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MiR-103a-3p Contributes to the Progression of Colorectal Cancer by Regulating GREM2 Expression

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Purpose: Our research aimed to investigate the influence of miR-103a-3p on the growth and apoptosis of colorectal cancer (CRC) cells.
Materials and Methods: Bioinformatics was employed to analyze differentially expressed microRNAs and predict target genes. qRT-PCR was applied to detect the expression of miR-103a-3p in CRC and normal cells. HCT116 and Caco-2 were chosen, and miR-103a-3p mimics, miR-103a-3p inhibitor, as well as specific siRNAs targeting *GREM2*, were constructed. We subsequently evaluated alternations in cell proliferation, cell cycle and cell cycle regulators, apoptosis, and related proteins (Bcl-2 and Bax) by CCK-8 testing, Western blotting, luciferase reporter, colony formation, and Annexin V-FITC/PI. Possible binding sites for miR-103a-3p on the 3'UTR of GREM2 were checked with luciferase assay, and the impact of GREM2 on miR-103a-3p activity was also validated with above biological function testing. Additionally, the effect of miR-103a-3p knockdown in CRC cells and the molecular mechanism of miR-103a-3p targeting GREM2 were also studied.
Results: Bioinformatics analysis revealed that miR-103a-3p expression increased remarkably in CRC, and targeted regulatory correlation existed between miR-103a-3p and GREM2. MiR-103a-3p inhibitor significantly impeded proliferative capacity and caused cell cycle arrest, as well as apoptosis, in HCT116 and Caco-2 cells. Consistent with this finding, overexpression of GREM2 showed similar effects to miR-103a-3p inhibition. Moreover, we demonstrated that miR-103a-3p connected target GREM2 and GREM2 knockdown reversed the effects of miR-103a-3p inhibitor on HCT116 and Caco-2 cell proliferation, cell cycle, and apoptosis. Further study showed that miR-103a-3p targeting GREM2 appeared to affect CRC progression via the transforming growth factor-β pathway.
Conclusion: MiR-103a-3p could augment CRC progression by targeting GREM2 and that miR-103a-3p/GREM2 could be potential novel targets for CRC therapy.

Key Words: Colorectal cancer, miRNA-103a-3p, GREM2, cell proliferation, apoptosis

INTRODUCTION

Colorectal cancer (CRC) ranks third among all diagnosed cancers and has the highest mortality only after lung cancer.[1] CRC carcinogenesis is complex, involving complicated epigenetic and hereditary variations, along with environmental factors.[2-4] Therefore, further studies on its molecular mechanisms are necessary to deepen understanding of CRC.

MicroRNAs (miRNAs) negatively modulate messenger (m) RNAs at the post-transcriptional stage.[2,3] They are potent regulators of cell growth and apoptosis.[5] Growing numbers of miRNAs have been shown to have strong associations with CRC progression[6] and are regarded as theranostic targets for various cancers or other diseases.[7,8] Among them, miR-103a-3p, a tumor-promoting miRNA, has been shown to be capable of facilitating malignant transformation in several types of cancers.[9-11] MiR-103a-3p levels in tumor samples[12,13] or plasma[14] have been found to hold prognostic and predictive value in CRC. Despite the strong connections between miR-103a-3p in CRC, exact

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mechanisms undergirding miR-103a-3p in CRC progression remain unclear.

Our research investigated the expression of miR-103a-3p in CRC and explored its role in cell growth and apoptosis. We discovered miR-103a-3p is dramatically upregulated in HCT116 and Caco-2 cells and that miR-103a-3p stimulates cell proliferation and suppresses apoptosis by targeting GREM2. This research provides a potential novel direction for CRC therapeutic intervention.

Table 1. qRT-PCR Primers

Name	Sequence (5'-3')
miR-103a-3p F	GCGAGCAGCATTGTACAGGG
miR-103a-3p R	AGTGCAGGGTCCGAGGTATT
GREM2 F	GAAGCTTTCCCTGTCCTTGTTC
GREM2 R	CCAGTCACTCTTGAGGTACTTG
U6 F	CTCGCTTCGGCAGCACA
U6 R	AACGCTTCACGAATTTGCGT
GAPDH F	GACCTGACCTGCCGTCTAG
GAPDH R	AGGAGTGGGTGTCGCTGT

F, forward primer; R, reverse primer.

MATERIALS AND METHODS

Bioinformatics analysis

CRC relevant miRNA data (cancer: n=50; normal: n=41) and mRNA data (cancer: n=6; normal: n=6) were obtained from the Gene Expression Omnibus (GEO) online database. Differential analysis was executed via the "limma" package, threshold as |logFC| >2, adj. *P* value <0.05 (for mRNA). FunRich (version 3.13, http://www.funrich.org) was employed for target prediction of differentially expressed miRNA (DE miRNA). Finally, MCODE was employed to identify clusters (highly connected for CRC development) in a miRNA-mRNA interaction network.

Cell culture

Normal NCM460 cells[15] and Caco-2, HT29, HCT116, and SW620 CRC cells[16] were employed in this study. These cells were bought from the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640.

Vector construction

MiR-103a-3p mimics, miR-103a-3p inhibitor, NC-miRNA, overexpression-NC (OE-NC), siGREM2, and GREM2 vectors constructed by Gene Pharma (Shanghai, China) were transfected into HCT116 and Caco-2 cells applying Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with the instructions provided by the manufacturer.

qRT -PCR

Total RNA from cells was obtained. Reverse transcription was conducted applying RNA reverse transcription kits. cDNA was synthesized applying the Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). Prime Script RT Master Mix Kit and SYBR Green reagent (Takara, Beijing, China) were employed to determine miR-103a-3p and GREM2 expression, respectively. U6 and GAPDH were applied as internal controls. mRNA levels were computed using the $2^{-\Delta\Delta Ct}$ method. The primers applied are shown in Table 1.

