Neurotensin Signaling Activates MicroRNAs-21 and -155 and Akt, Promotes Tumor Growth in Mice, and Is Increased in Human Colon Tumors

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BACKGROUND & AIMS: Neurotensin promotes inflammation and colon cancer via the neurotensin-1 receptor (NTR1). MicroRNAs (miRs) regulate protein synthesis by degrading or preventing translation of mRNAs. We analyzed expression of 365 different microRNAs by human colonic epithelial cells (NCM460) after activation of NTR1. METHODS: We performed microarray analysis of mRNA expression by neurotensin-stimulated NCM460 cells that overexpressed NTR1. Nuclear factor-κB (NF-κB) binding sites were identified and tumorigenesis was assessed using soft agar assays and xenograft analysis of severe combined immunodeficiency mice. Targets of neurotensin-regulated microRNAs were identified via bioinformatic, real-time polymerase chain reaction, and immunoblot analyses. We analyzed RNA samples from human normal colon and tumor samples. RESULTS: Neurotensin stimulated differential expression of 38 microRNAs, including miR-21 and miR-155, which have been associated with tumor growth and contain NF-κB binding sites. Neurotensin expression increased colony formation by HCT-116 cells. Blocking miR-21 and/or miR-155 prevented colony formation (P < .001). In mice, intraperitoneal administration of neurotensin increased the growth rate of HCT-116 xenograft tumors; blocking miR-21 and/or miR-155 slowed this tumor growth. Neurotensin activated Akt in HCT-116 cells; this effect was inhibited by blocking miR-21 and/or miR-155 (P < .001). Neurotensin activated Akt through miR-155-mediated suppression of the phosphatase protein phosphatase 2A catalytic subunit alpha (PPP2CA). Levels of phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 1 (SOCS1) mRNA, potential targets of miR-21 and miR-155, respectively, were down-regulated by these miRs. Levels of NTR1, miR-21, and miR-155 increased significantly in human colon tumor samples, compared with normal tissues, whereas PPP2CA, SOCS1, and PTEN mRNAs were reduced significantly. CONCLUSIONS: NTR1 activation stimulates expression of miR-21 and miR-155 in colonocytes, via Akt and NF-κB, to downregulate PTEN and SOCS1 and promote growth of tumors in mice. Levels of NTR1, miR-21, and miR-155 increase in human colon tumor samples and correlate with tumor stage.

Keywords: Neuropeptide; Colorectal Cancer; Signal Transduction; Tumor Suppressor.

During the past decade, hundreds of genes encoding small 19- to 25-nucleotide single-stranded RNA molecules called microRNAs (miRNAs) have been discovered.1 miRNAs negatively regulate gene expression by binding 3’ untranslated regions (UTRs) of transcripts,2 leading to messenger RNA (mRNA) degradation, or inhibition of translation into protein.3 More than one third of human genes are regulated by miRNAs.4 miRNAs are reported to play a role in the pathogenesis of diseases associated with disordered genome function such as cancer.5,6 In fact, the expression pattern of 217 miRNAs defines a cancer type better than 16,000 mRNAs, providing a much more efficient tool for diagnosis and targeted treatment.7 Thus, modulation of miRNA expression may influence human disease states, including colon cancer.8

Neurotensin (NT), a 13 amino acid neuropeptide, originally isolated from the calf hypothalamus,9 is expressed in the central nervous system and the gastrointestinal tract.10 In the latter, NT acts through its cognate receptor NTR1 to stimulate colonic cell proliferation, mitogen-activated protein kinase, and nuclear factor-κB (NF-κB) activation,11 promoting colon cancer12,13 and intestinal inflammation.14,15 On intestinal cells, NT also regulates the expression of several genes.15

The ability of neuropeptides to modulate expression of microRNAs in colonocytes remains to be understood. Here, we used microRNA microarray expression analysis to detect the miRNA signature of NTR1 activation in human colonic NCM460 epithelial cells overexpressing NTR1. After NT treatment we found functional NF-κB–binding sites on miR-21 and miR-155, known modulators of human cancer.16,17 Selective inhibition of these

Abbreviations used in this paper: miRNA, microRNA; mRNA, messenger RNA; NF-κB, Nuclear factor-κB; NT, neurotensin; PCR, polymerase chain reaction; siRNA, small interfering RNA; UTR, untranslated region.

