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miR-145-5p restrained cell growth, invasion, migration and tumorigenesis via modulating RHBDD1 in colorectal cancer via the EGFR-associated signaling pathway

Ying Niu, Jinping Zhang, Yalin Tong, Jiansheng Li, Bingrong Liu*

Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, PR China

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ABSTRACT

miR-145-5p has been reported to be downregulated and described functioning as a tumor suppressive gene in colorectal cancer (CRC), yet its detailed regulatory function and mechanism in malignant progression of the disease have not been thoroughly understood. In our study, miR-145-5p and rhomboid domain containing 1 (RHBDD1) in CRC tissues and cells were examined by qRT-PCR and western blot. MTT, colony formation, wound healing, Transwell invasion, and flow cytometry assays were performed to evaluate the malignant phenotypes of CRC cells. Xenograft tumor, qRT-PCR, and western blot assays were applied to validate the roles and mechanism of miR-145-5p in CRC *in vivo*. The interaction between miR-145-5p and RHBDD1 was investigated by luciferase reporter assay and western blot. The changes of the EGFR/Raf/MEK/ERK pathway were detected by western blot. We found miR-145-5p was lowly expressed and low miR-145-5p predicted poor prognosis in CRC, while RHBDD1 was greatly enhanced in CRC cells and tissues. RHBDD1 silencing resulted in inhibiting cell proliferative, invasive, and migratory potentials as well as elevating apoptotic ones in CRC cells. miR-145-5p was inversely related with RHBDD1 expression in CRC tissues. miR-145-5p was found to directly bind to RHBDD1 and restrained its expression in CRC cells. miR-145-5p overexpression repressed CRC cell proliferation, invasion, migration and induced apoptosis, and these effects were reversed by RHBDD1 upregulation. Moreover, in CRC xenograft tumor, its growth was impeded by miR-145-5p via suppressing RHBDD1. Furthermore, miR-145-5p inhibited the expression of EGFR, p-MEK1/2 and p-ERK1/2, *in vitro* and *in vivo* by targeting RHBDD1. In conclusion, our study revealed that miR-145-5p overexpression inhibited tumorigenesis in CRC by downregulating RHBDD1 via suppressing the EGFR-associated signaling pathway (EGFR/Raf/MEK/ERK cascades).

1. Introduction

Colorectal cancer (CRC) is considered as the most frequently occurred malignancies and rendered the 4th dominant cause of cancer-associated mortalities. Statistics estimated around 400,000 freshly diagnosed patients and approximate 200,000 mortalities in China in 2015 (Ahmed et al., 2014; Chen et al., 2016). Furthermore, the occurrence of CRC has rapidly increased in developing countries, which may be attributed to environmental and lifestyle changes (Sjo et al., 2011). Currently, regardless of significant advances in the clinical treatments for CRC, such as radiotherapy, chemotherapy, and surgical resection, the poor prognosis for CRC patients still cannot be reversed, especially in the ones with advanced stage CRC, whose five-year overall survival (OS) rate is fewer than 10% owing to high local recurrence and distant metastasis (Amano et al., 2014; Brenner et al., 2014). The carcinogenesis and development of CRC are considered as a consequence of accumulation of alternations in inactivated tumor suppressor genes and activated oncogenes (Song and Chan, 2017). Accordingly, it is desperately required to better understand the mechanisms involved in CRC progression and develop novel therapeutic approaches for CRC.

Recently, the microRNAs (miRNAs) functional mechanisms in CRC progression have attracted increasing attention. Accumulated evidence has shown that miRNAs are important regulator for various biological process, including cell differentiation, proliferation, apoptosis, as well as metastasis (Calin and Croce, 2006). A growing number of experimental data have revealed the abnormal expression of miRNAs in multiple cancers including CRC and their dysregulation is associated with tumor development by functioning as either tumor suppressors or oncogenes (Xuan et al., 2015). miR-145-5p, located at 5q32, is well-

* Corresponding author at: No. 1 Jianshe East Road, Zhengzhou, 450052, Henan, PR China.
E-mail address: bingrongcn@163.com (B. Liu).

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documented to be underexpressed and exert tumor-suppressive effects in diverse malignancies, including non-small cell lung cancer (NSCLC) (Li et al., 2018), bladder cancer (Zhang et al., 2018a), as well as CRC (Qin et al., 2015). However, the detailed mechanism related to miR-145-5p tumorigenesis inhibitory function in CRC has not been thoroughly understood.

Rhomboid family proteins include active rhomboids, inactive rhomboids lacking catalytic residues, and mitochondrial rhomboids (Urban, 2006). Parasite replication and invasion, mitochondrial membrane remodeling, and growth factor signaling are main functions of the rhomboid family proteins (Freeman, 2014). Rhomboid domain containing 1 (RHBDD1), a rhomboid family member, plays a crucial part in cell apoptosis regulation (Wang et al., 2008). RHBDD1 has been suggested to be enhanced and related with survival in CRC patients (Han et al., 2015; Song et al., 2015). Moreover, RHBDD1 promotes CRC growth and metastasis (Song et al., 2015; Zhang et al., 2018b), while lentivirus-mediated knockdown of RHBDD1 inhibits CRC cell growth (Han et al., 2015). By bioinformatics analysis, we found the RHBDD1 was a putative target of miR-145-5p. However, the cooperation of miR-145-5p and RHBDD1 in CRC stays to be further explored.

In the current study, we determined the interaction between miR-145-5p and RHBDD1 and their cooperation in CRC progression.

2. Materials and methods

2.1. Patient tissues

Thirty-five paired CRC specimens and peritumoral specimens were collected from patients undergoing surgery at the First Affiliated Hospital of Zhengzhou University from March 2012 to April 2013. These biopsies were immediately frozen using liquid nitrogen following the surgery, followed by storage in a −80 °C refrigerator prior to RNA extraction. CRC patients were classified using the TNM staging system according to the international standard criteria. All CRC patients received no chemo-therapy or radio-therapy prior to the surgery and written consents were signed. The experimental protocols were sanctioned by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University. The patient clinicopathological features were shown in Supplementary Table 1.

