TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Inflammation and DNA Methylation—Dependent Down-Regulation of miR-34b-5p Mediates *c-MYC* Expression and CRL4^{DCAF4} E3 Ligase Activity in Colitis-Associated Cancer

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miRNAs, a well-known group of noncoding RNAs, contribute to the pathogenesis of multiple diseases, including colitis-associated cancer (CAC). Our recent findings indicate that proinflammatory cytokines up-regulate *c-MYC* level, which subsequently activates cullin 4A and 4B (*CUL4A/4B*) and CRL4^{DCAF4} E3 ligases and promotes ubiquitination of suppression of tumorigenicity 7 in CAC. Herein, we identified and proved that miR-34b-5p can directly target *c-MYC*. *In vitro* oncogenic phenotype analyses and *in vivo* tumor formation assay indicated that miR-34b-5p overexpression could markedly decrease cell proliferation, colony formation, cell invasion, and tumor volumes. Overexpression of *c-MYC* *in vitro* could reverse the oncogenic phenotypes caused by miR-34b-5p up-regulation. In addition, the down-regulation of miR-34b-5p in CAC was dependent on the coregulation of the inflammatory microenvironment and DNA methylation. Collectively, our findings demonstrate that intracellular inflammation and DNA hypermethylation suppress miR-34b-5p expression, which limits its inhibitory effect on *c-MYC* and initiates the downstream events, including the induction of CRL4^{DCAF4} E3 ligase activity. The activated CRL4^{DCAF4} E3 ligase ubiquitinates suppression of tumorigenicity 7 and results in its degradation, eventually leading to the CAC tumorigenesis. (*Am J Pathol* 2020, 190: 674–688; <https://doi.org/10.1016/j.ajpath.2019.11.013>)

Colitis-associated cancer (CAC), an important subtype of colorectal cancer (CRC), is mainly developed by bowel diseases (eg, ulcerative colitis and Crohn disease) derived from inflammation.^{1,2} Clinical and experimental evidence suggests that the inflammatory gastrointestinal microenvironment significantly increases the chances of developing CAC through contributing to tumor initiation, progression, and metastasis.^{3,4} Intracellular inflammatory status can activate multiple tumorigenesis-associated pathways, such as the phosphatidylinositol-3 kinase/AKT serine/threonine kinase 1 signaling axis,⁵ the Janus kinase/STAT pathway,⁶ the NF- κ B-mediated signaling,^{7,8} and the Wnt/ β -catenin signaling.⁹ On the other hand, several transcription factors, including NF- κ B family members and *c-MYC*, can activate the expression and production of a variety of

proinflammatory cytokines, such as *IL-1B*, *IL-6*, tumor necrosis factor- α (*TNFA*), and transforming growth factor- β (*TGFB*), which in turn aggravate the inflammatory response.^{7,10} Intracellular inflammatory status induces *c-MYC* level, which further binds to the promoters of cullin 4A (*CUL4A*) and *CUL4B* and induces their expression.¹¹ The increased *CUL4A/4B* proteins form two CRL4^{DCAF4} (CRL4A^{DCAF4} and CRL4B^{DCAF4}) E3 ligases with DNA damage binding protein 1, ring box 1, and DNA damage binding protein 1— and *CUL4*-associated factor 4 (DCAF4).

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CRL4^{DCAF4} E3 ligases ubiquitinate the tumor suppressor suppression of tumorigenicity 7 (ST7) and cause its degradation.¹¹ However, the regulatory mechanism of *c-MYC* up-regulation under inflammatory conditions remains unknown.

miRNAs are emerging as an important group of non-coding RNAs, and they are identified to target and repress the expression of multiple genes involved in different biological processes.^{12,13} A great number of oncogenic and suppressive miRNAs are involved in the tumorigenesis of CRC, and the well-known miRNAs include let-7,¹⁴ miR-21,¹⁵ miR-29,¹⁶ miR-30a,¹⁷ miR-34a,¹⁸ miR-101,¹⁹ miR-135a/b,^{20,21} miR-200c,²² miR-342,²³ and miR-451.²⁴ Molecularly, miRNAs bind to the 3'-untranslated region of their target genes and negatively control the expression of their targets. For instance, Saridaki et al¹⁴ found that Let-7a could specifically target the oncogene Kirsten rat sarcoma (*KRAS*). Tang et al¹⁶ found that miR-29a promotes the metastasis of CRC by controlling the expression of matrix metalloproteinase 2 (*MMP2*) and cadherin 1 (*CDH1*). Nagel et al²⁵ found that miR-135a/5b function as oncogenes to inhibit adenomatous polyposis coli (*APC*) expression in CRC. However, there is no study showing that *c-MYC* is regulated by an miRNA in the pathogenesis of CAC.

Although a variety of miRNAs have been proved to contribute to tumorigenesis of CRC, the identified miRNAs are different because of the use of different experimental materials and detection techniques. To discover miRNAs that specifically target *c-MYC*, we performed a microarray analysis with three-paired cancerous tissues and adjacent noncancerous tissues from three CAC patients with tumor-node-metastasis stage III. Overall, 30 up-regulated miRNAs and 36 down-regulated miRNAs were identified. The targets of these differentially expressed miRNAs were then predicted via miRDB database (<http://mirdb.org>, last accessed November 2, 2019), and one of the down-regulated miRNAs, known as miR-34b-5p, was found to potentially target *c-MYC*. It was further verified whether miR-34b-5p could target *c-MYC* directly, investigating the mechanism underlying miR-34b-5p down-regulation in CRC cancerous tissues and evaluating the molecular and physiological effects of changes in miR-34b-5p expression.

Materials and Methods

Cell Culture and Transfection

The human noncancerous epithelial cell line HCEC-1CT was purchased from Evercyte (Vienna, Austria) and cultured in the medium, as described previously.¹¹ Six human CRC cell lines, including Caco-2, DLD-1, HCT-15, HCT-116, HT-29, and SW480, were obtained from ATCC (Manassas, VA). These six cancer cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO; catalog number F2442) and 1% penicillin-streptomycin antibiotics

(Sigma-Aldrich; catalog number P4333) in a 37°C incubator filled with 5% CO₂. Cell transfection with plasmids, siRNAs, miRNA-mimic, or inhibitor was conducted using a Lipofectamine 2000 reagent (Thermo Fisher Scientific, Waltham, MA; catalog number 11668019) following the manufacturer's protocol. Briefly, cells were seeded into a 24-well plate to reach 70% to 80% confluence. After washing twice with phosphate-buffered saline (PBS), cells were transfected with 1 µg plasmids (pCDNA3-2 × Flag-p65, pCDNA3-2 × Flag-p53, and pCDNA3-2 × Flag-*c-MYC*), 5 pmol miR-34b/c-5p-mimic (or anti-miR-34b/c-5p) (Thermo Fisher Scientific; catalog numbers AM10743, MH10743, MC12343, and MH11039), or 5 pmol siRNAs, including sip65 (Sigma-Aldrich; catalog number SASI_Hs01_00171091) and sic-MYC (Sigma-Aldrich; catalog number SASI_Hs01_00222676), using Lipofectamine 2000, followed by incubation at 37°C for another 24 hours. Cells were then applied to the required experiments.

