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MicroRNA-383 acts as a tumor suppressor in colorectal cancer by modulating CREPT/RPRD1B expression

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Abstract

CREPT (Cell-cycle-related and expression-elevated protein in tumor)/RPRD1B, a novel protein that enhances the transcription of Cyclin D1 to promote cell proliferation during tumorigenesis, was demonstrated highly expressed in most of tumors. However, it remains unclear how CREPT is regulated in colorectal cancers. In this study, we report that miR-383 negatively regulates CREPT expression. We observed that CREPT was up-regulated but the expression of miR-383 was down regulated in both colon cancer cell lines and colon tumor tissues. Intriguingly, we found that enforced expression of miR-383 inhibited the expression of CREPT at both the mRNA and protein level. Using a luciferase reporter, we showed that miR-383 targeted the 3'-UTR of CREPT mRNA directly. Consistently we observed that over expression of miR-383 shortened the half-life of CREPT mRNA in varieties of colorectal cancer cells. Furthermore, restoration of miR-383 inhibited cell growth and colony formation of colon cancer cells accompanied by inhibition of expression of CREPT and related downstream genes. Finally, we demonstrated that stable over expression of miR-383 in colon cancer cells decreased the growth of the tumors. Our results revealed that the abundant expression of CREPT in colorectal cancers is attributed to the decreased level of miR-383. This study shed a new light on the potential therapeutic therapy strategy for colorectal cancers using introduced miRNA.

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CONFLICTS OF INTEREST

The authors have no conflict of interest to disclose.

Keywords

CREPT; colorectal cancer; miR-383; tumor growth; colony formation

1. INTRODUCTION

We have reported that CREPT (Cell-cycle-related and expression-elevated protein in tumor, Gene ID: 58490, also named RPRD1B or C20ORF77) is highly expressed in many tumors.¹ CREPT family proteins include p15RS (also named RPRD1A) in mammals and Rtt103 in yeast.¹⁻³ We proposed that CREPT participated in the transcription regulation of *CCND1* to promote cell cycle progress,¹ while p15RS inhibits the cell proliferation by inhibiting Wnt signaling.^{3,4} Our previous observations showed that CREPT bound to both the promoter region and the region before the Poly-A signal in the termination region of the *CCND1* gene.¹ We discovered that CREPT mediated chromatin loop formation for the *CCND1* gene, which may facilitate the recycling of RNAP II during the transcription of genes in mammalian cells.^{1,5} Recently, our lab revealed that CREPT participated in the transcription of Wnt/ β -catenin signal target genes through the β -catenin and TCF4 complex.⁶ We found that CREPT interacted with both β -catenin and TCF4, and enhanced the association of β -catenin with TCF4, resulting in activation of Wnt signaling, which further promotes tumor cell growth.⁶ Consistent with our findings, Jung et al⁷ reported that *CREPT*, together with seven other genes, was significantly elevated in SV40-immortalized cells and NSCLC tissues. Additionally, Wang et al⁸ reported that CREPT was significantly up-regulated in endometrial cancer tissues and promoted tumor growth by accelerating cell cycle. Using the monoclonal antibody against CREPT,⁹ She et al¹⁰ reported that high expression of CREPT is correlated with poor prognosis in retroperitoneal leiomyosarcoma, similar to our previous observations that CREPT is related to prognosis in gastric cancers.¹ CREPT is also reported to be involved in the DNA damage repairing, indicating a role in regulation of genome stability.^{11,12} Together, accumulating evidence suggests that CREPT plays an important role in tumorigenesis by elevated expression. However, the molecular mechanisms responsible for the regulation of CREPT expression remain unclear.

MicroRNAs (miRNAs) are short endogenous non-coding RNAs that are involved in many cellular functions including regulation of cell proliferation, apoptosis, differentiation, and metabolism.^{13,14} miRNAs regulate gene expression by binding to complementary sequences, preferentially within the 3'-untranslated region (3'-UTR) of target messenger RNA (mRNA), to inhibit translation by promoting RNA degradation or destabilization.¹⁵⁻¹⁷ Usually, miRNAs target mRNAs with an average 3' length of 1600 nucleotides but rarely target mRNA with a 3' containing less than 1000 nucleotides.¹⁸

Our preliminary analysis indicated that CREPT mRNA contains a long 3' (2486 nucleotides), implying a post-transcriptional regulation by miRNAs. In this study, we found that miR-383 directly targets the 3'-UTR of CREPT mRNA. We observed an inverse correlation between the expressions of miR-383 and CREPT in colorectal tumor cell lines and human colorectal tumor tissues. Our study illuminates a novel mechanism of CREPT expression regulation in colorectal cancers.

