MicroRNA-346 Mediates Tumor Necrosis Factor α-Induced Downregulation of Gut Epithelial Vitamin D Receptor in Inflammatory Bowel Diseases

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Background: We recently reported that the gut epithelial vitamin D receptor (VDR) signaling inhibits colitis through inhibition of intestinal epithelial cell apoptosis, and the level of colonic epithelial VDR is markedly reduced in patients with inflammatory bowel diseases (IBD). VDR downregulation promotes colitis, but the mechanism underlying VDR downregulation in IBD is unknown.

Methods: VDR expression was analyzed in colon cancer cells under proinflammatory cytokine treatment. VDR as a target of miR-346 was confirmed using colon cancer cell culture. The relationship among inflammation, miR-346, and VDR was assessed in human IBD biopsies and experimental colitis models.

Results: We showed that tumor necrosis factor α (TNF-α) suppresses VDR expression while simultaneously upregulating miR-346 in human colon cancer cells. Further studies demonstrated that miR-346 inhibits VDR by a specific target sequence in the 3′ untranslated region of the human VDR transcript, and blockade of miR-346 with a hairpin inhibitor abrogates the ability of TNF-α to inhibit VDR, confirming that TNF-α downregulates VDR by inducing miR-346. Consistently, in human IBD biopsies, the reduction of epithelial VDR is associated with increased immune cell infiltration and elevation of TNF-α and miR-346. In an experimental model of colitis, mucosal VDR expression is reduced over time with the progression of colitis, inversely correlated with the induction of TNF-α and miR-346 in the mucosa.

Conclusions: These data suggest that during mucosal inflammation TNF-α induces miR-346, which downregulates epithelial VDR. Mucosal VDR reduction in turn compromises the integrity of the mucosal epithelial barrier, further driving mucosal inflammation and colitis development.

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Key Words: vitamin D, vitamin D receptor, TNF-α, miR-346, mucosal inflammation, colitis

The gut mucosal epithelial barrier separates the body from luminal microorganisms and antigens. Dysfunction of the barrier leads to increased translocation of luminal substances to the lamina propria, triggering inflammatory response.[1,2] In fact, impaired gut mucosal barrier is a significant pathogenic factor for inflammatory bowel diseases (IBD),[3] which include ulcerative colitis (UC) and Crohn's disease. It is known that inflammatory cytokines produced from mucosal inflammation can in turn increase mucosal permeability by altering intercellular tight junction and inducing apoptosis of intestinal epithelial cells (IECs).[3,4] This vicious cycle of mucosal events drives mucosal inflammation and promotes the development of colitis.

The vitamin D receptor (VDR) is a nuclear hormone receptor that mediates the biological activities of the vitamin D hormone, 1,25-dihydroxyvitamin D.[5] VDR is highly expressed in the epithelial cells of the gastrointestinal tract. Recently, we demonstrated that the epithelial VDR signaling plays a critical role in maintaining the integrity of the mucosal epithelial barrier by suppressing inflammation-induced IEC apoptosis.[6] In experimental colitis models, *Vdr* deletion exaggerates colonic inflammation, whereas transgenic overexpression of VDR in the epithelial cells renders the transgenic mice highly resistant to colitis. This anticolitic activity of the epithelial VDR signaling is independent of the VDR actions in the nonepithelial immune compartment.[6] Importantly, we observed that in patients with both UC and Crohn's disease, epithelial VDR levels are reduced by more than 50% in the lesion, independent of the serum vitamin D status.[6] Based on the observations from the experimental colitis models

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and human biopsies, we reason that epithelial VDR reduction compromises the gut mucosal barrier and contributes to the development of IBD; however, the molecular mechanism underlying epithelial VDR downregulation in IBD is unclear. The goal of this study was to investigate this important issue. Our results indicate that epithelial VDR downregulation is driven by colonic inflammation, and this process is mediated by miR-346.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatment, and Transfection

HCT116 and HT29 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were treated with tumor necrosis factor α (TNF-α; 100 ng/mL), interleukin 1β (IL-1β; 10 ng/mL), or IL-6 (100 ng/mL) for 0 to 48 hours, followed by isolation of total RNA or total protein lysates for analyses. In some experiments, cells were pretreated with actinomycin D (5 nM) before these cytokine treatments. In other experiments, cells were transfected with plasmids or microRNA oligo mimics using Lipofectamine 2000 (Life Technologies, Grand Island, NY) as detailed in each experiment.

Human Biopsies

Collections of colonic biopsies from patients with IBD and non-IBD controls were reported previously.[6] The collection of these biopsies was approved by the Institutional Review Board of the University of Chicago (Chicago, IL) and by the Institutional Ethical Committee of China Medical University (Shenyang, Liaoning, China), respectively. Study subjects were recruited with written informed consent from participants or their guardians. Biopsies were subjected to histological and immunohistochemical analyses. Total RNAs and lysates were prepared from the biopsies for analyses.

Experimental Colitis Models

IL-10$^{-/-}$ mice, which develop spontaneous chronic intestinal inflammation due to T cell–mediated aberrant immune response,[7,8] were obtained from Jackson Laboratory. Colonic mucosal VDR levels were examined at 3 months of age in wild-type and IL-10$^{-/-}$ mice. In other experiments, colitis was induced with 2,4,6-trinitrobenzene sulfonic acid (TNBS) in 8- to 12-week-old C57BL/6 mice as previously described.[6,9] After overnight fasting, mice were treated under anesthesia with 100 mg/kg TNBS (Sigma, St. Louis, MO) dissolved in 50% alcohol by intrarectal injection using a 1-mL syringe fitted with an 18-gauge stainless steel gavage needle, with 50% alcohol treatment as control. The mice were killed at days 0, 1, 2, and 3 following TNBS treatment, and colonic mucosal levels of VDR protein, TNF-α, and miR-346 transcripts were analyzed by Western blotting and real-time polymerase chain reaction (PCR). All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Chicago.