Tissue Collection

Twenty-four paired cancerous and adjacent noncancerous colon tissues were collected from 24 CRC patients who were not derived from inflammatory bowel disease and 24 CAC patients, respectively. All these patients underwent surgery in the Department of Integrated Traditional and Western Medicine, West China Hospital of Sichuan University (Chengdu, China). The basic information of these CRC and CAC patients is shown in [Supplemental Table S1](#). According to the requirement of the ethics committee of Sichuan University, all patients signed a consent form before their surgeries.

ELISA Data

The enzyme-linked immunosorbent assays (ELISAs) were performed following a previous protocol.¹¹ Briefly, blood samples from 24 CRC patients (same patients as tissue collection) and 24 controls (same volunteers as the previous study¹¹) were applied to ELISAs to measure the concentrations of cytokines, including IL-1β, IL-4, IL-6, and TNF-α. The ELISA kits were purchased from Abcam Company (Cambridge, MA), and they included ab214025 (IL-1β), ab46063 (IL-4), ab46027 (IL-6), and ab46087 (TNF-α). In addition, the ELISAs were also performed to measure the concentrations of IL-6 and TNF-α in the supernatant of cell culture medium of p65-overexpression cells.

Microarray Analysis and miRNA Quantification by qPCR Analysis

The microarray procedures followed a previous protocol.²⁶ Briefly, three-paired cancerous and noncancerous tissues from CAC patients under tumor-node-metastasis stage III were applied to miRNA isolation using a RNeasy Mini Kit (Qiagen, Shanghai, China; catalog number 217004). The

purified miRNA (500 ng per sample) was subjected to microarray analysis using a GeneChip miRNA 4.0 Assay (Thermo Fisher Scientific; catalog number 902445) following a protocol provided by the manufacturer. The chip slide was scanned with a GeneChip Scanner (Thermo Fisher Scientific; 3000 7G system). The expression of eight miRNAs, including miR-301a, miR-155-5p, miR-495-5p, miR-192-5p, miR-34b-5p, miR-205-3p, miR-34a, and miR-34c-5p, was verified by real-time quantitative PCR (qPCR) analyses in 24-paired tissues from CRC and CAC patients. The TaqMan miRNA assays included 478796 (miR-301a), 483064 (miR-155-5p), 478945 (miR-495-5p), 480876 (miR-192-5p), 478050 (miR-34b-5p), 478760 (miR-205-3p), 481304 (miR-34a), and 478052 (miR-34c-5p).

Total RNA Extraction and qPCR Analysis

Total RNA was extracted from biopsies and cultured cells with a TRIzol kit (Sigma-Aldrich; catalog number T9224) following the guidelines of the manufacturer. For each sample, 500 ng of total RNA was applied to synthesize cDNA by reverse transcription with a high-capacity kit (Thermo Fisher Scientific; catalog number 4368814). After dilution of 100-fold, cDNAs were used to determine gene expression levels by qPCR with the following primers: *CUL4A*, 5'-ACCATTGATGGAATCCTACTGC-3' (forward) and 5'-ACCTTTGGCCTTCGGCAGCATA-3' (reverse); *CUL4B*, 5'-ACTTCGTGCAGGCAACAAAGA-3' (forward) and 5'-CAGCATCTACAGATGCACTCT-3' (reverse); *c-MYC*, 5'-ACCACCAGCAGCGACTCT-3' (forward) and 5'-GCGTAGTTGTGCTGTGTGT-3' (reverse); *DCAF4*, 5'-TGATGACGAGTCTCCGTCA-3' (forward) and 5'-TCCGTCTGTCTTCTTCTTG-3' (reverse); *ST7*, 5'-GTCTCAGTAAGCCACTTGC-3' (forward) and 5'-GTCA-TAGTAAGTAAGAGGCT-3' (reverse); *TP53* (*p53*), 5'-AGCACTAAGCGAGCACTGCCCAA-3' (forward) and 5'-TTCCAGCCTGGGCATCCTTGAG-3' (reverse); *RELA* (*p65*), 5'-CAAGTGTCTTCCATCATGGA-3' (forward) and 5'-TGAGCAAGGAAAGAGCCGGCAGAG-3' (reverse); and β -actin, 5'-AGAGCTACGAGCTGCCTGAC-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse).

Western Blot Analysis

The immunoblot analysis method was the same as described previously.¹¹ Briefly, tissues and cells were lysed with 1× radioimmunoprecipitation assay buffer (Sigma-Aldrich; catalog number R0278) containing 1× protease inhibitor (EDTA-free cocktail; Sigma-Aldrich; catalog number S8820). The resulting protein was quantified, and equal amounts of total proteins were loaded into 10% SDS-PAGE gels. After electrophoresis, proteins were transferred from gels to polyvinylidene fluoride membranes (GE Healthcare, Chicago, IL; catalog number 10600057). After blocking in 5% milk, the polyvinylidene fluoride membranes were

probed with primary antibodies at 4°C overnight. The primary antibodies were purchased from Abcam Company, and they included anti-CUL4A (catalog number ab92554), anti-CUL4B (catalog number ab67035), anti-DCAF4 (catalog number ab83655), anti-c-MYC (catalog number ab39688), anti-Flag (catalog number ab49763), and antiglyceraldehyde-3-phosphate dehydrogenase (catalog number ab9483). After washing five times with PBS with Tween 20 buffer, the polyvinylidene fluoride membranes were further incubated with secondary antibodies (Thermo Fisher Scientific; catalog numbers 31430 and 31460). Protein signals on the membranes were determined using a Pierce ECL kit (Thermo Fisher Scientific; catalog number 32106).

ChIP Assay

The chromatin immunoprecipitation (ChIP) assay was performed following a protocol, as described previously.¹¹ Briefly, a total of 5×10^7 cells per immunoprecipitation reaction were used for cross-linking DNA and proteins with 1% formaldehyde (Sigma-Aldrich; catalog number 47608) at 37°C for 15 minutes. The reaction was then terminated by adding 1 mol/L glycine to reach a final concentration of 0.125 mol/L. Cells were then washed twice with PBS and applied to ChIP assays using a kit (Sigma-Aldrich; catalog number 17-295) following the guidelines of the manufacturer. The antibodies used for immunoprecipitation included anti-c-MYC and IgG (Sigma-Aldrich; catalog number 12136). After purification, DNA was used for measuring the relative enrichment of c-MYC on the promoters of *CUL4A* and *CUL4B* by qPCR analyses with the following primers: *CUL4A*, 5'-TTATTACCACGTCTTGGTAA-3' (forward) and 5'-AACGCATATTCTTTCCAC-3' (reverse); and *CUL4B*, 5'-AGATGAGACGGAGTGAGAGCC-3' (forward) and 5'-TCTGGCTTGTATTAGGAC-3' (reverse).