2. MATERIALS AND METHODS

2.1 Cell culture

CCD-841 CoN (normal colon epithelial), 293T, and human colon cancer cell lines SW-480, HCT-116, DLD-1, HT-29 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and passaged according to the ATCC protocol. HCT116 β /W cell line is a derivative from wild type HCT-116 cell by introduction of a heterozygous mutant β -catenin in HCT-116.¹⁹ Human colon carcinoma cell lines KM12C, KM12SM, and KM12L4a were kindly provided by Prof. X.F. Sun (Linköping University, Linköping, Sweden). The cell line KM12C derived from a patient with stage II colon cancer. The cell line KM12SM was derived from a spontaneous liver metastatic lesion arisen from the injection of KM12C into the cecum of nude mice. KM12L4a was produced by repeated intraspleen injection and harvesting of the liver metastases in nude mice.^{20,21} All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U of penicillin-streptomycin in a humidified atmosphere of 95% air and 5% CO₂ incubator at 37°C.

2.2. Patients and tissue samples

A total of 18 frozen primary tumor samples and their corresponding adjacent non-tumor tissues were obtained from colon cancer patients at the First Hospital in Qinhuangdao, China. Patient consent and approval by the local ethics committee was obtained. Tissue samples were collected during surgery, immediately frozen in liquid nitrogen or fixed in neutral formalin buffer solution for histological detection (Patient and tumor characteristics are described in Supplementary Table S1).

2.3. miR-383 mimic transfection

KM12SM and HCT116 β /W cells were transfected with miR-383 mimics, miR-383 inhibitor, or a miR-383 mimics control (negative control, NC) (Thermo Fisher Scientific, Waltham, MA) using Lipofect-amine RNAi-MAX Reagent (13778, Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Cells were collected for further assays 24 or 48 h after transfection.

2.4. Reporter plasmids and the luciferase reporter assay

To construct a luciferase reporter plasmid, the wildtype 3'-UTR of CREPT, including the predictive miR-383 binding site, was amplified using primers (Supplementary Table S2) and cloned into a psiCHECK2.0 Dual-Luciferase Reporter system (C8021, Promega, Madison, WI). To generate sensor vectors with mutations (psiCHECK2.0-CREPT-3'-MUT) or a deletion (psiCHECK2.0-CREPT-3'-DEL) in the predicted miR-383 binding site within CREPT 3'-UTR, we used primers outlined in Supplementary Table S2 by Phusion High-Fidelity DNA Polymerase (M0530L, New England Biolabs, Ipswich, UK). The sensor vectors with mutation or deletion were submitted to Majorbio Bio-Pharm Technology Co., Ltd (Shanghai, China) for DNA sequencing. For luciferase reporter assays, HCT116 β /W cells, or KM12SM cells were seeded in a 24-well plate and then co-transfected with the psiCHECK2.0-CREPT-3'-WT (or MUT, DEL) construct and miR-383 mimic or a negative

control. Firefly and Renilla luciferase activities were quantified 48 h after transfection using the Dual-Luciferase Reporter Assay System (E1980, Promega, Madison, WI) and Renilla luciferase activity was normalized to Firefly luciferase activity. Luciferase assays were repeated three times in independent experiments.

To determine whether miR-383 has any effect on Wnt/ β -catenin signaling pathway, KM12SM, or HCT116 β /W cells were transiently transfected with the indicated plasmids using Vigofect (T001, Vigofect Inc. Beijing, China) according to the manufacturer's instructions. In summary, 0.1 μ g of reporter plasmid super Top-luc reporter (104802, Biovector Inc., Beijing, China) and 5 ng of internal control plasmid pRL-TK (VQP0126, Promega) were transfected into the cells cultured in a 24-well plate. For the expression of TCF4, miR-383 mimic, NC mimic, or miR-383 inhibitor was co-transfected into the cells. An empty plasmid was used to balance the total amount of DNA. Luciferase activity was assayed after 24 h of transfection using a Dual-Luciferase reporter assay system (E1980, Promega). The luciferase activity was normalized by Firefly against Renilla luciferase activity and presented as mean $\pm$ standard deviation (SD).

2.5. RNA extraction and quantitative real-time PCR

For miRNA quantification, total RNAs including microRNAs were extracted from culture cells using EasyPure miRNA Kit (ER601-01, Transgen, Beijing, China) according to the manufacturer's instruction, and then cDNA was synthesized from 5 ng of total RNA using the miRNA First-Strand cDNA Synthesis SuperMix (AT301, Transgen, Beijing, China). The expression levels of miR-383 were quantified using TransScript Green miRNA Two-Step qRT-PCR SuperMix (AQ202-01, Transgen, Beijing, China). The relative miR-383 expression levels after normalization to U6 were calculated. The primers are shown in Supplemental Table S2. For CREPT, CyclinD1, CDK4, and CDK6 mRNA quantifications, total RNA was isolated using an RNAiso Plus (D9108, TAKARA, Dalian, China) according to the manufacturer's protocol, and 500 ng RNA was used for reverse transcription using PrimeScript1 RT Master Mix (RR036A, TAKARA, Dalian, China). Quantitative RT-PCR (qRT-PCR) assays included 0.4 mM each primer and 12.5 ng cDNA in SYBR Premix ExTaqTM II Tli RnaseH Plus (RR820A, TAKARA, Dalian, China). PCR reactions were performed on an ABI StepOne Plus (Applied Biosystems, MA). Quantification of gene expression was based on the Ct method and was normalized to GAPDH gene levels. The primers are shown in Supplemental Table S2. Melting curve analysis were performed to control PCR product specificities and to exclude nonspecific amplification.

