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Silencing the long noncoding RNA NORAD inhibits gastric cancer cell proliferation and invasion by the RhoA/ROCK1 pathway

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Abstract. – OBJECTIVE: The current study aimed to examine the role and mechanism of a conserved long noncoding RNA termed NORAD (noncoding RNA activated by DNA damage, also named LINC00657) in gastric cancer (GC) progression.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to determine the expression level of relevant genes in GC cell lines. Cell proliferation was examined by cell counting kit-8 (CCK-8) assays. Cell migration and invasion were detected by transwell migration and invasion assays. Protein levels of the indicated genes were detected by Western blotting. Cell apoptosis was examined by flow cytometry.

RESULTS: Results showed that NORAD knockdown decreased cell proliferation, migration and invasion but increased cell apoptosis. NORAD knockdown affected the expression of genes related to apoptosis and Epithelial-Mesenchymal Transition (EMT). In addition, NORAD's depletion resulted in reduced Ras Homolog Family Member A (RhoA) and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) expression. Furthermore, NORAD's expression was positively correlated with RhoA and ROCK1 expressions in GC based on The Cancer Genome Atlas (TCGA) database.

CONCLUSIONS: Our results demonstrate the oncogenic role of NORAD in gastric cancer progression.

Key Words:
Gastric cancer, NORAD, Proliferation, Metastasis, EMT.

Introduction

Gastric Cancer (GC), characterized by a poor clinical outcome, is one of the most common gastrointestinal malignancies in East Asia and the third leading cause of cancer-related death worldwide[1-3]. Despite numerous improvements in the diagnosis and treatment of GC, the 5-year survival rate remains poor[4]. Due to the lack of early diagnosis, most patients are diagnosed with advanced-stage GC[5], but chemotherapy provides little benefit for these patients[6]. Therefore, it is essential to identify novel biomarkers and therapeutic targets for GC that could help improve patient quality of life and survival.

Long noncoding RNAs (lncRNAs), a novel class of gene regulators, are transcripts longer than 200 nucleotides that lack protein-coding potential[7]. In recent decades, many thousands of functional lncRNAs have been identified. Emerging evidence has shown that lncRNAs play critical roles in cancer's cell migration, invasion, proliferation, and survival[8]. Several lncRNAs that are dysregulated in GC have been shown to affect tumour progression. For example, LINC01606 acts as a competing endogenous RNA (ceRNA) by sponging miR-423-5p to activate the Wnt/β-catenin signaling pathway, finally leading to the suppression of GC, cell migration, and invasion[9]. EGFR-AS1 promotes GC cell growth by increasing EGFR mRNA stability[10]. The lncRNA hepatocyte nuclear factor 1 homeobox A antisense RNA 1 (HNF1A-AS1) promotes cell cycle progression by binding to miR-661[11]. However, the functions and mechanisms of lncRNAs that are critical for GC progression remain largely unknown.

Recently, a conserved lncRNA called NORAD (noncoding RNA activated by DNA Damage, also named LINC00657) was shown to play an oncogenic role in various cancers. For instance, NORAD is upregulated in bladder cancer, and its high expression indicates a poor prognosis[12]. NORAD is induced by hypoxia and promotes pancreatic cancer cell migration and invasion by functioning as a ceRNA[13]. In addition, NORAD is upregulated in oesophageal squamous cell carcinoma (ESCC) and is associated with poor prognosis[14]. However, whether and how NORAD

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participates in the development and progression of GC remains to be elucidated.

In the current study, we focused on the function of NORAD in GC; then, we examined the effects of NORAD knockdown on the proliferation, migration, and invasion of GC cells. We demonstrated that NORAD depletion resulted in reduced RhoA and Rho-associated coiled-coil containing protein kinase 1 (ROCK1) expression. Furthermore, NORAD expression was positively correlated with RhoA and ROCK1 expression in GC based on the TCGA database. Our study indicates that NORAD may be a potential novel diagnostic and therapeutic target for GC.

Materials and Methods

Patient Specimens

Fresh GC tissue samples, tumour tissues and adjacent non-tumour tissues (NATs) were collected from 65 GC patients who underwent radical gastrectomy at the Department of General Surgery, Pudong New Area People's Hospital affiliated with Shanghai University of Medicine & Health Science between 2010 and 2013. All diagnoses of GC and lymph node metastasis were histologically examined. Fresh GC tissue samples were processed within 15 min of surgical removal, frozen and stored at –80°C until further use. NATs at least 2 cm from the tumour border were dissected and confirmed to lack tumour cells by visualization under a microscope. This study was approved by the Ethics Committee of Pudong New Area People's Hospital. The clinical and pathological features of these patients are described in Table I.

Table I. Patient demographics and clinical characteristics.

Characteristic	Number of patients
Patients	65
Male	42
Female	23
Age (years)	43-87, median=65
Tumour size (cm)	0.8-9.0, median=2.75
Histology differentiation	
Well	10
Moderate	18
Poor	37
Borrmann type	
Early stage	8
I+II type	10
III+IV type	47
Local invasion	
T3-T4	37
Lymph node metastasis	
Yes	45
No	20
TNM stage	
I-II	20
III-IV	45

TNM, tumour-node-metastasis, based on the American Joint Committee on Cancer/International Union Against Cancer staging manual (7th edition, 2009).

