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

ZILING FANG[1*], MIN ZHONG[1*], YI WANG[1,2], XIANG YUAN[1], HUI GUO[1], YANGYANG YAO[1], MIAO FENG[1], JUN CHEN[1], JIANPING XIONG[1] and XIAOJUN XIANG[1]

[1]Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006; [2]Department of Radiotherapy and Chemotherapy, Ningbo No. 2 Hospital, Ningbo, Zhejiang 315010, P.R. China

Received May 17, 2018; Accepted November 5, 2018

DOI: 10.3892/ijo.2018.4646

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 CRL4B 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

Correspondence to: Dr Xiaojun Xiang or Dr Jianping Xiong, Department of Oncology, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Donghu, Nanchang, Jiangxi 330006, P.R. China
E-mail: xjxiang_nc@163.com
E-mail: jpxiong_nc@sina.com

*Contributed equally

Key words: cullin 4B, microRNA-381, microRNA-489, gastric cancer, proliferation and invasion, Wnt/β-catenin pathway

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 $2^{-\Delta\Delta Cq}$ 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 regent (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 AGAAGUCAUUAAUTT-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 μg/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	2.9x.4	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: TGGTGTCGTGGAGTCG
CUL4B	F: TGGAAGTTCATTTACCACCAGAGATG R: TTCTGCTTTTAACACACAGTGTCCTA
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 $2x10^5$ 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 M5e; Molecular Devices, Sunnyvale, CA, USA) every 24 h for 5 days. The data was derived from triplicate samples and are presetned 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 $4.5x10^5$ per well, were seeded in 6-well plates and serum-starved for 24 h once fully confluent. A sterile 200-μl 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-μm pore size; BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assay, cells were suspended in 200 μl serum-free medium and seeded in the upper chambers of Transwell inserts pre-coated with Matrigel. Medium (500 μl) 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±2˚C with 40-70% humidity. The mice were randomly allocated to 1 of 4 groups (6 mice in each) and inoculated with $5x10^6$ 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 x $width^2$)/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 ± 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

