The mRNA Stability Factor HuR Inhibits MicroRNA-16 Targeting of COX-2

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Abstract

Commonly observed in colorectal cancer is the elevated expression of the prostaglandin (PG) synthase COX-2. In normal intestinal epithelium, the COX-2 mRNA is targeted for rapid decay through the 3′-untranslated region (3′-UTR) adenylate- and uridylate (AU)-rich element (ARE), whereas in tumors ARE-mediated decay is compromised. Here we show that the COX-2 ARE can mediate degradation through microRNA (miRNA)-mediated regulation. We identified miR-16 to bind the COX-2 3′-UTR and inhibit COX-2 expression by promoting rapid mRNA decay. In colorectal cancer cells and tumors, miR-16 levels were decreased approximately twofold and miR-16 expression in cancer cells attenuated COX-2 expression and PG synthesis. The COX-2 ARE is also bound by the RNA-binding protein HuR. In colorectal cancer tumors, HuR is overexpressed and localized within the cytoplasm, where it promotes ARE-mRNA stabilization. Under conditions of HuR overexpression, miR-16 was unable to promote rapid mRNA decay through the COX-2 ARE. Ribonucleoprotein immunoprecipitation of HuR showed direct association with miR-16 that was reversed when cytoplasmic trafficking of HuR was inhibited. Furthermore, this interaction between HuR and miR-16 promoted the downregulation of miR-16. These new results identify miR-16 as a central posttranscriptional regulator of COX-2 and show the ability of elevated levels of HuR to antagonize miR-16 function. Along with insight into altered ARE-mediated mRNA decay observed in colorectal cancer, these findings provide a new explanation for tumor-derived loss of miR-16.

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Introduction

Colorectal cancer is the third most common form of cancer in both incidence and mortality. Although colorectal cancer prevalence has decreased over the past decade due to early detection, it is anticipated that approximately 141,000 new cases will occur in 2011. In colorectal tumors, various genetic alterations allow for aberrant activation of signaling pathways resulting in enhanced expression of many growth- and inflammation-associated immediate-early response genes. Through their overexpression, these factors can contribute to colorectal cancer etiology at virtually all stages of tumorigenesis.

A critical point in the regulation of many inflammatory cytokines, growth factors, and proto-oncogenes occurs through posttranscriptional mechanisms that promote rapid mRNA decay. A prominent cis-acting RNA element present in a majority of these cancer-associated transcripts is the adenylate- and uridylate (AU)-rich element (ARE) contained within the mRNA 3′-untranslated region (3′-UTR). In normal intestinal epithelium, this RNA element serves to target these mRNAs for rapid decay through recognition of ARE-binding proteins and thus limit expression of potentially pathogenic factors. However, loss of ARE-mediated posttranscriptional regulation is observed in colorectal cancer cells and tumors, indicating the significance of this mechanism in carcinogenesis.

The COX enzymes carry out the rate-limiting step in prostaglandin (PG) synthesis and overexpression of the inducible isoform, COX-2, has been shown to occur at multiple stages of colon carcinogenesis, making it a prominent target of chemoprevention. In normal cells, COX-2 expression levels are potently regulated through the ARE present in its 3′-UTR. Whereas, under conditions of neoplastic transformation, the ability of the COX-2 ARE to promote posttranscriptional regulation is compromised. This is, in part, due to overexpression of the ARE-binding protein HuR in colorectal cancer cells and tumors, in which elevated HuR levels can impede ARE-mediated mRNA decay. These findings and others show increased expression of HuR to occur in a variety of human cancers and promote ARE-containing gene expression.
The mechanism by which HuR promotes COX-2 mRNA stabilization seems to be linked to its subcellular localization (11). In nonstressed cells, HuR is predominantly localized to the nucleus (>90%) and can shuttle between the nucleus and cytoplasm (13), whereas in tumor cells and tissues, HuR overexpression is accompanied by its accumulation in the cytoplasm (6, 7, 12). It is hypothesized that the ability of HuR to promote mRNA stabilization requires its translocation to the cytoplasm, where it binds target ARE-containing mRNAs and interferes with their rapid decay (14, 15); however, the mechanism by which cytoplasmic HuR mediates mRNA stabilization remains to be resolved.

Current work has shown the ability of HuR to control microRNA (miRNA)-mediated posttranscriptional regulation. miRNAs are small noncoding RNAs approximately 21 to 24 nucleotides in length that primarily bind to the 3′-UTR of targeted transcripts through imperfect base pairing, and this interaction is most often associated with regulated expression of the target mRNAs through mRNA degradation and/or translational suppression (16). More recently, HuR has been shown to rescue an ARE-containing mRNA from miRNA-mediated regulation in response to stress (17). In subsequent studies, HuR has further been shown to inhibit translation of specific mRNAs by promoting the recruitment of repressor miRNAs to the targeted 3′-UTR (18, 19). Through these contrasting roles, HuR is becoming recognized as a key factor in influencing miRNA-mediated regulation (20, 21).

In this article, we show that the ARE-containing 3′-UTR of COX-2 is targeted by the miRNA miR-16 leading to downregulation of COX-2 expression by altering mRNA stability. However, the inability of miR-16 to control COX-2 expression is compromised in the presence of elevated HuR. The ability of HuR to antagonize miR-16 involved a direct HuR/miR-16 interaction that was dependent on the presence of a functional 3′-UTR ARE and resulted in decreased miR-16 levels in colorectal cancer cells and tumors. These findings offer what we believe are new insights into mechanisms that govern COX-2 regulation in colorectal cancer and coordinated cross-talk between ARE-mediated decay and miRNA pathways critical for posttranscriptional regulation of cancer-associated gene expression.

