GSE1 predicts poor survival outcome in gastric cancer patients by SLC7A5 enhancement of tumor growth and metastasis

Received for publication, November 26, 2017, and in revised form, January 14, 2018 Published, Papers in Press, January 24, 2018, DOI 10.1074/jbc.RA117.001103

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Edited by Eric R. Fearon

Gastric cancer remains a malignancy with poor survival outcome. We herein report that GSE1, a proline-rich protein, possesses a role in the progression of human gastric cancer. The expression of GSE1 was observed to be much higher in human gastric cancer tissues compared with normal gastric tissues, and GSE1 expression correlated positively with lymph node metastasis, histological grade, depth of invasion, and clinical stage in gastric cancer patients. Moreover, GSE1 expression was also associated with decreased post-operative relapse-free survival and overall survival in the cohort. The forced expression of GSE1 in gastric cancer cell lines resulted in increased cell proliferation, increased colony formation, enhanced cell migration, and invasion. Furthermore, forced expression of GSE1 also increased tumor size and enhanced lung metastasis in xenograft models. The depletion of endogenous GSE1 with shRNAs decreased the oncogenicity and invasiveness of gastric cancer cells both *in vitro* and *in vivo*. In addition, GSE1 was determined to be a direct target of miR-200b and miR-200c. Furthermore, GSE1 positively regulated the downstream gene *SLC7A5* (also known as *LAT-1*), which was scanned and verified from mRNA sequencing. GSE1 therefore possesses an oncogenic role in human gastric cancer, and targeted therapeutic approaches to inhibit GSE1 function in gastric cancer warrant further consideration.

Gastric cancer is one of the leading causes of cancer-related mortality worldwide (1, 2). Patients with gastric cancer are commonly diagnosed at an advanced stage, and their median 5-year survival rates are no more than 20% (3). The current optimal approach for gastric cancer therapy is surgical resection with curative intent and adjuvant chemotherapy or radiotherapy. However, the recurrence rate of gastric cancer remains high, with poor prognosis (4, 5). Therefore, further study of the molecular mechanisms promoting gastric cancer progression is essential to unveil new diagnostic or prognostic markers and therapeutic targets to improve clinical outcomes.

GSE1 (human Gse1 coiled-coil protein), a proline-rich protein, also known as KIAA0182, was first isolated and identified using ion-trap mass spectrometry (6). Little is known about the roles of GSE1 in human diseases thus far. In our previous study, GSE1 was demonstrated to possess an oncogenic function in human breast cancer cells (7). We have also previously reported that GSE1 is negatively regulated by miR-489-5p and promotes both proliferation and metastasis in breast cancer cells (7). However, the role of GSE1 in human gastric cancer remains unclear.

In this study, we have defined a functional role for GSE1 in human gastric cancer cells. We have determined that GSE1 promoted cell proliferation, colony formation, migration, invasion, tumor growth, and metastasis both *in vitro* and *in vivo*. Moreover, gastric cancer patients with high tumor expression of GSE1 exhibited worse clinicopathological parameters and survival rates. SLC7A5 (solute carrier family 7 member 5, also named LAT-1 (L-type amino acid transporter 1), system L-type transporters, important in cell maintenance and proliferation (8, 9)) is positively regulated by GSE1 and partly mediates the role of GSE1 in progression of gastric cancer. In addition, we demonstrated that the expression of GSE1 is suppressed and

This work was supported in part by National Nature Science Foundation of China Grants 81472493, 81500373, 81502282, 81672609, and 81502975; Program for Excellent Talents and Scientific Research Program of Anhui Medical University Grant 2013xkj006; Anhui Provincial Academic and Technical Leader Reserve Candidate Grant 2016H074; Key Program of Outstanding Young Talents in Higher Education Institutions of Anhui Grant gxyqZD2016046; Natural Science Foundation of Anhui Province Grant 1608085MH193; Scientific Research Foundation of Anhui Medical University Grant 2015xkj043; and China Postdoctoral Science Foundation Grants 2016T90413 and 2015M581693. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains Fig. S1.

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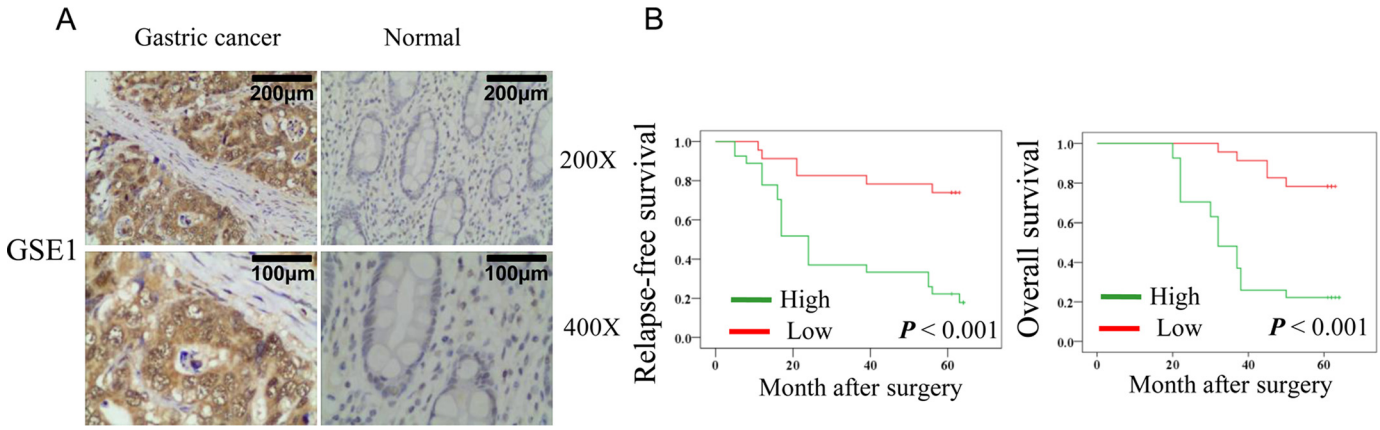


Figure 1. Expression of GSE1 in tissues from gastric cancer patients and the association between GSE1 expression and patients' survival rates. *A*, protein levels of GSE1 in gastric cancer tissues and normal gastric tissues were examined using immunohistochemistry. The magnifications of the photographs were ×200 and ×400. *B*, Kaplan–Meier curves were made to show the RFS and OS rates of gastric cancer patients with different GSE1 expression level.

directly targeted by miR-200b and miR-200c. Furthermore, GSE1 and SLC7A5 expression were positively correlated in gastric cancer tissues. *GSE1* and miR-200b/miR-200c expression were significantly and inversely correlated in gastric cancer tissues. Hence, we have provided evidence that GSE1 possesses an important function in the progression of human gastric cancer. GSE1 could potentially be utilized as a novel clinical diagnostic and therapeutic target for gastric cancer.

Results

Expression of GSE1 in human gastric cancer tissues and normal gastric tissues

We first determined the expression of GSE1 in 100 human gastric cancer tissues and 100 normal gastric tissues using immune histochemistry. Immunoreactive GSE1 protein was mainly located in the cytoplasm of gastric cancer cells and glandular epithelial cells (Fig. 1*A*). As shown in Table 1, 51 of 100 cases of normal gastric tissues were negative for expression of GSE1, 34 of 100 cases exhibited low expression of GSE1, 10 of 100 cases exhibited moderate expression of GSE1, and 5 of 100 cases exhibited high expression of GSE1. In contrast, 17 of 100 cases of gastric cancer tissues were negative for expression of GSE1, 27 of 100 cases exhibited low expression of GSE1, 37 of 100 cases exhibited moderate expression of GSE1, and 19 of 100 cases exhibited high expression of GSE1 ($p < 0.001$). Therefore, the expression levels of GSE1 in human gastric cancer tissues were higher than that in normal gastric tissues.

Association of GSE1 expression with clinicopathological parameters and survival of gastric cancer patients

We next examined the association of GSE1 expression with clinicopathological parameters, including patients' age, gender, tumor size, lymph node metastasis, histological grade, depth of invasion, and clinical stage. As shown in Table 2, the expression of GSE1 was positively correlated with patient lymph node metastasis ($p = 0.001$), histological grade ($p = 0.037$), depth of invasion ($p = 0.008$), and clinical stage ($p = 0.001$). However, there was no significant correlation between GSE1 expression and patients' age, gender, or tumor size ($p > 0.05$).

Furthermore, these gastric cancer patients were followed up for more than 5 years, and the association of GSE1 expression with their survival rate was analyzed by means of Kaplan–Meier analysis. Zero and 1 scoring of GSE1 were designated as low expression of GSE1; 2 and 3 scoring were designated as high expression of GSE1. In the 100 gastric cancer tissues, 44 exhibited low expression of GSE1, and 56 exhibited high expression of GSE1. As shown in Fig. 1*B*, both RFS rate ($p < 0.001$) and OS rate ($p < 0.001$) were significantly lower in tissues with high GSE1 expression compared with tissues with low GSE1 expression. This finding suggested that GSE1 is associated with poor prognosis in human gastric cancer.

Table 1
Expression of GSE1 in gastric cancer and normal tissues
$p < 0.001$.

Group	*n*	GSE1 expression			
		None	Low	Moderate	High
GC	100	17	27	37	19
Normal	100	51	34	10	5

Table 2
Association of GSE1 expression with clinicopathological parameters from gastric cancer patients

Parameter	*n*	GSE1 expression				*p* value
		None	Low	Moderate	High	
Age (years)						
≤60	34	10	7	13	4	0.075
>60	66	7	20	24	15	
Gender						
Male	54	11	14	20	9	0.760
Female	46	6	13	17	10	
Tumor size (cm)						
≤5	60	10	17	25	8	0.317
>5	40	7	10	12	11	
Lymph node metastasis						
No	34	15	17	1	1	0.001
Yes	66	2	10	36	18	
Grade						
I	11	1	6	1	3	0.037
II	48	7	10	25	6	
III	41	9	11	11	10	
Depth of invasion						
T1,2	40	13	9	13	5	0.008
T3,4	60	4	18	24	14	
Stage						
I–II	32	13	13	5	1	0.001
III–IV	68	4	14	32	18	

GSE1 stimulates cellular proliferation and oncogenicity of human gastric cancer cells

Gastric cancer cell lines BGC-823, HGC-27, AGS, and MKN-45 were used in this study. As shown in Fig. 2*A*, the basal level of GSE1 was high in HGC-27 and MKN-45 cells and was low in BGC-823 and AGS cells. Therefore, we have selected HGC-27 and MKN-45 cells to perform GSE1 depletion-related studies and selected BGC-823 and AGS cells to perform studies with forced expression of GSE1. The protein level of GSE1 decreased significantly after transfection with shGSE1-1 or shGSE1-2 in HGC-27 and MKN-45 cells (Fig. 2*B* and Fig. S1*A*) and increased significantly after transfection with an expression plasmid, pIRESneo3, encoding *GSE1* in BGC-823 and AGS cells (Fig. 2*C* and Fig. S1*E*).