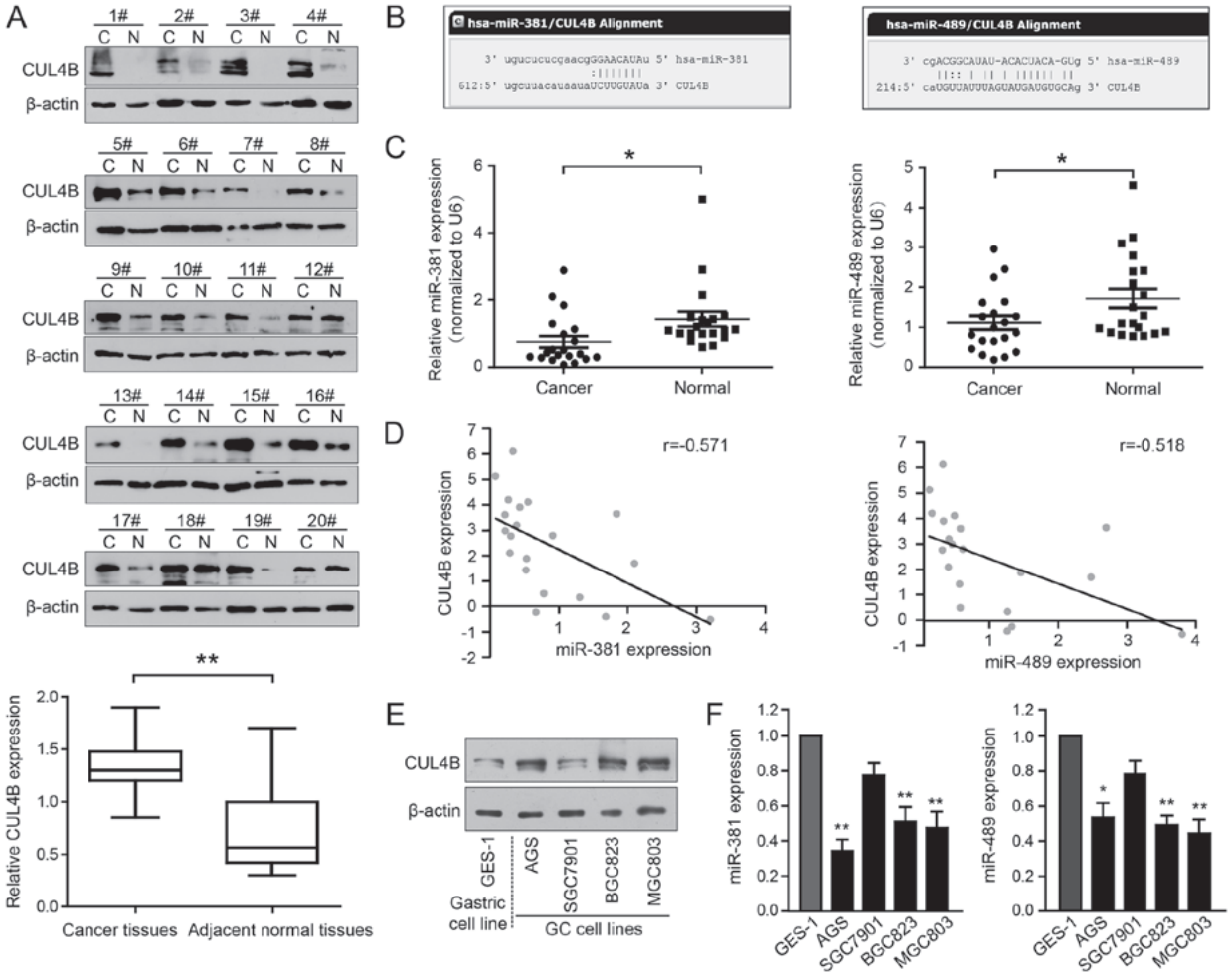

Figure 1. CUL4B expression inversely correlates with the miR-381 and miR-489 levels in gastric cancer (GC) tissues and cell lines. (A) CUL4B protein expression in 20 pairs of GC tissues (lanes labelled with 'C') and corresponding adjacent non-cancerous gastric tissues (lanes labelled with 'N') detected 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 ± 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