Materials and Methods

Cell culture, DNA transfection, siRNA, and miRNA transfection

HeLa, CaCo2, HT-29, LoVo, LS174T, and SKCO1 were purchased from American Type Culture Collection. HCA7 and Moser cells were kindly provided by S. Kirkland (Imperial College, London, United Kingdom) and R.D. Beauchamp (Vanderbilt University Medical Center, Nashville, TN), respectively. All cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS (Hyclone) except SKCO1, HCA7, and CaCo2 cell lines, which were maintained in MEM medium containing 10% (SKCO1, HCA7) or 20% (CaCo2) FBS. HeLa TetOFF/HuR-Flag and HeLa TetOFF/TTP-Flag cell lines were maintained in the presence of 2 μg/mL doxycycline (Dox; Clontech) as described (12); cells were grown in the absence of Dox for 48 hours to induce HuR-Flag or TTP-Flag expression. Where indicated, cells treated with the small molecule inhibitor for HuR, MS-444 (20 μmol/L; ref. 22) for the indicated times.

Transient transfections of cells with luciferase reporter constructs containing the COX-2 3′-UTR (10) or 1.8-kb COX-2 promoter (23) were accomplished using Lipofectamine Plus (Invitrogen) according to the manufacturer’s protocol. Where indicated, cells were transfected with the HuR expression vector pcDNA3-HuR-Flag or empty vector (6). After 3 hours, the media was changed and cells were sequentially transfected with miRNA for 48 hours. miRNA and siRNA transfection of cells using 50 nmol/L hsa-miRNA-16 mature miRNA duplex, random sequence negative control miRNA #2, predesigned siRNAs for COX-2, HuR, and negative control #1 siRNA (Ambion) were done using siQuest (Mirus) for 48 hours according to the manufacturer’s instructions. Where indicated, HeLa cells were stimulated with 10 ng/mL interleukin (IL)-1β (R&D Systems) for 24 hours prior to miRNA transfection.

RNA analysis

Total RNA was extracted using TRIzol reagent (Invitrogen). Northern blotting was done as described (10) and probed with P32-labeled DNA probes synthesized for COX-2 and actin (Promega). cDNA synthesis was done using 1 μg of total RNA in combination with oligo(dT) and Improm-II reverse transcriptase (Promega). Reverse transcriptase PCR (RT-PCR) was done using primers for COX-1 sense 5′-ACCTTCTACCGAGAGATGCTC-3′ and antisense 5′-TGACGCTCCAGAATGTCTCCA-3′ (407 bp product); COX-2 sense 5′-GTCACAGATGGCAAAATGCTG-3′ and antisense 5′-TAAGATAAACTGCCAGTG-3′ (500 bp product); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sense 5′-CCACCCATGGC- AAATTCCATGGCA-3′ and antisense 5′-TCTAGACTGCCAGGTCACTACC-3′ (598 bp product). PCR was done using 25 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 1 minute, and extension at 72°C for 1 minute. qPCR analysis was done as described (12) using the 7300 PCR Assay System (Applied Biosystems) with TaqMan probes for COX-2 and GAPDH (PTGS2, GAPDH; Applied Biosystems) and SYBR green PCR master mix (Applied Biosystems). The following primers were used in qPCR reactions: Luciferase; sense 5′-AAGGGTAGATTCCAGGGAGTTCCTC-3′ and antisense 5′-AGGCTCCTCAGAACAAGCTCCTC-3′; BCL2; sense 5′-ATGGTGTGGAGAGCGCTCAA-3′ and antisense 5′-ACAGTTTCACAAGGGCATCCTC-3′; VEGF, sense 5′-TACCCTCACAATGGCGAGTGG-3′ and antisense 5′-AAGATGTCCACCGGAGTCTC-3′; cyclin E1; sense 5′-ATCACCTCAAAAGTTGACACCAG-3′ and antisense 5′-AGGGGACTTAAAGCCATT-3′. GAPDH was used as a control for normalization. mRNA decay experiments were initiated by adding actinomycin D (ActD; 5 μg/mL; Fischer Scientific) to the growth medium at...
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specified times. For mRNA half-life assays in the presence of both HuR and miR-16, cells were cotransfected with COX-2 3′-UTR reporter plasmid and pcDNA3-HuR-Flag for 3 hours, followed by transfection with miR-16 for 48 hours. ActD treatment was done as described above.

For endogenous miRNA detection, 10 ng of total RNA was converted to cDNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems) with miRNA primers specific for mature hsa-miR-16, hsa-miR-15a, and the small nuclear protein RNU6B (U6) control for normalization (Applied Biosystems). qPCR detection of miRNAs was done using TaqMan probes designed for miR-16, miR-15a, U6, and 18S (Applied Biosystems).

Protein and PGE2 analysis

Western blots were done as described (12) using antibodies against COX-2 (160112; Cayman Chemical Company), COX-1 (H-62; Santa Cruz Biotechnology), HuR (3A2; Santa Cruz Biotechnology), nucleoporin (p62, BD Biosciences), and α-tubulin (DMA1; Sigma). Membranes were stripped and reprobed using β-actin antibody (Clone C4; MP Biomedicals). Detection and quantification of blots were carried out as previously described (10).