In monolayer cultures, shGSE1-1 or shGSE1-2 decreased the total cell number dramatically over a period of 5 days (both HGC-27 and MKN-45 cell lines) (Fig. 2*D* and Fig. S1*B*). Concordantly, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[4] assay showed a significant decrease in the cell viability over a period of 5 days after transfection with shGSE1-1 or shGSE1-2 in both HGC-27 and MKN-45 cells (Fig. 2*E* and Fig. S1*C*). Moreover, shRNA-mediated depletion of GSE1 in HGC-27 and MKN-45 cells significantly reduced cell colony formation, as indicated in Fig. 2*F* and Fig. S1*D* (HGC-27-shNC, 257 ± 33; HGC-27-shGSE1-1, 38 ± 4; HGC-27-shGSE1-2, 45 ± 5 ($p < 0.01$) and MKN-45-shNC, 651 ± 70; MKN-45-shGSE1-1, 318 ± 44; MKN-45-shGSE1-2, 330 ± 35 ($p < 0.01$)).

In contrast, the forced expression of GSE1 in BGC-823 and AGS cells dramatically enhanced total cell number and cell viability over a period of 5 days (Fig. 2 (*G* and *H*) and Fig. S1 (*F* and *G*)). Concordantly, BGC-823-GSE1 and AGS-GSE1 cells exhibited significantly enhanced cell colony formation compared with BGC-823-Vec and AGS-Vec cells, respectively (Fig. 2*I* and Fig. S1*H*). Thus, it is concluded that GSE1 stimulates proliferation and enhances the viability of human gastric cancer cells.

GSE1 promotes metastasis of human gastric cancer cells

Next, we evaluated the role of GSE1 in migration and invasion of human gastric cancer cells. After transfection with shGSE1-1 or shGSE1-2, both cell migration (HGC-27-shNC, 408 ± 55; HGC-27-shGSE1-1, 185 ± 30; HGC-27-shGSE1-2, 199 ± 39 ($p < 0.01$) and MKN-45-shNC, 156 ± 22; MKN-45-shGSE1-1, 62 ± 15; MKN-45-shGSE1-2, 78 ± 19 ($p < 0.01$)) and invasion (HGC-27-shNC, 171 ± 28; HGC-27-shGSE1-1, 31 ± 6; HGC-27- shGSE1-2, 37 ± 8 ($p < 0.01$) and MKN-45-shNC, 88 ± 20; MKN-45- shGSE1-1, 33 ± 8; MKN-45-shGSE1-2, 48 ± 11 ($p < 0.01$)) were abrogated in both HGC-27 and MKN-45 cells (Fig. 2 (*J* and *L*) and Fig. S1 (*I* and *K*). Moreover, depletion of GSE1 with shGSE1-1 or shGSE1-2 resulted in a retarded wound closure in HGC-27 and MKN-45 cells, as shown in Fig. 2*N* and Fig. S1*M*.

In contrast, the forced expression of GSE1 in BGC-823 and AGS cells dramatically increased cell migration (BGC-823-Vec, 18 ± 5; BGC-823-GSE1, 63 ± 9 ($p < 0.01$) and AGS-Vec, 35 ± 7; AGS-GSE1, 58 ± 13 ($p < 0.01$)) and invasion (BGC-823-Vec, 11 ± 5; BGC-823-GSE1, 39 ± 10 ($p < 0.01$) and AGS-Vec, 9 ± 6; AGS-GSE1, 26 ± 8 ($p < 0.01$)) compared with control, respectively (Fig. 2 (*K* and *M*) and Fig. S1 (*J* and *L*)). Concordantly, forced expression of GSE1 promoted a faster wound closure compared with the respective control BGC-823 and AGS cells (Fig. 2*O* and Fig. S1*N*). Therefore, GSE1 promotes the motile behavior of human gastric cancer cells.

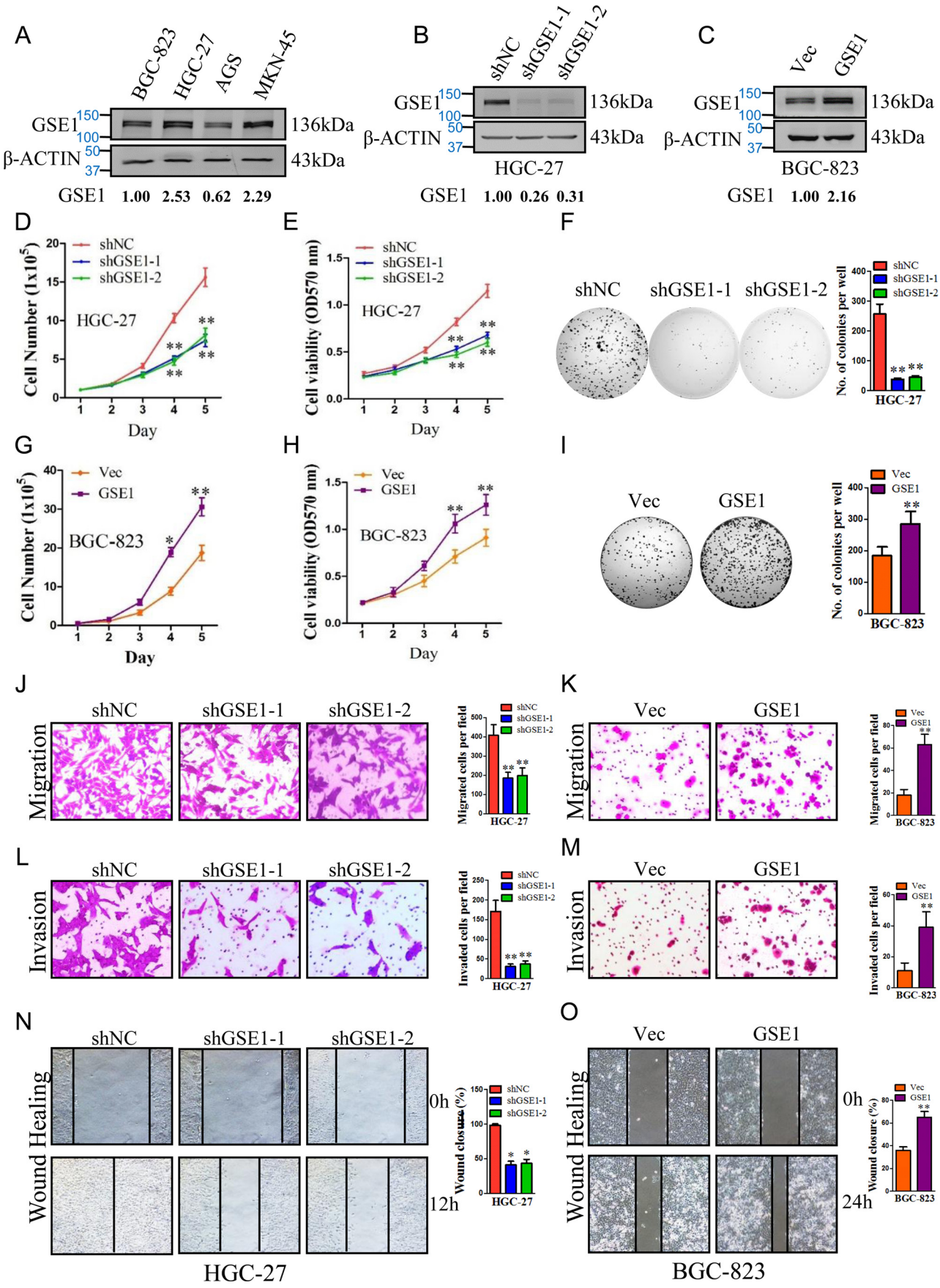
GSE1 promotes xenograft growth and metastasis of human gastric cancer cells

To determine the effects of GSE1 on tumor growth *in vivo*, HGC-27-shNC/HGC-27-shGSE1-1 (designated as HGC-27-shGSE1) and BGC-823-Vec/BGC-823-GSE1 cells were subcutaneously injected into the dorsal flank of nude mice. As shown in Fig. 3*A*, all of the eight sites injected with HGC-27-shNC cells formed palpable tumors, whereas only six of the eight sites injected with HGC-27-shGSE1 formed palpable tumors even until termination of the experiment. We measured the tumor size on a 3-day interval. The growth curves showed that HGC-27-shGSE1–generated tumors grew more slowly than HGC-27-shNC–generated tumors. After 21 days, HGC-27-shNC–generated tumors were more than 5 times the size of the HGC-27-shGSE1–generated tumors. Accordingly, the mean weight of tumors formed by HGC-27-shGSE1 cells was significantly lower than that of tumors formed by HGC-27-shNC cells ($p < 0.01$) (Fig. 3*B*). Moreover, the Ki-67–positive cell population in tumors formed by HGC-27-shGSE1 cells was significantly decreased compared with tumors formed by HGC-27-shNC cells ($p < 0.01$) (Fig. 3*C*).

In contrast, both BGC-823-Vec and BGC-823-GSE1 cells formed palpable tumors in all animals after less than a week. During the period of 21 days, BGC-823-GSE1–generated tumors grew much faster compared with BGC-823-Vec–generated tumors ($p < 0.05$). Tumors formed by BGC-823-GSE1 cells were more than 2 times the size of tumors formed by BGC-823-Vec cells at the end of the study (Fig. 3*D*). As such, the mean weight of tumors formed by BGC-823-GSE1 cells was significantly higher than that of tumors formed by BGC-823-Vec cells ($p < 0.01$) (Fig. 3*E*). Moreover, the Ki-67–positive cell population in tumors formed by BGC-823-GSE1 cells was significantly increased compared with tumors formed by BGC-823-Vec cells ($p < 0.01$) (Fig. 3*F*). These findings suggest that GSE1 promotes cell proliferation of gastric cancer cells and tumor growth *in vivo*.

Furthermore, we examined the effects of GSE1 on tumor metastasis *in vivo* by injecting HGC-27-shNC/HGC-27-shGSE1 and BGC-823-Vec/BGC-823-GSE1 into the venous circulation of mice. After 40 days, mice were killed, and their lungs were collected for histology. Five random sections of each mouse lung were examined for lung micrometastases. In the eight mice injected with HGC-27-shGSE1 cells, no lung metastases were observed, whereas four of eight mice injected with HGC-27-shNC cells exhibited lung metastases ($p = 0.021$). Meanwhile, lung metastases were observed in seven of eight

[4] The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; qPCR, quantitative PCR; IHC, immunohistochemistry; miRNA, microRNA; OS, overall survival; RFS, relapse-free survival; RNP-IP, ribonucleoprotein immunoprecipitation; Vec, vector.


