

miR-381 and miR-489 suppress cell proliferation and invasion by targeting CUL4B via the Wnt/ β -catenin pathway in gastric cancer

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Abstract. Accumulating evidence has highlighted the critical role of cullin 4B (CUL4B) in driving tumourigenesis in several malignancies, including gastric cancer (GC); however, the mechanisms underlying CUL4B upregulation remain unclear. The dysregulation of microRNAs (miRNAs or miRs) is known to be involved in tumourigenesis. In this study, we report that the expression of miR-381 and miR-489 is downregulated and is negatively correlated with that of CUL4B in GC tissues and cell lines. Further analysis verified that miR-381 and miR-489 directly targeted CUL4B. CUL4B silencing inhibited cell proliferation, migration and invasion by inactivating the Wnt/ β -catenin pathway. miR-381/miR-489 overexpression recapitulated the effects of CUL4B silencing, while CUL4B restoration negated the suppressive effects induced by the ectopic expression of miR-381/miR-489. Furthermore, miR-381/miR-489 exerted tumour suppressive functions by inactivating the Wnt/ β -catenin pathway through the targeting of CUL4B. Taken together, the findings of this study suggest that the miR-381/miR-489-mediated expression of CUL4B modulates the proliferation and invasion of GC cells via the Wnt/ β -catenin pathway, which indicates that the miR-381/miR-489-CUL4B axis is critical in the control of GC tumourigenesis.

Introduction

Gastric cancer (GC) is among the most lethal malignancies worldwide, and has a particularly high mortality rate in East Asian countries (1). Despite numerous advances in its

treatment, the overall survival of patients with GC remains poor, as the majority are diagnosed at an advanced stage for which there is a lack of effective therapies (2). Therefore, there is an urgent need to investigate the molecular mechanisms underlying gastric tumourigenesis in order to facilitate the clinical management of GC and identify novel therapeutic targets for patients with GC.

Cullin 4B (CUL4B) is a scaffold protein responsible for assembling DDB1, RBX1 and substrate component to form the CUL4B ubiquitin ligase complexes (3), contributing to ubiquitin-mediated proteolysis and tumourigenesis (4,5). Mounting evidence has highlighted the upregulation and oncogenic potential of CUL4B in various types of cancer, including hepatocellular carcinoma, lung cancer, colon cancer and bladder cancer (6-10). Mechanistically, CUL4B epigenetically represses a series of tumour suppressors, including insulin-like growth factor-binding protein 3 (IGFBP3), phosphatase and tensin homolog (PTEN) and p16 through physical interaction with the SIN3A/HDAC and SUV39H1/HP1/DNMT3A complexes (11,12). Yuan *et al* demonstrated that CUL4B positively regulated the Wnt/ β -catenin signalling pathway by repressing Wnt pathway antagonists in liver cancer (13). Moreover, CUL4B can control cell cycle progression in osteosarcoma cells by targeting p21 for ubiquitination and degradation (14). To date, the mechanisms underlying CUL4B upregulation in GC have not yet been fully elucidated, although CUL4B has recently been shown to be associated with a poor prognosis and tumour progression in this malignancy (15).

Over the past few decades, a large body of evidence generated regarding microRNAs (miRNAs or miRs) has unveiled a new and promising strategy with which to control target gene expression that may translate into clinical applications. miRNAs post-transcriptionally regulate the expression of their target genes by binding to 3' untranslated regions (3'-UTRs) (16-18). Of note, we as well as others have demonstrated that aberrantly expressed miRNAs are closely linked to human malignancies, including GC (19-21). miR-381 has been shown to inhibit cell growth and invasion, while a low expression of miR-381 has been shown to be associated with lymph node metastasis, an advanced clinical tumour stage and a poor survival (22). miR-489 has been shown to be downregulated in the GC tissues and cell lines, compared with

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their normal counterparts (23). However, the molecular basis for the regulatory effects of miR-381 and miR-489 targeting CUL4B in GC progression have not yet been fully elucidated.

The Wnt/ β -catenin pathway is dysregulated by mutations or copy number alterations in multiple human cancers, and regulates malignant progression, such as cell growth, apoptosis, migration and invasion. The Wnt/ β -catenin pathway is also highly activated in gastric tumourigenesis. Nevertheless, whether miR-381 and miR-489 participate in the Wnt/ β -catenin pathway remains unclear.

In this study, we discovered miR-381 and miR-489 to be novel, negative regulators of CUL4B in gastric carcinogenesis. The expression of miR-381/miR-489 was found to be downregulated and to inversely correlate with that of CUL4B in GC tissues and cell lines. The overexpression of miR-381 and miR-489 suppressed cell proliferation, invasion and epithelial-mesenchymal transition (EMT). Moreover, we verified that the restoration of CUL4B expression negated the miRNA-induced suppression of proliferation and invasion, and this miR-381/489-CUL4B axis was shown to regulate GC progression through the inactivation of the Wnt/ β -catenin pathway. On the whole, our data reveal the importance of the miR-381/489-CUL4B axis in gastric carcinogenesis, which may provide novel insight into the molecular mechanisms underlying GC development, and may lead to the development of novel therapeutic strategies in the future.

Materials and methods

Clinical tissue samples. All patients with GC in this study were treatment-naïve prior to surgery. Primary GC tissues and corresponding normal gastric tissues were obtained from 20 patients undergoing surgical resection at the Department of Surgery of the First Affiliated Hospital of Nanchang University (Nanchang, China) from December, 2016 to November, 2017. Following resection, the fresh tissue samples were frozen in liquid nitrogen and stored at -80°C . The clinicopathological characteristics of the patients were confirmed by two experienced pathologists and are summarized in Table I. This study was approved by the Independent Ethics Committee of the First Affiliated Hospital of Nanchang University and complied with the Declaration of Helsinki (Approval no. #021-2017). All patients agreed to participate in this study and provided written informed consent.

Cell lines and cell culture. Three GC cell lines (AGS, MGC803 and BGC823), the normal gastric epithelial cell line, GES-1, and 293 cells were obtained from Sun Yat-Sen University Cancer Center (Guangzhou, China), and the GC cell line, SGC7901, was purchased from the Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (both from HyClone, Logan, UT, USA) in a humidified chamber at 37°C in an atmosphere containing 5% CO_2 .

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). RNA was isolated from fresh human tissues or harvested cells using TRIzol reagent (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's

instructions, as previously described (24). Complementary DNA was synthesised using a PrimeScript RT Reagent kit (Takara, Ohtsu, Japan), and quantitative PCR (qPCR) was performed using the ABI 7500 Real-Time Fast PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR-Green qPCR SuperMix (Invitrogen/Thermo Fisher Scientific). Primers targeting miR-381, miR-489 and CUL4B were purchased from GenePharm (Shanghai, China). U6 or GAPDH were used as internal controls, and all results were calculated using the $2^{-\Delta\Delta\text{Cq}}$ method (25). The primer sequences are presented in Table II.