Cell Culture

The AGS, BGC-823, HGC-27, and MGC-803 GC cell lines and the human gastric epithelial cell line GES-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). AGS cells were cultured in F-12 medium (HyClone, GE Healthcare, Chicago, IL, USA), and GES-1, BGC-823, HGC-27 andMGC-803 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI1640) medium (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a 5% CO_2 atmosphere. All media were supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics (Sigma-Aldrich, St. Louis, MO, USA).

Colony Formation Assay

For colony formation assays, AGS and BGC-823 cells infected with negative control shRNA (shNC) or shRNA targeting NORAD (shNORAD) were plated in 12-well plates at a density of 200 cells/well. When the colonies contained >50 cells (approximately 2 weeks later), cells were stained with 0.5% crystal violet (Beyotime, Shanghai, China) for 20 min after fixation with 4% paraformaldehyde for 20 min. Then, cells were washed with PBS three times and air-dried at room temperature.

Cell Proliferation Assay

Cell viability was determined by Cell Counting Kit-8 (CCK-8) assays (Beyotime, Shanghai, China). AGS and BGC-823 cells that were transfected with the indicated siRNAs were plated in a 96-well plate ($1.5x10^3$ *cells/well*). At the indicated time points (0 d (6 h after transfection), 1 d, 2 d, 3 d, 4 d, and 5 d), 10 μ LCCK-8 solution was added to each well, and the plates were placed in the incubator for 2 h. The absorbance at 450 nm was detected by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). All experiments were performed three times.

Cell Apoptosis Assay

48 h after transfection (shNORAD or shNC), the transfected AGS and BGC-823 cells were trypsinized, and cell apoptosis was measured using the Annexin V-FITC/Propidium Iodide Apoptosis De-

tection Kit (BD Biosciences, San Jose, CA, USA). Cells were then washed with cold PBS and incubated with 5 μL propidium iodide (PI) and 5 μL annexin V-FITC for 15 min at room temperature in the dark. Afterwards, cells were immediately subjected to flow cytometry analysis (FACSCalibur, BD Biosciences, San Jose, CA, USA). The experiment was repeated three times.

Migration and Invasion Assays

Migration and invasion assays were used to detect the metastatic ability of GC cells. Briefly, shNORAD-infected AGS and BGC-823 cells were seeded into the upper chamber of an insert (8-μm pore size; Corning, New York, NY, USA) with Matrigel for invasion assays or without Matrigel for migration assays. The lower chamber was filled with 600 μL of the indicated medium supplemented with 10% FBS. After incubation for 24 h, cells were fixed and stained with 0.1% crystal violet. After removing the cells on the upper membrane with a cotton swab, those that invaded through the membrane were counted and imaged under a microscope in five random fields. The experiments were performed in triplicate.

Lentivirus-Mediated RNA Interference

The specific siRNA sequence targeting NORAD was 5'-AAGCCACCTTTGTGAACAGTA-3', as previously reported[12], and the negative control sequence was 5'-TTCTCCGAACGTGTCACGT-3'. Recombinant lentiviruses containing shNORAD or shNC were obtained from Genechem (Shanghai, China). The cells were selected with puromycin for 7 d after infection with lentivirus for 72 h. Then, total RNA was extracted, and the expression level of NORAD was analyzed by qRT-PCR.

RNA Isolation and qRT-PCR Assays

Total RNA was extracted from GC cells using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was prepared using the PrimeScript RT Reagent Kit (TaKaRa, Otsu, Shiga, Japan). Quantitative Real Time-PCR (qRT-PCR) was conducted with SYBR Green Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) on a 7500 Fast Real-time PCR machine according to the manufacturer's protocol. The results were normalized to the expression level of GAPDH, and relative fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences were as follows: NORAD forward, 5'-GCCATTGGGCGAGACCTACCT-3', and reverse, 5'-GTTCGGGACTTCGCTCACCTT-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3', and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'. Each experiment was performed in triplicate.

Western Blot Analysis

AGS and BGC-823 cells were treated with shNORAD or shNC lentivirus for 48 h, total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China), and the protein concentration was measured using a BCA protein assay kit (Beyotime). The sample was boiled for 10 min. Sample lysates (40 μg of protein) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After being blocked with 5% fat-free milk in Tris-Buffered Saline and Tween 20 (TBST), the membranes were incubated with the specific antibodies listed below at 4°C overnight, washed three times with TBST, and then incubated with the corresponding HRP-conjugated secondary antibody (Abcam, Cambridge, MA, USA) for 1 h at room temperature. Protein bands were visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA).