A
BGC-823
HGC-27
AGS
MKN-45
GSE1
β-ACTIN
136kDa
43kDa
GSE1 1.00 2.53 0.62 2.29
B
shNC
shGSE1-1
shGSE1-2
HGC-27
GSE1 1.00 0.26 0.31
C
Vec
GSE1
BGC-823
GSE1 1.00 2.16
D
Cell Number (1x10^5)
Day
HGC-27
E
Cell viability (OD570 nm)
F
No. of colonies per well
G
BGC-823
H
I
J
Migration
Migrated cells per field
K
L
Invasion
Invaded cells per field
M
N
Wound Healing
0h
12h
Wound closure (%)
O
24h

mice injected with BGC-823-GSE1 cells, whereas only three of eight mice injected with BGC-823-Vec cells exhibited metastases ($p = 0.039$). Moreover, the total number of lung micrometastases was much lower in mice injected with HGC-27-shGSE1 cells compared with mice injected with HGC-27-shNC cells ($p < 0.05$), whereas the number of lung micrometastases was much higher in mice injected with BGC-823-GSE1 cells compared with mice injected with BGC-823-Vec cells ($p < 0.01$). (Fig. 3, *G* and *H*). Hence, GSE1 also promotes tumor metastasis of gastric cancer cells *in vivo*.

GSE1 regulates the expression of SLC7A5 in gastric cancer cells

To identify the downstream mechanism of GSE1 in human gastric cancer cells, we performed mRNA sequencing in HGC-27-shNC and HGC-27-shGSE1-1 cells (designated as HGC-27-shGSE1) to search for genes potentially regulated by GSE1. As shown in Fig. 4*A*, the expression of *PER3*, *CTH*, *LAPTM5*, *DPM3*, *RAB3GAP2*, *AKR1C2*, and *SLC7A5* decreased significantly, and the expression of *TNFRSF1B*, *TINAGL1*, *ADC*, *IFI44L*, *IFI44*, *PALMD*, and *CSF1* increased significantly after transfection with shGSE1-1 in HGC-27 cells. Among these genes, *SLC7A5* showed the greatest reduction after depletion of GSE1. This is consistent with reports that SLC7A5 contributes to gastric cancer malignant behavior (8, 10).

To confirm mRNA-sequencing results, protein levels of SLC7A5 were examined in HGC-27 and MKN-45 cells after transfection with shGSE1 or shNC and in BGC-823 and AGS cells after transfection with *GSE1*-expressing plasmid or vector control plasmid. The observations were consistent with previous results, whereby GSE1 and protein levels of SLC7A5 decreased significantly after transfection with shGSE1 compared with shNC both in HGC-27 and MKN-45 cells (Fig. 4, *B* and *C*). Concordantly, both GSE1 and SLC7A5 increased dramatically after forced expression of GSE1 compared with vector-transfected control cells (Fig. 4, *D* and *E*). As such, this suggests that GSE1 positively regulates the expression of SLC7A5 in human gastric cancer cells.

GSE1 increased SLC7A5 transcript stability through a post-transcriptional mechanism

RT-quantitative PCR (qPCR) and mRNA decay assays were performed to determine the mechanism of regulation of SLC7A5 by GSE1 in HGC-27 and MKN-45 cells after transfection with shGSE1 or shNC and in BGC-823 and AGS cells after transfection with *GSE1*-expressing plasmid or vector control plasmid. As shown in Fig. 5*A*, shGSE1 dramatically decreased the mRNA levels of *SLC7A5* in both HGC-27 and MKN-45 cells compared with shNC. Moreover, the decay rate of *SLC7A5* mRNA dramatically increased after transfection with shGSE1 compared with shNC in both HGC-27 and MKN-45 cells (Fig. 5*B*). Concordantly, the mRNA levels of *SLC7A5* increased significantly after transfection with *GSE1*-expressing plasmid compared with vector control in both BGC-823 and AGS cells (Fig. 5*C*), and forced expression of GSE1 retarded *SLC7A5* mRNA decay compared with vector control (Fig. 5*D*). Therefore, GSE1 positively regulated the expression of SLC7A5 in human gastric cancer cells by increasing transcript stability in a post-transcriptional manner.

In addition, a ribonucleoprotein immunoprecipitation (RNP-IP) assay was performed using anti-GSE1 antibody, and the mRNA levels of *SLC7A5* and negative control gene *HER2* were detected. There was no significant change of *SLC7A5* mRNA enriched between anti-GSE1 antibody and control IgG, and similarly with the negative control gene *HER2*. There was also no significant difference in *SLC7A5* and *HER2* mRNA total levels between the anti-GSE1 antibody group and IgG group (Fig. 5*E*). Therefore, we excluded the possibility that *SLC7A5* mRNA was regulated by GSE1 through a direct binding mechanism. GSE1 might therefore regulate SLC7A5 indirectly.

SLC7A5 stimulates cellular proliferation and metastasis of human gastric cancer cells

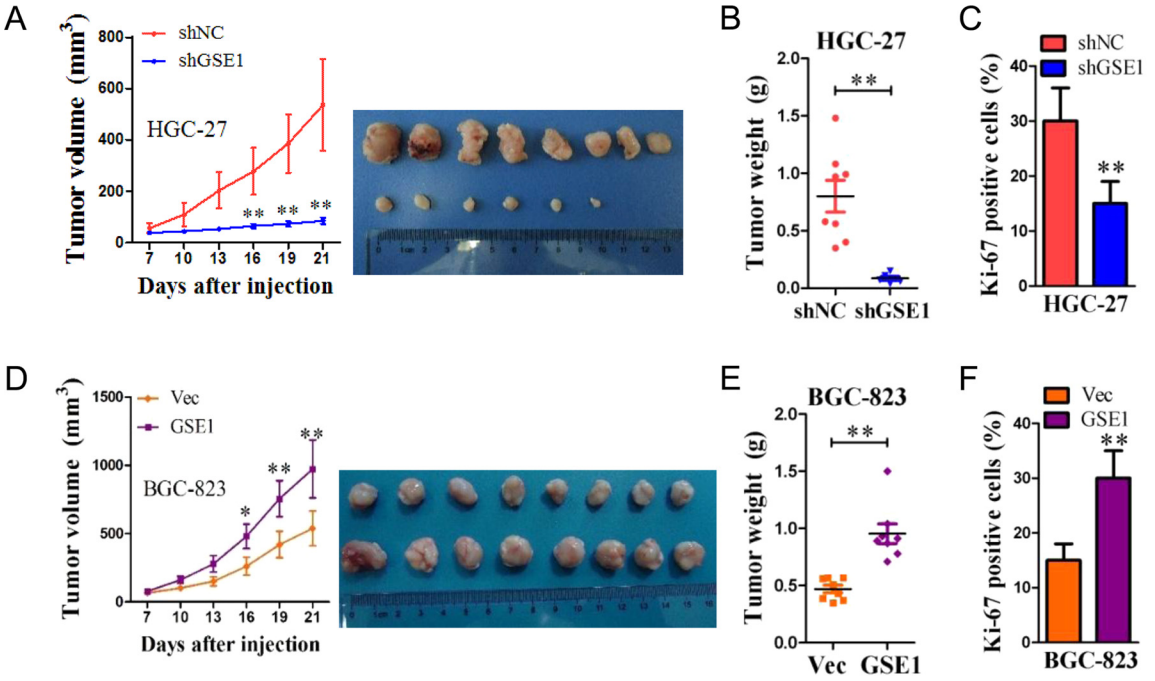
Next, we used shSLC7A5-1 and shSLC7A5-2 to deplete endogenous SLC7A5 in HGC-27 and MKN-45 cells. Protein levels of SLC7A5 decreased significantly after transfection with shSLC7A5-1 or shSLC7A5-2 in both HGC-27 and MKN-45 cells (Fig. 6*A*). The total cell number assay and MTT assay determined that cell growth and cell viability decreased significantly after depletion of SLC7A5 with shSLC7A5-1 or shSLC7A5-2 in both HGC-27 and MKN-45 cells during a period of 5 days (Fig. 6, *B* and *C*). Moreover, colony formation was reduced dramatically in HGC-27 and MKN-45 cells after transfection with shSLC7A5-1 or shSLC7A5-2 (Fig. 6*D*).

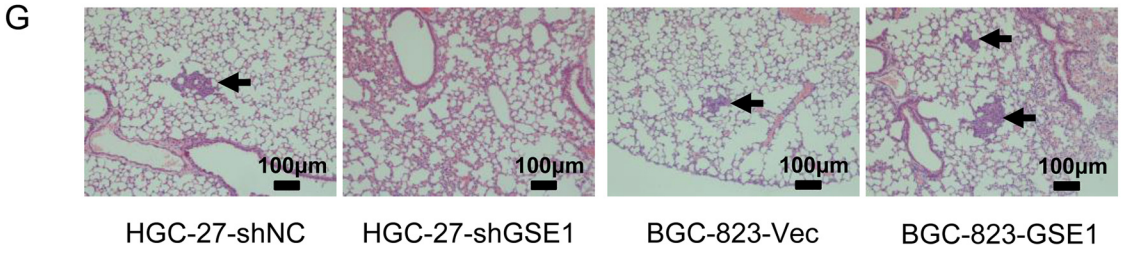
To determine the function of SLC7A5 in migration and invasion of human gastric cancer cells, cell migration, cell invasion, and wound-healing assays were carried out in HGC-27 and MKN-45 cells. After transfection with shSLC7A5-1 or shSLC7A5-2, both cell migration and invasion decreased significantly in HGC-27 and MKN-45 cells (Fig. 6, *E* and *F*). Accordingly, the depletion of SLC7A5 retarded wound closing both in HGC-27 and MKN-45 cells (Fig. 6*G*). This indicates that SLC7A5 promotes both proliferation and metastasis of human gastric cancer cells.

GSE1 stimulates oncogenic behaviors of human gastric cancer cells through SLC7A5

To determine whether GSE1-enhanced oncogenic behaviors of human gastric cancer cells were mediated by SLC7A5, cell function experiments were performed in BGC-823 and AGS

Figure 2. GSE1 promotes cell proliferation and metastasis of human gastric cancer cells. *A*, protein levels of GSE1 were examined in human gastric cancer cells BGC-823, HGC-27, AGS, and MKN-45 by using Western blotting. *B* and *C*, HGC-27 cells were transfected with shGSE1-1, shGSE1-2, or shNC. BGC-823 cells were transfected with *GSE1*-overexpressing pIRESneo3 plasmid or Vec control plasmid. Protein levels of GSE1 were examined using Western blotting. β-Actin was used as a control. In HGC-27-shNC, HGC-27-shGSE1-1, HGC-27-shGSE1-2, BGC-823-Vec, and BGC-823-GSE1 cells, a total cell number assay was performed with an original cell number of 1×10^5 and tested every day for 5 days (*D* and *G*); an MTT assay was performed to evaluate cell viability (*E* and *H*); a cell colony formation assay was performed with an original cell number of 1000 and tested after 10–15 days (*F* and *I*); cell migration and invasion were determined using a Transwell chamber (*J*–*M*); and a wound-healing assay was performed (*N* and *O*), with the photographs showing significant differences after 24 h. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.





