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ORIGINAL ARTICLE

Silencing of RhoC induces macrophage M1 polarization to inhibit migration and invasion in colon cancer via regulating the PTEN/FOXO1 pathway

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Abstract

Ras homologue family member C (RhoC) is an oncogene in diverse types of human cancers, whereas its regulatory mechanisms involving macrophage polarization is rarely investigated. This study is designed to explore the regulatory role of RhoC in colon cancer and the underlying molecular mechanisms involving macrophage polarization. We detected RhoC expression by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot, and analysed the biological function of RhoC knockdown in CC cells by the MTT, wound healing and transwell assay. Macrophage polarization-associated markers, genes associated with migration, phosphatase and tensin homologue (PTEN) and forkhead box O (FOXO) were determined by qRT-PCR and western blot. The xenograft tumour mouse model was used to assess the role of RhoC in vivo. RhoC is highly expressed in CC cells. The cell viability, invasion and migration abilities of CC cells were reduced by knockdown of RhoC. RhoC knockdown promoted M1 polarization, inhibited M2 polarization and decreased levels of genes associated with migration (matrix metalloproteinase-2 and matrix metalloproteinase-9). Silencing of RhoC inhibited tumour growth and expression of genes associated with migration in the xenografted model. In addition, silencing of RhoC promoted PTEN/FOXO1 expression, and PTEN inhibitor (SF1670) reversed the inhibitory effects of RhoC silencing. We demonstrated that silencing of RhoC reduced CC cells invasion and migration, and tumour growth by suppressing M2 macrophage polarization via regulating the PTEN/FOXO1 pathway.

KEYWORDS

colon cancer, FOXO1, macrophage polarization, PTEN, RhoC

1 | INTRODUCTION

Colon cancer (CC) is one of the most high incidence malignancies worldwide.[1] It is generally believed that CC is caused by many factors, such as lifestyle, genetic mutations, environmental and genetic factors.[2] Current treatments for CC include chemotherapy, radiation therapy, surgery, immunotherapy and targeted therapy.[2,3] Additionally, 15%–25% of CC patients are diagnosed with metastases, and metastatic patients have a 5-year survival

rate of approximately 10%.[4,5] Consequently, it is necessary to explore the molecular mechanisms that inhibit the development of CC and provide new strategies to enhance the survival rate of CC.

Ras homologue family member C (RhoC) is a member of the Rho GTPase family. Recently, RhoC has been shown to be up-regulated in various tumours, and may be associated with tumour progress and metastasis. Examples of this are gastric cancer (GC), breast cancer (BC), and oral squamous cell carcinoma (OSCC).[6–8] For instance, silencing of RhoC suppressed GC cell proliferation, migration and invasion, and resulted in a marked inhibition of GC tumour growth in a xenograft mice model.[7] Gao et al.[8] have reported that decreased RhoC markedly inhibited the invasion and migration abilities of OSCC cells. In addition, studies have reported that high expression of RhoC correlates with poor prognosis in CC.[9] Although up-regulation of RhoC has been found to promote CC cell invasion and migration, while inhibition of RhoC showed opposite effects,[10] the exact mechanism whereby RhoC regulates migration and invasion in CC remains unclear.

Tumour-associated macrophages (TAMs) have been reported to affect multiple aspects of CC, such as tumour angiogenesis and metastasis.[11] TAMs mainly exhibit two phenotypes with different roles. The M1 type plays pro-inflammation and anti-tumour roles and is characterized by the high expression of inducible nitric oxide synthase (iNOS), tumour necrosis factor-alpha (TNF-α) and cyclooxygenase-2 (COX2), while M2 type plays anti-inflammatory and pro-tumour roles and expresses high levels of CD206, Arginase (Arg-1) and interleukin-10 (IL-10).[12–14] However, it is still unclear whether RhoC regulates CC progress through promoting M2 macrophage polarization. Therefore, the role and mechanism of RhoC in macrophage polarization need to be further explored in CC.

Phosphatase and tensin homologue (PTEN) is a tumour suppressor gene, and loss of function of PTEN occurs in multiple types of cancer, such as CC, glioblastoma and breast cancer.[15] For example, PTEN has been reported to be involved in the invasion, and migration of glioma. In addition, Liang et al.[16] have reported that rescue of PTEN expression contributed to reduced tumour proliferation and increased apoptosis in CC. Forkhead box O (FOXO) is a transcription factor that acts downstream of PTEN.[17] Inhibition of FOXO is involved in various cellular functions, such as cell metabolism, cell survival and cell cycle progression.[18] Studies have demonstrated that PTEN regulates the premature activation of oocyte and tumour cell growth through FOXO factors.[19,20] However, there are few studies on the relationship between RhoC and PTEN/FOXO1 pathway in CC progress.

In this study we investigated the role of RhoC on CC and the interaction of RhoC with macrophages polarization in CC. RhoC silencing inhibited CC tumour growth and migration by reducing cell viability, suppressing invasion and migration, and promoting M1 polarization by PTEN/FOXO signalling pathway.

