Int J Clin Exp Med 2016;9(8):15844-15851
www.ijcem.com /ISSN:1940-5901/IJCEM0027581

Original Article

microRNA-138 suppressed proliferation and invasion of gastric cancer via RhoC

Jie Kan[1*], Bingyu Guan[2*], Jun Han[1], Xinguo Wang[1], Hao Wu[3]

[1]Department of Oncology, Qinghai Provincial People's Hospital, 2 Gonghe Road, Xining 810007, Qinghai Province, PR China; [2]Department of Orthopedic, The First People's Hospital of Xining, 3 Huzhu Road, Xining 810000, Qinghai Province, PR China; [3]Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, PR China. [*]Equal contributors.

Received March 6, 2016; Accepted July 4, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Gastric cancer (GC) is the fourth most predominant malignancy worldwide and remains the second most common cause of cancer-related death globally. Recent studies indicated that miRNAs have been involved in the tumorigenesis pathways in GC. In this study, we investigated the effect and molecular mechanism of miR-138 in human GC. The effects of miR-138 on the GC cell growth and invasion were first studied. Then the correlation of miR-138 with RhoC was confirmed by dual luciferase reporter assay. We found that the overexpression of miR-138 resulted in inhibition of GC growth and reversion of invasion. RhoC was identified as the target of miR-138. Altogether, we conclude that miR-138 can modulate gastric cancer cell growth and invasion by suppressing the expression of RhoC and the activation of PI3K/Akt pathway. These data represent a crucial step towards the understanding of the novel roles and molecular mechanism of miR-138, RhoC in GC progression. Therefore, miR-138 may serve as a useful therapeutic agent for miRNA-based GC therapy.

Keywords: Gastric cancer, RhoC, miR-138, proliferation, invasion

Introduction

Gastric cancer (GC) is the fourth most predominant malignancy worldwide and remains the second most common cause of cancer-related death globally [1]. Therefore, a better understanding of the molecular mechanisms of gastric cancer progression may lead to important improvement in the development of new therapeutic agents [2]. Recently, studies on the effect of microRNAs on gastric cancer have shown great progress [3-5].

MicroRNAs (miRNAs) are a class of endogenous small non-coding RNAs that control the target gene expression [6]. Several miRNAs have been classified and are aberrantly expressed in different cancer types. Overexpression or loss of expression of these miRNAs associates with tumor cell proliferation, apoptosis, survival, differentiation and invasion [7]. To data, many miRNAs have been found to exert significant regulatory effects in gastric cancer. However, little is known about miR-138 which acts as a tumor suppresser in several cancer types. Loss of miR-138 expression may partially contribute to the gain of hTERT protein expression via targeting the hTERT 3'-untranslated region (3'-UTR) in thyroid carcinoma [8]. miR-138 acts as a tumor suppresser and may serve as a therapeutic target for head and neck squamous cell carcinoma patients at risk of metastasis [9]. miR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene [10]. The down-regulation of miR-138 can regulate CCND3 and function as a tumor suppressor in hepatocellular carcinoma [11]. miR-138 functions as a tumor-suppressive miRNA and that down-regulation of miR-138 contributes to constitutive NF-kappaB activation and esophageal squamous cell carcinoma progression [12]. miR-138 inhibits tumor growth through repression of EZH2 in non-small cell lung cancer [13]. miR-138 negatively regulates non-small cell lung cancer cells through the interaction with cyclin D3 [14]. miR-138 suppresses larynx carcinoma metastases through ZEB2 inhibition [15]. Whether miR-138 acts as a tumor suppresser in gastric cancer?

To further understand the regulatory mechanisms of miR-138 in GC progression, we in this study chose gastric cancer HGC-27 and MGC-803 cells. First, we examined the effect of miR-138 on the GC cell growth and found that the overexpression of miR-138 inhibited cell growth. Importantly, we found that miR-138 overexpression induced the reversion of invasion with decreased Snail expression and increased E-cadherin expressions. Furthermore, we demonstrated that RhoC was direct functional targets of miR-138 in the progress of GC. Our study not only makes a contribution to the understanding of the roles and molecular mechanisms of miR-138 in GC progression, but also the data may be translated into new therapeutics and/or prognostic biomarkers for GC.