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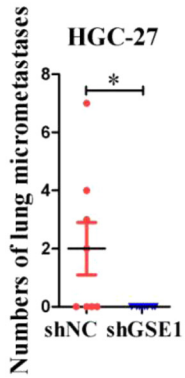
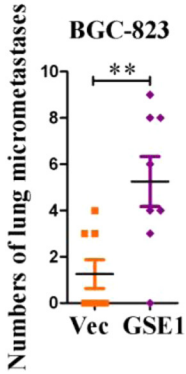
	HGC-27-shNC	HGC-27-shGSE1	*P* value χ2 test
Lung metastasis	4/8	0/8	0.021



	BGC-823-Vec	BGC-823-GSE1	*P* value χ2 test
Lung metastasis	3/8	7/8	0.039



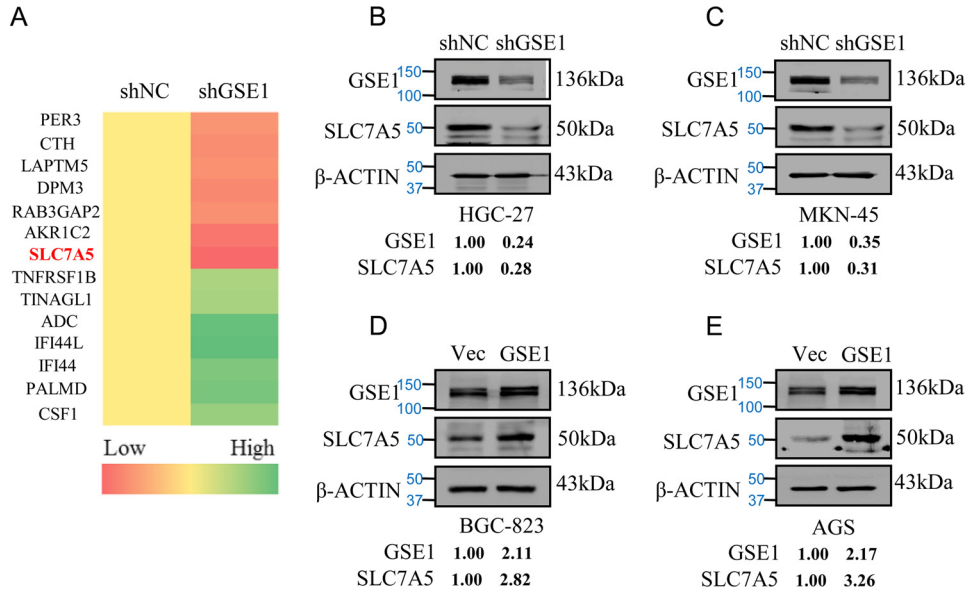


Figure 4. GSE1 regulates the expression of SLC7A5 in gastric cancer cells. *A*, heat map depicting the mRNA expression profile of mRNA sequencing in HGC-27-shNC and HGC-27-shGSE1-1 (designated as HGC-27-shGSE1) cells. *Yellow squares*, base level of mRNAs; *red squares*, down-regulated mRNAs; *green squares*, up-regulated mRNAs. *B* and *C*, protein levels of GSE1 and SLC7A5 were examined using Western blotting in HGC-27 and MKN-45 cells transfected with shGSE1-1 (designated as shGSE1) or shNC. *D* and *E*, protein levels of GSE1 and SLC7A5 were examined using Western blotting in BGC-823 and AGS cells transfected with *GSE1*-overexpressing plasmid or Vec control. *β*-actin was used as a control.

cells with forced expression of GSE1 and SLC7A5 depletion by shSLC7A5-1 (designated as shSLC7A5)/shNC and in HGC-27 and MKN-45 cells with GSE1 depletion by shGSE1-1 (designated as shGSE1)/shNC and forced expression of SLC7A5.

In BGC-823 and AGS cells, protein levels of GSE1 increased significantly after transfection with *GSE1* plasmid + shNC or *GSE1* plasmid + shSLC7A5 compared with control. Whereas protein levels of SLC7A5 increased significantly after transfection with *GSE1* plasmid + shNC compared with control, this increase was abrogated by transfection with GSE1 plasmid + shSLC7A5 (Fig. 7*A*). Consistent with previous results, cell proliferation (measured using total cell number assay), cell viability (measured using an MTT assay), cell colony formation, cell migration, cell invasion, and cell wound closing all increased significantly in both BGC-823 and AGS cells with the forced expression of GSE1. However, the depletion of SLC7A5 specifically abolished the enhanced oncogenic behaviors of BGC-823 and AGS cells as a consequence of the forced expression of GSE1 (Fig. 7, *B–G*).

In HGC-27 and MKN-45 cells, protein levels of GSE1 decreased significantly after transfection with shGSE1 + Vec plasmid or shGSE1 + *SLC7A5* plasmid compared with control. Whereas protein levels of SLC7A5 decreased significantly after transfection with shGSE1 + Vec plasmid compared with control, this decrease was abrogated by transfection with shGSE1 + *SLC7A5* plasmid (Fig. 8*A*). Consistent with previous results, cell proliferation (measured using a total cell number assay), cell viability (measured by using an MTT assay), cell colony formation, cell migration, cell invasion, and cell wound closing all decreased significantly in both HGC-27 and MKN-45 cells with the depleted expression of GSE1. However, the forced expression of SLC7A5 specifically abolished the decreased oncogenic behaviors of HGC-27 and MKN-45 cells as a consequence of the depleted expression of GSE1 (Fig. 8, *B–G*). Therefore, we conclude that GSE1 stimulates oncogenic behaviors of human gastric cancer cells associated with tumor progression partly through specific regulation of SLC7A5.

GSE1 is a direct downstream target of miR-200b and miR-200c in gastric cancer cells

To determine upstream mechanisms involved in GSE1 regulation, we used TargetScans to search for potential miRNAs directly targeting GSE1 in gastric cancer cells. MiR-200b and miR-200c were predicted to possess direct binding sites at the *GSE1* 3′-UTR (5′-CAGUAUU-3′). We generated luciferase reporter plasmids containing the *GSE1* 3′-UTR and *GSE1* 3′-UTR in which the binding site of miR-200b and miR-200c was mutated into 5′-CUCUACC-3′ (Fig. 9*A*). Fig. 9*B* showed that luciferase reporter activity in HGC-27 cells was significantly decreased after co-transfection with luciferase reporter plasmid containing *GSE1* wildtype 3′-UTR and miR-200b or miR-200c mimics compared with control. There was no significant difference in the luciferase reporter activity between the cells co-transfected with luciferase reporter plasmid containing *GSE1* mutated 3′-UTR/miR-200b or miR-200c mimics and the cells co-transfected with luciferase reporter plasmid containing *GSE1* mutational 3′-UTR/NC miRNA mimics (Fig. 9*B*). Fur-

Figure 3. GSE1 promotes tumor growth and metastasis of human gastric cancer cells in nude mice. *A* and *D*, HGC-27-shNC/HGC-27-shGSE1-1 (designated as HGC-27-shGSE1) and BGC-823-Vec/BGC-823-GSE1 cells were subcutaneously injected into nude mice. Every cell was injected at eight sites. Tumor volumes were evaluated every 3 days, and tumors were harvested and tumor growth curves were made after 21 days. *B* and *E*, weights of transplanted tumors were evaluated. *C* and *F*, Ki-67–stained percentage in sections of xenograft tumors was evaluated. *G*, HGC-27-shNC/HGC-27-shGSE1 and BGC-823-Vec/BGC-823-GSE1 cells were injected into the venous circulation of mice. After 40 days, mice were killed, and their lungs were collected. Hematoxylin and eosin–stained sections showed the lung micrometastases as indicated by *arrows*. *H*, numbers of mice with lung micrometastases of tumors and numbers of lung micrometastases in five random sections of each mouse were calculated. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