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miRNAs inhibited the ability of NT to promote colon cancer in vitro and in vivo.

Materials and Methods

Antibodies and Reagents

We used the following antibodies and reagents: phospho-NF-κB/p65 (Ser536) (1:500, 3031; Cell Signaling, Inc, Beverly, MA), NF-κB p65 (1:1,000, ab7970; Abcam, Inc, Cambridge, MA), PTEN (1:300, 9559; Cell Signaling, Inc), PPPP2CA (1:1000, TA300307; Origene, Rockville, MD), SOCS1 (1:750, sc-9021; Santa Cruz Biotech, Inc, Santa Cruz, CA), β-actin (1:1000, 130065; Santa Cruz Biotech, Inc), BAY-117082 (cat no. 196870; Calbiochem, Darmstadt, Germany), and NT (cat. no 048-03; PhoenixBiotech, Inc, Jamestown, NY).

Transduction of NCM460 Cell Line With NTR1 (NCM460-NTR1)

The human neurotensin receptor 1 (NTR1) gene was isolated from pCR2.1 with Eco RV and inserted into lentiviral backbone CMV-IRESF-PGK-Puro at the Eco RV site, 5′ to IRES. Lentiviral particles expressing NTRS1 were generated by transient co-transfection of 293T cells with a 3-plasmid combination, as described previously. After transduction, NCM460-NTR1 cells were maintained as described earlier.

MicroRNA Expression Analysis

Expression of 365 microRNAs were evaluated with microRNA profiling assays (TLDA human miRNA v1.0; Applied Biosystems, Carlsbad, CA) at the Dana Farber Molecular Diagnostics Facility. Results were validated using the mirVana quantitative reverse-transcription polymerase chain reaction (PCR) miRNA Detection Kit and quantitative reverse-transcription PCR Primer Sets, according to the manufacturer’s instructions (Ambion, Inc, Austin, TX). The internal control used was RNU48 expression.

Transfection Experiments

HCT-116 and DLD1 cells were transfected with 100 nmol/L small interfering RNA (siRNA) against NTR1#1 (s9767; Ambion, Inc) or 100 nmol/L siRNA against NTR1#2 (s9769; Ambion) or 100 nmol/L antisense microRNAs for miR-21 (as-miR-21) and miR-155 (as-miR-155) (Ambion, Inc) using siPORT NeoFX (Ambion). Transfection with 90 nmol/L siRNA control (AM4611; Ambion, Inc) or 100 nmol/L antisense miRNA negative control (AM17010; Ambion, Inc) was used as control. No cell toxicity was detected owing to the transfection agent. RNA was extracted at different time points after siRNA transfection for real-time PCR analysis. β-actin was used for loading control and transfections were performed in triplicate.

Luciferase Assays

HCT-116 and DLD1 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with firefly luciferase reporter constructs containing the 3′UTR of PTEN (HmtT015535; GeneCopoeia, Inc, Rockville, MD), PPPP2CA (HmtT014115; GeneCopoeia, Inc), or SOCS1 (S203417; Switchgear Genomics, Menlo Park, CA), and treated with NT (100 nmol/L). Cell extracts were prepared 24 hours after transfection, and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

Identification of NF-κB Sites in MicroRNA Regulatory Areas

Lever and Phylogeny cis-regulatory module algorithms were developed previously. To identify NF-κB-binding motifs (5 kb upstream and 2 kb downstream of microRNAs) we performed the following: (1) used Lever algorithm to assess motifs enriched in cis-regulatory modules; (2) incorporated phylogenetic information from 12 mammalian species (mouse, rat, human, rabbit, chimp, macaque, cow, dog, armadillo, tenrec, opossum, and elephant) and selected high-conservation score sites (>100) with the Phyl–cis-regulatory module algorithm, and (3) mapped conserved binding sites in regions of interest. Loci of the top predicted and conserved NF-κB binding motifs are shown in Supplementary Table 1.