2.2. Cell culture and transfection

The human CRC cell lines (Caco-2, DLD-1, LoVo, SW480 and T84) and normal colon epithelial cell line FHC were bought from Procell (Wuhan, China). All cells were maintained in DMEM (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA) together with 1% penicillin/streptomycin (Sigma, St. Louis, MO, USA) in a 5% CO_2 humidified incubator.

RHBDD1 short hairpin (sh) (sh-RHBDD1) sequence targeting RHBDD1 was designed and the validated sequence was 5′-GCTGGGAT TCTTGTTGGACTA-3′. When 293 T cell confluence was about 70%, the 20 μg modified plasmid (lv- sh-RHBDD1), 20 μg packing plasmids were cotransfected into cells to gain lentiviruses using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 2×10^5 SW480 or Caco-2 cells were plated into 24–well plate, and after 24 h, the lentiviruses were used to infected SW480 or Caco-2 cells. sh-RHBDD1, RHBDD1 overexpressing plasmid (pcDNA-RHBDD1), miR-145-5p antagomir (anti-miR-145-5p), miR-145-5p mimics (miR-145-5p), and their corresponding controls (sh-NC, anti-miR-NC, and miR-NC) were bought from GenePharma Co., Ltd. (Shanghai, China). All vectors were transiently delivered to SW480 and Caco-2 cells using Lipofectamine 2000.

2.3. qRT-PCR

RNAiso Plus (TaKaRa, Tokyo, Japan) was used to isolate tissues samples and cell total RNA. RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) was subsequently applied to produce single-strand cDNA. For the detection of miR-145-5p and RHBDD1 mRNA, qPCR was carried out using miRNA qPCR Assay Kit (CWBIO, Beijing, China) and Power SYBR Green PCR Master Mix (Takara) using a CFX96 real-time PCR System (Bio-Rad, Hercules, CA, USA). The $2^{-\Delta\Delta Ct}$ method was employed to compute miRNA/mRNA fold changes.

2.4. Western blot

Total proteins were isolated using cold radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Beijing, China). Ten percent SDS-PAGE was used to separate the isolated cell lysates, and NC membranes were used to electro-transfer the blots. Following being blocked with 5% skimmed dry milk, the membrane was immunoblotted with the following primary antibodies: monoclonal antibodies (anti-EGFR (dilution: 1:1500; ab52894, Abcam, Cambridge, MA, USA), anti-MEK1/2 (dilution: 1:20000; ab178876, Abcam)) and polyclonal antibodies (anti-β-actin (dilution: 1:1000; ab5694, Abcam), anti-RHBDD1 (dilution: 1:500; SAB3500406, Sigma-Aldrich (Shanghai) Trading Co.Ltd), anti-p-MEK1/2 (dilution: 1:1000; ab194754, Abcam), and anti-p-ERK1/2 (dilution: 1:1000; ab17942, Abcam)), and respective horseradish peroxidase – conjugated secondary antibodies. Last, a BeyoECl Plus kit (Beyotime) was applied to visualize the protein bands.

2.5. Cell proliferation assay

MTT assay was conducted to determine the cell proliferative abilities. In brief, 1×10^4 cells were placed into 96-well plates per well. After cultivation for consecutive (0, 1, 2 3) days at 37 °C, MTT reagent was added to stop the reaction, and proliferate rate was determined by measuring the optical density at 570 nm wavelength.

2.6. Colony formation assay

Following trypsinization, 500 transfected SW480 and Caco-2 cells were seeded and incubated for 14 days. Then, 70% ethanol was used to fix the colonies and 0.1% crystal violet (Sigma-Aldrich) was employed to stain them. Afterwards, visible colonies (> 50 cells/colony) were recorded.

2.7. Apoptosis assay

Annexin V-FITC apoptosis detection kit (BioLegend, San Diego, CA, USA) was taken to assess the cell death rate in treated cells. Forty-eight hours post-transfection, 1×10^5 cells were incubated with binding buffer supplemented with PI and Annexin V-FITC. The apoptotic cells were analysis by the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.8. Transwell assay

Following transfection, 4×10^4 SW480 and Cao-2 cells suspended in serum-free medium were added to the upper compartments (Corning-Costar, Lowell, MA, USA), while the lower ones were filled up with complete culture medium. Twenty-four hours post-incubation, 4% paraformaldehyde was used to fix the invaded cells, and 0.1% crystal violet was used to stain these cells. Five random fields were selected for counting the invaded cells under light microscopy (Olympus).

2.9. Wound-healing assay

The treated 5×10^5 SW480 and Caco-2 cells were seeded and incubated to 90% confluence. A linear scratch on the cell surface was made by a sterilized 200 μl pipette tip. Last, an inverted microscope was used to photograph the generated wound gas, 0 or 48 h after

incubation.

2.10. Luciferase reporter assay

The 3′UTR of RHBDD1 resting the sequence complementary to miR-145-5p (WT) and its mutant-type (MUT) were made and the pGL3-promoter vector ((Promega, Madison, WI, USA)) was used to load them, namely RHBDD1-WT and RHBDD1-MUT. Constructed reporter vectors together with miR-145-5p, anti-miR-145-5p, or matched controls were used to transfect Caco-2 and SW480 cells with 70% confluence using Lipofectamine™ 2000 (Invitrogen). A Dual Luciferase Reporter Assay System *(Promega)* was employed to value the Renilla luciferase activity, 48 h after transfection.

2.11. Xenograft tumor

The procedures in this section were conferred with the Animal Ethics Committee of the First Affiliated Hospital of Zhengzhou University. CRC xenograft models were generated by subcutaneously injecting 2×10^7 treated cells into the armpits of athymic nude mice (6-week-old, BALB/c, Beijing Hua Fukang Bioscience). Every 4 days, tumor volumes were monitored and calculated with: volume (mm^3) = (length × width^2)/2. Mice were sacrificed at day 28 after injection and weighed. Then, tumor tissues were excised, weighed and subjected to protein and RNA extraction.

2.12. Data analysis

All data were displayed as mean ± standard deviation (SD). GraphPad Prism software (GraphPad Prism version7; San Diego, CA, USA) was used to compute the analyses, and the significance of differences was analyzed by one-way analysis of variance analysis (ANOVA) or Student's *t*-test.