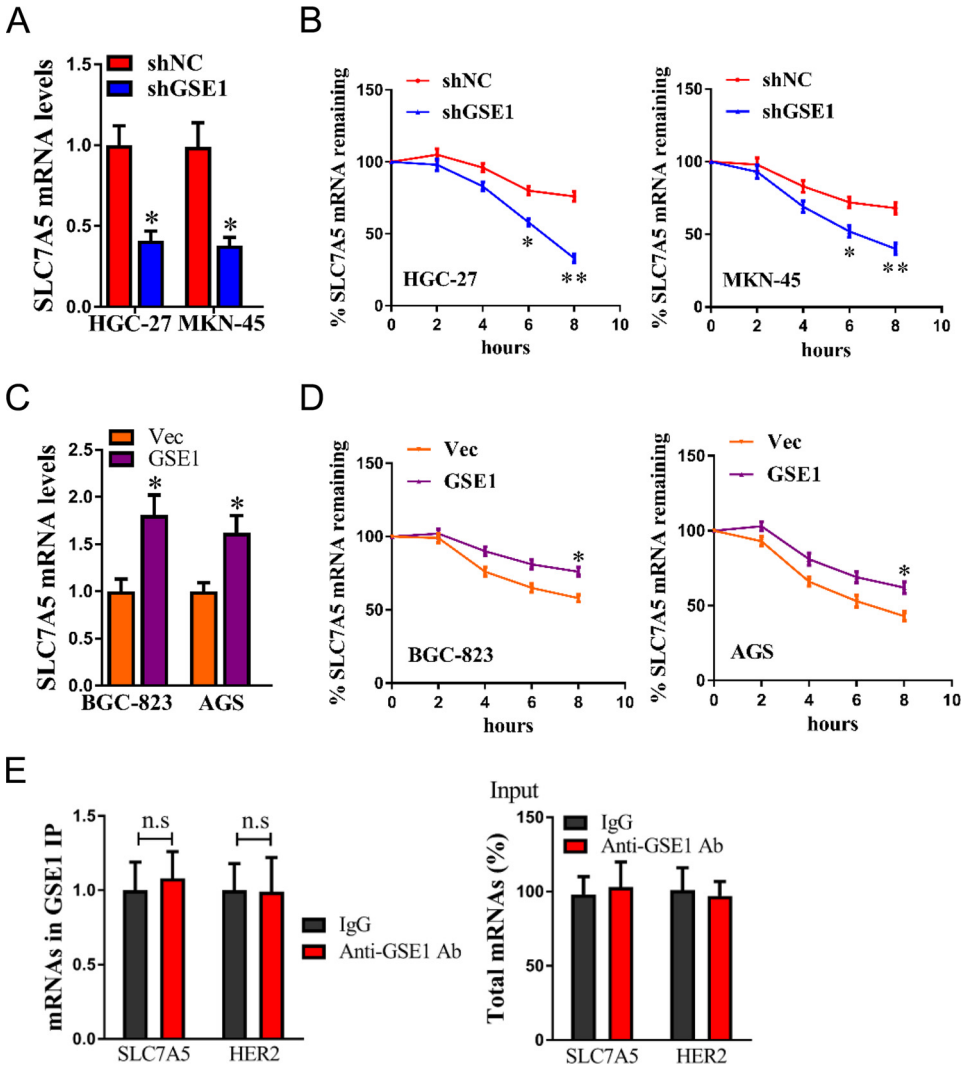


Figure 5. GSE1 increased SLC7A5 transcript stability through a post-transcriptional manner. *A*, mRNA levels of *SLC7A5* after transfection with shGSE1 and shNC in HGC-27 and MKN-45 cells were examined using RT-qPCR. *B*, an mRNA decay assay was performed in HGC-27 and MKN-45 cells after transfection with shGSE1 and shNC. mRNA levels of *SLC7A5* were detected using RT-qPCR at 0, 2, 4, 6, and 8 h after actinomycin D (10 μg/ml) treatment. *C*, mRNA levels of *SLC7A5* after transfection with *GSE1*-overexpressing plasmid or Vec control in BGC-823 and AGS cells were examined using RT-qPCR. *D*, mRNA decay assay in BGC-823 and AGS cells after transfection with *GSE1*-overexpressing plasmid or Vec control. *GAPDH* was used as a control for RT-qPCR. *E*, RNP-IP assay. mRNAs of *SLC7A5* and negative control gene *HER2* were enriched by anti-GSE1 antibody or control IgG and were analyzed by RT-qPCR. The total input mRNAs of *SLC7A5* and *HER2* were also examined. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

thermore, both miR-200b and miR-200c decreased the protein expression of GSE1 in HGC-27 and MKN-45 cells (Fig. 9*C*). Hence, miR-200b and miR-200c regulated the expression of GSE1 through binding sites in the 3′-UTR of *GSE1*.

Expression of SLC7A5, miR-200b, and miR-200c in human gastric cancer tissues and normal gastric tissues

We next examined the expression of SLC7A5 in the cohorts of 100 human gastric cancer tissues and 100 normal gastric tissues using immunohistochemistry. Immunoreactive SLC7A5 protein was mainly located in the cytoplasm with membranous enhancement of gastric cancer cells and glandular epithelial cells. A higher expression of SLC7A5 was observed in gastric cancer tissues compared with normal gastric tissues (gastric cancer tissues: 20% negative, 32% low, 30% moderate, 19% high; normal gastric tissues: 48% negative, 36% low, 10% moderate, 6% high ($p < 0.001$)) (Fig. 10*A* and Table 3). miR-200b and miR-200c expression levels were also examined in fresh gastric cancer tissues and normal gastric tissues using RT-qPCR. As shown in Fig. 10*B*, the expression of both miR-200b and miR-200c were much lower in gastric cancer tissues compared with normal gastric tissues (both $p < 0.01$).

Moreover, we analyzed the correlation between GSE1 and SLC7A5 protein levels in the 100 paraffin-embedded gastric cancer tissues (Table 4) and the correlation between *GSE1* mRNA and miR-200b/miR-200c levels in the 60 fresh gastric cancer tissues (Fig. 10*C*). There was a statistically positive correlation between GSE1 and SLC7A5 expression in gastric cancer tissues ($p = 0.003$, Pearson's correlation coefficient = 0.297) (Table 4). In contrast, *GSE1* and miR-200b expression were negatively correlated in fresh gastric cancer tissues ($p < 0.0001$, Pearson's correlation coefficient = −0.65047). *GSE1* and miR-200c expression were also negatively correlated in these gastric cancer tissues ($p < 0.0001$, Pearson's correlation coefficient = −0.54332) (Fig. 10*C*).

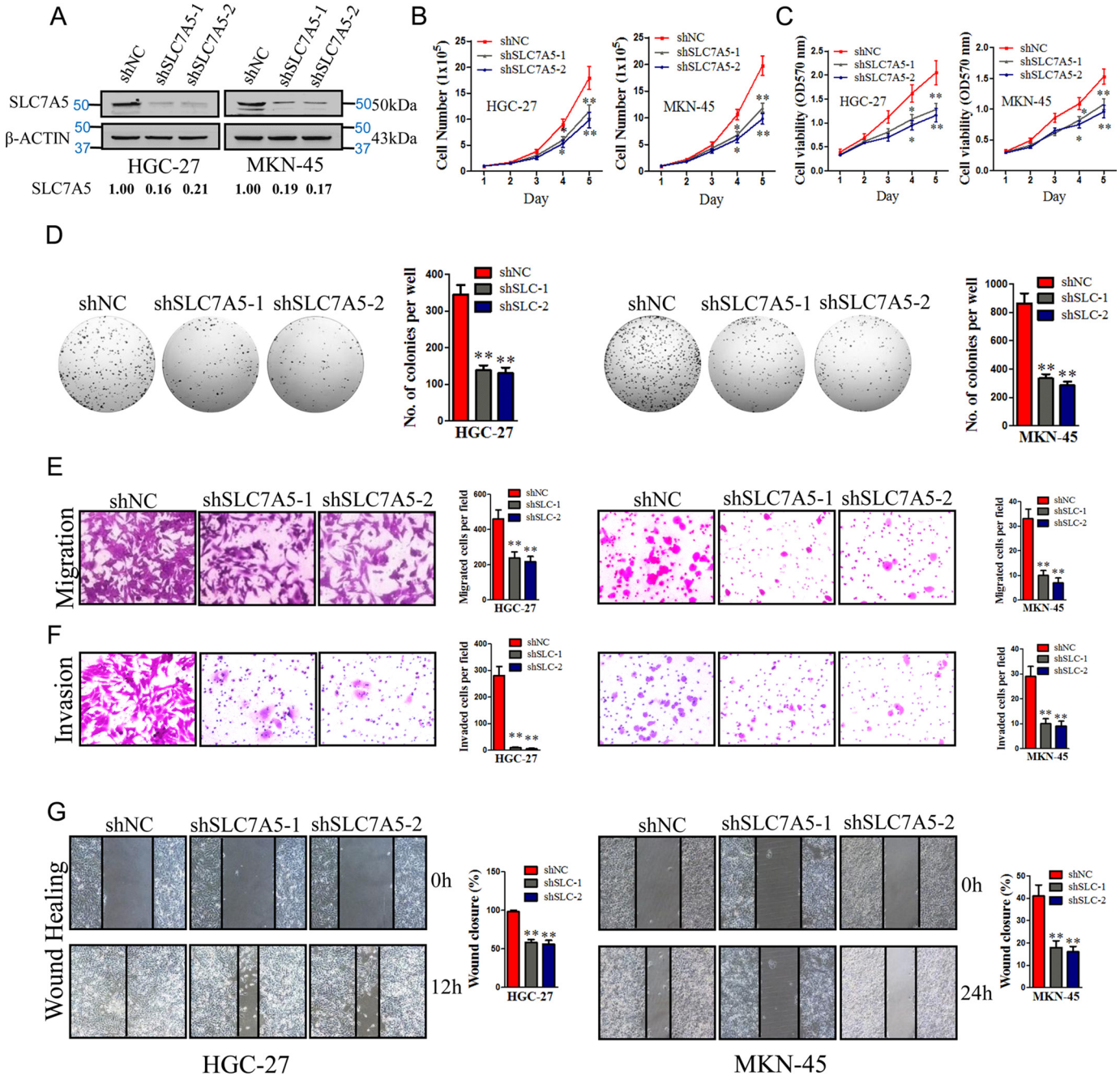


Figure 6. SLC7A5 promotes cell proliferation and metastasis of human gastric cancer cells. HGC-27 and MKN-45 cells were transfected with shGSE1-1, shGSE1-2, or shNC. *A*, protein levels of SLC7A5 were examined using Western blotting. β-Actin was used as a control. A total cell number assay (*B*), MTT assay (*C*), and cell colony formation assay (*D*) were performed to evaluate cell proliferation. A cell migration assay (*E*), cell invasion assay (*F*), and wound-healing assay (*G*) were carried out to evaluate cell metastasis. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

Discussion

Herein, we systematically examined the functional role of GSE1 in human gastric cancer. The forced expression of GSE1 in gastric cancer cells was observed to promote cell proliferation, colony formation, migration, and invasion *in vitro* and increase tumor growth and metastasis in xenograft models. Concordantly, the depletion of GSE1 in gastric cancer cells reduced the oncogenic properties of gastric cancer cells both *in vitro* and *in vivo*. Moreover, the expression of GSE1 protein was higher in gastric cancer tissues compared with normal gastric tissues, and the expression level of GSE1 positively associated with lymph node metastasis, histological grade, depth of invasion, and clinical stage in gastric cancer patients. High levels of GSE1 were also associated with both low relapse-free survival (RFS) and overall survival (OS) rates of gastric cancer patients. As reported previously, GSE1 possesses an oncogenic function in human breast cancer cells with promotion of both cell proliferation and metastasis. Moreover, the expression level of GSE1 was observed to be increased in breast cancer tissues and predicted poor prognosis in breast cancer patients (7). These published results are consistent with our current findings. To the best of our knowledge, this study is the first to

