miR-29b represses intestinal mucosal growth by inhibiting translation of cyclin-dependent kinase 2

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ABSTRACT **The epithelium of the intestinal mucosa is a rapidly self-renewing tissue in the body, and defects in the renewal process occur commonly in various disorders. microRNAs (miRNAs) posttranscriptionally regulate gene expression and are implicated in many aspects of cellular physiology. Here we investigate the role of miRNA-29b (miR-29b) in the regulation of normal intestinal mucosal growth and further validate its target mRNAs. miRNA expression profiling studies reveal that growth inhibition of the small intestinal mucosa is associated with increased expression of numerous miRNAs, including miR-29b. The simple systemic delivery of locked nucleic acid–modified, anti–miR-29b-reduced endogenous miR-29b levels in the small intestinal mucosa increases cyclin-dependent kinase 2 (CDK2) expression and stimulates mucosal growth. In contrast, overexpression of the miR-29b precursor in intestinal epithelial cells represses CDK2 expression and results in growth arrest in G1 phase. miR-29b represses CDK2 translation through direct interaction with the *cdk2* mRNA via its 3′-untranslated region (3′-UTR), whereas point mutation of miR-29b binding site in the *cdk2* 3′-UTR prevents miR-29b–induced repression of CDK2 translation. These results indicate that miR-29b inhibits intestinal mucosal growth by repressing CDK2 translation.**

Monitoring Editor
William P. Tansey
Vanderbilt University

Received: May 28, 2013
Revised: Jul 22, 2013
Accepted: Jul 24, 2013

INTRODUCTION

The homeostasis of the intestinal mucosa epithelium is preserved through strict regulation of cell proliferation, migration, differentiation, and apoptosis (Wildhaber *et al.*, 2003; Radtke and Clevers, 2005; Wang, 2007; Puleo *et al.*, 2011). Undifferentiated intestinal epithelial cells (IECs) continuously replicate within the crypts and differentiate as they migrate up the luminal surface of intestine to replace lost cells (Simons and Clevers, 2011). The epithelium of the human small intestine undergoes ~10^{11} mitoses/d, and this rapid and dynamic epithelial renewal is tightly regulated by numerous factors, including the presence of food in the gut and cellular polyamines (Johnson, 1988; Wang *et al.*, 2007). Food is one of the strongest stimulants of mucosal growth (Steiner *et al.*, 1968), whereas fasting results in mucosal atrophy of the intestine (Steiner *et al.*, 1968; Tabata and Johnson, 1986). Polyamines (spermidine and spermine and their precursor putrescine) are absolutely required for normal intestinal mucosal growth (Wang *et al.*, 1993; Li *et al.*, 2002; Casero and Marton, 2007), and decreasing the levels of cellular polyamines by α-difluoromethylornithine (DFMO) represses IEC proliferation in vitro and suppresses growth of the intestinal mucosa in vivo (Wang *et al.*, 1991; Liu *et al.*, 2009).

Posttranscriptional processes, in particular altered mRNA turnover and translation, are major mechanisms by which mammalian cells control gene expression in response to stress (Garneau *et al.*, 2007; Mendell and Olson, 2012). Changes in mRNA stability and translation are governed predominantly by microRNAs (miRNAs) and RNA-binding proteins through interaction with *cis* elements located at the 3′-untranslational regions (UTRs) of target mRNAs

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-05-0287) on July 31, 2013.

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Abbreviations used: CDK2, cyclin-dependent kinase 2; CR, coding region; DFMO, D,L-α-difluoromethylornithine; FBS, fetal bovine serum; FISH, fluorescence in situ hybridization; IEC, intestinal epithelial cell; IP, immunoprecipitation; LNA, locked nucleic acid; miRNA, microRNA; Q-PCR, quantitative PCR; UTRs, untranslational regions.

(Kedde *et al.*, 2007; Kim *et al.*, 2009; Omer *et al.*, 2009). miRNAs are small, noncoding RNAs of ~22 nucleotides that posttranscriptionally repress the expression of target genes and regulate a variety of cellular processes (Kedde *et al.*, 2007; Omer *et al.*, 2009; Léveillé *et al.*, 2011; Muddashetty *et al.*, 2011; Liu *et al.*, 2012; Mendell and Olson, 2012). Generally, miRNAs act by binding to the 3′-UTRs of target mRNAs, destabilizing them and/or inhibiting their translation (Eulalio *et al.*, 2008; Leung and Sharp, 2010), and the differential abundance of given miRNAs during disease progression suggests an especially significant role for miRNAs in human pathologies. Recently, miRNAs have also emerged as master regulators of gut epithelial homeostasis, and miR-222 and miR-503 have been shown to modulate IEC proliferation and apoptosis (Xiao *et al.*, 2011; Ye *et al.*, 2011; Cui *et al.*, 2012).

miR-29b is a member of the miR-29 family, known to target several mRNAs and to be implicated in many cellular functions (Ru *et al.*, 2012; Wang *et al.*, 2013). miR-29b regulates DNA methylation-related reprogramming events by targeting *dnmt3a* and *dnmt3b* (Guo *et al.*, 2012) and promotes fibrosis by altering expression of collagen isoforms (Roderburg *et al.*, 2010; Qin *et al.*, 2011). miR-29b modulates cell proliferation and apoptosis (Huang *et al.*, 2013; Li *et al.*, 2013) and also plays a role in the development of abdominal aortic aneurysm in mouse (Maegdefessel *et al.*, 2012). The abnormal expression of miR-29b is associated with tumorigenesis and cancer progression (Cortez *et al.*, 2010; Huang *et al.*, 2013), and miR-29b alters the tumor microenvironment to repress metastasis (Melo and Kalluri, 2013). Here our results indicate that miR-29b represses growth of the small intestinal mucosa by inhibiting expression of cyclin-dependent kinase 2 (CDK2). Moreover, miR-29b directly interacts with the *cdk2* mRNA via its 3′-UTR and to repress CDK2 translation. These findings provide a strong rationale for developing therapeutic strategies directed at miR-29b in order to promote intestinal mucosal growth under critical pathological conditions.

