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Adenosine 2B Receptor Expression Is Post-transcriptionally Regulated by MicroRNA

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We have reported that epithelial adenosine 2B receptor ($A_{2B}AR$) mRNA and protein are up-regulated in colitis, which we demonstrated to be regulated by tumor necrosis factor α (TNF- α). Here, we examined the mechanism that governs $A_{2B}AR$ expression during colitis. A 1.4-kb sequence of the $A_{2B}AR$ promoter was cloned into the pFRL7 luciferase vector. Anti-microRNA (miRNA) was custom-synthesized based on specific miRNA binding sites. The binding of miRNA to the 3'-untranslated region (UTR) of $A_{2B}AR$ mRNA was examined by cloning this 3'-UTR downstream of the luciferase gene in pMIR-REPORT. In T84 cells, TNF- α induced a 35-fold increase in $A_{2B}AR$ mRNA but did not increase promoter activity in luciferase assays. By nuclear run-on assay, no increase in $A_{2B}AR$ mRNA following TNF- α treatment was observed. Four putative miRNA target sites (miR27a, miR27b, miR128a, miR128b) in the 3'-UTR of the $A_{2B}AR$ mRNA were identified in T84 cells and mouse colon. Pretreatment of cells with TNF- α reduced the levels of miR27b and miR128a by 60%. Over expression of pre-miR27b and pre-miR128a reduced $A_{2B}AR$ levels by >60%. Blockade of miR27b increased $A_{2B}AR$ mRNA levels by 6-fold *in vitro*. miR27b levels declined significantly in colitis-affected tissue in mice in the presence of increased $A_{2B}AR$ mRNA. Collectively, these data demonstrate that TNF- α -induced $A_{2B}AR$ expression in colonic epithelial cells is post-transcriptionally regulated by miR27b and miR128a and show that miR27b influences $A_{2B}AR$ expression in murine colitis.

There are 4 adenosine receptors: A_1AR , $A_{2A}AR$, $A_{2B}AR$, and A_3AR . $A_{2B}AR$ ³ is the predominant adenosine receptor that is expressed in human colonic epithelial cells and colonic epithelial-derived cell lines, such as T84 (1). In intestinal epithelial cells, $A_{2B}AR$ couples with G_{α_s} and activates adenylate cyclase. In the colonic epithelium, $A_{2B}AR$ regulates vectorial electrogenic ion secretion, a secretory pathway that results in movement of isotonic fluid into the lumen (2–4). Up-regulation of ion secretion during inflammation is believed to be an impor-

tant process in inflammation-associated diarrhea (5, 6). In addition to its effect on ion transport, $A_{2B}AR$ mediates luminal secretion of interleukin-6 by polarized colonic epithelial cells, which results in neutrophil activation (7). Similarly, adenosine induces apical secretion of fibronectin, which potentiates the adhesion and invasion of *Salmonella typhimurium* through chemokine secretion (8).

We have observed that $A_{2B}AR$ mRNA and protein expression are up-regulated in human and animal models of inflammatory bowel disease (9). We have demonstrated that deletion of the $A_{2B}AR$ gene protects against murine colitis (10). In addition, $A_{2B}AR$ antagonists mitigate colonic inflammation in murine models of colitis (11), suggesting that $A_{2B}AR$ is a therapeutic target in inflammatory bowel disease and other intestinal inflammatory diseases. In this study, we examined the regulation of $A_{2B}AR$ expression in the colon by TNF- α , a critical proinflammatory cytokine that plays an important role in the pathogenesis of inflammatory bowel disease and whose antagonism constitutes an effective therapeutic strategy for inflammatory bowel disease (12).

EXPERIMENTAL PROCEDURES

Materials—pFRL7 luciferase vector was a gift from Richard A. Hodin (13). TNF- α was from R&D Systems. The First-strand cDNA Synthesis kit was from Fermentas (catalogue no. K1612; Hanover, MD). Invitrogen Ncode microRNA (miRNA) First-strand cDNA Module kit was catalogue no. 45-6612. iQ SYBR Green Supermix was from Bio-Rad.

Pre-miRNAs and anti-miRNAs for 27b, 128a, and let 7c, and the lipid-based SiPort NeoFX transfection reagent for transfection of pre-miRNAs were purchased from Ambion (Austin, TX). The cAMP immunoassay kit was from Applied Biosystems (Bedford, MA), isobutyl-1-methylxanthine was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), adenosine was from Research Biochemicals International (Natick, MA), and dextran sodium sulfate (DSS, molecular weight 50,000) was from ICN Biomedicals (Aurora, OH).

Prediction of miRNA Targets for $A_{2B}AR$ Gene—To identify the targets of human miRNAs for the $A_{2B}AR$ receptor, we used PicTar software, an algorithm that identifies miRNA targets. The miRNA target database contains all animal miRNA sequences from miRBase. The sequence database was scanned against the 3'-untranslated regions (3'-UTRs) that have been predicted in all available species in Ensemble. The interface links each miRNA to a list of predicted gene targets. $A_{2B}AR$ has four putative miRNA target sites (miR27a, miR27b, miR128a, and

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³ The abbreviations used are: $A_{2B}AR$, adenosine 2B receptor; TNF- α , tumor necrosis factor α ; miRNA, microRNA; DSS, dextran sodium sulfate; UTR, untranslated region; qPCR, quantitative PCR.

