Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

Molecular Cancer Therapeutics

MicroRNA-26b Represses Colon Cancer Cell Proliferation by Inhibiting Lymphoid Enhancer Factor 1 Expression

Zichao Zhang[1], KyoungHyun Kim[3], Xiao Li[1], Myriam Moreno[1], Thad Sharp[1], Michael J. Goodheart[2], Stephen Safe[4], Adam J. Dupuy[2], and Brad A. Amendt[1]

Abstract

microRNAs (miR) can act as oncogenes and tumor suppressors and several miRs are associated with cancer development and progression through the modulation of multiple cellular processes. *miR26b* is downregulated in several cancers and tumors and *miR26b* directly targets the lymphoid enhancer factor 1 (*Lef1*) *3′UTR* and inhibits endogenous *Lef1* expression. We report that *miR26b* expression is associated with human colon cancer through the regulation of *LEF1* expression in colon cancer cells. Analyses of multiple colon cancer cell lines revealed an inverse correlation between *miR26b* and *LEF1* expression. Normal human colon cells express low levels of *LEF1* and high levels of *miR26b;* however, human colon cancer cells have decreased *miR26b* expression and increased *LEF1* expression. We demonstrate that *miR26b* expression is a potent inhibitor of colon cancer cell proliferation and significantly decreases LEF1 expression. The LEF1-regulated genes *cyclin D1* and *c-Myc* were indirectly repressed by *miR26b* and this was consistent with decreased proliferation. *miR26b* overexpression in SW480 colon cancer cells also inhibited tumor growth in nude mice and this was due to decreased tumor growth and not apoptosis. Analyses of human colon cancer databases also demonstrated a link between *miR26b* and *LEF1* expression. *c-Myc* expression is associated with multiple cancers and we propose that *miR26b* may act as a potential therapeutic agent in reducing cancer cell proliferation through repressing LEF1 activation of *c-Myc* and *cyclin D1* expression. *Mol Cancer Ther; 13(7); 1942–51. ©2014 AACR.*

Introduction

microRNAs (miR) are a group of endogenous noncoding RNAs that posttranscriptionally regulate expression of protein-coding genes by recognizing specific mRNAs with complementary sequence (1, 2). miRs imperfectly match the 3′ untranslated region (UTR) of target mRNAs and inhibit translation and/or promote degradation. miRs can also target regions other than 3′UTR to trigger degradation of target mRNAs or repress protein synthesis. miRs are estimated to represent about 1% of all genes in human genome and the importance of their biologic functions are being intensively investigated (3–5). miRs are associated with cancer as either oncogenes or tumor-suppressor genes and in cancer cells they are either up- or downregulated (6–8). Many miRs upregulated in colon adenomas or adenocarcinomas have been reported (6). Recent reports have shown that *miR26b* is underexpressed in human breast cancer, parathyroid tumor, oral lichen planus disease, glioma cells, hepatocellular carcinomas, nasopharyngeal carcinomas, primary squamous cell lung carcinomas, and squamous cell carcinoma of the tongue (9–16). Recently, two new target genes of *miR26b* have been reported in glioma cells (EphA2) and human breast cancer cells (SLC7A11; refs. 9, 12). EphA2 is an erythropoietin-producing hepatocellular A receptor and activation of this receptor tyrosine kinase is associated with cancer cell growth (12). SLC7A11 is a solute carrier family seven member 11 that may play a role in providing resistance to apoptosis in cells (9). Finally, human embryonic stem cells and metastatic colorectal cancer cells also express *miR26b*, which may regulate *TAF12*, *PTP4A1*, *CHFR*, and *ALS2CR2* gene expression (17).

Wnt signaling is one of the most important and best characterized signaling pathways involved in embryonic development, cell proliferation, and cancer progression. The canonical Wnt signaling pathway is activated when Wnt ligand binds to cell surface receptor Frizzled and lipoprotein receptor-related protein (LRP), which activate Dishevelled and releases β-catenin from its destroying complex formed by Axin, adenomatous polyposis coli (APC), and GSK3β. The released β-catenin enters nucleus and binds to Lef/Tcf transcription factors to activate downstream gene expression (18). Among those components, many of them are potential targets of miR regulation.

Authors' Affiliations: [1]Craniofacial Anomalies Research Center, and [2]Department of Anatomy and Cell Biology, University of Iowa, Iowa City, Iowa; [3]Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio; and [4]Texas A&M Health Science Center, Houston, Texas

Current address for Z. Zhang: Department of Surgery, Gynecology Service, Memorial Sloan-Kettering Cancer Center, New York, NY.

Corresponding Author: Brad A. Amendt, Department of Anatomy and Cell Biology, University of Iowa, 51 Newton Road, 1-675 BSB, Iowa City, IA 52242. Phone: 319-335-3694; Fax: 319-335-7770; E-mail: brad-amendt@uiowa.edu

doi: 10.1158/1535-7163.MCT-13-1000

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

In the case of colorectal cancer, mutation of Wnt signal components is estimated to cause approximately 90% of the cancer (19). Most are associated with truncated APC and point mutations of phosphorylation sites in β-catenin involved in its degradation. Targeting the Wnt/β-catenin pathway, especially the downstream transcriptional regulation, would be effective in therapeutic treatment. We previously demonstrated that *miR26b* was a potent inhibitor of *LEF1* expression (20). Given the critical role of LEF1 in canonical Wnt signaling, colon cancer progression (21), and human sebaceous tumors (22), we asked whether *miR26b* repression of LEF1 would inhibit colon cancer cell proliferation.

Materials and Methods

Expression and reporter constructs

hsa-miR26b precursor (addition of six thymine deoxyriboside at 3′) was cloned from the genomic DNA of HEK 293 cell and was linked to the U6 promoter in pLL3.7 backbone. Human *LEF1* cDNA was cloned from total mRNA of HEK 293 cell and was inserted downstream of the *EF1α* promoter in pWPI backbone. The 7xTopFlash reporter plasmid was constructed into luciferase vector by inserting seven T-cell factor/lymphoid enhancer factor (LEF/TCF)–binding sites upstream of the minimal thymidine kinase (TK) promoter (20). The 7xFopFlash-negative control contains mutations within each LEF/TCF–binding site. All constructs were confirmed by DNA sequencing.

Lentiviral vector generation and cell proliferation assays

A second-generation Lentiviral vector system was used. The packaging vectors are psPAX2 and pMDG. The gene expression vectors were transfected with packaging vectors into HEK 293 FT cells to generate lentiviral vectors as previously described (23). The virus containing cell medium was harvested 48 hours after transfection. The viral vectors were concentrated by ultracentrifuge at 26,000 rpm for 2 hours at 4°C and added to the SW480 cell medium with 8 μg/mL polybrene. Infected cells were subject to the cell proliferation assay. Cells (150,000) were seeded in 60-mm plates on day 0 and then trypsinized and counted after 24, 48, 72, and 96 hours by a Coulter Z1 cell counter as previously described (24). Experiments were run in triplicate.