Cell Treatments

Cells (1×10^6) were treated with different concentrations of lipopolysaccharide (LPS; Sigma-Aldrich; catalog number L4524), including 0, 0.1, 0.4, 0.8, 1.2, and 2.0 $\mu\text{g/mL}$; different concentrations of IL-6 (Sigma-Aldrich; catalog number I3268), including 0, 40, 80, 120, 160, and 200 ng/mL; and different concentrations of TNF- α (Sigma-Aldrich; catalog number 11371843001), including 0, 4, 8, 12, 16, and 20 ng/mL. After incubation at 37°C for 4 hours, cells were collected and used in the required experiments. In addition, cells (1×10^6) were also treated with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine (AZA), and a histone deacetylase, trichostatin A. Cells were treated with 3.0 $\mu\text{mol/L}$ AZA or 0.5 $\mu\text{mol/L}$ trichostatin A at 37°C for 12 hours, followed by washing with PBS buffer twice and applying to the required experiments.

In Vitro Oncogenic Phenotype Analysis

The *in vitro* oncogenic phenotype analyses conducted in this study included cell proliferation and invasion, as well as colony formation. The methods of these experiments were the same as in a previously described protocol.¹¹ For cell proliferation assays, the same number of cells (1×10^3) was placed into a 12-well plate, and cell viability was detected daily for 5 days using an MTT kit (Abcam Company; catalog number ab211091) following a protocol provided by the manufacturer. To determine colony formation, the same number of cells (0.5×10^3) was placed equally into a 6-well plate and grown in the serum-free Dulbecco's modified Eagle's medium for 2 weeks at 37°C. Cell colonies were washed twice with PBS, fixed with 4% paraformaldehyde (Sigma-Aldrich; catalog number 158127) for 20 minutes, and stained with 0.5% crystal violet (Sigma-Aldrich; catalog number C0775) for 30 minutes. After rinsing five times, colonies were subjected to imaging. For cell invasion assays, the same volume of cell suspension (1×10^5 cells) in serum-free Dulbecco's modified Eagle's medium was placed into the top chambers of a 24-well Boyden chamber (R&D Systems, Minneapolis, MN; catalog number 3455-024-K). The bottom chambers were supplemented with 0.5 mL of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The Boyden chambers were placed at 37°C for 72 hours, and cells growing in the surface of bottom chambers were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet for 30 minutes. After rinsing five times, colonies were subjected to imaging.

DNA Methylation Detection

DNA methylation levels in tissues and cultured cells were performed according to a method known as quantitative methylation-specific PCR, which is designed to amplify bisulfite-modified methylated DNA sequences.²⁷ In brief, genomic DNA from clinical tissues and cultured cells was extracted using a kit (Sigma-Aldrich; catalog number 11814770001) following the guidelines of the manufacturer. For each sample, 1 µg of purified DNA was treated with the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA; catalog number D5007) to convert cytosine to uracil. DNA was then applied to quantitative methylation-specific PCR assays using a TaqMan kit (Thermo Fisher Scientific; catalog number 4440038) with the following primers: forward, 5'-GAATTTGGGTTTTATTTTTTAGG-3'; and reverse, 5'-CCAAACCCTAAACTAAGTCTCTC-3'.

In Vivo Tumor Formation and Inhibition Assays

For the tumor formation assay, cell suspensions of HCT116-control, HCT116 + miR-34b-5p-mimic, and HCT116-anti-miR-34b-5p in 100 µL of PBS buffer were injected into 6-week-old nude mice (female; $n = 10$ for each cell line),

which were purchased from Shanghai SLAC Laboratory Animal Co, Ltd (Shanghai, China) and maintained according to a protocol approved by the Institutional Animal Care and Use Committee of Sichuan University. Tumor volumes were determined using a fine caliper at a 5-day interval for 30 days. Tumor volumes were determined using a formula: volume = (length $\times$ width²)/2.

For the tumor growth inhibition assay, cell suspension of HCT-116 was first injected in 6-week-old nude mice (female; $n = 30$). After 20 days of injection, mice with similar tumor volumes (nearly 250 mm³) were randomly separated into three groups ($n = 5$ in each group), which were then injected with PBS, miR-34b-5p-mimic (20 nmol/L), or anti-miR-34b-5p (20 nmol/L) at a 5-day interval. Similarly, tumor volumes were also examined at 5-day intervals for 30 days.

Statistical Analysis

All experiments were independently performed by three different laboratory members. The data were analyzed using two-tailed *t*-tests or analysis of variance for each time point or post-hoc testing between different groups with the software of StatView version 5.0 (SAS Institute, Cary, NC). $P < 0.05$ was considered significant.

Results

miR-34b-5p Is Significantly Down-Regulated in Cancerous Colon Tissues from CAC Patients

Amplified *c-MYC* can simultaneously activate the expression of *CUL4A/4B* through binding to their promoters.¹¹ However, it is unclear how *c-MYC* is activated during this process. Given that miRNAs are important factors that contribute to the pathogenesis of CRC,^{14–25} this study aimed to determine whether miRNAs could directly target *c-MYC* in the tumorigenesis of CAC. Therefore, RNA was isolated from three-paired cancerous and adjacent noncancerous colon tissues and a microarray analysis was conducted to identify aberrantly expressed miRNAs with a GeneChip miRNA 4.0 chip, which efficiently covers miRNAs with the most up-to-date content. In total, 66 miRNAs were obtained that were consistently up-regulated or down-regulated in three cancerous tissues relative to their expression in noncancerous control tissues (Supplemental Table S2). Among these miRNAs, 30 were up-regulated, whereas the remaining 36 were decreased (Supplemental Table S2). The top 20 differentially expressed miRNAs, including 10 up-regulated and 10 down-regulated miRNAs, are listed in Figure 1A. To determine if these differentially expressed miRNAs were specific in CAC cancerous tissues but not in other types of CRC cancerous tissues, as well as to determine the accuracy of our microarray data, the expression of six miRNAs was measured, including three up-regulated (miR-301a, miR-155-5p, and miR-495-5p) and

three down-regulated (miR-192-5p, miR-34b-5p, and miR-205-3p) miRNAs in the 24-paired tissues from CRC patients who were not preceded by inflammatory bowel disease and had no obviously increased proinflammatory cytokines (Supplemental Figure S1) and 24-paired tissues from CAC patients, respectively. As shown in Figure 1, B–D, the qPCR results indicated that the average levels of miR-301a (approximately 4.1-fold induction), miR-155-5p (approximately 5.9-fold induction), and miR-495-5p (approximately 6.4-fold induction) were dramatically increased in cancerous colon tissues from CAC patients compared with those in the paired adjacent noncancerous colon tissues. In contrast, the average levels of miR-192-5p (approximately 3.2-fold deduction), miR-34b-5p (approximately 4.3-fold deduction), and miR-205-3p (approximately 4.7-fold deduction) were markedly decreased in CAC cancerous colon tissues (Figure 1, E–G). However, no significant change was observed in the expression of miR-301a, miR155-5p, and miR-205-3p in the cancerous tissues from CRC (Supplemental Figure S2). Only the up-regulation of miR-495-5p expression (approximately 1.6-fold induction) and down-regulation of miR-192-5p (approximately 2.1-fold deduction) and miR-34b-5p (approximately 1.5-fold deduction) in CRC cancerous tissues (Supplemental Figure S2) were observed. Comparing the expression of these six miRNAs in CRC and CAC

cancerous tissues, their differentially expressed patterns suggested that these miRNAs might play important regulatory roles in CAC.