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miR128b) in the 3'-UTR of A_{2B}AR mRNA. Fig. 3 shows the binding sites and target miRNA alignments for the A_{2B}AR 3'-UTR.

Cloning of 3'-UTR of A_{2B}AR with Predicted miRNA Target Sequences—The 402-bp 3'-UTR of A_{2B}AR, encompassing the predicted miRNA sequences, was inserted into the multiple cloning site of the pMIR-REPORT luciferase miRNA expression reporter (Applied Biosystems catalogue no. AM5795) with HindIII and SpeI.

miRNA Reverse Transcription—Total RNA was extracted using TRI reagent (MRC, Inc.). Reverse transcription was performed per the Invitrogen Ncode miRNA first-strand cDNA module kit protocol.

Nuclear Run-on Assay—Nuclear run-on assay was performed according to the protocol of Dr. Yan (14). Briefly, nuclei were isolated from T84 cells that were untreated or treated with 10 ng/ml TNF- α for 2–4 h. Cells (5×10^7) were pelleted in a test tube and washed twice with ice-cold phosphate-buffered saline. Cells were then lysed on ice for 10 min in lysis buffer, containing 0.3 M sucrose, 0.4% (v/v) Nonidet P-40, 10 mM Tris-HCl at pH 7.4, 10 mM NaCl, and 3 mM MgCl₂.

After centrifugation (15 min at 500 \times g), the nuclear pellet was resuspended and subjected to an additional 5-min lysis to remove any remaining intact cells. Following centrifugation, the nuclei were purified by centrifugation through a 2.0 M sucrose cushion. The nuclei were resuspended in 300 μ l of transcription buffer (50 mM Tris-HCl (pH 8.0), 150 mM KCl, 5 mM MgCl₂, 0.5 mM MnCl₂, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol).

After sequential pretreatment with 1 μ l of 50 μ g/ml RNase A and 2.5 μ l (100 units) of RNasin (Promega, Madison, WI), the *in vitro* elongation reaction was initiated with 0.25 mM each ATP, GTP, CTP, and UTP. The reaction proceeded for 25 min at 30 °C. After incubation with RNase-free DNase, RNA was extracted with phenol-chloroform, precipitated with ammonium acetate and isopropyl alcohol, washed with 70% ethanol, and dissolved in water. cDNA was synthesized with the Fermentas First-strand synthesis system and amplified by real-time PCR using A_{2B}AR-specific primers and 18S as an internal control.

Cell Culture—T84 cells were grown and maintained in culture as described (15) in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 medium, supplemented with penicillin (40 mg/liter), streptomycin (90 mg/liter), and 5% fetal bovine serum. Confluent stock monolayers were subcultured by trypsinization.

Experiments were performed using cells plated for 7–8 days on permeable supports of 4.5-cm² inserts. Inserts (0.4- μ m pore size; Costar, Cambridge, MA) were rested in wells that contained medium until steady-state resistance was achieved, as described (16, 17). Caco2-BBe cells and HT29-Cl.19A cells were grown in Dulbecco's modified Eagle's medium, supplemented with 14 mmol/liter NaHCO₃, 10% fetal bovine serum, and penicillin/streptomycin (Invitrogen).

Real-time PCR—Real-time PCR was performed using the iCycler sequence detection system (Bio-Rad). Briefly, 50 ng of reverse-transcribed cDNA was amplified (40 cycles of 95 °C for 15 s and 55 °C for 1 min), using 10 μ M gene-specific primers.

The primers were: mouse A_{2B}AR forward, ATGCAGCTA-GAGACGCAAGA and reverse, GTTGGTGGGGGTCTGTA-ATG; human A_{2B}AR forward, TTCTGCACTGACTTCT-ACGG and reverse, TTTATACCTGAGCGGGACAC; mature miR27b forward, TTCACAGTGGCTAAGTTCTGC and reverse, universal qPCR primer provided in the kit (Invitrogen Ncode miRNA First-strand cDNA synthesis and reverse transcription qPCR kit); mature miR128a forward, TCACAGTGA-ACCGTCTCTTTT and reverse, universal qPCR primer.

Internal controls were: human18S forward, CCCCTCGAT-GCTCTTAGCTGAGTGT and reverse, CGCCGGTCCAAG-AATTTACCTCT; mouse 36B4 forward, TCCAGGCTTTG-GGCATCA and reverse, CTTTATCAGCTGGACATCACT-CAG; human 5.8S forward, AGGACACATTGATCATCGAC-ACTTCGA and reverse, universal qPCR primer; mouse SnoRNA55 forward, TGACGACTCCATGTGTCTGAGCAA and reverse, universal qPCR primer. Fold induction was calculated using the Ct method ($\Delta\Delta\text{CT} = (\text{Ct target} - \text{Ct internal control}) \text{ treatment} - (\text{Ct target} - \text{Ct internal control}) \text{ non-treatment}$), and the final data were derived from $2^{-\Delta\Delta\text{CT}}$.