Cell culture, transient transfections, luciferase, and β-galactosidase assays

The CCD 841 CoN, SW480, DLD-1, Colo 320, and HEK 293 cells were obtained from the ATCC (cultured and frozen immediately for storage and used within 3 months; cell cultures used in revision experiments were subsequently thawed and cultured and genotypes were confirmed to previous cell cultures) and cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. SW480 cells were seeded in 12-well plates and transfected by Fugene HD (Roche) with 500 ng of reporter plasmid, 500 ng of expression plasmid, and 50 ng SV-40–driven β-galactosidase plasmid. Transfected cells were incubated for 48 hours and then lysed for reporter activities and protein content by Bradford assay (Bio-Rad) as previously described (25). Luciferase activity was measured with reagents from Promega and β-galactosidase activity was assessed by Galacto-Light Plus reagents (Tropix). Experiments were run in triplicate and all luciferase activities were normalized to β-galactosidase activity.

Real-time PCR analyses

Cells were cultured in T-75 flasks and two flasks of 80% confluent cells were harvested by scraping and miR isolation. Total RNA, including miR from cells, was prepared using the miRNeasy Mini Kit (Qiagen). Mulitple isolations were performed and separate qPCR assays were run on each sample ($n = 3$). The amount and integrity of the RNA samples were assessed by measurement at 260 and 280 nm and gel analyses. Quantitative real-time PCR for miR26b mature expression was done with TaqMan MicroRNA assay probes (Applied Biosystems), including U6B as a reference gene. Total RNA was reverse transcribed into cDNA by the iScript Select cDNA Synthesis Kit (Bio-Rad). Real-time PCR was carried out in a total reaction of 25 μL containing 12.5 μL iQ SYBR Green Supermix, 0.1 μmol/L forward primer, 0.1 μmol/L reverse primer, 0.25 μL cDNA template in the MyiQ Single color Real-Time Detection System and analyzed by the MyiQ Optical System Software 2.0 (Bio-Rad). *β-Actin* served as a reference gene for normalization of *LEF1*, *c-MYC*, and *cyclin D1* mRNA levels and $\Delta\Delta C_t$ values are reported. The thermal cycling profile consisted of 95°C for 4 minutes followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 18 seconds. Samples were run in triplicate. No-template control was run in each experiment. Melting curve analyses were performed to confirm amplification specificity of the PCR products. PCR primers are LEF1 F 5′-TTCCTTGGTGAACGAGTCTG-3′, R 5′-CTCTGGCCTTGTCGTGGTAG-3′; cyclin D1 F 5′-ACACGCGCAGACCTTCGTTG-3′, R 5′-GTAGGACAGGAAGTTGTTGG-3′; c-MYC F 5′-GATTCTCTGCTCTCCTCGAC-3′, R 5′- GTGATCCAGACTCTGACCTT-3′. *β-Actin* primers were described previously (26).

Western blot assays

Endogenous LEF1 isoforms were identified in colon cells and HEK 293 cells using the LEF1 antibody (Cell Signaling Technology; C12A5). c-MYC and cyclin D1 expressions in colon cells were detected using c-MYC antibody (Cell Signaling Technology; D84C12) and cyclin D1 antibody (Cell Signaling Technology; 92G2). GAPDH antibody was from Santa Cruz Biotechnology (sc-32233). Approximately 20 to 30 μg of cell lysates was analyzed in Western blots. Following SDS gel electrophoresis, the protein were transferred to PVDF filters (Millipore), immunoblotted, and detected

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

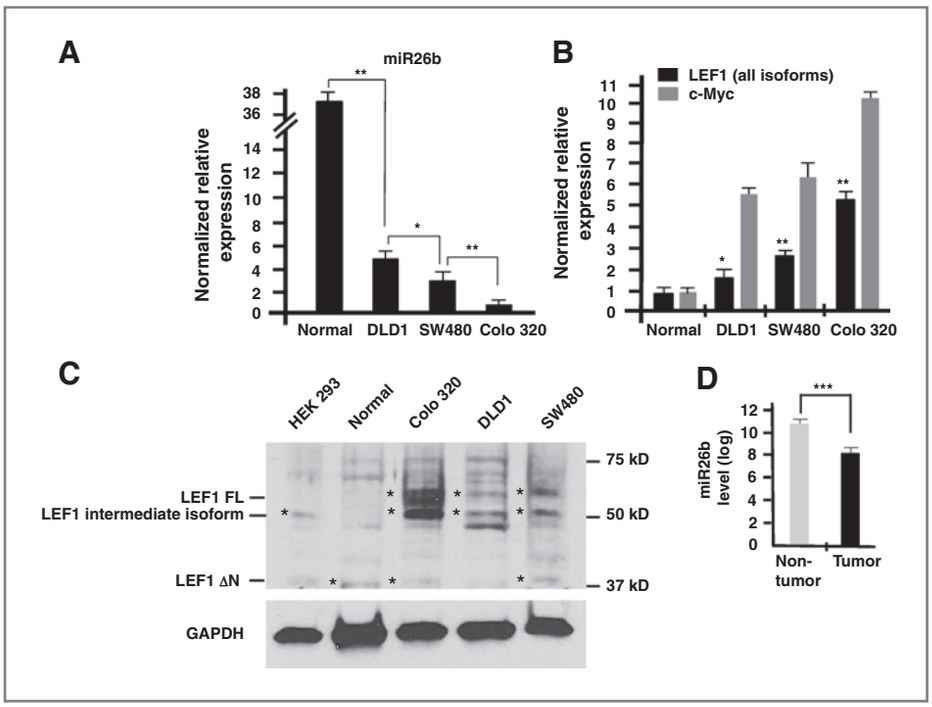

Figure 1. Endogenous LEF1 and *miR26b* expression among colon cancer cell lines. A and B, relative expression of *miR26b* and *LEF1*, *c-Myc* mRNA reveals endogenous *miR26b* and *LEF1*, *c-Myc* transcripts in a noncancer colon epithelial cell line (CCD 841 CoN) and colon cancer cell lines DLD-1, SW480, and Colo 320. *miR26b* expression is inversely correlated with *LEF1* and *c-Myc* expression. C, Western blot analysis of LEF1 expression in HEK 293 and colon cell lysates. The colon cancer cells express multiple LEF1 isoforms. The relative protein levels were consistent with mRNA levels. D, *miR26b* expression in human colon cancer tissue and normal tissue obtained from NCBI gene expression Omnibus database; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

by specific antibodies and ECL plus reagents from GE Healthcare.