PRIMARY ANTIBODIES USED IN THIS STUDY

Antibody	*WB*	*Specificity*	*Company*
Cleaved caspase-3 (#9664)	1:500	Rabbit Monoclonal	Cell Signaling Technology
RhoA (ab187027)	1:5000	Rabbit Monoclonal	Abcam
ROCK1 (ab45171)	1:1000	Rabbit Monoclonal	Abcam
E-cadherin (#3195)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
Bcl-2 (#4223)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
N-cadherin (#13116)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
Bax (#5023)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
GAPDH (ab181602)	1:5000	Rabbit Monoclonal	Abcam
Vimentin (#5741)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
ZEB1 (#3396)	1:1000	Rabbit Monoclonal	Cell Signaling Technology
Cleaved PARP (ab32064)	1:1000	Rabbit Monoclonal	Abcam

Statistical Analysis

SPSS 19.0 software and GraphPad Prism version 6.0 were used for statistical analysis. The Student's *t*-tests (two-tailed) were applied for the statistical analysis of NORAD gene expression determined by qRT-PCR and of the cell proliferation, apoptosis, colony formation, migration and invasion data. Data are presented as the mean ± Standard Deviation (SD) unless otherwise stated. Post hoc test Tukey's HSD was used to validate ANOVA for pairwise comparison. A *p*-value less than 0.05 was considered statistically significant.

Results

Lentivirus-Mediated Inhibition of NORAD in the AGS and BGC-823 Cell Lines

To dissect the role of NORAD in GC cells, we examined NORAD's expression in the human normal gastric *epithelial cell line* GES-1 and four GC cell lines by qRT-PCR. As shown in Figure 1A, NORAD was upregulated in most GC cells, of which AGS and BGC-823 cells harboured the highest NORAD expression. Therefore, in the subsequent study, we silenced NORAD in these two cell lines using lentiviruses containing a specific shRNA targeting NORAD (shNORAD) or a negative control sequence (shNC). The transfection efficiency was greater than 90% in both cell lines, as demonstrated by the percentage of GFP-positive cells (Figure 1B and 1C). The qRT-PCR analysis showed that NORAD transcript levels were dramatically downregulated in AGS and BGC-823 cells expressing shNORAD compared with those expressing shNC (Figure 1D).

Downregulation of NORAD attenuates GC cell proliferation

CCK-8 assays were performed in AGS and BGC-823 cells expressing shNORAD or shNC to examine the effect of NORAD on GC cell proliferation. The results revealed that NORAD knockdown significantly decreased GC cell proliferation (Figure 2A). Furthermore, colony formation assays revealed that fewer colonies formed in the shNORAD group than in the shNC group (Figure 2B). Collectively, these results showed that NORAD promotes the proliferation of GC cells.

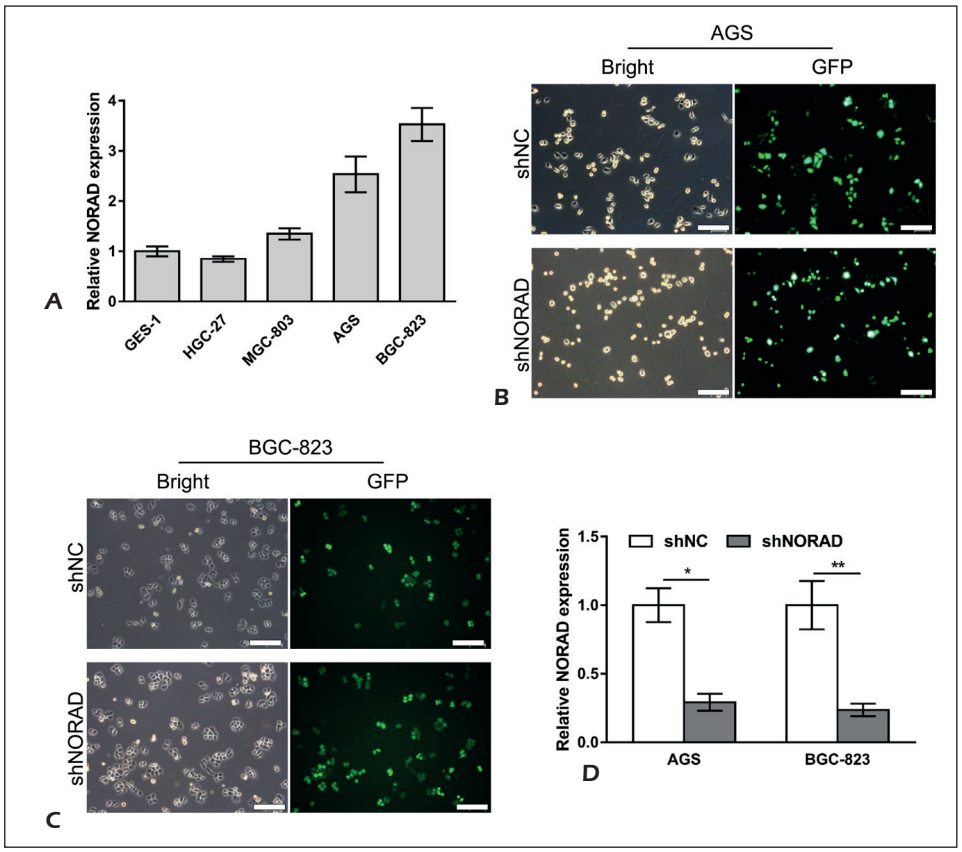

Figure 1. Lentivirus-mediated depletion of NORAD in gastric cancer cells. **A**, NORAD expression levels in the indicated gastric cancer cell lines. **B**, Representative images of AGS and BGC-823 cells infected with negative control lentivirus (shNC) or shNORAD lentivirus (Scale bar, 200 μm). **C**, The expression levels of NORAD in the indicated cells were examined by qRT-PCR, and GAPDH was used as the internal control. **p*<0.05, ***p*<0.01.