Experimental Animals—The Institutional Animal Care and Use Committee of Emory University approved all procedures that were performed on the animals. In addition, all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. Public Health Service.

Six-week-old C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at our facility. They were maintained on a 12-hour light-dark cycle and allowed free access to pelleted diet and tap water at controlled temperatures (25 ± 2 °C).

Induction of DSS-Colitis—Colitis was induced in male C57BL/6 mice by *ad libitum* oral administration of 3% (w/v) DSS in tap water for 6 days. Age-matched male wild-type C57BL/6 mice that received tap water served as controls. Mice were observed daily and evaluated for changes in body weight and the development of clinical symptoms.

Transient Transfection of Pre-miR and Anti-miR—To study the regulation of A_{2B}AR by miRNA, pre-miR27b and/or pre-miR128a (A_{2B}AR target miRNAs) and a plasmid that contained the associated miR27b and miR128a binding sites in A_{2B}AR (pMIR-3'-UTR) were co-transfected into subconfluent Caco2-BBe cells. The binding of miRNAs to A_{2B}AR 3'-UTR mRNA was reflected by pMIR-REPORT luciferase activity. Anti-miR27b and/or anti-miR128a was introduced into HT29 cells to study the blockade of miR27b and miR128a. Endogenous A_{2B}AR mRNA levels were measured by real-time PCR 48 h after transfection. The positive control for blocking efficiency was anti-miR let 7c, which regulates high mobility group A2 (HMGA-2) mRNA.

cAMP Measurement—HT29-Cl.19A cells were washed and equilibrated with Hanks' solution; then, the cells were untreated or treated with 100 μ M adenosine (basolateral). cAMP measurements were made in whole cell lysates in the presence of 1 mM 3-isobutyl-1-methylxanthine using a competitive cAMP immunoassay kit according to the manufacturer's protocol. Luminescence was read on a Luminoscan Ascent (Thermo Labsystems, Needham Heights, MA).

Regulation by MicroRNA A_{2B}AR

To study the suppression of endogenous miRNA function, anti-miR27b and anti-miR128a (Ambion AM17000, 17100) were introduced into HT29 cells, and A_{2B}AR signaling was measured by estimating the levels of adenosine-mediated cAMP production with and without transfection of the blockers.

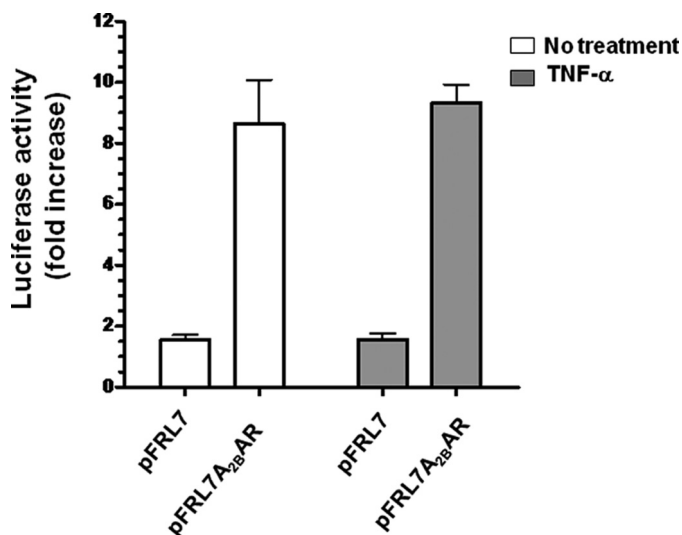


FIGURE 1. TNF- α does not affect A_{2B}AR promoter activity. The 1.4-kb fragment upstream of the A_{2B}AR coding region was cloned into the pFRL7 luciferase vector. Cells were transfected with pFRL7 1.4 A_{2B}AR or empty vector and untreated or treated with 20 ng/ml TNF- α for 6 h. Cells were lysed, and luciferase activity was measured. Data represent mean \pm S.E. (error bars) of four independent experiments; $n = 6$. Open bars represent no treatment, and gray bars represent TNF- α treatment.