Xenograft model

Male nude mice (Foxn1^{nu}; ages 6–7weeks) were purchased from Harlan and were maintained in laminar flow cabinets under pathogen-free conditions. SW480 cells (1×10^7 cells) stably expressing scramble shRNA (control) or miR26b in serum-free DMEM were injected into either side of flank area of nude mice. The mice were weighed, and tumor sizes were measured every third day with calipers for calculation of tumor volumes, $V = LW^2/2$, where L and W were length and width, respectively. After 33 days, mice were sacrificed and the tumors were collected for weighting and analysis of the expression levels of *miR26b* and other related genes. Tumors were flash-frozen in LN2, tissue was crushed and lysed, and RNA extracted using the Qiagen miRNeasy Extraction Kit (Qiagen).

TUNEL assay

SW480 cells stably expressing scramble shRNA or miR26b were grown to 75% confluence on glass slides and incubated for 2 hours with or without 0.5 mmol/L H_2O_2. The TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) assay was performed with the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instruction.

Statistical analysis

Statistics of growth curves were performed by the repeated measures of ANOVA. Other statistics were performed by the two-sample t test. P values less than 0.05 were considered to be significant.

Results

miR26b expression is inversely correlated with LEF1 expression in several colon cancer cell lines

The nontumor colon epithelial cell line (CCD 841 CoN; from the ATCC) was set as a control to compare the relative expression of *LEF1* and *miR26b* in noncancer colon versus colon cancer cells. *miR26b* was significantly downregulated in colon cancer cell lines compared with the normal cells. miR26b expression in normal cells was approximately 7.4, 12.3, and 37 times higher than observed in DLD-1, SW480, Colo 320 colon cancer cells, respectively (Fig. 1A). In contrast, *LEF1* isoform expression was upregulated in the cancer cells at both the mRNA level and protein level (Fig. 1B and C). A gradient of *LEF1* expression was detected in which normal cells <DLD-1 <SW480 <Colo 320 (Fig. 1B and C) and the data indicated an inverse relationship between *miR26b* and *LEF1* isoform expression in these cell lines. *c-Myc*, a *LEF1* target gene, was also correlated with increased *LEF1* expression (Fig. 1B). This inverse correlation suggests an inhibitory role of *miR26b* on *LEF1* expression and indicated that *miR26b* may have a potent regulatory role during cancer

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

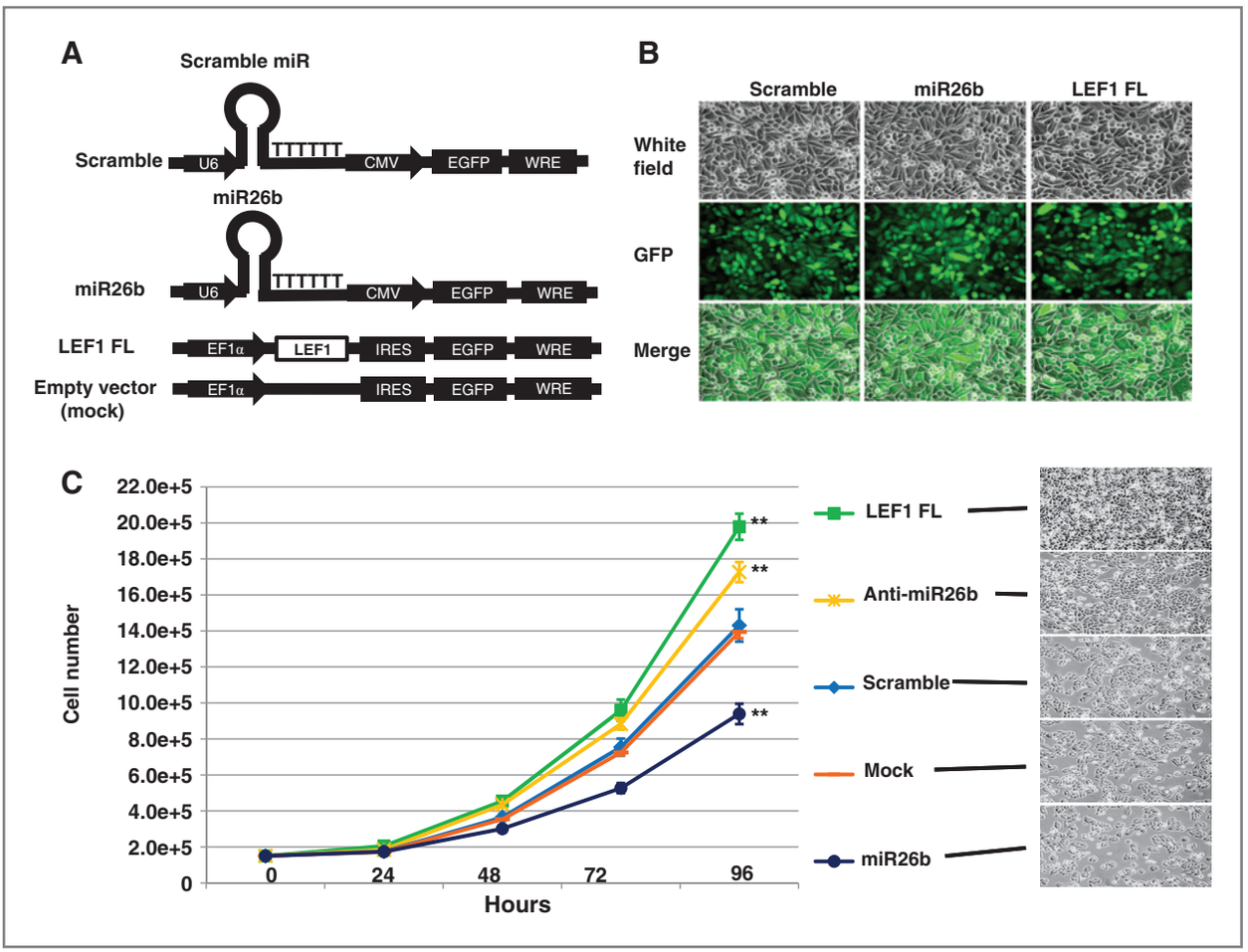

Figure 2. *miR26b* represses colon cancer cell proliferation. A, schematic of *miR26b* and *LEF1* overexpression lentiviral vectors. Human *miR26b* and scrambled miR expression are driven by the U6 promoter of pLL3.7 backbone. Human *LEF1 FL* mRNA (full-length isoform) is driven by the *EF1α* promoter of pWPI backbone. All vectors have GFP selection marker. B, GFP fluorescence pictures showing that infected SW480 cells have GFP expression. C, growth curves of SW480 cells with indicated genes expression. Of note, 150,000 cells were seeded in 60-mm plates on day 0 and counted after 24, 48, 72, and 96 hours. Experiments were run in triplicate. Error bars, SEM. Photos of cells in the plates on day 4 are shown on the right side. *LEF1 FL* cell growth was compared with mock-transfected cells and miR26b and anti-miR26b cells were compared with scrambled control; **, $P < 0.01$.

