

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

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

### 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  $\beta$ -catenin from its destroying complex formed by Axin, adenomatous polyposis coli (APC), and GSK3 $\beta$ . The released  $\beta$ -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:** <sup>1</sup>Craniofacial Anomalies Research Center, and <sup>2</sup>Department of Anatomy and Cell Biology, University of Iowa, Iowa City, Iowa; <sup>3</sup>Department of Environmental Health, University of Cincinnati, Cincinnati, Ohio; and <sup>4</sup>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

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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  $\beta$ -catenin involved in its degradation. Targeting the Wnt/ $\beta$ -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 *EF1 $\alpha$*  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 7xTopFlash-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  $\mu$ g/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 $\beta$ -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 Eugene HD (Roche) with 500 ng of reporter

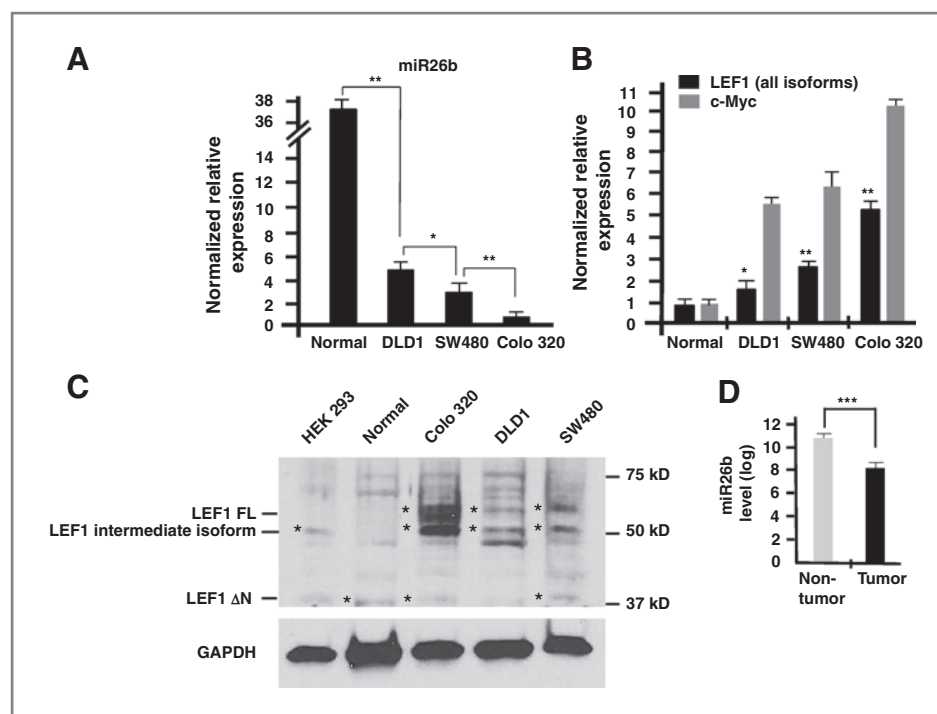
plasmid, 500 ng of expression plasmid, and 50 ng SV-40-driven  $\beta$ -galactosidase plasmid. Transfected cells were incubated for 48 hours and then lysed for reporter activities and protein content by Bradford assay (Bio-Rad) as previously described (25). Luciferase activity was measured with reagents from Promega and  $\beta$ -galactosidase activity was assessed by Galacto-Light Plus reagents (Tropix). Experiments were run in triplicate and all luciferase activities were normalized to  $\beta$ -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). Multiple 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  $\mu$ L containing 12.5  $\mu$ L iQ SYBR Green Supermix, 0.1  $\mu$ mol/L forward primer, 0.1  $\mu$ mol/L reverse primer, 0.25  $\mu$ L cDNA template in the MyiQ Single color Real-Time Detection System and analyzed by the MyiQ Optical System Software 2.0 (Bio-Rad).  $\beta$ -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'-CTCTGGCCTT-GTCGTGGTAG-3'; *cyclin D1* F 5'-ACACGCGCAGAC-CTTCGTTG-3', R 5'-GTAGGACAGGAAGTTGTTGG-3'; *c-MYC* F 5'-GATTCTCTGCTCCTCGAC-3', R 5'-GT-GATCCAGACTCTGACCTT-3'.  $\beta$ -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  $\mu$ g of cell lysates was analyzed in Western blots. Following SDS gel electrophoresis, the protein were transferred to PVDF filters (Millipore), immunoblotted, and detected