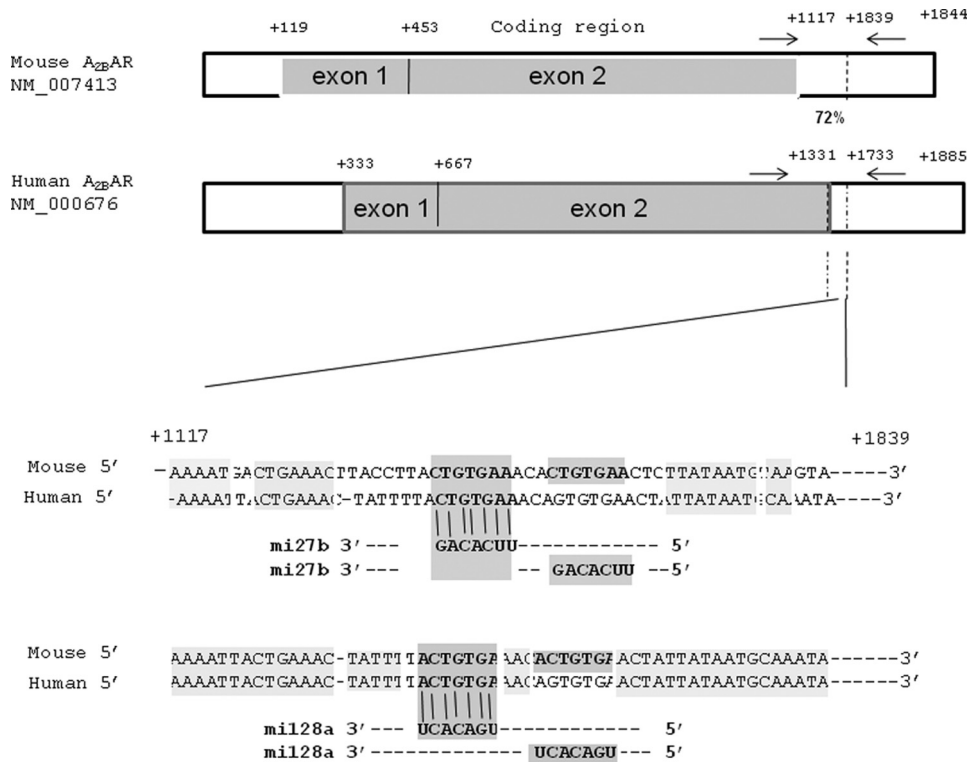


FIGURE 2. Schematic of miR27b and miR128a target sites in the 3'-UTR of A_{2B}AR. We performed an on-line search of the miRNA using the pictar and targets can databases and identified four putative miRNA target sites (miR27a, miR27b, miR128a, and miR128b) in the 3'-UTR of A_{2B}AR mRNA. The binding sites and target miRNA alignment for the A_{2B}AR 3'-UTR are shown.

Statistical Analysis—All data are expressed as means \pm S.E. Statistical analysis was performed by Student's *t* test or analysis of variance (Prism 4.0; GraphPad, San Diego, CA). A *p* value < 0.05 was regarded as significant.

RESULTS

TNF- α Does Not Affect A_{2B}AR Promoter Activity—Our previous studies demonstrated that A_{2B}AR mRNA and protein were up-regulated in cells that were pretreated with TNF- α . To understand better the regulation of A_{2B}AR, we performed experiments using a luciferase reporter construct that contained the A_{2B}AR promoter (pFRL7 1.4A_{2B}AR). The 5'-flanking region of A_{2B}AR was obtained from *Homo sapiens* chromosome 17S from the PAC (P1-derived artificial chromosome) RPCI-1 121M24 map 17p11 (a gift from Dr. R. Reinhardt, Max Planck Institute for Molecular Genetics, Berlin, Germany) by PCR, yielding a 1.4-kb fragment of the region. The human A_{2B}AR promoter sequence was submitted to NCBI GenBank under accession number DQ 351340. The isolated 1.4-kb and 2.5-kb fragments were subsequently cloned into the pFRL7 luciferase vector (13) at the KpnI and NcoI restriction sites to yield the pFRL7 1.4 A_{2B}AR plasmid. The transcriptional start site, as determined by rapid amplification of cDNA ends using the Gene Racer kit (Invitrogen), was 119 bp upstream of the translational start site.

COS-7 or Caco2-BBe cells were transfected with pFRL7 1.4A_{2B}AR and treated 72 h later with TNF- α for 6 h. As shown in Fig. 1, the relative luciferase activity in pFRL7 1.4 A_{2B}AR total lysates (11.6 ± 1.3) was ~ 10 times greater than that in empty vector transfectants (1.9 ± 0.13). TNF- α , however, did not increase A_{2B}AR promoter activity (11.01 ± 0.3), despite the increase in A_{2B}AR mRNA levels (9). Also, TNF- α had no effect on luciferase activity from a longer 2.5-kb fragment of the A_{2B}AR promoter.

TNF- α Does Not Increase mRNA Transcription—To clarify the effect of TNF- α on A_{2B}AR transcription, we performed nuclear run-on assays using nuclei from vehicle- or TNF- α -treated T84 cells. After *in vitro* elongation with dNTPs, the RNA was extracted, and novel A_{2B}AR mRNA synthesis was quantified by real-time PCR using A_{2B}AR-specific primers. There was no significant difference in A_{2B}AR mRNA levels between vehicle- and TNF- α treated nuclei. These data indicate that TNF- α does not affect A_{2B}AR transcription and suggest that TNF- α regulates A_{2B}AR by a post-transcriptional mechanism. This experiment was repeated three times, each yielding similar results.

TNF- α Down-regulates miR27b and miR128a—One of the primary mechanisms by which gene expression is post-transcriptionally regulated is miRNA. We determined whether A_{2B}AR was regulated by miRNA. First, we performed an online search of miRNAs using the pictar and targets can databases and identified four putative miRNA target sites (miR27a, miR27b, miR128a, and miR128b) in the 3'-UTR of A_{2B}AR mRNA (Fig. 2). All four miRNAs were expressed in T84 cells.

To determine the effect of TNF- α on miRNA and A_{2B}AR mRNA levels, we pretreated T84 cells for various times with TNF- α (10 ng/ml). Pretreatment of T84 cells with TNF- α increased A_{2B}AR mRNA levels by ~35-fold, ($*p < 0.03$, $n = 6$) (Fig. 3A) compared with untreated cells.