3. Results

3.1. miR-145-5p was underexpressed and predicted poor prognosis in CRC

Firstly, qRT-PCR was used to examine the miR-145-5p level in collected biopsies. When comparing to the miR-145-5p in adjacent normal tissues, that in CRC tissues was greatly reduced (Fig. 1A and B). As shown in Fig. 1A and B, miR-145-5p expression was greatly reduced in CRC tissues comparing to that in adjacent normal tissues. Moreover, our results presented that patients with advanced stage CRC displayed a notable lower miR-145-5p relative to that in the early stages (Fig. 1C). Further, CRC patients with lower miR-145-5p were correlated with a reduced median survival than other patients (Fig. 1D). Meanwhile, the squelched miR-145-5p expression was strongly correlated with lymph node metastases ($P = 0.01$), and T stage ($P = 0.028$) (Table 1). Also, qRT-PCR analysis showed that miR-145-5p was dramatically lowly expressed in CRC cells (Caco-2, DLD-1, LoVo, SW480 and T84 relative to FHC, especially in SW480 and Caco-2 cells (Fig. 1E). That the miR-145-5p expression serves as a prognostic biomarker in CRC patients has been implicated.

Table 1
Association between miR-145-5p expression and clinicopathologic parameters in 35 colorectal cancer patients.

Characteristics	miR-145-5p expression		X^2	*P*
	Low (n = 17) n (%)	High (n = 18) n (%)		
Age (years)			1.697	0.193
< 65	13 (76.47)	10 (55.56)		
≥ 65	4 (23.53)	8 (44.44)		
Sex			0.697	0.404
Male	10 (58.82)	13 (72.22)		
Female	7 (40.18)	5 (27.78)		
Histology			0.349	0.555
Adenocarcinoma	12 (70.59)	11 (61.11)		
Mucinous	5 (29.41)	7 (38.89)		
T stage			4.804	0.028
T1/T2	6 (35.29)	13 (72.22)		
T3/T4	11 (64.71)	5 (27.78)		
Lymph node metastases			6.556	0.010
Yes	13 (76.47)	6 (33.33)		
No	4 (23.53)	12 (66.67)		
Differentiation			1.588	0.208
Well-moderate	11 (64.71)	15 (83.33)		
Poor	6 (35.29)	3 (16.67)		

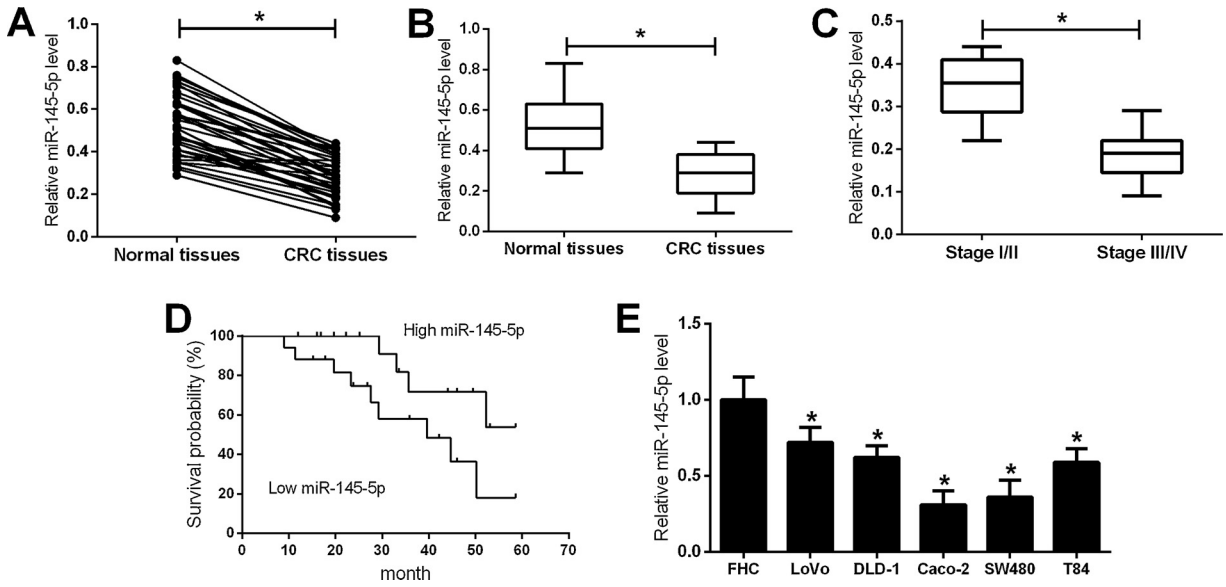

Fig. 1. Expression pattern and prognosis of miR-145-5p in CRC. (A and B) miR-145-5p expression in 35 paired CRC tissues and peritumoral healthy tissues was examined by qRT-PCR. (C) miR-145-5p expression in patients with TNM stage I/II and TNM stage III/IV CRC was analyzed. (D) Correlation between high/low miR-145-5p expression and survival in the cohort by Kaplan-Meier survival curve analysis. (E) miR-145-5p expression in CRC cell lines (LoVo, DLD-1, Caco-2, SW480 and T84) and normal colon epithelial cell line FHC was estimated by qRT-PCR. *$P < 0.05$.

