



Repression of MicroRNA Function Mediates Inflammation-associated Colon Tumorigenesis

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See Covering the Cover synopsis on page 465.

BACKGROUND & AIMS: Little is known about the mechanisms by which chronic inflammation contributes to carcinogenesis, such as the development of colon tumors in patients with inflammatory bowel diseases. Specific microRNA (miRNAs) can function as suppressors or oncogenes, and widespread alterations in miRNA expression have been associated with tumorigenesis. We studied whether alterations in miRNA function contribute to inflammation-associated colon carcinogenesis.

METHODS: We studied the effects of inflammatory cytokines, such as tumor necrosis factor, interleukin-1 α (IL1A), and IL1 β (IL1B), on miRNA function, measured by activity of reporter constructs containing miRNA-binding sites in their 3' untranslated regions, in human 293T embryonic kidney, Caco-2, HT29, and HCT116 colon carcinoma cells, as well as *dicer*^{+/+} and *dicer*^{-/-}, and *Apobec3*^{+/+} and *Apobec3*^{-/-} mouse embryonic fibroblasts. Cells were analyzed by immunoblots, immunohistochemistry, and flow cytometry. We generated transgenic mice expressing reporter constructs regulated by LET7B, MIR122, and MIR29b response elements; some mice were given injections of miRNA inhibitors (anti-MIR122 or anti-LET7B), a negative control, or tumor necrosis factor. Liver tissues were collected and analyzed by immunoblotting. Reporter mice were given azoxymethane followed by dextran sulfate sodium to induce colitis and colon tumors; some mice were given the ROCK inhibitor fasudil along with these agents (ROCK inhibitors increase miRNA function). Colon tissues were collected and analyzed by immunohistochemistry, immunoblots, and fluorescence microscopy. **RESULTS:** Incubation of cell lines with inflammatory cytokines reduced the ability of miRNAs to down-regulate expression from reporter constructs; *dicer* was required for this effect, so these cytokines relieve miRNA-dependent reductions in expression. The cytokines promoted degradation of APOBEC3G, which normally promotes miRNA loading into argonaute 2-related complexes. Mice with colitis had reduced miRNA function, based on increased expression of reporter genes. Administration of fasudil to mice did not reduce the severity of colitis that developed but greatly reduced the numbers of colon tumors formed (mean 2 tumors/colon in mice given fasudil vs 9 tumors/colon in mice given control agent). We made similar observations in IL10-deficient mice.

CONCLUSIONS: We found inflammatory cytokines to reduce

the activities of miRNAs. In mice with colitis, activities of miRNAs are reduced; administration of an agent that increases miRNA function prevents colon tumor formation in these mice. This pathway might be targeted to prevent colon carcinogenesis in patients with inflammatory bowel diseases.

Keywords: IBD; Mouse Model; Gene Regulation; Post-Transcriptional Regulation.

Epidemiologic and experimental data suggest a close link between inflammation and tumorigenesis. Immune cells typically infiltrate solid tumors, and inflammation impacts most, if not all, stages of tumorigenesis.¹ Clinically, inflammatory bowel disease, such as ulcerative colitis, is an important risk factor for colon cancer.^{2–4} However, the molecular mechanisms underlying the link between chronic inflammation and tumorigenesis have not been fully clarified.⁵ In addition, although anti-inflammatory molecules, such as anti-tumor necrosis factor (TNF α), anti-interleukin (IL) 6, or anti-IL1 β (IL1B), have been used clinically to prevent inflammation-associated colon tumorigenesis,^{6–8} no definite methods for preventing such tumorigenesis have been established, and novel strategies based on an understanding of the molecular mechanisms may be required.

MicroRNAs (miRNAs) are endogenous approximately 22-nucleotide RNAs that mediate important gene regulation events by base-pairing with messenger RNAs (mRNAs) and directing their repression.⁹ Primary miRNAs, which possess stem-loop structures, are transcribed by RNA polymerase

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Abbreviations used in this paper: Ago2, argonaute 2; AOM, azoxymethane; DSS, dextran sulfate sodium; GEO, Gene Expression Omnibus; GFP, green fluorescent protein; IL, interleukin; IL1A, interleukin 1 α ; IL1B, interleukin 1 β ; MEF, mouse embryonic fibroblast; miRNA, microRNA; mRNA, messenger RNA; RISC, RNA-induced silencing complex; ROCK, Rho-associated, coiled-coil containing protein kinase; TNF, tumor necrosis factor; UTR, untranslated region.

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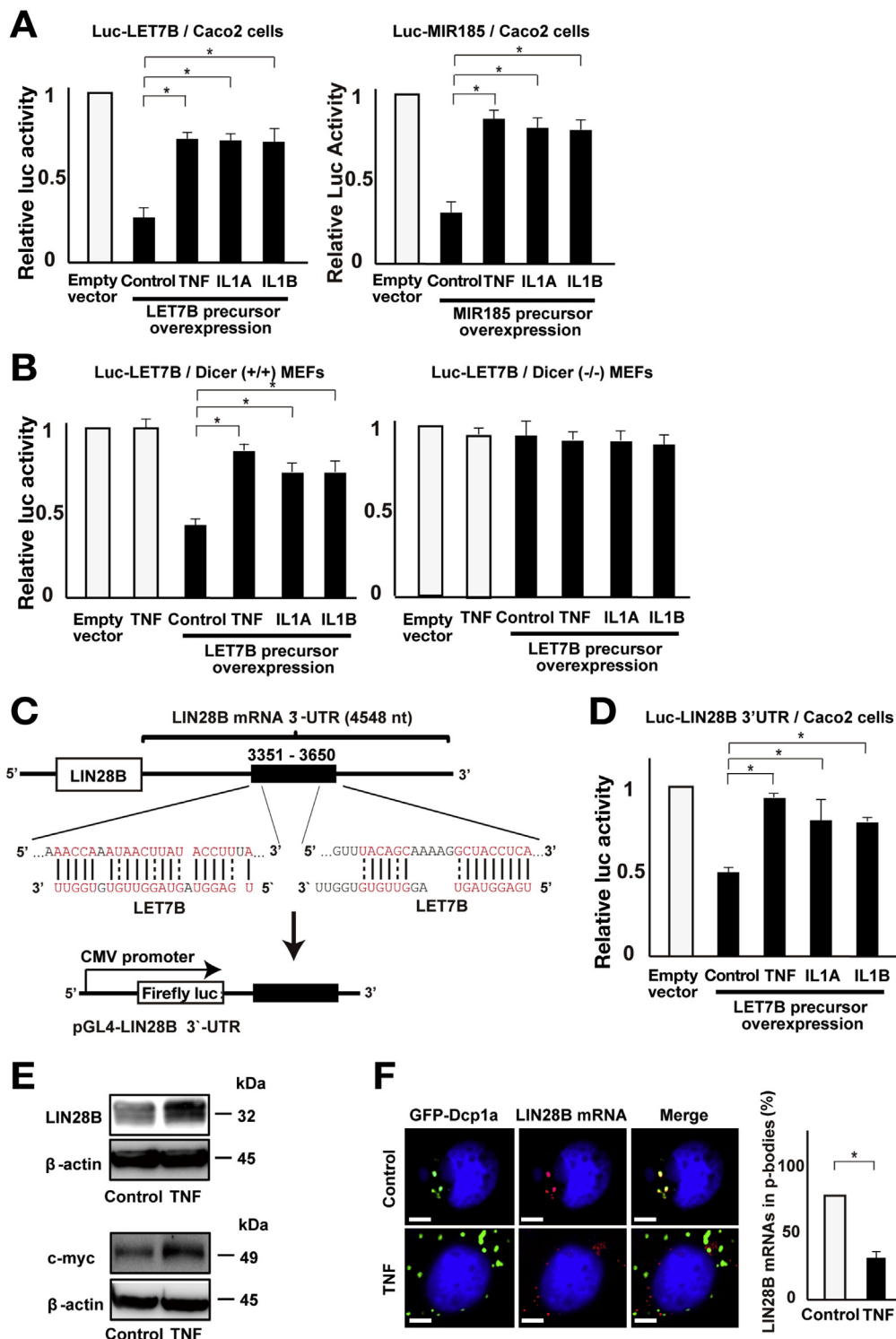
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IL¹⁰ and are processed in the nucleus.¹¹ The processed products, pre-miRNAs, are transported to the cytoplasm via exportin-5.^{12,13} The pre-miRNAs are further cleaved into mature miRNAs by Drosha and Dicer RNA polymerase III. Mature miRNA duplexes are loaded onto an RNA-induced silencing complex (RISC) and are unwound into the single-stranded mature form.¹⁴⁻¹⁶

Although specific miRNAs can function as either tumor suppressors or oncogenes in tumor development, a general

reduction in miRNA expression is commonly observed in human cancers.¹⁷⁻²⁰ In addition, impaired global miRNA expression, but not complete loss of miRNAs, has been shown to promote cellular transformation and tumorigenesis.²¹⁻²⁴

Similar to reduced miRNA levels, impaired miRNA function may be crucial in the tumorigenesis. The efficiency of miRNA-mediated gene suppression can be reduced when cells are exposed to stress.²⁵ In addition, our previous study showed that small interfering RNA-mediated mRNA decay,



which shares certain features with miRNA-mediated gene suppression, was inhibited when cells underwent inflammatory stress via TNF or IL1B stimulation.²⁶ Therefore, inflammatory stress might globally inhibit miRNA function. It is important to determine whether such inhibition can promote tumorigenesis, similar to global reduction in miRNA expression, when studying inflammation-associated carcinogenesis.

