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NOD2 Expression is Regulated by microRNAs in Colonic Epithelial HCT116 Cells

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Abstract

Background—Crohn's disease (CD) is associated with defective sensing of pathogens in genetically susceptible individuals. Nucleotide-binding oligomerization domain containing 2 (*NOD2*) mutations in coding regions are strongly linked to CD pathogenesis. Our laboratory has reported that microRNAs (miRNAs) are differentially expressed in CD. However, miRNA regulation of *NOD2* remains unknown. This study was designed to determine whether miRNAs regulate *NOD2* expression as well as downstream nuclear factor kappaB activation and inflammatory responses in colonic epithelial HCT116 cells.

Methods—*NOD2* and miRNA expression in stimulated HCT116 cells were assessed by quantitative reverse transcription–polymerase chain reaction. Regulation of *NOD2* expression by miRNAs was determined by luciferase reporter construct assays and transfection of specific miRNA mimics. Regulation of *NOD2* signaling and immune response by miRNAs was assessed by transfection of mimics followed by muramyl dipeptide stimulation.

Results—Muramyl dipeptide-induced increases in *NOD2*, interleukin-8, and CXCL3 expression were inversely associated with miRNA expression. Overexpression of miR-192, miR-495, miR-512, and miR-671 suppressed *NOD2* expression, muramyl dipeptide-mediated NF- κ B activation, and messenger RNA expressions of interleukin-8 and CXCL3 in HCT116 cells. A single-nucleotide polymorphism (rs3135500) located in the *NOD2* 3'-untranslated region significantly reduced miR-192 effects on *NOD2* gene expression.

Conclusions—To our knowledge, this is the first report demonstrating that miRNAs regulate *NOD2* and its signaling pathway. Four miRNAs downregulate *NOD2* expression, suppress NF- κ B activity, and inhibit interleukin-8 and CXCL3 messenger RNA expression. Treatment of CD with miRNAs may represent a potential anti-inflammatory therapeutic strategy in CD patients with and without *NOD2* gene mutations.

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KeywordsmicroRNAs; NOD2; MDP; NF- κ B

Inflammatory bowel disease (IBD) is a chronic relapsing and remitting inflammatory condition of the gastrointestinal tract. It comprises 2 major disease subtypes, including Crohn's disease (CD) and ulcerative colitis (UC). IBD is characterized by a dysregulation of the inflammatory response to intestinal microbiota in genetically predisposed individuals. Although both forms of IBD share some common features, each disease has its unique clinical course, genetic associations, and gene expression patterns.¹ Mucosal inflammation in UC is continuous and only involves the superficial layer of the colon, whereas inflammation in CD is discontinuous and transmural and affects any part of the gastrointestinal tract, with the terminal ileum most commonly involved.² Since 2005, genome-wide association studies have identified a number of shared and distinct genetic susceptibility loci for CD and UC.^{3–5} In addition, genome-wide messenger RNA (mRNA) expression profiles have demonstrated that mRNA transcripts are differentially expressed in CD and UC.^{6–8} Furthermore, microRNA (miRNA) expression studies have revealed common and unique miRNA signatures for CD and UC.^{9–12} Differential protein expression signatures were also identified in CD and UC patients.¹³

Although the intestinal microbiota are in constant interaction with the host epithelial and immune cells, the intestinal epithelium is the first line of defense against pathogens. Therefore, maintaining intestinal immune homeostasis is critical.¹⁴ The detection of invading pathogens by pattern recognition receptors is important in initiating innate immune response. Pattern recognition receptors comprised of cell surface receptors, such as Toll-like receptors (TLRs) and C-type lectin receptors, and intracellular receptors, such as RIG-I-like receptors and nucleotide-binding domain leucine-rich repeat containing receptors.¹⁵ Pattern recognition receptors are germline-encoded, evolutionarily conserved receptors that distinguish pathogen-associated molecular patterns. Pathogen-associated molecular patterns include lipopolysaccharide, a major component of outer membrane of gram-negative bacteria, peptidoglycan, a main component of gram-positive bacterial cell wall, flagellin, and microbial nucleic acids.