Cells transfected with luciferase reporter constructs were lysed in reporter lysis buffer (Promega) and assayed using the Luciferase Assay System (Promega). Reporter gene activities were normalized to total protein; all results represent the average of triplicate experiments. Where indicated, pGL3-Basic and pGL3-Enhancer vectors were used as controls.

PG E2 (PGE2) levels in culture media were analyzed by ELISA (R&D Systems). Where indicated, cells were pretreated with 10 μmol/L NS-398 (Cayman) for 1 hour, after which the media was removed and cultures were incubated for 20 minutes with serum-free media containing 10 μmol/L arachidonic acid (Cayman) in serum-free media. Relative PGE2 levels were normalized to total protein levels and are an average of 3 experiments.

Ribonucleoprotein immunoprecipitations

Immunoprecipitation (IP) of ribonucleoprotein complexes (RNP-IP) was done as described (24). Briefly, 100 μL of protein A/G Plus agarose beads (Santa Cruz) were washed with NT2 buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl2, 0.05% NP-40) and then coated with 30 μg anti-HuR antibody (3A2), anti-Argonaute 2 (AGO2) (C34C6; Cell Signaling), or control IgG diluted in 200 μL of NT2 buffer for 16 hours. Washed beads were incubated with equal amounts of cytoplasmic lysates obtained from cells lysed in polyol lysis buffer (20 mmol/L Tris-HCl pH 7.6, 5 mmol/L MgCl2, 150 mmol/L NaCl, 1 mmol/L DTT, 0.5% NP-40, 100 U/mL RNasin (Promega), 0.2 mmol/L phenylmethylsulfonyl-fluoride, 10X Protease Inhibitor Cocktail (Sigma)) for 30 minutes on ice followed by centrifugation for 30 minutes at 13,200 rpm, 4°C. NT2 buffer was added to a final volume of 1 mL and IP reactions were incubated for 16 hours at 4°C. Reactions were washed 5X with NT2 buffer and total RNA was isolated from immunoprecipitates using 1 mL TRIzol per IP reaction after final wash and then used for cDNA synthesis. Analysis of mRNA and miRNA in RNP-IP samples was done as described above.

In vitro miRNA-binding assay

Direct binding of recombinant HuR to miR-16 was measured in homogeneous solution using 2D-FIDA (fluorescence intensity distribution analysis) as described previously (22, 25). Briefly, 5′-TMR labeled miR-16 (5′-TMR-UAAGCACCCGUAAAUAUGGCC; Microsynth) was incubated with increasing concentrations of recombinant full-length HuR (prepared as described in Meisner and colleagues; ref. 25) in a buffer of PBS pH 7.2, 5 mmol/L MgCl2, 0.2% (w/v) Pluronic F-127 (Invitrogen), 1 mmol/L alpha-methylene ATP for at least 20 minutes at room temperature. The fluorescence anisotropy was measured using 2D-FIDA, and the Kd was determined by fitting the fluorescence anisotropy data to an equation describing fluorescence anisotropy in dependence of 1:1 complex formation, as derived from the law of mass action (25).

Human and mouse tissue samples

Human colon tumors and histologically normal tissue were obtained from surgical remnants from patients with colorectal cancer through the University of South Carolina Center for Colon Cancer Research Tissue Bank. The protocol was approved by the Institutional Review Board of the University of South Carolina. Tissue was snap-frozen in liquid nitrogen, and total RNA was isolated using TRIzol from approximately 50 mg of tissue.

APC–MIN+ mice 15 weeks of age obtained from the Center for Colon Cancer Research (University of South Carolina) were sacrificed and tumors were identified in small intestinal tissue. Tumor specimens were excised with scissors and pooled into 10 tumors per mouse, whereas adjacent normal epithelium was scraped from the muscular layer. All tissue samples were snap-frozen and total RNA was isolated using TRIzol.

Statistical analysis

The data are expressed as the mean ± SEM. Student t test was used to determine significant differences. P values less than 0.05 were considered significant.

Results

miR-16 regulates COX-2 expression

Our prior work had shown the ARE present in the COX-2 3′-UTR to be a key cis-acting element regulating expression levels on a posttranscriptional level (10). On the basis of previous work indicating miR-16 to be a pleiotropic regulator of ARE-containing mRNAs, and its ability to target reporter constructs bearing the COX-2 3′-UTR (26, 27), we sought to determine whether miR-16 may be involved in ARE-mediated regulation of COX-2. The ability of miR-16 to regulate endogenous COX-2 expression was shown using HeLa cells stimulated with IL-1β to induce COX-2
expression for 24 hours and then transfected with mature hsa-miR-16 or a random sequence negative control miRNA for 48 hours. As seen in Fig. 1A, expression of miR-16 in IL-1β–treated HeLa cells reduced COX-2 mRNA approximately 2-fold. This miR-16–dependent loss of COX-2 mRNA was reflected in a similar approximately 2-fold decrease in COX-2 protein expression and associated PGE2 synthesis (Fig. 1B and C). The ability of miR-16 to attenuate PGE2 synthesis was consistent with specific targeting of COX-2 because expression of COX-1 mRNA or protein were not impacted by miR-16 (Fig. 1A and B).