Chromatin Immunoprecipitation

By using published procedures, chromatin fragments from untreated and NT-treated (0.5, 1, and 6 h) HCT-116 and DLD1 cells were immunoprecipitated with 8 μg of antibody against NF-κB/p65 (ab7970; Abcam, Inc), DNA was extracted (Qiagen Purification Kit; Qiagen, Valencia, CA), and real-time PCR was performed for NF-κB/p65 binding sites in microRNA promoters.

Primers

miR-21 forward 5′-AAATTTGGGAGGACTCCAAGC-3′, reverse 5′-GAAAGGAAAAATGATGTCAGTGCAA-3′ (PCR product: 119 bp); miR-20 forward 5′-GGCAGAAACACAGGAAGCA-3′, reverse 5′-TGGTCATATCTTCAAGCACA-3′ (PCR product: 102 bp); and miR-155 forward 5′-GAATATATATCCCCGTTGTTG-GAA-3′, reverse 5′-TCCAAAAGAGACCTGAACTTAAA-3′ (PCR product: 102 bp). Negative control: chromatin immunoprecipitation in the HNRPA2 gene, as described previously. HNRPA2 primers: forward 5′-ACCGCCCTGACGTAGCGGA-3′, reverse 5′-CAACTCTGCGAGGAGCACCT-3′.

Anchorage-Independent Growth Assay

Transfections were performed as described earlier for 48 hours. Triplicates of 5 × 10⁵ cells were mixed 4:1 (vol/vol) with 2.0% agarose in growth medium (final, 0.4% agarose). Cell mixtures were plated on a layer of growth medium (0.5% agarose), fed every 6–7 days with growth medium (0.4% agarose), and colonies were counted after 15 days. Experiments were repeated 3 times. Statistical significance was calculated using the Student t test.

Invasion Assays

HCT-116 and DLD1 cells were treated with NT (100 nmol/L) and 100 nmol/L antisense-microRNAs (as-miR-21, as-miR-155, as-miR-NC). Assays were conducted according to the manufacturer’s protocol, using Matrigel invasion chambers (PharMingen, San Diego, CA) and 10% fetal bovine serum as chemotactrant. Invading cells were fixed and stained with 4′,6-di-aminido-2-phenylindole (Vector Laboratories, Inc, Burlingame, CA). Ten fields/insert were scored and standard deviation was measured. Assays were repeated 3 times. Statistical significance was calculated using the Student t test.

Enzyme-Linked Immunosorbent Assays

HCT-116 and DLD1 cells were exposed to medium or medium containing NT (20, 50, 100 nmol/L × 0.5 for 1, 3, and 6 h). In some experiments the cells were treated with control siRNA (100 nmol/L) or siRNA-NTR1 (100 nmol/L) for 24 hours.
Nuclear extracts were prepared and p65 was detected using the NF-κB/p65 Active Enzyme Linked Immunosorbent Assay (Cell Signaling) according to the manufacturer’s instructions. Samples were loaded in triplicate and data are presented as mean ± standard deviation. AKT phosphorylation (S473) (DuoSet IC Enzyme Linked Immunosorbent Assay, DYC887; R&D Systems, Minneapolis, MN), phospho-GSK3β(S9) (DYC-1590; R&D Systems), and phospho-p65(S536) (7834; Cell Signaling) were assessed by enzyme linked immunosorbent assay in cells transfected with as-miR-21 (100 nmol/L) and/or as-miR-155 (100 nmol/L) for 24 hours and treated with NT (100 nmol/L) for 24 hours.