Western blot analysis. Briefly, the cells were lysed in lysis buffer consisting 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.5% Nonidet P-40 (NP-40), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM pepstatin A and 1 mM leupeptin. Equal amounts of clear cell lysates were separated by 8 or 10% SDS-PAGE polyacrylamide gel electrophoresis on 8 or 10% gels and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with a 5% milk solution at room temperature, before being incubated at 4°C with the following primary antibodies overnight: CUL4B (diluted 1:1,000; 60151-1-Ig; Proteintech, Rosemont, IL, USA), β -catenin (1:1,000; #9562), cyclin D1 (1:1,000; #2978), c-Myc (1:1,000; #5605) (all from Cell Signaling Technology, Danvers, MA, USA), E-cadherin (1:1,000; ab1416), N-cadherin (1:1,000; ab18203), Vimentin (1:1,500; ab8978), β -actin (1:2,000; ab119716) (all from Abcam, Cambridge, MA, USA) and GAPDH (1:2,000; 14C10; Santa Cruz Biotechnology, Dallas, TX, USA). The following day, the membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse; Cell Signaling Technology; #7074, 1:3,000 and #7076, 1:4,000) for 1 h at room temperature. The signal was detected with super sensitive reagent (Thermo Fisher Scientific), and β -actin or GAPDH was used as an internal control. Quantification of the protein bands were analysed using ImageJ software (Rawak Software, Stuttgart, Germany).

Cell transfection, lentiviral transduction and stable cell lines. miR-381 and miR-489 mimics and the corresponding negative control, CUL4B small interfering RNA (siRNA), control siRNA, lentiviral vectors of hsa-miR-381, hsa-miR-489 and hsa-miR-negative control were purchased from GenePharm. The siRNA sequences were as follows: siRNA-NC, 5'-UUCUCCGAACGUGUCACGUTT-3'; and siRNA-CUL4B, 5'-CCACCC AGAAGUCAUUAUUTT-3'. The transfection of oligonucleotides into target cells was performed using TurboFect reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Certain cells, as indicated, were transduced using lentivirus-containing supernatant for 24 h in the presence of 2.5 $\mu\text{g}/\text{ml}$ polybrene. Following infection, puromycin selection was used to establish stable cell lines. An expression vector carrying a CUL4B sequence lacking the 3'-UTR (CUL4B-no UTR) was constructed by inserting the coding sequence into the psiCHECK-2 vector (Promega, Madison, WI, USA). RT-qPCR and western blot analysis were performed to confirm successful transfection. The data shown were derived from triplicate samples.

Table I. The clinicopathological characteristics of the patients with gastric cancer.

Patient no.	Age (years)	Sex	Tumor size (cm)	Depth of invasion	Differentiation (well, moderate or poorly)	TNM stage	Lymph node metastasis (N ₀ or N _x)
1	54	Female	3x3.1	T ₂	Poorly	III	N ₃
2	57	Female	4x2.5	T ₃	Moderate	II	N ₀
3	62	Female	2x1.5	T ₃	Moderate	I	N ₂
4	56	Male	5.5x4.1	T ₃	Well	II	N ₀
5	62	Female	2.4x2.8	T ₂	Poorly	I	N ₀
6	57	Female	5.8x2.7	T ₄	Poorly	III	N ₁
7	63	Male	2.6x3.2	T ₂	Moderate	III	N ₃
8	60	Male	3.5x2.1	T ₃	Moderate	II	N ₁
9	51	Female	5.9x3.0	T ₁	Well	I	N ₀
10	66	Male	2.1x1.8	T ₄	Poorly	III	N ₀
11	54	Male	4.2x2.0	T ₁	Moderate	I	N ₀
12	68	Male	5.4x2.9	T ₁	Moderate	I	N ₀
13	77	Female	4.6x2.6	T ₄	Moderate	I	N ₃
14	73	Male	2.8x2.2	T ₂	Poorly	II	N ₁
15	50	Male	5.5x3.3	T ₁	Well	I	N ₀
16	64	Female	3.2x2.2	T ₁	Well	I	N ₀
17	42	Male	2.3x2.1	T ₄	Poorly	III	N ₂
18	70	Male	5.1x3.2	T ₁	Moderate	I	N ₀
19	52	Male	2.9x4	T ₄	Poorly	IV	N ₃
20	49	Male	5.4x2.7	T ₁	Well	I	N ₀

The TNM stage of the patients was defined according to the AJCC staging system (7th edition, 2010) for GC (50).

Table II. Primer sequences used in RT-qPCR.

Gene	Primer sequence
miR-381	F: TGGTACTTAAAGCGAGGTTGC R: GGTCATGCACACACATACCAC
miR-489	F: ACACTCCAGCTGGGGTGACATCACATA R: TGGTGTCTGTTGGAGTCG
CUL4B	F: TGGAAGTTTCATTTACCACCAGAGATG R: TTCTGCTTTTAAACACACAGTGCCTA
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT
GAPDH	F: GATTCCACCCATGGCAAATTC R: AGCATCGCCCCACTTGATT

F, forward; R, reverse.

Dual-luciferase reporter assay. The identification of upstream regulatory miRNAs targeting CUL4B was performed using two independent miRNA databases: miRanda (<http://www.microrna.org/>) and TargetScan (<http://www.targetscan.org>). The full-length 3'-UTR of the CUL4B gene and a variant sequence were amplified by PCR and cloned into the pGL3 luciferase reporter vector (Promega). The wild-type plasmid (CUL4B-Wt-3'-UTR)

contained the full-length CUL4B 3'-UTR, including the sequences complementary to miR-381 and miR-489. The mutant plasmids (CUL4B-Mut-3'-UTR) carried variant 3'-UTR sequences created by replacing 'CUUGUAU' with 'GAACAUA' in the sequence complementary to miR-381 or 'UAUGAUGU' with 'ACACUACA' in that complementary to miR-489. The luciferase reporter assay was performed as previously described. 293 cells were seeded at 2x10⁵ cells per well in 12-well plates and transiently co-transfected with 1 µg reporter plasmid and 100 nM miR-381 mimic, miR-489 mimic, or negative control using TurboFect reagent for 48 h. Luciferase activity was measured using a Dual-Luciferase Reporter Assay kit (E1910; Promega) according to the manufacturer's instructions. *Renilla* luciferase activity was used for normalization, and all experiments included 3 biological replicates.

Cell Counting kit-8 (CCK-8) assay. To assess long-term cell viability, a CCK-8 assay (Dojindo Molecular Technologies, Rockville, MD, USA) was used according to the manufacturer's instructions as previously described (26). Following transfection for 24 h, the cells were seeded in 96-well plates. Cell viability was measured by the addition of WST-8 at a final concentration of 10% and measuring the absorbance of samples at 450 nm in a microplate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA, USA) every 24 h for 5 days. The data was derived from triplicate samples and are presented as means ± standard errors of the mean (SEM).