To establish whether the effect of miR-16 on COX-2 expression was mediated through a direct miRNA:mRNA association, ribonucleoprotein IP (RNP-IP) was done to determine whether COX-2 mRNA would associate with the RISC complex. IL-1β–stimulated HeLa cells transfected with miR-16 or control miRNA were used to isolate mRNAs associated with the RISC complex. qPCR was used to quantify COX-2 mRNA levels normalized to the respective IgG control and done in triplicate.

Fig. 1. miR-16 regulates COX-2 expression. A, HeLa cells were treated with IL-1β for 24 hours to induce COX-2 expression prior to being transfected with mature miR-16 or control miR for 48 hours; untreated (−) and nontransfected (NT) cells are indicated. COX-2 and COX-1 mRNA was detected by Northern blot and RT-PCR, respectively. Actin and GAPDH mRNA are shown as loading controls. Data shown represent 3 experiments. B, Western blot of COX-2 and COX-1 protein expression; actin served as a loading control. C, COX activity measured by PGE2 production in IL-1β–stimulated HeLa cells transfected with miR-16. Pretreatment of cells for 1 hour with 10 μmol/L NS-398 was used as a positive control for COX-2–specific inhibition. Relative PGE2 levels were normalized to total protein levels and are an average of 3 experiments. *, P < 0.023. D, HeLa cells were stimulated with IL-1β 24 hours prior to transfection with miR-16 for 48 hours, RNP-IP of Ago2 or control IgG was done to isolate mRNAs associated with the RISC complex. qPCR was used to quantitate COX-2 mRNA levels normalized to the respective IgG control and done in triplicate. *, P < 0.008. RT-PCR for COX-2 in RNP-IP reactions is shown in bottom panel. Total RNA from IL-1β–treated HeLa cells was used as a positive RT-PCR control. E, RNP-IP of Ago2 or control IgG from HeLa cells and qPCR of a validated miR-16 target, BCL-2, served as a positive control, and GAPDH served as a negative control.
miR-16 targets the COX-2 3′-UTR to promote rapid decay

To further investigate the relationship between miR-16 and COX-2 expression, luciferase reporter constructs bearing the full-length 1,455 nucleotide COX-2 3′-UTR (Luc+3′-UTR), the conserved ARE region of the 3′-UTR (Luc+ARE), or the ARE region deleted from the 3′-UTR (LucΔARE) were utilized (ref. 10; Fig. 2A). Two potential miR-16–binding sites were predicted within the COX-2 3′-UTR (denoted miR-16a, and miR-16b, Fig. 2A) using the 8 bp ARE complementary sequence of miR-16 as a prerequisite for targeting AU-rich elements, as single AUUUA motifs are resistant to miR-16–mediated regulation (26). Reporter constructs were transfected into HeLa cells along with miR-16 for 48 hr. Shown in Fig. 2B, miR-16 inhibited luciferase expression by approximately 2-fold in the presence of the full-length COX-2 3′-UTR. Similar levels of inhibition were observed in the presence of a single miR-16 site indicating the ability of miR-16 to target both the miR-16a and miR-16b 3′-UTR sites with similar efficiencies, and optimal inhibition was observed to occur in the presence of both miR-16 sites, suggesting a synergistic effect of multiple miRNA sites. An approximately 2-fold reduction in luciferase mRNA was observed in the presence of miR-16, consistent with miR-16–dependent reduction of luciferase activity derived from the Luc+3′-UTR reporter (Fig. 2C). To ensure that the effects of miR-16 were dependent on the presence of the COX-2 3′-UTR, a reporter construct containing a 1.8-kb COX-2 promoter (23) was cotransfected into HeLa cells with either miR-16 or the negative control. Shown in Fig. 2D, overexpression of miR-16 had no effect on COX-2 promoter or control SV40 promoter/enhancer activity, indicating miR-16 targeting of COX-2 is 3′-UTR dependent and not a consequence of indirect transcriptional interference.

Recent findings have implicated miRNA-mediated mRNA decay to be a predominant mechanism of miRNA-mediated regulation of gene expression (29), and the results shown in Fig. 1 indicate a decrease in COX-2 mRNA levels in the presence of miR-16. To determine whether miR-16–mediated COX-2 mRNA loss was due to elevated mRNA decay, HeLa cells cotransfected with Luc+3′-UTR reporter construct and miR-16 were treated with actinomycin D (ActD) to halt transcription and determine mRNA half-life. miR-16 promoted accelerated decay of Luc+3′-UTR mRNA, with more than 2-fold reduction in mRNA half-life as compared with control miRNA (Fig. 2E). The stability of the luciferase control vector was unaltered in the presence of miR-16 or control miR (data not shown). These results substantiate that targeted downregulation of COX-2 by miR-16 occurs via the 3′-UTR to promote enhanced mRNA degradation.