Materials and methods

Patient tissue specimens

A total of 34 specimens from GC patients were collected for this study. All specimens had been histologically and clinically diagnosed at Qinghai Provincial People's Hospital. For the use of these clinical materials for research purposes, prior patient's consents and approval from the Institutional Research Ethics Committee were obtained.

Cell culture

Human gastric cancer HGC-27 and MGC-803 cell lines were grown in RPMI-1640 (Thermo scientific, USA) containing 20% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomysin in a humidified atmosphere of 5% CO_2 at 37°C.

Cell transfection

miR-138 mimics (GenePharma, Shanghai, China) at a final concentration 50 nM were transfected into HGC-27 and MGC-803 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were analysed as required.

Cell proliferation assay

Cells were seeded into 96-well plates and transfected with miR-138 mimics or control. After transfection, cell proliferation was measured by Cell Counting Kit-8 (CCK-8) (Dojindo, Tokyo, Japan) according to the instructions. Cell Counting Kit-8 reagent was added at 0, 24, 48, and 72 h respectively and incubated at 37°C for 2 h. The OD (optical density) 450 nm value was detected by using a microplate reader (Bio-Rad, Richmond, USA).

Invasion assay

A total of 10^5 cells/well, suspended in serum free medium, were seeded into the upper chambers with 8.0 m PET membrane inserts (Corning Costar, MA, USA), which have been coated with 30% of BD Matrigel matrix (BD Biosciences, USA). The bottom chambers were added complete medium. After 48 hours incubation, cells remaining in the upper chamber were removed. Cells adhering to the lower membrane were fixed with methanol and stained with crystal violet before counted. Three independent experiments were performed.

Dual luciferase reporter assay

pmirGLO containing the wild-type or mutated potential target gene 3'-UTR and miR-138 mimics were cotransfected into HGC-27 and MGC-803 cells by Lipofectamine 2000 Transfection Reagent. Relative Renilla luciferase activity normalized to firefly luciferase activity was measured after transfection using the Dual-GloLuciferase Assay System (Promega, USA) on a SpectraMax L spectrophotometer (Molecular Devices, USA).

Real-time PCR assay

The relative expression level of miR-138 (normalized to U6) was determined using Hairpin-itTM miRNA real-time PCR Detection Kit (GenePharma, Shanghai, China) according to the manufacturer's protocol in a StepOnePlus™ real-time PCR instruments (Applied Biosystems, San Diego, USA). However, the relative expression levels of RhoC (normalized to β-actin) were determined using SYBR Green (Takara, Japan). The primers for RhoC were shown as below: Forward primer: 5'-GGAGGTCTACGTCCCTACTGT-3', Reverse primer: 5'-CGCAGTCGATCATAGTCTTCC-3'. The relative expression levels of the miRNA and mRNAs were determined using the 2-ΔΔCt analysis method, where U6 was used as an internal reference for miR-138 and β-actin for mRNAs. All reactions were performed in triplicate.

