MicroRNA-342 inhibits colorectal cancer cell proliferation and invasion by directly targeting DNA methyltransferase 1

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Abbreviations: cDNA, complementary DNA; CRC, colorectal cancer; DMEM, Dulbecco’s modified Eagle’s medium; DNMT1, DNA methyltransferase 1; FBS, fetal bovine serum; mRNA, messenger RNA; MT-MT, 3-(4,5-dimethylthiazole-2-yl)-2,5-bisphenyl tetrazolium bromide; PCR, polymerase chain reaction; siRNA, small interfering RNA.

Overexpressed DNA methyltransferase 1 (DNMT1) strongly contributes to tumor suppressor gene silencing in colorectal cancer (CRC). However, the underlying mechanism of DNMT1 overexpression is still unclear. MicroRNAs (miRNA) have been implicated as gene regulators controlling diverse biological processes, including carcinogenesis. In this study, we investigated whether some miRNA is involved in the regulation of DNMT1 and thus play a functional role in CRC. Our results showed that miR-342 was downregulated in CRC tissues and cell lines. Restoration of miR-342 resulted in a dramatic reduction of the expression of DNMT1 both at messenger RNA and protein levels by directly targeting its 3′ untranslated region. This in turn reactivated ADAM23, Hint1, RASSF1A and RECK genes via promoter demethylation. Furthermore, the enhanced expression of miR-342 could significantly inhibit SW480 cell proliferation in vitro (P=0.006). Further investigation demonstrated G2/M cell cycle arrest in SW480 cells, which was associated with an upregulation of p21 and downregulation of cyclinE and CDK2. Overexpression of miR-342 also inhibited SW480 cell invasion. The in vivo antitumor effect was evaluated in SW480 cells with lentivirus-mediated expression of miR-342. Results showed that overexpression of miR-342 significantly inhibited tumor growth and lung metastasis in nude mice (P=0.034). Our findings describe a new mechanism for the regulation of DNMT1 and aberrant DNA hypermethylation in CRC. This is also the first report to demonstrate that miR-342 may act as a tumor suppressor gene in CRC development. The newly identified miR-342/DNMT1 link provides a new, potential therapeutic target for the treatment of CRC.

Introduction

Colorectal cancer (CRC) accounts for 13% of all cancers and is one of the major causes of mortality worldwide (1). Despite innovative therapeutic strategies applied to CRC treatment, the prognosis for CRC patients has not significantly changed in the last 20 years. In past decades, studies have reported alternative factors involved in the pathogenesis of this malignancy, including genetic mutations in certain oncogenes or tumor suppressor genes (KRAS, APC, DCC, Smad-2 and Smad-4) and changes in the p53, β-catenin, transforming growth factor-β and WNT transduction pathways (2). Epigenetic variation, including hypermethylation of various tumor suppressor genes, has also been implicated in CRC development and progression (3). This suggests that colorectal carcinogenesis results from an accumulation of genetic and epigenetic alterations.

Recent work has demonstrated that DNA methylation is associated with transcriptional silencing and the loss of tumor suppressor gene function and thus plays an important role during carcinogenesis (4). DNA methylation is mainly carried out by three DNA methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3B. DNMT3A and DNMT3B are responsible for establishing de novo DNA methylation, whereas DNMT1 is the main enzyme responsible for replicating the DNA methylation pattern in genomic DNA during replication (5). Emerging studies have reported that overexpression of DNMT1 has been detected in several types of human cancers (7). Examination of clinical tissue samples showed that overexpression of DNMT1 in precancerous cancers is frequently associated with chronic inflammation and/or persistent infection with viruses or other pathogenic microorganisms, such as Epstein–Barr virus, hepatitis B or C viruses (HBV, HCV) and human papillomavirus (8). Furthermore, the elevated incidence of nuclear DNMT1 immunoreactivity was significantly associated with the degree of pancreatic intraepithelial neoplasia dysplasia (9). Also, increased protein expression of DNMT1 is significantly correlated with the malignant potential and poor prognosis of human hepatocellular carcinomas, with aggressiveness of pancreatic cancers and with poor tumor differentiation in gastric cancers (10,11). Indeed, several studies have demonstrated that the level of DNMT1 expression in colorectal polyps and cancers is significantly higher compared with their corresponding noncancerous mucosa (12–14) and have found that human colorectal carcinogenesis is accompanied by a progressive dysregulation of DNMT1 expression (15). However, the underlying mechanism of DNMT1 overexpression in human cancer cells especially in CRC remains unknown.