miR-34b-5p Can Directly Target *c-MYC* in Vitro

The regulatory mechanism of miRNAs mainly involves inhibiting the expression of their targets through direct binding to the 3'-untranslated region.^{14–24} Thus, this study focused on the down-regulated miRNAs to identify whether at least one of them could target *c-MYC*. After predicting the potential targets of these 36 down-regulated miRNAs in miRDB, it was identified that miR-34b-5p (23 nucleotides in length) could target the 3'-untranslated region of *c-MYC* through the seed sequence AGGCAG (Figure 2A). To further determine whether miR-34b-5p is also down-regulated in CRC cells, six CRC cell lines, HCT-15, HT-29, HCT-116, DLD-1, Caco-2 and SW480, were selected and then both the *c-MYC* mRNA levels and miR-34b-5p levels in these cell lines were measured. Consistent with our previous results,¹¹ *c-MYC* expression was up-regulated in all six CRC cell lines in comparison to that in HCEC-1CT cells (Figure 2B). Interestingly, the expression of miR-34b-5p in these cells was completely inverse to the *c-MYC* level (Figure 2C). In addition, this study also determined if *c-MYC* expression was negatively correlated

Figure 1 Identification and verification of differentially expressed miRNAs in cancerous colon tissues of colitis-associated cancer (CAC) patients. **A:** Heat maps of 20 differentially expressed miRNAs. Three paired cancerous (CAC) and adjacent noncancerous (control) colon tissues from CAC patients with tumor-node-metastasis stage III were used for RNA isolation and microarray analysis. The top 20 differentially expressed miRNAs, including 10 up-regulated and 10 down-regulated miRNAs, are shown. The reference bar is shown. **B–G:** Real-time quantitative PCR was performed to verify the expression of miRNAs. **B–G:** Three up-regulated miRNAs, miR-301a (**B**), miR-155-5p (**C**), and miR-495-5p (**D**), and three down-regulated miRNAs, miR-192-5p (**E**), miR-34b-5p (**F**), and miR-205-3p (**G**), were selected; and their expression levels in colon tissues from 24 CAC patients were measured. *** $P < 0.001$.

Figure 2 *c-MYC* is a direct target of miR-34b-5p. **A:** The 3'-untranslated region (UTR) of *c-MYC* contains a putative miR-34b-5p binding site. The potential binding position (positions 128 to 134 after the TGA codon) is indicated by a red line. The sequence of miR-34b-5p and its binding sequence to the *c-MYC* 3'-UTR are shown. Blue text represents the seed sequence of miR-34b-5p. Red text represents the binding sequence of miR-34b-5p in the promoter of *c-MYC*. **B** and **C:** The relative *c-MYC* mRNA level and miR-34b-5p level in colorectal cancer (CRC) cell lines. Real-time quantitative PCR (qPCR) analyses were performed to measure the *c-MYC* (**B**) and miR-34b-5p (**C**) levels in six CRC cell lines, including HCT-15, HCT-116, HT-29, DLD-1, Caco-2, and SW480. **D** and **E:** The relative expression levels of miR-34b-5p and *c-MYC* in cells with overexpression or down-regulation of miR-34b-5p. HCEC-1CT cells were transfected with miRNA-NC (control), miR-34b-5p-mimic, and anti-miR-34b-5p. The isolated RNA was used to measure the miR-34b-5p (**D**) and *c-MYC* (**E**) expression levels using qPCR. **F:** The effects of miR-34b-5p on the expression of *c-MYC* downstream targets. Three cell lines, HCEC-1CT, HT-29, and HCT-116, were transfected with miRNA-NC (control), miR-34b-5p-mimic (mimic), and anti-miR-34b-5p (anti-miR), respectively. The resulting cells were used to examine the protein levels of *c-MYC*, CUL4A, CUL4B, DCAF4, and ST7 by immunoblots. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 versus HCEC-1CT; †*P* < 0.05, ††*P* < 0.001. Nt, nucleotide.

with miR-34b-5p *in vivo*. Accordingly, the expression of *c-MYC* in 24-paired CAC tissues was measured to find that *c-MYC* was induced approximately 14-fold in CAC cancerous tissues (Supplemental Figure S3A). The correlation analysis indicated that the expression of miR-34b-5p and *c-MYC* was negatively correlated (Supplemental Figure S3B). The inverse levels of miR-34b-5p and *c-MYC* in CAC cancerous tissues and CRC cell lines, together with the functional mechanism of miRNAs, implied that miR-34b-5p might directly target *c-MYC*. To detect this possibility, miR-34b-5p-mimic and anti-miR-34b-5p were expressed in HCEC-1CT, HCT-116, and HT-29 cells, and the effect of miR-34b-5p expression changes on *c-MYC* mRNA levels was evaluated. Cells transfected with miR-34b-5p-mimic had similar miR-34b-5p levels (increased approximately ninefold above baseline), whereas HCT-116 and HT-29 cells transfected with anti-miR-34b-5p had a much lower miR-34b-5p level (approximately 0.1-fold) than HCEC-1CT cells (Figure 2D). After verifying the successful overexpression and

suppression of miR-34b-5p in HCEC-1CT, HCT-116, and HT-29 cells, the expression of *c-MYC* was also measured. The results indicated that miR-34b-5p overexpression dramatically decreased *c-MYC* mRNA level, whereas inhibition of miR-34b-5p resulted in a significant induction of *c-MYC* expression (Figure 2E). These results demonstrated that *c-MYC* was a direct target of miR-34b-5p in CRC cells. Previously, *c-MYC* has been reported to be targeted by miR-34b/34c.^{28,29} In our microarray results, only the down-regulation of miR-34b-5p, but not miR-34c (Supplemental Table S2), was observed. To verify if miR-34a (a member of the miR-34 family) and miR-34c were also differentially expressed, their expression was measured in 24-paired CAC tissues. The results showed that miR-34c expression was slightly down-regulated in cancerous tissues, but not miR-34a (Supplemental Figure S4, A and B). The study then determined if miR-34c could target *c-MYC*. Similarly, the potential targets of miR-34c in a database were also predicted (<http://www.mirdb.org>). *c-MYC* was not identified instead of *MYCN* (N-MYC, a member of MYC family) in

Figure 3 Overexpression of miR-34b-5p significantly inhibits the *in vitro* oncogenic phenotypes. **A:** Overexpression of miR-34b-5p decreases cell proliferation. HCT-116-miR-NC (control), HCT-116-miR-34b-5p-mimic, HCT-116-miR-34b-5p-mimic + c-MYC-OE, HCT116-anti-miR-34b-5p, and HCT116-anti-miR-34b-5p + c-MYC-KD cells were applied to determine cell proliferation at 4 hours (0 days) and 1, 2, 3, 4, and 5 days via an MTT assay with absorbance measurement at 590 nm. **B and C:** Overexpression of miR-34b-5p decreases the colony formation ability. The cells used in **A** were seeded in 6-well plates and cultured in serum-free medium for 2 weeks. Cell colonies were then fixed, stained with crystal violet, and imaged (**B**). The colony numbers were quantified (**C**). **D and E:** Overexpression of miR-34b-5p decreases the cell invasion ability. The invasion ability of the cells used in **A** was examined using Boyden chamber assays. Invaded cells were fixed, stained with 0.1% crystal violet, and imaged (**D**); and cell numbers were quantified (**E**). *** $P < 0.001$. Original magnification, $\times 40$ (**D**).

the candidate targets of miR-34c (data not shown). Then, the effect of overexpression or repression of miR-34c on the expression of *c-MYC* and *MYCN* was evaluated. Overexpression or repression of miR-34c-5p significantly repressed or increased *MYCN* expression, but not *c-MYC* (Supplemental Figure S4C), which suggested that *c-MYC* was not a direct target of miR-34c-5p. Thus, the following studies only focus on miR-34b-5p.

