18. Merzdorf CS, Sive HL. The zic1 gene is an activator of Wnt signaling. Int J Dev Biol. 2006;50:611-617.

19. Pourerebrahim R, Houtmeyers R, Ghogomu S, et al. Transcription factor Zic2 inhibits Wnt/beta-catenin protein signaling. J Biol Chem. 2011;286:37732-37740.

20. Fujimi TJ, Hatayama M, Aruga J. Xenopus Zic3 controls notochord and organizer development through suppression of the Wnt/beta-catenin signaling pathway. Dev Biol. 2012;361:2173-2179.

21. Liu L, Hu X, Sun D, Wu Y, Zhao Z. ZIC5 facilitates the growth of hepatocellular carcinoma through activating Wnt/beta-catenin pathway. Biochem Biophys Res Commun. 2018;503:2173-2179.

22. Degreef I, De Smet L, Sciot R, Cassiman JJ, Teijpar S. Immunohistochemical evidence for Zic1 coexpression with beta-catenin in the myofibroblast of Dupuytren disease. Scand J Plast Reconstr Surg Hand Surg. 2009;43:36-40.

23. Zang ZJ, Cutcutache I, Poon SL, et al. Exome sequencing of hepatocellular carcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. Nat Genet. 2012;44:570-574.

24. Deng YZ, Yao F, Li JJ, et al. RACK1 suppresses gastric tumorigenesis by stabilizing the beta-catenin destruction complex. Gastroenterology. 2012;142:812-823.e815.

25. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Sci Signal. 2014;7:re8.

26. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119:1420-1428.

27. Mosimann C, Hausmann G, Basler K. Beta-catenin hits chromatin: regulation of Wnt target gene activation. Nat Rev Mol Cell Biol. 2009;10:276-286.

28. Voulgari A, Pintzas A. Epithelial-mesenchymal transition in cancer metastasis: mechanisms, markers and strategies to overcome drug resistance in the clinic. Biochim Biophys Acta-Rev Cancer. 2009;1796:75-90.

29. Howe LR, Watanabe O, Leonard J, Brown AM. Twist is up-regulated in response to Wnt-1 and inhibits mouse mammary cell differentiation. Cancer Res. 2003;63:1906-1913.

30. Zhang N, Wei P, Gong A, et al. FoxM1 promotes beta-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer Cell. 2011;20:427-442.

31. Townsley FM, Thompson B, Bienz M. Pygopus residues required for its binding to Legless are critical for transcription and development. J Biol Chem. 2004;279:5177-5183.

32. Lu Y, Xie S, Zhang W, et al. Twal/Gid8 is a beta-catenin nuclear retention factor in Wnt signaling and colorectal tumorigenesis. Cell Res. 2017;27:1422-1440.

33. Li J, Sutter C, Parker DS, Blauwkamp T, Fang M, Cadigan KM. CBP/p300 are bimodal regulators of Wnt signaling. EMBO J. 2007;26:2284-2294.

34. Yang S, Liu Y, Li MY, et al. FOXP3 promotes tumor growth and metastasis by activating Wnt/beta-catenin signaling pathway and EMT in non-small cell lung cancer. Mol Cancer. 2017;16:124.

35. Daniels DL, Weis WI. ICAT inhibits beta-catenin binding to Tcf/Lef-family transcription factors and the general coactivator p300 using independent structural modules. Mol Cell. 2002;10:573-584.

36. Koyabu Y, Nakata K, Mizugishi K, Aruga J, Mikoshita K. Physical and functional interactions between Zic and Gli proteins. J Biol Chem. 2001;276:6889-6892.

37. Najdi R, Holcombe RF, Waterman ML, et al. Polycomb complex PRC1 preserves intestinal stem cell identity by sustaining Wnt/beta-Catenin transcriptional activity. Cell Stem Cell. 2016;18:91-103.

38. Chiaczkiera F, Rossi A, Jammula S, et al. Polycomb complex PRC1 preserves intestinal stem cell identity by sustaining Wnt/beta-Catenin transcriptional activity. Cell Stem Cell. 2016;18:91-103.

39. Zhang N, Wei P, Gong A, et al. FoxM1 promotes beta-catenin nuclear localization and controls Wnt target-gene expression and glioma tumorigenesis. Cancer Cell. 2011;20:427-442.

40. Pourerebrahim R, Van Dam K, Bauters M, et al. ZIC1 gene expression is controlled by DNA and histone methylation in mesenchymal proliferations. FEBS Lett. 2007;581:5122-5126.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Ge Q, Hu Y, He J, et al. Zic1 suppresses gastric cancer metastasis by regulating Wnt/beta-catenin signaling and epithelial-mesenchymal transition. The FASEB Journal. 2020;34:2161–2172. https://doi.org/10.1096/fj.201901372RR