MicroRNAs (miRNAs) are an evolutionarily conserved class of small noncoding RNAs (20–24 nucleotides) (16) that regulate gene expression via complete or partial matching to target genes at the 3′ untranslated region (UTR), causing suppression of protein translation or messenger RNA (mRNA) degradation (17). Recent studies have shown that up to 30% of human genes and most genetic pathways are regulated by miRNAs (18). Over 500 different miRNAs have been identified in human cells (19), and it is becoming clear that abnormal miRNA expression is associated with various human diseases, including various types of cancer (20,21). Therefore, it has been proposed that miRNAs may function as either oncogenes or tumor suppressors (22,23). Consistent with this idea, a number of overexpression and silencing miRNAs have been described that focus on colorectal carcinogenesis (24,25); miR-196a, miR-21 and miR-17–92 are upregulated, and miR-101, miR-143, miR-145 and miR-200 are downregulated (26). Although several miRNAs have been identified, only a few miRNAs contributing to human CRC have been experimentally validated. In the current study, we investigated whether specific miRNA is involved in the regulation of DNMT1 and thus play a functional role in CRC. Here, DNMT1 was identified as the direct target of miR-342. Enforced expression of miR-342 markedly reduced the expression of DNMT1 by directly targeting its 3′ UTR. Additionally, restoration of miR-342 was able to inhibit CRC cell proliferation and metastasis both in vitro and in vivo. Thus, our results not only revealed that overexpressed DNMT1 in human CRC could be the result of downregulation of miR-342 but also identified the important roles of miR-342 in CRC pathogenesis and implicate its potential application in cancer therapy.
obtained from the American Type Culture Collection. The CRC cell lines and 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), and FHC cells were grown in DMEM:F12 (containing 10 ng/ml cholera toxin, 0.005 mg/ml insulin, 0.005 mg/ml transferrin and 100 ng/ml hydrocortisone) medium supplemented with 10% fetal bovine serum (FBS; Invitrogen). The lentiviral vector pLV-miR-342 (50 nM) or control miRNA (50 nM) using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after transfection, and firefly and Renilla luciferase activities were analyzed with the Dual-Luciferase® Reporter Assay System, according to the manufacturer’s instructions (Promega).

**Drug treatment with 5-aza-2’-deoxycytidine**

Cells were grown in medium containing final concentrations of 10 μM 5-Aza-2’-deoxycytidine (Sigma) for 48 h. Genomic DNA, total RNA and cellular proteins were extracted and subjected to different analysis.

Methylation-specific PCR analysis

Genomic DNA was isolated from tumor cells using the TIANamp Genomic DNA Kit (TIANGEN BIOTECH, Beijing, China). DNA was treated with the EZ DNA Methylation-Gold Kit TM (ZYMO Research Co., Orange, CA) according to the manufacturer’s instructions. Methylation-specific PCR was performed using TaKaRa Taq™ Hot Start Version (TaKaRa Bio, China). Real-time PCR was performed using an Applied Biosystems 7900 Fast Real-Time PCR system (Applied Biosystems). The miR-342 expression 3Ct values from each sample were calculated by normalizing with an internal control (RNU6B), and relative expressions were calculated using the formula 2-ΔΔCt values (ΔΔCt = Ctarget - Ccontrol).

**Cell transfection**

The oligonucleotides miR-342 (5'-UCUCACACAGAUAUUGCCCGGU-3'), scramble (negative control, 5'-UCUCUCCAGACGUUCUGAGTTT-3') and siDNMT1 (5'-CCAGUAGCAGGUGUCUCCCTT-3', 5'-GGGAAACCGUGCUAUGGT-3') (27) were purchased from Gene Pharma Co., China. The plasmid pcDNA3.1-Myc-DNMT1, expressing full-length DNMT1, was obtained from Dr T.Kouzarides (Wellcome Trust/Cancer Research UK Institute and Department of Pathology, Cambridge, UK). For transient transfection, SW480 cells were seeded in 24-well plates (1 × 10^4 cells per well) at 60% confluence and transfected with 50 nM of oligonucleotides or 0.8 μg plasmid after 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 6 h of incubation at 37°C, the transfection medium was replaced with 2 ml of complete medium containing 10% FBS. Cells were collected for western blot, proliferation and invasion assays at 48, 48, and 16 h after transfection, respectively.