Mouse Experiments

HCT-116 wild-type cells (5 × 10⁴) or cells transfected with as-miR-NC or as-miR-155 and as-miR-21 were injected subcutaneously in the right flank of athymic nude mice (Charles River Laboratories, Wilmington, MA; n = 5 per group). Tumor volume (V [mm³] = axb²/2, where a equals the largest diameter and b equals the perpendicular diameter, was monitored every 5 days for 35 days. At approximately 100 mm³ (15 days), mice were treated with NT (100 nmol/L) (days 15, 20, and 25). Injections of as-miR-NC, as-miR-155, and as-miR-21 (5 mg/kg) were performed next to the tumor every 5 days (starting on day 5), suppressing microRNA expression levels greater than 80%.

RNA Expression Studies From Patient Samples

RNAs purchased were as follows: 11 normal colon tissues (Biochain, Hayward, CA; and Origene) and 14 colon cancer tissues (Origene). RNAs extracted were as follows: 7 normal colon tissues, 20 colon cancer tissues (Translational Pathology Core Laboratory, Department of Pathology, University of California, Los Angeles, CA). mRNA expression of NTR1, PTEN, SOCS1, PP2CA, miR-21, and miR-155 were determined by real-time PCR. Samples were run in triplicate and data represent mean ± standard deviation.

Results

NT Regulates the Expression of MicroRNAs in Colon Epithelial Cells

NT promotes colon cancer¹²,¹³ and intestinal inflammation¹⁴,¹⁵ by mechanisms involving regulation of gene expression. To assess changes in the expression of miRNAs in response to NT, nontransformed human colonic epithelial NCM460 cells overexpressing NTR1 (NCM460-NTR1) were treated with 10⁻⁷ mol/L NT for 30 minutes or 6 hours and miRNA arrays were performed. NT altered the expression levels of 38 miRNAs (8 up-regulated and 30 down-regulated) (Figure 1A). Hierarchical clustering analysis revealed that 5 different clusters of miRNAs were affected by NT. Specifically, the up-regulated miRNAs were divided into miRNAs that showed early and stable responses and miRNAs that showed dynamic continuous responses (Figure 1B). Furthermore, NT-associated down-regulated miRNAs were divided into miRNAs showing an early and transient response, miRNAs with a dynamic continuous suppression, and miRNAs with an early and stable response (Figure 1C), suggesting a dynamic interplay between NT signaling and miRNA expression. Validation of these results was performed using a real-time SYBR Green miRNA assay (Applied Biosystems) (Supplementary Figure 1).

To connect NTR1 stimulation with colon cancer development, we compared the NTR1 expression levels in colon cancer cell lines with NCM460 cells (Figure 2A). Seven of the 8 cell lines showed a marked increase in NTR1 mRNA levels (5- to 20-fold) (P < .00001). We chose the 2 cancer cell lines with the highest NTR1 mRNA levels (HCT-116, DLD1) and assessed mRNA expression of microRNAs at 0.5 and 6 hours after NT treatment. The microRNAs tested were the top 7 up-regulated and down-regulated microRNAs in response to NT treatment (Figure 1). Both cell lines showed increased expression levels of miRNAs that were up-regulated and decreased expression of miRNAs that were down-regulated in NCM460-NTR1 cells in response to NT (Figure 2B and C). We next used siRNAs to knock-down NTR1 expression and assessed expression of highly up-regulated miRNAs (miR-21, miR-210, and miR-155) and down-regulated miRNAs (miR-148a, miR-139, and miR-125b) in response to NT. In NTR1 silenced cells, NT did not affect the expression of miRNAs, suggesting that NTR1 is necessary for the effects of NT on the regulation of miRNA expression (Figure 2E and Supplementary Figure 2).