Histology and Immunohistochemical Staining

Freshly dissected mouse colon samples or human colon biopsies were fixed overnight with 4% formaldehyde in phosphate-buffered saline (pH 7.2), processed and embedded in paraffin wax. Tissues were cut into 4-μm sections. Colonic morphology was examined by standard hematoxylin and eosin staining. To examine VDR and inflammatory status, sections were stained with antibodies against VDR, CD4, CD11b (Santa Cruz Biotechnology, Dallas, TX), or TNF-α (Abcam, Cambridge, MA) as primary antibodies, respectively, followed by incubation with fluorescein isothiocyanate–conjugated anti–immunoglobulin G antibodies. Antigens were then visualized using a fluorescence microscope.

Western Blot

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P membranes. Western blot analyses were carried out as previously described[10] using antibodies against VDR (Santa Cruz Biotechnology) or β-actin (Sigma) as internal control. Total cell lysates, including cytosolic and nuclear proteins, were used for VDR assessment.

Real-Time PCR

Total RNAs were extracted using TRIzol reagents (Life Technologies). First-strand complementary DNAs (cDNAs) were synthesized using a ThermoscriptRT kit (Life Technologies) for messenger RNA (mRNA) transcript analysis, or using NCode VILO miRNA cDNA Synthesis Kit (Life Technologies) for miR-346 quantitation. Conventional PCR was carried out in a BioRad DNA Engine (BioRad, Hercules, CA). Real-time PCR was performed in a Roche LightCycler 480II Real-Time PCR System using SensiFAST SYBR No-Rox PCR kits (Bioline, Taunton, MA). Relative amounts of transcripts were calculated using the $2^{-\Delta\Delta Ct}$ formula. For mRNA transcript analysis, GAPDH was used as the internal control; for microRNA analysis, U6 was used as the internal control for normalization. Each data point was normalized to corresponding control values in data presentation. PCR primer sequences are provided in Table 1.

Plasmids

A 1.6 kb DNA fragment within 3′ untranslated region (3′UTR) between nucleotides 1444 to 3062 in human VDR cDNA was amplified by PCR using primers 5′TGATCTAGAGTGTTTGGCAATGAGATCTCCTGACTAGGA3′ (forward) and 5′CTGATCTAGAGAGCAAGCTGGACCAATACGTTCTGCAGCT3′ (reverse) and cloned into the XbaI site at the C-terminal downstream of the luciferase reporter gene in pGL3-Luc Control plasmid (Promega, Chicago, IL). The resultant plasmid was designated as pGL3-Luc-3′UTR. pGL3-Luc-3′UTR-Mut, in which the miR-346 target sequence (5′AGUGGGGGCAGAGGGCAGAGG3′) within the 3′UTR fragment was mutated at the seed sequence (5′AGUGGGGGCAGAaaatgacGG3′), was generated by site-directed mutagenesis. pCDH-miR-346 that expresses human miR-346 sequence was provided by Dr. Y-Y Mo (University of Mississippi Medical Center).

Luciferase Reporter Assays

HCT116 cells were cotransfected with luciferase reporter and plasmids or hsa-miR-346 oligo mimic using Lipofectamine 2000 as

TABLE 1. Primer Sequences Used in the Study

Primer Name	Forward (5′-3′)	Reverse (5′-3′)
hTNF-α	CGAGTGACAAGCCTGTAGC	GGTGTGGGTGAGGAGCACAT
hIL-6	AAATGCCAGCCTGCTGACGAAC	AACAACAATCTGAGGTGCCCATGCTAC
hIL-1β	GCTGAGGAAGATGCTGGTTC	GTGATCGTACAGGTCGCATCG
hGAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
mB2M	ACCGGCCTGTATGCTATCCAGAAA	ATTTCAATGTGAGGCGGGTGGAAC
mTNF-α	TCAGCCTCTTCTCATTCCTG	CAGGCTTGTCACTCGAATTT
mVDR	GATGCCCACCACAAGACCTA	CGGTTCCATCATGTCCAGTG
mmu-mir-346(5p)	TGTCTGCCCGAGTGCCTGCCTCT	
U6	GATGACACGCAAATTCGTGAA	
hsa-mir-346(5p)	TGTCTGCCCGCATGCCTGCCTCT	
hsa-mir-34a	TGGCAGTGTCTTAGCTGGTTGT	
hsa-mir-125b	TCCCTGAGACCCTAACTTGTG	
hsa-mir-17-3	ACTGCAGTGAAGGCACTTGTAG	

indicated in each experiment. Some transfected cells were treated with $1,25(OH)_2D_3$ (20 nM). After 24 hours, transfected cells were lysed and luciferase activity determined using the Dual-Luciferase Reporter assay system (Promega) as reported previously.[11]

Statistical Analysis

Data values were presented as mean ± SEM. Statistical comparisons were carried out using unpaired 2-tailed Student's *t* test or 1-way analysis of variance as appropriate, with $P < 0.05$ being considered statistically significant.