2 | MATERIALS AND METHODS

2.1 | Cell culture

The human CC cell lines (RKO, HCT-8 and SW480), normal colon NCM460 cells, and monocyte cell line THP-1 were obtained from the Cell Bank of the Chinese Academy of Sciences, and cultured in RPMI 1640 (Invitrogen) containing 10% FBS, 1% streptomycin/penicillin at 37°C in 5% CO_2 and 95% humidity.

2.2 | Transfection

shRNA targeting RhoC (sh-RhoC) and negative control (sh-NC) were obtained from GenePharma. sh-RhoC or sh-NC were transfected with SW4800 and HCT-8 cells using Lipofectamine 3000 reagent (Invitrogen) at 37°C for 48 h according to the instructions of the manufacturer. Subsequently, the transfected cells were employed in the next experiments.

2.3 | Macrophage polarization

THP-1 was incubated with phorbol 12-myristate 13-acetate (PMA, 200 nM) (Sigma-Aldrich) for 24 h and differentiated into macrophages. The supernatant was collected after transfected CC cells were grown for 24 h in a serum-free medium and centrifuged for 20 min at 1500 *g*. After being treated with conditioned medium from CC cells for 24 h, macrophages transformed into TAMs. The non-contact transwell system (pore size 0.4 m, Corning, Cambridge) was used to co-cultivate TAMs and CC cells. TAMs cultured in the top chamber communicated with comparable CC cells in the bottom chamber for 48 h, after which the CC cells were removed and analysed further.

2.4 | Western blot assay

Protein was extracted using a cell lysis buffer (Sigma-Aldrich). The samples were resolved by 10% SDS-PAGE and then transferred to the PVDF membranes. The

membranes were blocked with 5% skim milk at 25°C for 1 h, and incubated with the primary antibodies against RhoC (1:1000, ab180785, Abcam), MMP-2 (1:1000, ab92536, Abcam), PTEN (1:1000, ab267787, Abcam), FOXO1 (1:1000, ab179450, Abcam), p-FOXO1 (1:1000, ab131339, Abcam), MMP-9 (1:1000, ab76003, Abcam) and β-actin (1:1000, ab8227, Abcam) overnight at 4°C. Subsequently, the membranes were washed and incubated with HRP-conjugated secondary antibody (1:5000) for 1 h at 25°C. The enhanced chemiluminescence western blotting detection kits (Sigma-Aldrich) were used to analyse the blots. β-actin was employed as a protein loading control.

2.5 | Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted in SW480 and HCT-8 cells using TRIzol reagent (Sigma-Aldrich). According to the instructions of manufacturer, reverse transcription was performed using a Reverse Transcriptase kit, and qRT-PCR was performed using an SYBR® Green Master Mix Kit (Thermo Fisher Scientific) and was analysed by the Mastercycler ep realplex detection system (Eppendorf). The relative mRNA expression was normalized against β-actin using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in Table 1.

2.6 | 3-[4,5-dimethyl-2-thiazolyl]-2,5 diphenyl-2H-tetrazolium bromide (MTT) assay

The viability of SW4800 and HCT-8 cells was measured using an MTT kit (Sigma-Aldrich). Cells were cultured for 24 h into 96-well plates at 37°C, and 20 μl of MTT (2.5 mg/ml) was added to the wells and maintained for 4–6 h. Then, the absorbance (450 nm) was measured by a microplate reader (Labcompare) and recorded at 0, 24, 48 and 72 h.

2.7 | Wound healing assay

Cells were cultured for 24 h in a serum-free medium in 6-well plates. Pipette tips are used to scrape cells and create interstitial spaces. After 24 h, the dead cells were washed out and photomicrographs were instantly taken.

2.8 | Transwell assay

The invasion of SW4800 and HCT-8 cells was analysed by transwell (8 μm pore, Corning, Inc.). The upper chamber was pre-coated for 5 h with 1 μg/μl Matrigel (BD Biosciences), cells were added (5×10^4 cells) and cultured at 37°C overnight. The lower chamber contained medium with 10% FBS. Cells in the lower chamber were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 10 min at 25°C. The invasion cells were counted by light microscopy.

2.9 | Animals

Thirty BALB/c male nude mice (5 weeks, 18–20 g, Shanghai SLAC Laboratory Animal Co., Ltd.) were used in this study. The study was conducted in compliance with the National Institutes of Health's guidelines for the care and use of laboratory animals and approved by the ethics Committee of Harrison International Peace Hospital. The mice were randomized into three groups ($n = 10$) and were subcutaneously injected with HCT-8 cells (40 μl, 0.5×10^6 cells) transfected with sh-RhoC and sh-NC. They were euthanized after 28 days. Tumour weight was measured and volume was calculated every 7 days using the formula (length × width^2)/2.