It is known that overexpression of *c-MYC* can activate the expression of *CUL4A/4B* and enhance the ubiquitination and degradation of ST7 by CRL4^{DCAF4} E3 ligases.¹¹ This study next sought to evaluate whether miR-34b-5p overexpression and suppression could affect the expression of CRL4B complex components and their substrate ST7. To this end, the protein levels of CUL4A/4B, *c-MYC*, DCAF4, and ST7 in cells expressing miR-34b-5p or anti-miR-34b-5p were examined. As expected, miR-34b-5p overexpression

caused a dramatic decrease in the protein levels of *c-MYC*, CUL4A/4B, and DCAF4 but a significant accumulation of ST7 (Figure 2F and Supplemental Figure S5). In contrast, miR-34b-5p repression significantly increased the protein levels of *c-MYC*, CUL4A/4B, and DCAF4, but there was a dramatic decrease of ST7 (Figure 2F and Supplemental Figure S5). The regulatory role of miR-34b-5p on *c-MYC* expression suggests that aberrant expression of miR-34b-5p might cause tumorigenesis in CAC patients.

Overexpression or Repression of miR-34b-5p Affects the Enrichment of *c-MYC* on the Promoters of *CUL4A/4B*

Because overexpression or suppression of miR-34b-5p could cause inverse effects on *c-MYC* and its targets *CUL4A/4B*, it is hypothesized that the down-regulation or

up-regulation of *CUL4A/4B* was due to decreased or increased binding of *c-MYC*, respectively, to their promoters. To determine this possibility, ChIP assays were conducted in HCEC-1CT-control, HCEC-1CT-miR-34b-5p-mimic, and HCEC-1CT-anti-miR-34b-5p cells. After verifying the successful overexpression or suppression of miR-34b-5p in HCEC-1CT cells (Supplemental Figure S6A), ChIP assays were performed using anti-*c-MYC* antibody. miR-34b-5p overexpression markedly decreased the enrichment of *c-MYC* on the promoters of *CUL4A/4B*, whereas suppression of miR-34b-5p markedly increased this enrichment (Supplemental Figure S6, B and C).

In Vitro Overexpression of miR-34b-5p in CRC Cells Dramatically Impairs Oncogenic Phenotypes

Above results showed that miR-34b-5p overexpression could inhibit the expression of *c-MYC*, implying the possibility of inhibiting CRC cell growth through overexpressing miR-34b-5p. To verify this possibility, HCT116-control, HCT116-miR-34b-5p-mimic, HCT116-miR-34b-5p-mimic + *c-MYC*-OE, HCT116-anti-miR-34b-5p, and HCT116-anti-miR-34b-5p + *c-MYC*-KD cells were generated. After verifying the expression of miR-34b-5p and *c-MYC* (Supplemental Figure S7), cells were applied to evaluate oncogenic phenotypes. The cell viability determination revealed that miR-34b-5p overexpression in HCT-116 cells dramatically repressed cell proliferation in comparison to that of control cells (Figure 3A). Interestingly, overexpression of *c-MYC* in HCT116-miR-34b-5p-mimic cells could reverse this growth inhibition (Figure 3A). Moreover, a slight induction (approximately 20%) in the cell proliferation in cells expressing anti-miR-34b-5p in comparison to control cells (Figure 3A) was observed. Knockdown of *c-MYC* in HCT116-anti-miR-34b-5p cells resulted in a significant decrease in cell proliferation to the same level in HCT116-miR-34b-5p cells (Figure 3A). In addition, colony formation and cell invasion abilities were also evaluated using these cells. Similar to the cell proliferation assay results, a significant decrease (approximately 80%) in colony numbers and invaded cell numbers was seen in cells overexpressing miR-34b-5p compared with those seen in control cells and anti-miR-34b-5p-transfected cells (Figure 3, B–E). Overexpression of *c-MYC* in HCT116-miR-34b-5p-mimic cells reversed the colony numbers and invaded cell numbers (Figure 3, B–E), whereas knockdown of *c-MYC* in HCT116-anti-miR-34b-5p caused a decrease in colony numbers and invaded cell numbers (Figure 3, B–E).

Injection of miR-34b-5p *in Vivo* Markedly Inhibits Tumor Formation and Growth

The significant inhibition of oncogenic phenotypes by miR-34b-5p-mimic transfection led this study to determine the *in vivo* effect of miR-34b-5p overexpression on tumor formation and growth. For this purpose, nude mice were

primarily injected with HCT116-control, HCT116-miR-34b-5p-mimic, and HCT116-anti-miR-34b-5p cells, followed by examining the tumor volumes at 5-day intervals for 30 days. Consistent with the *in vitro* effects, *in vivo* tumor formation and growth results demonstrated that mice injected with HCT116-miR-34b-5p-mimic cells had much smaller tumor volumes than mice injected with the other two cell lines (Figure 4A). In addition, mice injected with HCT116-anti-miR-34b-5p cells exhibited slightly increased tumor volumes in comparison to mice injected with HCT116-control cells (Figure 4A). Next, the protein levels of *c-MYC*, *CUL4A/4B*, and ST7 in tumor tissues were measured from mice injected with the three cell lines. As expected, the protein levels of *c-MYC* and *CUL4A/4B* were dramatically down-regulated in tumor tissues from mice injected with HCT116-miR-34b-5p-mimic cells compared with those in tumors derived from the other two cell lines (Figure 4B). However, the protein level of ST7 was significantly increased in miR-34b-5p-mimic-derived tumors (Figure 4B). Next, it was evaluated whether miR-34b-5p-mimic had a therapeutic effect on tumors. Mice were injected with HCT-116 cells to generate tumors. After 20 days, mice that had tumors with similar volumes were divided into three groups and injected with PBS, 20 nmol/L miR-34b-5p-mimic, or 20 nmol/L anti-miR-34b-5p. Tumor volumes were measured for 30 days, and results suggested that tumor growth in mice injected with miR-34b-5p-mimic was dramatically inhibited in comparison to the other two groups of mice (Figure 4C). However, tumors were not fully eliminated, even at 30 days after injection (Figure 4C). Compared with the PBS-injected mice, the anti-miR-34b-5p-injected mice had a slight increase in tumor volumes (Figure 4C). Similarly, the protein levels of *c-MYC*, *CUL4A/4B*, and ST7 in tumor tissues were examined from three groups of mice. The Western blot analysis results showed that the *c-MYC* and *CUL4A/4B* levels were significantly lower in tumor tissues from mice injected with miR-34b-5p-mimic than in those from mice injected with the other two treatments (Figure 4D). However, the protein level of ST7 was significantly increased in tumors treated with miR-34b-5p-mimic (Figure 4D).