Western blot

Cells were split by radioimmunoprecipitation assay with protease inhibitor. Proteins were detached and transferred to PVDF membranes. Target proteins were detected by specific antibodies [GREM2, 1:100; transforming growth factor-β (TGF-β), 1:1000; cyclin D1, 1:1000; CDK2, 1:1000; P21, 1:1000; P27, 1:2000; Bcl-2, 1:1000; Bax, 1:1000; GAPDH, 1:1000; Abcam Inc, Cambridge, UK] followed by HRP adherent secondary antibodies using enhanced chemiluminescence.

Luciferase reporter

Cells were transfected with pmir-GLO vector (Promega, Madison, WI, USA) including wild-type/mutant GREM2 3'UTR, miR-103a-3p mimics/NC-miRNA. They were transfected for 48 h and then detected via dual-luciferase kit (Beyotime Institute of Biotechnology, Shanghai, China).

CCK-8 assay

Cells were evaluated using CCK-8 reagent (Dojindo, Tokyo, Japan). 5×10^3 cells were added in each well of a 96-well plate. At 0, 24, 48, and 72 h, 10 μL of CCK-8 were mixed, and optical density was measured.

Colony formation assay

Cells were inoculated into 6-well plates and cultivated in RPMI-1640 at 37°C for 2 weeks. Cells were washed with PBS, fastened by 4% paraformaldehyde, and treated with 1% crystalline violet staining for observation.

Apoptosis and cell cycle assays

Cells were stored in 75% ethanol at -20°C after 48 h of incubation. Next, HCT116 and Caco-2 were mixed in propidium iodide (PI) for 30 min. For apoptosis testing, the transfected cells were labeled with Annexin V-FITC and PI for 48 h. They were determined using flow cytometry (BD Biosciences, Franklin Lake, NJ, USA), and the data were analyzed using CellQuest (BD Biosciences).

Statistical analysis

The experimental data generated in this study were analyzed by t-test or one-way ANOVA with GraphPad prism 8.0 software (GraphPad Software, San Diego, CA, USA), and $p<0.05$ was considered statistically significant.

RESULTS

MiR-103a-3p is highly expressed in CRC

Through miRNA differential expression analysis, 295 DE miRNAs were obtained from the normal control and the CRC tissues from the GEO (Fig. 1A), among which 83 were upregulated and 212 were downregulated. Also, 869 DE mRNAs were obtained between the normal control and CRC tissues (Fig. 1B), including 366 upregulated and 503 downregulated mRNAs. FunRich analysis identified target miRNAs and genes (Fig. 1C). The results of MCODE revealed that CRC development was regulated by *NOVA1*, *RBM24*, *TFCP2L1*, and *GREM2* (Fig. 1D). Among target miRNAs, miR-107 has been shown to be involved in CRC development in previous research.[17] However, few reports of miR-103a-3p have been made in CRC. Therefore, the involvement of miR-103a-3p and its target genes in the development of CRC was thoroughly explored.