RESULTS

Changes in miRNA expression profiles during intestinal mucosal atrophy

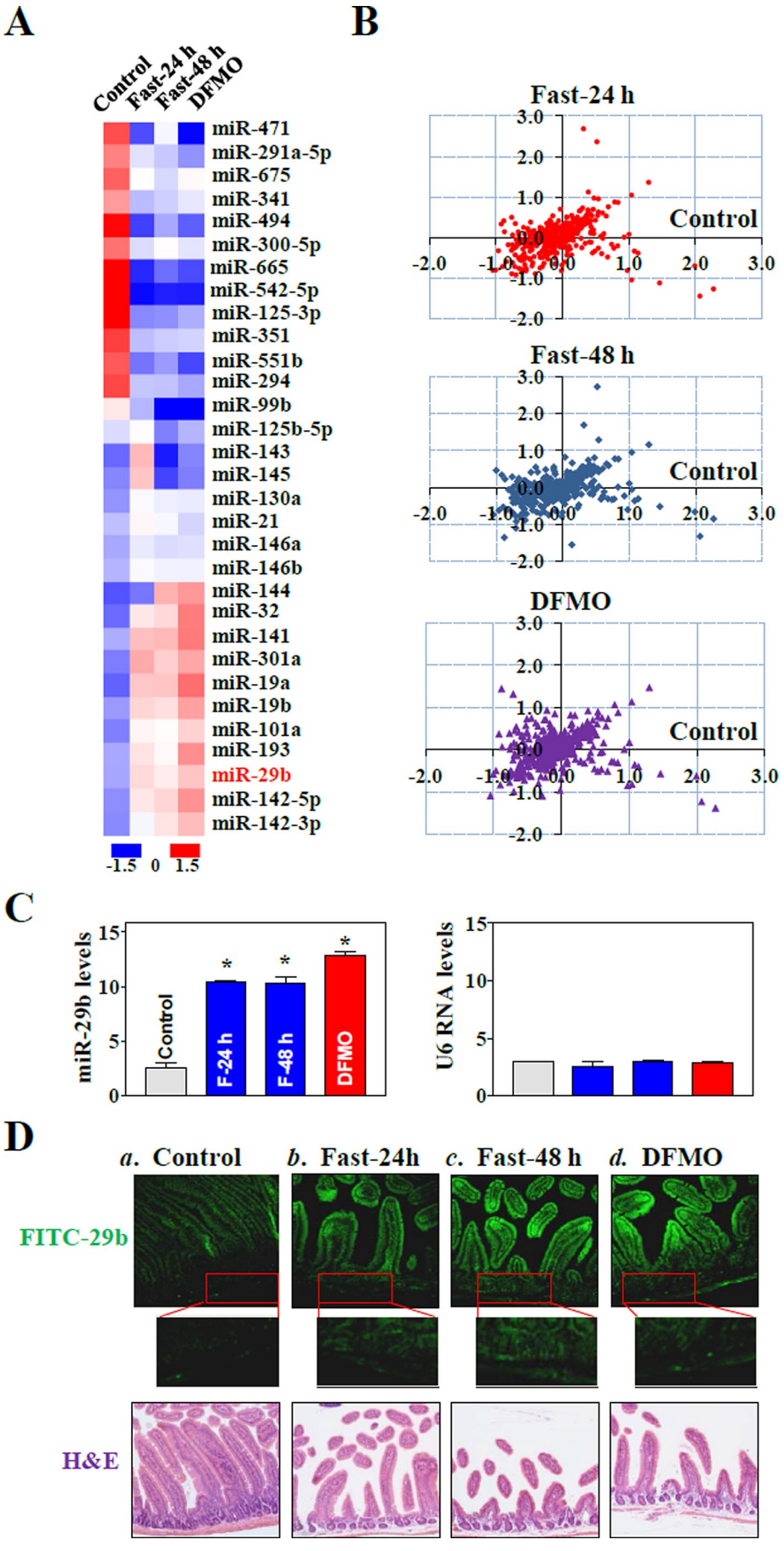
To determine the involvement of miRNAs in the regulation of intestinal mucosal growth, we carried out microarray-based interrogation of global miRNA expression on the mucosa of the small intestine in two mucosal atrophy models induced by fasting (Steiner *et al.*, 1968) or polyamine depletion by DFMO (Wang *et al.*, 1991). A comparison of the miRNA expression profiles in the mucosa in control mice relative to mice fasted for 24 or 48 h revealed that 27 miRNAs were differentially expressed during growth inhibition of the small intestinal mucosa (Figure 1, A and B). In fasted mice, expression of 16 miRNAs, including miR-471, miR-291a-5p, miR-675, and miR-341, decreased after a 24- or 48-h period of fasting, whereas expression of 11 miRNAs, including miR-29b (miR-29b-3p), miR-144, miR-32, miR-141, and miR-301a, increased. Generally, changes in global miRNA expression profiles in the small intestinal mucosa from mice exposed to DFMO for 6 d were similar to those observed in fasted mice. Although a sizable subset of miRNAs showed altered abundance after a period of fasting or polyamine depletion by DFMO, the present study was highly focused on miR-29b, based on its strong dependence on inhibition of the mucosal growth and the computationally predicted interactions with mRNAs encoding cell proliferation–regulatory proteins such as CDK2 and cyclin D1. Therefore real-time quantitative PCR (Q-PCR) analysis was used to confirm these changes and verify that the levels of miR-29b increased significantly after fasting and polyamine depletion (Figure 1C). In contrast, there were no significant changes in the levels of mucosal *u6* RNA after various treatments. We also examined the expression of other members of the miR-29 family, miR-29a and miR-29c, and demonstrated that neither fasting nor polyamine depletion by DFMO altered their levels in the small intestinal mucosa. To determine the localization of miR-29b, we examined the presence of miR-29b along the entire villus-crypt column by fluorescence in situ hybridization (FISH) assays and found that in the mucosa of the small intestine miR-29b was present in enterocytes along the entire villus in control mice, but there was little or no miR-29b present in crypt cells (Figure 1Da, top). In fasted and DFMO-treated mice, however, miR-29b was increased in cells of both the villi and crypts (Figure 1D, b–d, top). Consistent with our previous studies (Steiner *et al.*, 1968; Tabata *et al.*, 1986) and others (Wang *et al.*, 1991), fasting and polyamine depletion by DFMO caused a significant inhibition of intestinal mucosal growth, as shown in Figure 1D (bottom). These findings indicate that mucosal atrophy in the small intestine is associated with increase in levels of miR-29b, especially in cells of the crypts.