miR-34b-5p Can Be Suppressed by an Intracellular Inflammation

Proinflammatory cytokines can induce the expression of *c-MYC* and its two targets *CUL4A/4B*.¹¹ Thus, this study aimed to examine whether treatments with proinflammatory cytokines could affect miR-34b-5p levels. For this purpose, HCEC-1CT cells were initially treated using six different concentrations of LPS (0, 0.1, 0.4, 0.8, 1.2, and 2.0 µg/mL), a well-known stimulus to induce the production of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Interestingly, results showed that miR-34b-5p expression was gradually suppressed with the increase of LPS concentrations (Figure 5A). Consistent with previous

Figure 4 Overexpression of miR-34b-5p significantly inhibits tumor growth *in vivo*. **A:** Overexpression of miR-34b-5p significantly inhibits tumor formation and growth *in vivo*. HCT-116 cells were transfected with miRNA-NC (control), miR-34b-5p-mimic, and anti-miR-34b-5p separately. The resulting cells were injected intradermally into the flanks of nude mice. Tumor volumes were measured with fine calipers at 5-day intervals for 30 days. **B:** Overexpression of miR-34b-5p significantly decreases the protein levels of c-MYC and CRL4B E3 ligase components. Tumor tissues from mice in **A** were used to measure the protein levels of c-MYC, CUL4A, CUL4B, DCAF4, and ST7 via immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. **C:** Injection of miR-34b-5p-mimic significantly inhibits tumor growth *in vivo*. HCT-116 cells were injected intradermally into the flanks of nude mice. Twenty days after injection, mice with similar tumor volumes (approximately 250 mm³) were randomly divided into three groups and were then injected with miRNA-NC (control), miR-34b-5p-mimic, or anti-miR-34b-5p. Tumor volumes were measured with calipers at 5-day intervals. **D:** Injection of miR-34b-5p-mimic significantly decreases the levels of c-MYC and CRL4B E3 ligase components. Tumor tissues from mice in **C** were used to measure the protein levels of c-MYC, CUL4A, CUL4B, DCAF4, and ST7 via immunoblotting. GAPDH was used as the loading control. *n* = 5 in each group (C). **P* < 0.05, ****P* < 0.001.

results, a dose-dependent induction of *c-MYC* and its targets *CUL4A/4B* was also observed (Figures 5, B and C).¹¹ Next, HCEC-1CT cells were directly incubated using recombinant proinflammatory cytokines, including IL-6 (0, 40, 80, 120, 160, and 200 ng/mL) and TNF- α (0, 4, 8, 12, 16, and 20 ng/mL). Results also indicated that both IL-6 and TNF- α could suppress miR-34b-5p expression in a dose-dependent manner but result in inverse effects on the expression of *c-MYC* and *CUL4A/4B* (Figure 5, D–I).

It is well-known that LPS stimulus can activate toll-like receptor 4 (TLR4)/NF- κ B axis signaling and promote the expression and production of proinflammatory cytokines.³⁰ Results in Figure 5 suggested that the repression of miR-34b-5p might be controlled by NF- κ B because IL-6 and TNF- α were the downstream targets of NF- κ B. To determine this possibility, *p53*, a subunit of NF- κ B, was overexpressed in HCEC-1CT cells. The qPCR results indicated that overexpression of *p53* significantly induced the expression of *IL-1B*, *IL-6*, and *TNFA* (Supplemental Figure S8A). In addition, the concentrations of IL-6 and TNF- α were also measured in the supernatant of medium after cell culture. The ELISA results showed that both IL-6 and TNF- α concentrations were dramatically increased in the supernatant after culturing *p53*-OE cells (Supplemental Figure S8, B and C). These results suggested that overexpression of *p53* might affect miR-34b-5p downstream events. To verify this hypothesis, miR-34b-5p levels and mRNA levels of *c-MYC* and

CUL4A/4B were measured. The results indicated that miR-34b-5p was repressed in *p53*-OE cells, whereas *c-MYC* and *CUL4A/4B* were significantly induced (Supplemental Figure S8D). Moreover, protein levels of critical members of TLR4/NF- κ B signaling to visualize the activation of this pathway *in vivo* were also examined. Our results showed that the total protein levels of TLR4, myeloid differentiation primary response 88, TNF receptor-associated factor 6, and the nuclear proportion of *p53* were significantly accumulated in the three-paired cancerous tissues from CAC patients (Supplemental Figure S8E and Supplemental Figure S9). These results suggested that the activation of TLR4/NF- κ B signaling increased the production of proinflammatory cytokines, which mediated the expression of miR-34b-5p.

miR-34b is controlled by *p53*.³¹ Meanwhile, LPS and IL-6 have been reported to repress *p53* expression.³¹ Thus, it is speculated that inflammatory stimuli might primarily inhibit the expression of *p53*, which further mediated miR-34b-5p level. To verify this possibility, the effects of LPS and IL-6 on *p53* expression in HCEC-1CT cells is initially examined. As shown in Supplemental Figure S10, A and B, both LPS and IL-6 treatments caused a dose-dependent repression of *p53*. Moreover, the expression of *p53* in tissues from 24 CAC patients was also examined. A significant down-regulation of *p53* mRNA level in cancerous CAC tissues (Supplemental Figure S10C) was observed. The *p53* was then overexpressed or knocked down in HCEC-1CT cells,

Figure 5 Intracellular inflammation suppresses the expression of miR-34b-5p. **A:** Lipopolysaccharide (LPS) treatment suppresses miR-34b-5p expression. HCEC-1CT cells were treated with different concentrations (0, 0.1, 0.4, 0.8, 1.2, or 2 $\mu\text{g/mL}$) of LPS for 4 hours. The treated cells were subjected to RNA isolation, followed by real-time quantitative PCR (qPCR) analysis, to measure miR-34b-5p level. **B** and **C:** LPS treatment induces the expression of *c-MYC*, *CUL4A*, and *CUL4B*. The cells used in **A** were used to measure *c-MYC* (**B**) and *CUL4A* and *CUL4B* (**C**) expression. **D:** Recombinant IL-6 treatment suppresses miR-34b-5p expression. HCEC-1CT cells were treated with different concentrations (0, 40, 80, 120, 160, or 200 ng/mL) of IL-6 for 4 hours. The treated cells were subjected to RNA isolation, followed by qPCR analysis, to measure miR-34b-5p level. **E** and **F:** IL-6 treatment induces the expression of *c-MYC*, *CUL4A*, and *CUL4B*. The cells used in **D** were used to measure *c-MYC* (**E**) and *CUL4A* and *CUL4B* (**F**) expression. **G:** Recombinant tumor necrosis factor (TNF)- α treatment suppresses miR-34b-5p expression. HCEC-1CT cells were treated with different concentrations (0, 4, 8, 12, 16, or 20 ng/mL) of TNF- α for 4 hours. The treated cells were subjected to RNA isolation, followed by qPCR analysis, to measure miR-34b-5p level. **H** and **I:** TNF- α treatment induces the expression of *c-MYC*, *CUL4A*, and *CUL4B*. The cells used in **G** were used to measure *c-MYC* (**H**) and *CUL4A* and *CUL4B* (**I**) expression. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus 0.

followed by examining the miR-34b-5p level and mRNA levels of *c-MYC* and *CUL4A/4B*. The results indicated that overexpression of *p53* caused the induction of miR-34b-5p but a decrease of *c-MYC* and *CUL4A/4B*, whereas knock-down of *p53* resulted in the reverse effects (Supplemental Figure S10D). In addition, the effect of IL-6 stimulus in *p53*-OE and *p53*-KD cells was also evaluated. The qPCR results showed that IL-6-treated *p53*-OE and *p53*-KD cells markedly decreased miR-34b-5p level but significantly increased *c-MYC* and *CUL4A/4B* mRNA levels compared with the untreated *p53*-OE and *p53*-KD cells, respectively (Supplemental Figure S10E).