Pretreatment with TNF- α reduced the levels of miR27b and miR128a by 60% ($*p < 0.001$, $n = 6$) (Fig. 3B), whereas miR27a and miR128b levels were unaffected (data not shown). These data establish a direct correlation between A_{2B}AR mRNA and the miR27b and miR128a miRNAs.

Pre-miR27b and -miR128a and Anti-miR27b and -miR128a Regulate A_{2B}AR mRNA Levels and Function—Pre-miRNA precursors are small, partially double-stranded RNAs that mimic endogenous precursor miRNAs. To examine the regulation of

A_{2B}AR by miR27b and miR128a, we transfected T84 cells with pre-miR27b or pre-miR128a. Forty-eight hours after transfection, the cells were harvested, and A_{2B}AR mRNA levels were determined by real-time PCR. As shown in Fig. 4A, transfection of pre-miR27b or pre-miR128a decreased A_{2B}AR mRNA levels by 50% compared with untransfected cells (pre-miR27b 43.4 ± 4.83 , $*p < 0.001$; pre-miR128a 30.3 ± 6.1 , $*p < 0.003$), whereas 18S mRNA (control) was unaffected by pre-miR27b or pre-miR128a transfection. A commercial negative control pre-miRNA, containing a nontargeting sequence that had no homology to A_{2B}AR, had no effect on A_{2B}AR mRNA levels. These results demonstrate that miR27b and miR128a influence A_{2B}AR mRNA levels.

Next, we sought to determine the effect of inhibition of miR27b or miR128a on A_{2B}AR mRNA. Cells were transfected with custom-synthesized blockers of the miR27b or miR128a binding sites, and A_{2B}AR mRNA levels were measured by real-time PCR.

As shown in Fig. 4B, anti-miR27b and anti-miR128a increased A_{2B}AR mRNA levels by 5.3 ± 0.9 -fold ($*p < 0.01$) and 2.2 ± 0.02 -fold ($*p < 0.005$), respectively, compared with vector-transfected cells. 18S mRNA did not change, demonstrating the specificity of the miR blockers for A_{2B}AR mRNA. These data support the results of the pre-miRNA experiments and further demonstrate that miR27b and miR128a regulate A_{2B}AR mRNA levels.

To determine whether the effects of miR27b and miR128a on A_{2B}AR mRNA levels were reflected in functional alterations in A_{2B}AR, we measured adenylate cyclase activity in HT29-Cl.19A cells that were transfected with anti-miR27b or anti-miR128a. Forty-eight hours after transfection, cells were treated

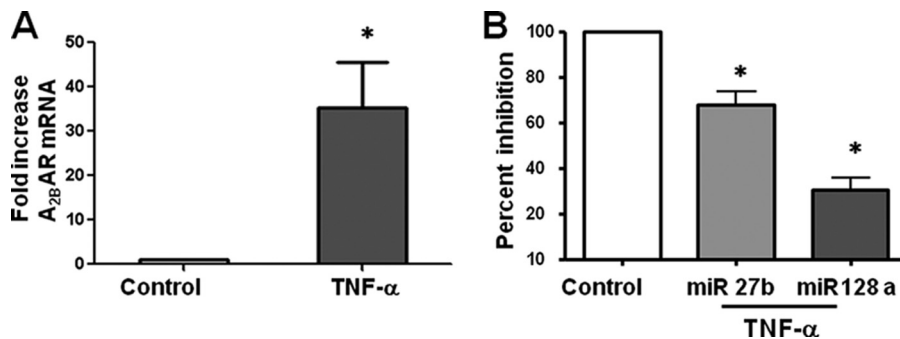


FIGURE 3. TNF- α pretreatment down-regulates miR27b and miR128a levels. A, T84 cells were pretreated with or without TNF- α . Cells were harvested after 2 h, and total RNA was extracted from the cells. A_{2B}AR mRNA levels were determined by real-time PCR. Data represent the mean \pm S.E. (error bars) of two independent experiments; $n = 6$. B, T84 cells were pretreated with or without TNF- α , and total RNA was extracted. miR27b and miR128a levels were measured using specific primers and normalized to 5.8S. Data represent mean \pm S.E. of two independent experiments; $n = 6$.