progression. *miR26b* targets all *LEF1* isoforms as they contain identical 3′UTRs and *miR26b*–binding sites. Furthermore, we analyzed *miR26b* expression in 54 human colon cancer specimens compared with 20 normal colon specimens from the NCBI Gene Expression Omnibus public database (GSE30454; ref. 27). These data show decreased *miR26b* expression ($P < 0.005$) in human colon cancer tissue (Fig. 1D). Because SW480 cells exhibited moderate expression of both *LEF1* and *miR26b*, we used these cells for overexpression and knockdown treatments of both genes in cell proliferation assays.

miR26b represses colon cancer cell proliferation

To test whether *miR26b* effected colon cancer proliferation, we established a variety of overexpression, knockdown, and rescue cell lines and analyzed their proliferation (Fig. 2). Human *miR26b* and control scramble shRNA were inserted downstream of the U6 promoter in pLL3.7 plasmid. Human *LEF1 FL* mRNA (full-length isoform) was inserted downstream of the *EF1α* promoter in pWPI plasmid (Fig. 2A). The gene expression vectors were transfected with packaging vectors into HEK 293 FT cells to generate lentiviral vectors. The virus containing cell medium was harvested 48 hours after transfection. The viral vectors were concentrated and added to the SW480 cell medium with 8 μg/mL polybrene. Approximately 100% infection efficiency was achieved, as indicated by expression of selection marker EGFP in virus-treated cells (Fig. 2B). Three stable cell lines were established: the control scramble cell line, the miR26b overexpression cell line, and the LEF1 FL overexpression cell line. These cell lines, together with SW480 cells transfected with anti-miR miRNA inhibitor (anti-miR26b; Ambion) and empty vector (mock) cells, were subjected to cell proliferation assays and the growth

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

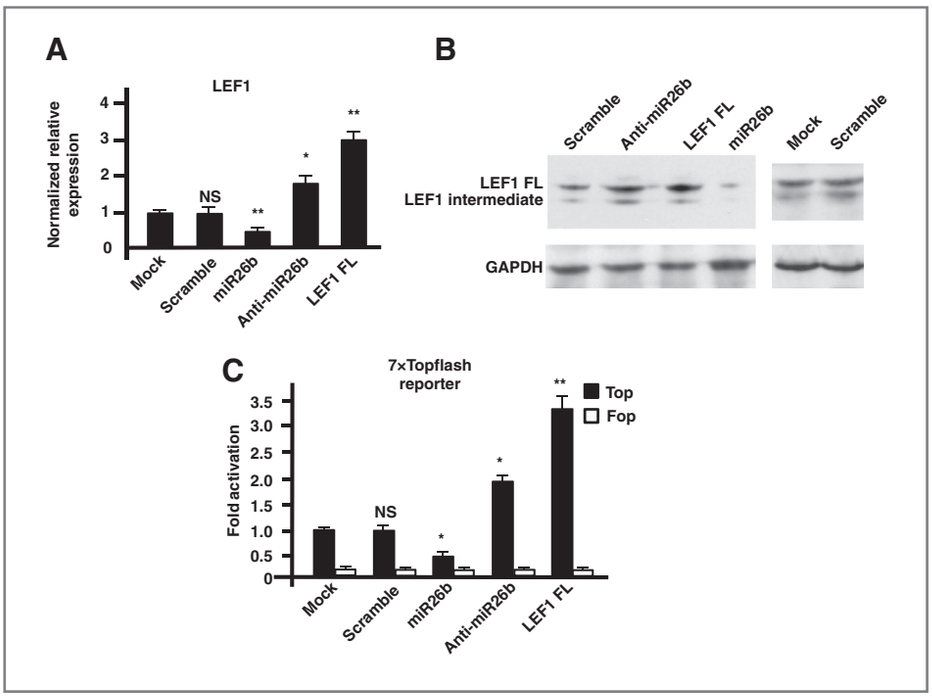

Figure 3. *miR26b* represses endogenous *LEF1* expression in SW480 cells. A, *LEF1* mRNA levels were significantly decreased in *miR26b* overexpression cells, whereas it was increased in anti-miR26b–treated cells. *LEF1* mRNA levels were significantly increased in *LEF1* overexpression cells as expected, whereas coexpression of LEF1 and *miR26b* reduced *LEF1* expression to normal levels. Real-time PCR normalized to *β-actin*, $n = 3$. B, Western blot analysis showing that LEF1 protein levels were reduced in *miR26b* overexpression cells compared with control cells, whereas it was increased in anti-miR26b–treated cells and LEF1 overexpression cells. LEF1 and *miR26b* combined overexpression cells revealed no change in LEF1 expression. C, Topflash reporter assays show that transfection of miR26b vector reduced Topflash activity, whereas transfection of anti-miR26b and LEF1 vector increased Topflash activity. Mutated reporter (Fopflash) served as a negative control. SV-40 promoter–driven β-galactosidase activity served as a control for transfection efficiency, $n = 3$. Error bars, SEM; *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

rates over 96 hours are summarized in Fig. 2C. *LEF1 FL* overexpression in SW480 cells significantly increased growth compared with the control cells. In contrast, *miR26b* overexpression decreased growth. The anti-miR26b–treated cells also exhibited increased growth *albeit* not as high as cells in which *LEF1 FL* was overexpressed. These data suggest that *miR26b* is a potent inhibitor of colon cancer cell growth through regulation of *LEF1* expression.

miR26b represses endogenous LEF1 expression in colon cancer cells

To demonstrate that *LEF1* isoform expression was targeted by *miR26b* in the cells used for cell proliferation assays, LEF1 expression was analyzed by real-time PCR and Western blots. *LEF1* mRNA levels were significantly decreased in the *miR26b*-overexpressing cells compared with control cells, whereas *LEF1* mRNA was significantly increased in cells transfected with anti-miR26b or overexpressing *LEF1 FL* (Fig. 3A). Western blots showed that LEF1 protein and mRNA levels were similar in these cell lines (Fig. 3B). A Topflash reporter assay was used to examine the overall Wnt/β-catenin signal activity. SW480 cells were transfected with 7xTopflash reporter plasmid and indicated expression plasmids, together with SV-40 β-galactosidase as a control. Transfection with *miR26b*-decreased Topflash activity compared with control cells, whereas anti-miR26b and LEF1 transfection increased reporter activity. The Fopflash plasmid with a mutation in each LEF/TCF–binding site was used as a negative control (Fig. 3C). These data indicated that *miR26b* had a potent inhibitory effect on LEF1 expression as well as Wnt/β-catenin signal activity in colon cancer cells.

miR26b represses expression of the LEF1 target genes *cyclin D1* and *c-MYC*

Wnt/β-Catenin signaling regulates cell proliferation through several different mechanisms (18, 19). One of the most important mechanisms is through regulation of cyclin D1 and c-Myc, which are essential regulators of cell proliferation and direct targets of the LEF1/β-catenin transcription complex (28–30). Therefore, we wanted to determine whether expression of these two genes was altered in our lentiviral-infected cells, which exhibit different rates of cell proliferation (Fig. 2C). Real-time PCR showed that *cyclin D1* and *c-Myc* mRNA levels were decreased in *miR26b*-overexpressing cells compared with control cells, whereas they were increased in anti–*miR26b*-transfected and LEF1 FL–overexpressing cells (Fig. 4A and B). Western blots showed that there were parallel changes in c-Myc and cyclin D1 protein and mRNA levels