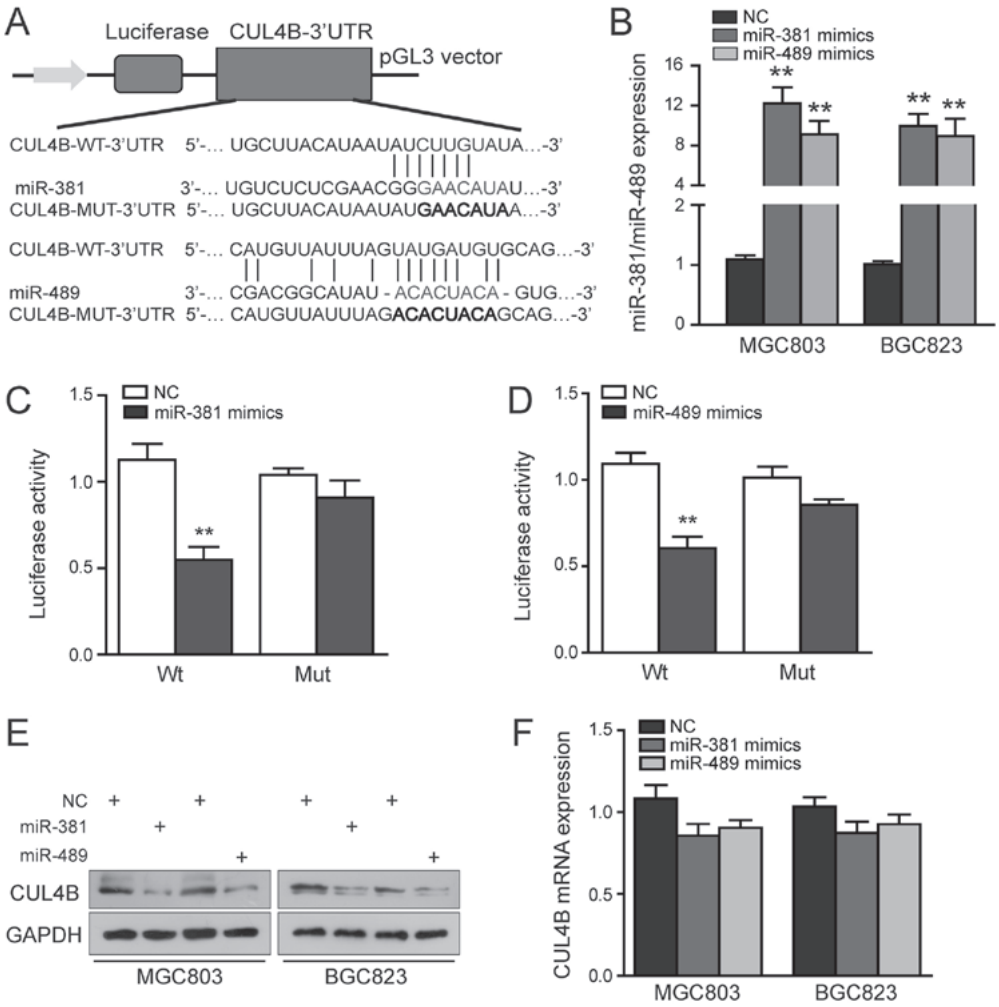

Figure 2. CUL4B is directly targeted by miR-381 and miR-489. (A) Wild-type (Wt) and mutant (Mut) CUL4B 3'-UTR sequences were cloned into a pGL3 reporter vector. Mutant constructs were generated by altering the seed regions within the CUL4B 3'-UTR, as indicated in bold. (B) miR-381 and miR-489 expression 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 ± 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 ($5x10^6$ 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

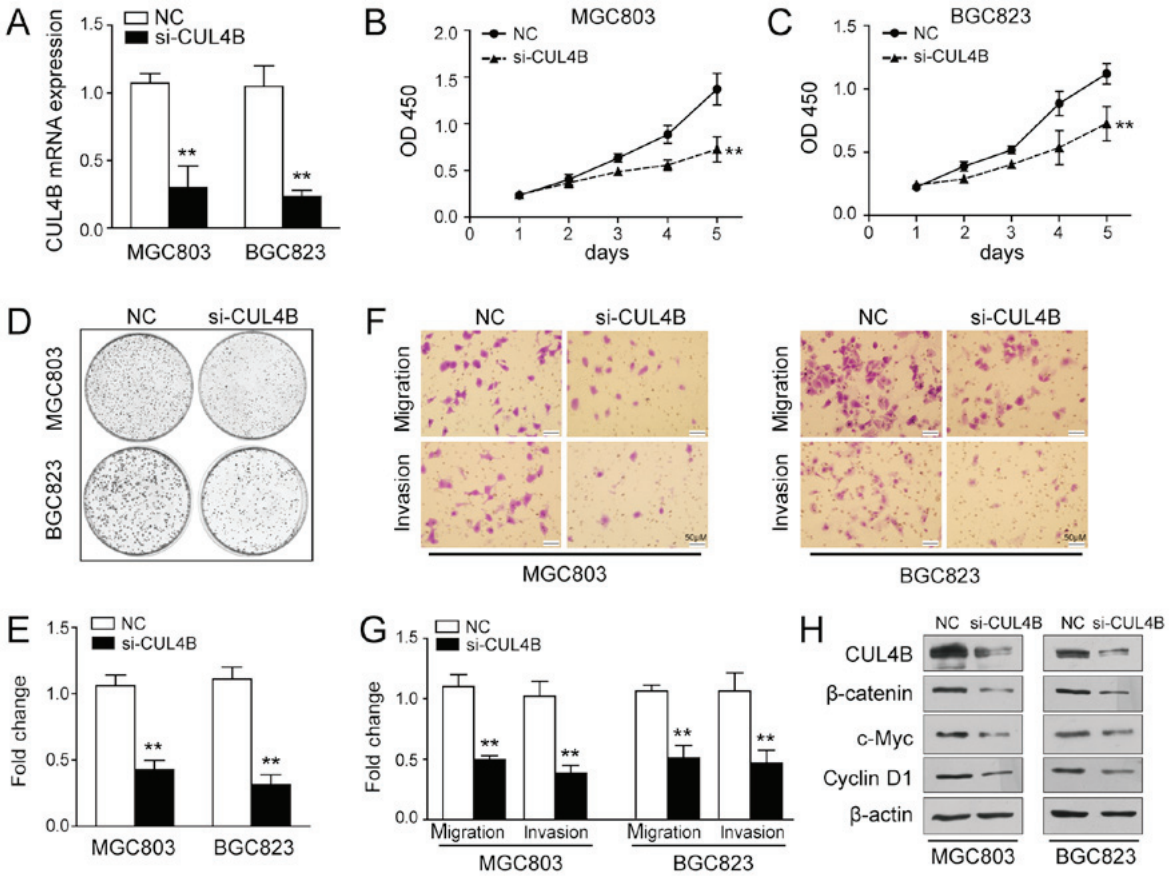

Figure 3. CUL4B silencing inhibits the proliferation, migration and invasion of gastric cancer (GC) cells via inactivating the Wnt/β-catenin pathway. (A) RT-qPCR analysis of CUL4B mRNA expression in GC cells following transfection with CUL4B siRNAs or the corresponding negative control for 24 h. (B and 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, x200). (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 ± 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