RESULTS

To test the hypothesis that inflammation downregulates VDR expression, we examined the effect of proinflammatory cytokines on VDR expression in colon cancer cells. As shown in Figure 1, among the cytokines tested (TNF-α, IL-1β, and IL-6), only TNF-α substantially suppressed VDR protein levels (by >45%) in HCT116 cells, a human colon cancer cell line (Fig. 1A, B). Further investigations showed that TNF-α suppressed VDR expression in dose-dependent (Fig. 1C, D) and time-dependent (Fig. 1E, F) manners. TNF-α also suppressed VDR in HT29 cells, another human colon cancer cell line (Fig. 1G, H), in which TNF-α was able to reduce VDR by more than 60%. These observations are relevant because TNF-α is a major inflammatory cytokine involved in the pathogenesis of IBD, and anti-TNF therapy is widely used in IBD management.[12,13]

MicroRNAs (miRNAs) are naturally occurring small noncoding RNAs that control target genes by translational repression or mRNA degradation.[14,15] As TNF-α quickly reduced VDR mRNA, we speculated that miRNAs might be involved in the downregulation of VDR. By in silico analysis (using miRanda: www.microrna.org), we identified several putative miRNA binding sites within the 3′UTR of hVDR cDNA, including sites for miR-17-3p, miR-34a, and miR-346. Previous studies showed that miR-125b targets hVDR.[16] Thus, we studied these 4 miRNAs. As shown in Figure 2, among these 4 miRNAs, miR-346 and miR-125b were markedly induced by TNF-α in HCT116 cells (Fig. 2A), suggesting that TNF-α might suppress VDR by inducing miR-346 and/or miR-125b. However, when the cells were transfected with miR-125b–specific hairpin inhibitor to inhibit miR-125b, TNF-α was still able to repress VDR; in contrast, when miR-346 was inhibited, TNF-α was no longer able to downregulate VDR (Fig. 2B, C). These observations strongly suggest that miR-346, not miR-125b, mediates the repressive effect of TNF-α on VDR expression. Consistent with this conclusion, the time course of miR-346 induction by TNF-α closely correlated with the time course of VDR downregulation by TNF-α in HCT116 cells (Fig. 3A, B). Moreover, when the cells were pretreated with actinomycin D, which blocks intracellular RNA synthesis, the induction of miR-346 as well as the suppression of VDR by TNF-α was markedly attenuated (Fig. 3C, D), suggesting that new RNA synthesis is required for VDR suppression. It is not surprising that miR-125b is unlikely a major mediator for VDR reduction, because even the previously published study was not able to demonstrate that miR-125b can indeed suppress VDR protein.[16]

These results prompted us to confirm that miR-346 can indeed directly target VDR. Figure 4A shows the target sequences of miR-346 in the 3′UTR of human and mouse VDR cDNAs. These sequences are highly conserved between human and mouse and have a high degree of complementarity with human and mouse miR-346s, respectively, particularly within the seed sequence at the 3′ end (Fig. 4A). Transfection of HCT116 cells with hsa-miR-346 oligo mimic substantially reduced hVDR (by >50%), and the potency was comparable with that of hVDR-specific siRNA (si-hVDR) that served as a positive control (Fig. 4B, C). To address whether miR-346 can suppress the transactivating activity of VDR, we cotransfected HCT116 cells with

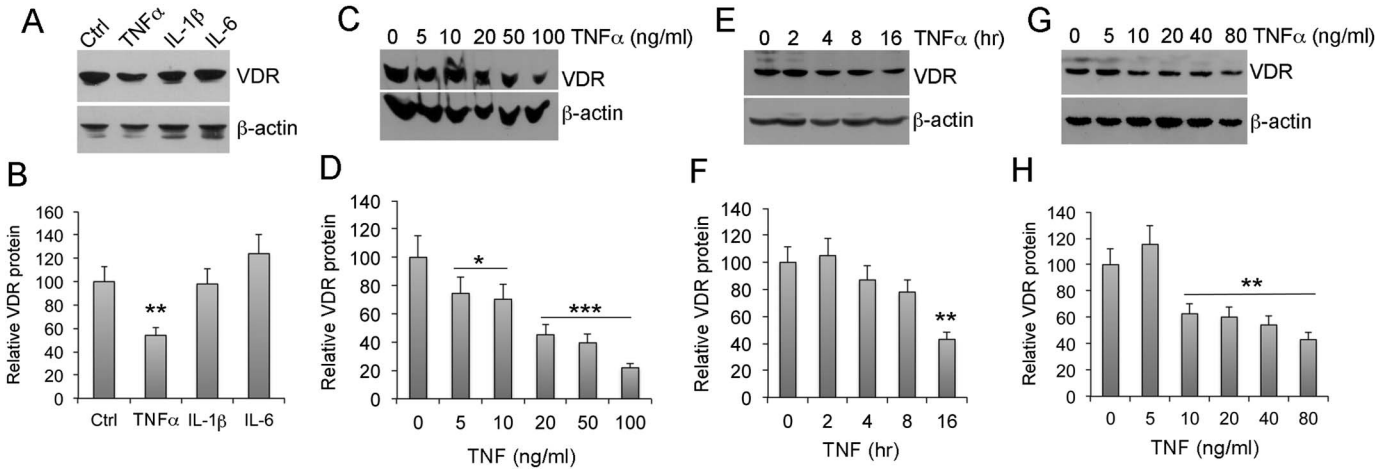

FIGURE 1. TNF-α suppresses VDR expression. A and B, HCT116 cells were treated with TNF-α (100 ng/mL), IL-1β (10 ng/mL), IL-6 (100 ng/mL), or phosphate-buffered saline control (Ctrl) for 24 hours. Total cell lysates were prepared. VDR expression was measured by Western blotting (A) and quantified by densitometry (B). C and D, HCT116 cells treated with increasing doses of TNF-α as indicated for 24 hours. VDR expression was quantified by Western blotting (C) and densitometry (D). E and F, HCT116 cells were treated with 100 ng/mL TNF-α for different times as indicated. VDR expression was quantified by Western blotting (E) and densitometry (F). G and H, HT29 cells were treated with increasing doses of TNF-α as indicated for 24 hours. VDR expression was quantified by Western blotting (G) and densitometry (H). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Ctrl or 0. All values are relative to corresponding control or 0 values.

p3xVDRE-Luc reporter plasmid and control miRNA oligo mimic, hsa-miR-346 oligo mimic or hVDR-specific siRNA, followed by $1,25(OH)_2D_3$ treatment. Although $1,25(OH)_2D_3$ stimulated luciferase activity in cells transfected with control miRNA, $1,25(OH)_2D_3$ induction of luciferase activity was markedly attenuated in cells transfected with miR-346 or hVDR-siRNA (Fig. 4D), indicating that miR-346 indeed inhibited VDR's transactivation activity.