Nucleotide-binding oligomerization domain containing 2 (*NOD2*) is a member of the nucleotide-binding domain leucine-rich repeat containing receptors family. It was the first CD susceptibility gene identified.^{16,17} Individuals who have *NOD2* mutations are more susceptible to CD.¹⁸ *NOD2* is expressed in antigen presenting cells, such as monocytes, macrophages, and dendritic cells, Paneth cells, and numerous epithelial cell types of colonic and pulmonary origin.¹⁹ *NOD2* confers responsiveness to the ligand muramyl dipeptide (MDP), a degraded bacterial cell wall component, peptidoglycan. Activation of *NOD2*-dependent pathways leads to the induction of NF- κ B as well as the activation of JNK (c-Jun N-terminal kinase) and p38 MAPK. *NOD2* activation has been shown to influence the expression and secretion of pro-inflammatory and anti-inflammatory cytokines, including interleukin (IL)-1 β , TNF α , IL-6, IL-8, IL-10, and IL-12p40.^{20–23} Dysfunctional nuclear factor-kappaB (NF- κ B) activation impairs production of regulatory cytokines and

chemokines and induces death of intestinal epithelial cells and impairment of the intestinal barrier.²⁴

The miRNAs are a group of small (~22 nucleotide) non-coding RNAs that function as key regulators of mRNA stability, degradation, and translational efficiency.²⁵ miRNAs have been implicated in many biological processes, including development, cell proliferation and differentiation, metabolism, apoptosis, and autophagy.^{25–28} miRNAs are also involved in the pathogenesis of a number of diseases, such as cancer, metabolic disorders, IBD, and other inflammatory and autoimmune diseases.^{9–11,26,27,29–32} Our laboratory has previously identified that the IBD-associated miRNA, miR-192, regulates the inflammatory cytokine-induced expression of macrophage inflammatory peptide-2 α in intestinal epithelial cells.⁹ Interestingly, although the regulation of NOD2 by miRNAs has not yet been investigated, in silico analysis of *NOD2* 3'-untranslated region (3'UTR) reveals 11 putative miRNA binding sites that include 2 putative miR-192 binding sites (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), implicating miR-192 and other miRNAs in the regulation of NOD2 expression and inflammatory responses.

In this study, we hypothesized that miRNA-mediated repression of NOD2 expression reduces MDP–NOD2 interactions and inhibits NOD2/NF- κ B signaling cascade and immune responses in HCT116 cells. We identified that miR-122, miR-192, miR-495, and miR-671 inversely correlated with NOD2 expression in response to MDP stimulation. Ectopic expression of miR-192, miR-495, miR-512, and miR-671 in HCT116 cells suppressed NOD2 expression, NF- κ B activation, and immune responses. In addition, mutating the seed regions of both miR-192 binding sites in the 3'UTR of *NOD2* influenced NOD2 gene expression. Furthermore, an immune disease-associated single-nucleotide polymorphism (SNP) (rs3135500) in the second miR-192 binding site also resulted in altered NOD2 expression. Our findings suggest that miRNAs may play a critical role in the regulation of NOD2 expression and function.

Materials and Methods

Cell Culture

THP-1, HCT116, HT-29, Caco-2, SW620, and T84 cell lines were previously obtained and used for this study. THP-1 cells were cultured in RPMI-1640 medium. HCT116 cells were cultured in McCoy's 5A medium. HT-29 and Caco-2 cells were cultured in Dulbecco's modified Eagle's medium. SW620 cells were cultured in Leibovitz's L-15 medium. T84 cells were cultured in Dulbecco's modified Eagle's medium/Ham's F12 50/50 medium. All cell lines were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in 5% CO₂. For experiments in which HCT116 cells were stimulated with MDP, medium alone (control) or medium containing MDP (Sigma–Aldrich, St. Louis, MO) at 50 μ g/mL was added and cells were harvested at respective time points for RNA and protein analyses.

Quantitative Reverse Transcription–Polymerase Chain Reaction for mRNA

Total RNA was isolated using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's protocol. RNA concentrations were determined using a Nano-Drop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). One microgram of total RNA was converted to complementary DNA (cDNA) using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Quantitative polymerase chain reaction (qPCR) analysis was performed using QuantiFast SYBR Green PCR kit (Qiagen, Valencia, CA). The qPCR amplifications were performed on the LightCycler 480 real-time PCR system (Roche, Indianapolis, IN). The expression of GAPDH was used as internal control. The primers for NOD2 were 5'-GATTGGCTGCCTTCCTTCTA-3' (forward) and 5'-GAGCGTCTCTGCTCCATCAT-3' (reverse). The primers for IL-8 were 5'-CTGCGCCAACACAGAAATTA-3' (forward) and 5'-TGAATTCTCAGCCCTCTTCAA-3' (reverse). The primers for CXCL3 were 5'-GAAGTCATAGCCACACTCA-3' (forward) and 5'-GCTCCCCCTTGTTAGTATCTTT-3' (reverse). The primers for GAPDH were 5'-CGACCACTTTGTCAAGCTCA-3' (forward) and 5'-AGGGGAGATTCAGTGTGGTG-3' (reverse).