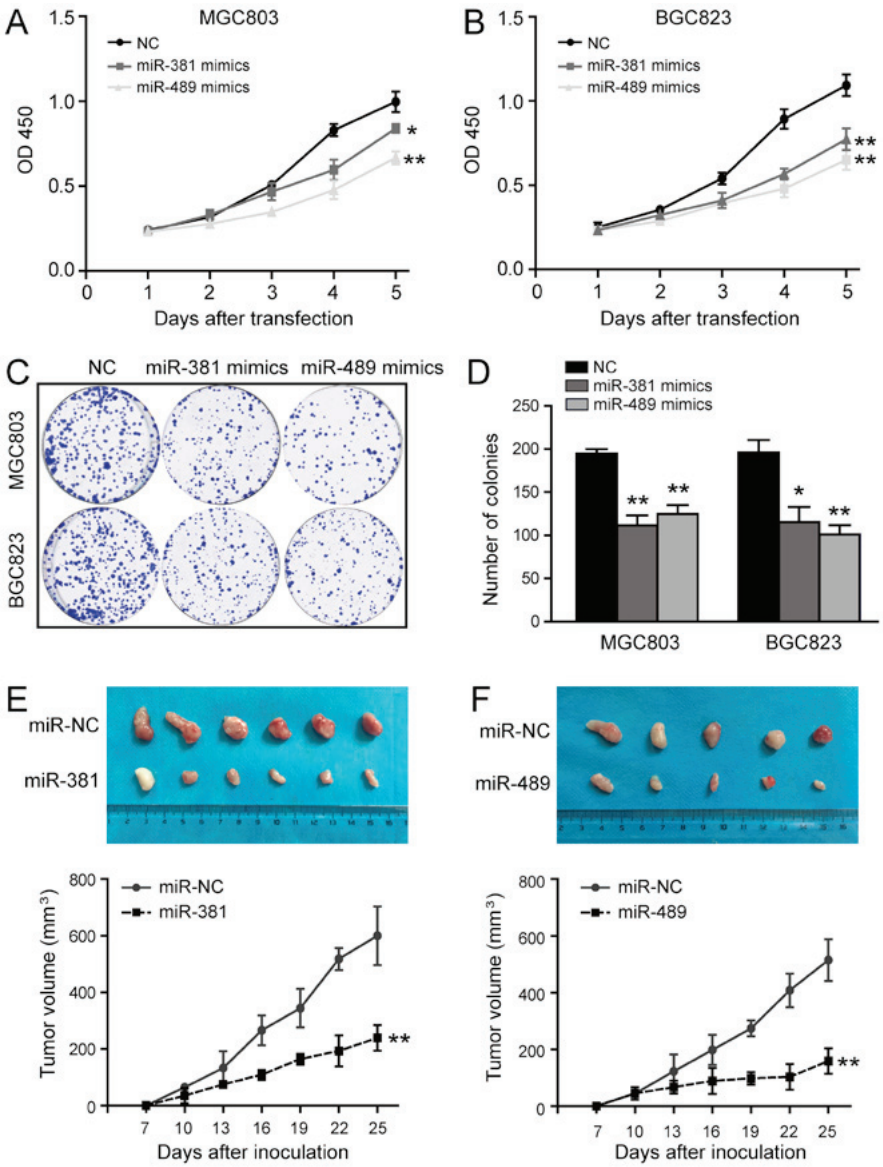

Figure 4. Ectopic expression of miR-381 or miR-489 inhibits gastric cancer (GC) cell proliferation *in vitro* and *in vivo*. (A and B) Following transfection with miR-381 or miR-489 mimics or negative control, the proliferation of GC cells was evaluated by CCK-8 assay. (C and D) Effects of miR-381 and 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