Colony formation assay. The colony formation assay was carried out as previously described (24). The MGC803 and BGC823 cells (500 per well) were seeded in a 60-mm dish 24 h post-transfection and cultured in RPMI-1640 medium containing 10% FBS for 10 days. Colonies comprising >50 cells were then fixed with 10% formaldehyde for 30 min and stained with 8.0% crystal violet (#R40052; Thermo Fisher Scientific) for a further 30 min at room temperature. Each experiment was repeated 3 times.

Wound healing assay. To evaluate cell motility, the indicated cells, at a density of 4.5×10^5 per well, were seeded in 6-well plates and serum-starved for 24 h once fully confluent. A sterile 200- μ l pipette tip was then used to scratch the culture to form a wound. The cells were subsequently carefully washed with phosphate-buffered saline and cultured in serum-free medium. Images were captured at 0 and 48 h after scratching to evaluate wound closure under a microscope (#CKX41; Olympus America, Inc., Center Valley, MA, USA). Percentage wound closure was calculated by comparing the wound area at a given time to that at 0 h. These experiments were performed at least 3 times.

Transwell assay. The Transwell assay was performed as described previously (26). For the migration assay, the indicated cells were seeded in the upper chambers of Transwell inserts with non-coated membranes (8- μ m pore size; BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assay, cells were suspended in 200 μ l serum-free medium and seeded in the upper chambers of Transwell inserts pre-coated with Matrigel. Medium (500 μ l) containing 15% FBS was added to the lower chambers as a chemoattractant. Following 72 h of incubation at 37°C, cells that had invaded the bottom surface of the membranes were fixed and stained with 10% crystal violet at room temperature. These experiments were performed using 3 biological replicates.

Mouse xenograft experiments. A total of 24 (6 weeks old; weighing, 18–21 g) female NOD SCID mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were supplied with free sterilized water and food at $22 \pm 2^\circ\text{C}$ with 40–70% humidity. The mice were randomly allocated to 1 of 4 groups (6 mice in each) and inoculated with 5×10^6 MGC803 cells stably expressing miR-negative control, hsa-miR-381, or hsa-miR-489 by subcutaneous injection into both anterior flanks. Tumour growth in two dimensions was monitored every 3 days using electronic digital callipers (Thermo Fisher Scientific). Tumour volume was calculated with the following formula: Tumour volume (mm^3) = (length \times width²)/2. The mice were euthanised, and tumours were harvested and weighed. All animal experiments were approved by the Animal Ethics Committee of Nanchang University. The maximum tumour diameter allowed per tumour was 1.5 cm.

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All data are presented as the means \pm SEM, and each experiment was repeated at least 3 times. Comparisons between 2 groups were made using a Student's t-test, while one-way ANOVA with a post hoc Dunnett or Bonferroni correction test was used when

analysing >2 groups. The association between miRNA expression and CUL4B protein expression was analysed by Pearson's correlation coefficient. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of CUL4B negatively correlates with that of miR-381 and miR-489 in GC tissues and cell lines. Our preliminary immunohistochemical data revealed that CUL4B was upregulated in GC tissues compared with paired adjacent normal gastric tissues (data not shown). In the present study, we found that 15 of the 20 GC samples (75%) examined exhibited a higher CUL4B protein expression than the matched adjacent non-cancerous gastric tissues (Fig. 1A; $P < 0.01$). To investigate the underlying mechanisms of CUL4B upregulation, we searched for potential miRNAs that target CUL4B using bioinformatics analysis (<http://www.microrna.org/microrna/home.do>). We screened out several miRNAs that may regulate CUL4B expression according to the SVR score and conserved status. Moreover, with TargetScan (www.Targetscan.org) for the second screening, miR-381 and miR-489 attracted our attention. The reasons for the selection of miR-381 and miR-489 for further investigation in this study were as follows: i) Two algorithms both predicted that miR-381 and miR-489 were potential regulatory miRNAs that could target CUL4B; and ii) these two miRNAs have been reported to be involved in cancer progression (27–29). As shown in Fig. 1B, potential miR-381 and miR-489 target sites are present in the CUL4B 3'-UTR. As the expression patterns of miRNAs are often the opposite of those of their target genes, we examined miR-381 and miR-489 expression in the above-mentioned 20 tissue sample pairs by RT-qPCR. As clearly illustrated in Fig. 1C, the miR-381 and miR-489 levels were consistently downregulated in the GC tissues compared with the non-cancerous controls. Correlation analysis indicated that the expression of miR-381 and miR-489 was negatively correlated with CUL4B protein expression in the 20 clinical GC samples (Fig. 1D; $r = -0.571$ and $r = -0.518$, respectively; $P < 0.01$). The expression of these miRNAs and CUL4B was also examined in the AGS, BGC823, MGC803 and SGC7901 human GC cells and in GES-1 normal gastric epithelial cells. Of note, CUL4B protein expression was much higher in the AGS, BGC823 and MGC803 cells, but lower in the SGC7901 cells, and was inversely associated with miR-381 and miR-489 expression (Fig. 1E and F). Collectively, these data suggest that the downregulation of miR-381 and miR-489 may play an important role in the pathogenesis of GC by affecting CUL4B expression.

CUL4B is a direct target of miR-381 and miR-489. To determine whether CUL4B is a direct target of miR-381 and miR-489 in GC, fragments of its 3'-UTR containing the predicted binding sites of these miRNAs or variants of these sequences were separately subcloned into the pGL3 vector (Fig. 2A). Luciferase reporter assays were then performed to verify the binding of the CUL4B 3'-UTR by these two miRNAs. As depicted in Fig. 2B, the miR-381 and miR-489 levels were significantly increased in the MGC803 and BGC823 cells upon transfection. We also found that the ectopic expression of miR-381 or miR-489 exerted a significant inhibitory