**Western blot analysis**

Total cellular proteins were extracted and separated in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, and western blot analysis was performed according to standard procedures. β-Actin on the same membrane was used as a loading control. The primary antibodies used included monoclonal anti-DNMT1 (1:3000; Abcam, Cambridge, MA), anti-p21 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclinE (1:1000; Santa Cruz Biotechnology), anti-cyclinD2 (1:1000; Santa Cruz Biotechnology) and anti-β-actin (1:2000; Santa Cruz Biotechnology). Proteins were visualized using the ECL procedure (Amersham Biosciences, Sweden).

**Semiquantitative reverse transcription–PCR**

Total RNA was extracted using the TRIZol Reagent® (DSBIO, Guangzhou, China) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI). cDNA was amplified by AmpliTaq® Gold DNA polymerase (Applied Biosystems) and gene-specific primers. The sequences of the primers are available in supplementary Table 1 is available at Carcinogenesis Online. β-Actin was used as an internal control. Specific PCR products were examined by electrophoresis in 2% agarose gels.

**Luciferase assay**

For luciferase reporter experiments, a fragment of the 3’ UTR of DNMT1 mRNA (region 5029–5236, from NM_001130823) and a mutant variant were cloned into the EcoRI site of pGL3-control vector downstream from the stop codon of luciferase and the new vectors were named pGL3-DNMT1 WT and pGL3-DNMT1 MUT, respectively. The following primers were used to amplify specific fragments: DNMT1 WT, forward-5’-TATGTTACCTCTTAGTACCCCTGTGTTCTGGC-3’ and reverse-5’-GGCGTCTAGATCACTTAAACTTAACTAAGGCGAACCTCA-3’. and DNMT1 MUT, Forward-5’-TTATGTTACCTCTTAGTACCCCTGTGTTCTGGC-3’ and Reverse-5’-GGCGTCTAGATCACTTAAACTTAACTAAGGCGAACCTCA-3’.

**Carcinogenesis**

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In vivo proliferation and metastasis assays
Female athymic BALB/c nude mice (4–5 weeks old) were purchased from the Animal Center of Chinese Academy of Science (Shanghai, China). All animal studies were conducted in accordance with National Institutes of Health animal use guidelines and the current Chinese regulations and standards on the use of laboratory animals. To determine the proliferation capacity of the cell lines stably expressing miR-342 in vivo, a total of \(1 \times 10^6\) cells were injected subcutaneously into nude mice \(n = 6\). The tumor volume was evaluated using the following formula: tumor volume \(= 4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)\). To investigate experimental lung metastasis, tumor cell suspensions \(1.5 \times 10^6\) cells per mouse) were injected unto the lateral tail veins of each anesthetized nude mouse \(n = 6\). Six weeks after injection, the animals were killed; lungs were dissected and paraffin embedded, and 5 \(\mu\)m sections were stained with hematoxylin and eosin. The metastases were counted in a double-blind manner with the aid of a dissecting microscope as described previously (28). To detect the expression of DNMT1, RECK and p21 in vivo, we carried out immunohistochemistry analysis according to standard procedures.

Statistical analysis
The statistical significance of the in vitro studies was analyzed using Student’s t-test (two tailed). Comparisons of the expression levels of protein immunoreactivity among different groups were analyzed by the Mann–Whitney U-test or Kruskal–Wallis test. Differences with \(P\) values of <0.05 are considered significant.

Results
Reduced miR-342 expression in CRC tissues and cell lines
Firstly, we assessed the expression of miR-342 in 15 pairs of matched human CRC tissue specimens using miRNA TaqMan real-time PCR. As shown in Figure 1A, miR-342 was expressed at a lower level in each tumor sample compared with the corresponding matched normal tissue, although the degree of downregulation varied from one tissue to another. The expression of miR-342 was further assessed in three CRC cell lines (SW480, HT29 and HCT116) and the normal colon epithelium cell line FHC. The results showed that the level of miR-342 was lower in all three CRC cell lines compared with FHC (Figure 1B), and the expression of miR-342 in SW480 cells was reduced compared with FHC by \(\sim 12\)-fold.