In this study, we examined miRNA function during inflammatory stimulation *in vivo* and *in vitro* and found that inflammation suppresses miRNA function by decreasing miRNA loading onto argonaute 2 (Ago2)-related RISC. Using Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor, which we previously found enhances remaining miRNA function by promoting miRNA-mediated mRNA decay,²⁷ we showed that enhancing miRNA function suppressed colitis-associated tumorigenesis in mice, as a model system of colon tumorigenesis associated with ulcerative colitis. This suggests that global impairment of miRNA function in chronic inflammation may have a causal role in inflammation-associated oncogenesis, and enhancement of miRNA function is a potential method for preventing inflammation-associated tumorigenesis.

Materials and Methods

Detailed protocols are provided in the [Supplementary Materials and Methods](#), and primer sequences are shown in [Supplementary Table 1](#).

Statistical Analysis

Significant differences between groups were determined with Student *t* test when variances were equal. When variances were unequal, Welch's *t* test was used. A *P* value <.05 was considered statistically significant.

Results

Obligate Haploinsufficient Role of Dicer in Cancer

There are already many reports describing the reduced expression of global miRNA levels or the obligate haploinsufficient role of Dicer in cancer.^{20–23} Consistently, increased numbers of tumors were observed in *Dicer*^{+/-} mice after treatment with AOM alone, which had reduced

miRNA levels²³ and global miRNA function impairment ([Supplementary Figure 1A](#)). However, *Dicer*^{Δ/Δ} mice did not show such increased tumorigenesis, confirming that Dicer works in an obligate haploinsufficient manner²³ ([Supplementary Figures 1A](#)). Although obligate haploinsufficiency of Dicer in cancer is almost certain, the molecular mechanism of oncogenesis induced by global miRNA dysfunction has remained largely unknown. To gain a better understanding of the oncogenic mechanism induced by impaired miRNA function, we established colon cancer cell lines with controllable expression of Dicer protein by doxycycline ([Supplementary Figures 1B and C](#)). Comprehensive gene expression profiles were determined by complementary DNA microarrays using wild-type cells, cells with complete deletion of Dicer, and cells with intermediate Dicer protein expression levels, which resemble reduced global miRNA function ([Supplementary Figure 1D](#), accession no. GSE 85909 in Gene Expression Omnibus database). Based on the results, gene sets and intracellular signaling pathways that were changed specifically in the cells with intermediate Dicer protein levels were identified ([Supplementary Figure 1E](#), and [Supplementary Tables 2 and 3](#)). Although further analyses will be required, these gene sets and intracellular signaling pathways may be involved in oncogenesis induced by the obligate haploinsufficiency of Dicer or global miRNA functional impairment. These results, together with previous reports that Dicer-deficient cells and mice are prone to transformation and tumorigenesis,^{20–23} suggest that the functional impairment of global miRNAs may promote colon tumorigenesis.

Inflammatory Stimuli Reduce MicroRNA function

We previously showed that small interfering RNA function was inhibited by inflammatory stimuli.²⁶ To determine whether inflammation also interferes with miRNA-mediated gene repression, we used luciferase reporter constructs containing miRNA-binding sites in their 3'-untranslated regions (UTRs) to evaluate the effect of inflammatory cytokines on miRNA-mediated inhibition of gene expression. Treatment of cells with TNF, IL1A, or IL1B reversed representative miRNA-mediated inhibition of luciferase reporter expression, such as LET7B or MIR185 ([Figure 1A](#) and [Supplementary Figure 2A](#)). These effects were

Figure 1. Inflammatory stimuli reduce miRNA function. (A) miRNA function was restored by various inflammatory cytokines. Luciferase reporter constructs carrying corresponding miRNA binding sites in their 3'-UTRs were used to examine miRNA function. The suppressive effects of miRNA precursor expression (LET7B, *left panel*; MIR185, *right panel*) on luciferase activity were reversed by inflammatory cytokines in Caco-2 cells. Data represent the mean ± SD of 3 experiments. **P* < .05. (B) Restoration of miRNA function by inflammatory stimuli was miRNA-dependent. Reporter activity suppression by the LET7B precursor was reversed by inflammatory stimuli in wild-type cells but not in Dicer-knockout cells. Data represent the mean ± SD of 3 experiments. **P* < .05. (C) A luciferase reporter carrying a region of the LIN28B 3'-UTR (nucleotides 3351–3650) containing 2 putative LET7B target sites was used to assess the effect of LET7B on endogenous gene sequences. Putative binding sequences are indicated in *red*. (D) The effect of miRNA on a reporter carrying wild-type LIN28B sequences in Caco-2 cells was reversed by various inflammatory cytokines. Data represent the means ± SD of 3 experiments. **P* < .05. (E) LIN28B and c-myc expression, both with 3'-UTRs that are LET7 targets, were up-regulated in Caco-2 cells by TNF. (F) *Left panels*: LIN28B mRNA was relocated from P-bodies to the cytosol after stimulation of Caco-2 cells with TNF. P-bodies were visualized by measuring GFP-Dcp1a (*green*), and LIN28B was visualized with *in situ* hybridization (*red*). 4',6-Diamidino-2-phenylindole (*blue*) was used to stain nuclei. Bar = 25 μm. *Right panels*: The percentages of LIN28B dots co-localized with P-bodies are shown based on 60 cells from 3 independent experiments. Data represent the means ± SD. **P* < .05.

Dicer-dependent, as they were not observed in *Dicer*-knockout mouse embryonic fibroblasts (MEFs) (Figure 1B and Supplementary Figures 2B and C), confirming that these inflammatory cytokines relieve miRNA-dependent gene repression. The effects were not observed when using reporters with mutations in the miRNA response elements, suggesting that they were miRNA function-specific (Supplementary Table 4). Other miRNAs examined showed similar tendencies in detecting endogenous miRNA function, without miRNA precursor overexpression (Supplementary Tables 4 and 5).

In addition, cytokine-mediated de-repression of miRNA-targeted genes was observed when using a reporter construct bearing natural LIN28B 3'-UTR sequences, which are known targets of LET7B (Figures 1C and D, Supplementary Figure 2D, and Supplementary Table 4).^{28,29} TNF consistently increased endogenous LIN28B protein levels in Caco-2 cells (Figure 1E). LIN28B protein expression levels, reporter activities with 3'-UTR sequences, and c-myc protein expression, another let-7-target,^{20,29,30} were also up-regulated by TNF, IL1A, or IL1B stimulation (Figure 1E and Supplementary Figures 2D and E). This up-regulated expression was not due to changes in the promoter activities of the target mRNAs (Supplementary Figures 2F and G). Experiments using other inflammatory stimuli showed that, of 13 cytokines examined, TNF, IL1A, and IL1B significantly inhibited miRNA function (Supplementary Tables 4 and 5). Cytokine-induced inhibition of miRNA function was unlikely to be executed at the level of miRNA expression because published analyses of miRNA profiles excluded this possibility.²⁵ We also confirmed that most miRNA expression levels were unchanged by TNF, IL1A, or IL1B treatment using miRNA microarrays (GEO accession no.: GSE37288; Supplementary Figure 2H).