NT Controls MicroRNA Expression Through NF-κB Activation in Colon Cancer Cells

Activation of NF-κB is observed in many cancers whereas NF-κB is a known target of NT in colonocytes.²³ We confirmed this effect (Supplementary Figure 3) and investigated whether the differential expression of miRNAs in response to NT involves NF-κB. By using Lever algorithm analysis we confirmed the existence of highly conserved NF-κB binding sites on the promoter region of miR-21, miR-210, and miR-155 (Supplementary Table 1). A time-course with various concentrations of NT showed a concentration-dependent increase in NF-κB activation detected by enzyme-linked immunosorbent assay for p65 activity (Figure 3A). Knock-down of NTR1 reversed this effect (Figure 3B).

Subsequently, cells were treated with NT (100 nmol/L, 0–6 h) and the enrichment of NF-κB in the promoter areas of miR-21, miR-210, and miR-155 was assessed by chromatin immunoprecipitation, indicating a significant increase for the promoters of miR-21 and miR-155 in both cell lines treated with NT for 0.5 and 6 hours (Figure 3C). Inhibition of NF-κB either via its pharmacologic inhibitor BAY117082 or via sip65 reversed the effect of NT (100 nmol/L, 6 h) on the expression levels of mature and primary transcripts of miR-21 and miR-155 in both cell lines (Figure 3D and E). Conversely, neither BAY117082 nor sip65 altered increased miR-210 expression in response to NT (Figure 3D). The exact site map of NF-κB binding sites are indicated in Supplementary Table 2. Moreover, we confirmed similar NTR1 signaling in relation to data described earlier in LoVo cells, expressing lower levels of NTR1 (Supplementary Figure 4).
NT Regulates AKT Activity in Colon Cancer Cells Through miR-21 and miR-155

We next determined whether NT affects the expression levels of direct targets of miR-21 and miR-155. Previous studies showed that miR-21 suppresses PTEN expression levels and miR-155 suppresses SOCS1 expression levels in cancer cells through direct binding in their 3’UTRs.8,24 We tested whether NT suppresses PTEN and SOCS1 levels in HCT116 and DLD1 cells. We found that NT inhibited the luciferase activities of PTEN and SOCS1 3’UTRs (Figure 4A), and suppressed PTEN and SOCS1 mRNA and protein levels (Figure 4B and C). Inhibition of
miR-155 or miR-21 by antisense–miR-155 and miR-21 blocked the effects of NT on PTEN and SOCS1 (Figure 4A–C). Because PTEN is a negative regulator of AKT activity, we tested whether NT affects AKT activity in colon cancer cells. Consistent with results from our laboratory and others, we found that NT increased AKT activation whereas miR-21 inhibition blocked this effect (Figure 4D). Interestingly, inhibition of miR-155 suppressed the effect of NT on AKT activation, and concurrent inhibition of miR-155 and miR-21 had a synergistic effect in suppressing AKT phosphorylation (Figure 4D).

**NT Activates AKT Through miR-155–Mediated PPP2CA Suppression**

Next, we examined how NT affects AKT phosphorylation through miR-155 in colon cancer cells. Bioinformatic analysis revealed that miR-155 may affect AKT activity through binding in the 3’UTR of
PPP2CA (Figure 5A), a well-known suppressor of AKT phosphorylation. Luciferase activity assay indicated that miR-155 suppresses PPP2CA, whereas antisense-miR-155 increases PPP2CA (Figure 5B). Furthermore, NT treatment suppressed PPP2CA mRNA and protein levels in both cell lines (Figure 5C and D). The increase of AKT phosphorylation by overexpression of miR-155 was comparable with PPP2CA inhibition by siRNA (Figure 5E).

AKT activation leads to activation of the NF-κB pathway and p65 phosphorylation. We found that inhibition of AKT in NT-treated colon cancer cells inhibited NF-κB activity (Figure 5F). Inhibition of miR-155 and miR-21 in NT-treated cells suppressed NF-κB phosphorylation (Figure 5F). Thus, NT induces a positive feedback loop between AKT and NF-κB in colon cancer cells.