DNMT1 is a direct target of miR-342
To identify the potential target genes of miR-342, we integrated bioinformatic algorithms including miRanda (29), PicTar (30) and TargetScan 4.0 (31). According to the prediction analysis, DNMT1 has a putative miR-342-binding site mapped to the 3’ UTR and is of particular interest. To validate the miRNA–target interactions, the expression of DNMT1 was evaluated in SW480 cells transfected with either miR-342 mimics or a nonspecific miRNA (as a negative control). As shown in Figure 2A, the transfection efficiency of SW480 was examined. Western blot and reverse transcription–PCR (RT–PCR) demonstrated that overexpression of miR-342 dramatically suppressed endogenous DNMT1 both at protein and mRNA levels compared with negative control miRNA (Figure 2B). In addition, miR-342 downregulated DNMT1 in a time-dependent manner in SW480 cells (Figure 2C). To further confirm whether DNMT1 is regulated by miR-342 through direct binding to its 3’ UTR, the DNMT1 3’ UTR (wild-type and mutant) was constructed and cloned downstream of the luciferase gene in a pGL3-control vector (Figure 2D). We performed a luciferase assay on 293T cells. As shown in Figure 2E, the reporter with the wild-type 3’ UTR of DNMT1 showed a markedly lower luciferase activity in cells expressing miR-342 compared with those expressing negative control miRNA \((P = 0.0032)\); however, we did not observe variations in the luciferase activity of cells cotransfected with pGL3-DNMT1-MUT and miR-342. Taken together, these results suggest that DNMT1 is a candidate target gene of miR-342 and that the 3’ UTR of DNMT1 is a functional target site for miR-342 in CRC cells.

Overexpression of miR-342 decreases the DNA methylation levels of ADAM23, Hint1, RASSF1A and RECK promoter regions in SW480 cells
To assess whether overexpressed miR-342 could lead to demethylation and induction of tumor suppressor genes through blocking DNMT1 expression in SW480. We first analyzed the mRNA expression levels of four TSGs, ADAM23, Hint1, RASSF1A and RECK. reverse transcription–PCR analysis showed the silencing of four genes expression in SW480 cells (Figure 3A). However, the expressions of these genes were detected in SW480 cells after transfection of miR-342 in comparison with the miRNA-negative control. To determine whether DNMT1 is associated with genes reactivation, DNMT1 knockdown with small interfering RNA (siRNA) was used. The results showed that these genes were found to be reactivated in SW480 cells with DNMT1 knockdown, and the similar effect was observed in the demethylating agent 5-Aza-2’deoxycytidine treatment cells. Furthermore, we also found that the silencing of these genes was not altered by overexpressing DNMT1 (Figure 3A). DNMT1 protein expression in SW480 cells with 5-Aza-2’deoxycytidine, siRNA and pcDNA3.1-DNMT1 treatment is shown by western Blot in Figure 3A.

To characterize the effects of the methylation changes on these genes expression, we examined the methylation status of the promoter region of ADAM23, Hint1, RASSF1A and RECK by using methylation-specific PCR in SW480 cells transfected with miR-342, siDNMT1, pcDNA3.1-DNMT1 or 5-Aza-2’deoxyecytidine treatment. As shown in Figure 3B, RASSF1A was found to be
completely hypermethylated, ADAM23, Hint1 and RECK partially methylated in SW480 cells. The inhibition of DNMT1 by miR-342 transfection reversed the promoter methylation of all four genes. The promoter demethylation of these genes by 5-Aza-2'deoxycytidine or siDNMT1 treatment was also confirmed. The results further showed that the methylation status of these genes was maintained in SW480 cells with cotransfection of miR-342 and pcDNA3.1-DNMT1. The level of promoter methylation status of ADAM23, Hint1 was increased in SW480 cells transfected pcDNA3.1-DNMT1 in comparison with SW480 cells.

MiR-342 inhibits CRC cell proliferation by targeting DNMT1

Because miR-342 is downregulated in CRC tissues and cell lines, we asked whether miR-342 plays a tumor-suppressive role in CRC development. To understand the functional role of miR-342, we evaluated the impact of miR-342 on cellular proliferation using MTT and clonogenic assays in SW480 cells. Our results showed that overexpression of miR-342 can suppress cellular proliferation by 44% 48 h after miR-342 mimics transfection (P < 0.006, Figure 4A). In contrast, the negative control miRNA had no effect on cellular proliferation. The clone assay also showed that overexpression of miR-342 significantly reduced the number of colonies formed and the size of the colonies after 15 days of culture compared with the negative control (P < 0.014, Figure 4B).