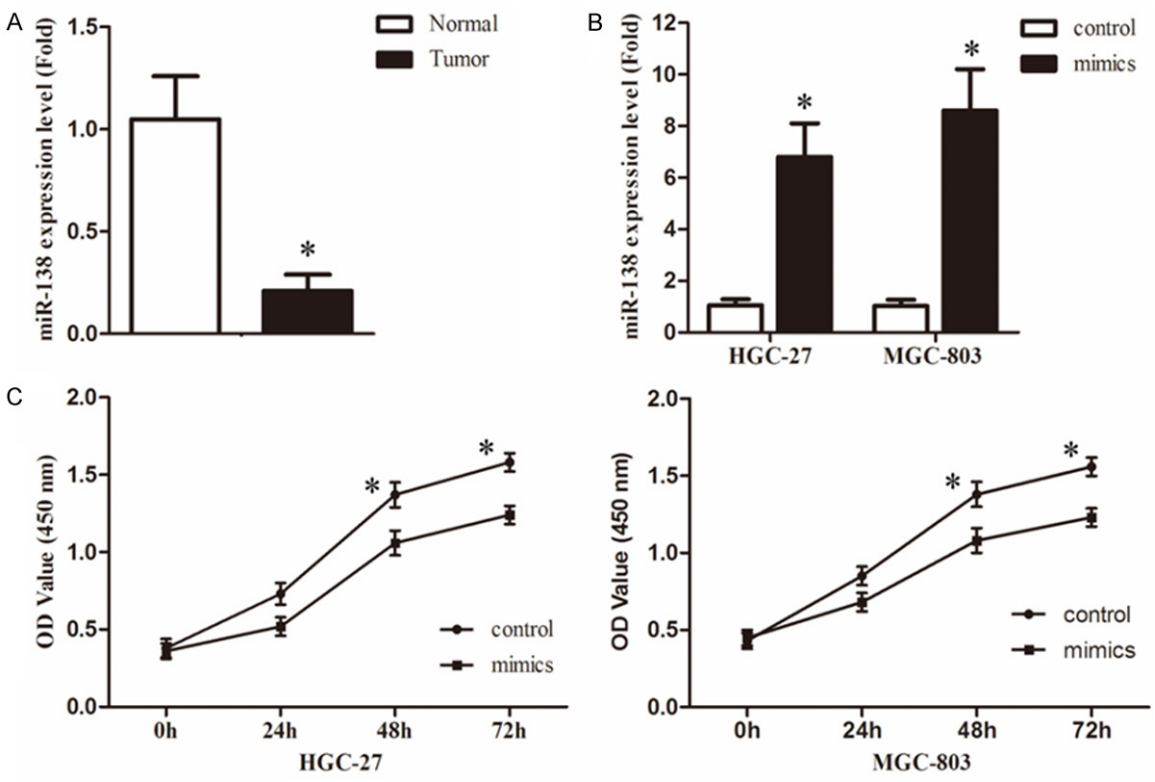

Figure 1. miR-138 was down-regulated and could suppress the proliferation in GC. A: Relative expression of miR-138 in non-tumor tissues (n = 12) and GC tissues (n = 22). Real-time PCR was used to determine the expression of miR-138 and normalized to U6 expression. Normal, non-tumor tissues; Tumor, tumor tissues. B: Relative expression of miR-138 in HGC-27and MGC-803 cells transfected by control and miR-138 mimics detected by real-time PCR and normalized to U6 expression. Data are means ± SD. *, $P < 0.05$. (n = 3). C: Proliferation of HGC-27and MGC-803 cells transfected by control and miR-138 mimics detected by CCK-8 assay. Data are means ± SD. *, $P < 0.05$. (n = 3).

Western blot

Total proteins were extracted from the cultured cells or tissues and quantified using a BCA Protein Assay Kit (Beyotime, Jiangsu, China) with BSA as a standard. Equal amounts of proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% BSA (5% w/v in PBS + 0.1% Tween 20) and incubated with primary antibodies at room temperature. The antibodies which are against RhoC, Snail, E-cadherin, and β-actin were used according to the manufacturer's instructions, and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were washed and incubated with respective secondary antibodies and were visualized by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Shown are the representative data from three individual experiments.

Statistical analysis

Data are presented as means ± SD of three independent experiments. Differences between groups were analysed by GraphPad Prism 5 software (GraphPad Software, CA, USA) with Student's t-test. Differences were considered statistically significant at $P < 0.05$.