Locked nucleic acid–modified mediated miR-29b silencing promotes intestinal mucosal growth

In an effort to define the in vivo biological importance of miR-29b, we decreased the levels of endogenous miR-29b by using systemically administered locked nucleic acid–modified (LNA) anti–miR-29b oligonucleotides. As shown in Figure 2A, in situ hybridization in the mucosa of the small intestine showed a predominant accumulation of the LNA anti–miR-29b in the crypt area in mice treated with anti–miR-29b but not in saline control. Minor fluorescein isothiocyanate staining of the LNA anti–miR-29b was also observed in the villi. To examine whether our LNA anti–miR-29b oligonucleotides could be used for miR-29b antagonism, we examined changes in the levels of miR-29b in the mucosa of the small intestine in mice after administration of LNA anti–miR-29b. Treatment with LNA anti–miR-29b for 4 consecutive days resulted in a sustained decrease in miR-29b in the small intestinal mucosa (Figure 2B); the levels of miR-29b were decreased by >90% in mice treated with LNA anti–miR-29b compared with those observed in animals treated with control LNA-scrambled oligonucleotides. Of interest, mucosal growth in the small intestine increased significantly in miR-29b–antagonized mice, as indicated by an increase in the lengths of villi and crypts (Figure 2, C and D, and Supplemental Figure S1). We also examined the effect of R-29b inhibition in other epithelial tissues in mice and found that treatment with LNA anti–miR-29b for 4 consecutive days did not significantly alter colonic mucosa growth or liver histology (Supplemental Figure S2). To determine the potential target mRNAs of miR-29b, our results further demonstrated that LNA-mediated miR-29b silencing robustly increased CDK2 expression, although it only marginally induced cyclin D1 levels. On the other hand, miR-29b silencing had no effect on CDK4 expression and decreased the levels of p21 protein (Figure 2E). These results strongly support the notion that miR-29b represses mucosal growth of the small intestine at least partially through a process involving reduction in CDK2 expression levels.

miR-29b interacts with and represses CDK2 mRNA translation

To investigate the exact targets of miR-29b in the intestinal epithelium, we performed experiments in cultured IECs (IEC-6 line). Consistent with our in vivo observations, both starvation by serum deprivation and polyamine depletion by DFMO increased the levels of miR-29b in IEC-6 cells (Figure 3, A and C). High-resolution in situ hybridization showed that in growth-arrested cells miR-29b accumulated primarily in the cytoplasm, rather than the nucleus, of IEC-6



cells (Figure 3, B and D). Using the program RNA22, we found that there is one computationally predicted binding site of miR-29b within the 3′-UTR of the *cdk2* mRNA (Figure 4A), suggesting a potential mechanism for the repression of CDK2 expression by miR-29b. To test this possibility, we first examined the association of miR-29b with the *cdk2* mRNA by RNA pull-down assays using biotin-labeled miR-29b (custom-made by Dharmacon; Figure 4Ba). To assess the transfection efficiency, we examined the levels of miR-29b and small nuclear RNA *u6* (which served as control) by Q-PCR analysis 24 h after the transfection. As shown in Figure 4Bb, cells transfected with the biotin-labeled miR-29b exhibited elevated miR-29b levels but displayed no changes in *u6* RNA levels (Figure 4Bc). The levels of *cdk2* mRNA were highly enriched in the pull-down materials from cells transfected with the biotin-labeled miR-29b but not from cells transfected with scrambled control oligomer (Figure 4C). The interaction of miR-29b with the *cdk2* mRNA is relatively specific, because increasing the levels of biotin–miR-29b failed to increase its binding to the *xiap* and *cdk4* mRNAs and only minimally induced binding to *ccnd1* mRNA (encoding cyclin D1). The levels of *xiap* and *cdk4* mRNAs in the pull-down materials were indistinguishable between cells transfected with biotin–labeled miR-29b and cells transfected with scrambled oligomers. These results indicate that the *cdk2* mRNA is a specific target of miR-29b.

The second set of experiments was designed to define the functional consequences of [miR-29b/*cdk2* mRNA] association. As shown in Figure 5, A and B, increasing the

FIGURE 1: Changes in miRNA expression in intestinal mucosal atrophy models induced by fasting or polyamine depletion. (A) Heat map depiction of miRNAs differentially expressed in the small intestinal mucosa in mice fasted for 24 or 48 h and mice treated with DFMO for 6 d. (B) Scatter plot of microarray analysis from the mucosa described in A. The $\log_{10}$-transformed signal intensities for each probe on both channels for controls and samples from mice fasted 24 h (top) or 48 h (middle) or DFMO treated (*bottom*). (C) RNA used in A was analyzed by Q-PCR. Values are means ± SEM of data from five or six animals. *$p < 0.05$ compared with controls. (D) Distribution of miR-29b (top) as measured by FISH using a miR-29b–specific oligomer in the mucosa described in A. Bottom, hematoxylin/eosin (H/E) staining of intestinal mucosa.