**NT Affects the Tumorigenicity and Invasiveness of Colon Cancer Cells Through Regulation of miR-21 and miR-155 Pathways**

Both miR-21 and miR-155 are implicated in cancer cell proliferation and invasiveness. Here, we assessed cancer cell colony formation in response to NT treatment and examined participation of these miRNAs. HCT-116, DLD1, and SW480 cells were treated with NT (100 nmol/L) in the presence or absence of as-miR-21 and/or...
as-miR-155 and the number of colonies was assessed 15 days after treatment. NT significantly increased the number of colonies for both cell lines whereas knock-down of miR-21 and/or miR-155 partially reversed this effect (Figure 6A). NT (100 nmol/L) also significantly increased the number of invading HCT-116, DLD1, and SW480 cells, which was partially reversed when miR-21 and/or miR-155 were knocked down (Figure 6B).

We then tested the effects NT on tumor growth in vivo. We injected athymic nude mice subcutaneously with wild-type or as-miR-155 and as-miR-21 transfected HCT-116 cells and monitored tumor size for 35 days. Where appropriate, NT (100 nmol/L) was administered intraperitoneally 15, 20, and 25 days after injection with HCT-116 cells. NT injection significantly increased the rate of tumor growth as compared with untreated animals, whereas knock-down of miR-155 and miR-21 in the tumor-forming cells partially reversed the effect (Figure 6C). NT inhibited PTEN, SOCS1, and PPP2CA mRNA levels in these tumors (day 35), but was not effective in tumors in which miR-155 and miR-21 were suppressed by antisense–miR-155 and antisense–miR-21 (Figure 6D).

**NT-MicroRNA Signaling Pathway in Human Colon Cancers**

In an attempt to assess the potential physiologic significance of our findings in human tumors, we quantified the expression levels of both miR-21 and miR-155 as well as their downstream targets PTEN, PPP2CA, and SOCS1 in 18 normal and 34 colon cancer human speci-
mens. We identified that NTR1, miR-21, and miR-155 were up-regulated significantly, whereas PPP2CA, SOCS1, and PTEN mRNA levels were reduced significantly in colon tumors relative to normal colon tissues (Figure 7A). Testing the correlation between NTR1 mRNA levels and the expression levels of the other genes in the circuit, we identified that NTR1 was correlated positively with miR-21 (r = 0.9364) and miR-155 (r = 0.8713), whereas NTR1 levels were correlated negatively with PTEN (r = −0.746), SOCS1 (r = −0.891), and PPP2CA (r = −0.8051) (Supplementary Figure 5). Importantly, the expression levels of all the genes correlated with tumor stage (Figure 7B).

Discussion

miRNA expression is deregulated across a broad spectrum of human cancers, including colon cancer,14,31–34 NT and its receptor NTR-1 have been implicated in the pathophysiology of colitis14,15,35,36 and colon cancer12,13 by mechanisms involving regulation of expression of several genes.37 However, the pathways involved in the mediation of these responses have not been fully elucidated, and participation of miRNAs in NTR1 signaling has not been studied. We report that NT binding to NTR1 in human colonocytes overexpressing this receptor differentially regulates expression of at least 38 miRNAs. We also show that NT/NTR1 interactions affect colon tumorigenesis in vitro and in vivo through stimulation of an inflammatory microRNA feedback network. Importantly, we detected that this microRNA circuit is activated in human colon cancer specimens and correlates with colon cancer tumor stage, suggesting the importance of NTR1-associated miRNA regulation in colon cancer pathophysiology.

Increased endogenous NTR1 expression in colon cancer cells lines is an initial indication of a relationship between
NT/NTR1 signaling and colon cancer. Assessment of HCT-116 and DLD-1 cells for expression of miRNAs with the highest dependency on NT treatment seen in NCM460-NTR1 cells showed that NT stimulation of these cells causes miRNA expression phenotypes similar to that of NT-treated NCM460-NTR1 cells.