Results

miR-138 was down-regulated in tissues from GC patients

To discover the expression of miR-138, real-time PCR was performed. Our results exhibited that the expression of miR-138 in tissues from GC patients was substantially lower than that in tissues from normal group (**Figure 1A**). These

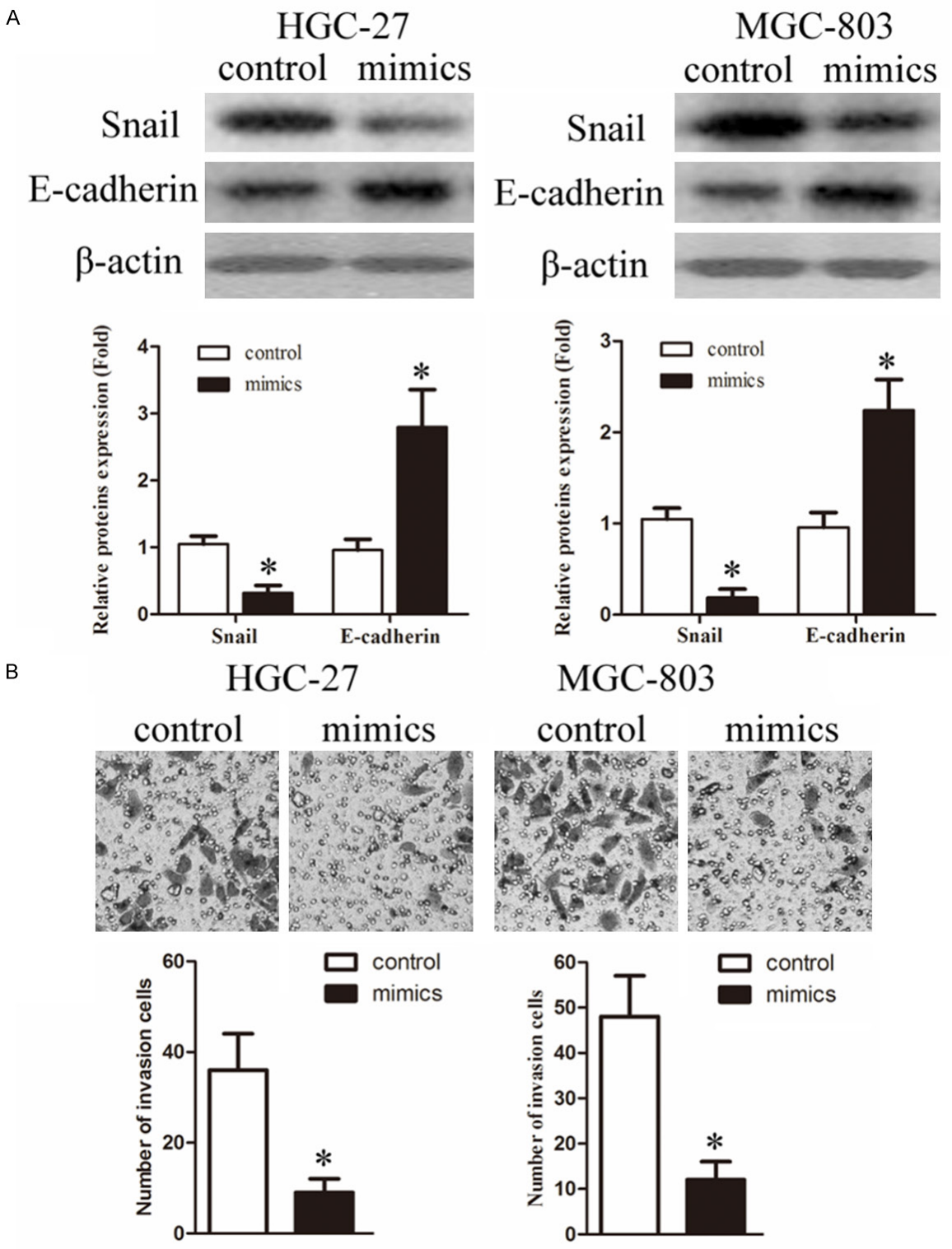

Figure 2. Overexpression of miR-138 reversed the invasion of GC cells. A: 48 hours after transfection, the total proteins were obtained from lysed cells and the expressions of Snail and E-cadherin were determined by Western blot. β-actin was used as a loading control. Data are means ± SD. *, $P < 0.05$. (n = 3). B: 24 hours after transfection, cell invasion was evaluated using Matrigel coated Transwell assay. Results are representative of three independent experiments. Data are means ± SD. *, $P < 0.05$.