Quantitative Reverse Transcription–Polymerase Chain Reaction for miRNA

One microgram of total RNA was converted to cDNA using the NCode VILO miRNA cDNA synthesis kit (Life Technologies) followed by qPCR using the NCode EXPRESS SYBR GreenER miRNA qRT-PCR kit (Life Technologies). The expression of miRNAs was calculated relative to RUNU6B, a ubiquitously expressed small nuclear RNA. The forward primer for miR-20a* was 5'-ACTGCATTATGAGCACTTAAAG-3'. The forward primer for miR-122 was 5'-GGAGTGTGACAATGGTGTGTTGTAA-3'. The forward primer for miR-124 was 5'-TAAGGCACGCGGT-GAATGCC-3'. The forward primer for miR-192 was 5'-CTGACC-TATGAATTGACAGCC-3'. The forward primer for miR-215 was 5'-ATGACCTATGAATTGACAGAC-3'. The forward primer for miR-342-5p was 5'-AGGGGTGCTATCTGTGATTGA-3'. The forward primer for miR-453 was 5'-AGGTTGTCCGTGGT-GAGTTCGCA-3'. The forward primer for miR-495 was 5'-AAACAAACATGGTGCCTTCTTT-3'. The forward primer for miR-512-5p was 5'-TCAGCCTTGAGGGCACTTTCA-3'. The forward primer for miR-671-5p was 5'-AAGCCCTG-GAGGGGCTGGAGGT-3'. The forward primer for RUNU6B was 5'-CGCAAGGATGACACGCAAATTCG-3'. A universal qPCR primer was used as the reverse primer.

Western Blot

Cells were lysed in cold RIPA buffer (Thermo Fisher Scientific, Rockford, IL) supplemented with 1% protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cell lysate was suspended in Laemmli sample buffer (Bio-Rad, Hercules, CA) containing 2-mercaptoethanol and boiled for 5 minutes. Protein concentration was determined with BCA protein assay (Thermo Fisher Scientific). After heat denaturation, total protein lysates were subjected to SDS–PAGE and transferred electrophoretically to PVDF membranes. Membranes were blocked in 5% nonfat milk in PBS with 0.1% Tween-20 (PBST) for 1 hour. The blots were incubated with goat anti-NOD2 (Novus Biologicals, Littleton, CO;

1:1000 dilution), rabbit anti-I κ B α (Santa Cruz, Dallas, TX; 1:1000 dilution), rabbit anti-p65 subunit of NF- κ B (Abcam, Cambridge, MA; 1:500 dilution), rabbit antitubulin (Cell Signaling, Danvers, MA; 1:1000 dilution), and mouse anti-GAPDH (Life Technologies; 1:1000 dilution) overnight at 4°C. After washing with PBST, blots were incubated with Alexa Fluor 680 (Life Technologies) and IRDye 800CW (LI-COR Biosciences, Lincoln, NE) conjugated secondary antibodies (1:10,000 dilution) for 1 hour. The band intensities were quantified using an Odyssey infrared imaging system (LI-COR Biosciences).

NOD2 3'UTR Constructs

The nucleotide sequence of the *NOD2* 3'UTR spanning 3181 to 4485 (RefSeq # NM_022162.1) was cloned into pmir-GLO dual-luciferase miRNA target expression vector (Promega, Madison, WI) by GenScript. The full-length (WT) pmirGLO-*NOD2*-3'UTR vector was used as a template to mutate the entire seed region of 1 or both putative miR-192 binding sites or to recreate the rs3135500 SNP. The modified seed sequences of each miR-192 binding site and SNP mutant are outlined in Figure 6A.

miRNA Mimic and Luciferase Construct Transfection

The miRNA mimic negative control (HMC0003) and mimics of miR-122 (HMI1002), miR-124 (HMI0086), miR-192 (HMI0311), miR-495 (HMI0610), miR-512-5p (HMI0640), and miR-671-5p (HMI0901) were obtained from Sigma. An miRNA mimic or a luciferase construct was transfected into HCT116 cells using Lipofectamine 2000 reagent (Life Technologies) according to the manufacturer's guidelines. At 24 hours posttransfection, cells were harvested for RNA and protein analyses or were harvested for measurement of luciferase activities.

Luciferase Reporter Assay

Cells were lysed in passive lysis buffer and then analyzed for the firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) on the GloMax-Multi Detection System: Luminometer (Promega) according to the manufacturer's instructions. The firefly luciferase activity was normalized to the renilla luciferase activity.

Statistical Analysis

GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA) was used for the statistical analysis. Statistical significance was determined by standard 2-tailed Student's *t* test (for comparison of 2 conditions) and 1-way analysis of variance with Dunnett's posttest (for multiple group comparisons). Results were reported as mean $\pm$ standard error of the mean. The *P* value < 0.05 was considered statistically significant.