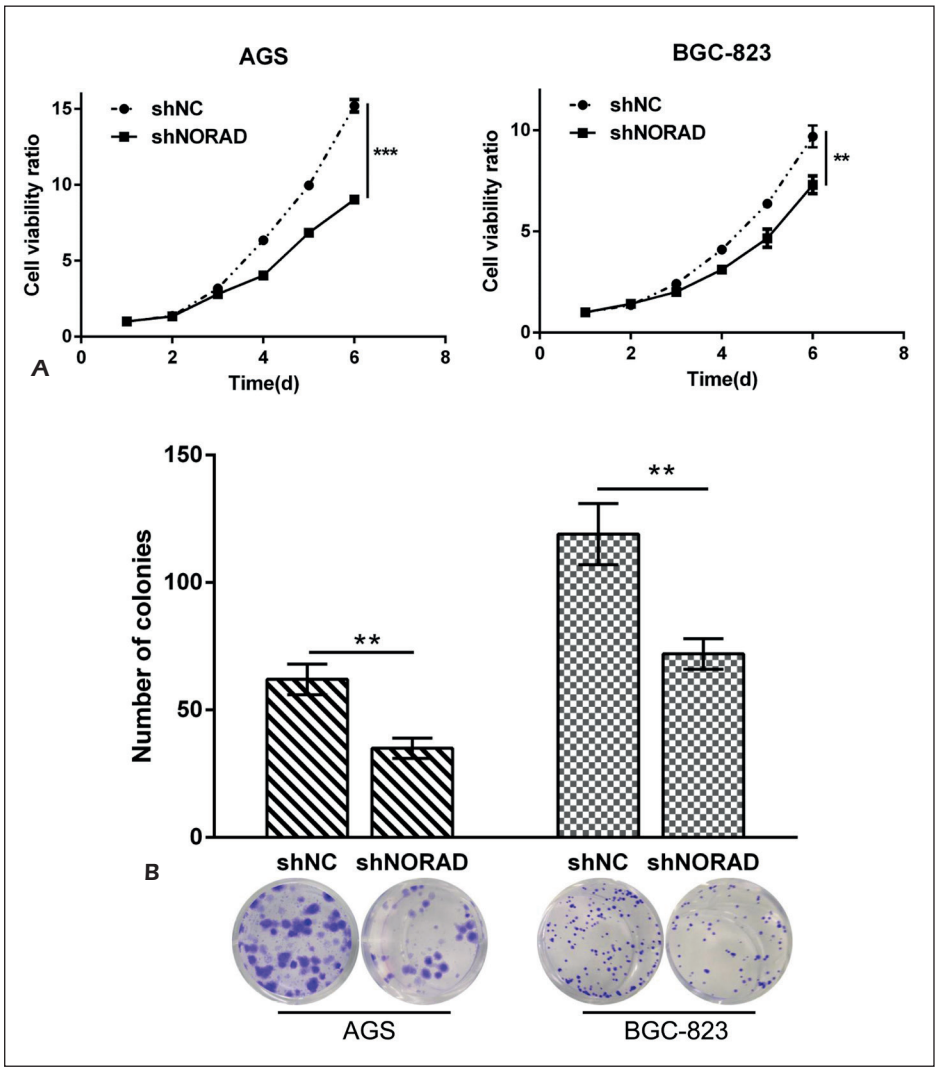

Figure 2. Downregulation of NORAD attenuated the proliferation of gastric cancer cells. **A**, CCK-8 assays showing the viability of AGS (left) and BGC-823 (right) cells after NORAD suppression. $^{**}p<0.01$, $^{***}p<0.001$. **B**, Colony formation analysis of AGS and BGC-823 cells infected with negative control (shNC) or shNORAD lentivirus. The plates were photographed after the cells were stained with crystal violet (upper panel). The colonies on each plate were counted and statistically analysed (lower panel). $^{**}p<0.01$.

Downregulation of NORAD Induces the Apoptosis of GC Cells by Modulating Apoptosis-Associated Genes

Next, we determined the effect of NORAD knockdown on GC cell apoptosis by flow cytometry (Figure 3A). NORAD knockdown significantly increased the percentage of apoptotic cells and decreased the proportion of surviving cells (Figure 3B). It is well known that apoptosis is orchestrated by pro-apoptotic and anti-apoptotic genes. Hence, we examined the effect of NORAD on the expression of apoptosis-related proteins. As shown in Figure 3C, the inhibition of NORAD induced increases in cleaved PARP, cleavedcaspase-3 and Bax expression levels and a decrease in Bcl-2 expression. Collectively, these data indicated that silencing NORAD induces cell apoptosis by regulating apoptosis-associated proteins.