Altered expression of miR-16 is observed in colorectal cancer allowing for increased COX-2

A characteristic feature observed in many human malignancies, including colorectal cancer, is dysregulated miRNA expression (30). Our findings indicating the ability of miR-16 to inhibit COX-2 expression suggested the possibility that miR-16 expression levels are altered during colorectal tumorigenesis, and this may contribute to increased COX-2 expression in tumors. This was evaluated by assaying miR-16 expression in normal colon and tumor tissue. In 16 of 18 tumor samples, decreased miR-16 levels were observed compared with normal tissue, averaging a 1.7-fold decrease in expression (Fig. 3A). To further validate these results, miR-16 levels were evaluated from normal small intestinal tissue and tumors isolated from an APCMin+ mouse, a murine model of gastrointestinal cancer in which COX-2 is overexpressed (31). Indicated in Fig. 3B, mouse intestinal tumors display a significant 1.4-fold decrease in miR-16 expression. Decreased miR-16 expression was also observed colorectal cancer cell lines compared with normal colon tissue (Fig. 3C). Interestingly, LoVo cells do not exhibit a considerable difference in miR-16 expression, which may account for the limited COX-2 expression observed in this colorectal cancer cell line (6, 12).

To determine the effects of miR-16 on COX-2 expression in colorectal cancer cells, the COX-2 overexpressing HCA7 and Moser lines (12) were transfected with miR-16 or control miRNA for 48 hours and examined for COX-2 mRNA expression. RT-PCR and qPCR analysis showed that COX-2 mRNA levels were attenuated approximately 2-fold in both cell lines in the presence of miR-16 compared with normal control, whereas COX-1 expression levels remained unchanged (Fig. 4A and B). The ability of miR-16 to target COX-2 protein expression and PGE2 synthesis was also evaluated. Expression of miR-16 in HCA7 cells elicited a 2-fold decrease in COX-2 protein levels and a significant decrease in PGE2 levels compared with the negative control (Fig. 4C). Moser cells (Fig. 4D) seemed to be more responsive to miR-16 overexpression in that COX-2 protein levels were diminished in the presence of miR-16, and PGE2 levels were decreased to the extent of treatment of cells with the COX-2 inhibitor NS-398. Similar effects were observed in the colorectal cancer cell line HT-29 (data not shown).

Overexpression of HuR antagonizes miR-16 activity

A common feature observed in colorectal tumors is elevated expression of the ARE-binding protein HuR, which is associated with overexpression of COX-2 (6, 7, 12, 32). On the basis of the proximity between the miR-16– and HuR-binding sites within the COX-2 3′-UTR, we sought to determine whether overexpression of HuR Antagonizes MicroRNA-16 to Promote COX-2 Expression
of HuR could affect miR-16 targeting of COX-2. The Luc+3'-UTR reporter construct was transfected into HeLa cells along with miR-16 and an HuR expression vector or empty vector control for 48 hours. Shown in Fig. 5A, miR-16 inhibited Luc+3'-UTR activity to a similar extent as shown above (Fig. 2B). However, in the presence of elevated HuR, the ability of miR-16 to regulate Luc+3'-UTR expression was abolished. As shown in Fig. 2, miR-16 can accelerate COX-2 mRNA decay through the 3'-UTR. To determine whether HuR...
overexpression might interfere with this process, HeLa cells were transfected with Luc+3’-UTR, miR-16, and the HuR expression vector. Cells were then treated with ActD, and Luc+3’-UTR mRNA half-life was determined by qPCR. Shown in Fig. 5B, overexpression of HuR was shown to stabilize Luc+3’-UTR mRNA (t1/2 > 200 min) in the presence of miR-16 or control miRNA. Experiments using Luc control vector lacking the 3’-UTR showed no effect on Luc mRNA stability by HuR or miR-16 (data not shown).

To determine whether the HuR-binding site contained within the functional COX-2 ARE (10) was necessary for HuR-mediated inhibition of miR-16, the LucΔARE reporter construct was transfected into HeLa cells along with miR-16 and HuR expression vector for 48 hours. In contrast to the Luc+3’-UTR vector, miR-16 was capable of attenuating LucΔARE activity regardless of HuR expression (Fig. 5C), suggesting that HuR binding to the COX-2 ARE is needed for its miR-16 inhibitory effect.

Evaluation of HuR’s suppression of miR-16 function upon other target mRNAs was examined using a Tet-responsive HuR-inducible HeLa cell line (HeLa-Tet-Off/HuR-Flag) to overexpress HuR (12). In these cells, the removal of Dox from the medium allows for overexpression of HuR that is observed in both the nucleus and the cytoplasm (Supplementary Fig. S1). Cells were grown in the presence or absence of Dox to control HuR overexpression and then stimulated with IL-1β for 24 hours followed by transfection of miR-16 or control miR. As shown in Fig. 5D, miR-16 target mRNAs COX-2, BCL-2 (28), VEGF (33), and cyclin E1 (34) displayed attenuated mRNA expression in miR-16-transfected cells in the absence of HuR overexpression. When HuR was overexpressed, inhibition of miR-16 function was observed with COX-2, BCL2, and VEGF transcripts but not with cyclin E1. Interestingly, COX-2, BCL2, and VEGF mRNAs all contain 3’-UTR ARE motifs, whereas cyclin E1 mRNA does not contain an ARE (35), further supporting that ARE binding by HuR to the same message in cis is needed for inhibition of miR-16.