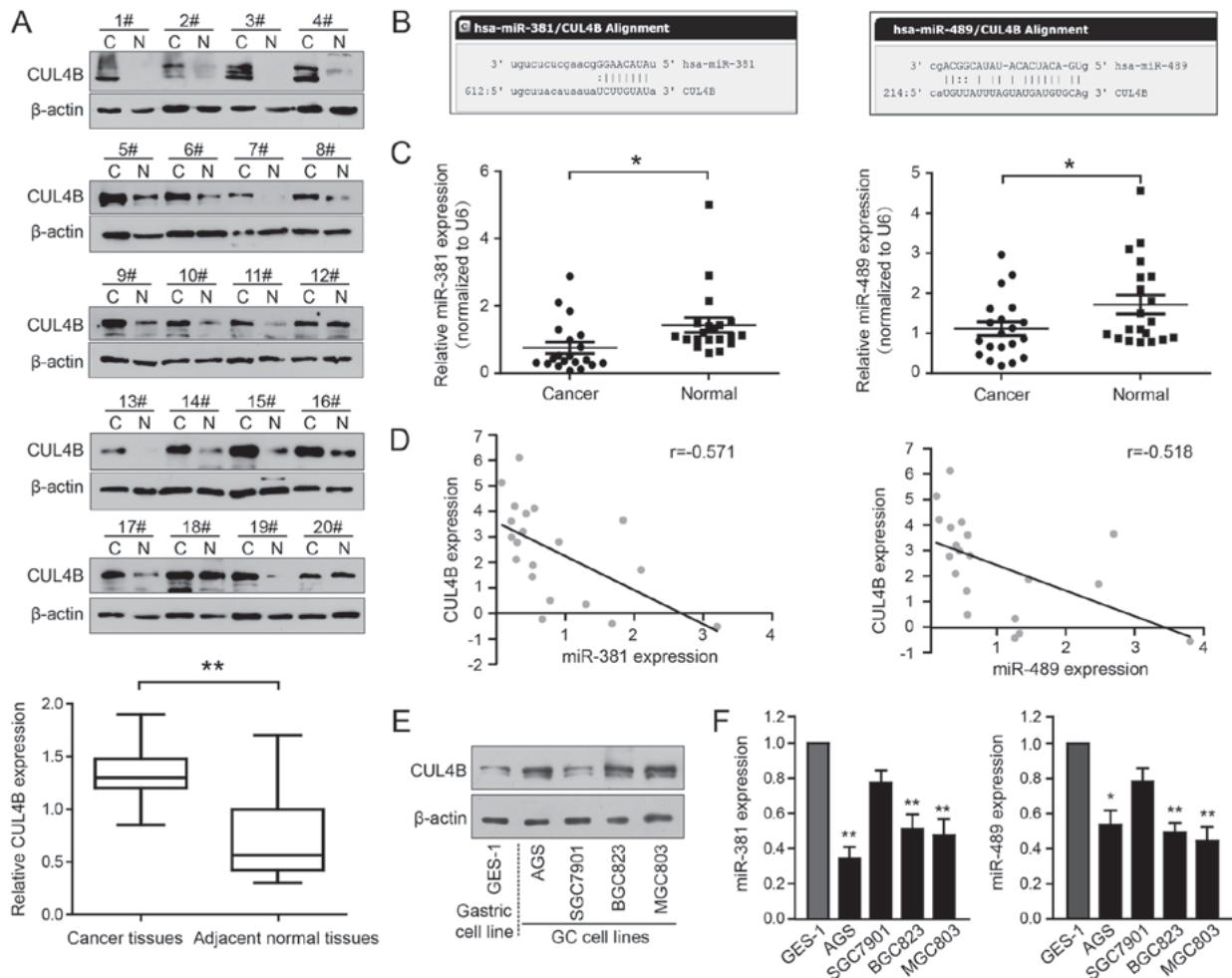


Figure 1. CUL4B expression inversely correlates with the miR-381 and miR-489 levels in gastric cancer (GC) tissues and cell lines. (A) CUL4B protein expression in 20 pairs of GC tissues (lanes labelled with 'C') and corresponding adjacent non-cancerous gastric tissues (lanes labelled with 'N') detected by western blot analysis. ** $P<0.01$, paired two-tailed Student's t-test. (B) The site within CUL4B with which the candidate regulatory miRNAs miR-381 and miR-489 was predicted to interact. (C) RT-qPCR analysis of miR-381 and miR-489 expression in gastric tumours and paired non-cancerous tissues. U6 was used as an internal control. * $P<0.05$, paired two-tailed Student's t-test. (D) Pearson's correlation analysis between CUL4B and miR-381/miR-489 expression levels in 20 paired GC specimens. (E) CUL4B expression in human gastric cell lines was measured by western blot analysis. (F) RT-qPCR analysis of miR-381 and miR-489 expression in GC cell lines. U6 was used as an internal control. One-way analysis of variance (adjusted for Dunnett's test). Data are presented as the means \pm SEM, $n=3$. * $P<0.05$ and ** $P<0.01$ compared to the corresponding controls.

effect on the luciferase activity of the CUL4B-Wt-3'-UTR plasmid in the 293 cells, but had no marked effect on that of the CUL4B-Mut-3'-UTR ($P<0.01$; Fig. 2C and D). As shown in Fig. 2E and F, miR-381 or miR-489 upregulation decreased CUL4B protein, but not mRNA expression in the MGC803 and BGC823 cells, suggesting that these miRNAs regulate CUL4B levels principally through translational repression. Collectively, these results strongly support the hypothesis that CUL4B is a direct target of miR-381/miR-489, and that they specifically and directly suppress CUL4B expression by binding to its 3'-UTR.

CUL4B silencing inhibits the proliferation, migration and invasion of GC cells by inactivating the Wnt/ β -catenin pathway. To determine the functional effects of CUL4B on GC cells, we examined cellular phenotypes following its knockdown using siRNA. Successful knockdown was confirmed by RT-qPCR (Fig. 3A) and western blot analysis (Fig. 3H). As clearly shown in Fig. 3B and C, the silencing of CUL4B suppressed GC cell growth. Consistent with this finding,

a significant decrease in the colony formation ability was detected in the cells in which CUL4B was knocked down when compared with the negative control ($P<0.01$; Fig. 3D and E). Moreover, CUL4B silencing markedly diminished the migratory and invasive abilities of the MGC803 and BGC823 cells ($P<0.01$; Fig. 3F and G). In previous studies, CUL4B was found to positively regulate the Wnt/ β -catenin pathway in pancreatic cancer and hepatocellular carcinoma, and the abnormal activation of this pathway has been shown to be involved in tumourigenesis (13,30). Therefore, in this study, we examined whether Wnt/ β -catenin signalling was affected by the silencing of CUL4B. As shown in Fig. 3H, the expression of β -catenin, c-Myc and cyclin D1 was downregulated in the MGC803 and BGC823 cells transfected with CUL4B siRNA. These data suggest that the silencing of CUL4B exerts a marked tumour suppressive effect on GC tumourigenesis via the inactivation of the Wnt/ β -catenin pathway.

miR-381 and miR-489 retard GC growth in vitro and in vivo. Given that miR-381 and miR-489 are frequently