Downregulation of NORAD Inhibits GC Cell Metastatic Behaviour

Next, we examined the role of NORAD in GC cell migration and invasion. Transwell migration and Matrigel invasion assays revealed a significant decrease in the number of invading and migrating cells in the shNORAD group compared to the shNC group (Figure 4A and 4B), indicating that NORAD knockdown inhibits the metastatic behaviour of AGS and BGC-823 cells. Next, Western blotting was applied to determine the expression of Epithelial-Mesenchymal Transition (EMT)-related proteins. The results showed that the downregulation of NORAD in AGS and BGC-823 cells increased E-cadherin protein levels and decreased those of N-cadherin, vimentin, and ZEB-1 (Figure 4C). Taken together, these data suggested that NORAD promotes GC cell migration and invasion.

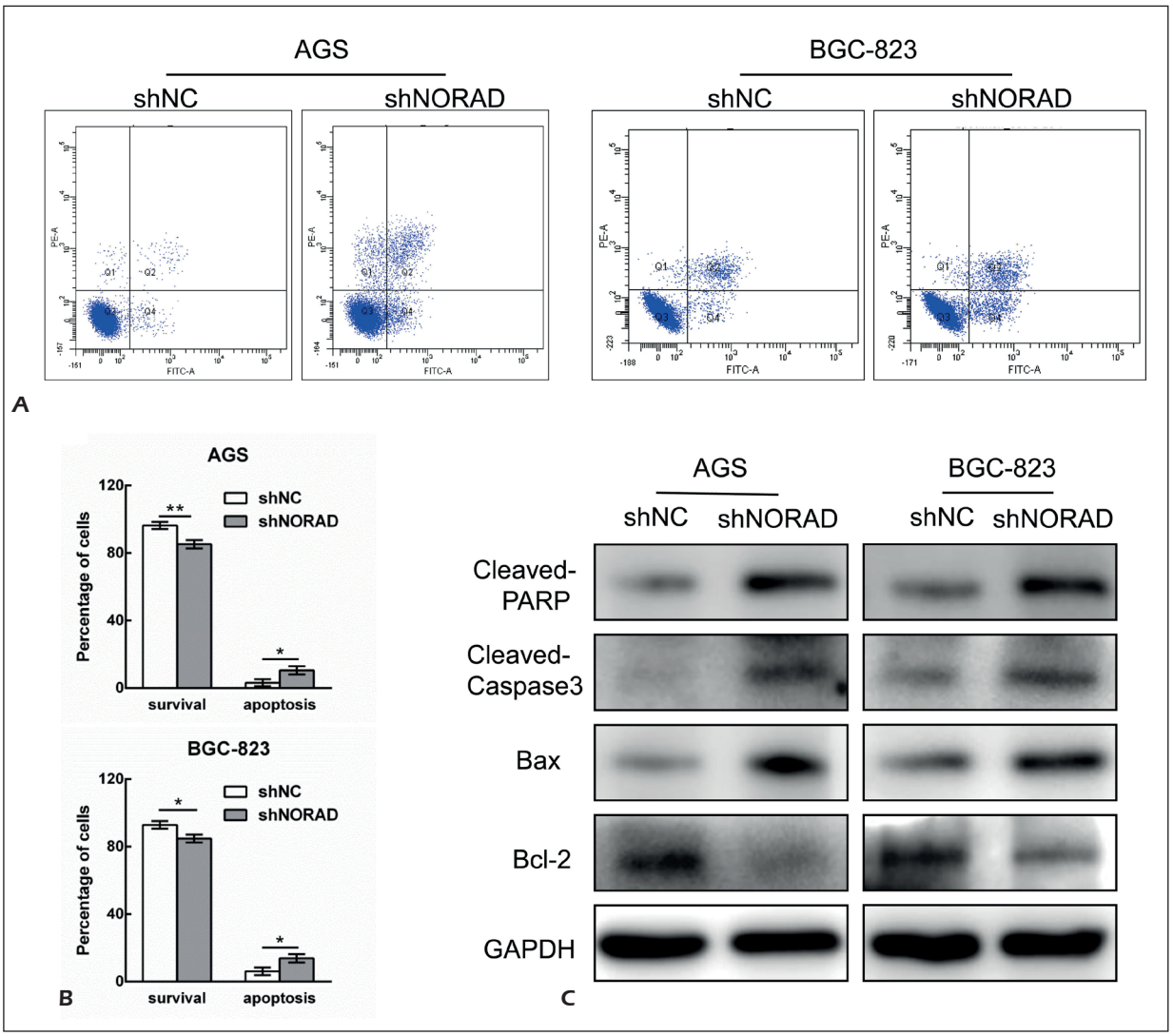

Figure 3. Inhibition of NORAD increased gastric cancer cell apoptosis. **A**, Flow cytometry analysis was performed in AGS and BGC-823 cells treated with shNC or shNORAD lentivirus using Annexin V-FITC and PI double staining. Q3 contains viable cells, and apoptotic cells are in Q2 and Q4. **B**, The percentages of live and apoptotic cells are presented as the mean ± SD. *$p<0.05$, **$p<0.01$. **C**, The expression levels of cleaved PARP, cleaved caspase 3, Bax and Bcl-2 were detected by Western blotting in AGS (left panel) and BGC-823 cells (right panel). The experiment was performed in triplicate.