According to bioinformatics analysis results in the GSE112264

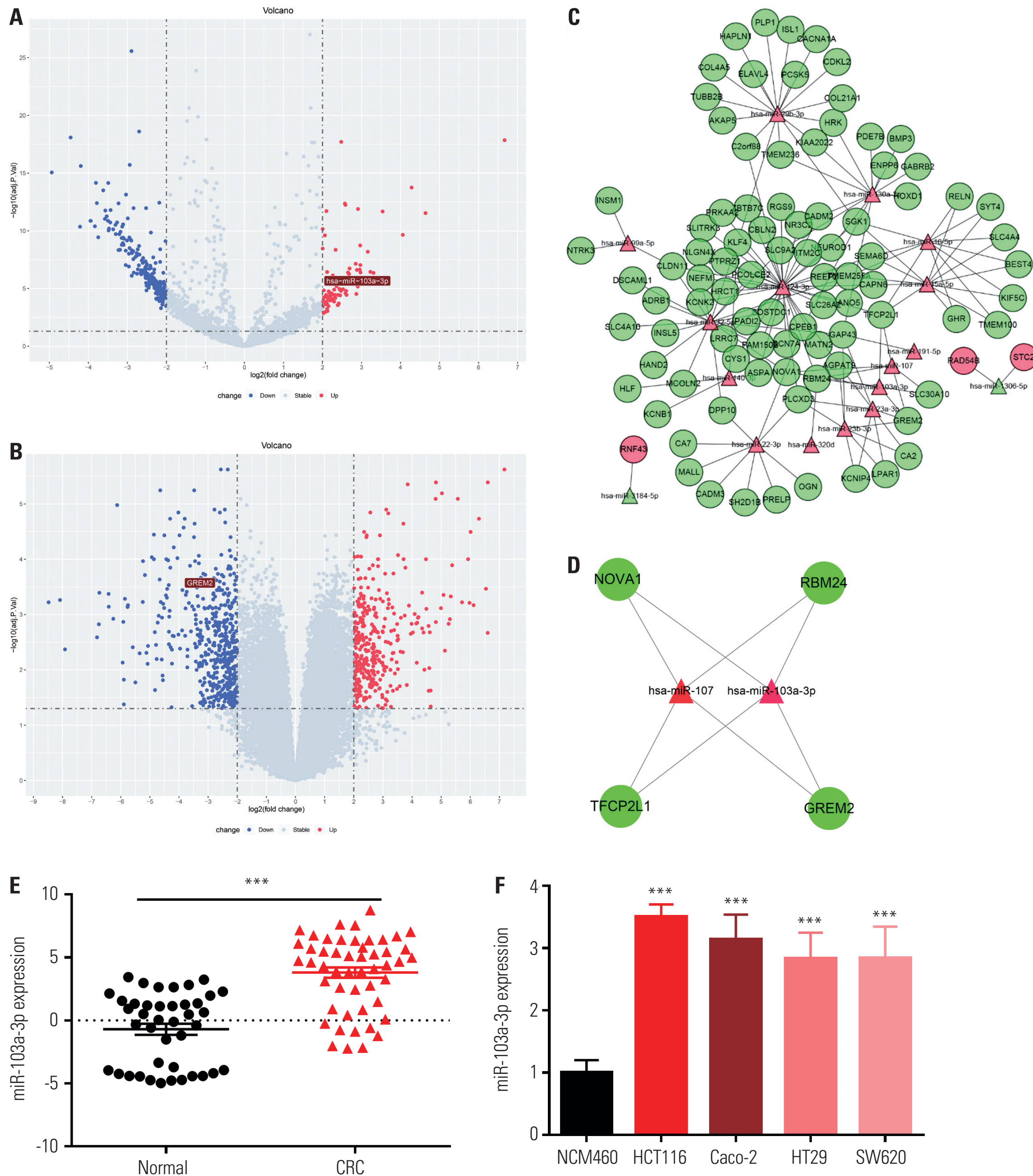

Fig. 1. MiR-103a-3p is upregulated in CRC. (A) Differential expression of miRNAs displayed in a volcano plot. (B) Differential expression of mRNAs displayed in a volcano plot. (C) FunRich analysis for target mRNA of differential miRNAs. (D) miRNA-target gene regulatory network. (E) The expressions of miR-103a-3p in the GSE112264 dataset. ***$p<0.01$ vs. normal. (F) qRT-PCR of miR-103a-3p expression in CRC and normal cells, with NCM460 cells as a control (***$p<0.001$). CRC, colorectal cancer.

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dataset (Fig. 1E), miR-103a-3p expression increased with CRC (p<0.001). Mounting reports have confirmed miR-103a-3p could promote the progression of gastric[10] and oral cancers.[18] Compared to NCM460 cells, miR-103a-3p was greatly enhanced in Caco-2, HT29, HCT116, and SW620 cells (Fig. 1F). Subsequent experiments were performed on HCT116 and Caco-2 cell lines wherein miR-103a-3p expression was highest.