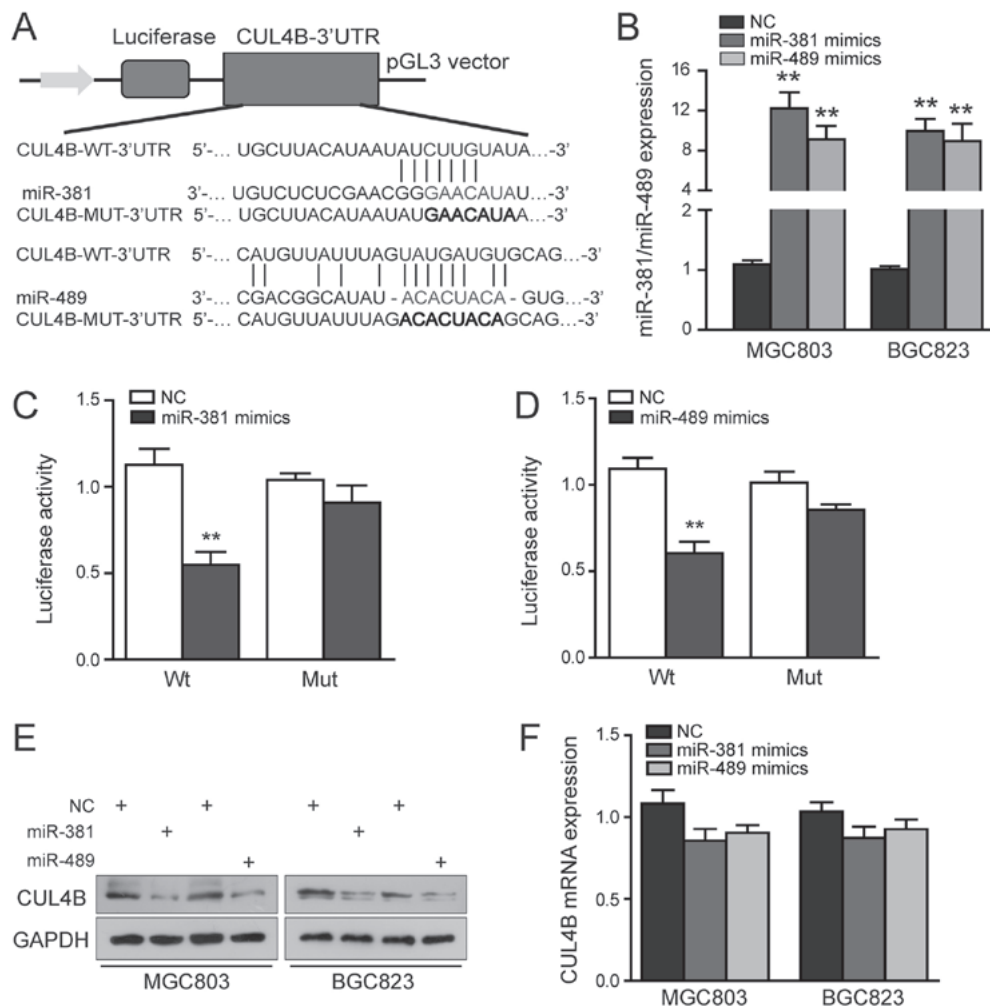


Figure 2. CUL4B is directly targeted by miR-381 and miR-489. (A) Wild-type (Wt) and mutant (Mut) CUL4B 3'-UTR sequences were cloned into a pGL3 reporter vector. Mutant constructs were generated by altering the seed regions within the CUL4B 3'-UTR, as indicated in bold. (B) miR-381 and miR-489 expression was measured by RT-qPCR at 48 h post-transfection. U6 was used as an internal control. (C and D) Relative luciferase activity in 293 cells was measured using a dual-luciferase reporter assay. (E) CUL4B protein expression in MGC803 and BGC823 cells was measured by western blot analysis post-transfection. (F) RT-qPCR analysis of CUL4B mRNA expression in the indicated cell lines 24 h post-transfection. Each bar represents the mean \pm SEM of 3 independent experiments, ** $P < 0.01$, compared to the negative control (NC); ANOVA with a post hoc Bonferroni correction test, $n = 3$.

downregulated in GC (22,23), we expressed these miRNAs ectopically to explore their biological functions. As illustrated in Fig. 4A and B, miR-381 and miR-489 overexpression reduced cell viability. Consistent with this, we observed a significant reduction in the number of colonies formed by the miRNA transfectants compared with the negative control (Fig. 4C and D). Subsequently, to determine whether these miRNAs can inhibit tumour growth *in vivo*, the MGC803 cells were infected with lentivirus expressing miR-381, miR-489, or negative control miRNA, and puromycin selection was used to establish lines stably expressing these constructs. These cells (5×10^6 per mouse) were then injected into the anterior flanks of nude mice. Compared with the miR-381 group, the miR-NC group developed significantly larger tumours ($P < 0.01$; Fig. 4E). Similarly, miR-489 overexpression resulted in the formation of markedly smaller tumour nodules and attenuated the growth of tumour xenografts in the nude mice ($P < 0.01$; Fig. 4F). Taken together, these results clearly suggest that the ectopic expression of miR-381/miR-489 strongly delays GC cell growth *in vitro* and *in vivo*.

miR-381 and miR-489 negatively regulate cell migration, invasion, and EMT of GC cells. We then evaluated the effects of miR-381 and miR-489 on GC cell migration and invasion using wound healing and Transwell invasion assays. The results of the wound healing assay revealed that the cells overexpressing miR-381/miR-489 took a much longer time to close the scratch wound than the controls ($P < 0.05$; Fig. 5A and B). Moreover, miR-381 or miR-489 overexpression significantly suppressed the invasive abilities of the MGC803 and BGC823 cells ($P < 0.01$; Fig. 5C and D). EMT is a critical process contributing to the initiation of cancer cell invasion; therefore, we also examined EMT markers in these two cell lines following miR-381 or miR-489 upregulation. Surprisingly, the levels of E-cadherin were increased and those of N-cadherin and Vimentin were reduced due to the elevated expression of miR-381 or miR-489 (Fig. 5E). Furthermore, the combination of miR-381 and miR-489 overexpression suppressed cell growth, migration and invasion more markedly (data not shown). The simultaneous overexpression of miR-381 and miR-489 exerted a more potent tumour-suppressive effect on the GC cells (data not shown). These data indicate a critical