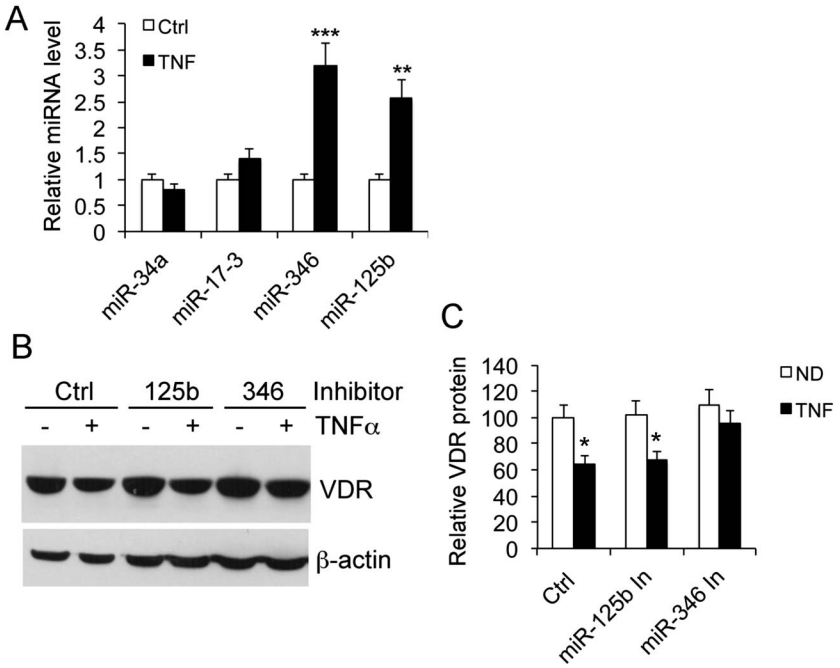

FIGURE 2. TNF-α inhibits VDR expression through miR-346 induction. A, HCT116 cells were treated with TNF-α (100 ng/mL) or phosphate-buffered saline control for 24 hours. Indicated miRNAs were quantified by real-time RT-PCR. $^{**}P < 0.01$, $^{***}P < 0.001$ versus corresponding Ctrl. B, HCT116 cells were transfected with control, miR-125b-specific or miR-346-specific hairpin inhibitor. After 24 hours, transfected cells were treated with (+) or without (−) 100 ng/mL TNF-α. Total cell lysates were prepared. VDR protein was analyzed by Western blotting. C, Densitometric quantitation of the Western blot data. In, inhibitor. $^{*}P < 0.05$ versus nontreated (ND). All values are relative to the control ND value.

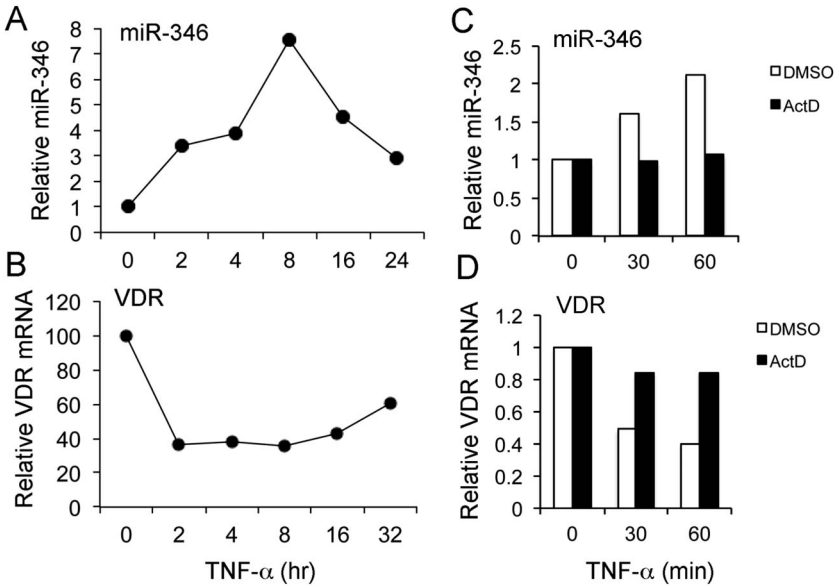

FIGURE 3. Inverse correlation between miR-346 induction and VDR suppression by TNF-α. A and B, HCT116 cells were treated with TNF-α (100 ng/mL) for different hours as indicated. Changes of miR-346 (A) and VDR mRNA transcript (B) over time were quantified by qRT-PCR. C and D, HCT116 cells were pretreated with actinomycin D (ActD, 5 nM) or dimethyl sulfoxide for 30 minutes followed by TNF-α (100 ng/mL) treatment for 0, 30 and 60 minutes. Levels of miR-346 (C) and VDR transcript (D) were quantified by qRT-PCR. Each experiment was repeated 3 times with similar results. Data from a single representative experiment are shown. All values are relative to corresponding 0 values.