Messenger RNA translocation to cellular processing bodies (P-bodies) has been suggested to be involved in miRNA-mediated translational suppression.²⁵ Our in situ hybridization revealed that LIN28B mRNA was concentrated in P-bodies, as demonstrated by its co-localization with the P-body marker green fluorescent protein (GFP)-Dcp1a in control cells, whereas TNF treatment led to relocalization of LIN28B mRNAs from P-bodies to the cytosol (Figure 1F). These data are consistent with a previous report that stress induces relocation of miRNA-targeted mRNA.²⁵ However, as miRNA-targeted mRNA localization to P-bodies is the final step of miRNA-mediated repression, their relocation may be either the cause or the consequence of the impairment of the miRNA-mediated repression pathway. Regardless, our results demonstrated that some inflammatory cytokines, such as TNF, exert a general suppressive effect on miRNA-mediated gene repression.

MicroRNA Function Is Impaired During Inflammation-Associated Colon Tumorigenesis in vivo

To monitor changes in miRNA function in vivo, we generated a GFP-based reporter transgenic mouse strain

bearing cytomegalovirus-promoter-driven GFP with LET7B, MIR122, and MIR29B response elements as representative miRNAs in the 3'-UTR of GFP mRNA (Figure 2A). In these mice, GFP expression levels were used to evaluate LET7B, MIR122, and MIR29B activity in vivo. To confirm that the reporter enabled monitoring of miRNA function, we first detected an increase in GFP reporter expression upon stimulation with TNF in MEFs derived from reporter mice (Supplementary Figure 3A). Next, we injected in vivo-ready anti-MIR122 or anti-LET7B oligonucleotides into the tail vein of these mice and determined the changes in hepatic GFP expression levels. As expected, GFP expression levels were enhanced by shutting down MIR122 or LET7B function using anti-miRNA oligonucleotides, whereas no enhancement was observed when negative control oligonucleotides were injected (Figure 2B), suggesting that the reporter mouse can be used to monitor miRNA function in vivo. In addition, we injected TNF into the tail vein and confirmed the enhanced GFP expression levels in the liver, suggesting that cytokine stimuli suppressed miRNA function, as observed in the in vitro experiments (Supplementary Figure 3B).

We treated 8- to 12-week-old reporter mice with a single dose of azoxymethane (AOM, 12.5 mg/kg), followed by 3 cycles of dextran sulfate sodium (DSS, administered in drinking water) to induce colitis-associated tumors (Figure 2C). AOM is a procarcinogen that forms O⁶-methylguanine upon metabolic activation³¹ and is commonly used to induce colorectal cancer in experimental animals.³² Repeated DSS administration causes chronic inflammation characterized by elevated inflammatory cytokine levels, such as TNF, IL1A, and IL1B,³³ which greatly enhances the incidence of AOM-induced tumors.³² GFP expression increased significantly, particularly in epithelial cells, during DSS-induced colitis, as determined with immunohistochemistry (Figure 2D) and Western blots using isolated colonic epithelial cells (Figure 2E), suggesting that miRNA function was impaired during colitis induction in vivo. Levels of cytokines, including TNF and IL1B, were indeed increased after inflammation was induced by DSS treatment in accordance with the increased GFP expression in the colonic epithelial cells (Supplementary Figures 3C and D). This was consistent with our in vitro data showing that inflammatory cytokines inhibited miRNA function (Figure 1). The miRNA function specificity confirmed that GFP expression levels in the colon of the mutant reporter mice with mutations in miRNA response elements did not significantly change before and after the induction of inflammation (Supplementary Figures 3E and F). These results suggest that functional impairment of global miRNAs by inflammatory stimuli can promote inflammation-associated tumorigenesis, through resembling obligate haploinsufficiency of *Dicer*.

MicroRNA Loading Into RNA-Induced Silencing Complex Decreases Under Inflammatory Stimuli

To determine the mechanisms by which inflammatory stimuli reduce miRNA function, we examined the expression

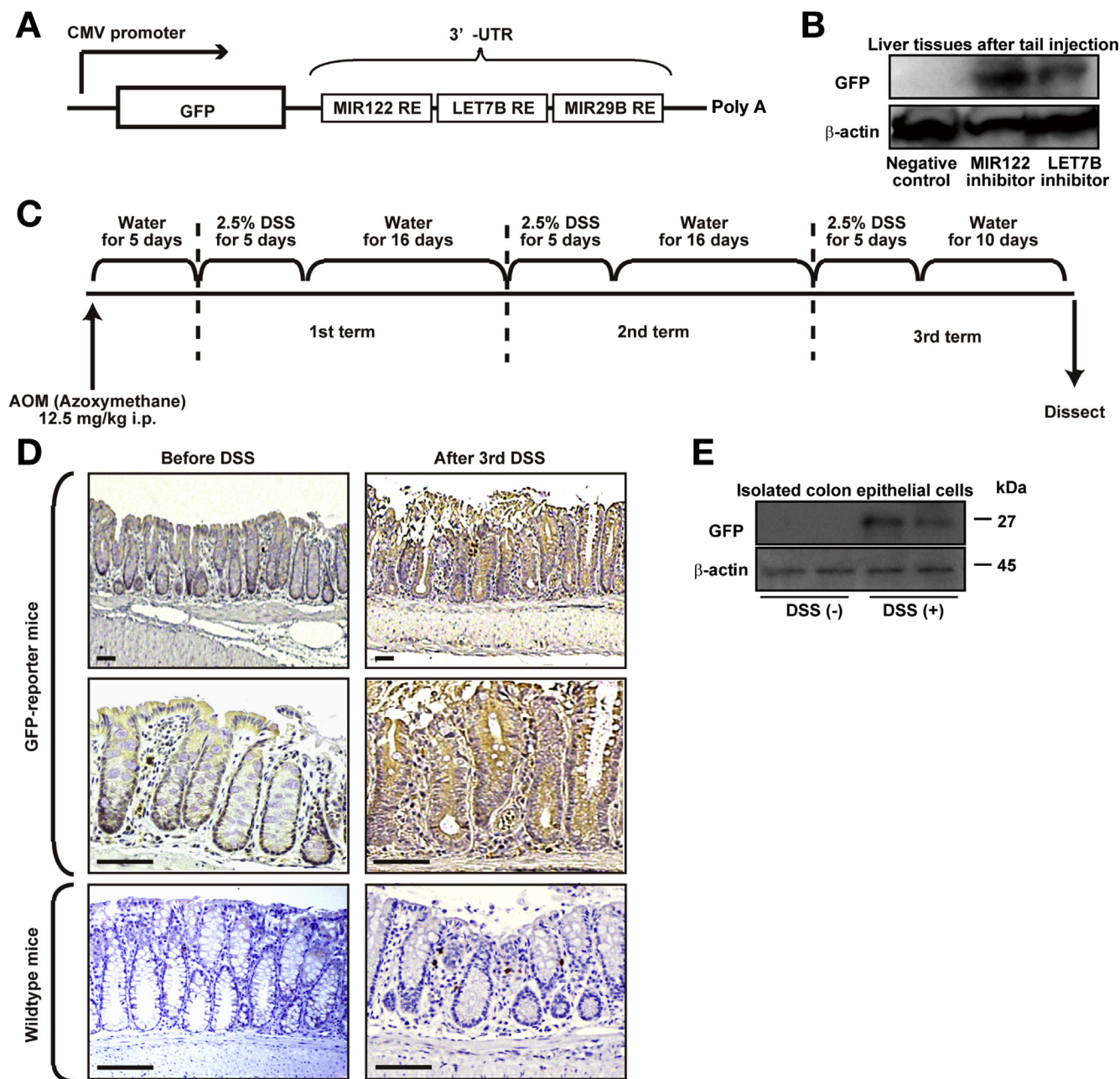


Figure 2. miRNA function is impaired during inflammation-associated colon tumorigenesis in vivo. (A) The construct for GFP-based reporter transgenic mice containing cytomegalovirus promoter-driven GFP complementary DNA with MIR29B, LET7B, and MIR122 target sequences in its 3'-UTR. (B) Representative images from 3 independent Western blot experiments for GFP expression in the liver 24 hours after injection of liposome-conjugated in vivo-ready anti-MIR122, anti-LET7B, or negative control oligonucleotides into the tail vein of reporter mice. GFP expression increased after administration of anti-MIR122 and anti-LET7B but not the negative control oligonucleotides. (C) Protocol for experimental inflammation-associated colon tumorigenesis used in this study. Colon tumors were induced by treatment with AOM plus DSS. (D) Immunohistochemical analyses of GFP expression in the colon before and after inducing inflammation. The sections shown are 30 mm proximal to the anal canal. Bars = 200 μm. Lower panels show magnified images of the glands in the upper panels. Similar results were obtained from 3 independent sets of mice. The bottom panels show anti-GFP stained images colon tissue of wild-type mice (non-reporter) as negative controls. (E) Representative Western blot images for GFP expression in isolated colonic epithelial cells before and after induction of inflammation. GFP expression increased after inducing inflammation.