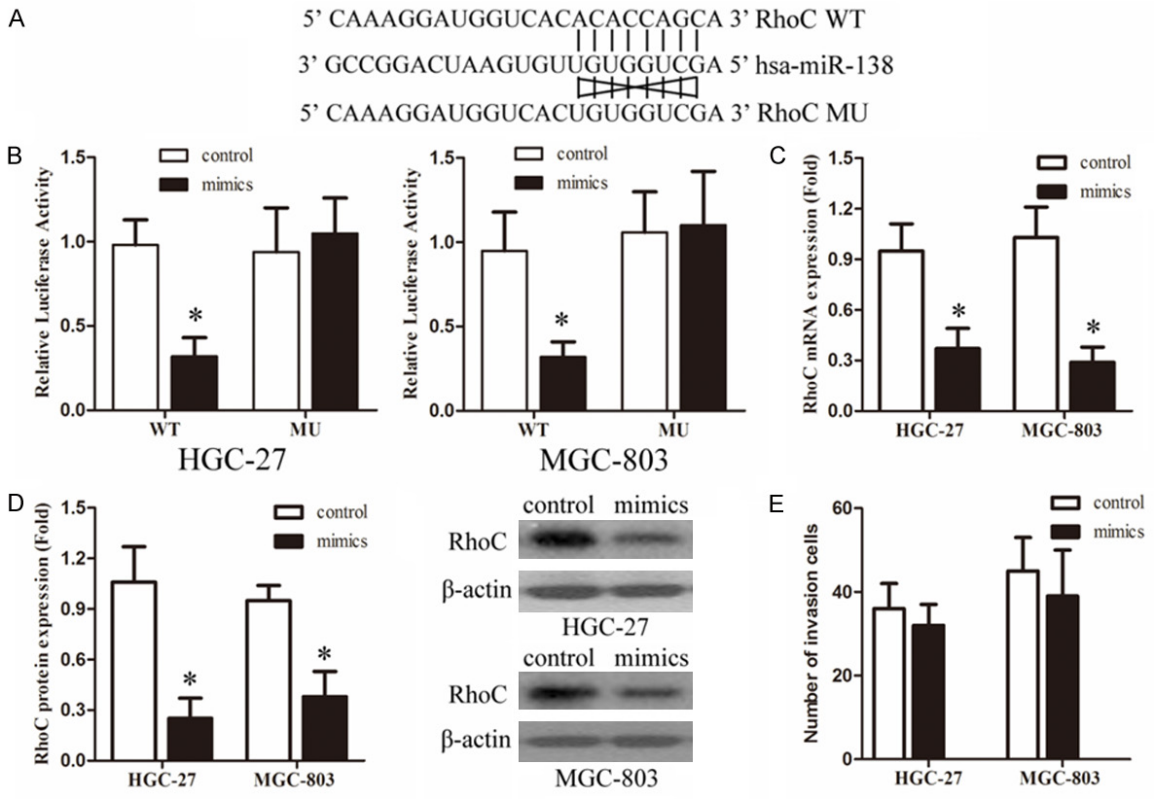

Figure 3. Identification of RhoC as a target of miR-138. A: Diagram of the luciferase reporter constructs. The predicted miR-138-targeting sequence was located in the 3'-UTR of the RhoC mRNA (WT-RhoC). Mutation was introduced into the target site for generating mutated reporter constructs (MU-RhoC). B: Dual luciferase reporter assay. Luciferase reporter constructs that included WT or MURhoC3'-UTR and miR-138 mimics or control were cotransfected into cells. 48 hours after co-transfection, luciferase activity was determined. Data are means ± SD. *, $P < 0.05$. (n = 3). C: The expression of RhoC mRNA in HGC-27 and MGC-803 cells transfected with miR-138 mimics or control was examined by real-time PCR assay, normalized to β-actin. Data are means ± SD. *, $P < 0.05$. (n = 3). D: The expression of RhoC protein in HGC-27 and MGC-803 cells transfected with miR-138 mimics or control was determined by Western blot. β-actin was used as an internal control. Results are representative of three independent experiments. Data are means ± SD. *, $P < 0.05$. E: 24 hours after transfection, cell invasion was evaluated using Matrigel coated Transwell assay. Results are representative of three independent experiments. Data are means ± SD.

data showed that miR-138 was down-regulated in GC tissues.