Results

Endogenous NOD2 is Functional in Colonic Epithelial Cells

We first analyzed the endogenous NOD2 mRNA and protein expression in colonic epithelial cell lines by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and Western blot, respectively. Specifically, using the THP-1 cell line as a positive control,³³ we

assessed NOD2 mRNA expression in Caco-2, HCT116, HT-29, SW620, and T84 cells (Fig. 1A). Although NOD2 expression was detected in all cell lines, the HCT116 cells expressed markedly greater NOD2 mRNA as compared with the other cell lines tested. Similarly, we compared NOD2 protein expression in HCT116, HT-29, and Caco-2 cells, with THP-1 cell line as a positive control (Fig. 1B). NOD2 protein expression was markedly greater in HCT116 cells but only minimally expressed in HT-29 and Caco-2 cells.

Previous reports indicate that NOD2 is functional in HCT116 cell.³⁴ First, we confirmed that NOD2 mRNA expression was inducible in HCT116 cells treated with MDP (Fig. 2A). Furthermore, our qRT-PCR analysis indicated that MDP (50 µg/mL) stimulation of NOD2 was time dependent with a maximal 2.4-fold increase in NOD2 mRNA expression observed 6 hours after MDP treatment. Second, we tested whether MDP can activate the NF-κB signaling pathway in HCT116 cells. We induced HCT116 cells with MDP and assessed phosphorylation of NF-κB and degradation of NF-κB inhibitor, alpha (IκBα) at different time points. Our result demonstrated that NF-κB is phosphorylated within the first 30 minutes of MDP induction, whereas IκBα degradation was seen after 60 minutes of MDP stimulation (Fig. 2B). To further study the function of NOD2 in HCT116 cells, we determined the regulatory effects of NOD2 on the inflammatory responses in HCT116 cells. We stimulated the cells with MDP and determined the expression of IL-8 and CXCL3 at different time points. The qRT-PCR analysis demonstrated that IL-8 and CXCL3 mRNA expressions were increased after MDP induction, with a maximal expression 2 hours after induction (Fig. 2C). These results confirmed that NOD2 is functional in HCT116 cells. Because we were able to confirm that NOD2 is highly expressed and functional in HCT116 cells, we used this cell line for our further examination of miRNA regulation of NOD2 in intestinal epithelial cells.

Expressions of miRNAs that Bind to NOD2 3'UTR in HCT116 Cells

An in silico analysis identified 10 miRNAs with putative binding sites in the 3'UTR of *NOD2* (Fig. 3A). We determined the endogenous expression of these 10 miRNAs by qRT-PCR (Fig. 3B). The 4 most highly expressed miRNAs in HCT116 cells were miRs-124, -192, -512, and -671 while the remaining miRNAs had low expression levels. Because we hypothesized that the MDP-induced increase in NOD2 expression may be influenced by a corresponding downregulation of regulatory miRNAs, we then determined which miRNAs exhibited altered expression in response to MDP (Fig. 3C). The results demonstrated that the expression of miRs-122, -192, -495, and -671 were significantly decreased in MDP-stimulated cells compared with unstimulated cells. Our data suggest an inverse correlation between NOD2 expression and the expression of these miRNAs in HCT116 cells, raising the possibility that NOD2 may be regulated by miRNAs and that the effect of MDP on NOD2 expression may result, in part, from a downregulation of these NOD2-associated miRNAs.

Regulation of NOD2 by miRNAs in Colonic Epithelial Cells

To identify whether miRNAs may regulate NOD2, we first determined whether *NOD2* 3'UTR may be influenced by endogenous miRNAs. A luciferase reporter construct bearing the *NOD2* 3'UTR was transfected into unstimulated HCT116 cells. This resulted in a 47% reduction in luciferase activity (Fig. 4A), indicating that the *NOD2* 3'UTR and endogenous

miRNAs can influence NOD2 gene expression ($P < 0.01$). Next, to determine which miRNAs may influence NOD2 gene expression, we co-transfected HCT116 cells with a luciferase reporter vector containing the *NOD2* 3'UTR and miRNA mimics (Fig. 4B). Our results demonstrated that miRs-122, -192, -512, and -671 individually suppressed luciferase activity, with miRs-192, -512, and -671 demonstrating the most significant effect. Meanwhile, miR-124 had no demonstrable effect on luciferase activity.