levels of miRNA pathway-related molecules (Dicer, Ago2, MOV10, Dead end protein homolog 1, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G [APOBEC3G; A3G], TNRC6 [ie, GW182], tripartite motif

containing 65, and HuR^{34–36}) in the presence or absence of TNF, IL1A, IL1B, IL6, or IL8. Of these, A3G protein levels were decreased exclusively by TNF, IL1A, and IL1B (Figure 3A), which are the cytokines that suppress miRNA

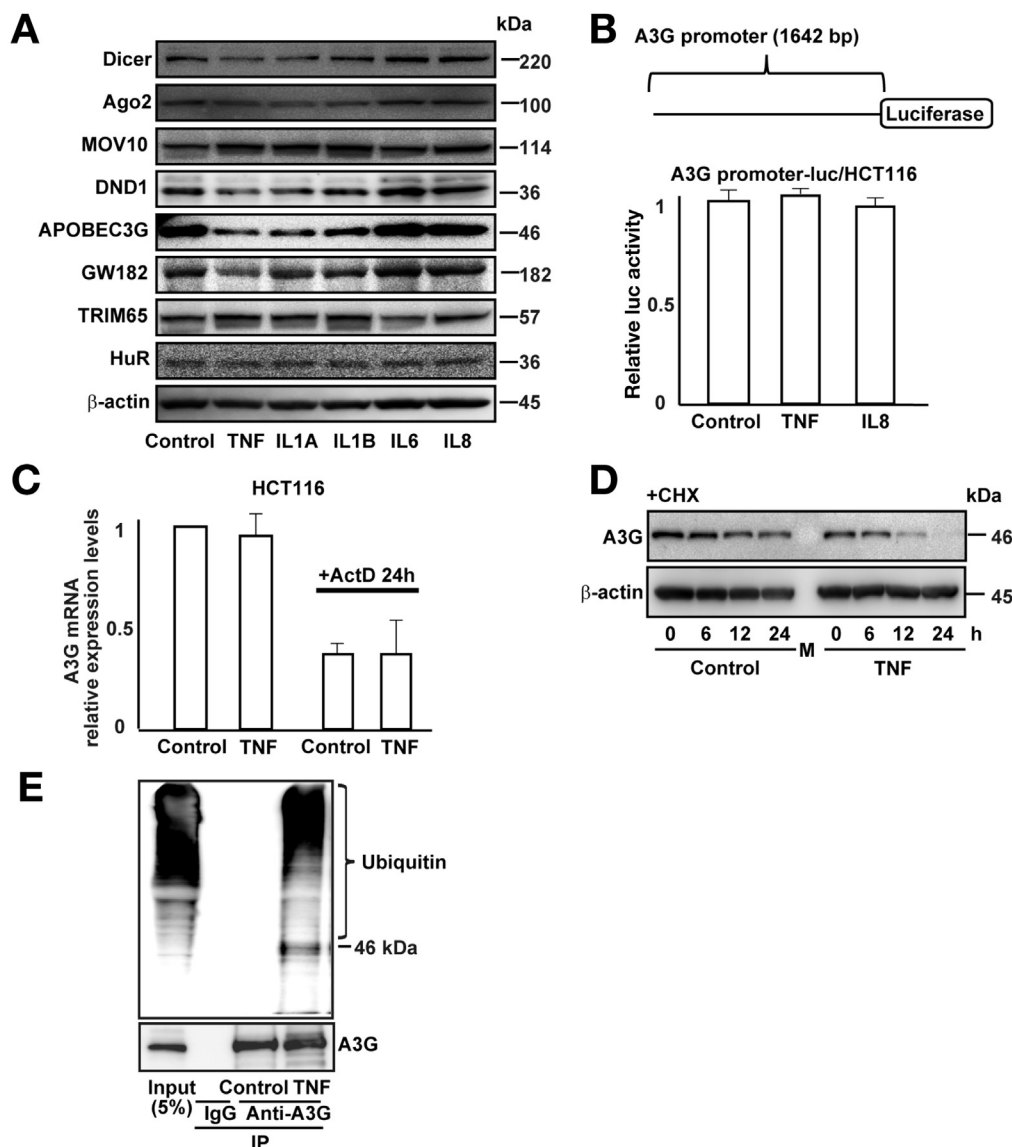


Figure 3. A3G protein levels decrease under cytokine stimulation. (A) HCT116 cells were treated with the indicated cytokines for 24 hours. Representative Western blot results are shown for 2 independent experiments. A3G protein levels decreased by TNF, IL1A, and IL1B treatment. Similar results were obtained using HT29 cells. (B) A3G promoter activities were not affected by TNF or IL8 treatment. The luciferase construct driven by the A3G promoter (*upper*) was transiently transfected into HCT116 cells, which were stimulated with cytokines for 24 hours before the assay. Relative luciferase levels were determined after the control sample levels were set to 1. Data represent the means \pm SD of 3 independent experiments. (C) A3G mRNA decay was not enhanced by TNF. A3G mRNA levels in HCT116 after exposure to TNF for 24 hours were determined by quantitative reverse transcription polymerase chain reaction. To exclude the effects of newly transcribed mRNAs, actinomycin D (ActD) was added for 24 hours. Data represent the means \pm SD of 3 independent experiments. (D) A3G protein degradation was enhanced by TNF. Representative images are shown for 2 independent experiments. M, marker. (E) Ubiquitination of A3G protein was enhanced by TNF. HCT116 cells were transiently transfected with HA-tagged ubiquitin-expressing plasmids. A3G proteins were precipitated with anti-A3G antibodies 3 hours after adding TNF. Ubiquitination was analyzed by Western blotting using anti-HA antibodies. The results shown are representative of 3 independent experiments, and 5% of the total cell lysate was used as an internal control ("input").

function (Figure 1). Although the promoter activities and mRNA levels of A3G did not decrease (Figures 3B and C), A3G protein degradation was significantly increased by TNF (Figure 3D) through enhanced ubiquitination (Figure 3E and Supplementary Figure 4A).

Consistent with previous reports that A3G is involved in Ago2-related complexes^{37–39} and regulates miRNA function,^{34,40,41} A3G interacted with Ago2. However, in parallel

with the decreased expression of A3G under TNF stimulation, the amount of A3G bound in Ago2-related complexes was also decreased under TNF stimulation, as revealed by immunoprecipitation and immunocytochemistry (Figures 4A and B). The *in vitro* binding assays showed that A3G bound with Dicer and Ago2, suggesting that they form a ternary complex (Supplementary Figure 4B). MicroRNA maturation was not affected by A3G in the