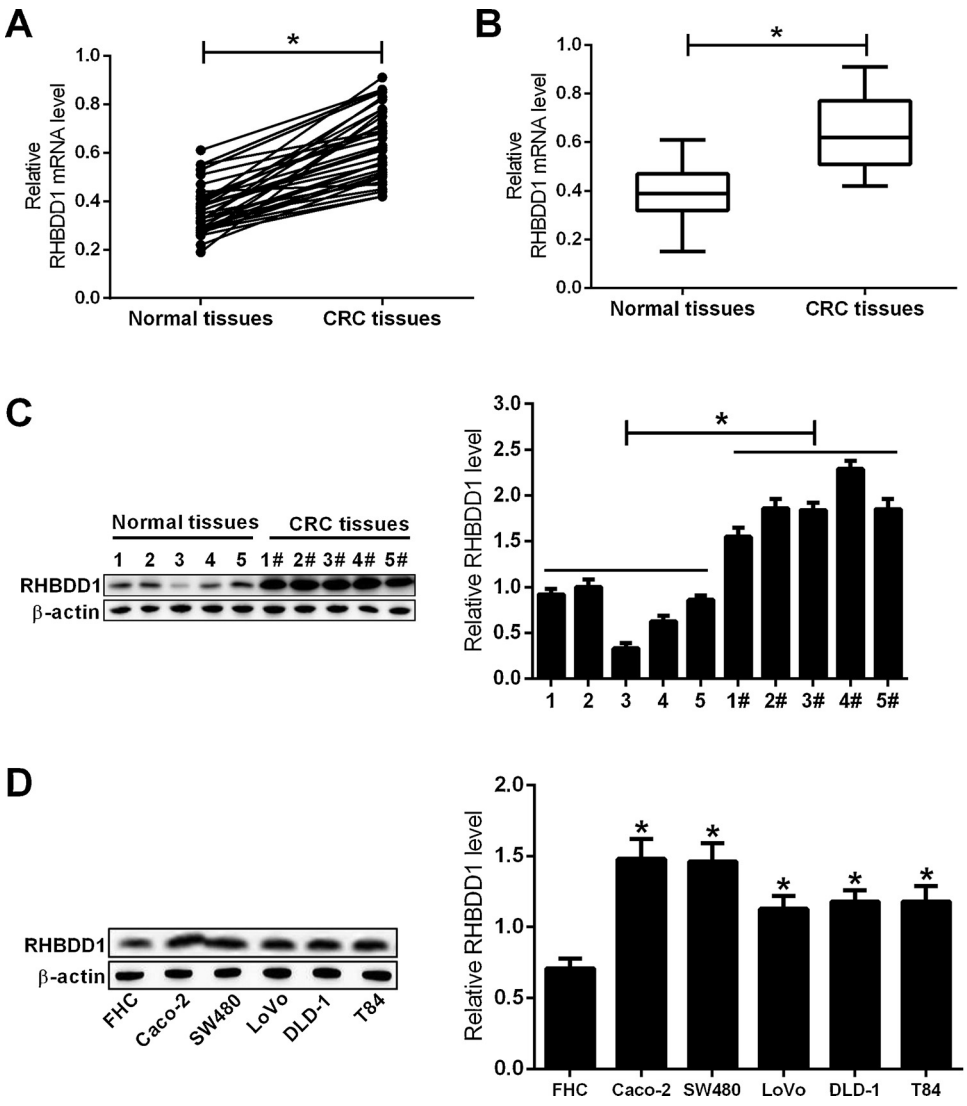

Fig. 2. Expression of RHBDD1 in CRC tissues and cells. (A and B) qRT-PCR analyses of RHBDD1 mRNA expression in 35 paired CRC tissues and adjacent healthy tissues. (C) Western blot of RHBDD1 protein level in clinical samples. (D) Western blot analyses of RHBDD1 protein level in CRC cells. *$P < 0.05$.

3.2. RHBDD1 expression was upregulated in CRC tissues and cells

Both RHBDD1 mRNA and protein levels were remarkably upregulated in 35 CRC tissues respective with that from the healthy ones (Fig. 2A-C). Meanwhile, in line with the result from clinical samples, CRC cell lines (Caco-2, DLD-1, LoVo, SW480 and T84) showed a marked high protein level of RHBDD1 versus that in FHC cells (Fig. 2D), particularly in SW480 and Caco-2 cells, which were thus chosen for subsequent experiments. These results suggested that the upregulated RHBDD1 might participate in CRC.

3.3. Knockdown of RHBDD1 inhibited the malignant phenotypes of CRC cells

Knockdown of RHBDD1 achieved by transfecting sh-RHBDD1 was used to determine its roles in CRC cells. Western blot analysis presented that RHBDD1 level was effectively reduced by sh-RHBDD1 in SW480 and Caco-2 cells in contrast to sh-NC group (Fig. 3A). MTT and colony formation assays demonstrated that RHBDD1 silencing remarkably impeded cell proliferative abilities in CRC cells in comparison with negative control group (Fig. 3B and C). Moreover, Transwell and wound healing analyses hinted that RHBDD1 depletion leaded to a dramatic drop in invasive and migratory abilities in Caco-2 and SW480 cells versus sh-NC-transfected group (Fig. 3D and E), respectively. Meanwhile, the CRC cell apoptosis was distinctly elevated following introduction with sh-RHBDD1 relative to sh-NC group (Fig. 3F). We concluded that RHBDD1 silencing suppressed CRC progression *in vitro*.

3.4. miR-145-5p directly targeted RHBDD1 in CRC cells

According to our online bioinformatics analysis, RHBDD1 and miR-145-5p share putative complementary binding regions. For the validation of direction interaction, luciferase reporter plasmids carrying the WT or MUT 3′UTR of RHBDD1 were constructed (Fig. 4A). The subsequent assay showed that the luciferase activity with RHBDD1-WT co-transfection was drastically repressed by miR-145-5p augmentation, while increased by miR-145-5p inhibition, but caused no noticeable change on those introduced with RHBDD1-MUT (Fig. 4B). Besides, a negative connection between miR-145-5p and RHBDD1 mRNA expression in CRC tissues was proved by correlation assay (Fig. 4C). Furthermore, RHBDD1 protein level was apparently suppressed by transfection with miR-145-5p but greatly promoted following introduction with anti-miR-145-5p in CRC cells (Fig. 4D) cells. Collectively, these data indicated that miR-145-5p targeted directly and inhibited RHBDD1.