NORAD Activates the RhoA/ROCK1 Pathway

It has been reported[13] that NORAD activates the RhoA/ROCK1 axis by acting as a ceRNA through competition for hsa-miR-125a-3p. We hypothesized that NORAD may exert its protumourigenic role by enhancing signaling through the RhoA/ROCK1 pathway. To test this hypothesis, we examined RhoA and ROCK1 protein levels after silencing NORAD in AGS and BGC-823 cells. Decreased RhoA and ROCK1 levels were observed when NORAD was downregulated (Figure 5A). Consistently, we observed positive correlations between NORAD expression and RhoA expression (Figure 5B) and ROCK1 expression (Figure 5C) in stomach adenocarcinoma (STAD) using the online web tool GEPIA[15] based on the TCGA database. Furthermore, GC patients with high NORAD expression had a shorter overall survival (OS), as revealed by the analysis of a public GEO database (GSE62254) using the online web portal Kaplan-Meier-plotter[16], indicating that NORAD may be an indicator of poor prognosis (Figure 5D). Moreover, we determined

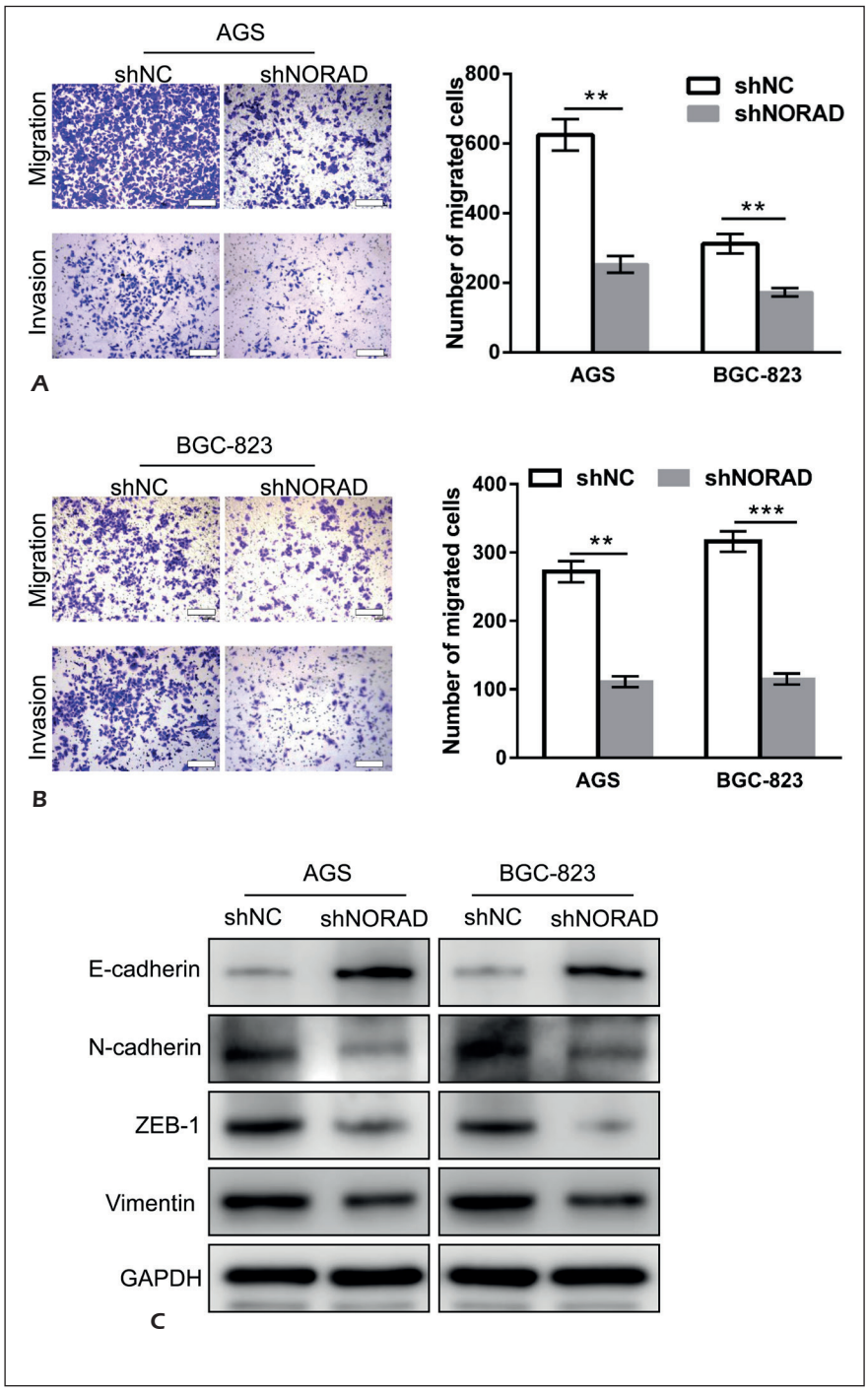

Figure 4. NORAD knockdown inhibited gastric cancer cell migration and invasion by orchestrating EMT-associated genes. The effect of NORAD knockdown on migration and invasion was evaluated in AGS and BGC-823 cells. **A**, Representative microscopic images of migrated and invasive AGS cells from the shNC and shNORAD groups are shown (left panel). Scale bar, 200 µm. The numbers of migrated and invaded AGS cells were calculated in five random fields (right panel), and the results are shown as the mean ±SD, $^{**}p<0.01$. **B**, Representative microscopic images of migrated and invasive BGC-823 cells from the shNC and shNORAD groups are shown (left panel). Scale bar, 200 µm. The numbers of migrated and invaded BGC-823 cells were determined in five random fields (right panel), and the results are presented as the mean ±SD, $^{**}p<0.01$. **C**, Effect of NORAD knockdown on the protein levels of E-cadherin, N-cadherin, ZEB1 and vimentin in AGS and BGC-823 cells. GAPDH served as a loading control.

NORAD expression in 65 GC specimens from patients using qRT-PCR analysis. As shown in Figure 5E, NORAD was overexpressed in GC tissues relative to NATs (p=0.012), and NORAD expression was positively correlated with Borrmann type (p<0.001) (Table II). In summary, NORAD predicts poor survival and activates the RhoA/ROCK1 signaling pathway.

Discussion

The development of GC is complicated and involves alterations in numerous oncogenes and tumour suppressors. Increasing evidence[17,18] suggests that lncRNAs play key roles in tumour initiation and development. Recently, NORAD was found to be upregulated in various types of