2.6. Western blot analysis

Western blot analysis was performed with a standard method.⁶ Cell pellets were washed in 500 μ L of PBS and centrifuged for 5 min at 12 000 g. Protein extracts (35 ng) prepared with lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. After blocking with 5% non-fat milk in PBS-Tween, the membranes were probed with the anti-CREPT (PA5-26915, Invitrogen), anti-CyclinD1 (Sc-20044, Santa Cruz Biotechnology, Santa Cruz, CA), anti-CDK4(2906, Cell Signaling, Boston, MA), and anti-CDK6 (13331, Cell Signaling) antibodies at the appropriate dilution in PBS-Tween overnight at 4°C. After

being completely washed for three times in PBS-Tween, the membranes were probed for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (31430, 31460, Thermo Fisher Scientific) at the appropriate dilution in PBS-Tween. Finally enhanced chemiluminescent visualization (ECL) system (NCI 5080, Pierce Biotechnology, Rockford, IL) was applied to the membrane to detect proteins after being washed three times again in PBS-Tween.²²

2.7. Cell growth assay

For cell growth assays, KM12SM cells in 6-well plate were transfected with miR-383 mimic, negative control (NC) mimic, and CREPT siRNA, respectively, and further re-seeded in a 24-well plate 24 h later. To verify miR-383 inhibit the cell growth mainly by targeting CREPT 3'UTR, we further co-transfected miR-383 and pcDNA3.1-CREPT without 3'UTR sequence, NC, and pcDNA3.1-CREPT, respectively. Cells were collected every day for 4 days and counted using a hemocytometer after trypan blue staining as previously reported.²³ Cell viability was determined using a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously.²⁴ In brief, cells were transfected with miR-383 mimic, NC mimic, CREPT siRNA, miR-383 together with pcDNA3.1-CREPT and NC together with pcDNA3.1, respectively, in a 6-well plate 24 h later, cells were re-seeded into a 96-well plate in triplicate. After 4–6 days (or until ~90% confluency of NC treatment group), cell medium was replaced with WST-8 (Sigma-Aldrich, Carlsbad, CA) dye for 1–5 h. The absorbance was quantified using a microplate reader at 450 nm.

2.8. Colony formation assay

Colony formation assays were performed using KM12SM cells transfected with NC mimic, miR-383 mimic, or siRNA CREPT in a 6-well plate in triplicate (4×10^2 cells/well). The cells were incubated for 2 weeks and stained with crystal violet. Colonies containing 50 cells or more were counted.²⁵

2.9. In vivo tumor growth assays

BALB/c-nu nude mice (aged 4 weeks) were purchased from Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Yanshan University. Mice that were housed under identical conditions were allowed free access to a standard diet and tap water and exposed to a 12 h light: 12 h dark cycle. The nude mice were injected s.c. with 5×10^6 KM12SM cells stably transfected pcDNA 3.1, pcDNA3.1-pre-miR-383, or pcDNA-miR-383-sponge, respectively, on the back of mice. Tumor growth was measured using calipers every two days. The tumor volume was calculated as $\text{Tumor Volume} = (\text{length} \times \text{width}^2)/2$ as was used previously.²⁴

2.10. Statistics

All results in this study were statistically presented as mean $\pm$ standard errors. Two-way ANOVA and chi-square test was used to analyze the significance of the cell growth curve and xenografts tumor growth studies. $P < 0.05$ was considered to be statistically significant by Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

RESULTS

3.1. CREPT is abundantly expressed in colon cancer cell lines and clinical samples

Our previous studies demonstrated that CREPT is highly expressed in many cancers as compared to that in adjacent non-tumor tissues.¹ In this study, we show that the mRNA level of CREPT is abundantly expressed in a panel of colorectal cancer cells while hardly detected in a normal colon epithelial cell line, CCD841 (Figure 1A). Similarly, the protein level of CREPT in the colorectal cancer cells remains at high level while no protein is shown in the normal cell (Figure 1B). In order to understand the clinical relevance in colorectal cancer, we analyzed the expression of CREPT mRNA in the tumor and adjacent normal tissues from 18 pairs of patient samples. The results showed that CREPT mRNA was elevated in 13 patient samples (72%) of colorectal tumors as compared to adjacent non-tumor tissues (Figure 1C). This is consistent with our previous observation that CREPT protein expression was highly expressed in 75% of patient colon cancer tissue samples.¹ All the results suggest that CREPT is abundantly expressed in colorectal cancers.