DNA Hypermethylation Occurs in the Promoter of miR-34b-5p

A variety of studies have reported that DNA hypermethylation occurs in the promoter of miR-34b-5p.^{32–34} Thus, this study next aimed to investigate this aspect in the pathogenesis of CAC. First, the CpG percentage in a 2000-bp length of miR-34b-5p promoter region was analyzed. A CpG island was found upstream of region [(-50)–(-550)] of miR-34b-5p (Figure 6A). Primers specifically recognizing this CpG island were then designed to detect the methylated DNA level by quantitative

methylation-specific PCR assays. Using three-paired tissues from CAC patients, methylated DNA level in cancerous tissues was found to be significantly higher (approximately 4.2-fold) than that in noncancerous tissues (Figure 6B). To further verify this observation, the methylated DNA level in six CRC cell lines was measured, and it was found that the average methylated DNA levels were also increased in HCT-15 (approximately 3.0-fold), HCT-116 (approximately 5.9-fold), HT-29 (approximately 4.1-fold), DLD-1 (approximately 2.1-fold), Caco-2 (approximately 2.5-fold), and SW480 (approximately 2.3-fold) cells (Figure 6C). Next, this study aimed to study the effect of DNA methylation inhibitor AZA on the expression of miR-34b-5p, *c-MYC*, and *CUL4A/4B*. For this purpose, HCEC-1CT and HCT-116 cells were treated with AZA and a histone deacetylase inhibitor, trichostatin A, was used as a control. The qPCR assay results indicated that AZA treatment significantly induced the expression of miR-34b-5p but markedly decreased the expression of *c-MYC* and *CUL4A/4B* in both HCEC-1CT and HCT-116 cells (Figure 6, D and E). These results suggested that DNA hypermethylation in the promoter of miR-34b-5p also contributed to its down-regulation in the tumorigenesis of CAC.

Inflammation and DNA Hypermethylation Coregulate the Expression of miR-34b-5p

The above results indicated that both inflammation and DNA hypermethylation contributed to the repression of miR-34b-5p. To further verify these results, the combined effects of AZA and IL-6 treatments on the expression of miR-34b-5p, *c-MYC*, and *CUL4A/4B* were evaluated. Accordingly, both HCEC-1CT and HCT-116 cells were treated with AZA or IL-6 alone and AZA + IL-6, followed by measuring miR-34b-5p, *c-MYC*, and *CUL4A/4B* levels. Consistent with the above results, AZA treatment alone increased miR-34b-5p level but decreased the expression of *c-MYC* and *CUL4A/4B* (Figure 7A). Conversely, IL-6 treatment alone repressed miR-34b-5p but induced the expression of *c-MYC* and *CUL4A/4B* (Figure 7A). The combined treatments of AZA and IL-6 could neutralize these two opposite effects on the expression of miR-34b-5p, *c-MYC*, and *CUL4A/4B* (Figure 7A). ChIP assays were then performed using anti-*c-MYC* antibody to evaluate the effects of AZA + IL-6 treatments on the enrichment of *c-MYC* on the promoters of *CUL4A/4B*. The qPCR results demonstrated that AZA treatment alone markedly decreased

Figure 6 DNA methylation suppresses the expression of miR-34b-5p. **A:** The promoter of miR-34b-5p contains a CpG island. A 2000-bp length of the miR-34b-5p promoter was selected and used to analyze GC percentage. A CpG island located upstream [−50–(−550)] of miR-34b-5p genomic locus was identified. **B:** The methylated DNA level in the CpG island increases in cancerous tissues. Three-paired noncancerous tissues (NTs) and cancerous tissues (CTs) from colitis-associated cancer patients under tumor-node-metastasis stage III were used to isolate genomic DNA and then treated with bisulfite. The resulting DNA was used to determine the DNA methylation level in the CpG island region of the miR-34b-5p promoter with quantitative methylation-specific PCR (qMSP). **C:** The methylated DNA level in the CpG island increases in colorectal cancer (CRC) cell lines. Genomic DNA from six CRC cell lines, including HCT-15, HCT-116, HT-29, DLD-1, Caco-2, and SW480, was treated with bisulfite, followed by qMSP assays, to determine the DNA methylation level in the CpG island region of miR-34b-5p promoter. **D:** 5-Aza-2'-deoxycytidine (AZA) treatment increases miR-34b-5p level. HCEC-1CT and HCT-116 cells were treated with 3 μ Mol/L AZA and 0.5 μ Mol/L trichostatin A (TSA) for 12 hours, respectively. Cells were then used to isolate RNA, followed by determining miR-34b-5p level by real-time quantitative PCR (qPCR). **E:** AZA treatment significantly decreases the expression of *c-MYC* and *CUL4A/4B*. The same cells used in **D** were used to isolate total RNA, followed by determining the expression of *c-MYC* and *CUL4A/4B* by qPCR. $^{**}P < 0.01$, and $^{***}P < 0.001$; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ versus HCEC-1CT.

(approximately 0.3-fold) the enrichment of *c-MYC* on the promoters of *CUL4A/4B*, whereas IL-6 treatment alone significantly increased (approximately fivefold) this enrichment (Figure 7B). The combined treatments of AZA and IL-6 resulted in the enrichment of *c-MYC* to approximately 1.6-fold (Figure 7B). These results suggested that both inflammation and DNA hypermethylation contributed to the repression of miR-34b-5p, thereby mediating the expression of *c-MYC* and *CUL4A/4B*.

Discussion

The up-regulation of the transcription factor *c-MYC* is a hallmark of multiple cancers.³⁵ However, the mechanism underlying *c-MYC* induction is still obscure in different cancers.³⁵ The inflammatory microenvironment in CAC patients is known to induce the expression of *c-MYC*.¹¹ Herein, a transcriptional mechanism mediated by an miRNA in which miR-34b-5p directly targeted *c-MYC* and thus affected *c-MYC* downstream signaling was found. Both *in vitro* and *in vivo* experiments suggested that overexpression of miR-34b-5p could dramatically repress tumor cell growth because it could dramatically disrupt *c-MYC*-mediated downstream signaling. More important, both inflammatory cytokines and DNA methylation

contributed to the down-regulation of miR-34b-5p. In inflammation-mediated regulation, the activation of TLR4/NF- κ B signaling promoted the production of inflammatory cytokines, which repressed p53 level and the p53-targeted miR-34b-5p. In addition, DNA hypermethylation in the promoter of miR-34b-5p also contributed partially to its down-regulation (Figure 8). This study provided evidence to support the cross-links of NF- κ B-, p53-, and *c-MYC*-mediated signaling pathways, which clearly enhance the understanding of how *c-MYC* was activated in CAC tumorigenesis.