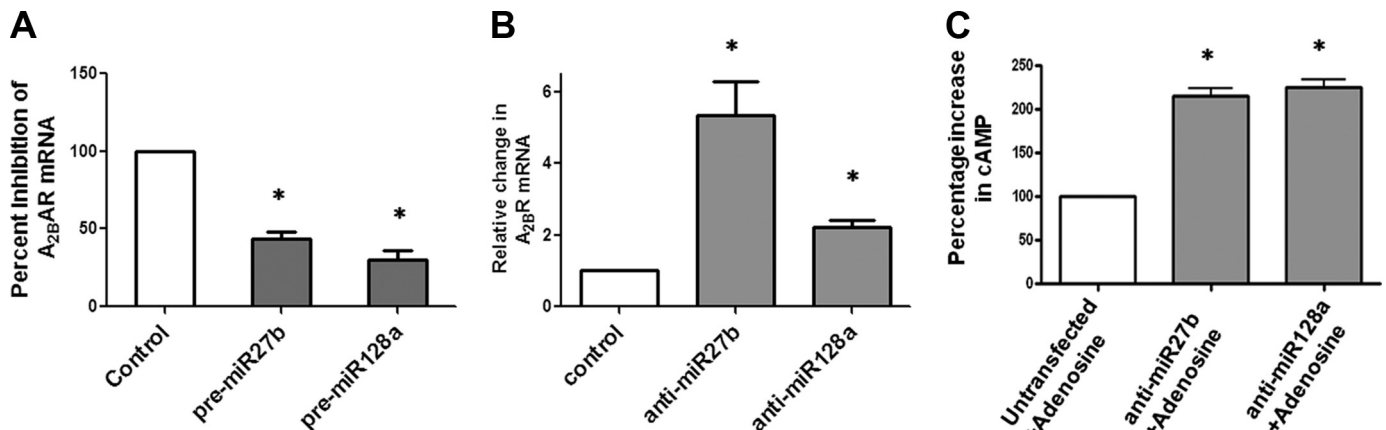


FIGURE 4. Pre-miR27b and -miR128a and anti-miR27b and -miR128a regulate A_{2B}AR mRNA levels and function. A, T84 cells were transfected with pre-miR27b and pre-miR128a. Total RNA was extracted 48 h after transfection. A_{2B}AR mRNA levels were measured by real-time PCR. Data represent mean \pm S.E. (error bars) of two independent experiments; $n = 6$. B, colonic epithelial cells were transfected with anti-miR27b and anti-miR128a. Cells were harvested after 48 h, and total RNA was extracted from the cells. A_{2B}AR mRNA levels were determined by real-time PCR. Data represent mean \pm S.E.; $n = 6$. C, colonic epithelial cells were transfected with anti-miR27b and -miR128a. Cells were stimulated with adenosine for 7 min 48 h after transfection. Total cell lysates were collected, and cAMP levels were estimated. Data represent mean \pm S.E.; $n = 10$.

Regulation by MicroRNA $A_{2B}AR$

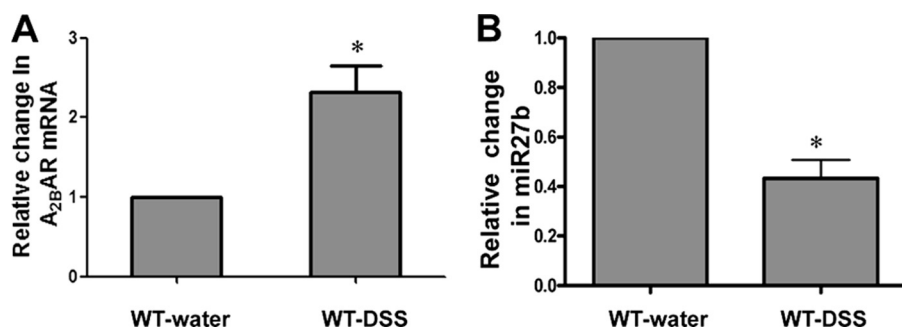


FIGURE 5. Increased levels of $A_{2B}AR$ mRNA during colitis correlate with decreased miR27b levels. Age- and gender-matched C57BL/6 mice were fed with 3% DSS for 7 days, after which mice were killed and examined for severity of colitis. Total RNA was extracted from the colons of these mice. $A_{2B}AR$ mRNA (A) and miR27b (B) levels were measured compared with control mice. Data are represented as $A_{2B}AR$ mRNA normalized to 18S, and miR27b is normalized to SnRNA55. Data are represented as mean \pm S.E. (error bars) of two independent experiments; $n = 3$. WT, wild type.