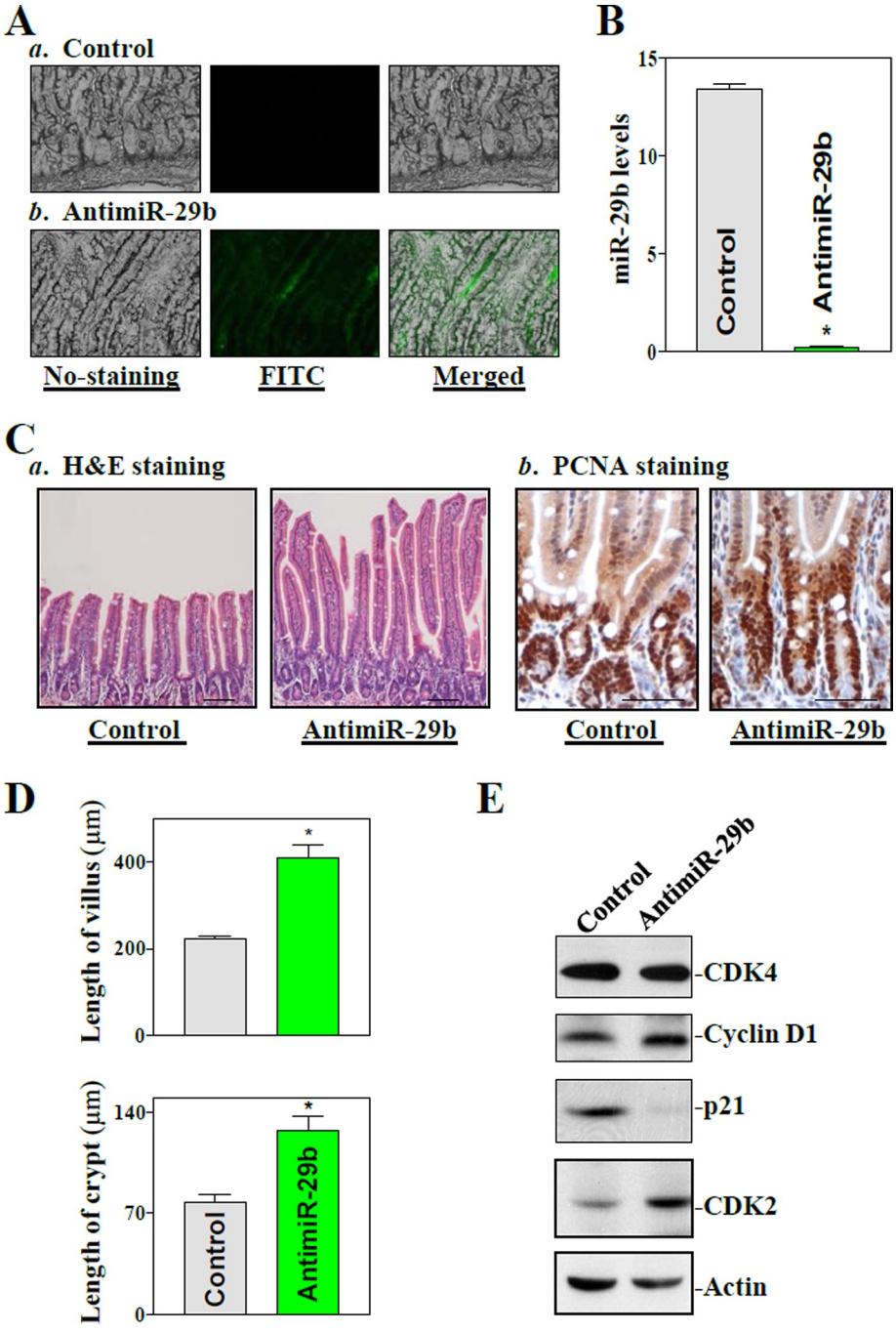


FIGURE 2: LNA-mediated miR-29b silencing promotes intestinal mucosal growth. (A) In situ detection of LNA anti–miR-29b in the small intestinal mucosa in mice treated with saline (a) or LNA-antimiR-29b (b). Mice were injected intraperitoneally with LNA anti–miR-29b, whereas control mice were injected with equal volume of saline. At 24 h after injection, small intestinal tissues were harvested for histological examination. (B) Levels of miR-29b in the small intestinal mucosa in mice injected intraperitoneally with LNA anti–miR-29b or control LNA-scrambled oligonucleotides for 4 consecutive days. On day 5, total RNA was isolated, and the levels of miR-29b were measured by Q-PCR analysis. Values are means ± SEM of data from five or six animals. *$p < 0.05$ compared with controls. (C) Photomicrographs of H/E (a) and proliferating cell nuclear antigen signals (b) in the mucosa described in B. Scale bar, 100 μm. (D) Changes in the length of villi (top) and crypt (bottom) of the mucosa described in B. *$p < 0.05$ compared with controls. (E) Expression of proliferation-associated proteins in the mucosa described in B.

levels of miR-29b by transfection with the miR-29b precursor (pre–miR-29b) decreased CDK2 protein levels. To examine whether miR-29b inhibited CDK2 expression by repressing the translation of *cdk2* mRNA, we examined changes in the level of new CDK2 protein synthesis after ectopic miR-29b overexpression and demonstrated that newly synthesized CDK2 protein decreased significantly in pre–miR-29b–transfected cells compared with cells transfected with scrambled oligomer (Figure 5Bb). Inhibition of CDK2 protein synthesis by miR-29b was specific, since there was no change in nascent GAPDH synthesis after miR-29b overexpression. To further define the role of miR-29b in the regulation of CDK2 translation, we examined the relative distribution of *cdk2* mRNA in individual fractions from polyribosome gradients after miR-29b overexpression. Although increasing the levels of miR-29b did not affect global polysomal profiles (Figure 5C), the abundance of *cdk2* mRNA associated with actively translating fractions (fractions 7–10) decreased dramatically in pre–miR-29b–transfected cells, with a significant shift of *cdk2* mRNAs to low-translating fractions (fractions 4–6; Figure 5D, top). In contrast, housekeeping *gapdh* mRNA distributed similarly in both groups (Figure 5D, bottom). We also examined the influence of miR-29b on *cdk2* mRNA stability and found that miR-29b overexpression by transfection with pre–miR-29b did not alter the half-life of *cdk2* mRNA (Supplemental Figure S3).

The third set of experiments was designed to determine whether this inhibitory effect was mediated through the *cdk2* 5′-UTR, coding region (CR), or 3′-UTR. Fractions of the *cdk2* 5′-UTR, CR, and 3′-UTR were subcloned into the pmirGLO dual-luciferase miRNA target expression vector to generate pmirGLO-CDK2-5′UTR, pmirGLO-CDK2-CR, and pmirGLO-CDK2-3′UTR reporter constructs (Figure 5E, schematic). miR-29b overexpression decreased the levels of cdk2-3′UTR luciferase reporter activity but failed to inhibit the activities of cdk2 5′UTR and CR reporters (Figure 5E, bottom), indicating that increasing the levels of miR-29b repressed *cdk2* mRNA translation through interaction with the *cdk2* 3′-UTR rather than with the 5′-UTR or CR. Furthermore, when three nucleotides within the predicted miR-29b–binding site located at the *cdk2* 3′-UTR were point mutated (Figure 5F, schematic), the miR-29b–elicited repression was completely prevented (Figure 5F, bottom). Taken together, these results indicate that miR-29b interacts with the *cdk2* mRNA via its 3′-UTR, thus repressing CDK2 translation.

miR-29b–modulated CDK2 expression plays a role in IEC proliferation

We further studied the biological functions of miR-29b–regulated CDK2 expression and demonstrated that it plays an important role in the regulation of IEC proliferation. As shown in Figure 6, A and B,

decreasing the levels of endogenous CDK2 by miR-29b overexpression resulted in growth arrest in G1 phase in IEC-6 cells. Populations in the S and G2/M phases decreased in pre–miR-29b–transfected cells, along with increases in G1 cells (Figure 6C), similar to growth arrest in CDK2-silenced cells (data not shown). Furthermore, in cells overexpressing miR-29b, transfection with the CDK2 expression vector lacking the *cdk2* 3′-UTR (and hence resistant to miR-29b–elicited repression) not only increased the levels of DDK-tagged CDK2 protein (Figure 6D), but also partially rescued miR-29b–induced growth inhibition (Figure 6E). These findings indicate that miR-29b represses IEC proliferation primarily by inhibiting CDK2 expression.

DISCUSSION

The inhibition of intestinal mucosal growth causes complex pathological processes. The identification of the underlying causes and successful medical treatment remains a major challenge, especially in surgical intensive care patients supported with total parenteral nutrition (Wildhaber *et al.*, 2003; Puleo *et al.*, 2011). Efforts to develop effective therapeutics to maintain mucosal epithelial integrity are extremely important. In the present study, we highlight the novel function of miR-29b as a biological repressor of mucosal growth in the small intestine and further show that miR-29b inhibits mucosal growth by repressing CDK2 expression. These findings advance our understanding of the molecular mechanism underlying gut mucosal epithelial homeostasis and can potentially provide innovative molecular therapy to promote mucosal growth under pathological conditions.