**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<sup>nu</sup>; ages 6–7 weeks) were purchased from Harlan and were maintained in laminar flow cabinets under pathogen-free conditions. SW480 cells ( $1 \times 10^7$  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<sub>2</sub>O<sub>2</sub>. 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.

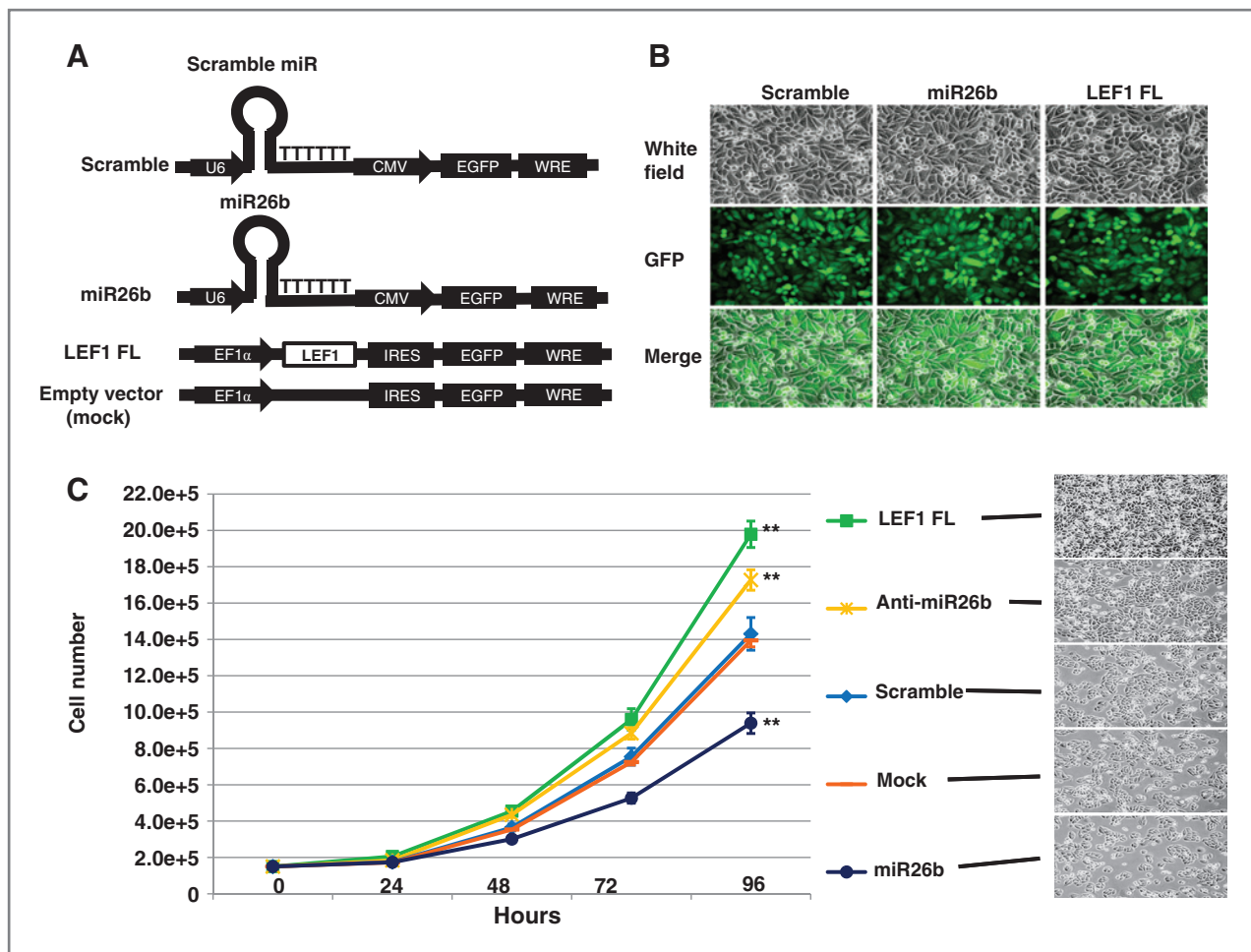
### Statistical analysis

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 down-regulated 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



**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 *EF1 $\alpha$*  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; \*\*,  $P < 0.01$ .

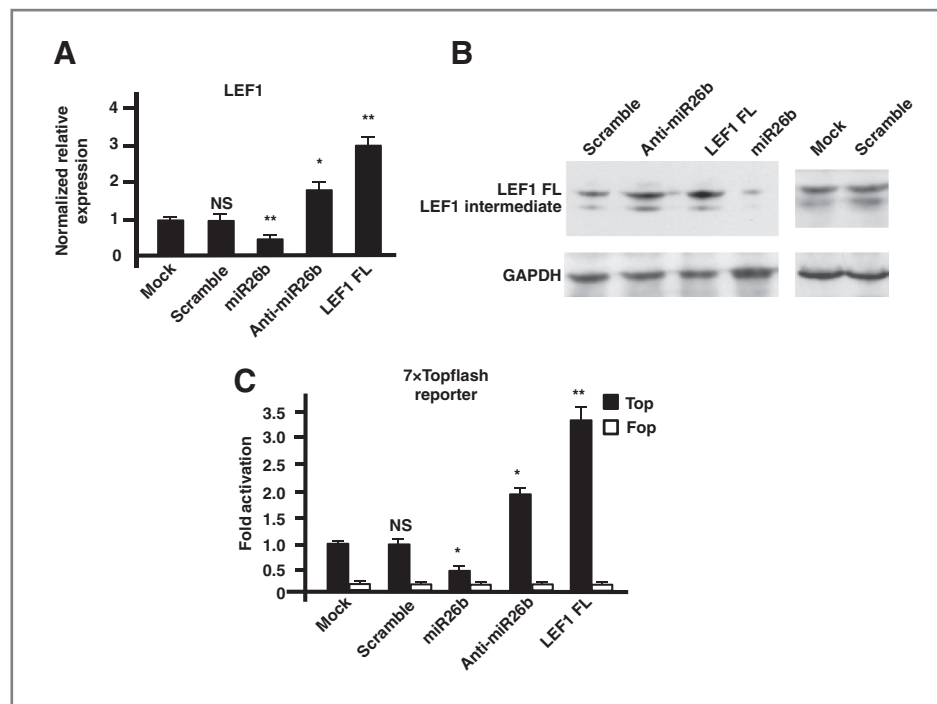
progression. *miR26b* targets all *LEF1* isoforms as they contain identical 3'UTRs and *miR26b*-binding sites. Furthermore, we analyzed *miR26b* expression in 54 human colon cancer specimens compared with 20 normal colon cancer specimens from the NCBI Gene Expression Omnibus public database (GSE30454; 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 *EF1 $\alpha$*  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  $\mu\text{g}/\text{mL}$  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





**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  $\beta$ -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  $\beta$ -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**