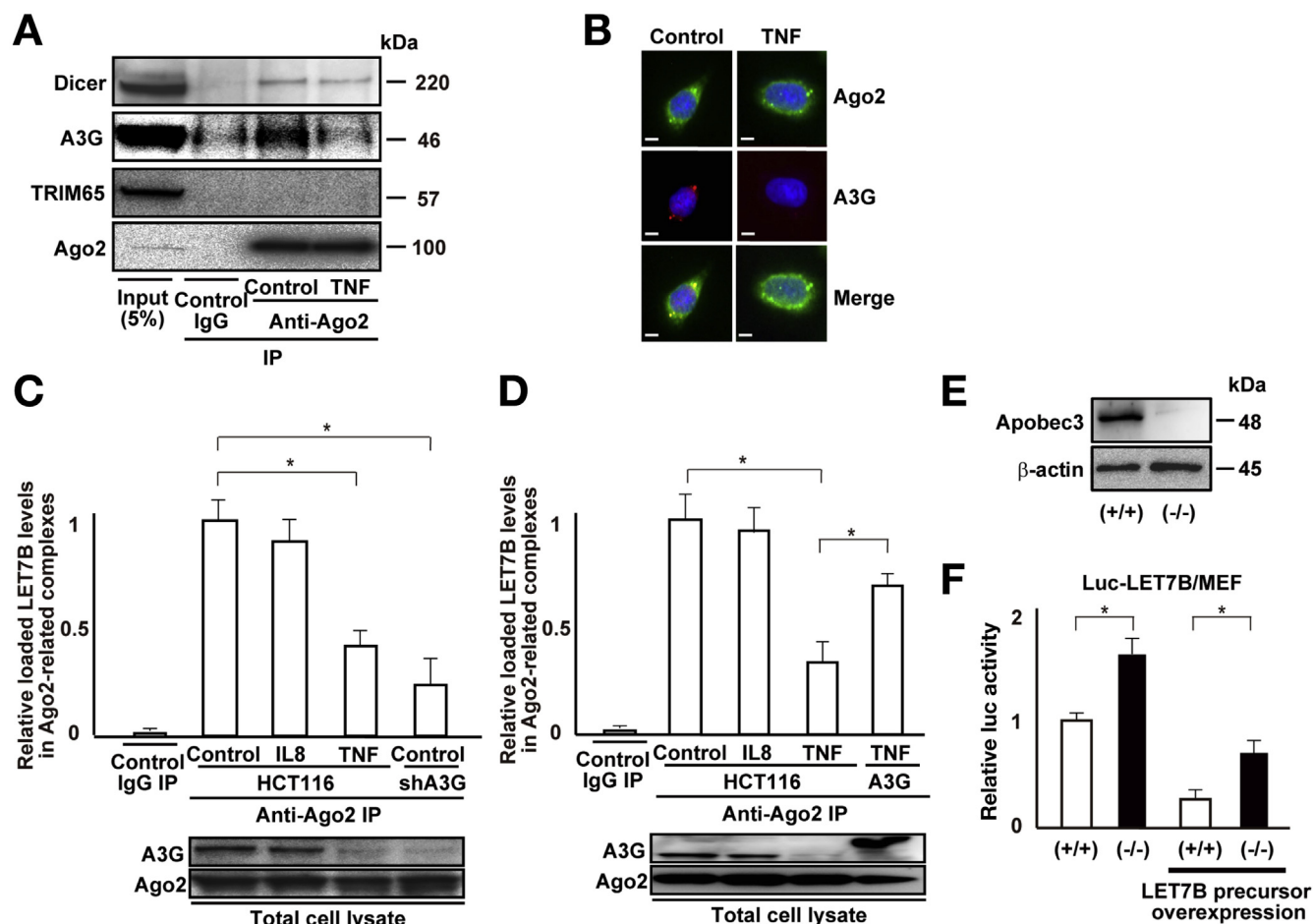


Figure 4. miRNA levels recruited into RISCs decrease under inflammatory cytokines. (A) A3G binds with Ago2. HCT116 cells were treated or untreated with TNF for 24 hours, followed by immunoprecipitation using mouse anti-Ago2 antibody. Mouse IgG was used as a negative control. Precipitated lysates were used for Western blots with antibodies against the indicated proteins, and 5% of the total cell lysate was used as an internal control ("input"). The results are representative of 4 independent experiments. (B) HCT116 cells transiently transfected with halo-tagged A3G-expressing plasmids were treated or untreated with TNF for 24 hours. Cells were immunostained using antibodies against Ago2 (green) and halo (red). Nuclei were stained with 4',6-diamidino-2-phenylindole. Representative images are shown for 3 independent experiments. Bar = 25 μ m. (C, D) TNF decreased miRNA loading into RISCs. RISCs in HCT116 cells were precipitated. LET7B levels in the complexes were determined with quantitative reverse transcription polymerase chain reaction. *Left:* A3G knockdown (shA3G) decreased the loading levels. *Right:* A3G overexpression reversed the decreased loading of LET7B induced by TNF. IL8 was used as a negative control. LET7B levels in RISCs from the control samples were set to 1. Data represent the means \pm SD of 3 experiments. The *lower panels* show A3G and Ago2 protein levels in the total cell lysate determined with Western blots. * P < .05. (E) Confirmation of APOBEC3 protein in wild-type (+/+) and *Apobec3*-deleted (-/-) MEFs by Western blotting. Representative images of 2 experiments are shown. (F) Reporter activity suppressed by LET7B was de-repressed in *Apobec3*-deleted MEFs. Similar effects were observed irrespective of LET7B precursor overexpression. Data represent the means \pm SD of 3 experiments. White bars: wild-type (+/+) MEFs; black bars: *Apobec3*-deleted (-/-) MEFs. * P < .05.

in vitro analyses, but recruiting the matured miRNA into Ago2-related complexes was promoted with the existence of A3G protein in vitro (Supplementary Figures 4C and D). Representative mature miRNA levels were not affected by TNF, irrespective of A3G overexpression (Supplementary Figure 4E), whereas mature miRNA levels loaded into RISCs decreased in cells treated with TNF, with similar effects observed in A3G knockdown cells (Figure 4C), which were antagonized by A3G overexpression (Figure 4D and Supplementary Figure 4F). Similar effects were observed for APOBEC3, a mouse homologue to human A3G.⁴² miRNA function was de-repressed in *Apobec3* gene-deleted MEFs

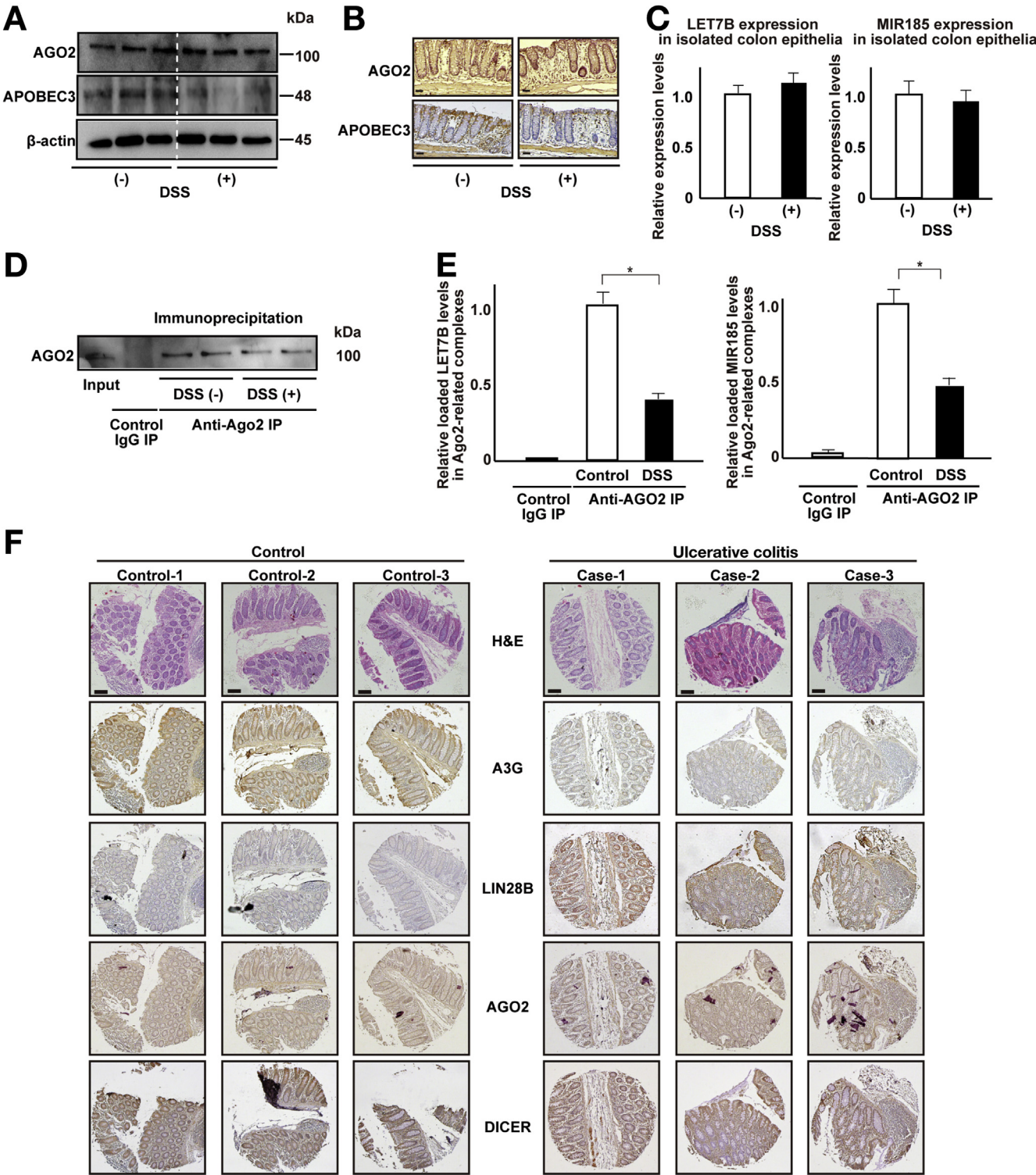
(Figures 4E and F, and Supplementary Figure 4G). Similar to A3G, APOBEC3 binds Ago2 (Supplementary Figure 4H), and the loading levels of mature miRNAs into RISCs were decreased in *Apobec3*-gene-deleted MEFs (Supplementary Figure 4I).