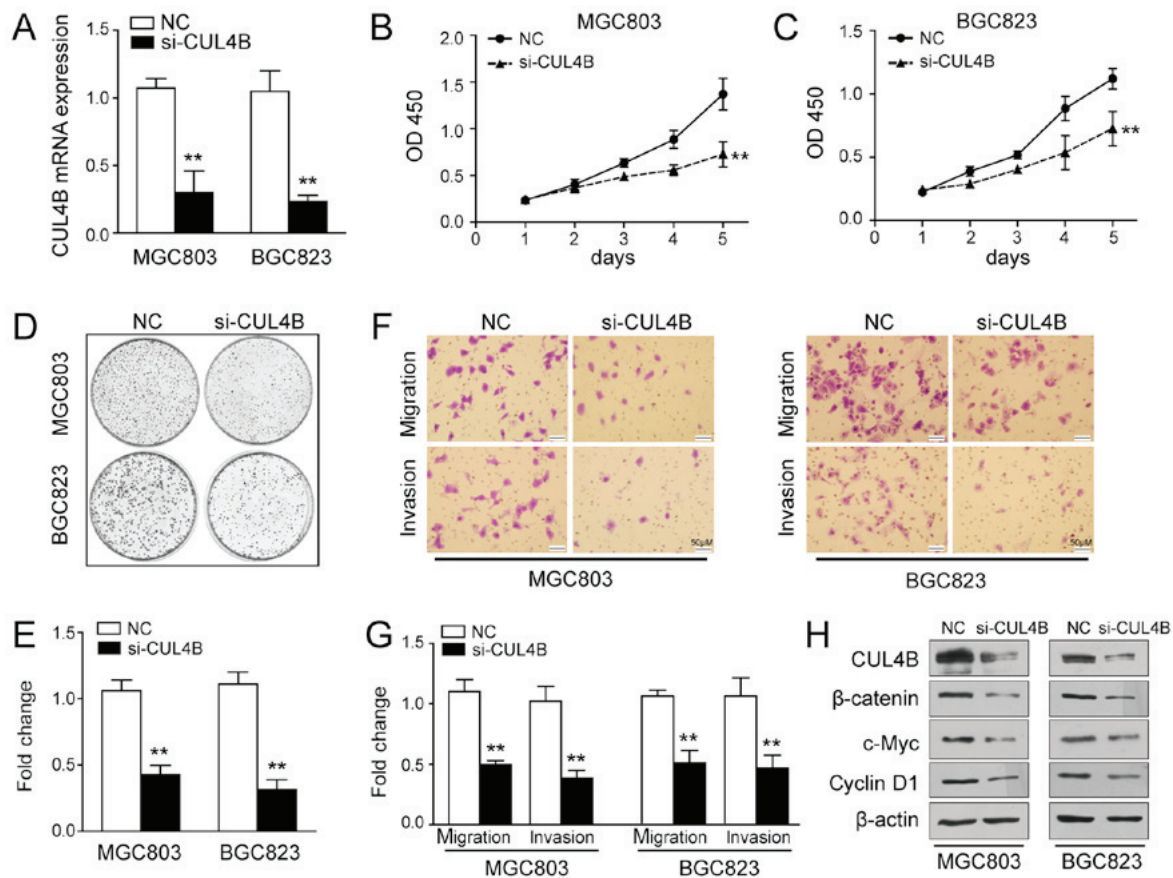


Figure 3. CUL4B silencing inhibits the proliferation, migration and invasion of gastric cancer (GC) cells via inactivating the Wnt/ β -catenin pathway. (A) RT-qPCR analysis of CUL4B mRNA expression in GC cells following transfection with CUL4B siRNAs or the corresponding negative control for 24 h. (B and C) CCK-8 assay of GC cells transiently transfected with CUL4B siRNAs or the negative control (NC). (D and E) Effects of CUL4B on cell colony formation ability. (F and G) Transwell assays were performed to test the migratory and invasive ability of cells following transfection with CUL4B siRNAs or the corresponding negative control (magnification, $\times 200$). (H) Western blot analysis of CUL4B, β -catenin, c-Myc and cyclin D1 following transfection with CUL4B siRNA or negative control for 48 h. All data are presented as the means \pm SEM of triplicate experiments, $n=3$. ** $P<0.01$ vs. NC group; t-test.

role of miR-381/miR-489 in cell migration, invasion and EMT, apart from cell proliferation.

CUL4B overexpression counteracts the phenotypic effects of miR-381 and miR-489. To further confirm that the functional effects of miR-381 and miR-489 in GC are mediated by the inhibition of CUL4B, we overexpressed a CUL4B sequence lacking the 3'-UTR in the miRNA-transfected cells. As shown in Fig. 6A, the restoration of CUL4B expression partially negated the effects of miR-381 and miR-489 on the protein levels of CUL4B and components of the Wnt/ β -catenin pathway. Furthermore, CCK-8, colony formation and Transwell invasion assays demonstrated that the restoration of CUL4B expression also counteracted the inhibitory effects of miR-381 and miR-489 on GC cell proliferation and invasion (Fig. 6B-F). Taken together, these results further demonstrate that the altered CUL4B expression is a main mediating factor in the functions of both miR-381 and miR-489.

Discussion

It has been well established that CUL4B is involved in the initiation and development of human malignancies, as it contributes to tumour progression by inducing cell proliferation, invasion and metastasis (31,32). In the current

study, we demonstrated that CUL4B was upregulated in GC tissues and cell lines, suggesting that it plays a role in GC tumourigenesis. Moreover, we identified the downregulation of miR-381 and miR-489 as a novel mechanism responsible, at least in part, for CUL4B overexpression in GC and the consequent activation of the Wnt/ β -catenin pathway.

There is much evidence of a critical role for miRNAs in gastric tumourigenesis via the modulation of cell proliferation, invasion, EMT and chemoresistance (33-35). Previous studies have indicated that some members of the Cullin protein family may be targeted by miRNAs (26,36). Moreover, CUL4B and miR-194 have been found to regulate each other directly in a coordinated manner (10). In the present study, we identified miR-381 and miR-489 as potential regulators of CUL4B using computational prediction tools. We also noted that the expression of these miRNAs was downregulated and inversely correlated with that of CUL4B in GC tumour samples and cell lines. More credibly, CUL4B was confirmed as a direct target gene of both miR-381 and miR-489 by western blot analysis, RT-qPCR and luciferase reporter assay. These data further uncovered a potentially critical role of miR-381 and miR-489 in the suppression of GC tumourigenesis, leading to CUL4B overexpression.

The altered expression of miR-381 and miR-489 has been strongly implicated in human tumourigenesis and cancer