Overexpression of miR-138 suppressed the proliferation of GC cells

To explore the role of miR-138 in GC cells, we studied the effects of miR-138 overexpression on the proliferation of HGC-27 and MGC-803 cells. Increased level of miR-138 was confirmed by real-time PCR assay (**Figure 1B**). As shown in **Figure 1C**, transfection of the miR-138 mimics into HGC-27 and MGC-803 cells noticeably suppressed the cell growth, examined by the CCK-8 assay. Taken together, our results indicate that miR-138 suppressed the growth in GC cells.

Overexpression of miR-138 reversed the invasion of GC cells

We next explored whether the expression of miR-138 could affect the invasion of GC cells. As shown in **Figure 2A**, miR-138 overexpression led to a substantial decrease in Snail expression and increase in E-cadherin expression. These genes are associated with the malignant property of invasion. Therefore, we further observed the effect of miR-138 on the invasion of GC cells by matrigel-coated transwell assay. As demonstrated in **Figure 2B**, the increased miR-138 level in HGC-27 and MGC-803 cells resulted in reduced cell invasion. In conclusion, our results showed that miR-138 reversed invasion via decreasing Snail expres-

sion and increasing E-cadherin expression in GC cells.

RhoC is the direct target of miR-138

To further recognize the molecular mechanism of miR-138-suppressed cell growth and invasion in GC cells; we used bioinformatic algorithms (TargetScan and miRanda) to predict the potential miR-138 target genes. Among them, RhoC was found to have putative miR-138 binding sites in its 3'-UTR, which is in agreement with the previous studies. To confirm whether RhoC is a direct target of miR-138, the 3'-UTR of RhoC that contains putative miR-138 binding site was cloned into pmirGLO vector at the downstream of the luciferase gene (WT-RhoC). The control reporter vector was also established in which the seed region of the miR-138 binding site was mutated (MU-RhoC) (**Figure 3A**). Then, HGC-27 and MGC-803 cells were cotransfected with plasmids and miR-138 mimics. The results showed that miR-138 overexpression decreased the luciferase reporter activities of WT, indicating that miR-138 could bind to the putative binding site in RhoC 3'-UTR. Mutation of the miR-138 binding site abolished the inhibition of miR-138 (**Figure 3B**). Additionally, real-time PCR and western blot analyses showed that miR-138 overexpression significantly decreased the levels of RhoC mRNA and protein expression in HGC-27 and MGC-803 cells (**Figure 3C** and **3D**). As demonstrated in **Figure 3E**, mutation of the miR-138 binding site abolished the reduction of cell invasion in HGC-27 and MGC-803 cells. These results showed that RhoC was a direct target of miR-138 in GC cells.

Discussion

The correlation between miR-138 and RhoC has been observed by multiple laboratories, but it has not been studied in gastric cancer. miR-138 plays an important role in tongue squamous cell carcinoma (TSCC) migration and invasion by concurrently targeting RhoC and ROCK2, and miR-138 may serve as a novel therapeutic target for TSCC patients at risk of metastatic disease [16]. Down regulation of RhoC by miR-138 results in de-activation of FAK, Src and Erk1/2 signaling pathway in head and neck squamous cell carcinoma [17]. In this study, we determined that the overexpression of miR-138 in HGC-27 and MGC-803 cells inhibited the cell growth and invasion by suppressing the expression of RhoC.