It is well established that NTR1 coupling activates the transcription factor NF-κB in NCM460-NTR1 and HCT116 human colonic epithelial cells. Lever algorithm analysis followed by chromatin immunoprecipitation assay revealed NF-κB binding sites on 3 of the miRNAs up-regulated in response to NT and indicated NF-κB

Figure 6. NT affects the tumorigenicity and invasiveness of colon cancer cells through regulation of miR-21 and miR-155 pathways. (A) Number of colonies and (B) invading cells in NT-treated HCT116, DLD1, and SW480 cells in which miR-21 and/or miR-155 were knocked down. (C) NT effects on tumor volume in a HCT116-xenograft model described in the Materials and Methods section. The P value indicates differences between NT/as-miR-NC–treated mice vs NT/as-miR-21/as-miR-155–treated mice. (D) PTEN, SOCS1, and PPP2CA mRNA levels in NT-treated HCT-116-xenograft tumors (day 35).
Figure 7. NT-microRNA signaling pathway in human colon cancers. (A) NTR1, PTEN, SOCS1, miR-21, and miR-155 expression levels assessed by real-time PCR in 18 normal and 34 (13 stage I, 7 stage II, and 14 stage III) colon cancer tissues. (B) Correlation between NTR1, PTEN, and SOCS1 mRNA levels and miR-21 and miR-155 expression levels and colon tumor stage. (C) NT-microRNA signaling pathway in colon cancer.
binding after NT treatment on the miR-21 and miR-155 promoter in HCT-116 and DLD-1 cells, in line with previous studies suggesting NF-κB as an important factor for the regulation of miR-21 and miR-155. Interestingly, miR-21 and miR-155 are up-regulated in inflamed intestinal mucosa of patients with ulcerative colitis and Crohn’s disease, whereas NTR1 expression is increased in the colon of patients with ulcerative colitis and animal models of intestinal inflammation. Together with studies showing NTR1 as a proinflammatory mediator in the intestine, these results suggest a possible link between NTR1 and miR-21 and miR-155 in the development of colitis.

Selective inhibition of miR-21 and/or miR-155 reduced the number of colonies formed after NT treatment of HCT-116 and DLD-1 cells and reduced tumor volume of HCT-116 xenografts in nude (nu/nu) mice treated with NT. These results strongly indicate that NT affects the tumorigenicity and invasiveness of colon cancer cells through miR-21- and miR-155-dependent pathways.

NT treatment of colon cancer cell lines inhibited PTEN and SOCS1 activity and suppressed PTEN and SOCS1 mRNA and protein levels by pathways involving, respectively, miR-21 and miR-155. Previous studies showed that suppression of PTEN and SOCS1 expression levels by miR-21 and miR-155, respectively, in cancer cells involves direct binding of these miRNAs in their 3’UTRs. PTEN is a negative regulator of AKT activity and AKT signaling is one of the primary pathways commonly implicated with cancer development. Consistent with results from our laboratory and others, NT increased AKT activity in colon cancer cells in a manner partially dependent on miR-21 and miR-155, suggesting that NT regulates AKT activity in colon cancer cells through miR-21 and miR-155.

The catalytic subunit of the serine/threonine phosphatase PP2A (PP2CA) suppresses AKT and may be an intermediate between NT/miRNA signaling and the regulation of AKT activity. Bioinformatic analysis of NTR1 potentially could bind in the 3’UTR of PPP2CA, altering its expression and ultimately affecting the regulation of AKT activity. We showed that NT causes AKT phosphorylation in part owing to direct interaction between miR-155 and the 3’UTR of PPP2CA, altering its expression and ultimately affecting the regulation of AKT activity. The identification of miR-155 as a macroRNA target of PPP2CA is consistent with results from our laboratory and others, providing a novel link between the NF-κB and AKT pathways in cancer (Figure 7C).