TABLE 1 Primers for qRT-PCR in this study

Gene	Forward (5′-3′)	Reverse (5′-3′)
RhoC	GGAGGTCTACGTCCCTACTGT	CGCAGTCGATCATAGTCTTCC
iNOS	GGGCAGCCTGTGAGACCTT	TGAAGCGTTTCGGGATCTG
TNF-α	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATGTGG
COX2	ACACACTCTATCACTGGCACC	TTCAGGGCGAAGCGTTTGC
CD206	AAGGCGGTGACCTCACAAG	AAAGTCCAATTCCTCGATGGTG
Arg-1	AACGGGAGGGTAACCATAAGC	GCGCATTCACAGTCACTTAGGT
IL-10	CCTCGTGGAGCCTCAGTTTTC	GAGCACGTCAGGTACATTTCAATT
β-actin	TGTTACCAACTGGGACGACA	GGGGTGTTGAAGGTCTCAAA

2.10 | Statistical analysis

Statistical data were presented as the mean ± SD. Two-group comparisons were analysed by Student's *t*-test, and multiple group comparisons were analysed using two-way ANOVA by Tukey's post-hoc test and one-way ANOVA by Tukey's pairwise post-hoc test using Prism 7.0 software (GraphPad). $p < .05$ was considered significant.

3 | RESULTS

3.1 | RhoC is up-regulated in CC cells

RhoC expression was detected in NCM460, SW480, HCT-8 and RKO cells. Western blot showed that RhoC was highly expressed in RKO, SW480 and HCT-8 cells compared with NCM460 cells (Figure 1, $p < .01$). Due to the relatively high expression level of RhoC, SW480 and HCT-8 cells were selected for subsequent experiments.

3.2 | RhoC silencing inhibits cell viability, migration and invasion

The effect of RhoC on CC cells was investigated by transfection of shRNA-RhoC in SW480 and HCT-8 cells. The expression of RhoC was decreased after RhoC silencing (Figure 2A, $p < .01$). RhoC silencing reduced the viability of SW480 and HCT-8 cells (Figure 2B, $p < .01$). In addition, knockdown of RhoC inhibited migration and invasion of SW480 and HCT-8 cells (Figure 2C,D, $p < .01$). Furthermore, western blot analysis showed that the levels of genes associated with migration (MPP-2 and MPP-9) were reduced by RhoC silencing in SW480 and HCT-8 cells (Figure 2E, $p < .01$). The data showed that RhoC silencing reduced cell viability, and suppressed migration and invasion by CC cells, thereby contributing to suppressing the progress of CC.

3.3 | RhoC silencing inhibits M2 polarization and promotes M1 polarization

The Human Protein Atlas (https://www.proteinatlas.org/) is used to predict the RhoC expression link it with known cell type markers in different single cell type clusters in CC tissue. As shown in Figure 3A, the expression of CD163 (marker of M2 macrophages) is up-regulated. Therefore, the role of RhoC silencing on the polarization of macrophages was explored in CC cells co-cultured with TAMs. The levels of M2 markers (CD206, Arg-1 and IL-10) were reduced (Figure 3B,C, $p < .01$), while M1 markers (iNOS, TNF-α, and COX2) showed higher expression in macrophages treated with CM from sh-RhoC-transfected CC cells (Figure 3D,E, $p < .01$). The data showed that inhibition of RhoC promotes polarization of M1 macrophage and inhibits M2 macrophage polarization.

3.4 | RhoC silencing inhibits cell viability, migration and invasion by the PTEN/FOXO1 pathway

Western blotting showed that RhoC silencing up-regulates the expression of PTEN and p-FOXO in HCT-8 cells (Figure 4A, $p < .01$). To clarify the mechanism of RhoC in CC, HCT-8 cells were treated with the PTEN inhibitor (SF1670, 2 μM). The data showed that SF1670 enhanced cell viability, promoted migration and invasion, and increased the expression of genes associated with migration (MPP-2 and MPP-9) in HCT-8 cells compared with sh-RhoC groups (Figure 4B–E, $p < .01$), indicating SF1670 reversed the inhibition effect of RhoC silencing on CC cells.

3.5 | SF1670 reverses the effects of RhoC silencing on macrophage polarization

Furthermore, whether RhoC silencing inhibits M2 macrophage polarization by regulating PTEN/FOXO was

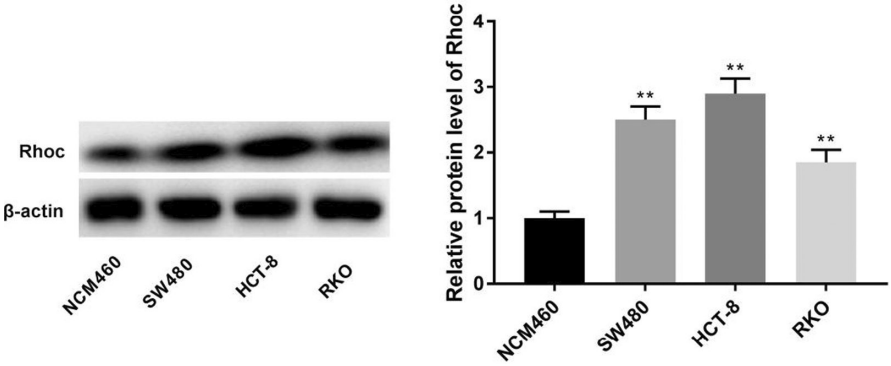

FIGURE 1 RhoC is up-regulated in CC cells. RhoC expression was detected in different cells by western blot. $^{**}p < .01$ versus NCM460 cells.