RhoC is a member of the Rho GTPase family that can regulate many cellular functions, most notably cytoskeletal organization, in response to extracellular factor [18]. Activation of RhoC leads to assembly of the actin-myosin contractile filaments into focal adhesion complexes that lead to cell polarity and facilitate motility [19]. Enhanced expression of RhoC has been reported to correlate with the progression of several cancer types to a metastatic phenotype, including breast carcinomas, non-small cell lung carcinoma, gastric carcinomas, squamous cell carcinomas of the head and neck, hepatocellular carcinoma, ovarian cancer [20-26]. Overexpression of the RhoC gene may be involved in the metastasis of gastric carcinomas and may be a good genetic marker for the prediction of a metastatic potential [23]. Blockade of RhoC activity may be a potential target in the development of novel strategies for treating metastases of head and neck cancer [24]. RhoC has a critical role in metastasis of hepatocellular carcinoma (HCC), implicating RhoC as a potential therapeutic target to block HCC metastasis [25]. Aberrant RhoC expression might be involved in EMT of ovarian cancer cells, initiated by TGF-β1 and VEGF [26]. RhoC mediates EGFR/PI3K/Akt/GSK3β signaling in head and neck squamous cell carcinoma to reduce E-cadherin expression, thus promoting a more invasive phenotype [27]. Therefore, our results indicated that miR-138 reversed the gastric cancer invasion by directly targeting RhoC.

Additionally, recent studies have demonstrated that RhoC plays an important role in STAT3 phosphorylation and the activation of core cancer stem cell transcription factors [28]. RhoC regulates the proliferation of gastric cancer cells through interaction with IQGAP1 [29]. Phosphoinositide 3-kinase (PI3K) and the downstream Akt/mammalian target of rapamycin (mTOR) pathway have essential roles in modulating cellular functions, activating molecules like ribosomal protein S6 kinase and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP1), which contribute to the regulation of cell size, proliferation, and survival [30, 31]. Several previous studies have demonstrated activation of the Akt/mTOR pathway and its contribution to cell survival and pro-

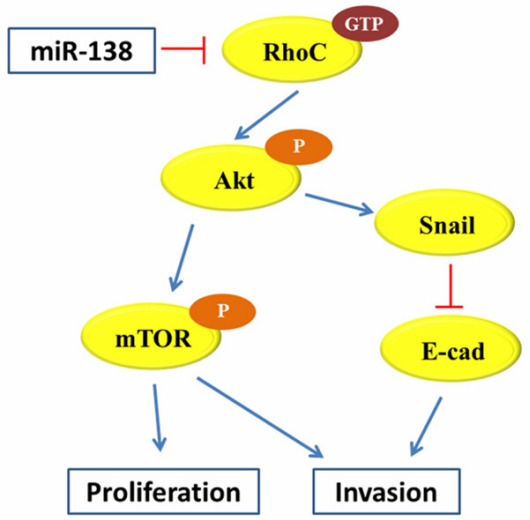

Figure 4. Proposed model for miR-138-mediated RhoC signaling in the regulation of the proliferation and invasion in GC.

liferation in several cancer types [32, 33]. Altogether, we conclude that miR-138 can modulate gastric cancer cell growth by suppressing the expression of RhoC and the activation of PI3K/Akt pathway.

In this study we studied the molecular mechanisms mediating the miR-138 suppressed cell proliferation and invasion in gastric cancer. More importantly, we found that miR-138 also played a role in gastric cancer by directly targeting RhoC, and might eventually suppressed PI3K/Akt and its downstream signaling pathway (**Figure 4**). These findings will help us understand the role and mechanism of miR-138 in gastric cancer.

Acknowledgements

This work was supported by a grant from Project of science and Technology Department of Qinghai Province (No. 2015-ZJ-751), and the National Natural Science Foundation of China (No. 81301898).

Disclosure of conflict of interest

None.

Address correspondence to: Hao Wu, Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, Jiangsu Province, PR China. Tel: +86-25-68136043, Fax: +86-25-83780826; E-mail: whdactor@163.com

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