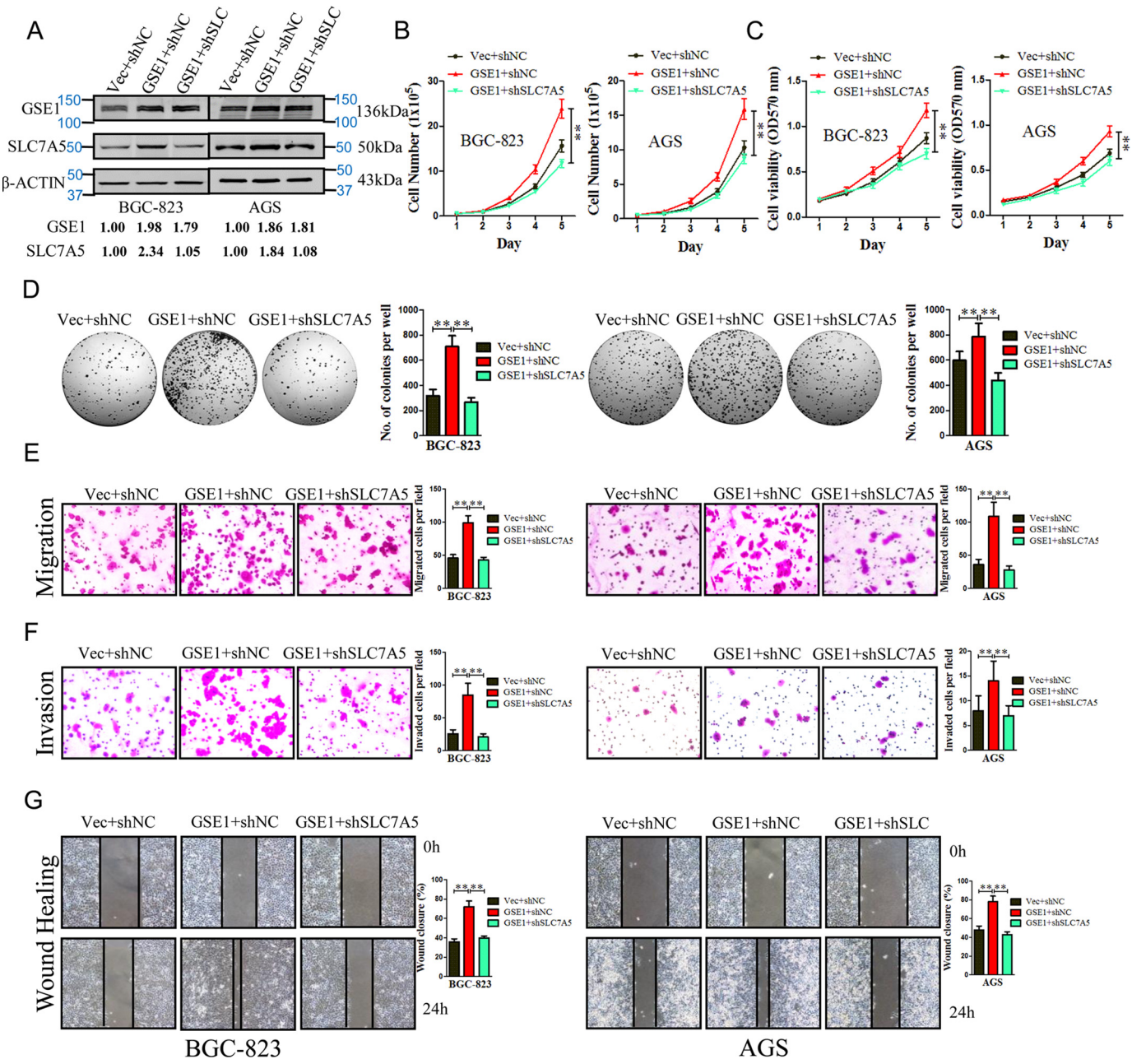


Figure 7. SLC7A5 mediates the oncogenic role of GSE1 in human gastric cancer cells (BGC-823 and AGS). BGC-823 and AGS cells with forced expression of GSE1 were co-transfected with shSLC7A5-1 (designated as shSLC7A5) or shNC. *A*, protein levels of GSE1 and SLC7A5 were examined using Western blotting. *β*-Actin was used as a control. A total cell number assay (*B*), MTT assay (*C*), and cell colony formation assay (*D*) were performed to evaluate cell proliferation. A cell migration assay (*E*), cell invasion assay (*F*), and wound-healing assay (*G*) were carried out to evaluate cell metastasis. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

report that GSE1 possesses an oncogenic role in human gastric cancer.

SLC7A5 was observed to be positively regulated by GSE1 and to partly mediate the functions of GSE1 in gastric cancer. Consistent with our results, Wang *et al.* (8) have reported previously that suppression of the expression of SLC7A5 in the MKN-45 gastric cancer cell line dramatically decreased cell proliferation, migration, and invasion. Furthermore, it has been reported that SLC7A5 expression was positively regulated by CRKL and mediated the oncogenic role of CRKL in the gastric cancer cell line SGC-7901 (11). SLC7A5 was also reported to be a direct target of miR-126 and to mediate the tumor-suppressive function of miR-126 in MKN-45 gastric cancer cells (10). Furthermore, we have observed that the protein level of SLC7A5 was significantly higher in gastric cancer tissues compared with normal gastric tissues. GSE1 and SLC7A5 were positively correlated in gastric cancer tissues. Ichinoe *et al.* (12) reported that gastric cancer cases with lymph node metastasis exhibit significantly higher SLC7A5 expression than cases without lymph node metastasis, and high expression of SLC7A5 correlated with high Ki-67 level and a significantly poorer prognosis compared with the low-SLC7A5 group. SLC7A5 expression has also been reported to be significantly associated with clinicopathologic features such as tumor size, lymph node metastasis, local invasion, and TNM stage in gastric cancer patients (8). These reports are concordant with our data herein. In addition,

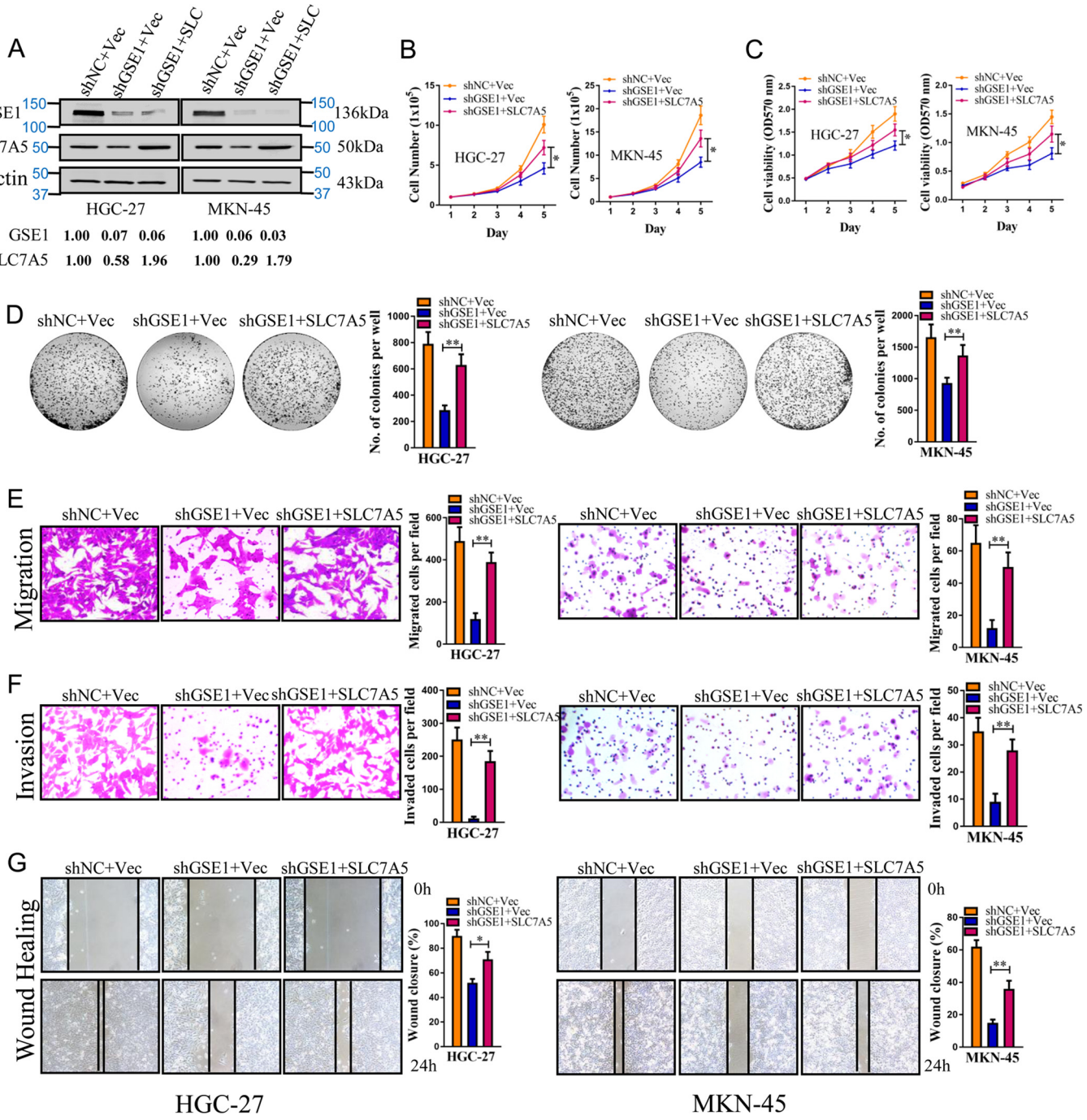


Figure 8. SLC7A5 mediates the oncogenic role of GSE1 in human gastric cancer cells (HGC-27 and MKN-45). HGC-27 and MKN-45 cells were co-transfected with shGSE1-1 (designated as shGSE1)/shNC and *SLC7A5* plasmid/Vec plasmid. *A*, protein levels of GSE1 and SLC7A5 were examined using Western blotting. *β*-Actin was used as a control. A total cell number assay (*B*), MTT assay (*C*), and cell colony formation assay (*D*) were performed to evaluate cell proliferation. A cell migration assay (*E*), cell invasion assay (*F*), and wound-healing assay (*G*) were carried out to evaluate cell metastasis. *, $p < 0.05$; **, $p < 0.01$. *Error bars*, S.D.

SLC7A5 was also reported to promote growth of human breast cancer cells (13). Furthermore, Furuya *et al.* (14) documented that SLC7A5 expression correlated with tumor size, nuclear grade, and Ki-67 labeling index in tissues from breast cancer patients. In T-cell lymphoblastic lymphoma/T-cell acute lymphoblastic leukemia, SLC7A5 supports tumor cell growth and survival (15). Moreover, SLC7A5 acts as a tumor promoter in other human cancers, including non-small cell lung cancer (16), colon cancer (17), prostate cancer (18), and cutaneous melanoma (19). In a complex study of various human cancers, Kaira *et al.* (20) reported that SLC7A5 correlated significantly with cell proliferation and angiogenesis at primary and metastatic sites of human neoplasms. Hence, SLC7A5 is an important tumor promoter in human cancers, and it mediates the oncogenic role of GSE1 in human gastric cancer cells. Moreover, we attempted to delineate the mechanisms for the regulation of SLC7A5 by GSE1 and determined that GSE1 increased the transcript stability of SLC7A5 in a post-transcriptional manner not dependent on direct binding.