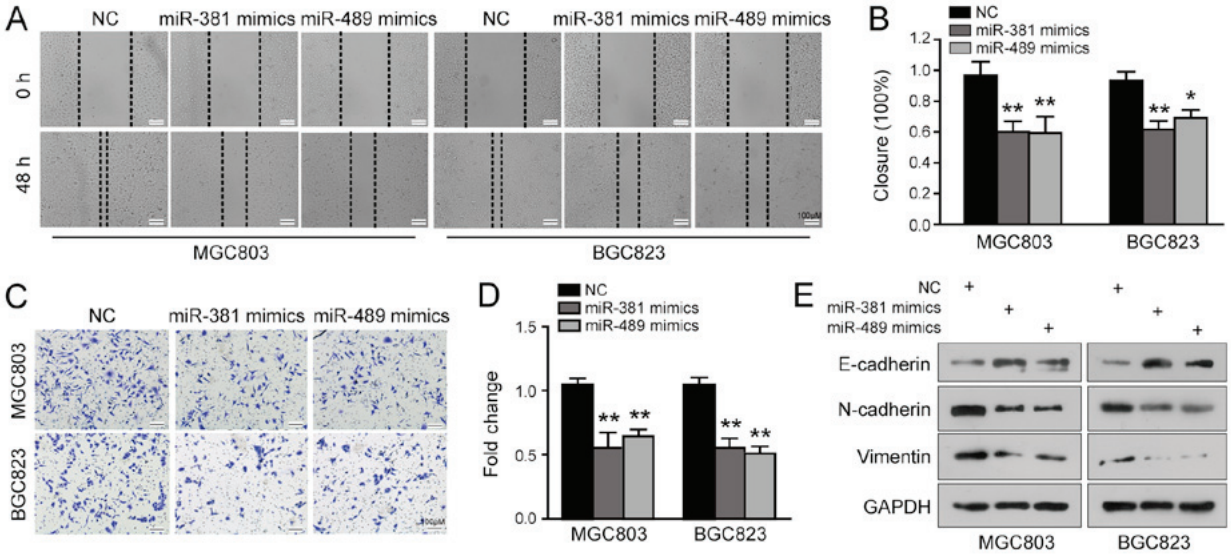

Figure 5. miR-381 and miR-489 regulate the migration, invasion and epithelial-mesenchymal transition (EMT) of gastric cancer (GC) cells. (A and B) Cell migration ability evaluated by wound healing assays (magnification, x100). (C and D) The indicated cells were subjected to Matrigel invasion assays. 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 ± SEM of triplicate experiments, n=3. *P<0.05 and **P<0.01 vs. the negative control (NC) group.