To further establish a link between miR-342 and DNMT1, cell proliferation after knockdown and overexpression of DNMT1 was examined. As shown in Figure 3C, knockdown of endogenous DNMT1 expression by small interfering RNA resulted in a dramatic inhibition of SW480 cell proliferation (reduced by 38.24%) (P < 0.0064, Figure 4C). In addition, concomitant overexpression of miR-342 and DNMT1 nullified the inhibitory effect of miR-342 on the cell proliferation of SW480 cells compared with cells transfected with miR-342 and the empty expression vector groups (P = 0.012, Figure 4D). However, there was no significant difference between the group that cotransfection-negative control miRNA with pcDNA3.1-DNMT1 and the group that cotransfection miR-342 with pcDNA3.1-DNMT1. These results demonstrated that miR-342 can inhibit CRC cell proliferation by targeting DNMT1.

MiR-342 induces G0/G1 cell cycle arrest in CRC cells

To dissect the mechanism of the antiproliferative effect of miR-342, we determined whether growth inhibition was associated with specific cell cycle control. Flow cytometric analysis showed that,
compared with the negative control, SW480 cells overexpressing miR-342 showed an increase in the number of cells in G0/G1 phase ($P = 0.0062$, Figure 4E). To examine the molecules involved in the cell cycle distribution, we analyzed the expression of p21, which functions as a regulator of cell cycle progression at G1 phase. Western Blot analysis showed that the p21 protein was induced...
in miR-342 overexpressing cells compared with negative control (Figure 4F). We further analyzed four predicted G0/G1 cell cycle-related proteins, cyclinD1, cyclinE, CDK2 and CDK6. As shown in Figure 3F, cyclinE and CDK2 protein levels were markedly reduced 24 h after miR-342 transfection. However, cyclinD1 and CDK6 protein expression remained unchanged (data not shown). These results indicated that the growth inhibitory effect of miR-342 was due to an induction of G0/G1 arrest.

**MiR-342 inhibits CRC cell invasion by targeting DNMT1**

To explore the effect of miR-342 overexpression on the invasion of CRC cells, a transwell assay was performed. SW480 cells transfected with miR-342 mimics or negative control miRNA were seeded into the chambers, and their invasive potential was determined after 16 h of culture. Transwell assays showed that the invasive capacity of SW480 cells overexpressing miR-342 was reduced by 36% compared with the negative control ($P = 0.0006$, Figure 5A).

**Fig. 5.** MiR-342 inhibits CRC cell invasion by targeting DNMT1. (A) Invasion assay of SW480 cells transfected with miR-342 or negative control. Representative images are shown on the left, and the average number of cells per field at the indicated time points is shown on the right. Data are the mean of three independent experiments ($^*P = 0.0006$). (B) Invasion assay of SW480 cells with DNMT1 knockdown by RNAi ($n = 3$, $^*P = 0.0003$). (C) Invasion assay of SW480 cells overexpressing DNMT1 with miR-342. Invasion analysis of the cells transfected with empty expression vector or miR-342 ($n = 3$, $^*P = 0.0034$).
To determine whether DNMT1 is a critical mediator of miR-342 in CRC cell metastasis, we downregulated DNMT1 expression using RNAi in SW480 cells. DNMT1 RNAi reduced the invasive potential of SW480 cells to an extent similar as that seen with miR-342 overexpression (~28% decreased, \( P = 0.0003 \), Figure 5B). Importantly, restoration of DNMT1 strongly increased SW480 cell invasion that be inhibited by miR-342. However, transfection of the empty expression vector did not have the same effect (Figure 5C). These results suggested that overexpression of miR-342 can significantly suppress CRC cell invasion and that DNMT1 is a direct and functional target for miR-342.