Based on the above results, we tested whether miRNA mimics could directly influence endogenous NOD2 mRNA and protein expression. Similar to our luciferase assay results, we observed that miRs-122, -192, -495, and -512 mimic transfection significantly decreased endogenous NOD2 mRNA expression (Fig. 4C), with the miR-512 mimic transfection resulting in the greatest reduction in NOD2 mRNA expression and miR-124 mimic transfection having no effect on NOD2 mRNA expression. Interestingly, miR-671 mimic transfection had no effect on endogenous NOD2 mRNA expression. When we examined miRNA mimic transfection effects on endogenous NOD2 protein expression by Western blot analysis, our densitometry analysis demonstrated that transfection of the miRs-192, -512, and -671, and to a lesser extent miR-495, mimics resulted in a marked decreased NOD2 protein expression (Fig. 4D). The effect of miRs-122 and -124 mimic transfection on NOD2 protein expression seemed less robust. Similarly, when we assessed the effect of miRNA mimic transfection on MDP-stimulated NOD2 expression after 6 hours, our results demonstrated that miRs-192, -495, -512, and -671 inhibited MDP-stimulated NOD2 protein expression as well (see Figure, Supplemental Digital Content 1, <http://links.lww.com/IBD/A346>). Overall, our results indicated that miRs-192, -512, and -671, consistently influence NOD2 expression. Results for miR-495 and miR-122 were less convincing. The miR-122 mimic transfection significantly reduced luciferase activity when co-transfected with the luciferase reporter vector containing the *NOD2*-3'UTR, influenced endogenous NOD2 mRNA expression and seemed to modestly reduce NOD2 protein expression. The miR-495 mimic transfection did not significantly reduce luciferase activity when co-transfected with the luciferase reporter vector containing the *NOD2*-3'UTR. However, the miR-495 mimic seemed to modestly reduce endogenous NOD2 mRNA and protein expression. Our results with the miR-124 mimic indicated that it, most likely, does not directly influence NOD2 mRNA or protein expression.

miRNAs Attenuate Innate Immune Responses via Suppression of NOD2 Signaling Pathway

Because miRNAs were demonstrated to influence NOD2 protein expression, we hypothesized that these miRNAs may influence downstream NOD2 function. To test this hypothesis, we examined the effect of NOD2-associated miRNAs on MDP-induced NF- κ B activity, transfected HCT116 cells with miRNA mimics, and stimulated cells with MDP. We assessed phosphorylation of NF- κ B at 30 minutes (Fig. 5A) and degradation of I κ B α at 60 minutes (Fig 5B). Our results demonstrated that mimic transfection of all 4 miRNAs with the greatest effect on NOD2 protein expression, miRs-192, -495, -512 and -671, resulted in reduced NF- κ B phosphorylation (Fig. 5A) and reduced I κ B α degradation (Fig. 5B). Meanwhile, transfection of a miR-124 mimic, with the least effect on NOD2 expression, resulted in only modest alterations in I κ B α degradation and no effect in NF- κ B

phosphorylation. Interestingly, transfection of the miR-122 mimic, while only having modest effects on NOD2 protein expression, had significant effects on NF- κ B phosphorylation and I κ B α degradation.

Next, we examined whether the NOD2-associated miRNA mimic transfection influenced downstream IL-8 and CXCL3 mRNA expression (Fig. 5C). Our results indicated that the transfection of mimics of miRs-192, -495, -512, and -671 all, to varying degrees, significantly reduced both CXCL3 and IL-8 mRNA expression. Interestingly, we found that both miR-122 and miR-124 mimic transfection also reduced CXCL3 and IL-8 mRNA expression.

Mutating the miR-192 Binding Sites on NOD2 Increase Luciferase Activities

Of the miRNAs that were found to influence NOD2 expression and function, miR-192 was of particular interest for 3 reasons: first, the differential expression of miR-192 has been noted in IBD⁹; second, there are 2 predicted miR-192 binding sites within the NOD2 3'UTR; third, an SNP (rs3135500) in the NOD2 3'UTR was found to be associated with ileal CD³⁵ and other immune-related diseases³⁶ and corresponds to the seed region of 1 of the 2 putative miR-192 binding sites. We next examined whether 1 or both putative miR-192 binding sites influenced NOD2 expression and whether the SNP within the putative miR-192 binding site could influence NOD2 gene expression. The sequence alignments of the 2 putative miR-192 binding sites and the SNP are shown in Figure 6A. To determine which miR-192 binding site may be influenced by endogenous miR-192, 2 constructs containing mutations in each of the putative miR-192 binding sites, a construct containing mutations in both binding sites and an additional construct containing the SNP (rs3135500) in the second putative miR-192 binding site were created (Fig 6A). Transfecting mutant constructs into HCT116 cells resulted in a restoration of luciferase activity (Fig. 6B). The results indicated that the first miR-192 binding site (nucleotides 129–150) ($P < 0.01$) and the second miR-192 binding site (nucleotides 1141–1160) ($P < 0.01$) were equally influential, resulting in a 32% restoration in luciferase activity. Of note, the construct containing mutations of both miR-192 binding sites (M1 + 2) exerted the greatest influence on the NOD2 expression in HCT116 cells (47% restoration in luciferase activity), indicating that both miR-192 bindings sites are functional and the cumulative effects of both miR-192 binding sites are additive.