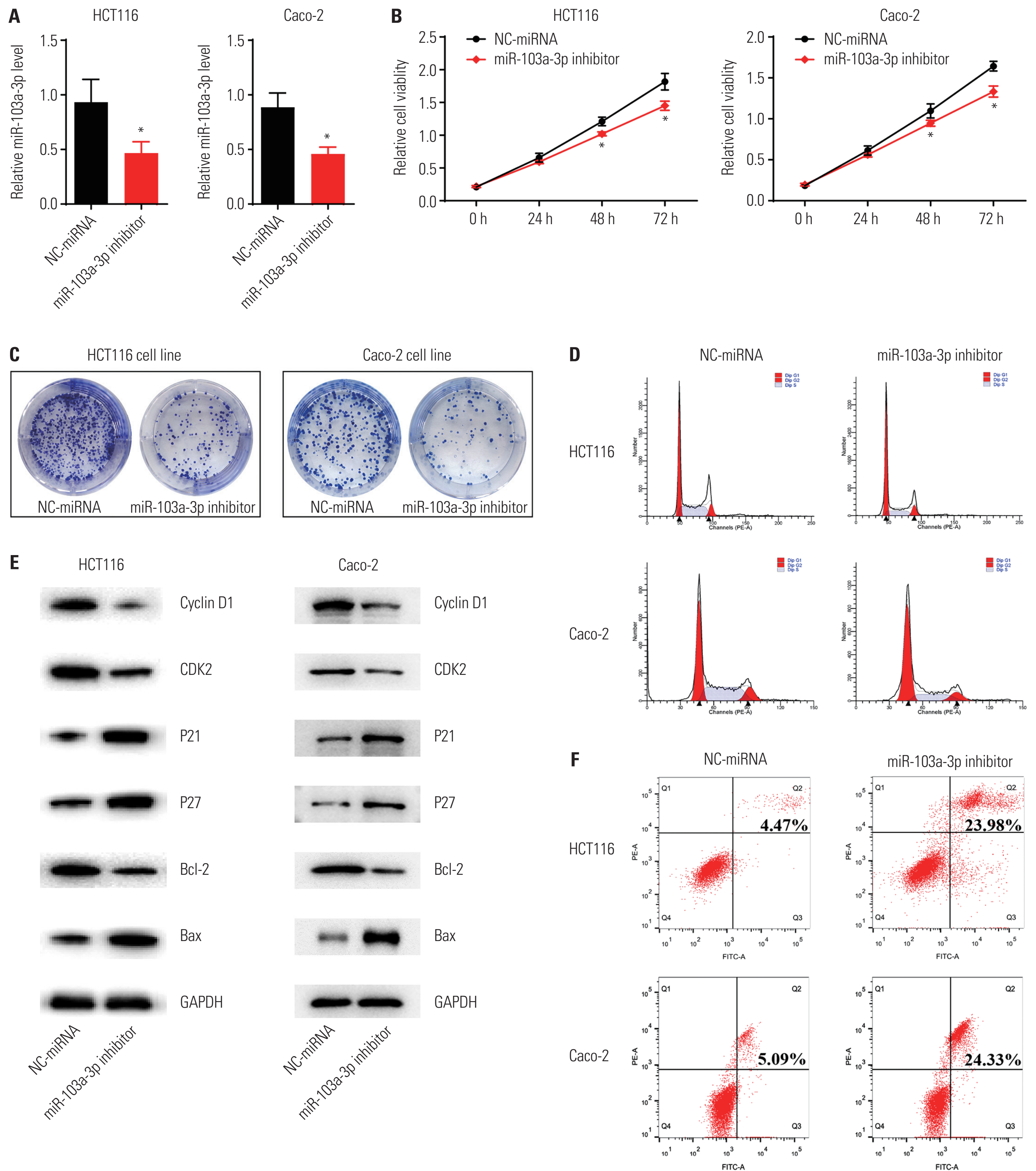

Fig. 2. MiR-103a-3p inhibitor affects the growth and apoptosis of HCT116 and Caco-2. (A) miR-103a-3p levels with qRT-PCR. *p<0.05 vs. NC-miRNA group. (B) Cell viability test. *p<0.05 vs. NC-miRNA group. (C) Colony formation assay. (D) Cell cycle distribution and statistical analysis. (E) Cell cycle and apoptosis. (F) Flow cytometric detection of apoptosis ratio.

MiR-103a-3p downregulation inhibits HCT116 and Caco-2 cell growth and promotes apoptosis

To examine miR-103a-3p activity in CRC, NC-miRNA or miR-103a-3p inhibitor was transfected into HCT116 and Caco-2 cells. The miR-103a-3p levels in the inhibitor group were drastically reduced, compared to the NC-miRNA group (Fig. 2A). CCK-8 assay revealed that miR-103a-3p downregulation suppressed the increase of the proliferation rate of cells upon transfection for 48 h (Fig. 2B). Also, colony formation experiments after transient transfection further demonstrated the reduced clonal ability of miR-103a-3p knockdown compared to NC-miRNA (Fig. 2C). Cell cycle analysis with flow cytometry indicated that miR-103a-3p inhibitor arrested G1 phase and abated S phase ratio after transfection for 48 h (Fig. 2D). Western blotting revealed that miR-103a-3p inhibitor downregulated G1/S-related regulatory factors with Cyclin D1 and CDK2, while P21 and P27 were upregulated in cells, compared with controls (Fig. 2E).

Cell apoptotic proportions were also assessed. In contrast to the NC-miRNA transfected group, the apoptosis rate of cells in miR-103a-3p inhibitor was markedly higher ($p<0.001$) (Fig. 2F). Meanwhile, upregulation of the apoptotic protein Bax and downregulation of the anti-apoptotic protein Bcl-2 in miR-103a-3p in the aforementioned groups were also observed (Fig. 2E). Therefore, these results demonstrated miR-103a-3p knockdown might inhibit cell proliferation by aggravating apoptosis in HCT116 and Caco-2.

GREM2 is a target of miR-103a-3p

Through FunRich and MCODE analysis, the relationships between miR-103a-3p and *NOVA1*, *RBM24*, *TFCP2L1*, and *GREM2* are shown to be important in CRC development (Fig. 1C and D). Of these targets, GREM2 expression was significantly lower in CRC patients in bioinformatics analysis ($p<0.001$) (Fig. 3A). Using the online tool TargetScan, we discovered that the only probable binding sites for miR-103a-3p in 3'-UTR of GREM2 were mutated (Fig. 3B). Luciferase assay indicated luciferase activity in cells co-transfected with GREM2-wt+miR-103a-3p