MiR-342 inhibits CRC growth and metastasis in vivo

To further investigate the contribution of miR-342 in vivo, we constructed a lentivirus vector to mediate the expression of miR-342 and established two stable cell lines, which were named LV-mir342-SW480 and LV-miRcontrol-SW480 (Figure 6A). These two cell lines were injected subcutaneously into the flanks of nude mice. Tumor progression was studied over time. At 4 weeks postimplantation, the mice were killed, and the tumors were removed. As shown in Figure 6B, the volume and weight of the tumors resulting from injection of LV-mir342-SW480 were significantly less than those of the LV-miRcontrol-SW480 cells (\( P = 0.034 \)). Furthermore, immunohistochemistry analyses revealed that tumors from LV-mir342-SW480 cells had decreased DNMT1 levels compared with LV-miRcontrol-SW480 cells. In contrast, the expression level of p21 was higher in cells overexpressing miR-342 (Figure 6C).

To explore the inhibition of in vivo tumor metastasis by miR-342, we implanted LV-miR342-SW480 and LV-miRcontrol-SW480 cells into nude mice through the lateral tail vein. As shown in Figure 6D, most mice injected with the control vector displayed a large number of lung metastatic nodules after 6 weeks of growth. In contrast, injection of cells with overexpressing miR-342 did not produce metastatic nodules in any of the mice. Taken together, these observations are consistent with the in vitro results and indicate that miR-342 has the ability to suppress CRC cell growth and metastasis in vivo.

Discussion

DNA methylation is associated with the silencing of tumor suppressor genes in cancers. Hypermethylation of various genes has also been implicated in CRC carcinogenesis. Inactivation of the cell cycle regulator CDKN2, and p21WAF1 (32,33), the histidine triad (HIT) nucleotide-binding protein 1 (HINT1) (34), the cell adhesion molecule ADAM23 (35) and the metastasis suppressor gene RECK (36) in colorectal cancers (CRC). One mechanism suggested for the abnormal hypermethylation of these genes is dysregulation of DNMT1. An increasing amount of evidence indicates that miRNAs are involved in aberrant mechanisms of DNA hypermethylation by regulating DNMTs. Previous studies reported that miRNA-29b can indirectly target DNMT1 and induce global DNA hypomethylation and expression of tumor suppressor genes in acute myeloid leukemia (37,38). Huang et al. (39) recently demonstrated that miRNA-152 can target DNMT1 and induce aberrant DNA methylation in HBV-related hepatocellular carcinoma. MiRNA-21 and miRNA-143 also can directly and indirectly target DNMT1 and contribute to DNA hypomethylation in Lupus CD4+ T cells (40). In the present study, we showed for the first time that miR-342 exerts its function by specifically targeting DNMT1 in CRC. We demonstrated that miR-342 frequently downregulated DNMT1 in CRC tissues and cell lines (Figure 1). Overexpression of miR-342 inhibited the expression of DNMT1 both at transcriptional and posttranscriptional levels (Figure 2B). Furthermore, we also provided evidence from the luciferase activity assay that DNMT1 is a direct target of miR-342, indicating that miR-342 is specifically involved in the regulation of DNMT1. This result suggests that DNMT1 may be regulated by different miRNAs in various cell types and argues in favor of the proposed combinatorial miRNA target regulation, in which different miRNAs are expressed in different cell types and may coordinately regulate one target gene (31). 5-Aza-2‘deoxycytidine has been approved by the United States Food and Drug Administration and has been extensively used as a demethylating agent for epigenetic investigations and also as a drug for cancer therapy. However, because 5-Aza-2‘deoxycytidine combines with DNA during the methylation reaction, the DNA damage effect derived from the formation of DNMT–DNA adducts may lead to cytotoxicity. MiR-342 has the same effect in inhibiting DNMT1. This identification contributes to our understanding of a potential demethylating agent for epigenetic cancer therapy.

In this work, we also provide insights about whether miR-342 is involved in the control of DNA methylation by targeting DNMT1 in CRC cells. In our study, four TSGs, RASSF1A, ADAM23 (modulate cell–cell and cell–extracellular matrix interactions and is involved in cell migration) (35), HINT1, histidine triad (function as a tumor suppressor in several tumor cell lines) (41-44) and RECK (a metastasis suppressor gene that can negatively regulate matrix metalloproteinase-2 and -9 activities and inhibit tumor invasion and metastasis) (45) were demonstrated to be silenced, the methylation level of their promoters was detected in SW480 cells. Inhibition of DNMT1 by miR-342 overexpressing markedly enhanced the demethylation, which in turn reactivated these four gene expressions, which was in line with the effect of treatment with the DNMT1 inhibitor 5-Aza-2‘deoxycytidine. Moreover, the demethylation effect of miR-342 can be counteracted by overexpression of DNMT1 (Figure 3B). These results indicate that DNMT1 is necessary and sufficient to maintain aberrant CpG island methylation of ADAM23, HINT1, RASSF1A and RECK genes and that miR-342 is required for restoring the hypomethylation status of these tumor suppressor genes by targeting DNMT1 in CRC cells.