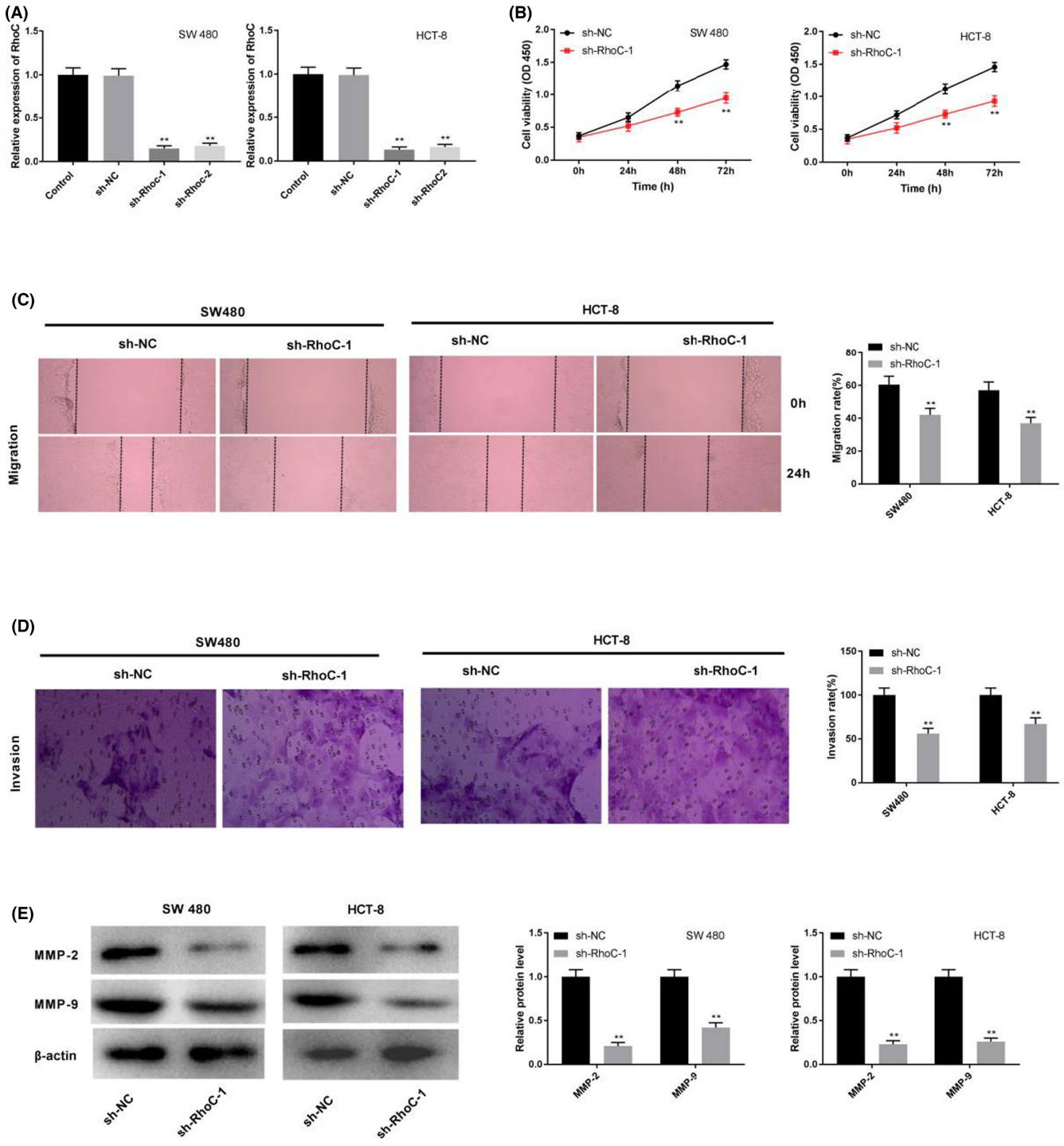

FIGURE 2 RhoC silencing inhibits cell viability, migration and invasion. (A). The RhoC expression in different groups was detected by RT-qPCR. (B). Cell viability was measured by MTT assay. (C). Wound healing assay was performed to detect the migration. (D). Transwell assay was performed to detect the invasion. (E). The levels of genes associated with migration (MMP-2 and MMP-9) were detected by western blot. $^{**}p < .01$ versus sh-NC.

explored. The data suggested that SF1670 reversed the effects of RhoC silencing on the expression of M2 markers (CD206, Arg-1 and IL-10) and M1 markers (iNOS, TNF-α and COX2) (Figure 5A,B, $p < .01$). The data indicated that sh-RhoC inhibited macrophage polarization by up-regulating the expression of PTEN and FOXO.