In this study we identified a novel miRNA–transcription factor feedback loop network regulating colon cancer growth and invasiveness. Previously, we described a regulatory feedback loop network between NF-κB, Lin28B, let-7, and interleukin-6 that operates in different cancer cell types, involved in the transformation process. Recently, another study identified a regulatory feed-forward circuit (KRAS-miR-143/miR-145) involved in pancreatic oncogenesis. These data suggest that miRNA–transcription factor networks are highly important for tumor initiation and progression and their modulation could have therapeutic potential.

Overall, we have identified a combination of increased NTR1, miR-21, and miR-155 levels, and lowered PTEN, PPP2CA, and SOCS1 levels in colon tumors, suggesting the importance of this signaling pathway in human colon carcinogenesis. The expression levels of these genes correlated with tumor stage, suggesting that activation of the NTR1 circuit is potentially important for tumor progression. Our results are consistent with previous reports indicating increased NT expression in human colon cancers, although NTR1 expression also is increased in colorectal cancer, as well as in inflammatory bowel disease–related colon cancer.

We show the ability of NT or any neuropeptide to modulate expression of specific miRNAs in human colonic epithelium that are functionally linked to colon cancer growth. Also, this report shows PPP2CA phosphatase as a target of miR-155. Further efforts should focus on the study of the regulation of the remaining miRNAs in response to NT, in the sense of other transcription factors involved and different signaling pathways that may be implicated in several intestinal disease states.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.07.038.

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Dimitrios Iliopoulos and Charalabos Pothoulakis contributed equally.

Conflicts of interest
The authors disclose no conflicts.

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Supplementary Materials and Methods

Cell Culture

Cell cultures were as follows: human colonic epithelial cells NCM460: M3D medium (InCell, San Antonio, TX), 10% (vol/vol) heat-inactivated fetal bovine serum, 10 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Carlsbad, CA), 37°C, 5% CO2; NCM460-NTR1 cells: same media/conditions with NCM460, 2 μg/mL puromycin (Sigma, Natick, MA); human colon cancer HCT116 cells: McCoy’s 5A medium, 10% (vol/vol) fetal bovine serum, 10 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 37°C, 5% CO2; human colon cancer DLD1 and SW480 cells: RPMI-1640 medium, 10% fetal bovine serum, 10 U/mL penicillin, and 100 μg/mL streptomycin. The Institutional Biosafety Committee approved all the procedures involving human cell lines.

Gel Electrophoresis and Immunoblotting

Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli, and transferred to polyvinylidene difluoride membranes in 25 mmol/L Tris, 192 mmol/L glycine. Membranes were blocked (phosphate-buffered saline, 10% nonfat dry milk, 0.05% Tween-20) and probed with antibodies followed by corresponding horseradish peroxidase-labeled secondary antibodies (1:1000). Blots were developed with enhanced chemiluminescence reagent (PerkinElmer Life Sciences, Waltham, MA) and exposed in an Eastman Kodak Co. 440 Image Station (Kodak, Rochester, NY). Data analysis was performed with Kodak 1D image analysis software.

Real-time PCR Analysis

Purified RNA from untreated and NT-treated HCT-116 and DLD1 cells and human colon tissues were reverse-transcribed and subjected to SYBR Green-based real-time PCR analysis.

Primers

Primers were as follows: actin forward: 5’-CCTG-TACGCCAACACAGTGCC-3’, reverse 5’-ATACCTCTGCTTGCTGATCC-3’; PTEN forward: 5’-CCGAAAGGTGTTGCTACCATTTCT-3’, reverse 5’-AAAATATTCTTCTTCTGAGCATTTCC-3’; SOCS1 forward 5’-GGGAGCCATCTCTCAAGCTAAGG-3’; PPP2CA forward 5’-TCAAGAGCCTCTTTCGGAAGAGCTT-3’, reverse 5’-GGCCATGCAATCTCCACAGACA-3’; NTR1 forward 5’-CGTGGACGGCAGACTTCA-3’, reverse 5’-CAGCCAGCGACCACAAAGG-3’.