3.2. Identification of CREPT-targeting miRNAs

To elucidate the molecular mechanism for the overexpression of CREPT in colorectal cancers, we determined to analyze how the CREPT mRNA is up-regulated. As high level of mRNA may be a product from accelerated transcription and increased stability of mRNA, we were in particular interested in whether CREPT mRNA is stabilized in tumors in this study. Interestingly, our preliminary analysis demonstrated that CREPT has a long 3'-untranslated region (UTR) of 2486 nucleotides, suggesting a possible post-transcriptional regulation by microRNAs (miRNAs). Therefore, we screened potential miRNA binding sites within the 3'-UTR of CREPT using two miRNA targeting prediction programs (miRanda, TargetScan). Intriguingly, seven overlapping miRNAs containing highly conserved putative binding sites within CREPT 3'-UTR were identified as miR-182, miR-383, miR-194, miR-192, miR-215, miR-300, and miR-381 (Figure 2A). We then examined the effect of these miRNAs on the CREPT expression and found that miR-383 has the strongest inhibitory effect on the CREPT protein (Figure 2B). We finally choose to focus on miR-383 for its potential ability in the regulation of CREPT expression in colorectal cancers.

In order to determine the role of miR-383 on the protein expression of CREPT, we transfected miR-383 in both KM12SM and HCT116 β /W cells. The results showed that miR-383 inhibited CREPT expression in a dose and time dependent manner (Figure 2C). In particular, in a 72 h transfection, higher amounts of miR-383 showed almost similar effect as siRNA on the inhibition of CREPT expression (Figure 2C, comparing line 12 to 9). Consistently, we observed that the mRNA level of CREPT was decreased by transfection of miR-383 for 24 h (Figure 2D). These results suggest that miR-383 is a negative regulator for CREPT expression.

To examine the level of miR-383 in the colorectal cancer cell lines where CREPT was abundantly expressed, we performed a qRT-PCR analysis. The results showed that miR-383 level was dramatically decreased in all the cancer cell lines in comparison with the normal epithelial cell CCD841 (Figure 2E). Furthermore, we observed that miR-383 mRNA

expression was decreased in the tumor tissues in 12 colorectal cancer patients comparing with that in the adjacent non-tumor tissues (Figure 2F). Interestingly, we also observed that miR-383 level was increased in four tumors. Overall, we performed an analysis on the correlation of CREPT and miR-383 expression in tumor patients and obtained a very strong reversed correlation ($P < 0.05$) (Figure 2G). All these results suggest that the expression of miR-383 is negatively correlated to the expression of CREPT in colorectal cancer cells and tissues, implying a negative regulation mechanism of CREPT expression by miR-383.

3.3. miR-383 regulates the expression of CREPT directly

To validate whether CREPT is a direct target of miR-383, we engineered a luciferase reporter containing the 3'-UTR of the CREPT mRNA. As controls, we generated luciferase reporters with a mutation or deletion within the predicted miR-383 binding site in the CREPT mRNA 3'-UTR (Figure 3A). The KM12SM and HCT116 β /W cells were transiently transfected with the CREPT 3'-UTR-reporters along with miR-383 or its mimics (negative control) for a luciferase assay. The results showed that transfection of different dosages of miR-383 led to a significant decrease in the reporter activity in comparison with the maximal amount of mimics in both KM12SM and HCT116 β /W cells (Figure 3B). At the same time, we observed that transfection of miR383 failed to inhibit the luciferase activity in the mutated reporters where miR383 binding site was mutated or deleted (Figure 3C). These results suggest that miR-383 inhibits the luciferase activity via a direct interaction with the CREPT 3'-UTR.

To reveal whether miR383 plays a role on the mRNA stability, we examined the half-life of CREPT mRNA. The results showed that ectopic miR-383 overexpression dramatically decreased the half-life of CREPT mRNA, while an inhibitor against miR-383 slightly prolonged the half-life (Figure 3D).

To further verify the effect of miR383 on the endogenous CREPT expression, we transfected miR-383 with or without a CMV-driven cDNA CREPT expression vector where no 3'-UTR exists. A Western blot analysis demonstrated that miR-383 inhibits the endogenous expression of CREPT, but fails to inhibit the exogenous CREPT expression (Figures 3E and 3F). Taken together, our results suggest that miR-383 negatively regulates CREPT expression directly by binding to its mRNA 3'-UTR.