3.6 | RhoC silencing inhibits tumour growth and migration

Furthermore, the role of RhoC was further analysed in xenograft tumour models. Our results showed that RhoC silencing suppressed tumour volume and weight

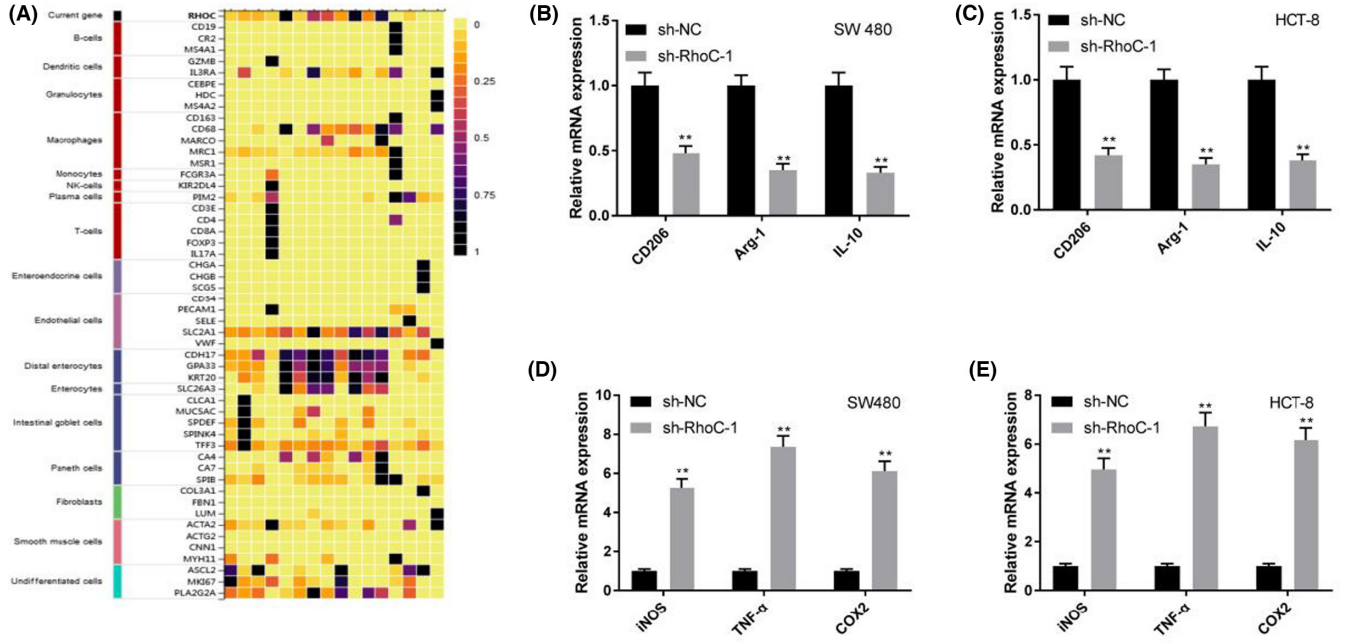

FIGURE 3 RhoC silencing inhibits M2 polarization and promotes M1 polarization. (A). Data are from the human protein atlas. (B, C). The levels of M2 markers (CD206, Arg-1 and IL-10) were determined by RT-qPCR. (D-E). The levels of M1 markers (iNOS, COX2 and TNF-α) were detected by RT-qPCR. $^{**}p < .01$ versus sh-NC.

(Figure 6A–C, $p < .01$). Consistently, RhoC silencing significantly decreased the levels of genes associated with migration (MPP-2 and MPP-9) (Figure 6D, $p < .01$). In vivo tests showed that RhoC promotes tumour growth, while RhoC silencing contributes to suppressing tumour growth.

4 | DISCUSSION

Colon cancer is one of the main reasons for high levels of cancer-related death, and its invasion and metastasis significantly affect the prognosis of CC patients.[21] At present, the molecular mechanism of the occurrence and development of CC has not been fully elucidated. In our study, we found RhoC is closely associated with CC progression by regulating cell viability, invasion, migration and macrophage polarization by targeting the PTEN/FOXO pathway.

Rho GTPases could regulate the cytoskeleton, affect cell mobility, polarity and division, and play a critical role in cell migration and invasion, which includes the highly homologous RhoA, RhoB and RhoC.[22] In recent years, the role of the RhoC has been widely studied in various cancers.[6] Studies have demonstrated that the abnormal activation of RhoC plays a key role in various tumour cell biological functions, such as cell differentiation, apoptosis, proliferation, invasiveness and metastatic ability.[23] Xie et al.[24] have reported that RhoC enhanced cell proliferation and inhibited apoptosis rate in hepatocellular carcinoma. Kaushal et al.[25] have revealed that the up-regulation of RhoC is linked to enhanced cancer cell invasion, migration and metastasis in BC cells. Furthermore, the expression of RhoC is elevated in human GC tissues, and knockdown of RhoC markedly restrained GC cell migration and invasion, reduced GC tumour development and lung metastasis in mouse models. Consistent with previous research, our study also showed that RhoC expression was up-regulated in CC cells and RhoC knockdown inhibited CC cell viability, migration and invasion in vitro and decreased tumour volume and weight in vivo. Consistently, RhoC mRNA expression was up-regulated in CC tissues, and overexpression of RhoC stimulated cell invasion and migration.[10] HomeoboxD10 could down-regulated RhoC to enhance CC cell apoptosis and constrain proliferation, migration and invasion.[26]