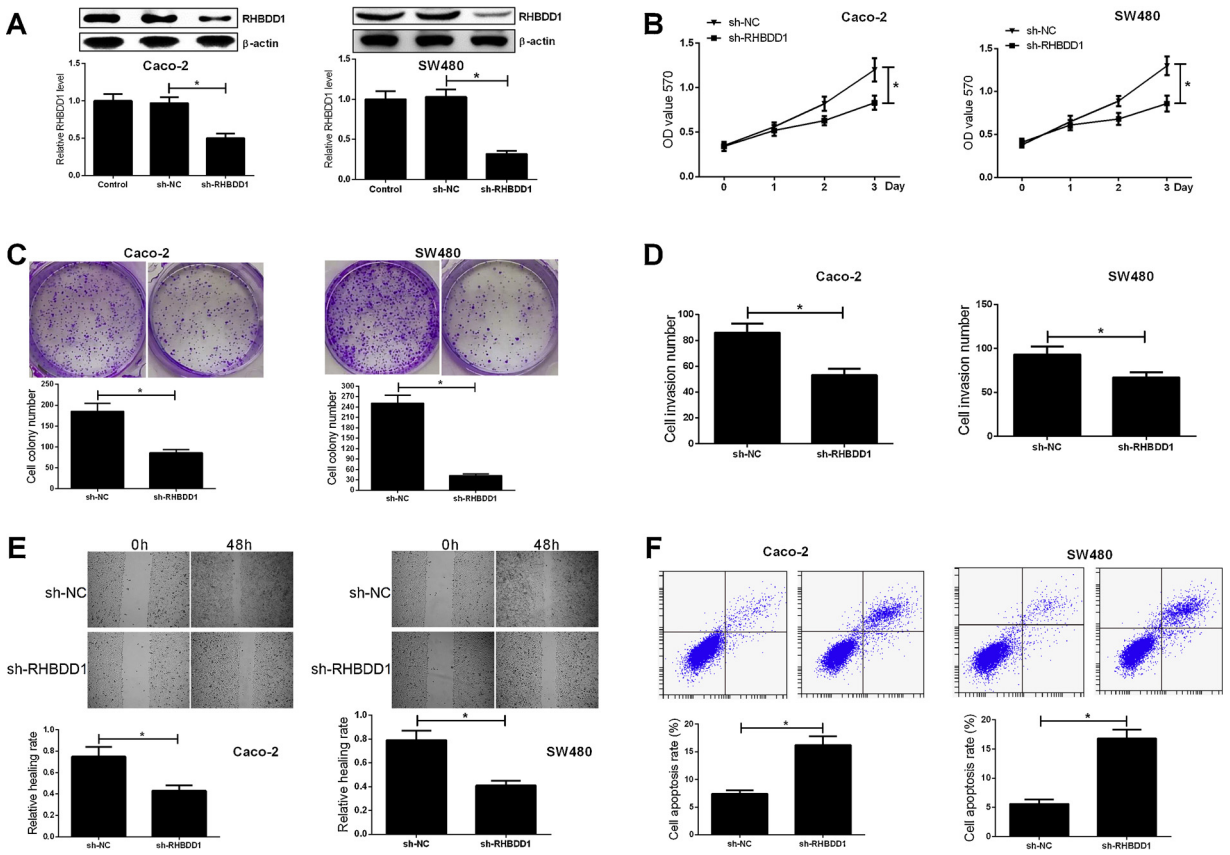

Fig. 3. RHBDD1 silencing alleviated the malignant phenotypes of CRC cells. sh-RHBDD1 or sh-NC was introduced to Caco-2 and SW480 cells. (A) Western blot analysis of the protein level of RHBDD1 in the introduced SW480 and Caco-2 cells. (B) The transfected CRC cell proliferation was assessed by MTT assay. (C) The treated cells colony forming ability was estimated by colony forming assay. (D) Transwell assay was used to estimate the cell invasion in treated cells. (E) Wound healing assay was performed to evaluate the migration in treated cells. (F) Flow cytometry assay was used to explore the apoptosis of treated cells. *$P < 0.05$.

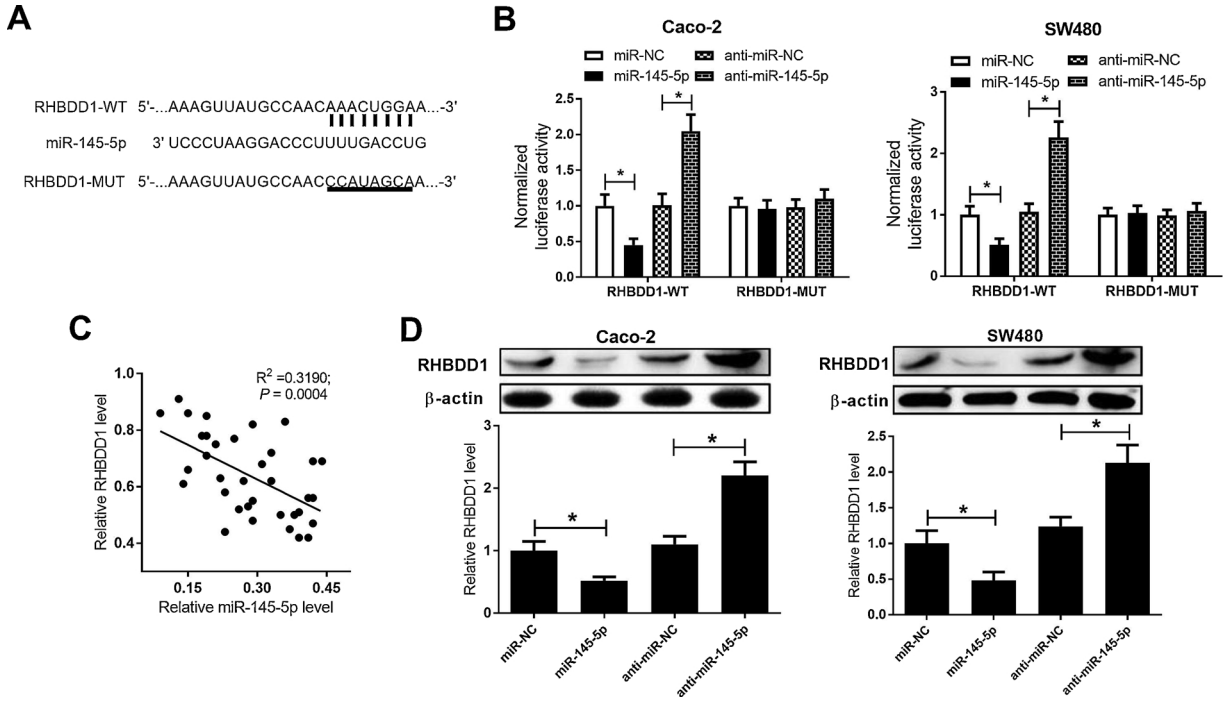

Fig. 4. The correlation between RHBDD1 and miR-145-5p in CRC cells. (A) The WT or MUT 3′UTR of RHBDD1 embracing sites that complementary to miR-145-5p. (B) Luciferase activity was measured by luciferase reporter assay in co-transfected cells. (C) Correlation between expression of miR-145-5p and RHBDD1 in CRC samples. (D) RHBDD1 level in CRC cells transfected with miR-145-5p, anti-miR-145-5p, or corresponding controls was determined by western blot. *$P < 0.05$.