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

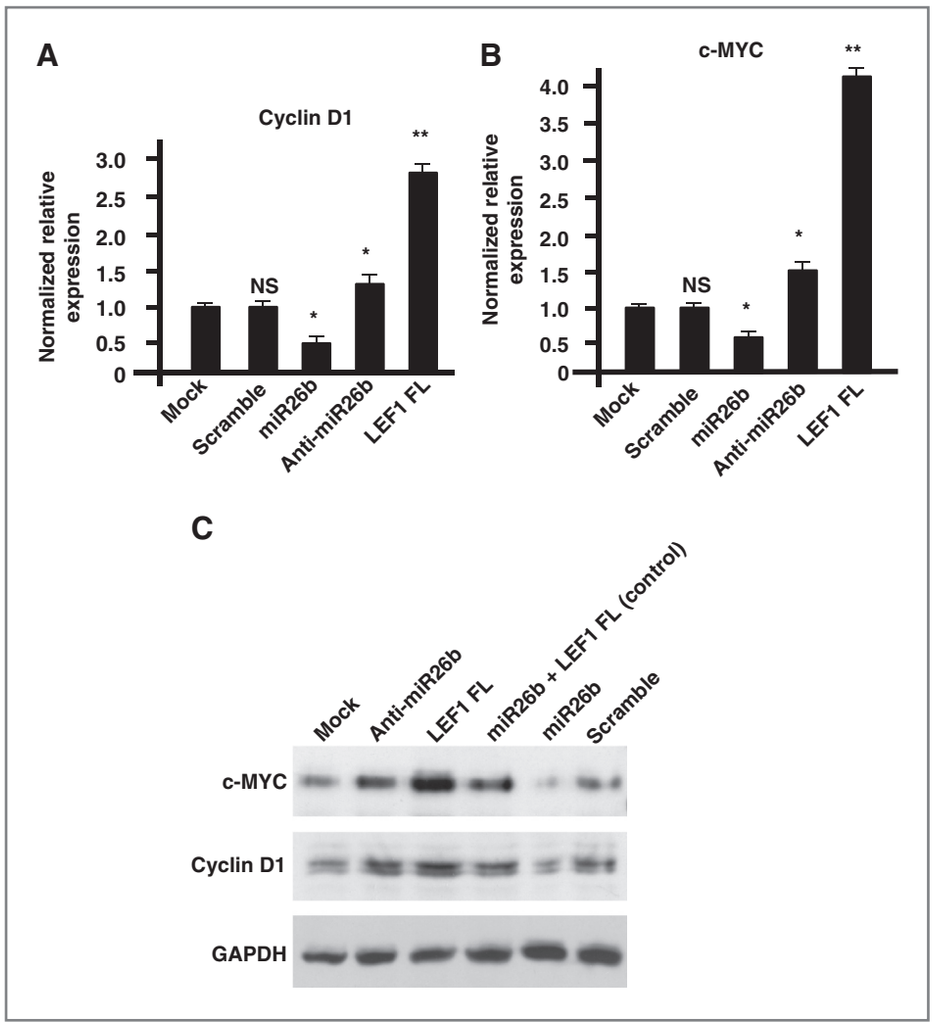

Figure 4. *miR26b* repressed expression of LEF1 target genes *cyclin D1* and *c-Myc*. A and B, real-time PCR results showing that *cyclin D1* and *c-Myc* mRNA levels were reduced in *miR26b* overexpression cells, whereas they were increased in anti-miR26b–treated cells. LEF1 overexpression cells had increased expression of *cyclin D1* and *c-Myc* as expected, whereas coexpression of LEF1 and *miR26b* reduced their expression to normal levels. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. C, a Western blot analysis with cyclin D1 and c-Myc antibodies showing that the protein levels were changed similar to mRNA levels. *miR26b* cells transfected with LEF1 FL cDNA were used as a positive control for LEF1 FL expression, which is not targeted by *miR26b*.

(Fig. 4C), suggesting that *miR26b* repressed c-Myc and cyclin D1 expression through inhibition of LEF1 in colon cancer cells. As a control *miR26b*-expressing cells were transfected with the *LEF1 FL* cDNA, which is not targeted by *miR26b* to demonstrate Lef1 FL expression by the Western blot analysis (Fig. 4C).

miR26b inhibits tumor growth in a xenograft nude mouse model

To test the effect of *miR26b* inhibition on growth of colon cancer cells *in vivo*, we injected male nude mice (Foxn1^{nu}; ages 6–7weeks) with SW480 cells (1×10^7 cells) stably expressing scramble shRNA (control) or *miR26b* in serum-free DMEM at either side of the flank area. Tumor sizes were measured every third day with calipers to calculate tumor volumes ($V = LW^2/2$; L, length; W, width; Fig. 5A). After 33 days, mice were sacrificed and the tumors were collected for weighing and expression of miR26b and other related genes. The average weight of miR26b tumors was about 50% of the control tumors (Fig. 5B and C) and real-time PCR analyses showed that miR26b expression was significantly increased in *miR26b* tumors, whereas *LEF1*, *c-Myc*, and *cyclin D1* were significantly decreased in tumors derived from *miR26b*-overexpressing SW480 cells (Fig. 5D and E). These data were consistent with the *in vitro* cell proliferation assay and real-time PCR results.

Tumor growth inhibition due to decreased proliferation not apoptosis

To test whether *miR26b* inhibited colon cancer cells through apoptosis, we performed TUNEL assays. SW480 cells stably expressing scramble miR (control) or *miR26b* were stained with Promega TUNEL Assay Kit and almost no apoptosis was detected in either control or *miR26b* cell lines (Fig. 6A and B). The same cell lines were treated with 0.5 mmol/L H_2O_2 for 2 hours as positive controls. After H_2O_2 treatment, apoptosis was induced in both cell lines (Fig. 6A and B). These data suggest that *miR26b* expression does not affect apoptosis of colon cancer cells in the