A3G Protein Levels in Colon Epithelia Decrease During Chronic Inflammation

Next, we examined mouse and human colon epithelial cells. APOBEC3 protein expression levels decreased in isolated mouse colonic epithelial cells with DSS-induced colon

inflammation in vitro (Figures 5A and B). Although representative mature miRNA levels in isolated colonic epithelial cells did not change significantly before and after induction of inflammation (Figure 5C and Supplementary Figure 5A), miRNA levels loaded into RISCs in the cells were significantly decreased after inflammation was induced (Figures 5D and E and Supplementary Figure 5B), similar to the effects observed after TNF treatment in vitro.

Whereas Ago2 and Dicer protein levels were comparable to those in the controls in human colon tissue, A3G protein expression levels were clearly lower in ulcerative colitis patients (Figure 5F and Supplementary Figure 5C). Concomitantly, LIN28B protein levels were up-regulated in consecutive sections of ulcerative colitis tissue (Figure 5F and Supplementary Figures 5D and E), consistent with the in vitro results.



Enhancing MicroRNA Function Suppresses Inflammation-Associated Colon Tumorigenesis

We reported previously that ROCK inhibitors enhance miRNA function.²⁷ Specifically, fasudil, a ROCK inhibitor, reversed miRNA function suppressed by TNF (Supplementary Figure 6A). To examine whether the pharmacologic augmentation of miRNA function can suppress tumorigenesis and help determine whether the global impairment of miRNA function during chronic inflammation has a role in tumor initiation or promotion, we applied fasudil to an AOM plus DSS-induced inflammation-associated colon tumor mouse model. Mice received a standard dose of fasudil in their drinking water (100 mg/kg/d) starting on day 6 of DSS administration.⁴³ Immunohistochemical detection of GFP in reporter mice confirmed a reduction in miRNA function during colitis and reversal by fasudil (Figure 6A), whereas no significant effects by fasudil were detected without DSS-induced colitis (Supplementary Figures 6B and C). The protein expression levels of LIN28B and c-myc, representative miRNA Let7 targets, which are often de-regulated during colon tumorigenesis,^{29,44,45} underwent changes similarly to those in GFP expression (Supplementary Figure 6C). The severity of inflammation was almost the same in the control and fasudil groups in terms of body weight loss (Figure 6B), final colon length (Figure 6C), and inflammatory scores in the histologic analysis (Figure 6D and Supplementary Figure 6D), likely because the inflammation is induced by bacteria in this model. However, there were significantly fewer colon tumors at day 62 in fasudil-treated mice (approximately 2 tumors per mouse) than in control mice (approximately 9 tumors per mouse) (Figures 6E and F). Although increased c-myc and LIN28B expression were observed in control mice, their expression was significantly lower in the background colon tissue of fasudil-treated mice (Supplementary Figures 6C, E, and F), suggesting that global impairment of miRNA function during chronic inflammation may lead to tumor initiation. We subsequently showed that fasudil had no effects on the promoter activities of such genes in vitro (Supplementary Figure 6G), suggesting that the suppressive effects of fasudil treatment on gene expression were

dependent on miRNA function. In addition, because the tumor-suppressive effects of fasudil and the suppression of induced LIN28B and c-myc expression in inflammatory colonic mucosa by fasudil were lost when Dicer-deficient mice were used (Supplementary Figures 6H and I), the effects were likely miRNA-dependent. In addition, fasudil did not suppress tumorigenesis in *Apc*^{Δ14/+} mice with no significant differences of gross tumor morphology, including size (Supplementary Figure 6J), in which colon tumors occur spontaneously at around 5–6 months after birth due to the disruption of the *Apc* gene,⁴⁶ suggesting that the tumor-suppressive effects of fasudil treatment in the inflammation-associated cancer model were not due to direct inhibitory effects on cell growth.

To confirm these results in another in vivo model, we used IL10-deficient mice (Supplementary Figure 7A) without DSS treatment. These mice develop spontaneous intestinal inflammation as a result of the IL10 deficiency.^{47–49} TNF and IL1B in the intestinal tissues increase with age because of the intestinal inflammation in this model (Supplementary Figure 7B). Although the severity of inflammation was nearly identical in the control and fasudil groups (Supplementary Figures 7B and C), there were fewer colon tumors at day 182 (26 weeks) in fasudil-treated mice (approximately 1 tumor per mouse) than in control mice (approximately 4 tumors per mouse) (Supplementary Figures 7D and E), consistent with the results of DSS-induced tumorigenesis. Together, these results suggest that inflammation-associated tumors can be suppressed by augmenting miRNA function.

Discussion

We report that miRNA function is generally suppressed during inflammation and that impairment of global miRNA function causes inflammation-associated tumorigenesis in a mouse colitis-induced tumor model. In addition, pharmacologic augmentation of miRNA function prevents inflammation-associated tumorigenesis. These results provide information on the pathogenesis of inflammation-associated tumorigenesis and identify new potential

Figure 5. A3G protein levels in colon epithelia decrease by chronic inflammation. (A) Ago2 and APOBEC3 protein levels in mouse colon epithelial cells before and after 3 cycles of DSS treatment, determined with Western blots. Representative images of 2 independent experiments using 3 mice in each group are shown. (B) APOBEC3 protein expression levels in mouse colon epithelia decreased after one cycle of DSS treatment, determined by immunohistochemistry. Ago2 protein was stained for comparison. Similar results were obtained from 3 independent sets of mice. Bars = 200 μ m. (C) Mature miRNA expression levels in mouse colon epithelial cells were similar before and after 1 cycle of DSS treatment, determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Relative expression levels are shown as the means \pm SD of 2 independent experiments using 3 mice in each group, after adjusting the values from the cells before DSS treatment to 1. **P* < .05. (D) The levels of Ago2 protein immunoprecipitated from isolated mouse colon epithelial cells before and after 1 cycle of DSS treatment were comparable, determined with Western blots. The results of 2 mice in each group are shown. Immunoprecipitation using control IgG was used as a negative control, and 10% of the samples before immunoprecipitation are shown as an internal control ("input"). (E) miRNA levels loaded into RISCs in mouse colon epithelial cells decreased after 1 cycle of DSS treatment. RISCs in the isolated mouse colon epithelial cells were immunoprecipitated. miRNA levels in the complexes were determined by qRT-PCR. Data represent the means \pm SD of 2 independent experiments using 2 mice in each group. Only minimal miRNAs were detected in the precipitants precipitated with control normal IgG. **P* < .05. (F) The protein expression status in human tissues from control and ulcerative colitis patients was determined using immunohistochemistry. Consecutive sections from each patient were stained (*in brown*) with antibodies against the indicated proteins. Representative results from 3 cases in each group are shown. Bars = 500 μ m.