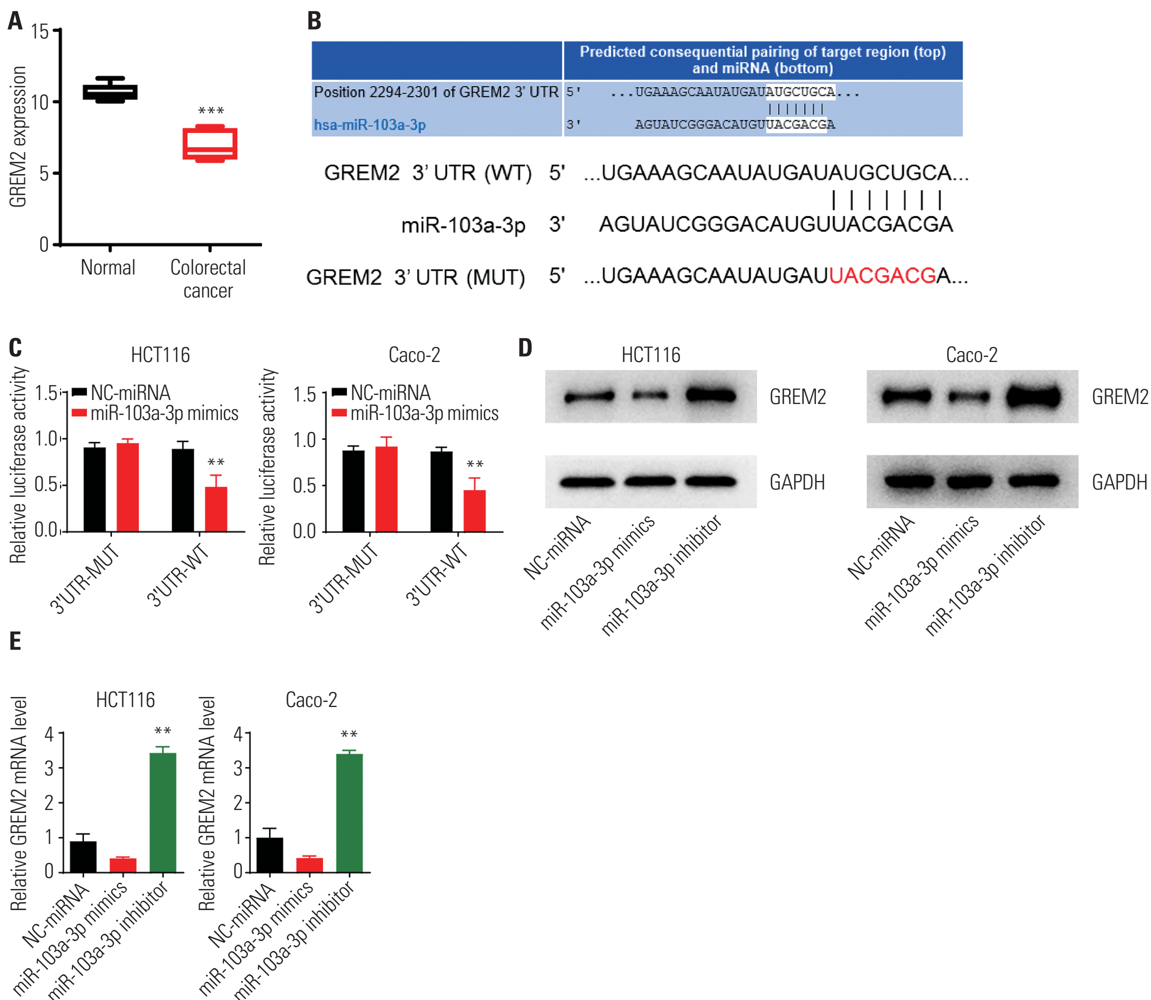

Fig. 3. GREM2 is a direct target gene of miR-103a-3p. (A) Levels of GREM2 in the GSE156355 dataset. (B) MiR-103a-3p was bound to the 3'-UTR of GREM2 according to TargetScan. (C) Luciferase reporter assays. (D) Western blotting of GREM2 protein expression. (E) qRT-PCR of GREM2 mRNA. $^{**}p<0.01$, $^{***}p<0.001$ vs. normal or NC-miRNA group.

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mimics was markedly reduced, compared with the GREM2-wt+NC-miRNA group (p<0.01). Inversely, the luciferase activity in GREM2-MUT+miR-103a-3p mimics cells exhibited no difference from that of the GREM2-WT+NC-miRNA group (Fig. 3C). Further experiments were conducted to research the influence of miR-103a-3p on GREM2 protein levels. Western blot-