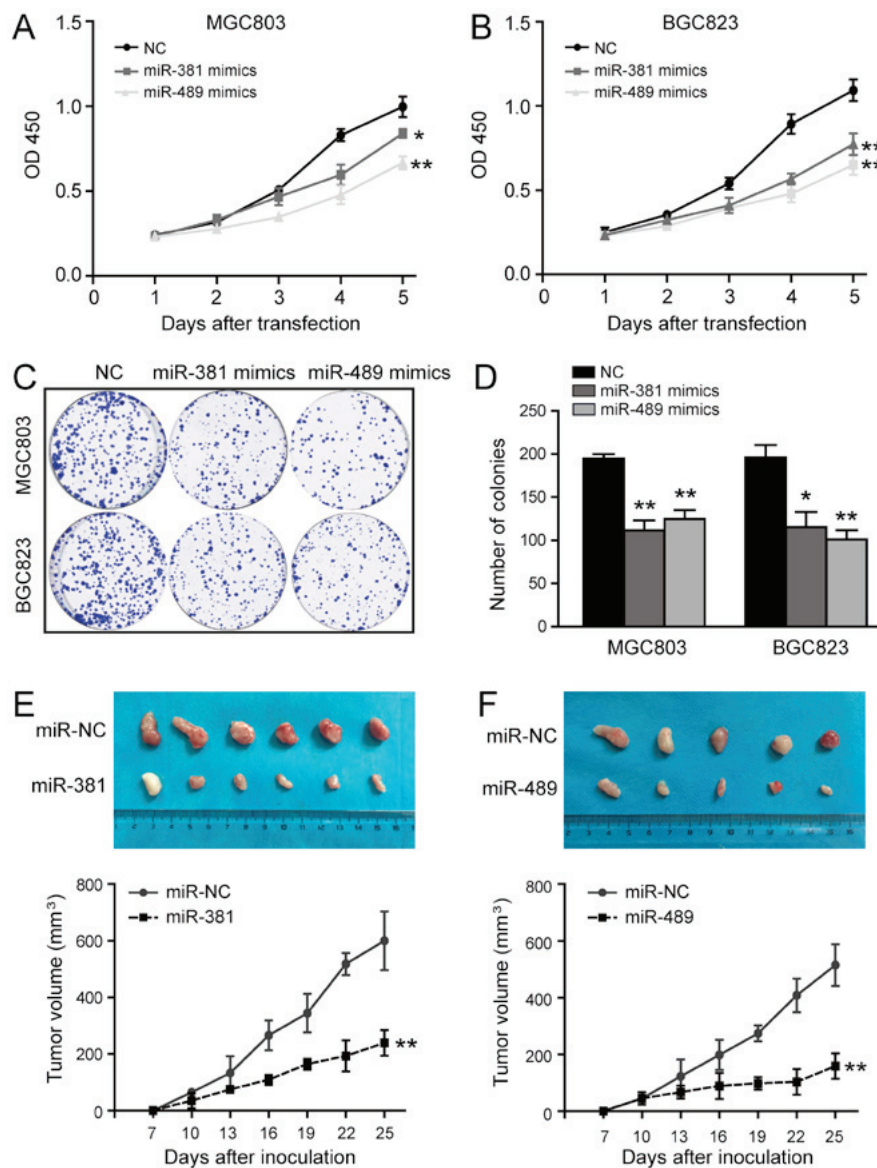


Figure 4. Ectopic expression of miR-381 or miR-489 inhibits gastric cancer (GC) cell proliferation *in vitro* and *in vivo*. (A and B) Following transfection with miR-381 or miR-489 mimics or negative control, the proliferation of GC cells was evaluated by CCK-8 assay. (C and D) Effects of miR-381 and miR-489 on colony formation. Representative micrographs (C) and quantification (D) of colonies formed by the indicated groups of cells. (E and F) Xenograft experiments were performed to confirm the effects of miR-381 and miR-489 on tumour growth *in vivo*. Each group was subcutaneously injected with MGC803 cells overexpressing miR-381, miR-489 or miR-NC. Tumours were measured every 3 days once detectable on day 7. One-way analysis of variance (adjusted for Bonferroni correction's test), $n=3$. * $P<0.05$ and ** $P<0.01$ vs. the miR-NC group.

progression. However, the reported expression patterns and roles of miR-381 in various types of cancer markedly differ. miR-381 has been shown to be downregulated in malignancies and exerts tumour suppressive effects on colon, breast and liver cancer and pituitary tumours (27,37-39). In lung cancer, this miRNA has been shown to inhibit cell migration and invasion by targeting LRH-1, where it acts as a prognostic marker (40). However, miR-381 may even function as an oncogenic miRNA in certain other malignancies, including glioma and osteosarcoma (41,42). miR-489 has also been identified as a tumour suppressor miRNA in multiple types of cancer, such as breast, ovarian, colorectal and non-small cell lung cancer (43-46). Recently, it has been reported in two different studies that miR-381 and miR-489 inhibit GC growth and metastasis by targeting TMEM16A and PROX1 (22,23), respectively. In the present study, we further expanded the

function of miR-381 and miR-489 in GC. We revealed that these two miRNAs are critical for cell proliferation, invasion, and the EMT process (Figs. 4 and 5), which recapitulates the effects stimulated by CUL4B silencing (Fig. 3). Of note, the combined overexpression of miR-381 and miR-489 suppressed GC cell growth, migration and invasion more markedly due to a greater inhibition of CUL4B protein expression (data not shown). For more conclusive results, we performed rescue experiments by overexpressing CUL4B in GC cells and evaluated the outcomes using CCK-8, colony formation, and Transwell invasion assays. Surprisingly, re-expression of CUL4B partially attenuated the effects of miR-381 and miR-489 on GC cell proliferation and invasion (Fig. 6). These data provide direct evidence that miR-381/miR-489 inhibit GC progression by suppressing CUL4B expression. In addition, it is worth noting that the ectopic expression of CUL4B did

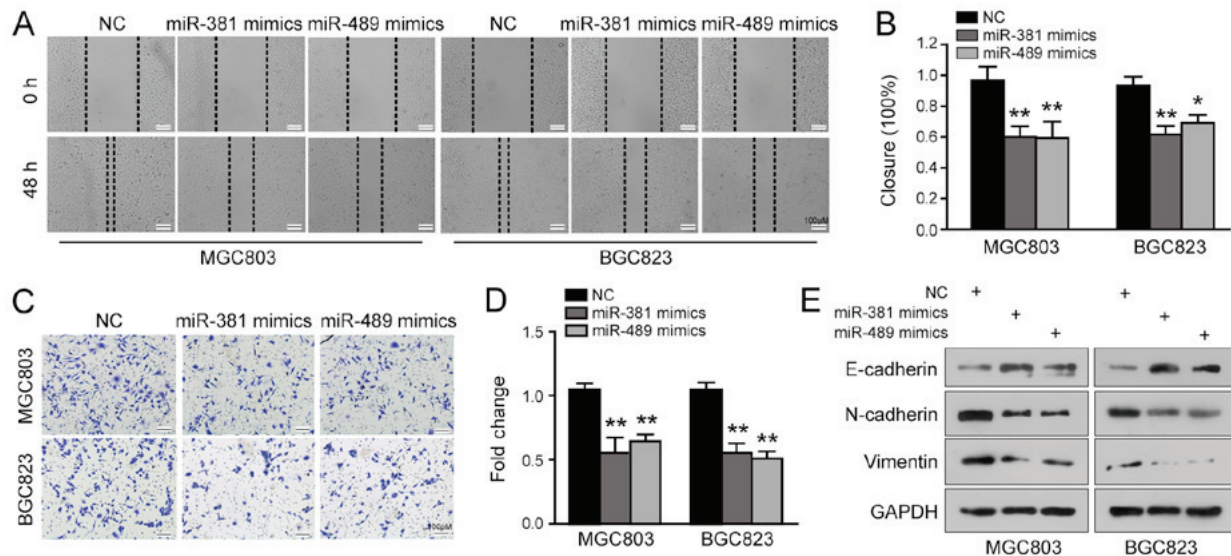


Figure 5. miR-381 and miR-489 regulate the migration, invasion and epithelial-mesenchymal transition (EMT) of gastric cancer (GC) cells. (A and B) Cell migration ability evaluated by wound healing assays (magnification, x100). (C and D) The indicated cells were subjected to Matrigel invasion assays. Representative images (C) and quantification (D) of invasive cells are shown (magnification, x100). (E) Western blot analysis of E-cadherin, N-cadherin and Vimentin following transfection with miR-381 or miR-489 mimics. One-way analysis of variance (adjusted for Bonferroni correction's test). All data are presented as the means \pm SEM of triplicate experiments, n=3. *P<0.05 and **P<0.01 vs. the negative control (NC) group.