MiRNAs have recently been demonstrated to potentially play a significant role in tumorigenesis. To date, hundreds of different miRNAs regulate many known oncogenic and tumor suppressor pathways involved in the pathogenesis of CRC. The miR-200 family suppresses the zinc finger E-box-binding homeobox promotes CRC cell epithelial-mesenchymal transition and invasion (46). MiRNA-192, miR-31 and miR-21 induce resistance to 5-fluorouracil and may be important clinical indicators of chemotherapy efficacy in CRC (47-49). In recent work, we have started to shed light on the importance of miR-342 in colorectal carcinogenesis. While miR-342 is upregulated in human acute promyelocytic leukemia (50), it has been shown to be downregulated in various types of cancers, including lung adenocarcinomas and breast cancer (51,52). In agreement with reports showing a reduction in the expression of miR-342 in CRC (53), we have also observed downregulation of miR-342 in CRC tissues and cell lines. Restoration of miR-342 can reduce CRC cell proliferation, invasion and migration (data not shown) in vitro. Importantly, in vivo xenograft tumor growth analysis revealed a significant decrease in tumor growth and metastasis following treatment with the miR-342 expression vector. To our knowledge, this is the first study showing that miR-342 regulates cellular proliferation and invasion in CRC cells. Grady et al. (53) observed that apoptosis mediated by miR-342 is reconstituted in HT29 CRC cells; however, in our study, we did not observe this effect. We demonstrated that the same miRNA performs different functions through distinct target genes depending on the tissue or cell type. Furthermore, we also noticed that overexpression of DNMT1 by the pcDNA3.1-DNMT1 plasmid nullifies the effect of suppression of cell proliferation and invasion by miR-342. Noticeably, the proliferative and invasive capacity of DNMT1 overexpression alone is not significantly changed in CRC cells (data not shown). Knockdown of DNMT1 gene expression by RNAi had an effect on in vitro proliferation and invasion that was similar to that of the restoration of miR-342. These properties support the notion that DNMT1 repression contributes to the tumor-suppressive effects of miR-342. However, the suppressive effect of miR-342 on proliferation and invasion is stronger than its effect in suppressing DNMT1 expression. This indicates that there might be additional miR-342 targets that act as tumor suppressors. These findings suggest that miR-342 plays the tumor-suppressive role is at least partly through decrease in DNMT1.
expression. The demethylation and reexpression of tumor suppressor genes downstream of DNMT1 are possible responsible for cell proliferation and invasion inhibition of CRC cells.

Importantly, we demonstrated that restoring miR-342 expression in CRC cells caused an inhibition of cell proliferation via G0/G1 cell cycle arrest (Figure 4E). In our study, we found that downregulation of
DNMT1 by overexpression of miR-342 promoted the expression of p21, which agrees with Oridate’s report that siRNA-mediated disruption of DNMT1 expression in head and neck squamous carcinoma cells resulted in an increase in p21 protein levels (S4). It is well known that p21 can bind to and inhibit the activity of cyclin–CDK2 complexes, thus functioning as a regulator of cell cycle progression at G1 phase. In our study, we observed that the level of cyclinE1 and CDK2 was decreased, accompanied by high expression of p21 after transfection of miR-342 (Figure 4F). We speculated that the induction of p21 could be the mechanism responsible for the antiproliferation effect of miR-342.

In summary, our results reveal that overexpression of the DNMT1 protein in CRC cells could be the result of decreased levels of miR-342. Through downregulating DNMT1 expression, miR-342 led to reactivation of tumor suppressor genes via promoter DNA hypomethylation.

Furthermore, miR-342 can significantly suppress CRC cell proliferation and invasion. The newly identified miR-342/DNMT1 link provides a new, potential therapeutic target for the treatment of CRC.

**Supplementary material**

Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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