3.4 Overexpression of miR-383 inhibits the CREPT-mediated transcriptional activity and Wnt signaling

As miR-383 targets CREPT for its expression, we reasoned that miR-383 might be able to regulate the expression of genes downstream of CREPT. As our previous study revealed that CyclinD1, CDK6, and CDK4 were up-regulated by CREPT,^{1,26} we determined to examine whether miR-383 functions on the regulation of these genes driving colorectal cancer cell proliferation. To test this hypothesis, we performed Western blot and qRT-PCR experiments to measure CyclinD1, CDK6, and CDK4 expression in miR-383 transfected KM12SM cell lines. The results showed that exogenous expression of miR-383 down regulated mRNA levels of CyclinD1 (Figure 4A), CDK6 (Figure 4B), and CDK4 (Figure 4C). It appeared that high dosage of miR383 reached the similar inhibitory effect on the mRNAs of these three

genes as the siRNA against CREPT did (Figure 4A–C comparing last column to the second). Simultaneously, protein levels of CyclinD1, CDK6, and CDK4 were decreased by over-expression of miR-383 (Figure 4D). These results suggest that miR-383 modulates the expression of CyclinD1, CDK6, and CDK4 through inhibition of CREPT expression.

As our previous study showed that CREPT enhances the activity of the β -catenin/TCF4 complex to initiate transcription of Wnt target genes,⁶ we next determined to examine whether miR-383 regulates the Wnt signaling pathway. For this purpose, we transfected miR-383 with a Wnt-responsive luciferase reporter, superTOP-luciferase, into both KM12SM and HCT116 β /W cell lines, where endogenous CREPT expressed was high (see Figure 1). A luciferase report experiment result demonstrated that transfection of miR-383 significantly decreased the luciferase activity either at the basal level or under the stimulation of Wnt conditional medium in both KM12SM and HCT116 β /W cells (Figure 4E). As a control, an inhibitor of miR-383 failed to decrease the luciferase activity (Figure 4E, last columns in each group). These results suggest that miR-383 inhibits Wnt signaling. Furthermore, we transfected constitutively active β -catenin with the reporter into the cells that presence of a vector-based CREPT vector, which exits no miR-383 targeted sequence. A luciferase reporter experiment showed the over-expression of miR-383 failed to inhibit the luciferase activity (Figure 4F). This result indicate that miR-383 inhibits Wnt/ β -catenin signaling pathway, by negatively regulating the expression of CREPT.

3.5. miR-383 induces growth inhibition in colon cancer cells

We next investigated whether miR-383 could suppress the growth of colon cancer cells by negatively regulating CREPT expression and whether its inhibition effect was mainly mediated by targeting CREPT mRNA 3'UTR sequence. To this end, we intended to compare the effect of miR-383 on the proliferation of KM12SM cells with the depletion of CREPT. An MTT experiment showed that the number of viable cells in miR-383 transfected cells was significantly decreased in comparison with that of NC mimic cells (Figure 5A, column 2 vs 1).Correlatively, the inhibitory effect of miR-383 was similar to that from a siRNA against CREPT (Figure 5A, column 3 vs 2). While when CREPT was overexpressed in cells by vector-based CREPT transfection (pcDNA3.1-CREPT, which has no 3'UTR sequence in the vector), the proliferation inhibitory effect of miR-383 was cancelled for there is no binding site of miR-383 on the vector-based CREPT mRNA. A quantitative cell proliferation experiment indicated KM12SM cells transfected with miR-383 or siRNA against CREPT grew significantly less than the mock cells transfected with a non-specific NC mimic ($P < 0.001$), and the growth inhibition effect of miR-383 was rescued by the vector based over expression of CREPT in cells (Figures 5B and 5C). In a colony formation assay, we observed that KM12SM cells transfected with miR-383 formed as much less colonies as those transfected with an siRNA against CREPT (Figures 5D and 5E). These results suggest that miR383 inhibits cell proliferation and colony formation; similar to that from depletion of CREPT, implying that the inhibition effect of miR-383 might be through, at least in part, by negatively regulating CREPT expression.

We further examined the effect of miR-383 on the cell cycle in colon cancer cells. A flow cytometry analysis showed that the percentage of G1 phase cells dramatically increased in

the miR-383 transfected cells ($67.862 \pm 4.12\%$) (Figure 5F2), higher than that in NC mimic transfected cells ($48.73 \pm 5.47\%$) (Figure 5F1). Inhibition of miR383 with its inhibitor mimic almost abolished the role on the increased G1 phase cells ($54.76 \pm 6.17\%$) (Figure 5F3). As a positive control, the percentage of G1 phase from cells transfected with an siRNA against CREPT was increased to $70.32 \pm 4.17\%$ (Figure 5F4), similar to the effect of miR-383 transfection. A quantitative presentation of the cell cycle changes in G1, S, and G2/M phases showed consistent results (Figure 5G). All of the results demonstrated that the overexpression of miR-383 prolonged the G1 phase and prevented cells from entering the S phase. This effect of miR-383 appears consistent with that from depletion of CREPT, implying a role of miR-383 through CREPT.