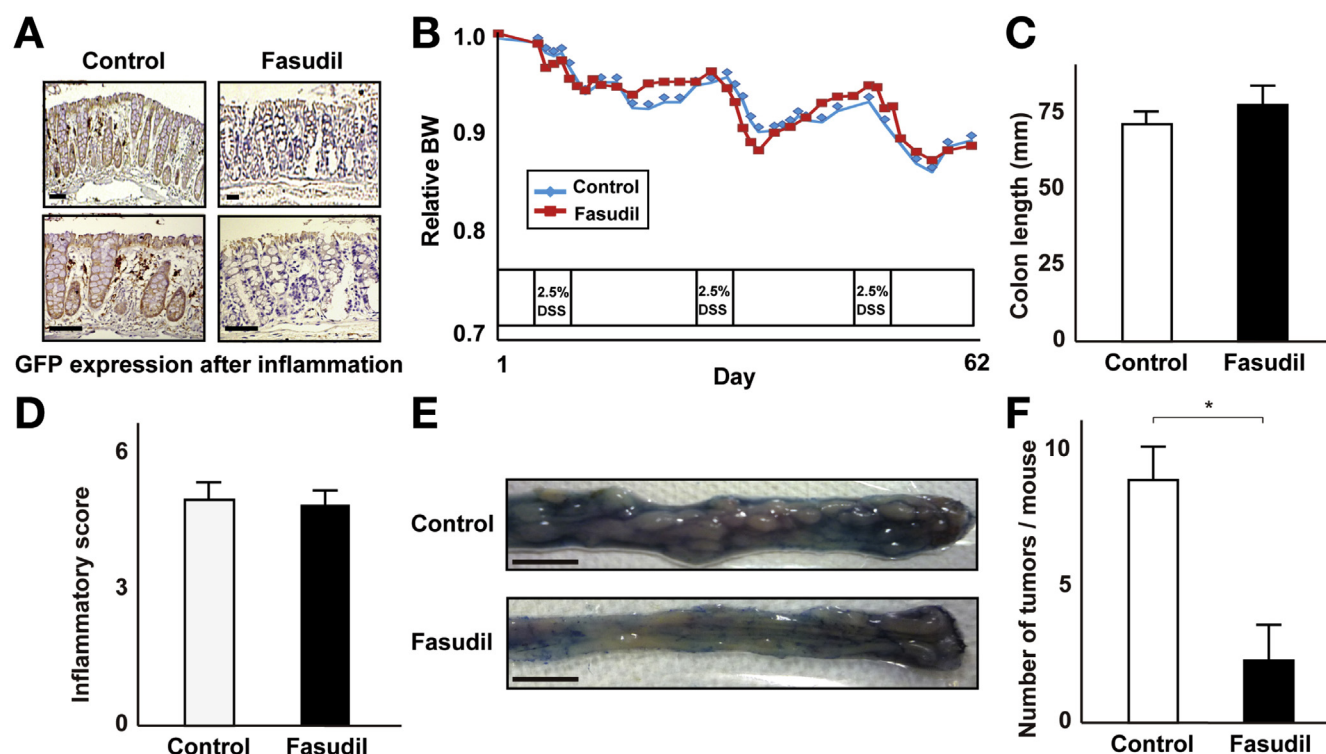


Figure 6. Enhancing miRNA function suppresses inflammation-associated colon tumorigenesis. (A) Fasudil, a ROCK inhibitor, enhanced miRNA function in mice after induction of colon inflammation, as determined by immunohistochemical analysis of GFP expression in reporter transgenic mice at day 62. The sections shown are 30 mm proximal to the anal canal. Bars = 200 μ m. The lower panels show magnified images of the glands in the upper panels. Similar results were obtained from 3 independent sets of mice. (B) Body weight changes in mice treated with fasudil ($n = 12$) and in nontreated controls ($n = 12$) during DSS-induced colon inflammation. Body weight loss was similar in the control and fasudil groups. (C) Colon length was measured on day 62 in control and fasudil-treated mice. Data represent the means \pm SD ($n = 12$ per group). The final colon length was similar in the control and fasudil groups. (D) Inflammatory scores of mice from each group ($n = 12$ per group). The inflammatory scores in the histologic analysis were similar in the control and fasudil groups. (E, F) Fewer tumors were detected in fasudil-treated mice. Representative colon images are shown in (E). Bars = 5 mm. Mean numbers of tumors per mouse \pm SD ($n = 12$ per group) are shown in (F). * $P < .05$.

methods for preventing colitis-induced tumorigenesis, such as ulcerative colitis–associated colon tumors.

A global reduction in miRNA levels is a general trait of human cancers and has a causal role in the transformed phenotype.^{17–20,22} This study demonstrated that global impairment of miRNA function under pathologic conditions also has a role in tumorigenesis, similar to the reduction in miRNA levels. Although numerous studies have revealed the critical tumor-promoting effects of global miRNA inhibition,^{17–20,22,23} elucidation of the mechanisms of tumorigenesis promotion by global miRNA inhibition is currently more difficult than showing the tumor-promoting or tumor-inhibiting effects of specific miRNAs. Because impairment of global miRNA function could increase the half-life of many mRNAs, tumorigenesis might have been enhanced because short-lived oncogene mRNAs were more greatly influenced than other mRNA species. While we identified the mRNAs and intracellular signaling pathways specifically affected by global miRNA inhibition in this study, the mechanisms underlying tumorigenesis induced by global miRNA impairment require further study.

The phenomena that chronic inflammation-associated miRNA function impairment may make colon mucosa

more prone to carcinogenesis are consistent with clinical observations in ulcerative colitis patients.^{3,4,50} Whereas we showed that colitis-associated tumorigenesis was enhanced by inflammation-associated inhibition of miRNA function as an ulcerative colitis–associated tumorigenesis model, other inflammation-associated tumors may also be promoted by miRNA function impairment. Because cellular stress, such as oxidative stress, can also relieve miRNA-mediated gene repression,²⁵ miRNA function impairment in such situations may also contribute to increase the tumor susceptibility.

It was shown in this study that A3G binds with Ago2, which is consistent with previous reports that A3G is involved in Ago2-related protein complexes.^{37–39} Although the functional involvement of A3G in miRNA function has been suggested,^{34,41} its precise role was undetermined. We report that A3G does facilitate the transfer of miRNAs into RISCs in vivo and in vitro and enhances miRNA function. A3G was originally reported to mediate intracellular resistance to lentiviruses, including human immunodeficiency virus 1,⁵¹ which is counteracted by the viral infectivity factor by inducing A3G polyubiquitination and proteasomal degradation.^{52,53} While we showed that TNF-induced A3G degradation was also mediated by enhancing its

ubiquitination, the precise mechanisms for the enhancement of ubiquitination by inflammatory stimuli remain to be elucidated. Nonetheless, because inflammation-induced miRNA functional impairment may be more common than expected, its pathologic roles are worth testing in various inflammatory diseases.

There are several limitations to our study. Because there is no appropriate method at present to examine miRNA function comprehensively, we primarily tested the function of specific miRNAs, such as LET7 and MIR185, as representatives, which were selected based on their involvement in colon carcinogenesis.^{54–57} However, chronic inflammation theoretically reduces global miRNA loading into Ago2-related RISCs, which could not be tested. Similarly, the affected mRNAs and intracellular signaling pathways by miRNA functional impairment were determined using cells with reduced expression of Dicer protein. While global miRNA expression is reduced in these cells, which theoretically resembles the state of global impairment of miRNA functions by inflammation, there remains the possibility that other factors are also involved in this experimental condition. Technical innovations are needed for precise determination and further examination of the changes in global miRNA functions.

While there are dozens of mouse models resembling human IBD, no single model completely reproduces all of the pathologic features of human IBD.⁵⁸ In this study, we used 2 different mouse models, IL10-deficient mice as a genetically engineered model and DSS-induced colitis as a chemically induced model, to test the preventative effects of ROCK inhibitor on inflammation-associated tumorigenesis. However, whether we can directly apply the results obtained from these mouse models to human IBD cases is still unknown.

In summary, we propose that attenuation of global miRNA function in chronic inflammation contributes to inflammation-associated colon tumorigenesis. From these results, manipulation of global miRNA activity may be useful for preventing inflammation-associated tumorigenesis, such as ulcerative colitis-associated tumorigenesis. Because fasudil, a ROCK inhibitor that we used here to enhance miRNA function,²⁷ is currently used clinically to treat cerebral vasospasm,⁵⁹ it may be applicable to prevent tumorigenesis in ulcerative colitis patients as drug repositioning. Further, determining the effects of combinatorial therapy of anti-inflammatory drugs and ROCK inhibitors on inflammation-associated tumorigenesis, as well as elucidating biomarkers to predict patient groups who will respond to such preventive methods, will also be interesting. Future experimental and clinical trials should test the efficacy of pharmacologic augmentation of miRNA function for preventing inflammation-associated tumorigenesis.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2016.10.043>.