Tumor-associated macrophages are an important component of the innate immune system and differentiate into different phenotypes under the stimulation of various factors, thereby performing different regulatory roles in the physiological and pathological processes.[27] Lee et al.[28] have reported that the inhibition of M2 macrophage polarization may exert anti-tumour effects in CC. Reduction of paxillin decreased cell proliferation and invasion in CC by suppressing the polarization of M2 macrophages, thereby preventing tumour progression.[29] Furthermore, soluble factors produced by M2 macrophages have been found to result in tumour cells disseminating, thereby promoting metastasis of CC.[30] Accordingly, we speculated that RhoC

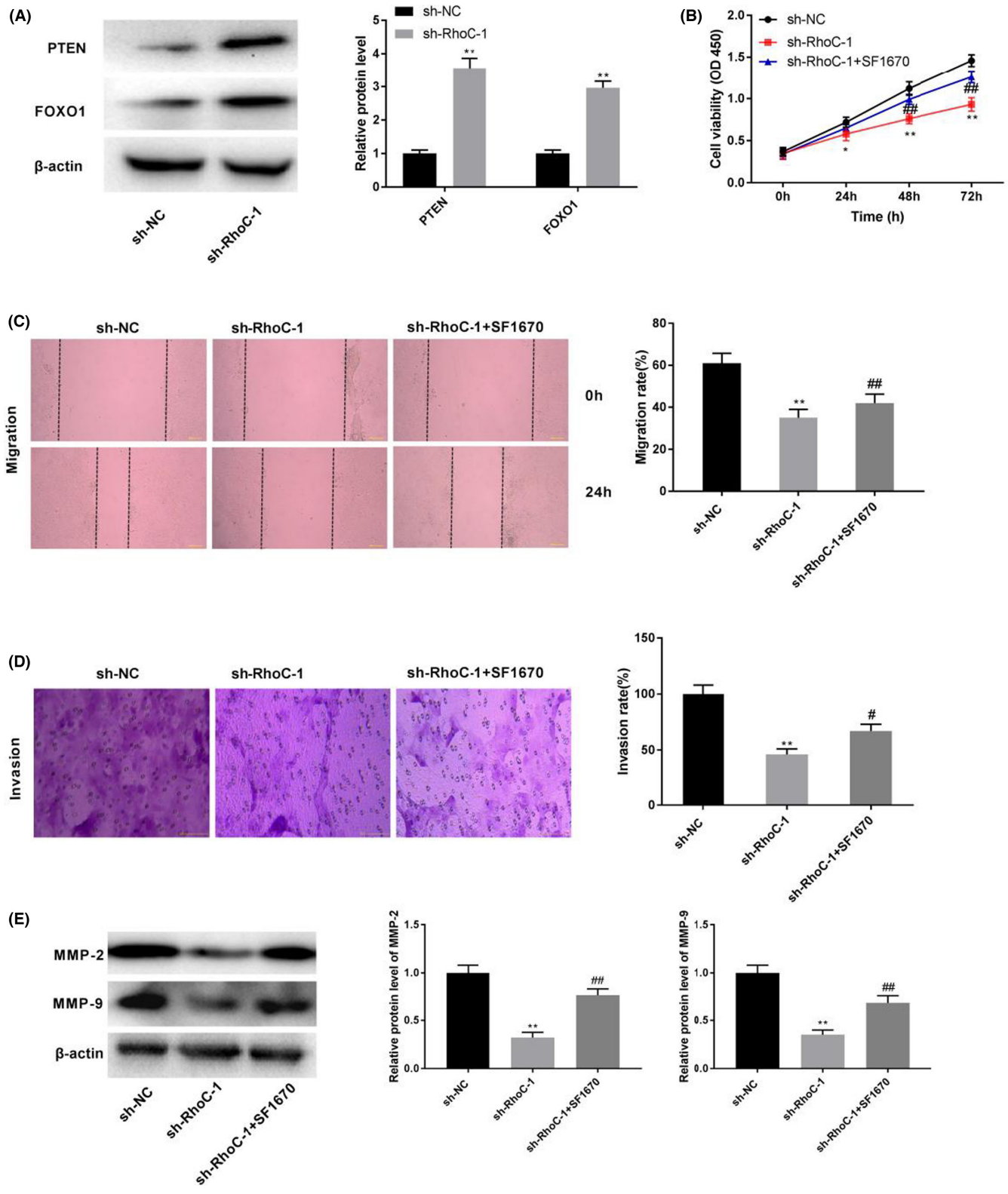

FIGURE 4 RhoC silencing inhibits cell viability, migration and invasion by the PTEN/FOXO1 pathway. (A). The expression of PTEN/FOXO1 was detected by western blot. (B). Cell viability was measured by MTT assay in different groups. (C–D). The migration and invasion were detected by wound healing assay and transwell assay in different groups. (E). The levels of genes associated with migration (MMP-2 and MMP-9) were detected in different groups by western blot. $^{*}p < .05$, $^{**}p < .01$ versus sh-NC. $^{\#}p < .05$, $^{\#\#}p < .01$ versus sh-RhoC-1.