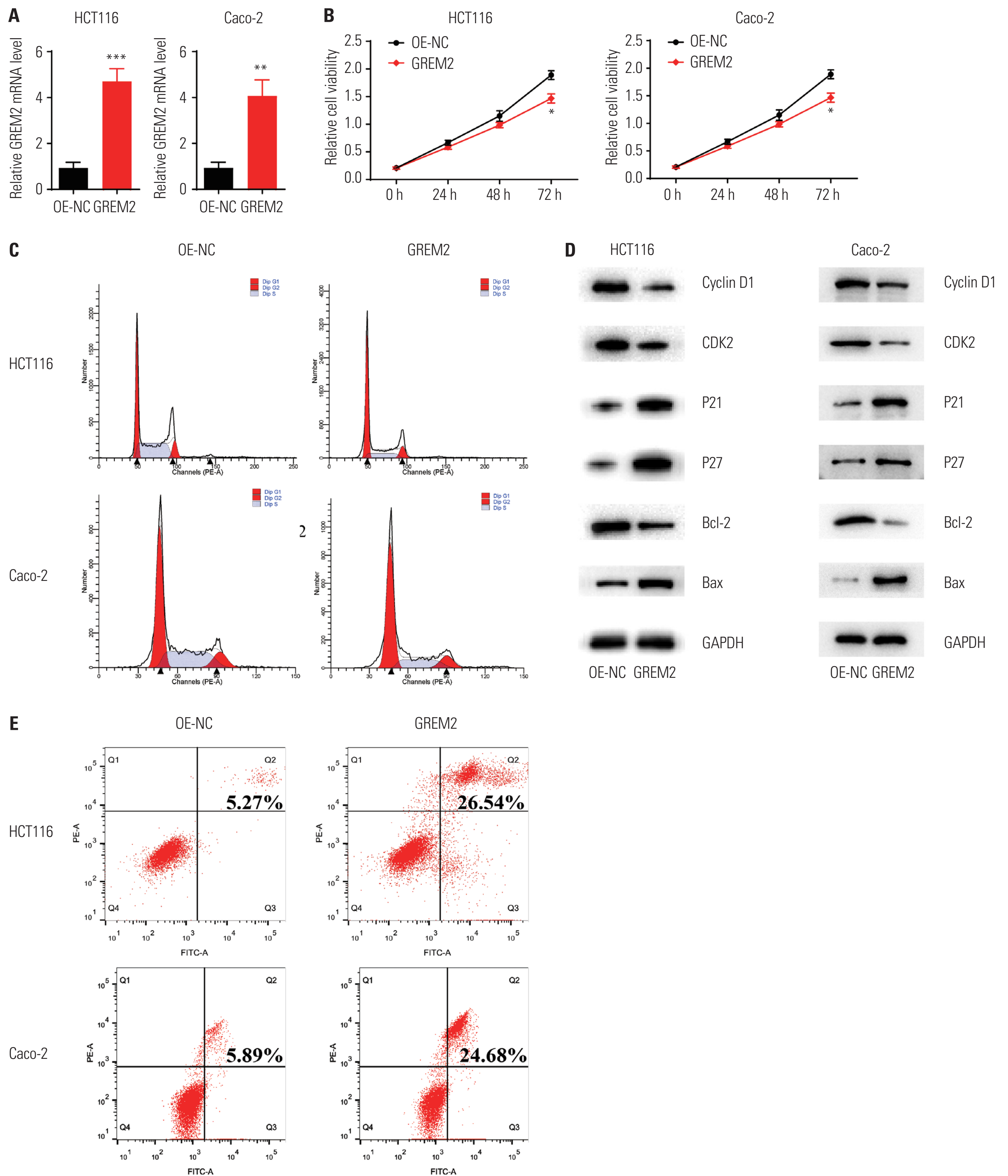

Fig. 4. Impact of overexpressed GREM2 on the growth and apoptosis of HCT116 and Caco-2. (A) GREM2 expression with qRT-PCR. (B) Cell viability assay. (C) Flow cytometry for cell cycle detection. (D) Cell cycle and apoptosis-related proteins. (E) Flow cytometric detection of apoptosis ratio. *p<0.05, **p<0.01, ***p<0.001 vs. OE-NC group. OE-NC, overexpression-NC.

ting revealed reduced GREM2 protein levels in the miR-103a-3p overexpression group, compared to NC-miRNA (Fig. 3D). Nevertheless, GREM2 protein expression in the miR-103a-3p inhibitor group was upregulated (Fig. 3D). qRT-PCR further confirmed these results (Fig. 3E). Altogether, these results confirmed that GREM2 is a target gene of miR-103a-3p in cells.

GREM2 overexpression inhibits HCT116 and Caco-2 cell proliferation and G1/S transition and promotes apoptosis

GREM2 activity in CRC development has not been reported. Herein, cells were transfected with vector coding for GREM2 to detect cell growth, cell cycle status, and apoptosis. qRT-PCR results showed that GREM2 protein levels in HCT116 and Caco-2 transfected with GREM2 were about five-fold higher than that in OE-NC (Fig. 4A). CCK-8 testing revealed that overexpression of GREM2 remarkably depressed the growth of HCT116 and Caco-2 cells at 72 h (Fig. 4B). Cell cycle testing showed that the proportions of transfected cells in G1 phase was visibly higher and those in S phase were relatively lower in contrast to OE-NC transfected cells (Fig. 4C). No difference in G2 phase was found in these two groups. Western blot analysis suggested that overexpressed GREM2 sequestered cyclin D1 and CDK2 levels and upregulated the expression of P21 and P27 (Fig. 4D). The apoptosis rate of cells transfected with GREM2 was pronouncedly reinforced, compared with the OE-NC (Fig. 4E). Apoptosis-related protein levels showed the same trend (Fig. 4D). The above results indicated that GREM2 overexpression could suppress HCT116 and Caco-2 proliferation by promoting apoptosis.

GREM2 knockdown eliminates the cancer-suppressing efficacy of miR-103a-3p inhibitor

Learning more about miR-103a-3p function in CRC through GREM2 as a cancer contributor, miR-103a-3p inhibitor+siGREM2 was co-transfected into HCT116 and Caco-2. In the way that was expected, GREM2 expression was downregulated by siGREM2 ($p<0.01$) (Fig. 5A). Compared with miR-103a-3p inhibitor alone, noticeably strengthened viability of HCT116 and Caco-2 co-transfected of miR-103a-3p inhibitor+siGREM2 from 48 h was seen ($p<0.05$) (Fig. 5B). The apoptosis rate (Fig. 5C) and Western blot analysis (Fig. 5D) showed that miR-103a-3p inhibitor+siGREM2 reduced apoptosis when compared with the miR-103a-3p inhibitor transfected group. Moreover, compared with NC-miRNA group, the ratio of HCT116 and Caco-2 in the G1, S, or G2 phase in the aforementioned group was almost the same (Fig. 5E). Western blot analysis of cycle modulating proteins highlighted similar trends (Fig. 5D). In summary, GREM2 knockdown rescued the impact of miR-103a-3p downregulation on the proliferation and apoptosis of cells.

MiR-103a-3p downregulation inhibits CRC development by targeting GREM2 to inhibit TGF-β signaling

Western blot revealed that GREM2 downregulated TGF-β protein expression in HCT116 and Caco-2, while siGREM2 upregulated TGF-β protein expression in cells, indicating that GREM2 could inhibit the TGF-β pathway (Fig. 6). Further results showed that in the cell lines of HCT116 and Caco-2, GREM2 was downregulated and TGF-β expression was upregulated by miR-103a-3p, while GREM2 expression was upregulated and TGF-β was downregulated by miR-103a-3p inhibitor. In a word, downregulation of miR-103a-3p inhibits proliferation and promotes apoptosis in HCT116 and Caco-2 by targeting GREM2 through TGF-β pathway.

DISCUSSION

Recent studies have indicated that miRNAs are essential moderators during cellular processes.[2] Our research confirmed miR-103a-3p as a promoter of CRC, which facilitated CRC progression by targeting GREM2.

Data concerning miR-103a-3p in CRC are limited. MicroRNA microarray expression analysis in CRC has implicated high levels of miR-103a-3p as a risk factor for worsening prognosis.[12,13] Elevated serum miR-103a-3p in CRC patients has further been shown to be a diagnostic biomarker.[14] Unfortunately, the underlying mechanism of miR-103a-3p in CRC development remains unclear. We discovered downregulated miR-103a-3p represses HCT116 and Caco-2 proliferation through arresting cell cycle at G1/S phase in vitro. All these data above strengthened the hypothesis that miR-103a-3p serves as a cancer accelerator in CRC.