We next examined whether an alteration of a single nucleotide in the second miR-192 binding site, corresponding to the rs3135500 SNP, could regulate NOD2 expression. Transfection of the SNP construct into HCT116 cells significantly restored luciferase activity ($P < 0.05$) (Fig. 6B) and this restoration of luciferase activity was as robust as the mutation of the entire seed region of the second miR-192 binding site. To further examine the influence of the SNP on miR-192 regulation of NOD2 expression, we co-transfected a miR-192 mimic with wild-type, M1 + 2, or SNP constructs into HCT116 cells. The result demonstrated that miR-192 mimic transfection reduced luciferase activity in cells transfected with wild-type construct. However, the ability of miR-192 mimic transfection to reduce luciferase activity was abolished in the HCT116 cells transfected with the M1 + 2 and SNP constructs (Fig. 6C), providing additional evidence that miR-192 regulates NOD2

expression and the rs3135500 SNP influences the ability of miR-192 to regulate NOD2 expression.

Discussion

Multiple regulatory mechanisms have been implicated in the regulation of NOD2 gene expression. These include regulatory elements in the promoter region of the *NOD2* gene, promoter methylation, and ubiquitination pathways.^{37–39} Specifically, NF- κ B regulatory elements have been shown to significantly influence intestinal epithelial NOD2 gene expression.^{40,41} In addition, it has been demonstrated that NOD2 gene expression was reactivated by the demethylating agent 5-aza-2' deoxycytidine in the phytohemagglutinin-stimulated peripheral blood mononuclear cells.³⁹ Recently, it has been reported that NOD2 can be degraded by TRIM27 through ubiquitination and proteasomal degradation.³⁷ Finally, environmental stimuli, such as cigarette smoking, have been shown to delay TNF α -induced NOD2 mRNA expression.⁴³

In this report, we present the first evidence that miRNAs also play a significant role in regulating NOD2 gene expression. Specifically, we demonstrated that miRs-192, -495, -512, and -671 influenced NOD2 gene expression as well as its downstream NF- κ B activity and immune responses in intestinal epithelial cells.

Modulation of miRNA expression in response to various pro-inflammatory components in primary human monocytes was previously investigated.⁴⁴ miR-129-5p was upregulated in response to MDP activation.⁴⁴ Thirteen biological processes targeted by miRNAs also respond to MDP stimulation.⁴⁴ Modulation of miRNA expression by TLR stimulation has been shown in several studies. Specifically, miR-155, miR-146, miR-132, miR-147, miR-9, miR-21, and miR-346^{45–48} are increased in response to TLR2 and TLR4 activation while miR-19a/b are decreased in response to TLR2 activation.⁴⁵ However, miRNAs can regulate TLR expression and its signaling pathway. Specifically, let-7i suppresses TLR4 expression in human cholangiocytes.⁴⁹

In our study, we identified miRs-192, -495, -512, and -671 as having direct influences on NOD2 gene expression. Of these miRNAs, only miR-192 has been associated with TLR expression changes. It has been shown that TLR-1, TLR-3, and TLR4a expressions were altered after overexpression of miR-192 in lipopolysaccharide-induced zebrafish liver (ZFL) cell line.⁵⁰ However, each of these miRNAs has been shown to influence other genes. Specifically, upregulation of miR-495 has been found to be associated with reduced methionine adenosyltransferase 1A (MAT1A) expression in hepatocellular carcinoma.⁵¹ Increased miR-512 expression has been found to be associated with cervical neoplasm,⁵² whereas miR-671 has been found to be associated with nonalcoholic fatty liver disease.⁵³

Of particular interest is the finding that miR-192 regulates NOD2 expression and function. We previously demonstrated that miR-192 is dysregulated in intestinal epithelial cells of patients with IBD and that miR-192 influences intestinal epithelial cell innate immunity via the direct regulation of macrophage inflammatory peptide 2 α expression.⁹ Here, we demonstrated that miR-192 significantly altered NOD2 mRNA and protein expression. In

addition, a miR-192 mimic significantly reduced NF- κ B phosphorylation and I κ B α degradation and downstream IL-8 and CXCL3 expression. These results suggest that miR-192 is a key miRNA in the pathogenesis of IBD and in the regulation of intestinal epithelial cell innate immune responses.