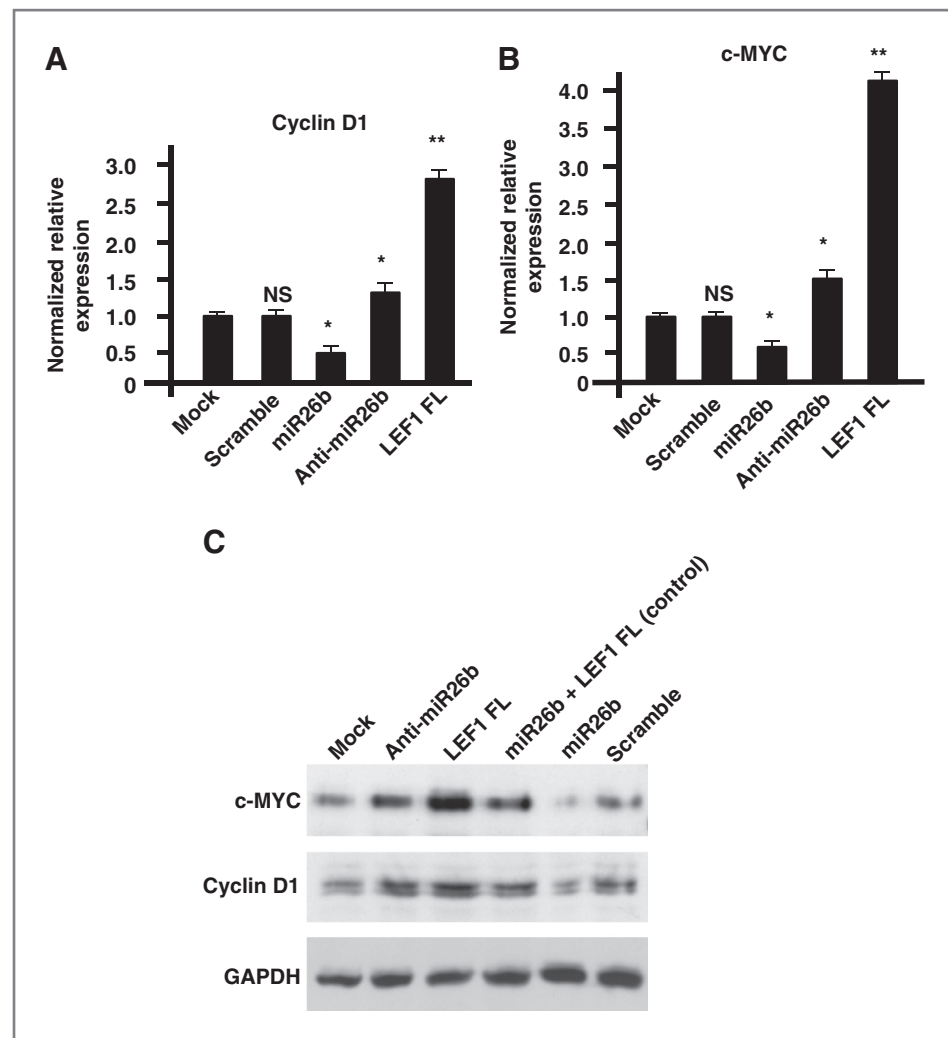
To demonstrate that *LEF1* isoform expression was targeted by *miR26b* in the cells used for cell proliferation assays, *LEF1* expression was analyzed by real-time PCR and Western blots. *LEF1* mRNA levels were significantly decreased in the *miR26b*-overexpressing cells compared with control cells, 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/ $\beta$ -catenin signal activity. SW480 cells were transfected with 7xTopflash reporter plasmid and indicated expression plasmids, together with SV-40  $\beta$ -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/ $\beta$ -catenin signal activity in colon cancer cells.