3.6. miR-383 inhibits tumorigenesis in vivo

To explore the potential role of miR-383 during tumorigenesis, we observed the growth process of a xenograft colon cancer. Injection of KM12SM cells with stably overexpressed pre-miR-383 resulted in a dramatic reduction in tumor size compared to mock cells where only empty vector was transfected (Figure 6A, blue line). Interestingly, the tumor size was increased in KM12SM cells with stably overexpressed miR-383 sponge (Figure 6A, red line), suggesting that inhibition of miR-383 accelerates the tumorigenesis. Our experiment showed that the tumor size from miR-383 stable expression cells was much smaller than the control tumors (Figure 6B), while the bodyweights of the mice among the groups remained unchanged (data not shown). Finally, we examined CREPT levels in the xenograft tumors from cells with transfection of miR-383. A Western blot analysis showed that CREPT protein level was significantly decreased in the tumors from pre-miR383-stably transfected cells (Figure 6C, middle lane and Figure 6D, column 2 vs 1), but increased in the tumors from cells with stable transfection of miR-383-sponge (Figure 6C, last lane and Figure 6D, column 3 vs 1). The results suggest the miR-383 inhibited the expression of CREPT during the tumorigenesis in the xenograft model. Furthermore, a real-time PCR analysis indicated that the mRNA level of CREPT was slightly decreased in the xenograft tumor from overexpression of pre-miR383 (Figure 6E). The decreased fold change of CREPT mRNA levels was less than that from the protein levels in the pre-miR-383 stable tumors (Figure 6D), implying that the role of miR-383 on the expression of CREPT might mainly occurs at the translation process. This echoes our observation that miR-383 binds to the 3'-UTR region of CREPT mRNA. Collectively, all the data suggest that miR-383 acts as a tumor suppressor by down-regulating the expression of CREPT during tumorigenesis.

4 DISCUSSION

In our previous study, we found that CREPT is preferentially highly expressed in diverse human tumors and the overexpression of CREPT accelerates tumor growth.¹ Other reports have focused on the function of CREPT, its downstream target genes and the mechanism responsible for CREPT mediated tumor progression.^{1,7,8,10} However, how CREPT expression during the tumorigenesis is regulated remained elusive. In this study, we found that CREPT mRNA has a long 3', accounting for about 64% of the whole length of mRNA. We speculated that this long 3' sequence provides a chance for the regulation of the expression after transcription. Indeed, we revealed that miR-383 binds to the 3' sequence to

down regulate mRNA stability and its translation, through its partial complementary binding, as occurs in most cases.^{27,28} We have provided strong evidence that miR-383 inhibits the expression of CREPT, in particular during the tumorigenesis. This mechanism of the regulation of gene expression has been reported widely on the regulation of tumorigenesis for colorectal cancers.^{29,30,31} Our study revealed a novel set of counterpart regulators where decreased expression of miR-383 attributes, at least in part, to the elevated expression of CREPT in tumors. Yet, we could not exclude the possibility that CREPT might be regulated at the transcriptional level during the tumorigenesis. Nevertheless, this study is the first report to demonstrate the regulation of CREPT expression in tumors.

Another issue in this study is that miR-383 may function on the regulation of tumorigenesis independent of CREPT. In our study, we found that miR383 is one of the miRNAs targeting CREPT mRNA. Although we did not examine the role of other miRNAs on the regulation of CREPT expression, this study provided clear evidence that miR-383 is a major one to down regulate the expression of CREPT at the post-transcriptional level. Indeed, our data showed that over-expression of miR-383 led to inhibition of the cell proliferation, colony formation, and cell cycle (Figure 5), as well as the downstream gene expression (Figure 4A–C), equivalent to the effect of CREPT depletion by as siRNA. While when we overexpressed CREPT by transfected vector-based CREPT, the proliferation inhibition effect of miR-383 was rescued for the 3'UTR sequence absence on the CREPT vector plasmid. These results suggest that miR-383 functions mainly on CREPT, or CREPT related features of tumorigenesis.

miR-383 has been reported in other human cancers. In testicular germ cell tumor, miR-383 was found as a negative regulator to directly target interferon regulatory factor-1.³² In medulloblastoma, miR-383 is down regulated and acts as a regulator controlling cell growth through targeting PRDX3.³³ miR-383 represses proliferation, migration, and angiogenesis of glioma cells via VEGF-mediated FAK and Src signaling pathway. It also targeted on AKT3 and further exerted its anti-proliferation, migration, and invasion by AKT pathway in thyroid cancer.³⁴ ROBO3 is a direct target of miR383, and Han et al³⁵ have found that a binding of ROBO3 to SFRP releases the Wnt/ β -catenin from its inhibition, resulting in increased Wnt/ β -catenin activity. While the inhibition of ROBO3 by miR-383 would restore SFRP's inhibition of the Wnt/ β -catenin pathway. Interestingly, the highest expression level of miR-383 was observed in all normal brain tissues,³⁶ suggesting its important role in maintaining brain tissue hemostasis. Consistent with our observation in this study, others reported that miR-383 directly targeted and negatively regulated the cell cycle-associated oncogene CCND1 in glioma cells.³⁷ Our data showed that miR-383 regulates the expression of CCND1 in response to Wnt signaling. We speculate that miR-383 on the glioma may also through CREPT expression, which eventually regulated the expression of CCND1 as reported previously.^{1,6} In this study, our results indicate that miR-383 inhibits Wnt/ β -catenin signaling pathway, by negatively regulating the expression of CREPT and further inhibit the proliferation, colony formation, and tumorigenesis. We speculated that miR-383 could exert its anti-proliferative effects by multiple signaling pathways through targeting multiple proteins in colorectal cancer. Further studies are needed to confirm this speculation.