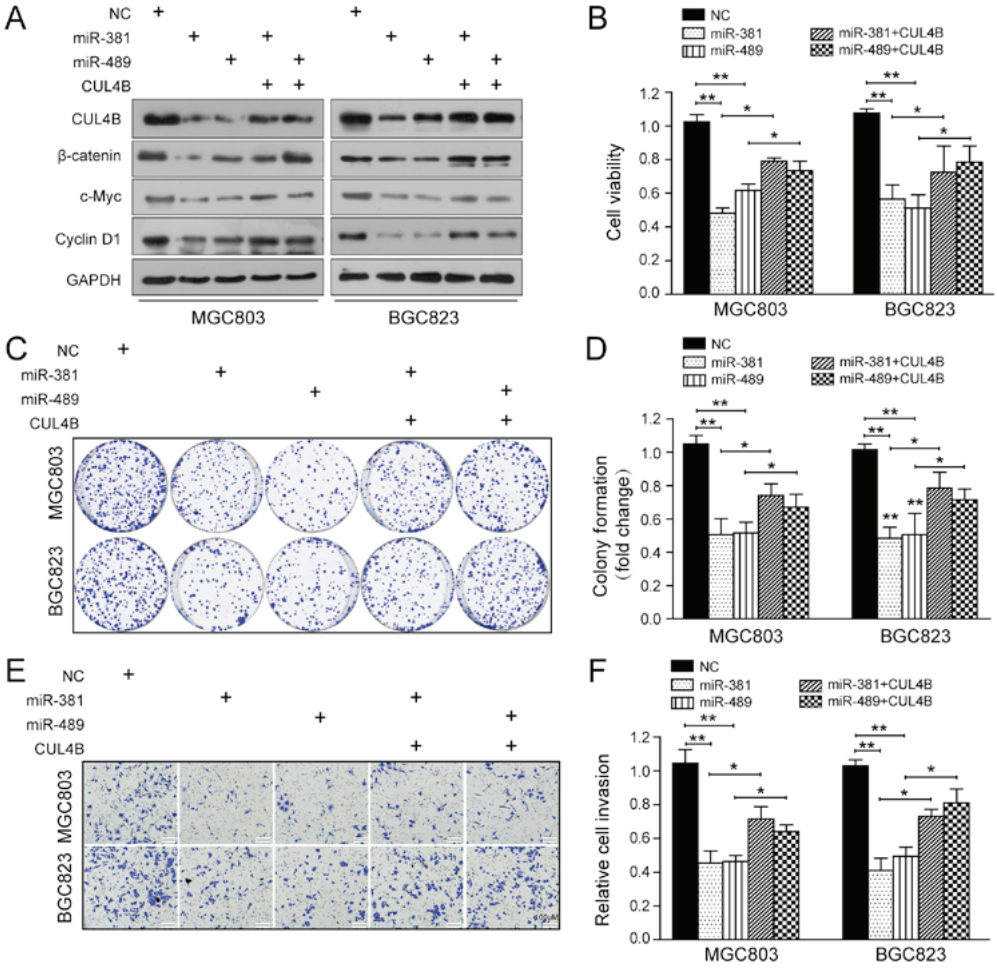

Figure 6. CUL4B counteracts the suppressive effects of miR-381 and miR-489 upregulation. (A) Western blot analysis of expression of CUL4B and its downstream Wnt/β-catenin pathway targets after transfection with miR-381/miR-489 mimics or negative control and CUL4B-no UTR (CUL4B without its 3'-UTR, shown 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 ± 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.

Acknowledgements

Not applicable.

Funding

The study was supported by the National Natural Science Foundation of China (no. 81660402), and grants from the Science and Technology Department of Jiangxi Province (nos. 20161ACB21018 and 20171BBH80027), and the Graduate Student Innovation Foundation of Jiangxi Province, China (YC2015-B010).

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.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Wadhwa R, Song S, Lee JS, Yao Y, Wei Q and Ajani JA: Gastric cancer-molecular and clinical dimensions. Nat Rev Clin Oncol 10: 643-655, 2013.
3. Petroski MD and Deshaies RJ: Function and regulation of cullin-RING ubiquitin ligases. Nat Rev Mol Cell Biol 6: 9-20, 2005.
4. Yi J, Lu G, Li L, Wang X, Cao L, Lin M, Zhang S and Shao G: DNA damage-induced activation of CUL4B targets HUWE1 for proteasomal degradation. Nucleic Acids Res 43: 4579-4590, 2015.
5. Qian Y, Yuan J, Hu H, Yang Q, Li J, Zhang S, Jiang B, Shao C and Gong Y: The CUL4B/AKT/β-catenin axis restricts the accumulation of myeloid-derived suppressor cells to prohibit the establishment of a tumor-permissive microenvironment. Cancer Res 75: 5070-5083, 2015.
6. Qu Z, Li D, Xu H, Zhang R, Li B, Sun C, Dong W and Zhang Y: CUL4B, NEDD4, and UGT1As involve in the TGF-β signalling in hepatocellular carcinoma. Ann Hepatol 15: 568-576, 2016.
7. Mok MT and Cheng AS: CUL4B: A novel epigenetic driver in Wnt/β-catenin-dependent hepatocarcinogenesis. J Pathol 236: 1-4, 2015.
8. Jiang T, Tang HM, Wu ZH, Chen J, Lu S, Zhou CZ, Yan DW and Peng ZH: Cullin 4B is a novel prognostic marker that correlates with colon cancer progression and pathogenesis. Med Oncol 30: 534, 2013.
9. Mao XW, Xiao JQ, Xu G, Li ZY, Wu HF, Li Y, Zheng YC and Zhang N: CUL4B promotes bladder cancer metastasis and induces epithelial-to-mesenchymal transition by activating the Wnt/β-catenin signaling pathway. Oncotarget 8: 77241-77253, 2017.
10. Mi J, Zou Y, Lin X, Lu J, Liu X, Zhao H, Ye X, Hu H, Jiang B, Han B, *et al*: Dysregulation of the miR-194-CUL4B negative feedback loop drives tumorigenesis in non-small-cell lung carcinoma. Mol Oncol 11: 305-319, 2017.
11. Hu H, Yang Y, Ji Q, Zhao W, Jiang B, Liu R, Yuan J, Liu Q, Li X, Zou Y, *et al*: CRL4B catalyzes H2AK119 monoubiquitination and coordinates with PRC2 to promote tumorigenesis. Cancer Cell 22: 781-795, 2012.
12. Yang Y, Liu R, Qiu R, Zheng Y, Huang W, Hu H, Ji Q, He H, Shang Y, Gong Y, *et al*: CRL4B promotes tumorigenesis by coordinating with SUV39H1/HP1/DNMT3A in DNA methylation-based epigenetic silencing. Oncogene 34: 104-118, 2015.