To confirm that miR-346 inhibits VDR through the target site in the 3′UTR, we constructed a luciferase reporter plasmid in which a 3′UTR fragment of hVDR cDNA (1444–3062) that contains the miR-346 target site was cloned to the 3′ downstream of the luciferase gene in pGL3-Luc Control plasmid (Fig. 4E). The resultant pGL3-Luc-3′UTR reporter displayed reduced luciferase activity when cotransfected with hsa-miR-346 mimic in HCT116 cells, in contrast to pGL3-Luc Control plasmid, which was not affected by miR-345 mimic (Fig. 4F). Moreover, when cells were cotransfected with pCDH-miR-346 and pGL3-Luc-3′UTR-Mut, a mutant plasmid reporter that carries mutations in the seed sequence, the mutant reporter's activity was no longer affected by ectopically expressed miR-346, in contrast to pGL3-Luc-3′UTR, whose activity was reduced by pCDH-miR-346 plasmid (Fig. 4G). Together these data demonstrate that VDR is a direct target of miR-346, and TNF-α suppresses VDR by inducing miR-346.

We then assessed the relevance of this regulatory relationship among colonic inflammation, miR-346 and epithelial VDR in patients with IBD. As shown by immunostaining, massive infiltration of $CD4^+$ and $CD11b^+$ cells were detected in the lamina propria of active UC lesion biopsies (Fig. 5A). Similarly, the number of lamina propria TNF-α–producing cells was also dramatically increased in the active colitis lesion (Fig. 5B). Consistently, real-time PCR quantitation showed that TNF-α mRNA transcript increased by >8-fold (IL-1β increased by >2-fold) (Fig. 5C), whereas VDR and ZO-1 expression decreased by >50% and >80%, respectively (Fig. 5D), in these lesion biopsies relative to adjacent normal tissues. Indeed, immunostaining data confirmed that the reduction of VDR in the lesion mostly occurred in IECs (Fig. 5E), and Western blot analysis confirmed that the reduction in VDR protein levels was >50% in the lesions (Fig. 4F, G). Importantly, miR-346 was induced by >2.5-fold in the lesion (Fig. 5H). Therefore, colonic inflammation and TNF-α are inversely correlated with VDR and positively correlated with miR-346 in patients with IBD, which is consistent with the notion that TNF-α downregulates VDR by inducing miR-346.

We further validated our conclusion using experimental colitis models. IL-10 null mice spontaneously develop chronic intestinal inflammation and colitis due to aberrant immune response.[7,8] As shown in Figure 6, colonic mucosal VDR levels were dramatically suppressed in IL-10 null mice compared with wild-type mice at 4 months of age (Fig. 6A), when the IL-10 null mice had developed severe mucosal inflammation. In TNBS-induced colitis model, severe mucosal inflammation develops within 3 days.[6] We found that in TNBS-treated mice colonic mucosal VDR protein levels gradually decreased over time in the first 3 days after TNBS treatment (Fig. 6B), which was accompanied by a dramatic, time-dependent induction of TNF-α transcript (Fig. 6C) and miR-346 (Fig. 6D) in the mucosa during this period. Again, these observations are consistent with our conclusion that TNF-α suppresses VDR expression through induction of miR-346.

DISCUSSION

The VDR is abundantly expressed in the intestine. The classic action of the VDR in the small intestine is to regulate transcellular calcium transport, but its role in the large intestine