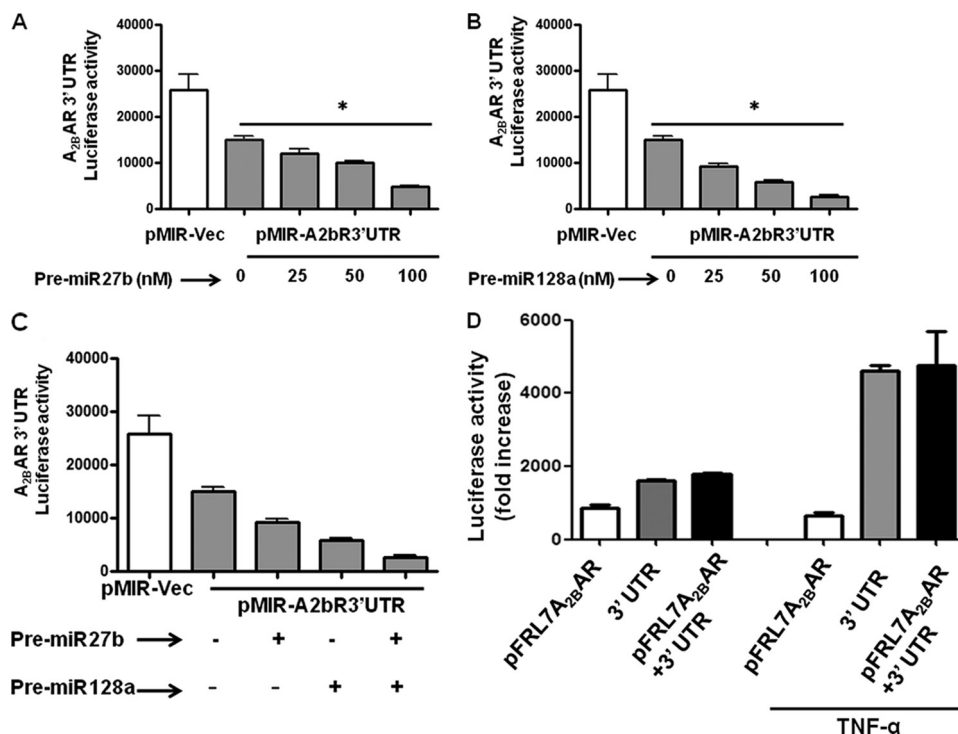


FIGURE 6. Pre-miR27b and pre-miR128a decrease $A_{2B}AR$ 3'-UTR luciferase activity, and addition of $A_{2B}AR$ promoter to 3'-UTR does not influence luciferase activity. The 3'-UTR of the $A_{2B}AR$ -predicted miRNA binding target was inserted into the multiple cloning site of the pMIR-REPORT luciferase miRNA expression reporter vector. Pre-miR27b and pre-miR128a were co-transfected with $A_{2B}AR$ 3'-UTR pMIR-REPORT. Dose-dependent effect of pre-miR27b (A), dose-dependent effect of pre-miR128a (B), and additive effect of pre-miR27b and pre-miR128a (C) were demonstrated by luciferase assay. Data are represented as mean \pm S.E. of two independent experiments; $n = 12$. D, transfection of 3'-UTR of $A_{2B}AR$ alone, pFRL7.1.4 $A_{2B}AR$ promoter alone, or 3'-UTR + pFRL7.1.4 $A_{2B}AR$ promoter together were transfected into Caco2-BBe cells with or without TNF- α treatment (20 ng/ml) for 6 h. Cells were lysed, and luciferase activity was measured. Data represent mean \pm S.E. (error bars); $n = 6$.

basolaterally with adenosine (100 μ M), and cAMP levels were measured. As shown in Fig. 4C, cAMP levels were significantly higher in miR27b (0.07 ± 0.01 , $*p < 0.02$) and miR128a transfectants (0.08 ± 0.01 , $*p < 0.016$) compared with untransfected cells (0.04 ± 0.004), suggesting that the up-regulation of $A_{2B}AR$ mRNA in anti-miR27b- and anti-miR128a-transfected cells effects an increase in $A_{2B}AR$ levels.

Elevations in $A_{2B}AR$ mRNA Levels during Colitis Correlate with Decreased miR27b Levels—We have shown that $A_{2B}AR$ mRNA and protein levels are increased during colitis. To deter-

mine whether miR27b or miR128a levels correlate inversely with $A_{2B}AR$ mRNA during colitis, we measured miR27b, miR128a, and $A_{2B}AR$ mRNA levels by real-time PCR in a DSS model of colitis. All mice that were fed 3% DSS for 7 days developed signs of severe colitis (clinical score: 9.4 ± 1.0 ; histological score: 9.1 ± 2.3), presented as bloody diarrhea and weight loss. Consistent with our previous observations (9), $A_{2B}AR$ mRNA levels increased 2.3 ± 0.3 -fold ($*p < 0.05$), compared with control mice that received water (Fig. 5A). Notably, miR27b levels decreased 33.4 ± 6.6 -fold ($*p < 0.005$) in colitic mice compared with control mice that received water (Fig. 5B). There was no change in miR128a levels (data not shown). These data demonstrate that there is an inverse correlation between miR27b and $A_{2B}AR$ mRNA, supporting evidence for a post-transcriptional mechanism of $A_{2B}AR$ regulation by miRNA.

Pre-miR27b and Pre-miR128a Decrease $A_{2B}AR$ 3'-UTR Luciferase Activity—To confirm that miRNA functions through an interaction with the $A_{2B}AR$ 3'-UTR, we co-transfected pre-miR27b and/or pre-miR128a with the $A_{2B}AR$ 3'-UTR luciferase reporter construct pMIR-REPORT. As shown in Fig. 6, A and B, introduction of pre-miR27b or pre-miR128a significantly inhibited 3'-UTR-dependent $A_{2B}AR$ luciferase activity in a dose-dependent manner (25–100 nM, $n = 8$, $*p < 0.0001$). Further, co-transfection of pre-miR27b (50 nM) and pre-miR128a (50 nM) produced a robust additive inhibitory effect on luciferase activity (Fig. 6C). We performed the luciferase assay with an additional group, in which we co-transfected the $A_{2B}AR$ 3'-UTR and the $A_{2B}AR$ promoter. We observed no additional increase in luciferase activity beyond that induced by the 3'-UTR alone (Fig. 6D), suggesting that $A_{2B}AR$ expression is regulated by miRNA.