The MYC family is a class of transcription factors, and it is composed of three members: *c-MYC*, MYCN, and L-MYC. These members function as oncogenes, and they are amplified in >50% of human cancers.³⁶ Because of the important role of the MYC family and its prevalence in cancers, researchers have made great efforts to screen small molecules that directly target these family members. Direct targeting is currently becoming a challenge owing to the undruggable structures of these proteins.³⁷ Thus, new strategies, such as disruption of the MYC/MAX transcription complex and inhibition of MYC-mediated transcription, are being developed.³⁷ Recently, several interesting findings have revealed that miRNAs can directly regulate *c-MYC* expression in different biological processes. For instance, it was found that Let-7a could target *c-MYC* in Burkitt

Figure 7 Inflammation and DNA methylation coregulate the expression of miR-34b-5p. **A:** The combined effects of 5-aza-2'-deoxycytidine (AZA) and IL-6 treatments on the expression of miR-34b-5p and its downstream molecules. HCEC-1CT and HCT-116 cells were treated with 3 μ M/L AZA, 200 ng/mL IL-6, and 3 μ M/L AZA + 200 ng/mL IL-6 for 12 hours. The resulting cells were used to isolate RNA, followed by determining miR-34b-5p, *c-MYC*, and *CUL4A/4B* levels. **B:** The combined effects of AZA and IL-6 treatments on the enrichment of *c-MYC* on the promoter of *CUL4A/4B*. The same cells used in **A** were used to perform chromatin immunoprecipitation (ChIP) assay with *c-MYC* and IgG antibodies. The purified DNA was applied to real-time quantitative PCR assays to determine the enrichment of *c-MYC* on the promoter of *CUL4A/4B*. * P < 0.05, ** P < 0.01, and *** P < 0.001.

Figure 8 A schematic of the inflammation and DNA methylation—dependent miR-34b-5p suppression and its downstream events in the tumorigenesis of colitis-associated cancer (CAC). Harmful stimuli activate the toll-like receptor 4 (TLR4)/NF-κB signaling axis and cause the translocation of the NF-κB subunits from the cytoplasm to the nucleus, where NF-κB subunits induce the expression of proinflammatory cytokine genes, such as *IL-1B*, *IL-6*, and *TNFA*. The matured proinflammatory cytokines in the cytoplasm inhibit the expression of p53, which can bind to the promoter of miR-34b-5p to regulate its expression. On the other hand, DNA hypermethylation occurs in the CpG island of miR-34b-5p and causes its down-regulation. The decreased miR-34b-5p impairs its inhibitory effect on *c-MYC* and causes the induction of *c-MYC* expression. The activated *c-MYC* further induces the expression of its targets *CUL4A* and *CUL4B*. The increased *CUL4A* and *CUL4B* proteins associate with DNA damage binding protein 1 (DDB1), ring box 1 (RBX1), and DCAF4 to form two CRL4^{DCAF4} E3 ligases, which ubiquitinate the tumor suppressor ST7 and cause its degradation, eventually resulting in the tumorigenesis of CAC. IKK: I-kappa-B kinase; MD2: myeloid differentiation protein-2; MyD88, myeloid differentiation primary response 88; TIRAP: TIR domain containing adaptor protein; TICAM1: toll-like receptor adaptor molecule 1; TNF-α, tumor necrosis factor-α; TRAF6: TNF receptor associated factor 6; TRAM: translocation associated membrane protein; Ub: Ubiquitination.

lymphoma cells.³⁸ Also, miR-449c could directly target *c-MYC* and inhibit the progression of non-small-cell lung cancer cells.³⁹ Also, DNA methylation-dependent repression of miR-449c eliminated its inhibitory effect on *c-MYC* and thus affected cell cycle progression.²⁶ These results remind us that miRNAs may contribute to the regulation of *c-MYC* in CAC. In this study, results clearly demonstrate that miR-34b-5p can directly target *c-MYC* and that miR-34b-5p overexpression markedly inhibits oncogenic phenotypes *in vitro* and suppresses tumor formation and growth *in vivo*. One interesting *in vivo* result was injection of mice with miR-34b-5p-mimic almost eliminated tumors (Figure 4). This promising result implies that injection of miR-34b-5p-mimic may be an effective approach to inhibit tumor growth in CAC patients.

In the human genome, miR-34 family consists of three members: miR-34a, miR-34b, and miR-34c.⁴⁰ They have been found to be differentially expressed and function as tumor suppressors in multiple cancers.^{29,40,41} The current understanding regarding the mechanisms of miR-34 member down-regulation in different cancers mainly includes

DNA hypermethylation in their promoter regions and p53 regulation.²⁹ In this study, a significant down-regulation of miR-34b-5p and a slightly decrease of miR-34c-5p was observed. It is strange because p53 can regulate both miR-34b and miR-34c, and the promoter of miR-34c also has CpG islands (Supplemental Figure S11). Because the overexpression or repression of miR-34c-5p does not significantly change the expression of *c-MYC* instead of affecting *MYCN*, this study only focused on revealing miR-34b-5p regulation. In addition to implicating epigenetic and transcription factor regulatory mechanisms, several publications have also suggested that the intracellular inflammatory status can regulate miRNA levels. For instance, repression of miR-194-5p mediated by inflammation status contributed to the degeneration of intervertebral disc by targeting *CUL4A/4B* simultaneously.⁴² Other studies found that proinflammatory cytokines could induce miR-625-5p level and cause the decrease of its target, *COL1A1* (collagen type I α 1), in the study of disc degeneration.⁴³ This study also found that an inflammatory microenvironment could reduce miR-34b-5p level. These results suggest

that the inflammatory status is an important stimulus regulating miRNA expression, and it may act as an important regulator of miRNA expression in inflammatory diseases. In addition to investigating miR-34b-5p, the targets of the other 35 down-regulated miRNAs were also predicted, and it was found that some of them can target genes involved in inflammation. For example, miR-495-3p, miR-1323, and miR-452-3p were potential to target *IL-1B*, *IL-6*, and *TNFA*, respectively (data not shown). Next steps include determining if these miRNAs are also involved in the tumorigenesis of CAC.

Conclusions

In summary, an inflammatory microenvironment and DNA hypermethylation suppressed the expression of miR-34b-5p, which abolished its inhibitory effect on *c-MYC* expression and caused the significant up-regulation of *c-MYC*. The increased *c-MYC* further activated the expression of *CUL4A/4B*, and their encoded proteins associated with DNA damage binding protein 1, ring box 1, and DCAF4 to assemble CRL4^{DCAF4} E3 ligases, which ubiquitinated ST7 and led to its degradation, eventually causing tumorigenesis. Importantly, the promising effect of miR-34b-5p-mimic on tumor growth inhibition provides a new strategy for CAC therapy.

Author Contributions

H.L. designed the experiments, organized the figures, and wrote the manuscript; C.Y. performed microarray, real-time quantitative PCR, Western blot analysis, *in vitro* oncogenic phenotype assays, and *in vivo* tumor formation assay; W.L. and H.H. assisted with cell cultures and cell treatments and performed chromatin immunoprecipitation assays.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.11.013>.

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