Recognizing specific miRNA targets is essential for elucidating miRNA function on tumorigenesis and development progression.[19,20] GREM2 was discovered as a target of miR-103a-3p by bioinformatics analysis. Luciferase activity verified the binding of miR-103a-3p to the 3'-UTR of GREM2. In HCT116 and Caco-2 lines, miR-103a-3p expression was inversely related to GREM2 protein levels. Furthermore, GREM2 knockdown diminished the influences of miR-103a-3p downregulation on proliferation and apoptosis in HCT116 and Caco-2. Therefore, miR-103a-3p possibly accelerates the CRC progress by depressing GREM2.

The exact function of GREM2 in disease is controversial, owing to incompatible results in different types of illnesses. In gastric cancer, GREM2 is reported to be an oncogene that regulates cell growth, apoptosis, invasion, migration, and tumorigenic ability. However, in diabetic nephropathy-related cells, highly expressed GREM2 not only raised the Bax/Bcl2 ratio but also boosted apoptosis in high-glucose milieu.[21] These findings demonstrated that the action of GREM2 is disease-specific. In the literature, it was reported that GREM2 is present at low lev-

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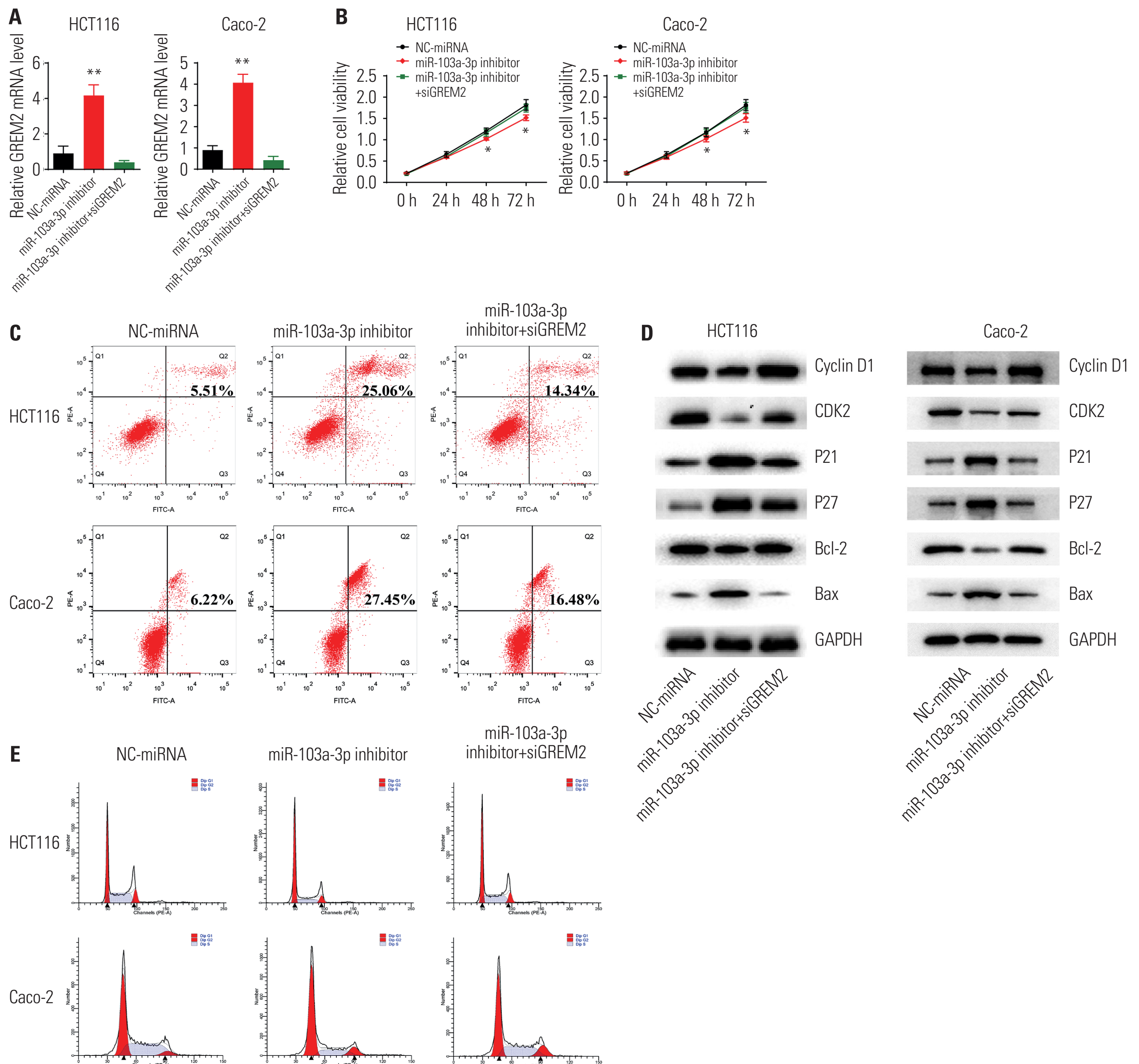

Fig. 5. GREM2 knockdown reduces the impact of miR-103a-3p downregulation on the proliferation and apoptosis of HCT116 and Caco-2. (A) GREM2 expression with qRT-PCR, $**p<0.01$ vs. NC-miRNA group. (B) CCK-8 for cell viability detection, $*p<0.05$ vs. NC-miRNA group. (C) Flow cytometric detection of apoptosis. (D) Western blots of expression of cell cycle and apoptosis-related proteins. (E) Cell cycle.

els in normal intestinal epithelium, yet cannot be detected in adenomas,[22] and genome-wide association study has indicated that GREM2 is likely to be a susceptibility gene in CRC.[23] These two works indicated that GREM2 can act as a tumor-suppressor in CRC. Research into mechanisms has revealed that GREM2 overexpression blocks HCT116 and Caco-2 proliferation and G1/S phase changes and promotes apoptosis.