The results reported here indicate that miR-29b inhibits the renewal of cells in the small intestinal epithelium in vivo as well as in culture. In fasted or DFMO-treated mice, miR-29b levels increased in the intestinal mucosa during growth inhibition. Although the miR-29b present in the crypts was undetectable in control mice, its levels increased by fasting and DFMO-induced polyamine depletion (Figure 1D), suggesting that miR-29b is involved in the pathogenesis of mucosal growth inhibition. This notion is strongly supported by our results obtained from studies using the LNA-modified antagomirs targeting miR-29b in vivo. Consistent with studies from others (Esau *et al.*, 2006; Elmen *et al.*, 2008), systemic administration of LNA-mediated antagonism of miR-29b had no acute or subchronic toxicities in mice but almost completely decreased the levels of endogenous miR-29b in the mucosal tissue (Figure 2B). Of importance, LNA-mediated miR-29b silencing stimulated mucosal growth, as indicated by an increase in the lengths of crypts and villi of the small intestine. On the other hand, ectopic overexpression of miR-29b by transfection with the pre–miR-29b inhibited cell proliferation and resulted in G1-phase growth arrest in cultured IECs.

The results presented here also show that the *cdk2* mRNA is a target of miR-29b and that [miR-29b/*cdk2* mRNA] association represses *cdk2* mRNA translation without affecting its stability. Through the use of various ectopic reporters bearing partial transcripts spanning the *cdk2* 5′-UTR, CR, and 3′-UTR with or without the miR-29b–binding site, our results further show that miR-29b interacted with the 3′-UTR of *cdk2* mRNA but not with the 5′-UTR or CR and that the nucleotides spanning positions 1694–1716 of the *cdk2* 3′-UTR was the predominant site through which miR-29b repressed *cdk2* mRNA translation. These findings are consistent with results that miR-29b associates with the mRNAs of *bcr/abl1*, *gata3*, *pdpn*, *col1a1*, *col3a1*, *col5a1*, *eln*, *dnmt3a*, and *dnmt3b*, thus destabilizing mRNAs and/or repressing their translation (Cortez *et al.*, 2010; Roderburg *et al.*, 2010; Qin *et al.*, 2011; Guo *et al.*, 2012; Maegdefessel *et al.*, 2012; Huang *et al.*, 2013; Li *et al.*, 2013; Melo and Kalluri, 2013). miRNAs

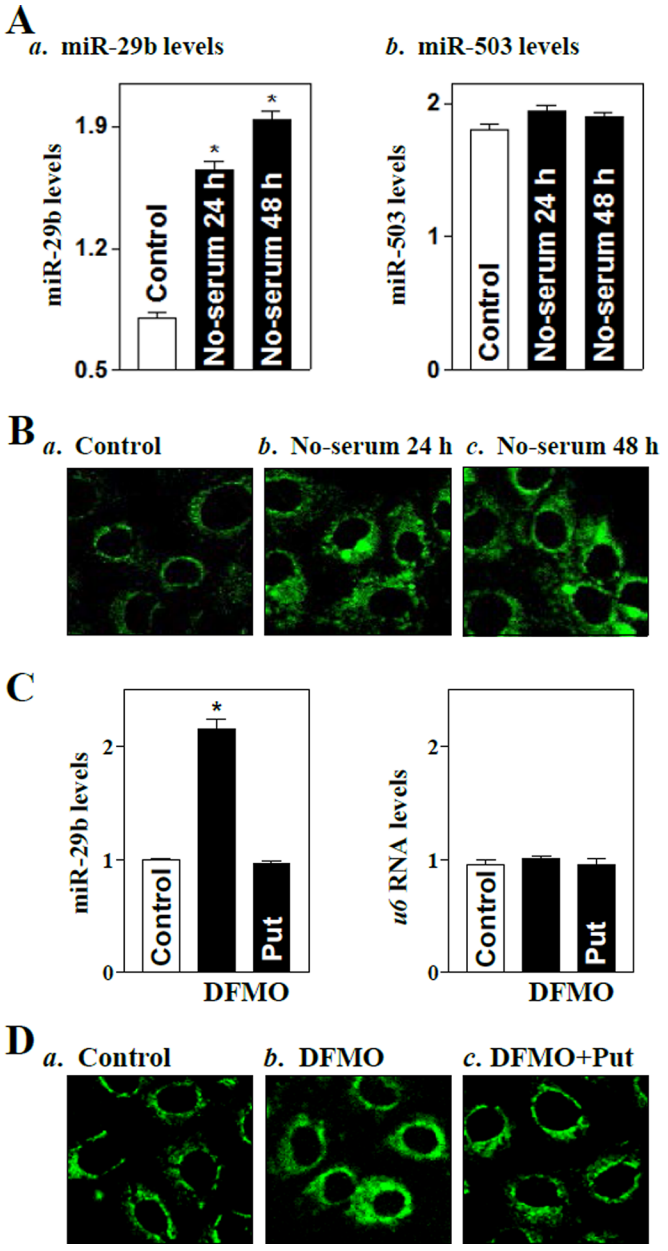


FIGURE 3: Changes in miR-29b in IEC-6 cells after serum deprivation or polyamine depletion. (A) The levels of miR-29 (a) and miR-503 (b) in cells exposed to the serum-free medium for 24 and 48 h. Values are means ± SEM of data from three separate experiments. *$p < 0.05$ compared with controls. (B) Distribution of miR-29b as measured by FISH in cells described in A. (C) The levels of miR-29b in IEC-6 cells exposed to DFMO or DFMO plus putrescine (Put) for 6 d. *$p < 0.05$ compared with controls and cells exposed to DFMO plus Put. (D) Distribution of miR-29b as measured by FISH in cells described in C.

commonly interact with the 3′-UTRs of target transcripts, although in some instances they also associate with the CR or 5′-UTR of target mRNAs for their regulatory actions. In this regard, we reported that miR-503 represses *cugbp1* mRNA translation by interacting with the *cugbp1* CR (Cui *et al.*, 2012) and miR-519 inhibits HuR translation also through association with the *HuR* CR (Abdelmohsen *et al.*, 2008).