Overexpression of HuR attenuates miR-16 expression

To better understand the basis of the observed inhibition of miR-16 by HuR, we determined whether miR-16 levels were altered by HuR overexpression. HeLa-Tet-Off/HuR-Flag cells were grown in the absence of Dox to induce...
expression of HuR and miR-16 levels were quantified by qPCR. As shown in Fig. 6A, overexpression of HuR led to a substantial decrease in miR-16 levels. This loss of miR-16 was not observed with overexpression of the ARE-binding protein TTP using Tet-responsive TTP-inducible HeLa cells (12), supporting a specific effect of HuR in promoting miR-16 reduction. To determine whether this effect on miR-16 is observed when HuR expression is attenuated, HCA7 cells were transfected with HuR siRNA for 72 hours and miR-16 levels were quantified by qPCR. As shown in Fig. 6B, a 2-fold reduction in HuR protein levels in response to HuR siRNA resulted in a more than 5-fold increase in miR-16 levels. In addition, various colorectal cancer cell lines were examined to determine whether endogenous HuR overexpression and cytoplasmic localization associated with decreased miR-16 levels. CaCo2, HCA7, Moser, and HT-29 cells all display nuclear and cytoplasmic localization of HuR consistent with HuR overexpression (12), whereas nuclear localization was observed in LoVo cells (Supplementary Fig. S2). Consistent with localization of HuR, miR-16 levels were attenuated in all colorectal cancer cell lines, except in LoVo cells (Fig. 3C), further supporting that HuR overexpression is needed to regulate miR-16 levels.

On the basis of these results, we speculated whether miR-16 downregulation might involve a direct HuR:miR-16

Figure 4. Overexpression of miR-16 targets COX-2 in colon cancer cells. colorectal cancer cell lines HCA7 and Moser were transfected with miR-16 or control miRNA for 48 hours. COX-2 and COX-1 mRNA expression was assayed by RT-PCR (A) and qPCR (B) using GAPDH as loading control. *, P < 0.05. C and D, COX-2 protein expression in miR-16-transfected HCA7 and Moser cells was examined by Western blot; actin served as a loading control. Data shown represent 3 experiments. Respective PGE2 levels from culture media were assayed as described above. *, P = 0.0405 for HCA7, P = 0.0289 for Moser.
interaction. To test this, cytoplasmic lysates were generated from Tet-responsive HuR-inducible cells grown in the presence or absence of Dox for 48 hours and subjected to RNP-IP using anti-HuR antibody or control IgG to determine whether HuR is bound to miR-16 when overexpressed and present in the cytoplasm. As shown in Fig. 6C, HuR associated with miR-16 only in immunocomplexes when HuR expression was induced [Dox (−) conditions]. A positive control, COX-2 mRNA was also detected by RT-PCR in HuR immunoprecipitates only when HuR was overexpressed (Fig. 6C). To evaluate the specificity of the HuR:miR-16 interaction, qPCR reactions were carried out to detect control U6 snRNA and miR-15a (Fig. 6C). miR-15a is transcribed cocistronically with miR-16 (26) and was not immunoprecipitated with HuR under any conditions indicating that HuR does not interact with the unprocessed pri-miR-16-1/15a transcript. Pre-miR-16-1 was also analyzed in immunoprecipitates by qPCR analysis using precursor-miRNA-specific forward and reverse primers targeting the stem-loop sequence present in pre-miR-16-1 and was not detected in HuR immunoprecipitates, indicating that the co-IP reflects an interaction of HuR with mature miR-16 (data not shown). This interaction between HuR and miR-16 was also shown to occur in vitro using a 2D-FIDA assay (22, 25), with a $K_d$ of 120 nmol/L observed between recombinant full-length HuR and labeled miR-16 (Fig. 6D).

To determine whether the presence of the ARE-containing COX-2 mRNA was a necessary component facilitating HuR:miR-16 interaction, HeLa-Tet-OFF/HuR-Flag cells were grown to induce HuR overexpression and transfected with a COX-2 or control siRNA, then assayed for the presence of miR-16 in HuR immunoprecipitates. As shown in Fig. 6E, the association between HuR and

Figure 5. Overexpression of HuR antagonizes the ability of miR-16 to target the COX-2 3′-UTR. A, HeLa cells were cotransfected with Luc−3′-UTR in addition to a vector expressing Flag-tagged HuR (−HuR) or empty vector, followed by transfection of miR-16 or control miRNA for 48 hours. Luc activity was normalized to the respective control and are the average of 3 experiments. * $P = 0.0106$. B, Luc−3′-UTR mRNA decay in HeLa cells cotransfected with HuR expression vector and miR-16 (open circles) or control miRNA (black circles) for 48 hours prior to treatment with ActD at indicated times. Luc mRNA decay was assayed by qPCR and normalized to GAPDH. C, HeLa cells were transfected as described in (A) except the Luc−ARE construct was used. Luc activity was normalized to the respective control and are the average of 3 experiments. * $P < 0.03$. D, HeLa-Tet-OFF/HuR-Flag were grown in the absence of Dox for 48 hours to induce expression of HuR. After the first 24 hours of HuR induction, cells were stimulated with IL-1β for 24 hours followed by transfection of miR-16 (grey bars), or control miRNA (black bars). After 48 hours, COX-2, BCL-2, VEGF, and cyclin E1 mRNA levels were analyzed by qPCR using GAPDH for normalization. * $P < 0.05$. 