Abnormal proliferation is a crucial early event in cancer development.[24] MiR-103a-3p boost cell proliferation through inhibiting ATF7 in gastric cancer.[10] It was discovered that miR-103a-3p knockdown attenuates proliferation and migration, and accelerates apoptosis in thyroid cancer.[11] Here, we proved that miR-103a-3p knockdown impaired HCT116 and Caco-2 proliferation and G1/S transition in CRC. It is widely accepted that the cell cycle plays a decisive part in cancer cell proliferation.[25] To exit silence and initiate the cell cycle, cells initially upregulate cyclin D1.[26] Meanwhile, CDK inhibitors (e.g., P21 and P27) suppress cell cycle G1/S phase transformation and bring about cell cycle arrest.[27,28] Here, we demonstrated that miR-103a-3p inhibitor reduced the levels of cyclin D1 and CDK2 and increased the levels of P21 and P27. Our research indicatet miR-103a-3p stimulates HCT116 and Caco-2 proliferation through regulating the cell cycle. Studies have reported that cell cycle arrest is closely related to apoptosis.[29] As expected, our

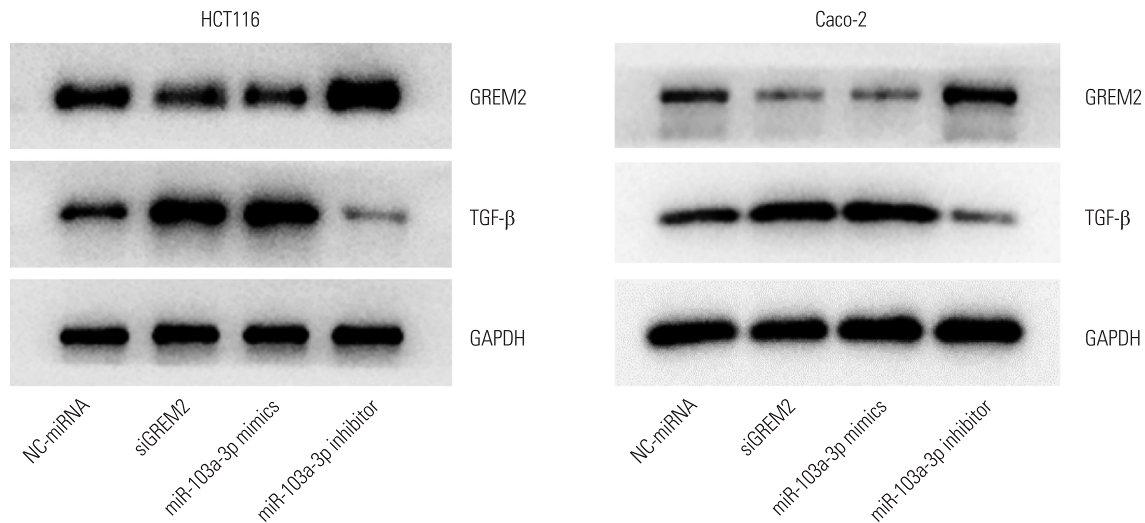

Fig. 6. Western blot analysis of TGF-β expression. TGF-β, transforming growth factor-β.

research demonstrated miR-103a-3p inhibitor advanced cell apoptosis of HCT116 and Caco-2. Interestingly, we confirmed that the apoptotic proteins Bcl-2 and Bax were modulated by miR-103a-3p inhibitor, and this is consistent with reports by Zhang, et al.[30] Highly expressed miR-103a-3p in CRC cells promotes the expression of HIF1A by targeting the core molecules LATS2 and SAV1 of the Hippo/YAP1 pathway and also improves CRC proliferation, invasion, migration, glycolysis, and angiogenesis.[31] However, GREM2 knockdown eliminated this effect. Related reports have indicated that GREM2 inhibits the enhancement of drug resistance in prostate cancer cells by regulating TGF-β pathway.[32] The downregulation of miR-103a-3p inhibits proliferation and apoptosis of HCT116 and Caco-2 by targeting GREM2 to TGF-β pathway was also indicated by our research.

In summary, miR-103a-3p was confirmed to be highly expressed in CRC cells (HCT116 and Caco-2 cell lines). In-depth investigation has revealed that in CRC cells, miR-103a-3p downregulation inhibits proliferation through G1/S transition and accelerates apoptosis by targeting GREM2 through TGF-β pathway. Our research might offer a basic mechanism for CRC progression and provide insights that miR-103a-3p/GREM2 is possibly a novel target for CRC therapy.

AVAILABILITY OF DATA AND MATERIAL

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization: Zongxiang Zhang. Data curation: Zongxiang Zhang and Xiaolian Zhu. Formal analysis: Zongxiang Zhang. Funding acquisition: Zongxiang Zhang. Investigation: Xiaolian Zhu. Methodology: Zongxiang Zhang and Xiaolian Zhu. Project administration: Xiaolian Zhu. Resources: Xiaolian Zhu. Software: Zongxiang Zhang and Xiaolian Zhu. Supervision: Xiaolian Zhu. Validation: Zongxiang Zhang. Visualization: Xiaolian Zhu. Writing—original draft: Zongxiang Zhang. Writing—review & editing: Zongxiang Zhang and Xiaolian Zhu. Approval of final manuscript: all authors.

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