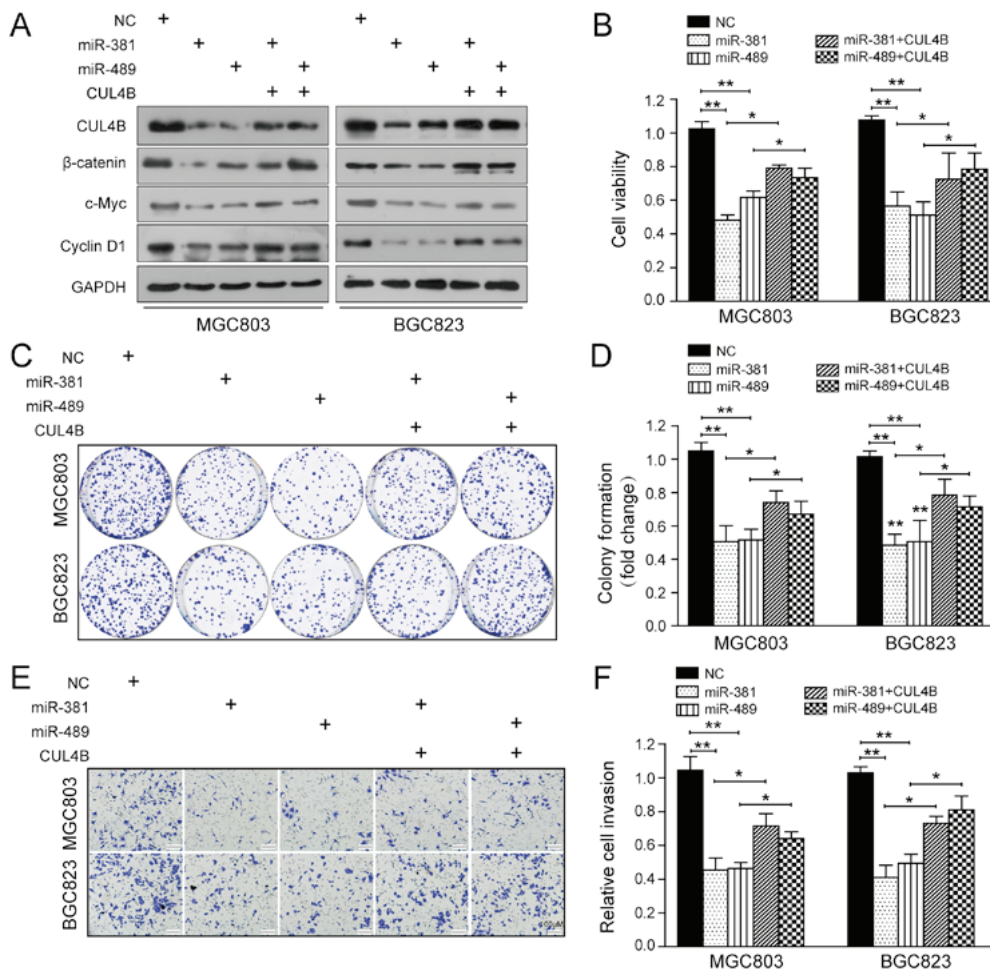


Figure 6. CUL4B counteracts the suppressive effects of miR-381 and miR-489 upregulation. (A) Western blot analysis of expression of CUL4B and its downstream Wnt/β-catenin pathway targets after transfection with miR-381/miR-489 mimics or negative control and CUL4B-no UTR (CUL4B without its 3'-UTR, shown as CUL4B). GAPDH was used as an internal control. (B) At the indicated time points post-transfection, the cell growth rate was measured using a CCK-8 assay. (C and D) Colony formation assays of gastric cancer (GC) cells after the indicated treatments. (E and F) Transwell invasion assays of GC cells following the indicated treatments (magnification, x100). Representative images (E) and quantification (F) of invasive cells in the indicated groups are shown. One-way analysis of variance (adjusted for Bonferroni correction's test). All data are presented as the means \pm SEM of triplicate experiments, n=3. *P<0.05 and **P<0.01.

not completely abrogate the observed miRNA-induced effects, suggesting that other downstream targets may also contribute to the functions of miR-381 and miR-489.

The involvement of Wnt/ β -catenin signalling in a wide variety of cellular processes, such as growth, differentiation, invasion and tumourigenesis (47), has been well documented. Our data, along with those of others, have revealed CUL4B to be a positive regulator of the Wnt/ β -catenin pathway (9,48). In this study, based on our finding that the behaviour of GC cells was affected by miR-381, miR-489 and CUL4B expression, we examined whether the Wnt/ β -catenin pathway was also involved in this interplay. Our results revealed that miR-381 or miR-489 overexpression markedly decreased the expression of β -catenin, c-Myc, and cyclin D1, suggesting that these miRNAs act as negative regulators of Wnt/ β -catenin signalling. Indeed, a previous study on renal cancer indicated that the gene encoding β -catenin is a target of miR-381 (49), although the association between miR-489 and the Wnt/ β -catenin pathway in tumourigenesis has not yet been clarified. Furthermore, in this study, we found that the re-establishment of CUL4B expression also negated the suppressive effects of miR-381 and miR-489 on Wnt/ β -catenin signalling. Taken together, these data suggest that miR-381/miR-489 inactivate the Wnt/ β -catenin pathway by targeting CUL4B. Thus, the inactivation of Wnt/ β -catenin signalling is also involved in the anti-tumour functions of miR-381 and miR-489 in GC.

In conclusion, the data from the present study demonstrate that the downregulation of miR-381 and miR-489 is responsible for the aberrant CUL4B overexpression in GC tumourigenesis. The miR-381/489-CUL4B axis greatly contributes to the initiation and progression of GC via the Wnt/ β -catenin pathway. Our results not only expand the target pool of miR-381 and miR-489, but also confirm a direct interaction between CUL4B and these miRNAs, highlighting the miR-381/489-CUL4B axis as a promising target for GC therapeutic strategies.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

ZF contributed to the analysis and interpretation of the data and the drafting of the manuscript. MZ contributed to the acquisition and analysis of the data. YW and XY contributed

to data analysis and technical support. HG and YY performed the western blot analysis of the gastric cancer clinical samples. MF and JC contributed to the acquisition of the data and revised the manuscript for important intellectual content. JX and XX conceived and designed, and supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Independent Ethics Committee of the First Affiliated Hospital of Nanchang University and complied with the Declaration of Helsinki (Approval no. #021-2017). All patients agreed to participate in this study and gave written informed consent. All animal experiments were approved by the Animal Ethics Committee of Nanchang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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