DISCUSSION

miRNAs are short RNAs in introns and intergenic regions that regulate gene expression. A large primary miRNA (pre-miRNA) is transcribed and processed to a pre-miRNA hairpin, which is converted in the cytoplasm into a mature miRNA that

targets genes that harbor complementary sites in their 3'- or 5'-UTRs (18, 19). miRNAs are up- and down-regulated in several disease conditions. We have demonstrated that A_{2B}AR protein levels increase after treatment with proinflammatory cytokines but that A_{2B}AR promoter activity is unaltered. We also examined miRNAs in bioinformatics databases that were complementary to the A_{2B}AR 3'-UTR to determine whether miR27b and miR128a regulated the levels of A_{2B}AR mRNA and protein.

In our previous studies, we observed the up-regulation of A_{2B}AR by TNF- α in colonic epithelial cells and in the DSS mouse model of colitis (9). Using a pFLR7 promoter-reporter construct that contained either the 1.4-kb and 2.5-kb fragments of the A_{2B}AR promoter, we found that TNF- α did not increase A_{2B}AR promoter activity (Fig. 1). A_{2B}AR, however, has been shown to be transcriptionally regulated during hypoxia through hypoxia inducible factor (HIF)-binding elements (20) and by *b-myb* (21) in endothelial cells and vascular smooth muscle cells, respectively. Our luciferase assays were performed with whole cell lysates, which should have harbored all of the transcription factors that regulate the promoter. Studies by our laboratory and others have noted the transcriptional regulation of genes by TNF- α by 4–6 h, suggesting that the transcriptional mechanism is active in our luciferase assay (22–24). Similarly, by nuclear run-on assay, we observed no increase in A_{2B}AR transcription in response to TNF- α . Although the involvement of other cis- or trans-elements and signaling pathways that regulate A_{2B}AR transcription further upstream of the 2.5-kb element cannot be ruled out with these assays, these data strongly suggest that TNF- α up-regulates A_{2B}AR through a post-transcriptional mechanism. Thus, we speculated that A_{2B}AR regulation was mediated by A_{2B}AR-specific miRNAs. A search of bioinformatics databases identified miR27b and miR128a as containing target sequences in the 3'-UTR of A_{2B}AR. Treatment of colonic epithelial cells with TNF- α inhibited miR27b and miR128a by 60%, which was associated with a 5-fold increase in A_{2B}AR mRNA levels (Fig. 4, A and B). Colonic tissue from mice with DSS-induced colitis experienced a 50% reduction in miR27b levels (Fig. 5B). These findings implicated miR27b and miR128a in the regulation of A_{2B}AR mRNA. Our studies were unable to determine the relative contribution of miR27b and miR128a because the consensus binding sites for them in the A_{2B}AR 3'-UTR overlap significantly (Fig. 2).

miRNAs constitute a prominent class of regulatory genes in animals, representing ~1% of all predicted genes, suggesting that sequence-specific, post-transcriptional regulatory mechanisms that are mediated by these small RNAs are ubiquitous (25–27). We tested this hypothesis using synthetic pre-miRNAs (miRNA precursor molecules) and a reciprocal set of anti-miRNAs. Pre-miRNAs are small, chemically modified, double-stranded RNA molecules that mimic endogenous mature miRNA molecules. These molecules are incorporated into the RNA-induced silencing complex, which is also responsible for miRNA activity. In contrast to miRNA expression vectors, pre-miRNAs can be used in dose-response studies because they can be introduced directly into the cell by transfection. Inside cells, pre-miRNAs up-regulate miRNA activity (28), enabling one to analyze miRNA function. Transfection of pre-

miRNA reduced A_{2B}AR mRNA levels in T84 cells (Fig. 4A). In reciprocal experiments, blockade of miR27b and miR128a, as a result of the introduction of specific anti-miRNAs, increased A_{2B}AR mRNA levels (Fig. 4B). Anti-miRNAs also enhanced adenosine-mediated cAMP production (Fig. 4C), indicating an increase in A_{2B}AR function and directly linking miR27b and miR128a to the regulation of A_{2B}AR expression (29). miRNAs have important functions in the regulation of genes. Several studies have demonstrated that miRNAs govern basic cellular functions. Studies by Care *et al.* (30, 31) noted that miR133 and miR1 regulate cardiomyocyte hypertrophy. In our experiments, co-transfection of pre-miR27b or pre-miR128a with an A_{2B}AR 3'-UTR reporter construct increased luciferase activity in a dose-dependent manner (Fig. 6, A and B). Co-transfection of pre-miR27b and pre-miR128a resulted in the additive inhibition of luciferase activity (Fig. 6C). By luciferase assay, co-transfection of the 3'-UTR of A_{2B}AR and the A_{2B}AR promoter resulted in no additional increase in luciferase activity beyond that induced by 3'-UTR alone, suggesting that miRNA plays the significant role in the regulation of A_{2B}AR expression (Fig. 6D).

Recent bioinformatic and experimental evidence suggests that a substantial proportion of genes (>30%) are subject to miRNA-mediated regulation (32). Our studies on the possible function of miRNAs in colonic inflammation demonstrate that miR27b and miR128a are critical regulators of A_{2B}AR expression. Because A_{2B}AR antagonism ameliorates colitis, our results suggest that the regulation of A_{2B}AR expression by targeting miRNAs might be an effective therapeutic strategy for colitis.

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