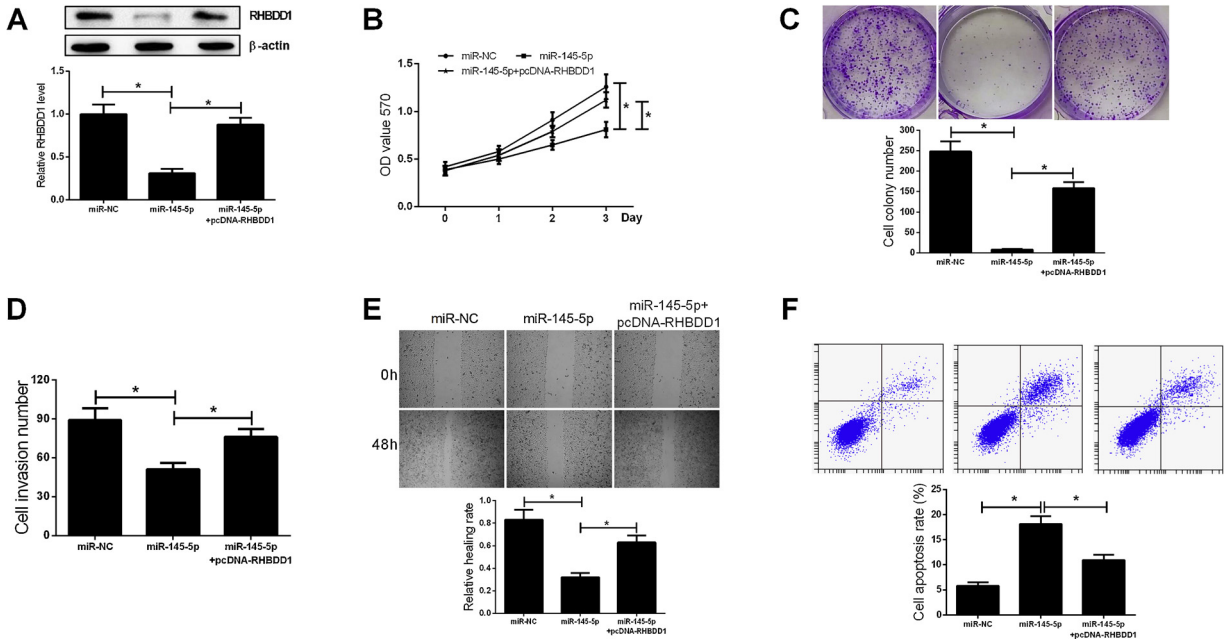

Fig. 5. Regulation of miR-145-5p or together with RHBDD1 on the malignant phenotypes of CRC cells. miR-NC, miR-145-5p, or miR-145-5p + pcDNA-RHBDD1 was transfected into SW480 cells. (A) Western blot analyses of RHBDD1 protein levels. (B) The proliferation of treated SW480 cells was detected by MTT assay. (C) Colony formation assay was used to evaluate the colony forming abilities. (D) Transwell assay was taken to assess cell invasion. (E) Cell migration was assessed by wound healing assay. (F) Flow cytometry analysis was used to detect the apoptosis. $^*P < 0.05$.

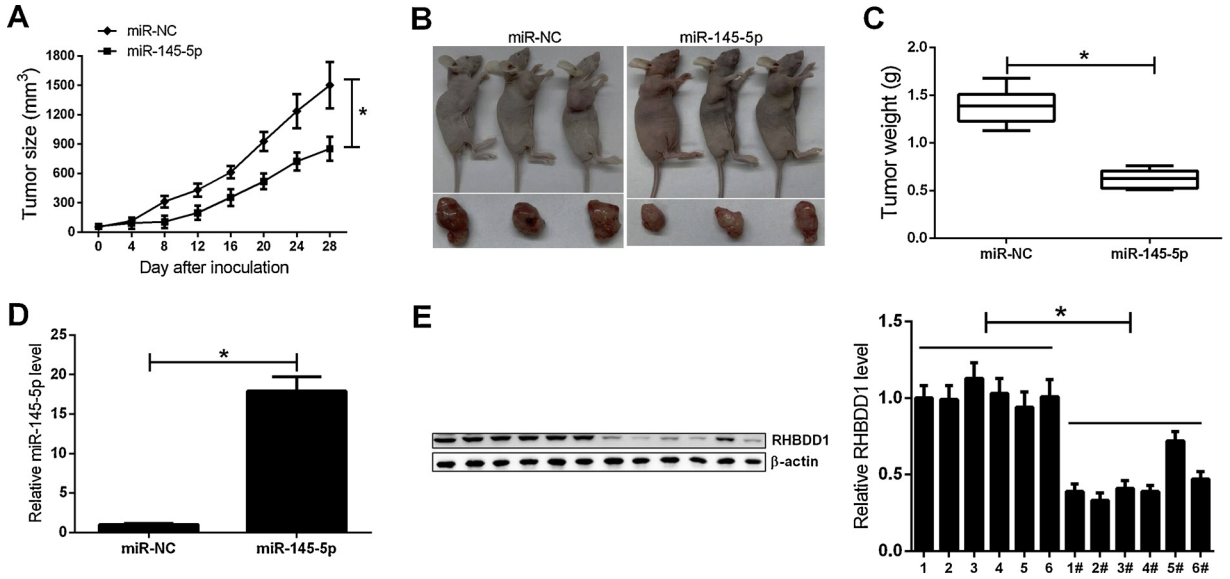

Fig. 6. miR-145-5p inhibited CRC tumor development and RHBDD1 expression. miR-145-5p or miR-NC-transfected SW480 cells were subcutaneously injected into armpits of the nude mice. (A) Every 4 days, tumor volume was assessed. (B and C) Tumor tissues were excised at day 28 after injection and weighed. (D) miR-145-5p expression level in the excised tumors was valued by qRT-PCR. (E) The RHBDD1 protein level in the xenograft was estimated by western blot. $^*P < 0.05$.