In conclusion, we revealed that miR-383 mediates binds to the 3'UTR of CREPT mRNA, leading to the unstable maintenance and impaired translation. In such a way, miR383 functions as a negative regulator to guard the cell with a low level of CREPT protein. Eventually, high level of miR-383 overcomes the oncogenic protein CREPT and prevent the cells from becoming tumor cells. As most of the tumor is induced by over-activation of Wnt signaling, we propose that the miR-383, by inhibition of CREPT expression, can block the Wnt signaling, at least in part, to decrease the expression of tumor related genes including CyclinD1, CDK4, and CDK6 (Figure 7). Our study provided a clue for diagnosis/prognosis of colorectal cancers using decreased level of miR-383.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

CREPT is over-expressed in colon cancer cell lines and clinical samples. A, qPCR showed that CREPT mRNA in a panel of colon cancer cell lines was significantly higher than in the normal colon epithelial cell line, CCD841. B, The protein level of CREPT in the colorectal cancer cells remains at high level but no protein is detected in the normal cell, CCD841. C, qPCR revealed that CREPT mRNA expression was significantly higher in 13 colon cancer tissues than in adjacent non-tumor tissues in 18 pairs of clinical samples. The results are expressed as means of three independent experiments $\pm$ SD

FIGURE 2.

CREPT expression is regulated by miRNAs. A, A Venn diagram showed highly conserved miRNAs targeting CREPT mRNA predicted by using TargetScan and miRanda softwares. B, A quantitative presentation for Western blotting analysis showed that miR-383 has the strongest inhibitory effect on the CREPT protein expression levels in both KM12SM cell line and HCT116β/W cell line. C, A Western blotting analysis showed that transient transfection of miR-383 mimic down-regulated CREPT protein expression. Endogenous CREPT protein levels were examined after the transfection of different dosages of miR-383

mimic in 48 or 72 h. NT, none treated cells; NC, negative control cells transfected with a non-related siRNA. A mixture of two siRNAs against CREPT was used as a positive control (indicated as siRNA). D, qRT-PCR results showed that transient transfection of miR-383 mimic inhibited the CREPT mRNA expression in KM12SM cell line. Different amounts of miR-383 were used for the transfection. E, A Taqman microRNA analysis demonstrated that mature miR-383 mRNA level was significantly decreased in a panel of colon cancer cell lines compared with that in CCD841 cell, a non-cancer cell line. F, qRT-PCR results showed that miR-383 mRNA was down-regulated in 12 of 18 colon cancer tissues compared with the paired adjacent-non-tumor tissues. Note that no significant difference between tumor tissues and paired non-tumor tissues was observed in 2 pairs of samples, and 4 pairs of samples showed up-regulated miR-383 in colon cancer tissues. The results are presented as means of three independent experiments $\pm$ SD. G, A correlation analysis revealed that CREPT and miR-383 expression in tumor patients appeared a very strong reversed correlation ($R = -0.495$, $P < 0.05$). A Chi-square analysis was performed. The percentage represented the population of the samples in the group

FIGURE 3.

miR383 regulates CREPT expression directly. A, Constructions of psiCHECK-2.0 that contains CREPT-3'UTR (WT), CREPT-3'UTR mutant (MUT, three mutated nucleotides in predicted miR-383 targeting sequence), and CREPT-3'UTR deletion (DEL, the whole predicted miR-383 targeting sequence was deleted). Using the Not I and Xho I restriction sites, CREPT 3'UTR was inserted into after the translation stop codon of Renilla luciferase gene (hRluc), followed by a poly A sequence. Note that hLuc driven by HSV-TK promoter was linked directly for the use of normalization of transfection efficiency. B, Luciferase reporter assays showed that miR-383 mimics inhibited the reporter activity of psiCHECK2.0-CREPT-3'UTR significantly in a dose dependent manner in different cell lines. NC, a base reporter activity normalized as 1.0. C, miR-383 mimic down-regulated luciferase activities from psiCHECK2.0-CREPT-3'UTR WT, but did not affect luciferase activity from psiCHECK2.0-CREPT-3'UTR MUT or DEL in both KM12SM and HCT-116

β/W cell lines. D, miR-383 mimic decreased the half-life of the CREPT mRNA. The half-life of the CREPT mRNA was calculated based on the regression of the level to the time after transfection. Note that miR-383 inhibitor (miR-383 Inh) prolonged the half-life time of CREPT mRNA. E, A Western blotting analysis showed that miR-383 mimic inhibited the endogenous CREPT expression, but not on exogenous CREPT expression. F, A quantitative presentation for the inhibition effect of miR383 on endogenous CREPT expression. All the results were presented as means of three independent experiments ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

FIGURE 4.