References

1. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. *Cell* 2010;140:883–899.
2. Feagins LA, Souza RF, Spechler SJ. Carcinogenesis in IBD: potential targets for the prevention of colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2009;6:297–305.
3. Ekblom A, Helmick C, Zack M, et al. Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 1990;323:1228–1233.
4. Collins RH, Feldman M, Fordtran JS. Colon cancer, dysplasia, and surveillance in patients with ulcerative colitis. A critical review. *N Engl J Med* 1987;316:1654–1658.
5. Yashiro M. Molecular alterations of colorectal cancer with inflammatory bowel disease. *Dig Dis Sci* 2015;60:2251–2263.
6. Popivanova BK, Kitamura K, Wu Y, et al. Blocking TNF- α in mice reduces colorectal carcinogenesis associated with chronic colitis. *J Clin Invest* 2008;118:560–570.
7. Lasry A, Zinger A, Ben-Neriah Y. Inflammatory networks underlying colorectal cancer. *Nat Immunol* 2016;17:230–240.
8. West NR, McCuaig S, Franchini F, et al. Emerging cytokine networks in colorectal cancer. *Nat Rev Immunol* 2015;15:615–629.
9. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;136:215–233.
10. Ambros V. The functions of animal microRNAs. *Nature* 2004;431:350–355.
11. Denli AM, Tops BB, Plasterk RH, et al. Processing of primary microRNAs by the Microprocessor complex. *Nature* 2004;432:231–235.
12. Yi R, Qin Y, Macara IG, et al. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 2003;17:3011–3016.
13. Lund E, Güttinger S, Calado A, et al. Nuclear export of microRNA precursors. *Science* 2004;303:95–98.
14. Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. *Genes Dev* 2005;19:2979–2990.
15. Mourelatos Z, Dostie J, Paushkin S, et al. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev* 2002;16:720–728.
16. Gregory RI, Chendrimada TP, Cooch N, et al. Human RISC couples microRNA biogenesis and post-transcriptional gene silencing. *Cell* 2005;123:631–640.
17. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–838.
18. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866.
19. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res* 2007;67:2456–2468.
20. Kumar MS, Lu J, Mercer KL, et al. Impaired microRNA processing enhances cellular transformation and tumorigenesis. *Nat Genet* 2007;39:673–677.

21. Kumar MS, Pester RE, Chen CY, et al. Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev* 2009;23:2700–2704.
22. Lambert I, Nittner D, Mestdag P, et al. Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis in vivo. *Cell Death Differ* 2010;17:633–641.
23. Yoshikawa T, Otsuka M, Kishikawa T, et al. Unique haploinsufficient role of the microRNA-processing molecule Dicer1 in a murine colitis-associated tumorigenesis model. *PLoS One* 2013;8:e71969.
24. Hata A, Lieberman J. Dysregulation of microRNA biogenesis and gene silencing in cancer. *Sci Signal* 2015;8:re3.
25. Bhattacharyya S, Habermacher R, Martine U, et al. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006;125:1111–1124.
26. Mols J, van den Berg A, Otsuka M, et al. TNF- α stimulation inhibits siRNA-mediated RNA interference through a mechanism involving poly(A) tail stabilization. *Biochim Biophys Acta* 2008;1779:712–719.
27. Yoshikawa T, Wu J, Otsuka M, et al. ROCK inhibition enhances microRNA function by promoting deadenylation of targeted mRNAs via increasing PAIP2 expression. *Nucleic Acids Res* 2015;43:7577–7589.
28. Guo Y, Chen Y, Ito H, et al. Identification and characterization of lin-28 homolog B (LIN28B) in human hepatocellular carcinoma. *Gene* 2006;384:51–61.
29. Viswanathan SR, Powers JT, Einhorn W, et al. Lin28 promotes transformation and is associated with advanced human malignancies. *Nat Genet* 2009;41:843–848.
30. Kim HH, Kuwano Y, Srikanth S, et al. HuR recruits let-7/RISC to repress c-Myc expression. *Genes Dev* 2009;23:1743–1748.
31. Pegg AE. Methylation of the O6 position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest* 1984;2:223–231.
32. Okayasu I, Ohkusa T, Kajiyama K, et al. Promotion of colorectal neoplasia in experimental murine ulcerative colitis. *Gut* 1996;39:87–92.
33. Onizawa M, Nagaishi T, Kanai T, et al. Signaling pathway via TNF- α /NF- κ B in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 2009;296:G850–G859.
34. Liu C, Zhang X, Huang F, et al. APOBEC3G inhibits microRNA-mediated repression of translation by interfering with the interaction between Argonaute-2 and MOV10. *J Biol Chem* 2012;287:29373–29383.
35. Li S, Wang L, Fu B, et al. Trim65: a cofactor for regulation of the microRNA pathway. *RNA Biol* 2014;11:1113–1121.
36. Kundu P, Fabian MR, Sonenberg N, et al. HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. *Nucleic Acids Res* 2012;40:5088–5100.
37. Wichroski MJ, Robb GB, Rana TM. Human retroviral host restriction factors APOBEC3G and APOBEC3F localize to mRNA processing bodies. *PLoS Pathog* 2006;2:e41.
38. Gallois-Montbrun S, Kramer B, Swanson CM, et al. Anti-viral protein APOBEC3G localizes to ribonucleoprotein complexes found in P bodies and stress granules. *J Virol* 2007;81:2165–2178.
39. Phalora PK, Sherer NM, Wolinsky SM, et al. HIV-1 replication and APOBEC3 antiviral activity are not regulated by P bodies. *J Virol* 2012;86:11712–11724.
40. Huang J, Liang Z, Yang B, et al. Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members. *J Biol Chem* 2007;282:33632–33640.
41. Ali S, Karki N, Bhattacharya C, et al. APOBEC3 inhibits DEAD-END function to regulate microRNA activity. *BMC Mol Biol* 2013;14:16.
42. Nair S, Sanchez-Martinez S, Ji X, et al. Biochemical and biological studies of mouse APOBEC3. *J Virol* 2014;88:3850–3860.
43. Higashi M, Shimokawa H, Hattori T, et al. Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats in vivo: effect on endothelial NAD(P)H oxidase system. *Circ Res* 2003;93:767–775.
44. Madison BB, Liu Q, Zhong X, et al. LIN28B promotes growth and tumorigenesis of the intestinal epithelium via Let-7. *Genes Dev* 2013;27:2233–2245.
45. Yochum GS, Sherrick CM, Macpartlin M, et al. A beta-catenin/TCF-coordinated chromatin loop at MYC integrates 5' and 3' Wnt responsive enhancers. *Proc Natl Acad Sci U S A* 2010;107:145–150.
46. Colnot S, Niwa-Kawakita M, Hamard G, et al. Colorectal cancers in a new mouse model of familial adenomatous polyposis: influence of genetic and environmental modifiers. *Lab Invest* 2004;84:1619–1630.
47. Kühn R, Löhler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263–274.
48. Sellon RK, Tonkonogy S, Schultz M, et al. Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun* 1998;66:5224–5231.
49. Berg DJ, Davidson N, Kühn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest* 1996;98:1010–1020.
50. Ullman TA, Itzkowitz SH. Intestinal inflammation and cancer. *Gastroenterology* 2011;140:1807–1816.
51. Sheehy AM, Gaddis NC, Choi JD, et al. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 2002;418:646–650.
52. Sheehy AM, Gaddis NC, Malim MH. The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nat Med* 2003;9:1404–1407.
53. Yu X, Yu Y, Liu B, et al. Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* 2003;302:1056–1060.
54. Akao Y, Nakagawa Y, Naoe T. let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull* 2006;29:903–906.

55. Piskounova E, Polyarchou C, Thornton JE, et al. Lin28A and Lin28B inhibit let-7 microRNA biogenesis by distinct mechanisms. *Cell* 2011;147:1066–1079.
56. **Zhang Z, Liu X, Feng B, Liu N**, et al. STIM1, a direct target of microRNA-185, promotes tumor metastasis and is associated with poor prognosis in colorectal cancer. *Oncogene* 2015;34:4808–4820.
57. Imam JS, Buddavarapu K, Lee-Chang JS, et al. MicroRNA-185 suppresses tumor growth and progression by targeting the Six1 oncogene in human cancers. *Oncogene* 2010;29:4971–4979.
58. Wirtz S, Neurath MF. Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev* 2007;59:1073–1083.
59. Shibuya M, Suzuki Y, Sugita K, et al. Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Results of a prospective placebo-controlled double-blind trial. *J Neurosurg* 1992;76:571–577.

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Conflicts of interest

The authors disclose no conflicts.

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