The specific molecular mechanisms by which miR-29b association with *cdk2* mRNA represses CDK2 translation is unknown. We

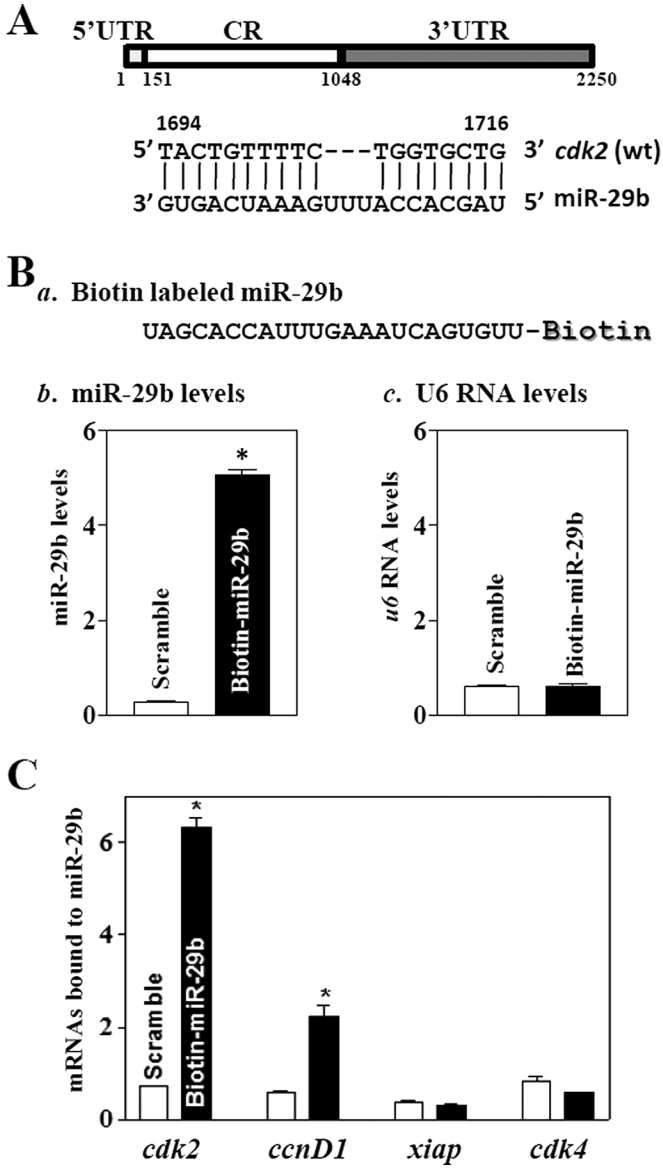


FIGURE 4: miR-29b associates with the *cdk2* mRNA. (A) Schematic representation of *cdk2* mRNA depicting predicted target site for miR-29b in its 3′-UTR. (B) Levels of biotinylated miR-29b were measured by Q-PCR analysis 24 h after transfection. Values are means ± SEM from three separate experiments. *$p < 0.05$ compared with scramble. (C) Binding of biotinylated miR-29b to *cdk2*, *cccnd1*, *xiap*, and *cdk4* mRNAs in the materials pulled down by biotin–miR-29b.

recently demonstrated that miR-222 and miR-503 inhibit CDK4 and CUGBP1 translation in IECs by enhancing the recruitment of *cdk4* and *cugbp1* mRNAs to processing bodies (P-bodies; Xiao *et al.*, 2011; Cui *et al.*, 2012), where mRNAs are sorted for translation repression and/or degradation (Garneau *et al.*, 2007; Mendell and Olson, 2012). Silencing P-body–resident proteins such as Ago2 or RCK prevents miRNA-induced repression of CDK4 and CUGBP1 translation, whereas ectopic overexpression of Ago2 enhances the inhibitory effects of miR-222 and miR-503 (Xiao *et al.*, 2011; Cui *et al.*, 2012). It is unclear, however, whether miR-29b induces subcellular localization of the *cdk2* mRNA to P-bodies in IECs and whether alterations in *cdk2* mRNA recruitment to P-bodies affect its translation rate. This possibility is actively investigated in our ongoing experiments.

The use of systemically administered LNA anti–miRs in exploring miRNA function sheds light on the development of novel therapeutic approaches to enhance intestinal mucosal growth in the clinical setting. Although the complete set of mRNAs controlled by miR-29b in the intestinal epithelium is unknown, the fact that inhibition of a single miRNA (i.e., miR-29b alone) by LNA anti–miRNA oligonucleotide can induce a significant mucosal hyperplasia in vivo highlights the power of individual miRNAs in modulating gut mucosal growth. These findings are particularly significant in the mucosa of the small intestine because it is the most rapidly self-renewing tissue in the body, and inhibition of mucosal growth occurs commonly in various pathological conditions (Puleo *et al.*, 2011; Simons and Clevers, 2011). Unlike most traditional therapeutics, in which drugs have specific cellular targets, the key component of miRNA modulation lies in regulation of entire functional gene networks (Mendell and Olson, 2012). Of course, this is also a weakness of the potential therapeutics, since unintended off-target effects may also occur.

In summary, miR-29b functions as a potent repressor of mucosal growth in the small intestine by targeting CDK2. LNA-mediated miRNA-29b silencing results in mucosal hyperplasia in vivo, whereas ectopic miR-29b overexpression causes IEC growth arrest in G1 phase. Our results further show that miR-29b interacts with and represses *cdk2* mRNA translation. Because CDK2 controls how cells enter into and progress through the cell cycle and is necessary for normal gut epithelial renewal (Smartt *et al.*, 2007), our findings suggest that miR-29b–mediated CDK2 repression contributes to inhibiting mucosal growth under critical pathological conditions, representing a novel therapeutic target for patients with mucosal atrophy.

MATERIALS AND METHODS

Chemicals and cell culture

Tissue culture medium and dialyzed fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA), and biochemicals were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies recognizing CDK2, CDK4, cyclin D1, p21, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences (Sparks, MD), and the secondary antibody conjugated to horseradish peroxidase was obtained from Sigma-Aldrich. LNA anti–miR-29b oligonucleotides that antagonize the intestinal mucosa–expressed miR-29b and control LNA–scrambled oligonucleotides were custom generated by Exiqon (Vedbaek, Denmark). The precursor of miR-29b (pre-miR-29b) was purchased from Applied Biosystems (Foster City, CA), and biotin-labeled miRNA-29b was custom-made by Dharmacon (Lafayette, CO). The IEC-6 cell line was used at passages 15–20 in experiments, and there were no significant changes in biological function or characterization of IEC-6 cells at passages 15–20 (Wang *et al.*, 1996; Liu *et al.*, 2005).