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

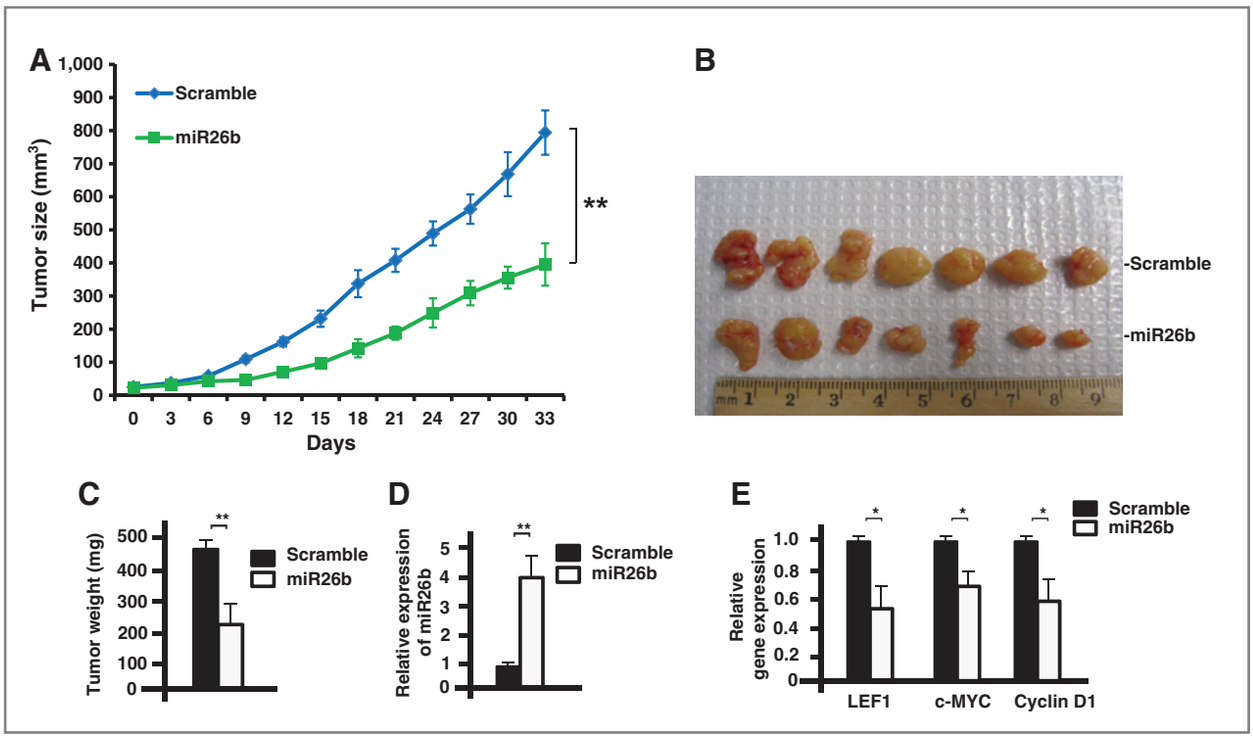

Figure 5. Effect of *miR26b* inhibition on growth of SW480 cells in the xenograft model. A, growth curves of control and *miR26b* tumors. Male nude mice were injected with SW480 cells (1×10^7 cells) stably expressing scramble shRNA (control) or *miR26b* in DMEM at either side of flank area. Tumor sizes were measured every third day to calculate tumor volumes ($V = LW^2/2$; *L*, length; *W*, width). The two curves were analyzed by ANOVA and the *P* value of <0.01. B, tumors collected for weighing and analyses of gene expression. C, the average weight of *miR26b* tumors was about half of the control tumors. D, *miR26b* expression was significantly increased in *miR26b* tumors measured by real-time PCR; $n = 3$. E, *LEF1*, *c-Myc*, and *cyclin D1* were significantly decreased in *miR26b* tumors measured by real-time PCR. $n = 3$, experiments were run in triplicate. Error bars, SEM; $n = 7$; *, $P < 0.05$; **, $P < 0.01$.

presence or absence of oxidative stress, indicating that *miR26b* primarily regulates colon cancer cell/tumor proliferation and not survival.

Discussion

The role of LEF1 in colon cancer

We previously demonstrated that *miR26b* not only targets the β-catenin–responsive isoform of *LEF1 FL* but also the dominant negative isoform (*LEF1ΔN*) and intermediate isoform because they share the same 3′UTR. Thus, *miR26b* also reduces expression of the dominant negative isoform. However, many colon cancer cells lack expression of the dominant negative isoform of *LEF1* (31). The full-length *LEF1* isoform is responsive to Wnt/β-catenin signaling in colon cancer cells (19). LEF1 and TCF belong to a family of DNA-binding transcription factors that interact with β-catenin to activate genes involved in cell proliferation, morphogenesis, epithelial–mesenchymal transition, and stemness, which can lead to cancer progression (32–36). The LEF1 full-length and intermediate isoforms are produced in colon cancers through Wnt signaling and the LEF1ΔN isoform is produced by an alternative promoter, which is inactive in some cancers (31, 37). Furthermore, β-catenin also activates *cyclin D1* expression in colon carcinoma cells through TCF/LEF1–binding elements in the cyclin D1 promoter (29, 30). The c-Myc promoter contains multiple Tcf-4–binding sites and is activated by β-catenin and presumably by LEF1 (28). These data demonstrate the role for LEF1/TCF and β-catenin in activating *cyclin D1* and *c-Myc* expression in cancer cells and our studies demonstrate that *miR26b* targets *LEF1* expression, which in turn regulates *cyclin D1* and *c-Myc* expression.

c-Myc regulation of miRs and cancer

Myc is a well-known oncogenic transcription factor that controls many cellular processes, including cell growth, differentiation, cell adhesion, and motility. Aberrant Myc activity is associated with many human cancers and Myc-mediated transactivation of target genes results in deregulated gene expression. Interestingly, some of these Myc target genes are miRs. Myc directly activates transcription of the polycistronic *miR17–92* cluster and *miR9* (38–40). The *miR17–92* cluster is classified as an oncogene as its expression is associated with several solid tumors (for reviews see refs. 41, 42). Conversely, Myc represses several miRs that are tumor suppressors, including let-7 family members, *miR34a*, *miR29* members, *miR26a*, and *miR15a/16-1*