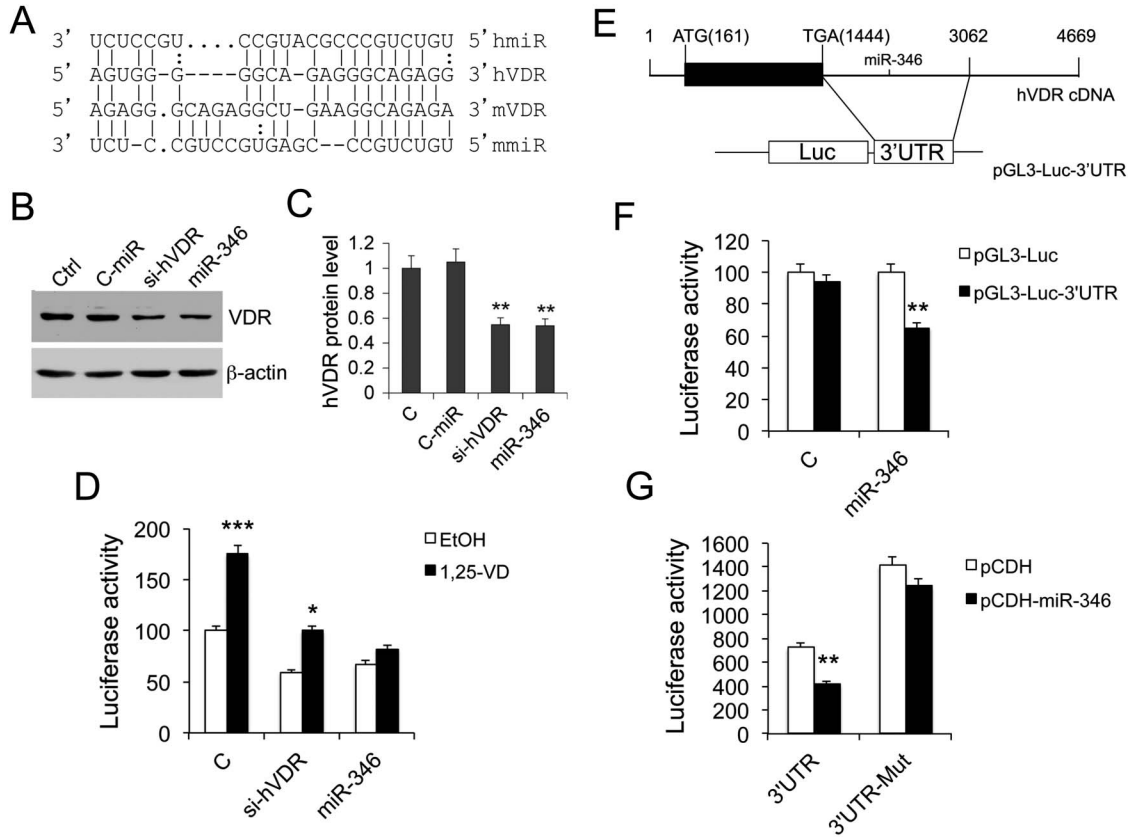

FIGURE 4. miR-346 inhibits VDR by targeting a 3′UTR site. A, Alignment of human and mouse miR-346 sequences and corresponding miR-346 target site in the 3′UTR of human and mouse VDR transcripts. B and C, HCT116 cells were untransfected (Ctrl or C) or transfected with control miRNA (C-miR), hVDR-specific siRNA (si-hVDR), or miR-346 oligo mimic. Total cell lysates were prepared. VDR protein levels were analyzed by Western blotting (B) and quantified by densitometry (C). $^{**}P < 0.01$ versus Ctrl; (D) HCT116 cells were cotransfected with p3xVDRE-Luc and control miRNA (C), hVDR-specific siRNA (si-hVDR) or miR-346 oligo mimic. After 24 hours, the cells were stimulated with ethanol (EtOH) or 1,25$(OH)_2D_3$ (20 nM), followed by luciferase activity assay. $^{*}P < 0.05$, $^{***}P < 0.001$ versus corresponding EtOH. E, Diagrammatic illustration of hVDR transcript including the protein-coding region (161–1444, black box) and the whole 3′UTR (1444–4669). Also shown is the construction of pGL3-Luc-3′UTR plasmid. F, HCT116 cells were co-transfected with pGL3-Luc or pGL3-Luc-3′UTR and control miRNA (C) or miR-346 oligo mimic, followed by luciferase activity assay. $^{**}P < 0.01$ versus pGL3-Luc. G, HCT116 cells were cotransfected with pGL3-Luc-3′UTR (3′UTR) or pGL3-Luc-3′UTR-Mut (3′UTR-Mut) and pCDH or pCDH-miR-346 as indicated. The pCDH-miR-346 plasmid expresses hsa-miR-346. After 24 hours, luciferase activity was quantified in the cell lysates. $^{**}P < 0.01$ versus corresponding pCDH.

is less clear. Our recent work demonstrated that colonic epithelial VDR signaling plays a key role in maintaining mucosal barrier integrity and protects against mucosal inflammation.[6] One of the major actions of epithelial VDR is to suppress inflammation-induced apoptosis of IECs. Consistent with this role, we observed that the colonic VDR level is substantially reduced in patients with IBD.[6] It is conceivable that epithelial VDR reduction increases the risk of mucosal barrier dysfunction and promotes mucosal inflammation. Thus, it is important to understand how mucosal VDR is downregulated in the context of mucosal inflammation and colitis.

In this report we demonstrated that inflammation plays a key role in the downregulation of the VDR in colonic epithelial cells. Proinflammatory cytokine TNF-α suppresses VDR expression, and the time course of VDR downregulation is inversely correlated with that of miR-346 upregulation. We validated that the VDR transcript contains a conserved 3′UTR sequence that is targeted by miR-346 to inhibit VDR expression. Moreover, we showed that colonic VDR downregulation is inversely correlated with TNF-α and miR-346 induction and lamina propria immune cell infiltration in human IBD biopsies. We also observed that, in experimental models of colitis, colonic VDR expression is gradually reduced with the progression of mucosal inflammation toward the development of colitis, which is closely associated with the induction of TNF-α and miR-346 in this process. Together the in vitro and in vivo evidence strongly supports the notion that TNF-α suppresses epithelial VDR through induction of miR-346.

Proinflammatory cytokines play key roles in promoting mucosal inflammation. Among a number of inflammatory cytokines commonly detectable in IBD, our study identified TNF-α as a key cytokine that negatively regulates epithelial VDR expression. Although it is possible that other cytokines not yet tested