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Figure 6. Overexpression of HuR attenuates miR-16 expression. A, HeLa-Tet-OFF/HuR-Flag and HeLa-Tet-OFF/TTP-Flag cells were grown in the absence of Dox for 48 hours to induce expression of HuR or TTP. Relative miR-16 levels were determined by qPCR using U6 as loading control and normalized to miR-16 levels in the absence of HuR or TTP overexpression. Data shown are the average of 3 experiments. *, P = 0.0028. B, HCA7 cells were transfected with siRNA against HuR (grey bars) or control siRNA (black bars) for 72 hours. Relative HuR mRNA and miR-16 levels were determined by qPCR using 18S for normalization. **, P = 0.0028; *, P = 0.0259. Western blot of HuR protein expression after siRNA transfection is shown using loading control actin. C, HeLa Tet-OFF/HuR-Flag cells were grown in the absence of Dox for 48 hours to induce HuR expression. Cytoplasmic extracts were subjected to RNP-IP of HuR or control IgG to isolate miRNAs associated with HuR. qPCR was used to quantitate miR-16, miR-15, and U6 RNA levels using the miR-16/IgG control IP for normalization in each respective condition. Lower panel reflects respective RT-PCR of COX-2 and GAPDH mRNA present in HuR RNP-IP reactions. Positive (+) control is total RNA amplified in RT-PCR reaction. D, 5’TMR–labeled miR-16 was incubated with increasing concentrations of recombinant full-length HuR. Fluorescence anisotropy was measured using 2D-FIDA, and the affinity was determined by fitting the fluorescence anisotropy data to yield a $K_d = 120 \pm 9 \text{nmol/L}$. E, HeLa Tet-OFF/HuR-Flag cells were grown in the absence of Dox and transfected with COX-2 or control siRNA for 48 hours. COX-2 mRNA was assayed by qPCR using GAPDH for normalization. *, P = 0.0001. Cytoplasmic extracts were subjected to RNP-IP of HuR or control IgG and qPCR was used to quantitate miR-16 present in immunoprecipitates; IgG control IPs were used for normalization.
miR-16 was lost in samples where COX-2 mRNA was attenuated.

**HuR:miR-16 interaction is dependent on HuR cytoplasmic localization**

Because the interaction between HuR and miR-16 was observed only under conditions in which HuR levels were elevated, we explored whether this interaction was dependent on HuR cytoplasmic localization. To accomplish this, HeLa Tet-OFF/HuR-Flag cells were grown in the absence of Dox to induce HuR expression, after which they were treated with a low-molecular weight inhibitor of HuR, MS-444. This compound has been previously shown to prevent the cytoplasmic trafficking of HuR by interfering with its homodimerization (22). Cytoplasmic lysates were obtained and subjected to HuR RNP-IP followed by qPCR analysis for miR-16. As depicted in Fig. 7A, treatment of cells with MS-444 resulted in a time-dependent decrease in the amount of miR-16 associated with HuR. Cellular fractionation further confirmed that the amount of HuR present in the cytoplasm decreased over time of compound treatment (Fig. 7B) and correlated with loss of miR-16 binding. As a control, total HuR and nucleoporin levels were assayed in cells treated with MS-444 to validate the observed changes in HuR cytoplasmic localization were not due to the loss of either HuR or nucleoporin (in preparation and Fig. 7C). These results indicated that heightened levels of cytoplasmic HuR can interact with mature miR-16 to promote its downregulation, and this interaction can be reversed by the addition of an HuR inhibitor that prevents its cytoplasmic localization.

**Discussion**

Data obtained from several sources clearly shows COX-2 overexpression to be a critical step contributing to various...
facets of colorectal cancer tumorigenesis (9), and growing evidence indicates that this overexpression is facilitated through loss of rapid ARE-mediated mRNA decay (11). Previously, we showed that elevated expression of the mRNA stability factor HuR in colon cancer promoted COX-2 mRNA stabilization by interfering with ARE-mediated decay (6, 12). The findings presented here identify miR-16 to be a key component in targeting COX-2 mRNA for rapid decay and overexpression of HuR directly interferes with this process. This effect was dependent on HuR being overexpressed and present in the cytoplasm, where it maintained the ability to bind both, the COX-2 mRNA and miR-16 and promote miR-16 downregulation as observed in colorectal cancer cells and tumors. In addition, HuR seems to suppress miR-16 function in an mRNA ligand-dependent manner, in that a functional ARE was needed for interference of miR-16 function.

Normal cell growth is associated with rapid decay of ARE-containing mRNAs and selective targeting of these transcripts, which compose 5% to 8% of the human transcriptome (36), is an essential way of controlling their expression. However, a number of observations have indicated that deficiencies in ARE-mediated decay occurs during neoplastic transformation of cells (8, 37), and current findings have shown a 3- to 4-fold enrichment in ARE-containing genes to occur during colorectal cancer tumorigenesis (38). These observations can be explained in part by the results presented here and indicate that ARE-mediated posttranscriptional regulation is an important regulatory mechanism lost during the early stages of colorectal cancer tumorigenesis.