13. Yuan J, Han B, Hu H, Qian Y, Liu Z, Wei Z, Liang X, Jiang B, Shao C and Gong Y: CUL4B activates Wnt/β-catenin signalling in hepatocellular carcinoma by repressing Wnt antagonists. J Pathol 235: 784-795, 2015.
14. Chen Z, Wang K, Hou C, Jiang K, Chen B, Chen J, Lao L, Qian L, Zhong G, Liu Z, *et al*: CRL4BDCAF11 E3 ligase targets p21 for degradation to control cell cycle progression in human osteosarcoma cells. Sci Rep 7: 1175, 2017.
15. Qi M, Jiao M, Li X, Hu J, Wang L, Zou Y, Zhao M, Zhang R, Liu H, Mi J, *et al*: CUL4B promotes gastric cancer invasion and metastasis-involvement of upregulation of HER2. Oncogene 37: 1075-1085, 2018.
16. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
17. Hammond SM: An overview of microRNAs. Adv Drug Deliv Rev 87: 3-14, 2015.
18. Lin S and Gregory RI: MicroRNA biogenesis pathways in cancer. Nat Rev Cancer 15: 321-333, 2015.
19. Xiang XJ, Deng J, Liu YW, Wan LY, Feng M, Chen J and Xiong JP: MiR-1271 Inhibits Cell Proliferation, Invasion and EMT in Gastric Cancer by Targeting FOXQ1. Cell Physiol Biochem 36: 1382-1394, 2015.
20. Xia JT, Chen LZ, Jian WH, Wang KB, Yang YZ, He WL, He YL, Chen D and Li W: MicroRNA-362 induces cell proliferation and apoptosis resistance in gastric cancer by activation of NF-κB signaling. J Transl Med 12: 33, 2014.
21. Ma DH, Li BS, Liu JJ, Xiao YF, Yong X, Wang SM, Wu YY, Zhu HB, Wang DX and Yang SM: miR-93-5p/IFNAR1 axis promotes gastric cancer metastasis through activating the STAT3 signaling pathway. Cancer Lett 408: 23-32, 2017.
22. Cao Q, Liu F, Ji K, Liu N, He Y, Zhang W and Wang L: MicroRNA-381 inhibits the metastasis of gastric cancer by targeting TMEM16A expression. J Exp Clin Cancer Res 36: 29, 2017.
23. Zhang B, Ji S, Ma F, Ma Q, Lu X and Chen X: miR-489 acts as a tumor suppressor in human gastric cancer by targeting PROX1. Am J Cancer Res 6: 2021-2030, 2016.
24. Fang Z, Zhang L, Liao Q, Wang Y, Yu F, Feng M and Xiang X and Xiong X: Regulation of TRIM24 by miR-511 modulates cell proliferation in gastric cancer. J Exp Clin Cancer Res 36: 17, 2017.
25. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
26. Han X, Fang Z, Wang H, Jiao R, Zhou J and Fang N: CUL4A functions as an oncogene in ovarian cancer and is directly regulated by miR-494. Biochem Biophys Res Commun 480: 675-681, 2016.
27. He X, Wei Y, Wang Y, Liu L, Wang W and Li N: MiR-381 functions as a tumor suppressor in colorectal cancer by targeting Twist1. Onco Targets Ther 9: 1231-1239, 2016.
28. Yang X, Ruan H, Hu X, Cao A and Song L: miR-381-3p suppresses the proliferation of oral squamous cell carcinoma cells by directly targeting FGFR2. Am J Cancer Res 7: 913-922, 2017.
29. Chen X, Wang YW, Xing AY, Xiang S, Shi DB, Liu L, Li YX and Gao P: Suppression of SPIN1-mediated PI3K-Akt pathway by miR-489 increases chemosensitivity in breast cancer. J Pathol 239: 459-472, 2016.
30. He YM, Xiao YS, Wei L, Zhang JQ and Peng CH: CUL4B promotes metastasis and proliferation in pancreatic cancer cells by inducing epithelial-mesenchymal transition via the Wnt/β-catenin signaling pathway. J Cell Biochem 119: 5308-5323, 2018.
31. Dong J, Wang XQ, Yao JJ, Li G and Li XG: Decreased CUL4B expression inhibits malignant proliferation of glioma in vitro and in vivo. Eur Rev Med Pharmacol Sci 19: 1013-1021, 2015.
32. Chen Z, Shen BL, Fu QG, Wang F, Tang YX, Hou CL and Chen L: CUL4B promotes proliferation and inhibits apoptosis of human osteosarcoma cells. Oncol Rep 32: 2047-2053, 2014.