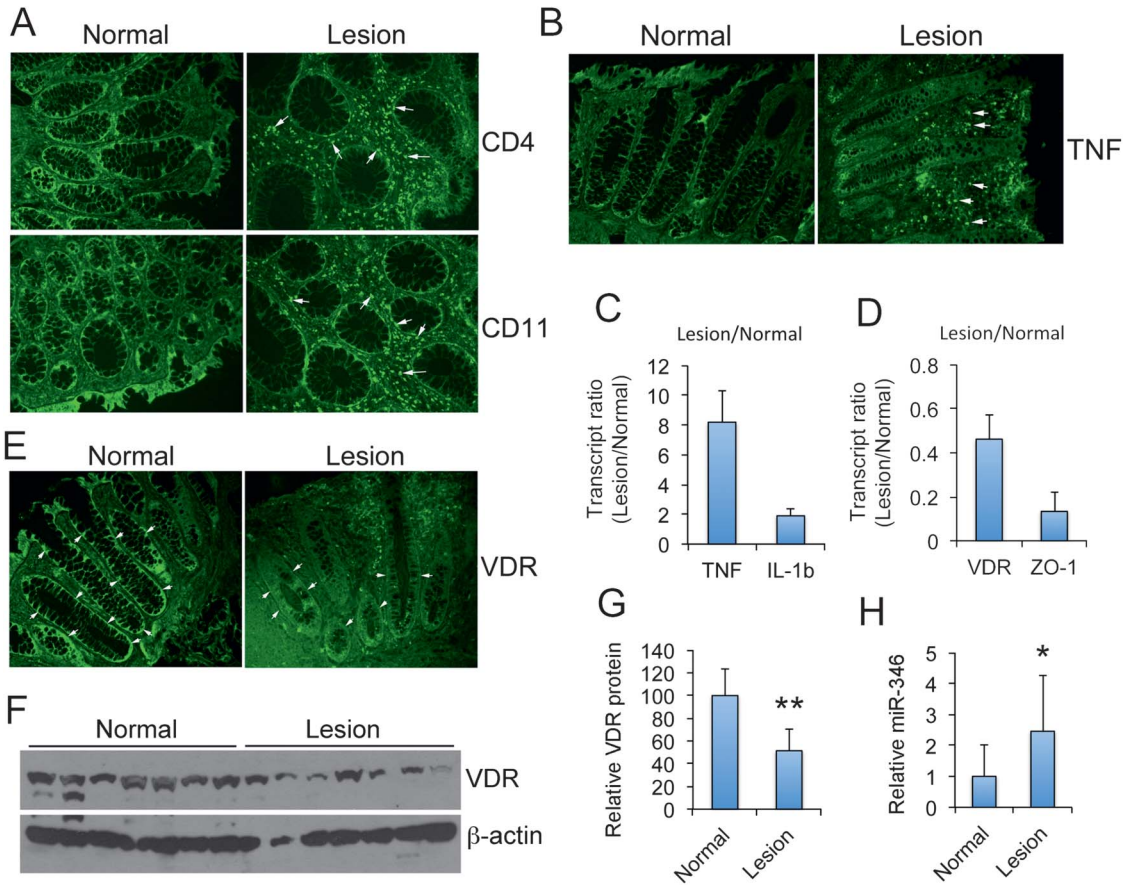

FIGURE 5. Immune cell infiltration and TNF-α expression are correlated with miR-346 induction and VDR suppression in human IBD biopsies. A, Immunostaining of active UC and adjacent normal colon biopsies with anti-CD4 or anti-CD11b antibodies. Arrows indicated examples of $CD4^-$ or CD11b-positive cells. B, Immunostaining of active UC and adjacent normal colon biopsies with anti-TNF-α antibodies. Arrows indicated examples TNF-α-producing cells. C, qRT-PCR quantitation of TNF-α and IL-1β transcripts in active UC lesion and adjacent normal colon biopsies. Data are expressed as ratio of lesion to normal tissue. D, qRT-PCR quantitation of VDR and ZO-1 transcripts in active UC and adjacent normal colon biopsies. Data are expressed as ratio of lesion to normal tissue. E, Immunostaining of active UC and adjacent normal colon biopsies with anti-VDR antibodies. Arrows indicated epithelial cells. F, Western blot analysis of VDR protein levels in active UC lesion (lesion) and adjacent normal colon biopsies (normal). Each lane represents 1 individual patient. G, Quantitative data of the Western blot images. $^{**}P < 0.01$ versus Normal. H, qRT-PCR quantitation of miR-346 in active UC lesion and adjacent normal colon biopsies. $^{*}P < 0.05$ versus normal.

may also be involved in the downregulation of VDR, TNF-α as an inflammatory mediator to inhibit VDR makes good sense because TNF-α plays a critical role in the development of colitis and anti-TNF therapy is a standard therapy in the management of IBD.[12,13] It is known that TNF-α induces mucosal barrier injury by promoting IEC apoptosis and disrupting the intercellular junctions between epithelial cells. PUMA, a proapoptotic BCL-2 member, and claudin-2, a tight junction protein, are examples of TNF-α targets in these processes.[17,18] Therefore, it is not surprising that VDR is another target for TNF-α to damage the mucosal barrier. Although both miR-125b and miR-346 are stimulated by TNF-α, we showed that only miR-346 is able to inhibit VDR. Although miR-346 is unlikely able to block VDR nuclear translocation, inhibition of VDR protein levels ought to be sufficient to reduce VDR's activity. Here we were not able to confirm the VDR-inhibiting activity of miR-125b reported previously.[16] In fact, no reduction of VDR protein by miR-125b was reported in this previous work. It seems that miR-125b is not an effective inhibitor of VDR. How TNF-α induces miR-346 is an interesting question that remains to be determined in future studies.

Our previous studies have shown that intestinal VDR signaling inhibits inflammation-induced apoptosis of IECs by blocking NF-κB activity,[6] but how mucosal inflammation affects epithelial VDR is unknown. That TNF-α suppresses VDR through miR-346 is thus a novel finding. Given the critical role of VDR in maintaining the integrity of mucosal epithelial barrier, this finding offers a mechanistic insight into how mucosal inflammation induces mucosal barrier damage. Mucosal inflammation downregulates VDR, which impairs the mucosal epithelial barrier. As a result, VDR reduction in the epithelial cells in turn promotes mucosal inflammation. This vicious cycle is