An objective of this study was to further elucidate defects in COX-2 posttranscriptional regulation present in colorectal cancer in the context of miRNA-mediated regulation. miR-16 was previously determined to promote degradation of ARE-containing transcripts through complementarity to ARE motifs that was independent of canonical miRNA 5’ seed sequence commonly observed in many examples of miRNA-mediated regulation (26, 39, 40). Within the COX-2 3’-UTR, there are 2 miR-16- binding sites that contain 8 bp homology to the miR-16 sequence UAAAUAUU previously determined to provide efficient targeting by miR-16 (26). Using 3’-UTR reporter constructs, the 2 regions containing either miR-16 site maintained similar efficiencies in attenuating reporter expression by accelerating rapid mRNA decay, in agreement with work examining COX-2 3’-UTR-mediated posttranscriptional regulation by miR-16 in response to diabetic stimuli in leukocytes (27). Furthermore, miR-16 was efficient in controlling endogenous COX-2 expression at both the mRNA and protein level, which subsequently resulted in reduced PGE2 levels in both IL-1β–stimulated HeLa and colon cancer cells. Interestingly, HCA7 cells seemed to be marginally resistant to miR-16 downregulation of COX-2 compared with other colon cancer cells. Previous work has shown that HCA7 cells possess a truncated COX-2 3’-UTR variant resulting from alternative polyadenylation and lacks 1 of the 2 miR-16-binding sites (miR-166a, ref. 41). Thus, the moderate sensitivity of HCA7 cells to miR-16 can be attributed to the presence of the truncated mRNA transcript, which was reported to be inherently more stable than the full-length transcript (41). These results show a central role for miR-16 in controlling COX-2 expression in varied physiologic and pathologic conditions.

A fundamental question about the role of miR-16 in colorectal cancer etiology pertains to identifying and understanding the factors that contribute to its loss in tumors. The results presented here show decreased miR-16 levels to be present in both human colorectal tumors and cell lines, along with loss in murine adenomas derived from the APC Min/+ mouse. These results are in agreement with those showing reduced miR-16 levels present in the colorectal microRNAome (42) and detection of lower miR-16 expression observed in the stool of advanced stage colorectal cancer patients (43). On the basis of these findings, we hypothesize that tumor-derived loss of miR-16 expression contributes to COX-2 overexpression observed during colorectal cancer tumorigenesis.

Various mechanisms have been reported that influence miR-16 expression in tumors. miR-16 is part of a poly-cistronic miRNA also encoding miR-15a located on chromosome 13q14. Although genomic loss of this region in chronic lymphocytic leukemia results in miR-16 loss (44), this region is not an observed site of genomic alterations in colorectal cancer (45). Expression of miR-16 initiates from transcription of the DLEU2 promoter and overexpression of c-Myc can suppress miR-16 expression through repression of the DLEU2 promoter (46). Finally, miR-16 maturation can be modulated by p53 through its interaction with the miRNA processing complex involving Drosha and p68 (47), indicating that loss of p53 in colorectal cancer tumors may contribute to decreased mature miR-16 levels owing to altered miRNA processing.

The mRNA stability factor HuR is overexpressed in a variety of cancers, particularly colorectal cancer, and this overexpression is characterized with increased cytoplasmic localization (12, 13). In this context, HuR can interfere with ARE-mediated decay; however, the manner in which HuR facilitates this process is still not understood. The findings presented here identify a new facet of this process in that HuR can antagonize miR-16 targeting of the COX-2 3’-UTR and promote mRNA stabilization. Our data indicate a mechanism in which HuR inhibition of miR-16 occurs by HuR directly binding to miR-16 and preventing its interaction with the COX-2 3’-UTR (Fig. 7D). This association between HuR and miR-16 seems facilitated by nucleation at the COX-2 ARE because deletion of the ARE allowed for miR-16 function through the site adjacent to the ARE (miR-166a). HuRmiR-16 interaction was not detected when the COX-2 transcript was downregulated by siRNA. In support of this, HuR was observed to interfere with miR-16 targeting of other ARE-containing transcripts, whereas miR-16 was capable of regulating non-ARE-containing target cyclin E1 mRNA regardless of HuR expression status. More importantly, the ability of HuR to inhibit miR-16 was only apparent under conditions observed in colorectal cancer.
cells and tumors in which HuR was overexpressed and located in the cytoplasm. Furthermore, inhibition of HuR cytoplasmic trafficking by MS-444 diminished its association with miR-16. On the basis of these observations, we hypothesize that a central event occurring during cellular transformation or in response to stress is the altered nucleo-cytoplasmic localization of HuR; the cytoplasmic presence of HuR allows it access to mature miR-16 at the COX-2 ARE, ultimately leading to pathogenic mRNA stabilization.

Unexpected in this study were the results indicating ability of HuR to promote miR-16 downregulation and that siRNA knockdown of HuR in colorectal cancer cells allows for increased miR-16 levels. Although this suggests a much more active role of HuR in RNA and specifically miRNA metabolism, it remains to be seen how this newly found activity of HuR might connect to its recently discovered RNA 3’-modifying enzymatic activity (48). Whether through a direct enzymatic action or not, our data strongly suggest a novel mechanism describing how HuR overexpression interferes with ARE-mediated mRNA decay. By virtue of its cytoplasmic localization in tumor cells, HuR actively contributes to miR-16 elimination, allowing for unrestricted HuR binding to the COX-2 ARE. This ability of HuR to regulate miRNA levels when overexpressed extends to other miRNAs such as miR-30 and let-7a (unpublished observations), which may, in part, explain the observed loss of specific miRNAs in tumors. The findings reported in this study shed a new light on the role of HuR in connecting miRNA and ARE-mediated posttranscriptional regulation and indicate it to be a viable therapeutic target that can impact both mRNA and miRNA expression levels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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