Multiple genetic analyses have implicated NOD2 in the pathogenesis of a number of chronic inflammatory diseases and autoimmune diseases, including CD,^{16,17} psoriasis,^{42,54} psoriatic arthritis,^{54,55} asthma,³⁶ sarcoidosis,⁵⁶ atopic dermatitis,⁵⁷ allergic rhinitis,⁵⁷ and Blau syndrome.⁵⁸ A number of studies have shown that SNPs in miRNA binding sites are associated with diseases^{32,59,60} and may significantly alter miRNA binding to associated gene transcripts.^{61,62} In particular, 8 SNPs in the *NOD2* gene were recently analyzed for associations with atopic phenotypes in a large German adult population (n = 1875).³⁶ Of particular interest in this report is that an SNP with an A allele at rs3135500 (G4384A) is located in the *NOD2* putative miRNA binding site for miR-192. The G4384A SNP was identified to be associated with an increased risk of asthma (odds ratio = 1.374, *P* = 0.023) and predicted to significantly alter miRNA binding to the *NOD2* transcript.³⁶ Here, we experimentally confirmed that the rs3135500 variant is a loss-of-function SNP by luciferase assay in HCT116 cells. Specifically, transfecting the *NOD2* 3'UTR vector possessing the rs3135500 SNP into HCT116 cells resulted in a higher baseline luciferase activity when compared with the WT vector and conferred the loss of response to miR-192 mimic transfection. It may be hypothesized that the rs3135500 variant may physiologically contribute to altered NOD2 expression in disease. Further studies are necessary to determine whether epithelial cells or other immune cells harboring the rs3135500 SNP demonstrate altered NOD2 expression and immune responses, contributing to the chronicity of inflammation as seen in CD and atopic disease.

Of note, although our data demonstrated that the transfection of mimics of miRs-192, -495, -512, and -671 influenced NOD2 expression, the proportional effects of these mimics on downstream NF- κ B activity and immune responses in intestinal epithelial cells did not correlate solely to NOD2 expression levels. There was much greater correlation to miRNA mimic effects on reducing NF- κ B phosphorylation and I κ B α degradation than downstream IL-8 and CXCL3 expression. It has been suggested that a single miRNA can target hundreds of mRNAs while a mRNA can be regulated by multiple miRNA.⁶³ It has also been demonstrated that several miRNAs react to 1 stimulus.⁴⁴ It is quite possible that each miRNA has multiple targets in the cytokine signaling pathways examined. This is notable in the case of IL-8, which has putative miR-124 binding site in the 3'UTR. The down-regulation of IL-8 in miR-124 overexpressed cells may be due to the direct effect of miR-124 on 3'UTR of *IL-8* rather than indirect effect from NOD2 signaling. Furthermore, the potential role of miRNAs with putative binding sites in the *NOD2* 3'UTR regulating other immune functions is indicated by the results obtained with the miR-124 mimic transfection, in which we observed very little direct effect on NOD2 expression but significant regulation of MDP-stimulated IL-8 and CXCL3 gene expression. Further studies are necessary to dissect all the gene targets of these NOD2-regulatory miRNAs.

In summary, we have observed that MDP enhances NOD2 expression and suppresses the expression of miRNAs with putative *NOD2* 3'UTR binding sites in HCT116 cells. We also

demonstrated that miR-192, miR-495, miR-512, and miR-671 are capable of regulating NOD2 expression in different degrees by binding to the putative complementary binding sites within the 3'UTR of *NOD2* mRNA. This raises the possibility that overexpression of more than 1 miRNA may increase the efficiency of the regulation of NOD2 expression. In addition, we have shown that these miRNAs inhibit NOD2 signaling pathway and expression of IL-8 and CXCL3 in response to MDP stimulation. Taken together, we have identified a novel mechanism in which MDP-mediated down-regulation of miRNAs leads to overexpression of NOD2, excessive NF- κ B activation, and amplification of innate immune responses and susceptibility to chronic inflammatory and autoimmune diseases. HCT116 findings observed in our study may not be true in other cell lines or primary cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

NOD2 mRNA and protein expression in colonic epithelial cell lines. A, NOD2 mRNA expression in various colonic epithelial cell lines (HCT116, HT-29, Caco-2, T84, and SW620), and positive control THP-1 cells was assessed by qRT-PCR. B, NOD2 protein expression in various colonic epithelial cell lines (HCT116, HT-29, and Caco-2) and positive control THP-1 cells was assessed by Western blot analysis.

Figure 2.