Plasmid construction

The expression vector containing DDK-tagged CDK2 protein was obtained from OriGene (Rockville, MD). The chimeric firefly luciferase reporter construct containing *cdk2* mRNA was described previously (Xiao *et al.*, 2011; Cui *et al.*, 2012). The full-length CDK2 5′-UTR, CR, or 3′-UTR and different 3′-UTR fragments were subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) to generate the pmirGLO-Luc-CDK2-5′UTR, pmirGLO-Luc-CDK2-CR, and pmirGLO-CDK2-3′UTR. Transient transfections were performed using the Lipofectamine reagent as recommended by the manufacturer (Liu *et al.*, 2009; Yu *et al.*, 2011). Luciferase activity was measured using the Dual Luciferase Assay System (Promega), and the levels of firefly luciferase

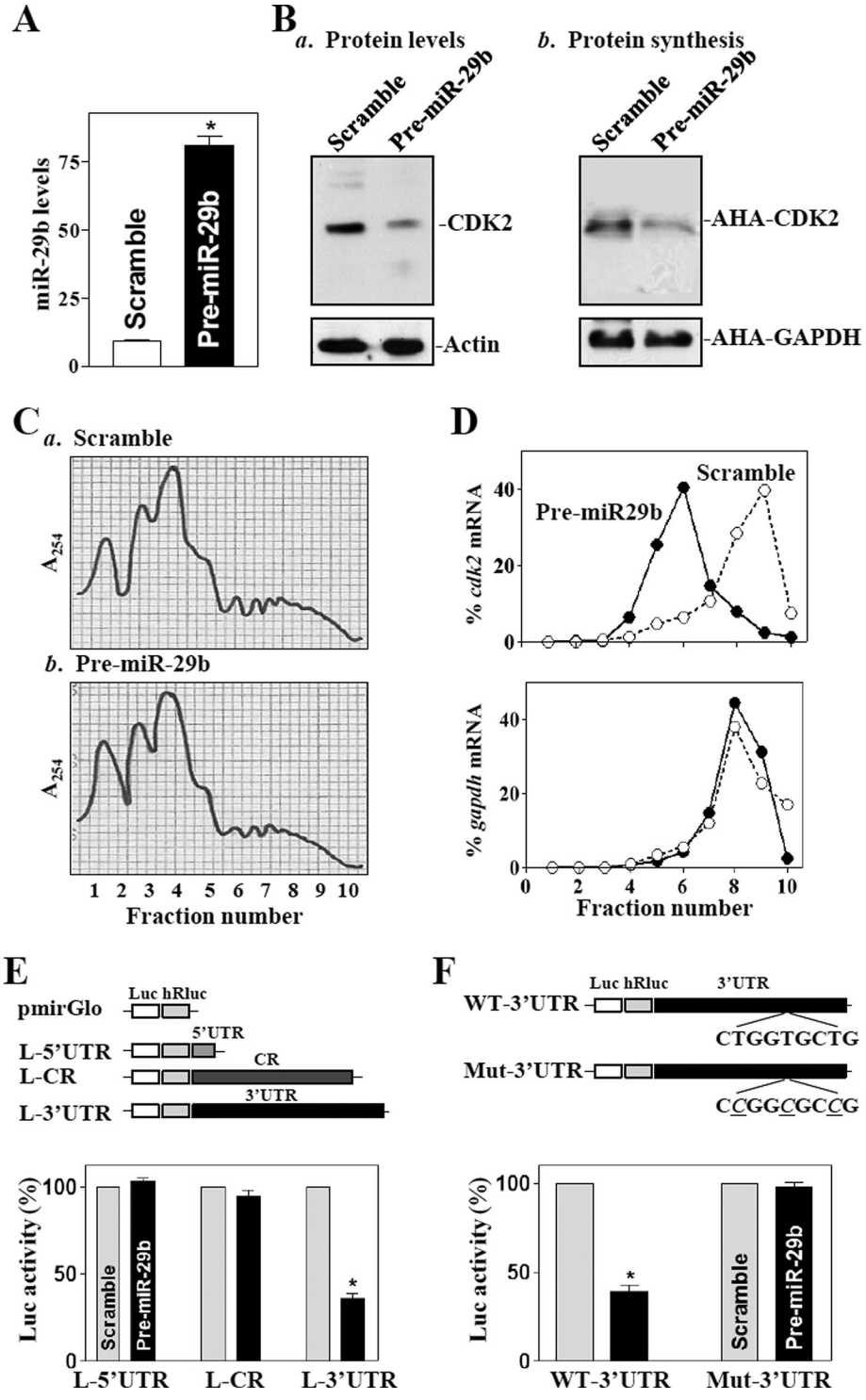


FIGURE 5: miR-29b inhibits CDK2 translation. (A) Levels of miR-29b 48 h after transfection of pre–miR-29b. Values are means ± SEM from three separate experiments. *$p < 0.05$ compared with scramble. (B) The levels of CDK2 protein (a) and its new synthesis (b) after ectopic miR-29b overexpression. After cells were exposed to L-azidohomoalanine (AHA), cell lysates were incubated with the reaction buffer containing biotin/alkyne reagent; the biotin-alkyne/azide–modified protein complex was pulled down by paramagnetic streptavidin-conjugated Dynabeads. (C) Polysomal profiles in cells described in A. Nuclei were pelleted, and the resulting supernatants were fractionated through a 10–50% linear sucrose gradient. (D) Distributions of *cdk2* (top) and *gapdh* (bottom) mRNAs in each gradient fraction prepared from cells described in C. The levels of *cdk2* and *gapdh* mRNAs were plotted as a percentage of the total *cdk2* or *gapdh* mRNA levels in the samples. (E) Levels of reporter activities after ectopic overexpression of miR-29b. Results are expressed as the means ± SEM data from three separate experiments. *$p < 0.05$ compared with cells transfected with scrambled RNA. (F) Effect of deletion of miR-29b–binding site (schematic) on luciferase reporter activity after ectopic miR-29b overexpression.

activity were normalized to *Renilla* luciferase activity. All of the primer sequences for generating these constructs are provided in Supplemental Table S1.

Murine studies

A/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and housed and handled according to guidelines approved by the institutional animal care and use committee. Animals were deprived of food but allowed free access to tap water for 24 or 48 h in the fasting model. In experiments with DFMO, mice first were injected intraperitoneally with 50 mg/100 g body weight, followed by 2% DFMO in their drinking water. In studies of LNA-mediated miR-29b silencing as described previously (Elmen *et al.*, 2008; Allen *et al.*, 2012), mice were injected intraperitoneally with LNA anti–miR-29b (500 μg/100 g body weight/d) for 4 consecutive days, whereas control mice were injected with an equal volume of saline. On day 5, a 4-cm small intestinal segment taken 0.5 cm distal to the ligament of Trietz was removed, and the mucosa was scraped with a glass slide for various measurements.