MiR-200b and miR-200c were herein demonstrated to directly target and suppress the expression of GSE1. In this

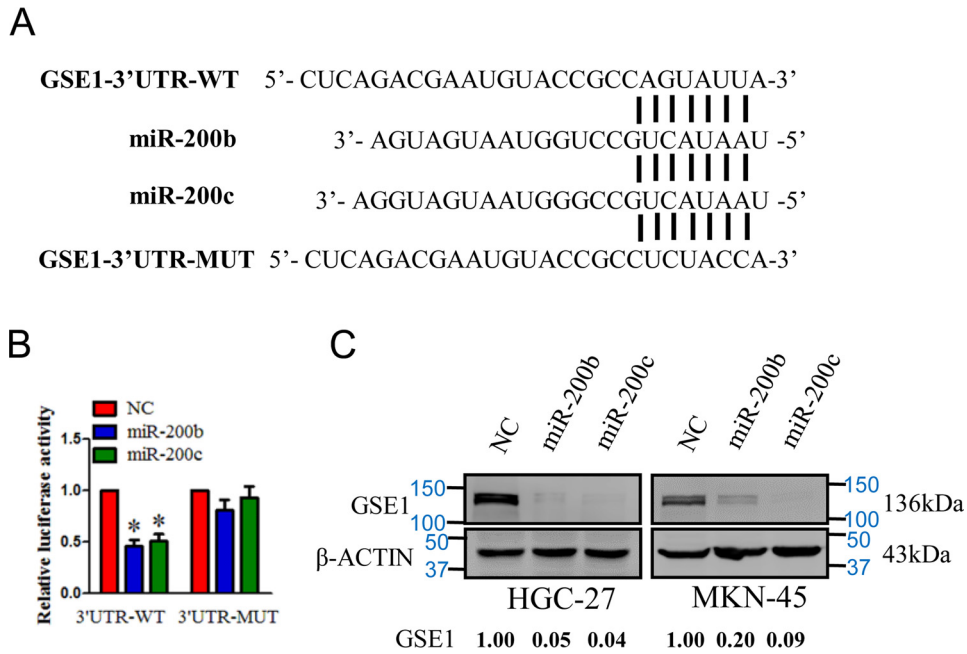


Figure 9. GSE1 is a direct target of miR-200b and miR-200c in gastric cancer cells. *A*, predicted binding site and mutant binding site between miR-200b/miR-200c and the 3′-UTR of *GSE1*. *B*, luciferase assay of HGC-27 cells co-transfected with miR-200b/miR-200c mimics and a luciferase reporter– containing *GSE1* wildtype 3′-UTR (*3′ UTR-WT*) or *GSE1* mutant 3′-UTR (*3′ UTR-MUT*). *C*, protein levels of GSE1 were examined using Western blotting in HGC-27 and MKN-45 cells transfected with miR-200b, miR-200c, and NC mimics. *Error bars*, S.D.

study, the expression levels of miR-200b and miR-200c were observed to be dramatically lower in gastric cancer tissues compared with normal gastric tissues. Moreover, in gastric cancer tissues, expression levels of *GSE1* and miR-200b/miR-200c were significantly and inversely correlated. As now established, miRNAs play important roles in tumor initiation, development, and progression, including in human gastric cancer (21–25). For example, miR-27a, miR-520c, and miR-224 possess oncogenic roles in human gastric cancer (26–28), whereas miR-132–3p, miR-134, and miR-29c exhibit tumor-suppressive functions in human gastric cancer (29–31). Herein, we identified that miR-200b and miR-200c potentially suppress the oncogenic behaviors of human gastric cancer cells by targeting GSE1. It has been previously reported that miR-200b decreases cell proliferation, invasion, and migration by targeting ZEB2 in human gastric cancer (32); miR-200c also suppresses gastric cancer progression by targeting ZEB1/2 and FN1 (33, 34). Our findings are consistent with these observations. Moreover, Chang *et al.* (35) reported that the expression levels of the miR-200 family, including miR-200b and miR-200c, were significantly lower in clinical samples of gastric cancer compared with paired non-cancerous tissues. Lower levels of miR-200b and miR-200c were associated with high histological grade and the presence of an intravascular cancer embolus (35). This report is also concordant with our data herein. In addition, miR-200b and miR-200c also contribute to tumor suppression in other human cancers, such as colon cancer, pancreatic cancer, breast cancer, and prostate cancer (36–42). Therefore, miR-200b and miR-200c are tumor suppressors that, at least in gastric cancer, act partly by decreasing the expression of GSE1.

In summary, we have shown for the first time the functional roles of GSE1 in human gastric cancer cells *in vitro* and in xenograft models *in vivo*. High expression of GSE1 correlated with poor survival outcome for gastric cancer patients. GSE1 was identified as a direct target of miR-200b and miR-200c, and increased SLC7A5 expression was demonstrated to mediate the oncogenic functions of GSE1. GSE1 is therefore a potential diagnostic and therapeutic target in human gastric cancer.

Experimental procedures

Clinical samples

One hundred paraffin-embedded gastric cancer tissues and 100 normal gastric tissues were collected at the Department of Pathology in the First Affiliated Hospital of Anhui Medical University (Hefei, Anhui, China). These tissues were from patients who underwent surgical resection between 2009 and 2010. Patients with other diseases or patients who underwent special therapies before surgery were excluded. The clinical- pathological parameters of these patients were determined according to the 2003 World Health Organization classification system. These gastric cancer patients were followed up for more than 5 years, and the deadline was December 2, 2016. Another 60 fresh gastric cancer tissues and 60 fresh normal gastric tissues were collected for the miRNA and mRNA study. These tissues were from patients who underwent surgical resection in 2016. This study plan was approved by the institutional review boards of Anhui Medical University and was carried out in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki). All patients had signed an informed consent form.

Immunohistochemistry (IHC)

IHC analyses of GSE1 and SLC7A5 protein expression in paraffin sections of tissues from gastric cancer patients and Ki-67 protein expression in paraffin sections of tumors formed from HGC-27-shNC/HGC-27-shGSE1 and BGC-823-Vec/BGC-823-GSE1 cells in mice were carried out using the Ultra

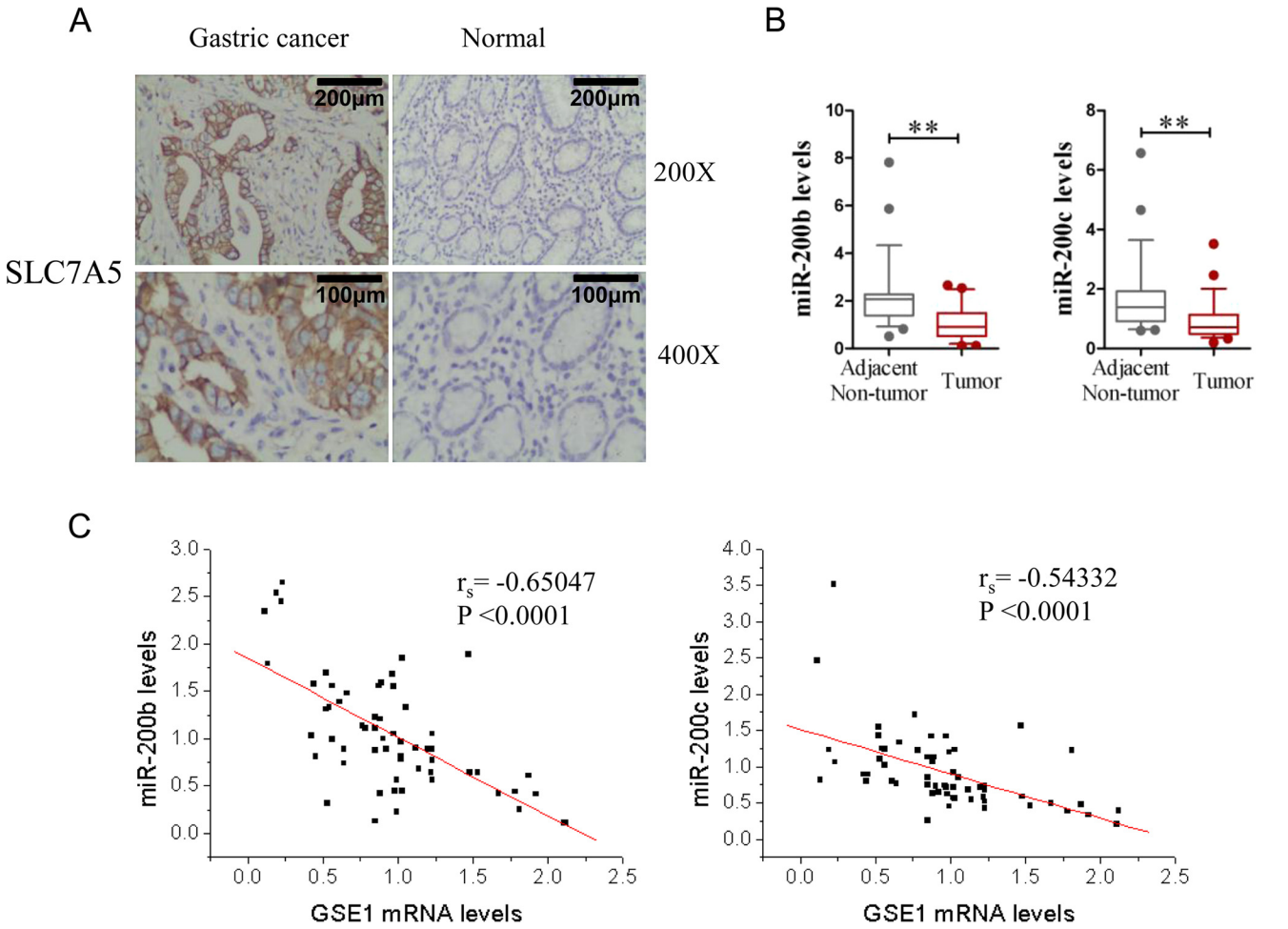


Figure 10. SLC7A5 and miR-200b/miR-200c expression levels in tissues from gastric cancer patients. *A*, protein levels of SLC7A5 in gastric cancer tissues and normal gastric tissues were examined using immunohistochemistry. The magnifications of the photographs were ×200 and ×400. *B*, miR-200b/miR-200c expression levels in gastric cancer tissues and normal gastric tissues were examined using RT-qPCR. *C*, *GSE1* mRNA levels in fresh gastric cancer tissues were examined using RT-qPCR, and the correlation between *GSE1* mRNA and miR-200b/miR-200c levels was analyzed. **, $p < 0.01$. *Error bars*, S.D.

Table 3
Expression of SLC7A5 in gastric cancer and normal tissues
*, $p < 0.001$.