3.5. CRC cell malignancy was repressed by miR-145-5p via targeting RHBDD1 in vitro

For the investigation of the connection of miR-145-5p and RHBDD on CRC cells, rescue experiments were performed in miR-145-5p-introduced SW480 cells through RHBDD1 overexpression. Western blot analysis revealed that miR-145-5p restoration obviously hindered RHBDD1 expression in SW480 cells, which was counteracted following reintroduction with pcDNA-RHBDD1 (Fig. 5A). As demonstrated by MTT and colony formation assays, ectopic expression of RHBDD1 remarkably abolished the inhibitory effect on cell proliferation mediated by ectopic miR-145-5p in SW480 cells (Fig. 5B and C). Meanwhile, promotion of miR-145-5p substantially impeded the invasive and migratory capacities in SW480 cells, while these effects were evidently weakened by forced expression of RHBDD1 (Fig. 5D and E), as demonstrated by wound healing assay and Transwell assay, Furthermore, miR-145-5p increase promoted apoptosis in SW480 cells, which was greatly undermined after RHBDD1 overexpression (Fig. 5F). Therefore, miR-145-5p restrained the CRC cell malignance via targeting RHBDD1.

3.6. miR-145-5p arrested CRC xenograft tumor growth via RHBDD1

Further exploration of the miR-145-5p biological function in CRC was processed by *in vivo* analyses. Forced expression of miR-145-5p

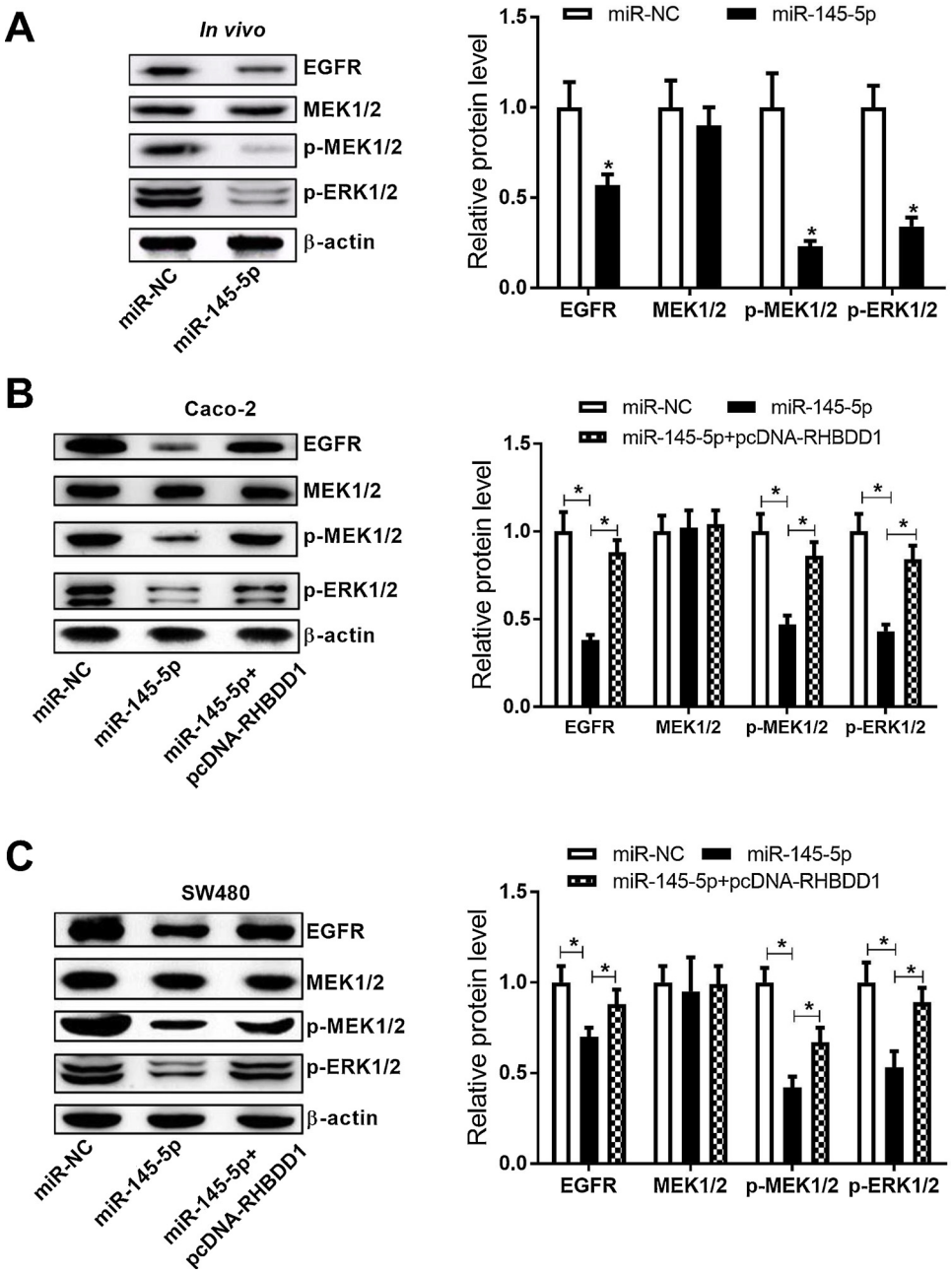

Fig. 7. Regulation of miR-145-5p or along with RHBDD1 on the EGFR/Raf/MEK/ERK signaling in CRC. Western blot analysis was applied to determine the protein levels of EGFR, MEK1/2, p-MEK1/2, and p-ERK1/2 in resected xenografted tumor tissues (A), Caco-2 (B) and SW480 (C) cells with miR-NC, miR-145-5p, or miR-145-5p + pcDNA-RHBDD1 transfection. $^{*}P < 0.05$.

dramatically suppressed tumor volume (Fig. 6A) and weight (Fig. 6B and C) comparing to their counterparts. Moreover, tumors from miR-145-5p overexpressing group exhibited a higher miR-145-5p expression than tumors from miR-NC group (Fig. 6D). Also, RHBDD1 expression was lower in the miR-145-5p overexpressing tumor in contrast to the level in miR-NC group (Fig. 6E). Collectively, miR-145-5p inhibiting the CRC xenograft tumor progression via regulating RHBDD1 was validated.