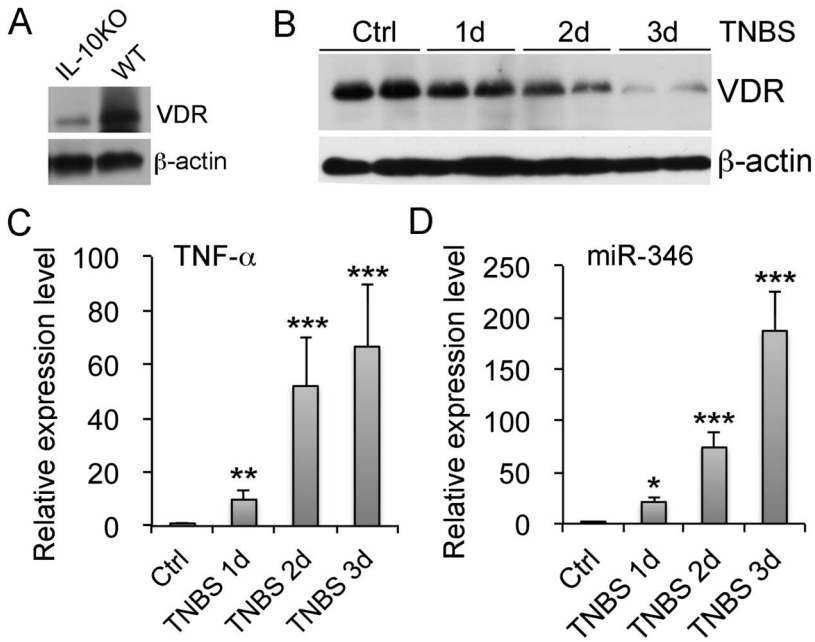

FIGURE 6. Colonic VDR expression is inversely correlated with mucosal TNF-α and miR-346 levels in experimental colitis mouse models. A, Western blot analysis of colonic mucosal VDR protein in wild-type and IL-10 null (IL-10KO) mice. B, Changes of colonic mucosal VDR protein levels in TNBS-treated mice from day 0 (control) to day 3. Total mucosal lysates were used for Western blotting. C, Changes of colonic mucosal TNF-α transcript levels from day 0 to day 3 in the TNBS mouse colitis model. D, Changes of colonic mucosal miR-346 levels from day 0 to day 3 in the TBNS mouse colitis model. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ versus Ctrl. All values are relative to corresponding control values.

thought to contribute to the progression of mucosal inflammation and promote the development of colitis. It is well known that the vitamin D hormone can induce VDR expression[19] and, through VDR, suppresses TNF-α production.[20] Thus, VDR and TNF-α seem to be mutually regulated by the other. Vitamin D therapy raises vitamin D hormone that induces and activates VDR, which shifts the balance to favor inhibition of TNF-α and blockade of IEC apoptosis. This is a mechanism by which vitamin D therapy suppresses mucosal inflammation and ameliorates IBD.[21,22] However, our data infer that anti-TNF therapy should alleviate the suppression of VDR by TNF-α and restore epithelial VDR levels to protect the barrier. Certainly this prediction requires further confirmation. Interestingly, a recent study reported that miR-346 controls TNF-α secretion in activated macrophages,[23] suggesting a feedback loop existing between TNF-α and miR-346. As the TNF-α-miR-346-VDR axis is not restricted in epithelial cells, the interplays among TNF-α, VDR, and miR-346 under various pathophysiological conditions in colonic epithelial cells as well as in other cell types and the resultant biological effects warrant further investigation.

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REFERENCES

1. Fasano A, Shea-Donohue T. Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat Clin Pract Gastroenterol Hepatol.* 2005;2:416–422.
2. Watson AJ, Chu S, Sieck L, et al. Epithelial barrier function in vivo is sustained despite gaps in epithelial layers. *Gastroenterology.* 2005;129:902–912.
3. Gibson PR. Increased gut permeability in Crohn's disease: is TNF the link? *Gut.* 2004;53:1724–1725.
4. Edelblum KL, Yan F, Yamaoka T, et al. Regulation of apoptosis during homeostasis and disease in the intestinal epithelium. *Inflamm Bowel Dis.* 2006;12:413–424.
5. Haussler MR, Whitfield GK, Kaneko I, et al. Molecular mechanisms of vitamin D action. *Calcif Tissue Int.* 2013;92:77–98.
6. Liu W, Chen Y, Golan MA, et al. Intestinal epithelial vitamin D receptor signaling inhibits experimental colitis. *J Clin Invest.* 2013;123:3983–3996.
7. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 1993;75:263–274.
8. Berg DJ, Davidson N, Kuhn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest.* 1996;98:1010–1020.
9. Wirtz S, Neufert C, Weigmann B, et al. Chemically induced mouse models of intestinal inflammation. *Nat Protoc.* 2007;2:541–546.
10. Li YC, Bolt MJG, Cao L-P, et al. Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. *Am J Physiol Endocrinol Metab.* 2001;281:E558–E564.
11. Yuan W, Pan W, Kong J, et al. 1,25-dihydroxyvitamin D3 suppresses renin gene transcription by blocking the activity of the cyclic AMP response element in the renin gene promoter. *J Biol Chem.* 2007;282:29821–29830.
12. Rutgeerts P, Sandborn WJ, Feagan BG, et al. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med.* 2005;353:2462–2476.
13. Colombel JF, Sandborn WJ, Rutgeerts P, et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology.* 2007;132:52–65.
14. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;116:281–297.

15. Zamore PD, Haley B. Ribo-gnome: the big world of small RNAs. *Science*. 2005;309:1519–1524.
16. Mohri T, Nakajima M, Takagi S, et al. MicroRNA regulates human vitamin D receptor. *Int J Cancer*. 2009;125:1328–1333.
17. Qiu W, Wu B, Wang X, et al. PUMA-mediated intestinal epithelial apoptosis contributes to ulcerative colitis in humans and mice. *J Clin Invest.* 2011;121:1722–1732.
18. Weber CR, Raleigh DR, Su L, et al. Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity. *J Biol Chem.* 2010;285:12037–12046.
19. Zella LA, Kim S, Shevde NK, et al. Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3. *Mol Endocrinol.* 2006;20:1231–1247.
20. Chen Y, Liu W, Sun T, et al. 1,25-dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages. *J Immunol.* 2013;190:3687–3695.
21. Cantorna MT, Munsick C, Bemiss C, et al. 1,25-dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease. *J Nutr.* 2000;130:2648–2652.
22. Ananthakrishnan AN, Khalili H, Higuchi LM, et al. Higher predicted vitamin D status is associated with reduced risk of Crohn's disease. *Gastroenterology*. 2012;142:482–489.
23. Semaan N, Frenzel L, Alsaleh G, et al. miR-346 controls release of TNF-alpha protein and stability of its mRNA in rheumatoid arthritis via tristetraprolin stabilization. *PLoS One.* 2011;6:e19827.