Group	*n*	SLC7A5 expression			
		None	Low	Moderate	High
Gastric cancer	100	20	32	30	18
Normal	100	48	36	10	6

Sensitive-SP kit (Maixin-Bio, Fuzhou, China) as described in our previous study (43, 44). Antibodies used in this study were rabbit polyclonal antibody against GSE1, rabbit polyclonal antibody against SLC7A5 (both 1:100; Proteintech Group, Inc., Chicago, IL), and mouse polyclonal antibody against Ki-67 (1:1; Zhongshan Goldenbridge Biotechnology Co., Beijing, China). The stained sections were evaluated using an Olympus microscope (Olympus America, Inc., Melville, NY). For examination of the protein levels of GSE1 and SLC7A5 in patient tissues, one sample for each patient was utilized. The IHC-stained sections were reviewed and scored independently by two experienced pathologists who had no knowledge of the patients' identities or clinical status, and both pathologists had similar accuracy rates. Expression levels of GSE1 and SLC7A5 in patients' tissues were evaluated using the 0, 1, 2, 3 scoring system. Sections with 0% stained cells were designated as no expression of GSE1 or SLC7A5 (score of 0); sections with 1–33% stained cells were designated as low expression of GSE1 or SLC7A5 (score of 1); sections with 34–66% stained cells were designated as moderate expression of GSE1 or SLC7A5 (score of 2); sections with 67–100% stained cells were designated as high expression of GSE1 or SLC7A5 (score of 3). In Kaplan–Meier analysis, scores of 0 and 1 of GSE1 were designated as low expression of GSE1; scores of 2 and 3 were designated as high expression of GSE1.

Cell lines and cell culture

Human gastric cancer cell lines BGC-823, HGC-27, AGS, and MKN-45 were used in this study. All cells were obtained from ATCC (American Type Culture Collection) (Manassas, VA) and cultured at 37 °C in a humidified atmosphere of 5% CO_2 as recommended.

Transfection of plasmid constructs and RNA oligonucleotides

In this study, we used mammalian expression vector pIRESneo3 (Invitrogen) to construct *GSE1*- and *SLC7A5*-overexpressing plasmids. *GSE1* coding sequence transcript (GenBank™ accession number NM_001134473.2) was cloned into

Table 4
Correlations of GSE1 and SLC7A5 expression in gastric cancer patients
Shown is a GSE_1_2 × SL_1 cross-tabulation.

GSE_1_2	SL_1				Total
	None	Low	Moderate	High	
None					
Count	2	11	2	2	17
Percentage within GSE_1_2	11.8	64.7	11.8	11.8	100.0
Percentage within SL_1	10.0	34.4	6.7	11.1	17.0
Percentage of total	2.0	11.0	2.0	2.0	17.0
Low					
Count	12	6	8	1	27
Percentage within GSE_1_2	44.4	22.2	29.6	3.7	100.0
Percentage within SL_1	60.0	18.8	26.7	5.6	27.0
Percentage of total	12.0	6.0	8.0	1.0	27.0
Moderate					
Count	4	10	14	9	37
Percentage within GSE_1_2	10.8	27.0	37.8	24.3	100.0
Percentage within SL_1	20.0	31.3	46.7	50.0	37.0
Percentage of total	4.0	10.0	14.0	9.0	37.0
High					
Count	2	5	6	6	19
Percentage within GSE_1_2	10.5	26.3	31.6	31.6	100.0
Percentage within SL_1	10.0	15.6	20.0	33.3	19.0
Percentage of total	2.0	5.0	6.0	6.0	19.0
Total					
Count	20	32	30	18	100
Percentage within GSE_1_2	20.0	32.0	30.0	18.0	100.0
Percentage within SL_1	100.0	100.0	100.0	100.0	100.0
Percentage of total	20.0	32.0	30.0	18.0	100.0

pIRESneo3 and was designated as pIRESneo3-GSE1. *SLC7A5* coding sequence transcript (GenBank™ accession number NM_003486.6) was cloned into pIRESneo3 and was designated as pIRESneo3- SLC7A5. shRNAs (including shGSE1-1, shGSE1-2, shSLC7A5-1, and shSLC7A5-2) and miR-200b/miR-200c mimics were synthesized by GenePharma (Shanghai, China). As described previously (43, 44), all plasmids, shRNAs, and miRNA mimics were transfected using lip2000 (Qiagen). Specific shRNA was used: shGSE1-1, 5′-GCCUACAUCUAUGAUGAGUTT-3′; shGSE1-2, 5′-GAGAUGAACAACAGUCCCATT-3′; shSLC7A5-1, 5′-GGAAGGGUGAUGUGUCCAATT-3′; shSLC7A5-2, 5′-GCAUUAUACAGCGGCCUCUTT-3′.

Western blot analysis

Western blot analysis was performed as described in our previous studies (43, 44). Rabbit polyclonal antibody against GSE1, rabbit polyclonal antibody against SLC7A5 (both 1:1000; Proteintech Group, Inc., Chicago, IL), and mouse β-actin monoclonal antibody (1:5000; Sigma) were used.

Cell proliferation and metastasis assays

Cell proliferation assays, including total cell number assay, MTT assay, and colony-formation assay, were performed essentially as described previously (43–45). Briefly, in the total cell number assay, cells were seeded into 6-well plates with an original cell number of 1×10^5 and subsequently counted daily for 5 days. In the MTT assay, cells were seeded into 96-well plates with an original cell number of 2000, and cell viability was tested ($A_{570\,nm}$) every day for 5 days. In the colony-formation assay, cells were seeded into 6-well plates with an original cell number of 1000 and tested after 10–15 days. Meanwhile, cell metastasis assays, including the cell migration assay, cell invasion assay, and wound-healing assay, were also performed as described previously (43–45). For the migration assay, HGC-27–derived cells were tested after 18 h; BGC-823–derived cells, MKN-45–derived cells, and AGS-derived cells were tested after 24 h. For the invasion assay, HGC-27–derived cells were tested after 24 h; BGC-823–derived cells, MKN-45–derived cells, and AGS-derived cells were tested after 48 h. For the wound-healing assay, pictures were shot at 0 h and after 24 h.

Xenograft analyses

HGC-27-shNC/HGC-27-shGSE1-1 (designated as HGC-27-shGSE1) and BGC-823-Vec/BGC-823-GSE1 cells (500×10^4 cells/125 μl/site) were subcutaneously injected into the dorsal flank of 4-week-old BALB/c-nu/nu mice (Shanghai Slaccas Co., Shanghai, China). Each cell was injected into eight sites, and palpable tumors formed after about a week. The length and width of the tumors were measured every 3 days. Tumor volume was calculated according to the formula, volume (mm^3) = $L \times W^2 \times \Pi/6$ (46). After 21 days, mice were sacrificed, and tumors were harvested. Weights of the tumors were measured, and Ki-67 protein expression in paraffin sections of these tumors was examined using IHC.

For tail-vein injection, HGC-27-shNC/HGC-27-shGSE1 and BGC-823-Vec/BGC-823-GSE1 cells (500×10^4 cells in 250 μl of PBS) were injected directly into the lateral tail vein of 4-week-old BALB/c-nu/nu mice. Every group contained eight mice. After 40 days, mice were sacrificed, and their lungs were harvested and prepared for histologic examination. Five random hematoxylin and eosin–stained sections of each mouse lung were reviewed and examined for lung micrometastases from injected tumor cells. The exact number of micrometastases in each mouse lung of the five random sections was also calculated.

Experiments on animals in this study were carried out according to the institutional animal care and use committee guidelines. Local institutional approval from the Animal Ethics Committee of Anhui Medical University was obtained before commencement of work (approval LLSC20160331).

mRNA sequencing

mRNA sequencing in HGC-27-shNC and HGC-27-shGSE1-1 cells (designated as HGC-27-shGSE1) to screen differentially expressed mRNAs was performed at Kangcheng Biotechnology Co. (Shanghai, China).

Luciferase reporter assay

A luciferase reporter assay was carried out to examine the targeted regulation of miR-200b and miR-200c on GSE1 in gastric cancer cells and was performed as described in our previous publications using the Dual-Luciferase reporter assay system (Promega Corp.) (44, 47). Both *Renilla* luciferase activity and firefly luciferase activity were measured in each sample. Firefly luciferase activity was used as a base control.

RT-qPCR

miRNAs were isolated from fresh tissues of gastric cancer patients. miR-200b and miR-200c expression levels were exam-

ined using RT-qPCR. As described previously, this was performed using TaqMan® microRNA assays (Applied Biosystems, Foster City, CA) (44, 46). *U6* was used as an endogenous miRNA control. mRNAs were isolated from cells after transfection for 48 h or directly from human tissues. mRNA levels of *GSE1* and *SLC7A5* were examined by RT-qPCR using SYBR Green Master Mix (Applied Biosystems) as described previously (44, 47). *GAPDH* was used as an endogenous control. The primers used were as follows: miR-200b, 5′-GCCGCTAATACTGCCTGGTAAT-3′ (forward) and 5′-GTGCAGGGTCCGAGGT-3′ (reverse); miR-200c, 5′-GCCGCTAATACTGCCGGGTAATG-3′ (forward) and 5′-GTGCAGGGTCCGAGGT-3′ (reverse); *U6*, 5′-TGGAACGATACAGAGAAGATTAGCA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse); *GSE1*, 5′-AGAGCACCACCAGGCCAGGAC-3′ (forward) and 5′-CGTGGGTGCAGCATGGAGC-3′ (reverse); *SLC7A5*, 5′-CCTGCCTGTGTTCTTCATCCTG-3′ (forward) and 5′-GACCACCTGCATGAGCTTCTG-3′ (reverse); *GAPDH*, 5′-TGCACCACCAACTGCTTAGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse).

mRNA decay assay

mRNA decay assay was performed to detect the interaction between GSE1 protein and *SLC7A5* mRNA. HGC-27, MKN-45, BGC-823, and AGS cells were treated with actinomycin D (10 μg/ml) 48 h after shGSE1/*GSE1*-expressing plasmid transfection, and then the cells were harvested at 0, 2, 4, 6, and 8 h after actinomycin D treatment. mRNA levels of *SLC7A5* were determined using RT-qPCR. *GAPDH* was used as an endogenous control.

RNP-IP RT-PCR

The binding between GSE1 protein and *SLC7A5* mRNA was examined using an RNP-IP RT-PCR assay as described previously (48). The GSE1 protein-*SLC7A5* mRNA complex was captured by anti-GSE1 antibody, and the *SLC7A5* mRNA was examined using RT-qPCR. IgG and *HER2* mRNA were used as negative controls.

Statistical analyses

At least three replicates were performed for each experiment, and the results represented the average. RT-qPCR, luciferase reporter assay, cell proliferation and metastasis assays, tumor growth in xenograft, and expression of miR-200b/miR-200c in tissues were analyzed using an unpaired two-tailed *t* test. Immunohistochemistry and clinicopathological parameter studies were analyzed using Pearson's χ^2 test. RFS and OS rate in patients were analyzed through Kaplan–Meier curves, and the significance of the differences was analyzed using a log-rank test. It was considered statistically significant when $p < 0.05$.

Author contributions—K.D., S.T., W.W., and Z.W. conceptualization; K.D., S.T., X.H., X.W., X.L., and W.W. data curation; K.D., S.T., X.H., X.W., X.L., P.L., W.W., and Z.W. formal analysis; K.D., S.T., X.H., W.W., and Z.W. funding acquisition; K.D., S.T., X.H., X.W., X.L., R.F., Y.Z., and W.W. investigation; K.D., S.T., and Z.W. writing-original draft.

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