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

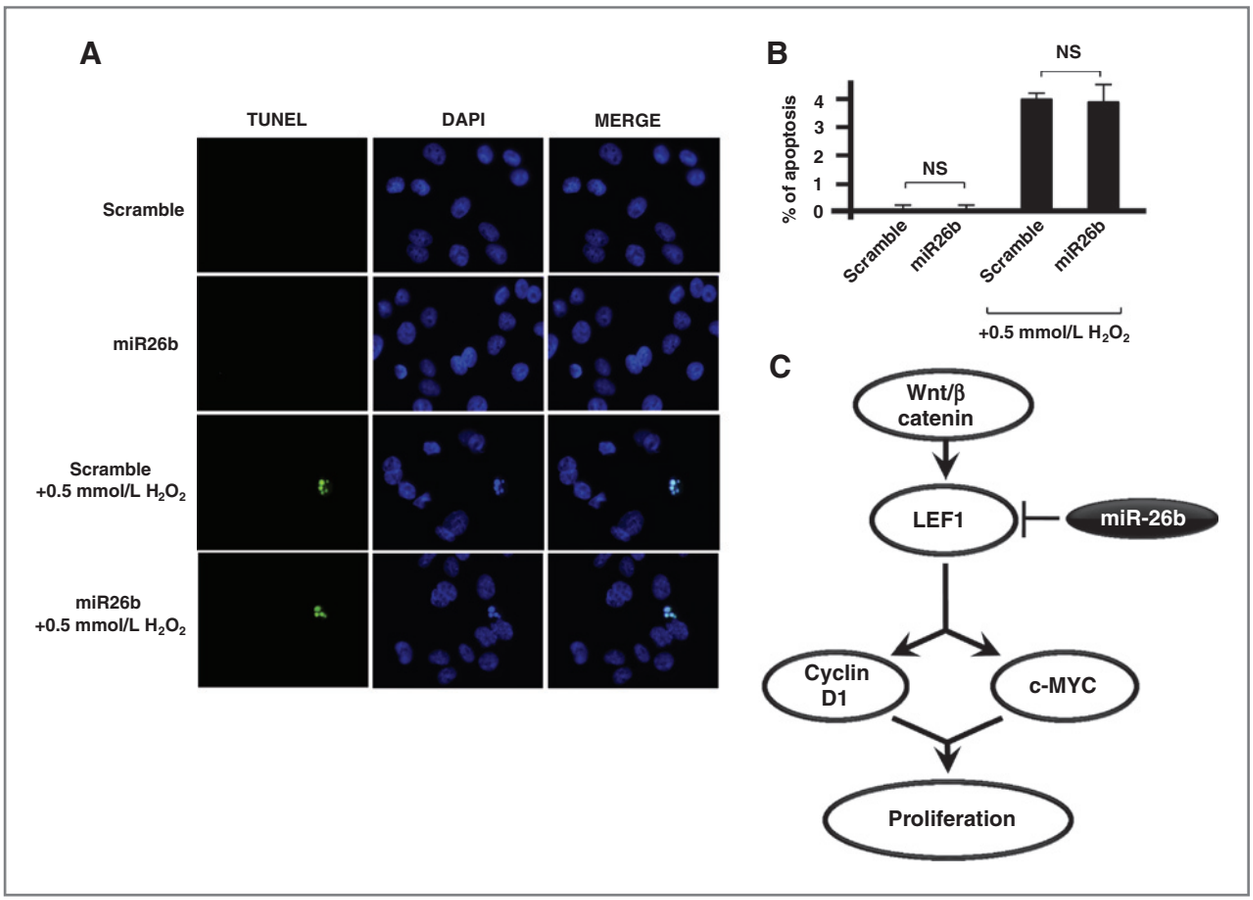

Figure 6. Apoptosis is not affected by *miR26b*. A, SW480 cells stably expressing scramble shRNA (control) or *miR26b* were treated with or without 0.5 mmol/L H_2O_2 for 2 hours and stained with the Promega TUNEL Assay Kit. B, almost no apoptosis was detected in either control or *miR26b* cell lines without H_2O_2. After H_2O_2 treatment, apoptosis was detected in both cell lines; $n = 6$; NS, not significant. C, a schematic model illustrates the underlying mechanism of *miR26b* repression of cancer cell proliferation through regulation of *LEF1*, *cyclin D1*, and *c-Myc*.

(43). Myc-regulated miRs control cell-cycle programs, apoptosis, angiogenesis, metabolism and, therefore, tumorigenesis through a variety of molecular mechanisms and gene targets (41, 42). *miR26a* overexpression has been used in an MYC-driven mouse hepatocellular carcinoma model to reverse disease progression (44). The molecular mechanism of *miR26a* involved decreased expression of *cyclin D1* and *D2* and induced cell-cycle arrest (44). Our data suggest that *miR26b* overexpression could potentially be used in patients as a therapeutic, targeting hepatocellular carcinoma in these patients or a combination of *miR26a* and *b*. Similar to *miR26a*, which is expressed at low levels in human liver cancer samples, *miR26b* is also expressed at low levels in colon cancer cells (Fig. 1A; ref. 17). Interestingly, there is a direct correlation with the severity of colon cancer with a decrease in *miR26b* expression and a concomitant increase in *LEF1* expression, suggesting that *miR26b* could be an effective therapeutic agent in treating this disease. *miR26b* was also decreased in LoVo colon cancer cells and *miR26b* overexpression in these cells suppressed cell growth and tumor growth *in vivo* (17). However, *LEF1* was not included as a *miR26b* target in the LoVo cells and tumors.

In summary, *miR26b* is decreased and *LEF1* expression is increased in colon cancer cells. We show an inverse correlation between *miR26b* and *LEF1* expression in multiple colon cell lines (Fig. 1A and B) and that *miR26b* has a potent inhibitory effect on colon cancer cell proliferation (Fig. 2). Furthermore, in patient colon cancer samples *miR26b* expression is decreased (Fig. 1D). Endogenous *LEF1* expression is significantly decreased by *miR26b* overexpression and the Wnt/β-catenin target genes *cyclin D1* and *c-Myc* are also repressed, and this represents the underlying mechanism of *miR26b*-dependent inhibition of colon cancer cell proliferation. Thus, *miR26b* is a tumor repressor that is a potent inhibitor of colon cancer cell proliferation that blocks Wnt/β-catenin–dependent activation of LEF1 and a model for tumor suppressor–like activity of *miR26b* is illustrated in Fig. 6C. These findings also have important therapeutic implications.

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

Given the prevalence of increased Wnt/β-catenin signaling in colon cancer patients, *miR26b* can be a potential candidate for gene therapy of multiple cancer types that express *LEF1*. The efficient delivery of miRs and other nucleic acids are being extensively investigated with some clinical trials already in progress (4, 5).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Z. Zhang, K.H. Kim, X. Li, T. Sharp, B.A. Amendt
Development of methodology: Z. Zhang, X. Li, T. Sharp, B.A. Amendt
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z. Zhang, K.H. Kim, X. Li, M. Moreno, T. Sharp, M.J. Goodheart, S. Safe, B.A. Amendt
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Zhang, K.H. Kim, X. Li, T. Sharp, S. Safe, A.J. Dupuy, B.A. Amendt
Writing, review, and/or revision of the manuscript: Z. Zhang, K.H. Kim, X. Li, M. Moreno, T. Sharp, M.J. Goodheart, S. Safe, A.J. Dupuy, B.A. Amendt
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Li, T. Sharp, B.A. Amendt
Study supervision: S. Safe, B.A. Amendt

Grant Support

This work was supported by the NIH grant DE13941 to B.A. Amendt.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 22, 2013; revised March 28, 2014; accepted April 21, 2014; published OnlineFirst April 30, 2014.