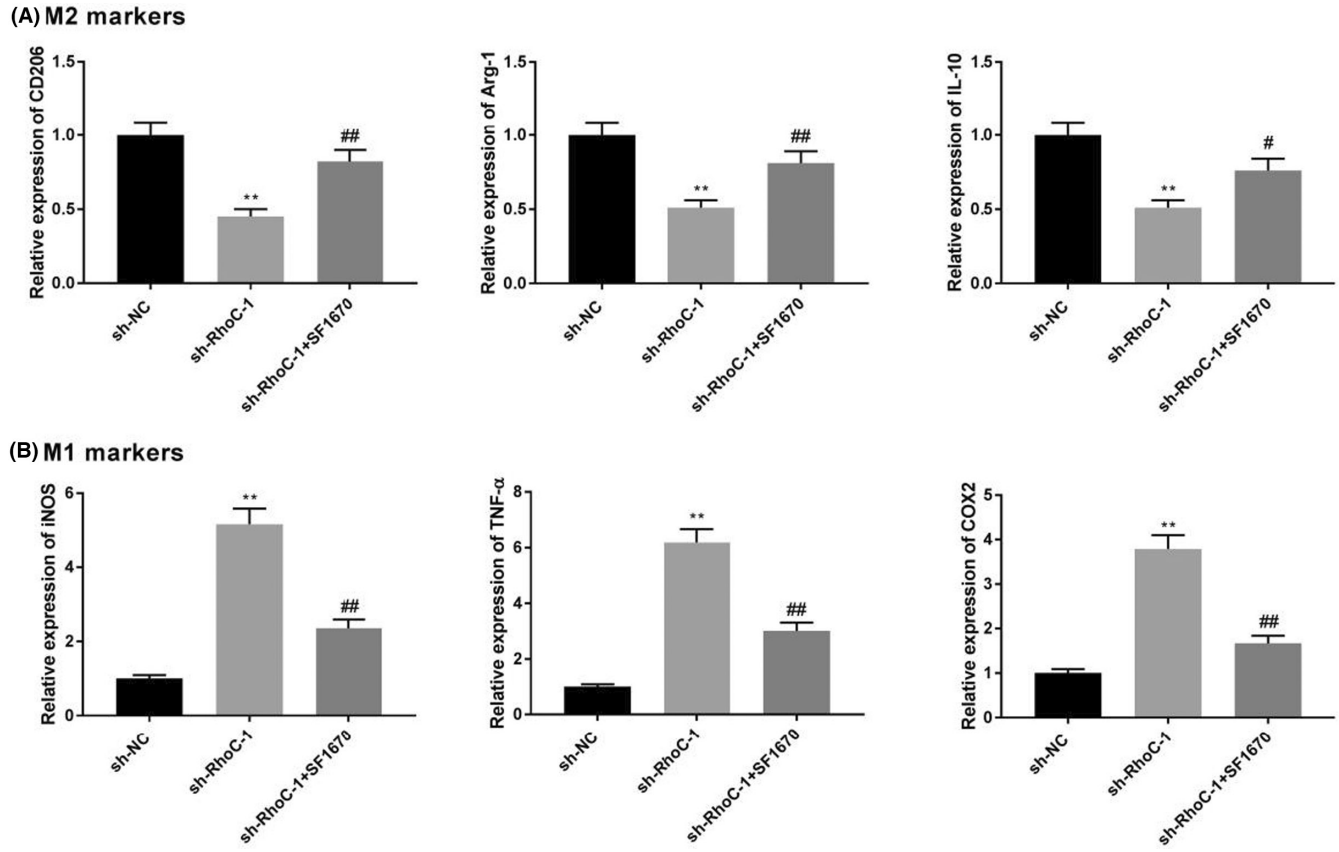

FIGURE 5 SF1670 reverses the effects of RhoC silencing on macrophage polarization. (A, B). The expression of M2 markers (CD206, Arg-1 and IL-10) and M1 markers (iNOS, COX2 and TNF-α) was determined by RT-qPCR in different groups. $^{**}p < .01$ versus sh-NC. $^{\#}p < .05$, $^{\#\#}p < .01$ versus sh-RhoC-1.