NOD2 is inducible and functional in HCT116 cells. A, Time-dependent NOD2 mRNA expression in MDP (50 μ g/mL) treated and untreated (Control) HCT116 cells was assessed by qRT-PCR. B, NF- κ B activation in HCT116 cells in response to MDP stimulation for indicated times was assessed via Western blot analysis on whole cell lysates using anti-p65 (for NF- κ B phosphorylation), anti-I κ B α (for I κ B α degradation), anti-tubulin, and anti-GAPDH antibodies. C, Time-dependent IL-8 and CXCL3 mRNA expression in HCT116 cells in response to MDP (50 μ g/mL) treatment was assessed by qRT-PCR. Results are mean $\pm$ standard error, n = 3. ** P < 0.01; *** P < 0.001 compared with 0 hour time point.

Figure 3.

Expression of putative NOD2-associated miRNAs in basal and MDP-treated HCT116 cells. A, Schematic representation of NOD2 mRNA with 10 putative miRNA binding sites. B, Endogenous expression of putative NOD2-associated miRNAs in HCT116 cells was analyzed using qRT-PCR (normalized to U6B). C, Expression of 6 putative NOD2-associated miRNAs in MDP-stimulated (50 μ g/mL) and unstimulated (Ctrl) HCT116 cells after 6 hours was assessed using qRT-PCR (normalized to U6B). Results are mean $\pm$ standard error, n = 3. * P < 0.05; ** P < 0.01 compared with control (Ctrl).

Figure 4.

miRNAs modulate NOD2 expression in HCT116 cells. A, Luciferase activity of HCT116 cells transfected with the pmirGLO empty vector (EV) and wild-type pmirGLO-*NOD2*-3'UTR (WT) reporter constructs. Luciferase activity was normalized to Renilla luciferase activity. B, Luciferase activity of HCT116 cells co-transfected with 50 μ g of the WT p*NOD2*-3'UTR and 50 nM control (Ctrl) mimic or miR-122, miR-124, miR-192, miR-495, miR-512, and miR-671 mimics. Luciferase activity was normalized to Renilla luciferase activity. C, The qRT-PCR quantifying NOD2 mRNA expression levels from HCT116 cells transfected with control (Ctrl) mimic or miR-122, miR-124, miR-192, miR-495, miR-512, and miR-671 mimics. D, Western blot analysis of NOD2 protein expression in HCT116 cells transfected with 50 nM control (Ctrl) mimic or miR-122, miR-124, miR-192, miR-495, miR-512, and miR-671 mimics. Densitometry levels were obtained using an Odyssey infrared imaging system and results are relative to the Ctrl mimic after normalization to the corresponding tubulin. For A, B, C, results are mean $\pm$ standard error, $n = 3$. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ compared with EV (A) and Ctrl (B and C).

Figure 5.

Decreased NF-κB phosphorylation and degradation of IκBα in HCT116 cells transfected with miRNA mimics. A, Western blot analysis for NF-κB phosphorylation in HCT116 cells transfected with miRNA mimics for 24 hours followed by MDP stimulation (50 μg/mL) for 30 minutes using anti-phospho-NF-κB (p65) and anti-GAPDH antibodies. Densitometry levels were obtained using an Odyssey infrared imaging system and results are relative to the Ctrl mimic after normalization to individual GAPDH levels. B, Western blot analysis for IκBα degradation in HCT116 cells transfected with miRNA mimics for 24 hours followed by MDP stimulation (50 μg/mL) for 60 minutes using anti-IκBα and anti-tubulin antibodies. Densitometry levels were obtained using an Odyssey infrared imaging system and results are relative to the Ctrl mimic after normalization to individual tubulin levels. C, The qRT-PCR analysis of IL-8 and CXCL3 mRNA expression in HCT116 cells transfected with 50 nM Ctrl, miR-122, miR-124, miR-192, miR-495, miR-512, and miR-671 mimics for 24 hours followed by 2 hours of MDP stimulation (50 μg/mL). Results are mean ± standard error, n = 3. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with Ctrl mimic.

Figure 6.

NOD2 miR-192 binding site mutation effects on reporter gene expression. A, Sequence alignments and binding site mutations in the constructs for miR-192 with the proximal putative miR-192 binding site (192-1) and the distal putative miR-192 binding site (192-2). M1 and M2 and SNP are mutant constructs for 192-1 and 192-2 binding sites, respectively. B, Luciferase reporter activity in HCT116 cells transfected with the WT, M1, M2, M1 + 2, and SNP reporter constructs. Results are relative to the WT construct. C, Luciferase reporter activity in HCT116 cells co-transfected with WT, M1 + 2, or SNP reporter constructs and the Ctrl or miR-192 mimics. Results are relative to the Ctrl mimic. Luciferase activity was normalized to Renilla luciferase activity. Results are mean $\pm$ standard error, n = 4. ** P < 0.01; *** P < 0.001.