3.7. miR-145-5p inhibited EGFR-associated signaling pathway in vivo and in vitro by downregulating RHBDD1

Previously, it was reported that RHBDD1 promoted CRC growth through activating the EGFR/Raf/MEK/ERK signaling cascades (Song et al., 2015). Herein, we studied the effect of miR-145-5p or along with RHBDD1 on the EGFR/Raf/MEK/ERK pathway in CRC. As shown in Fig. 7A-C, miR-145-5p expression augmentation prominently suppressed the EGFR, p-MEK1/2, and p-ERK1/2 protein levels in resected xenografted tumor tissues, Caco-2 and SW480 cells, which were notably restored following reintroduction with pcDNA-RHBDD1, suggesting that the EGFR/Raf/MEK/ERK signaling cascades was inactivated by miR-145-5p via targeting RHBDD1 *in vitro* and *in vivo*.

4. Discussion

Evidence has been accumulating that the miRNAs deregulation is essential for CRC formation and progression as tumor suppressors or promoters depending on the cellular context. Identifying CRC-associated miRNAs and their direct targets is helpful for understanding the regulation of CRC tumorigenesis, which may provide novel biomarkers

for diagnosis to improve the CRC prognosis.

miR-145, a newly identified miRNA, has been confirmed as tumor suppressor that is commonly downregulated in different cancers. For instance, miR-145 expression is lower in NSCLC clinical samples, and overexpressing miR-145 represses cell proliferative, migratory and invasive potentials and increases apoptotic abilities in NSCLC cells via inactivating the rapamycin (mTOR) pathway (Li and Zheng, 2017). miR-145 suppresses proliferative and migratory capabilities via inhibiting transforming growth factor-β1 (TGF-β1) in breast cancer cells (Ding et al., 2017). Under-expression of miR-145 in epithelial ovarian cancer tissues and cells and that the tumor suppressive function of miR-145 via targeting tripartite motif-containing protein 2 (TRIM2) were also annotated (Chen et al., 2015). Moreover, the roles of miR-145-5p in CRC have also been well-recorded in many previous studies. miR-145 is under-expressed in CRC tumors with whose promotion inhibited CRC growth and metastatic abilities via targeting Fascin-1 both *in vivo* and *in vitro* (Feng et al., 2014). miR-145 impedes CRC migratory and invasive abilities through the p21-activated kinases 4 (PAK4) pathway (Sheng et al., 2017). miR-145 enforced expression enhances cell response to cetuximab in colon cancer cells, leading to an increase of related cellular cytotoxicity (Gomes et al., 2016). Consistently with these literatures, a significant decrease of miR-145-5p expression in CRC cells and tissues was observed, which indicated that miR-145-5p may be used for CRC prognosis identification. Moreover, we found that promotion of miR-145-5p repressed the malignant phenotypes of CRC cells *in vitro* by inhibiting cell proliferative, invasive and migratory traits and promoting apoptotic traits, as well as hindered CRC tumor growth *in vivo*, suggesting the anti-tumor effects of miR-145-5p on CRC cells.

The potential mechanism of miR-145-5p exerting the anti-tumor effects on CRC was further explored by target prediction using online bioinformatics analysis. And our results showed that RHBDD1 was predicted to contain the binding sites complementary to miR-145-5p. RHBDD1, a newly identified rhomboid family member, its upregulation was observed in several malignancies, such as glioblastoma (Wei et al., 2014), breast cancer (Zhang et al., 2018c), and CRC (Han et al., 2015; Song et al., 2015). RHBDD1 has been suggested to promote breast cancer tumiogenesis by regulating cyclin-dependent kinase 2 (CDK2) and p-protein kinase B (Akt) levels (Zhang et al., 2018c). Moreover, tumor growth was inhibited and apoptosis was facilitated in RHBDD1 silenced hepatocellular carcinoma cells (Liu et al., 2013). Our study found that RHBDD1 was overexpressed in CRC cells and tissues, and RHBDD1 depletion hindered cell proliferative, migratory and invasive abilities and facilitated apoptotic ones in CRC cells, which was in line with the previous work (Han et al., 2015; Song et al., 2015; Zhang et al., 2018b). Furthermore, we demonstrated that RHBDD1 was directly regulated by miR-145-5p and RHBDD1 was inhibited in CRC *in vivo and in vitro*. And, a negative correlation between miR-145-5p and RHBDD1 expression in CRC was validated in this study. Rescue experiments revealed that the malignant phenotypes of CRC cells and tumors were depressed by miR-145-5p via regulating RHBDD1. Meanwhile, we further revealed that EGFR/Raf/MEK/ERK signaling was inactivated by miR-145-5p *in vitro* and *in vivo* via interacting with RHBDD1. The Ras/Raf/MEK/ERK signaling has been proved playing an essential function in regulating cell cycle progression, cell proliferation, as well as apoptosis (Chang et al., 2003). The hyper-activation of EGFR and its downstream Ras/Raf/MEK/ERK signaling in various tumors and implication in the progression of multiple tumors including CRC was also well-documented (Roberts and Der, 2007; Markman et al., 2010). Of note, it has been reported that RHBDD1 promoted CRC growth through activating the EGFR/Raf/MEK/ERK signaling pathway (Song et al., 2015). Accordingly, it is reasonable to infer that the CRC malignant phenotypes are suppressed by miR-145-5p targeted RHBDD1 and inactivation of the EGFR/Raf/MEK/ERK signaling.

In summary, our study proved the under-expression of miR-145-5p in CRC tissues and cells, which indicated poor prognosis. In addition, RHBDD1, which was highly enhanced in CRC cells and tissues, was identified as a miR-145-5p target. Furthermore, we revealed that the inhibition of CRC tumorigenesis was induced by miR-145-5p via RHBDD1 downregulation and EGFR-associated signaling inhibition. Our current study may provide the molecular basis for miR-145-5p as an indicator for prognosis and target for CRC therapy.

Declaration of Competing Interest

None.

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