Overexpression of miR-383 inhibits CREPT-mediated transcription and Wnt signaling. A, Exogenous expression of miR-383 downregulated the expression level of CyclinD1 mRNA in a dose dependent manner. The level of CyclinD1 mRNA was examined by RT-PCR from cells transfected with indicated plasmids. siRNA against CREPT was used as a positive control. B, Exogenous expression of miR-383 downregulated the expression levels of CDK6 in mRNA level in a dose dependent manner. The level of CDK6 mRNA was examined by RT-PCR from cells transfected with indicated plasmids. C, Exogenous expression of miR-383 downregulated the expression levels of CDK4 in mRNA level in a dose dependent manner. D, Exogenous expression of miR-383 downregulated the expression levels of CyclinD1, CDK6, and CDK4 at the protein level. Western blots were performed for the lysates of cells transfected with the indicated plasmids. NT, none treated cells; NC, negative control cells transfected with a non-related siRNA. A mixture of two siRNAs against

CREPT was used as a positive control (indicated as siRNA). E, Luciferase assays demonstrated that miR-383 significantly reduced Wnt signaling in both KM12SM and HCT116 β /W cells where endogenous CREPT with miR-383 targeted sequence at its 3'UTR is highly expressed (see Figure 1). A specific luciferase reporter in response to Wnt signal was used. Note that miR-383 inhibitor (383 Inh) failed to inhibit the reporter activity. F, Over-expression of miR-383 failed to inhibit the luciferase activity mediated by a vector-based CREPT, which had no miR-383 targeted sequence at the 3'-UTR. All the results were presented as means of three independent experiments $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

FIGURE 5.

miR-383 induces growth inhibition in colon cancer cells. A, MTT assays revealed that exogenous expression of miR-383 suppressed proliferation of KM12SM cells and the proliferation inhibition effect of miR-383 was cancelled by the overexpression of the vector-based CREPT, which had no miR-383 targeted sequence at the 3'-UTR. A mixture of two siRNAs against CREPT was used as a control. B, Cell growth curves showed that exogenous expression of miR-383 suppressed growth of KM12SM cells and the growth suppression effect of miR-383 was cancelled by the overexpression of vector-based CREPT in cells. C,

A quantitative presentation for the inhibition effect of miR383 on the cell growth of KM12SM cell line and the abolishment of the inhibition effect of miR-383 on the vector-based CREPT overexpression cell line. D, Representative micrographs of crystal violet-stained cell colonies. E, A quantitative presentation of the inhibition effect of miR-383 on the colony formation of KM12SM cells. F, Cell cycle analyses were performed by cell flow cytometry. Note that miR-383 arrested the cell cycle in the G0/G1 phase. Representative cell cycle diagrams of KM12SM cells transfected with NC mimic (F1); miR-383 mimic (F2); miR-383 inhibitor mimic (F3); and CREPT siRNA (F4). G, A quantitative presentation of the cell cycle changes in G1, S, G2/M phases. All the results are presented as means of three independent experiments $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

FIGURE 6.

miR-383 inhibits tumorigenesis in vivo. A, The growth curves of KM12SM cells which were stably overexpressed with miR-383, miR-383 sponges. The tumors were formed by the cells in BALB/c-nu nude mice. B, The average tumor size at the end of the experiment at Day 32. C, A Western-blotting analysis for the CREPT protein expression in KM12SM xenografts stably expressing pre-miR-383, miR-383 sponge, or blank vector, respectively. D, A quantitative presentation of the CREPT protein expression in KM12SM xenografts stably expressed pre-miR-383, miR-383 sponge or blank vector, respectively. E, The expression of CREPT and mature miR-383 mRNA in KM12SM xenografts stably expressing pre-miR-383, miR-383 sponge, or blank vector, respectively. The results are presented as means of three independent experiments $\pm$ SD. * $P < 0.05$, ** $P < 0.01$

FIGURE 7.

Working model. CREPT is highly expressed in tumors and functions to promote tumorigenesis. miR-383 targets the 3'UTR of CREPT mRNA to un-stabilize it thereafter to inhibit the activity of CREPT. As CREPT interacts with both β-catenin and TCF4 to enhance the association of β-catenin with TCF4, in response to Wnt signaling for the expression of targeted mRNA, cyclinD1, CDK4, and CDK6, miR383 also inhibit these gene expression. We proposed that miR-383 could act as a tumor suppressive microRNA, in part by negatively regulating CREPT expression and subsequently Wnt/β-catenin signaling pathway