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

Wnt/ $\beta$ -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*/ $\beta$ -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

**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).

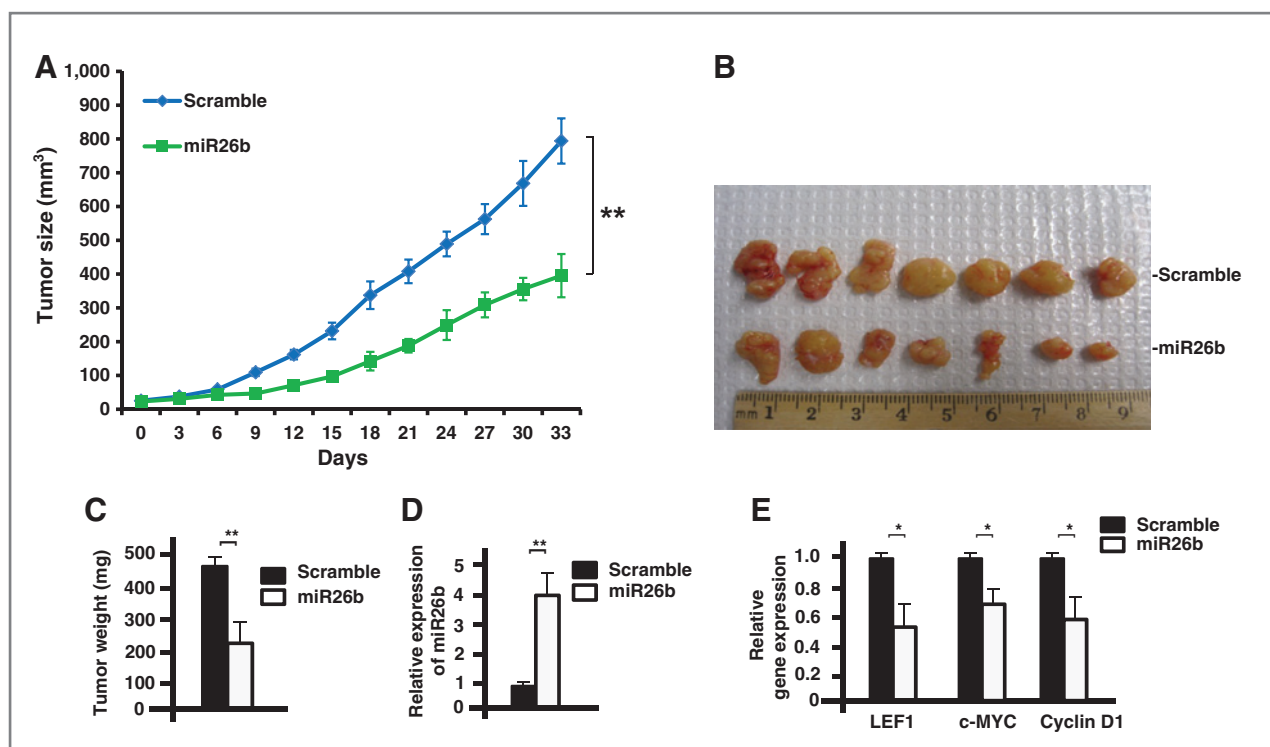
#### ***miR26b* inhibits tumor growth in a xenograft nude mouse model**

To test the effect of *miR26b* inhibition on growth of colon cancer cells *in vivo*, we injected male nude mice (Foxn1<sup>nu</sup>; ages 6–7 weeks) with SW480 cells ( $1 \times 10^7$  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<sub>2</sub>O<sub>2</sub> for 2 hours as positive controls. After H<sub>2</sub>O<sub>2</sub> 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



**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 ( $1 \times 10^7$  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