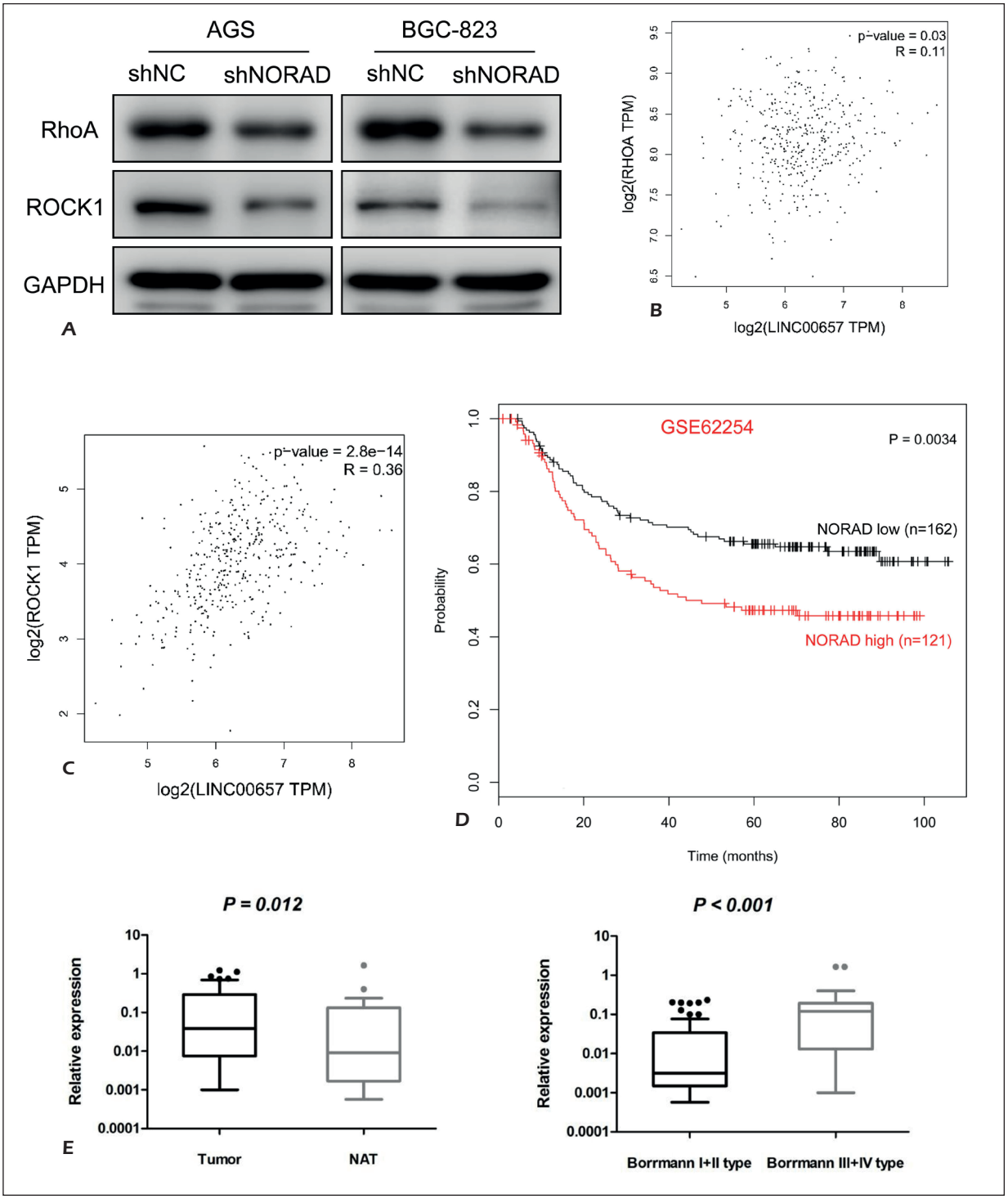

Figure 5. NORAD activates the RhoA/ROCK1 axis and predicts poor prognosis in gastric cancer. **A**, Western blot assay of RhoA and ROCK1 expression with GAPDH serving as a loading control. **B** and **C**, Scatter plots showing the association between NORAD expression and RhoA (**B**) or ROCK1 expression (**C**) using the online web portal GEPIA based on the TCGA STAD data repository. Pearson correlation coefficients (R) and *p*-values are shown. **D**, Kaplan-Meier survival analysis showed that high NORAD expression levels in STAD were significantly correlated with worse overall survival. The result was obtained by using the online web tool KM plotter based on GEO data (GSE62254). **E**, NORAD expression in 65 pairs of samples from GC patients (left) and classified as either Borrmann type I+II or Borrmann type III+IV (right). The expression levels were determined by qRT-PCR and normalized to those of an endogenous control (GAPDH). The data were analysed using the $2^{-\Delta CT}$ method.

Table II. Association of the expression of NORAD, a lncRNA, with the clinicopathological characteristics of GC.

Variable	Category	Relative NORAD expression		χ^2	*p*
		Low (32)	High (33)		
Age	<60	14	13	0.127	0.722
	≥60	18	20		
Sex	Male	15	27	8.676	0.003
	Female	17	6		
Tumour location	Upper stomach	5	0	5.640	0.130
	Middle stomach	10	12		
	Lower stomach	15	18		
	Mixed	2	3		
Borrmann type	Early stage	3	5	1.705	0.426
	I+II type	10	6		
	III+IV type	19	22		
Histology differentiation	Well	4	6	0.85	0.654
	Moderate	8	10		
	Poor	20	17		
Tumour invasion (AJCC)	***Tis-T_2***	***21***	***7***	***13.069***	***<0.001***
	T_3-T_4	***11***	***26***		
Lymph node metastasis	***Yes***	***13***	***32***	***24.213***	***<0.001***
	No	***19***	***1***		
TNM stage(AJCC)	***I-II***	***19***	***1***	***24.213***	***<0.001***

Bold values indicate statistical significance, $p<0.05$

cancer, including bladder cancer, colorectal cancer, pancreatic cancer and ESCC[12-14,19]. However, to the best of our knowledge, the biological and molecular characteristics of NORAD in GC cells still need to be elucidated.

To explore the function of NORAD in GC cells, we first examined NORAD expression in different GC cells and downregulated NORAD in the cell lines with higher relative expression. We found that NORAD depletion inhibited the proliferation and colony formation abilities of AGS and BGC-823 cells. Aberrant cell growth is a hallmark of tumourigenesis[20]. In addition, increased apoptosis was observed in shNORAD-transfected GC cells. In this research, we