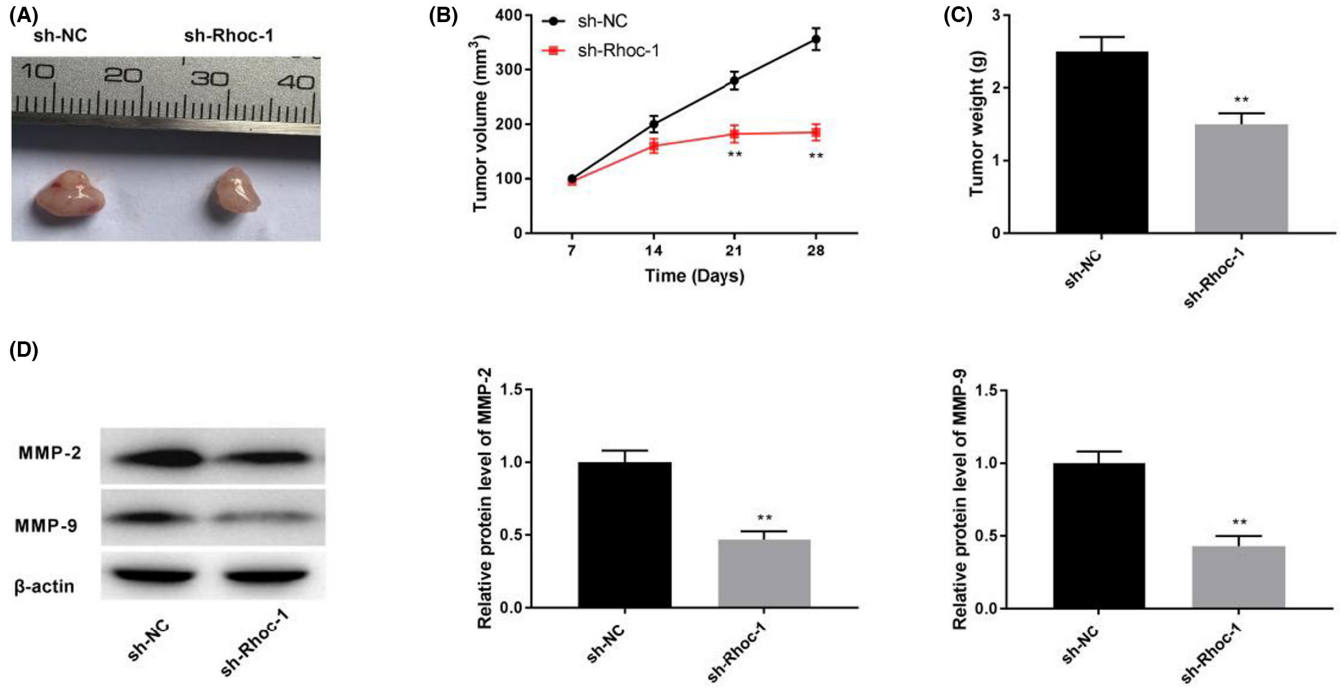

FIGURE 6 RhoC silencing inhibits tumour growth and migration. (A–C) Tumour volume and weight in different groups. (D) The expression of genes associated with migration was determined by western blot. $^{**}p < .01$ versus sh-NC.

can regulate CC development by regulating macrophage polarization. In this study, knockdown of RhoC reduced M2 marker expression, while promoting M1 marker expression. Taken together, we suggested that the silencing of RhoC slows down the progression of CC by inhibiting M2 macrophage polarization. If so, then targeting macrophage polarization via RhoC may be a potential strategy for CC therapy.

Phosphatase and tensin homolog has been shown to regulate intracellular signalling pathways that are important in tumour cell proliferation, cell cycle progression and invasiveness. Up-regulation of PTEN could reduce metastasis, while promoting apoptosis of glioma cells[31] and inhibition of PTEN promoted proliferation and metastasis in CC.[32] FOXO pathway is an essential regulator of cell survival, apoptosis, proliferation, and cell cycle.[33,34] FOXO dysfunction is associated with tumour progression and tumorigenesis.[35] Up-regulation of FOXO pathway in prostate cancer could suppress cell proliferation, invasion and retarded tumour progression in vivo,[36] and FOXO1 degradation may promote cell proliferation in CC.[37] Furthermore, FOXO1 has been reported to be involved in PMA-induced macrophage differentiation of leukaemia cells.[38] Long non-coding RNA ANCR could promote the invasion and migration of gastric cancer by inhibiting the expression of FOXO1 to inhibit macrophage M1 Polarization.[39] In this study, we found knockdown of RhoC increased the expression of PTEN and FOXO1. Furthermore, the feedback experiment showed that SF1670 reversed the inhibition effects of RhoC silencing on cell viability, invasion, migration of CC cells and M2 polarization. Consistently, PTEN up-regulation could regulate CC cell invasion,[40] and down-regulation of PTEN expression induced M2 macrophage polarization.[41]

In summary, we investigated the biological function and underlying mechanism of RhoC in CC cells and mice models. The results suggested that silencing of RhoC inhibited cell viability, migration, invasion and M2 polarization by regulating PTEN/FOXO expression, as well as suppressed tumour growth in vivo. These findings showed that RhoC might be used as a promising therapeutic target for CC, providing new options for CC treatment in the future.

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CONFLICT OF INTEREST

There are no conflicts of interest.

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