References

1. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 2004;5:522–31.
2. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–97.
3. Nelson P, Kiriakidou M, Sharma A, Maniatak E, Mourelatos Z. The microRNA world: small is mighty. Trends Biochem Sci 2003;28: 534–40.
4. Nana-Sinkam SP, Croce CM. Clinical applications for microRNAs in cancer. Clin Pharmacol Ther 2013;93:98–104.
5. Mendell JT, Olson EN. MicroRNAs in stress signaling and human disease. Cell 2012;148:1172–87.
6. Visone R, Croce CM. MiRNAs and cancer. AJP 2009;174:1131–8.
7. Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007;302:1–12.
8. Esquela-Kerscher A, Slack FJ. Oncomirs–microRNAs with a role in cancer. Na Rev 2006;6:259–69.
9. Liu XX, Li XJ, Zhang B, Liang YJ, Zhou CX, Cao DX, et al. MicroRNA-26b is underexpressed in human breast cancer and induces cell apoptosis by targeting SLC7A11. FEBS Lett 2011;585:1363–7.
10. Rahbari R, Holloway AK, He M, Khanafshar E, Clark OH, Kebebew E. Identification of differentially expressed microRNAs in parathyroid tumors. Ann Surg Oncol 2010;18:1158–65.
11. Danielsson K, Ebrahimi M, Wahlin YB, Nylander K, Boldrup L. Increased levels of COX-2 in oral lichen planus supports an autoimmune cause of the disease. J Eur Acad Dermatol Venereol. 2012;26: 1415–9.
12. Wu N, Zhao X, Liu M, Liu H, Yao W, Zhang Y, et al. Role of microRNA-26b in glioma development and its mediated regulation on EphA2. PloS ONE 2011;6:e16264.
13. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumors suppressors and oncogenes. Oncogene 2006;25:6188–96.
14. Weidhaas JB, Babar I, Nallur SM, Trang P, Roush S, Boehm M, et al. MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy. Cancer Res 2007;67:11111–6.
15. Jeong SH, Wu HG, Park WY. LIN28B confers radio-resistance through the posttranscriptional control of KRAS. Exp Mol Med 2009;41:912–8.
16. Arora H, Qureshi R, Jin S, Park AK, Park WY. miR-9 and let-7g enhance the sensitivity to ionizing rediation by suppression of NFKB1. Exp Mol Med 2011;43:298–304.
17. Ma YL, Zhang P, Wang F, Moyer MP, Yang JJ, Liu ZH, et al. Human embryonic stem cells and metastatic colorectal cancer cells shared the common endogenous human microRNA-26b. J Cell Mol Med 2011;15:1941–54.
18. Moon RT, Kohn AD, De Ferrari GV, Kaykas A. Wnt and β-catenin signalling: diseases and therapies. Nature 2004;5:689–99.
19. Paul S, Dey A. Wnt signaling and cancer development: therapeutic implication. Neoplasma 2008;55:165–76.
20. Zhang Z, Florez S, Gutierrez-Hartmann A, Martin JF, Amendt BA. MicroRNAs regulate pituitary development, and microRNA 26b specifically targets lymphoid enhancer factor 1 (Lef-1), which modulates pituitary transcription factor 1 (Pit-1) expression. J Biol Chem 2010;285:34718–28.
21. Kriegl L, Horst D, Reiche JA, Engel J, Kirchner T, Jung A. LEF-1 and TCF4 expression correlate inversely with survival in colorectal cancer. J Translational Medicine 2010;8:123–31.
22. Niemann C, Owens DM, Schettina P, Watt FM. Dual role of inactivating Lef1 mutations in epidermis: tumor promotion and specification of tumor type. Cancer Res 2007;67:2916–21.
23. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet 2003;33:401–6.
24. Lee S-O, Chintharlapalli S, Liu S, Papineni S, Cho SD, Yoon K, et al. p21 expression is induced by activation of nuclear nerve growth factor–induced Bα (Nur77)in pancreatic cancer cells. Mol Cancer Res 2009; 7:1169–78.
25. Amendt BA, Sutherland LB, Russo AF. Multifunctional role of the Pitx2 homeodomain protein C-terminal tail. Mol Cel Biol 1999;19:7001–10.
26. Zhang Z, Wlodarczyk BJ, Niederreither K, Venugopalan S, Florez S, Finnell RH, et al. Fuz regulates craniofacial development through tissue specific responses to signaling factors. PLoS ONE 2011;6:e24608.
27. Balaguer F, Moreira L, Lozano JJ, Link A, Ramirez G, Shen Y, et al. Colorectal cancers with microsatellite instability display unique miRNA profiles. Clin Cancer Res 2011;17:6239–49.
28. He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–12.
29. Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 1999;96:5522–7.
30. Tetsu O, McCormick F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
31. Li TWH, Ting JHT, Yokoyama NN, Bernstein A, van de Wetering M, Waterman ML. Wnt activation and alternative promoter expression of LEF1 in colon cancer. Mol Cell Biol 2006;26:5284–99.
32. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic Alterations during colorectal-tumor development. N Eng J Med 1988;319:525–32.
33. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–50.

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

34. Merrill BJ, Gat U, DasGupta R, Fuchs E. Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev 2001; 15:1688–705.
35. van Genderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, Bruhn L, et al. Development of several organs that require inductive epithelial-mesenchymal interactions is impared in LEF-1-deficient mice. Genes Dev 1994;8:2691–703.
36. Kim K, Lu Z, Hay ED. Direct evidence for the role pf beta-catenin/LEF-1 signaling pathway in induction of EMT. Cell Biol Int 2002; 26:463–76.
37. Hovanes k, Li TWH, Waterman ML. The human LEF-1 gene contains a promoter preferentially active in lymphocytes and encodes multiple isoforms derived from alternative splicing. Nuc Acids Res 2000;28: 1994–2003.
38. ODonnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature 2005;435: 839–43.
39. Mestdagh P, Fredlund E, Pattyn F, Schulte JH, Muth D, Vermeulen J, et al. MYCN/c-MYC-induced microRNAs repress coding gene networks associated with poor outcome in MYCN/c-MYC-activated tumors. Oncogene 2010;29:1394–404.
40. Ma L, Young J, Prabhala H, Pan E, Mestdagh P, Muth D, et al. miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin, and cancer metastasis. Nat Cell Biol 2010;12:247–56.
41. Bui TV, Mendell JT. Myc: maestro of microRNAs. Genes Cancer 2010; 1:568–75.
42. Frenzel A, Loven J, Henriksson MA. Targeting MYC-regulated miRNAs to combat cancer. Genes Cancer 2011;1:660–7.
43. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, et al. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet 2008;40:43–50.
44. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, et al. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell 2009;137:1005–17.

Published OnlineFirst April 30, 2014; DOI: 10.1158/1535-7163.MCT-13-1000

Molecular Cancer Therapeutics

MicroRNA-26b Represses Colon Cancer Cell Proliferation by Inhibiting Lymphoid Enhancer Factor 1 Expression

Zichao Zhang, KyoungHyun Kim, Xiao Li, et al.

Mol Cancer Ther 2014;13:1942-1951. Published OnlineFirst April 30, 2014.