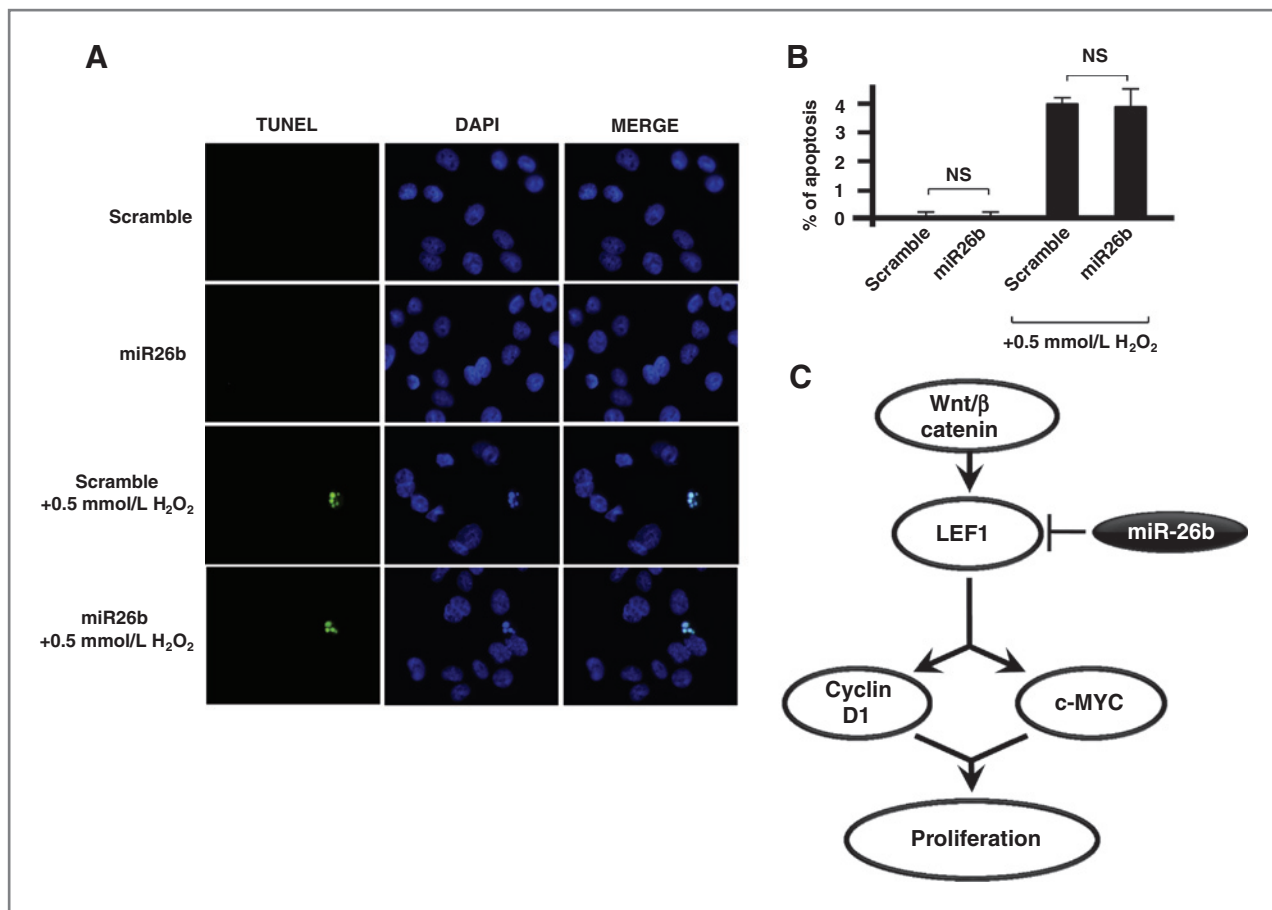
### The role of LEF1 in colon cancer

We previously demonstrated that *miR26b* not only targets the  $\beta$ -catenin-responsive isoform of *LEF1 FL* but also the dominant negative isoform (*LEF1 $\Delta$ N*) 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/ $\beta$ -catenin signaling in colon cancer cells (19). *LEF1* and TCF belong to a family of DNA-binding transcription factors that interact with  $\beta$ -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 *LEF1 $\Delta$ N* isoform is produced by an alternative promoter, which is inactive in some cancers (31, 37). Furthermore,  $\beta$ -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  $\beta$ -catenin and presumably by *LEF1* (28). These data demonstrate the role for *LEF1*/TCF and  $\beta$ -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*



**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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>. After H<sub>2</sub>O<sub>2</sub> 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/ $\beta$ -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/ $\beta$ -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.



Given the prevalence of increased Wnt/ $\beta$ -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

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