found that knockdown of NORAD significantly enhanced the levels of cleaved caspase-3 and cleaved PARP, which are considered central indicators of apoptosis[21]. The mitochondria-mediated apoptosis pathway is modulated by the apoptosis-promoting protein Bax and the anti-apoptotic protein Bcl-2[22]. Our results showed that NORAD knockdown in GC cells increased Bax expression and decreased Bcl-2 expression, indicating that shNORAD-induced apoptosis in GC cells might occur via the mitochondrial pathway.

EMT, a conserved cellular process characterized by the loss of epithelial cell polarity and the appearance of characteristics of interstitial cells, is involved in tumour metastasis[23]. Previous studies[13,19] in pancreatic and colorectal cancer demonstrated the metastasis-promoting role of NORAD. Consistently, our results showed that NORAD knockdown suppressed the migration and invasion of GC cells, accompanied by the increased expression of an epithelial marker (E-cadherin) and the decreased expression of mesenchymal markers (vimentin and N-cadherin). However, the detailed mechanisms responsible for NORAD-mediated metastasis remain to be further investigated.

Mechanisms of how NORAD regulates cancer progression remain mysterious to date. Lee et al[24] reported that NORAD regulates genomic stability by sequestering PUMILIO proteins, leading to the enhanced transcription of genes involved in mitosis and DNA replication. A more recent study reported that NORAD contributes to colorectal cancer progression by inhibiting miR-202-5p[19]. Li et al[13] found that NORAD could regulate the RhoA/ROCK1 pathway in pancreatic cancer through competition for hsa-miR-125a-3p. Consistent with this study, our results showed that knockdown of NORAD results in decreased RhoA and ROCK1 expression. Furthermore, positive correlations between NORAD expression and RhoA expression and ROCK1 expression were observed in STAD based on the TCGA database. However, further *in vivo* work needs to

be done, and the mechanism by which NORAD activates the RhoA/ROCK1 pathway requires further investigation.

Conclusions

We demonstrated that NORAD depletion negatively regulates GC cell proliferation, migration, and invasion through inactivating the RhoA/ROCK1 axis and that higher NORAD expression correlates with poorer prognosis. These results indicated that NORAD may be a novel prognostic marker and a novel promising therapeutic target for GC treatment.

Conflict of Interests

The authors report no conflicts of interest in this work.

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