33. Li Q, Li Z, Wei S, Wang W, Chen Z, Zhang L, Chen L, Li B, Sun G, Xu J, *et al*: Overexpression of miR-584-5p inhibits proliferation and induces apoptosis by targeting WW domain-containing E3 ubiquitin protein ligase 1 in gastric cancer. J Exp Clin Cancer Res 36: 59, 2017.
34. Zheng P, Chen L, Yuan X, Luo Q, Liu Y, Xie G, Ma Y and Shen L: Exosomal transfer of tumor-associated macrophage-derived miR-21 confers cisplatin resistance in gastric cancer cells. J Exp Clin Cancer Res 36: 53, 2017.
35. Petersen B and Kingsley K: Differential expression of miR-21, miR-133 and miR-155 from exosome fractions isolated from oral squamous cell carcinomas in vitro. J Med Discov 1: 16010, 2016.
36. Deng J, Lei W, Xiang X, Zhang L, Lei J, Gong Y, Song M, Wang Y, Fang Z, Yu F, *et al*: Cullin 4A (CUL4A), a direct target of miR-9 and miR-137, promotes gastric cancer proliferation and invasion by regulating the Hippo signaling pathway. Oncotarget 7: 10037-10050, 2016.
37. Liang Y, Zhao Q, Fan L, Zhang Z, Tan B, Liu Y and Li Y: Down-regulation of MicroRNA-381 promotes cell proliferation and invasion in colon cancer through up-regulation of LRH-1. Biomed Pharmacother 75: 137-141, 2015.
38. Ming J, Zhou Y, Du J, Fan S, Pan B, Wang Y, Fan L and Jiang J: miR-381 suppresses C/EBPα-dependent Cx43 expression in breast cancer cells. Biosci Rep 35: 35, 2015.
39. Zhang Q, Zhao S, Pang X and Chi B: MicroRNA-381 suppresses cell growth and invasion by targeting the liver receptor homolog-1 in hepatocellular carcinoma. Oncol Rep 35: 1831-1840, 2016.
40. Tian C, Li J, Ren L, Peng R, Chen B and Lin Y: MicroRNA-381 serves as a prognostic factor and inhibits migration and invasion in non-small cell lung cancer by targeting LRH-1. Oncol Rep 38: 3071-3077, 2017.
41. Wang Z, Yang J, Xu G, Wang W, Liu C, Yang H, Yu Z, Lei Q, Xiao L, Xiong J, *et al*: Targeting miR-381-NEFL axis sensitizes glioblastoma cells to temozolomide by regulating stemness factors and multidrug resistance factors. Oncotarget 6: 3147-3164, 2015.
42. Li Y, Zhao C, Yu Z, Chen J, She X, Li P, Liu C, Zhang Y, Feng J, Fu H, *et al*: Low expression of miR-381 is a favorite prognosis factor and enhances the chemosensitivity of osteosarcoma. Oncotarget 7: 68585-68596, 2016.
43. Wang X, Wang X, Gu J, Zhou M, He Z, Wang X and Ferrone S: Overexpression of miR-489 enhances efficacy of 5-fluoro-uracil-based treatment in breast cancer stem cells by targeting XIAP. Oncotarget 8: 113837-113846, 2017.
44. Wu H, Xiao Z, Zhang H, Wang K, Liu W and Hao Q: MiR-489 modulates cisplatin resistance in human ovarian cancer cells by targeting Akt3. Anticancer Drugs 25: 799-809, 2014.
45. Gao S, Liu H, Hou S, Wu L, Yang Z, Shen J, Zhou L, Zheng SS and Jiang B: MiR-489 suppresses tumor growth and invasion by targeting HDAC7 in colorectal cancer. Clin Transl Oncol 20: 703-712, 2018.
46. Xie Z, Cai L, Li R, Zheng J, Wu H, Yang X, Li H and Wang Z: Down-regulation of miR-489 contributes into NSCLC cell invasion through targeting SUZ12. Tumour Biol 36: 6497-6505, 2015.
47. Clevers H and Nusse R: Wnt/β-catenin signaling and disease. Cell 149: 1192-1205, 2012.
48. Zhang JQ, Chen S, Gu JN, Zhu Y, Zhan Q, Cheng DF, Chen H, Deng XX, Shen BY and Peng CH: MicroRNA-300 promotes apoptosis and inhibits proliferation, migration, invasion and epithelial-mesenchymal transition via the Wnt/β-catenin signaling pathway by targeting CUL4B in pancreatic cancer cells. J Cell Biochem 119: 1027-1040, 2018.
49. Chen B and Liu B: MiRNA-381 inhibits the invasion of renal carcinoma and the underlying mechanisms. Zhong Nan Da Xue Xue Bao Yi Xue Ban 40: 1053-1059, 2015 (In Chinese).
50. Washington K: 7th edition of the AJCC cancer staging manual: Stomach. Ann Surg Oncol 17: 3077-3079, 2010.