Fluorescence in situ hybridization

FISH was performed with the LNA microRNA FISH optimization kit from Exiqon as described (Elmen *et al.*, 2008). Briefly, the mucosa was fixed with 4% fresh paraformaldehyde overnight and embedded in paraffin for sections. The slides were deparaffinized and then incubated with proteinase-K. After washes with phosphate-buffered saline (PBS), the slides were dehydrated and hybridized with 50 nM double-digoxigenin (DIG) LNA probe for 1 h at 55°C. The slides were washed with saline–sodium citrate buffers and PBS and then incubated with 2% sheep serum blocking solution. Anti–DIG-fluorescein Fab fragments (Roche, Indianapolis, IN) were applied at 1:800 to the slides for 1 h at room temperature. After extensive rinsing with PBS-Tween, images were processed using an Axio Observer microscope (Carl Zeiss, Jena, Germany).

Reverse transcription and quantitative real-time PCR and miRNA array analysis

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen, Valencia, CA) and used in

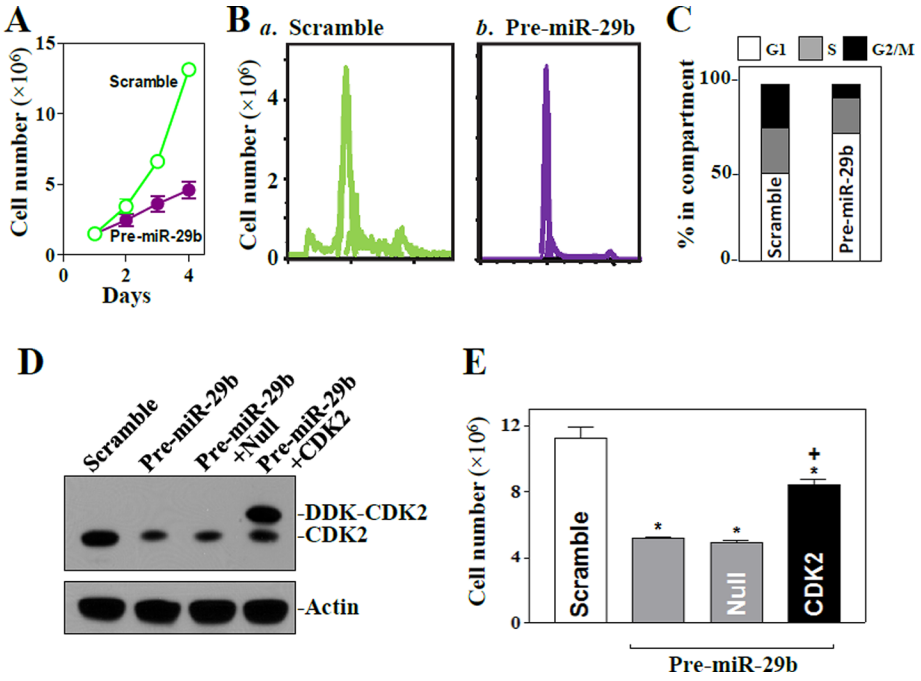


FIGURE 6: Effect of miR-29b–regulated CDK2 expression on cell proliferation. (A) Changes in cell growth after ectopic overexpression of miR-29b. Values are means ± SEM from three separate experiments. *$p < 0.05$ compared with cells transfected with scramble. (B) Flow cytometric analysis of cell cycle distribution after miR-29b overexpression for 72 h. (C) The relative G1, S, and G2/M compartments calculated from data described in B. (D, E) Changes in levels of CDK2 and cell growth after cotransfection the pre–miR-29b and CDK2 expression vector containing DDK-tagged CDK2 (DDK-CDK2). After cells were transfected with either control scramble or premiR-29b for 24 h, they were transfected with the CDK2 expression vector or control vector (Null). The levels of CDK2 protein (D) and cell growth (E) were examined 48 h after CDK2 overexpression. *,+$p < 0.05$ compared with scramble and pre–miR-29b, respectively.

real-time and PCR amplification reactions as described (Xiao *et al.*, 2011). Q-PCR was performed using 7500 Fast Real-Time PCR Systems with specific primers, probes, and software (Applied Biosystems, Foster City, CA).

For miRNA array studies, total RNA was purified with a miRCURY RNA isolation kit, and a miRCURY LNA array of miRNA profiling services was performed by Exiqon. The levels of miRNA-29b were also quantified by Q-PCR by using the TaqMan MicroRNA assay (Invitrogen).

Analysis of newly translated protein and polysome analysis

New synthesis of nascent CDK2 protein was detected by Click-iT protein analysis detection kit (Life Technologies, Grand Island, NY) and performed following the manufacturer's manual. Briefly, cells were incubated in methionine-free medium and then exposed to L-azidohomoalanine. After mixing of cell lysates with the reaction buffer containing biotin/alkyne reagent and $CuSO_4$ for 20 min, the biotin-alkyne/azide–modified protein complex was pulled down using paramagnetic streptavidin-conjugated Dynabeads. The pull-down material was resolved by 10% SDS–PAGE and analyzed by Western immunoblotting analysis using the antibody against CDK2 or glyceraldehyde-3-phosphate dehydrogenase.

Polysome analysis was performed as described (Yu *et al.*, 2013). Briefly, cells at ~70% confluence were incubated for 15 min in 0.1 mg/ml cycloheximide and then lifted by scraping in 1 ml of protein extraction lysis buffer and lysed on ice for 10 min. Nuclei were pelleted, and the resulting supernatant was fractionated through a 10–50% linear sucrose gradient to fractionate cytoplasmic components according to their molecular weights. The eluted fractions were prepared with a fraction collector (Brandel, Gaithersburg, MD), and their quality was monitored at 254 nm using a UV-6 detector (ISCO, Louisville, KY). After RNA in each fraction was extracted, the levels of each individual mRNA were quantified by Q-PCR in each of the fractions.

Biotin-labeled miR-29b pull-down assays

Biotin-labeled miR-29b was transfected into cells, and 24 h later whole-cell lysate was collected as described (Xiao *et al.*, 2011). Cell lysates were mixed with streptavidin-Dynal beads and incubated at 4°C on a rotator overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to reverse transcription, followed by Q-PCR analysis.

Statistics

Values are means ± SEM from three to six samples. Autoradiographic results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined by using Duncan's multiple-range test (Harter, 1960).

ACKNOWLEDGMENTS

This work was supported by Merit Review Grants to J.-Y.W. and J.N.R. from the U.S. Department of Veterans Affairs and by National Institutes of Health Grants DK57819, DK61972, and DK68491 to J.-Y.W. M.G. is supported by the National Institute on Aging–Intramural Research Program, National Institutes of Health. J.-Y.W. is a Senior Research Career Scientist, Medical Research Service, U.S. Department of Veterans Affairs.

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