Published in final edited form as:
Exp Cell Res. 2010 December 10; 316(20): 3512–3521. doi:10.1016/j.yexcr.2010.07.007.

MicroRNA *mir-16* is anti-proliferative in enterocytes and exhibits diurnal rhythmicity in intestinal crypts

Anita Balakrishnan[1,2,3,*], **Adam T Stearns**[1,2,4], **Peter J Park**[5,6], **Jonathan M. Dreyfuss**[5], **Stanley W Ashley**[1,2], **David B Rhoads**[2,7], and **Ali Tavakkolizadeh**[1,2,*]

[1] Department of Surgery, Brigham and Women's Hospital, Boston, Massachusetts, 02115

[2] Department of Surgery, Harvard Medical School, Boston, Massachusetts, 02115

[3] School of Clinical Sciences, Division of Gastroenterology, University of Liverpool, Liverpool, L69 3GE, United Kingdom

[4] Department of Physiology, Anatomy and Genetics, University of Oxford, Oxford OX1 2JD, United Kingdom

[5] Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115

[6] Harvard Medical School Center for Biomedical Informatics, Boston, MA, 02115

[7] Pediatric Endocrine Unit, MassGeneral Hospital for Children Boston, Massachusetts, 02114

Abstract

Background and aims—The intestine exhibits profound diurnal rhythms in function and morphology, in part due to changes in enterocyte proliferation. The regulatory mechanisms behind these rhythms remain largely unknown. We hypothesized that microRNAs are involved in mediating these rhythms, and studied the role of microRNAs specifically in modulating intestinal proliferation.

Methods—Diurnal rhythmicity of microRNAs in rat jejunum was analyzed by microarrays and validated by qPCR. Temporal expression of diurnally rhythmic *mir-16* was further quantified in intestinal crypts, villi, and smooth muscle using laser capture microdissection and qPCR. Morphological changes in rat jejunum were assessed by histology and proliferation by immunostaining for bromodeoxyuridine. In IEC-6 cells stably overexpressing *mir-16*, proliferation

*Corresponding authors: Anita Balakrishnan: Thorn 1503, Brigham & Women's Hospital, 20 Shattuck Street, Boston, MA 02115, United States Tel: +1 617 732 5163 Fax: +1 617 732 8305 anita.balakrishnan@doctors.org.uk. Ali Tavakkolizadeh: Department of Surgery, Brigham & Women's Hospital, 75, Francis Street, Boston, MA 02115, United States, Tel: +1 617 732 5500 Fax: +1 617 732 8305 atavakkoli@partners.org.

Disclosures: All authors confirm no conflicts of interest.

Transcript profiling: The raw microarray data have been submitted to GEO with the accession number: GSE20603 This is a GEO-generate link that the reviewers can use to access the data: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=txgtdamsseqmufo&acc=GSE20603

Author contributions to manuscript: AB, SWA, DBR and AT designed the studies; AB and ATS conducted the research; AB, PJP and JMD analyzed the data; AB, DBR and AT wrote the paper. All authors read and approved the final manuscript.

was assessed by cell counting and MTS assay, cell cycle progression and apoptosis by flow cytometry, and cell cycle gene expression by qPCR and immunoblotting.

Results—*mir-16* peaked 6 hours after light onset (HALO 6) with diurnal changes restricted to crypts. Crypt depth and villus height peaked at HALO 13-14 in antiphase to *mir-16*. Overexpression of *mir-16* in IEC-6 cells suppressed specific G1/S regulators (Cyclins D1-3, Cyclin E1 and Cyclin-dependent kinase 6) and produced G1 arrest. Protein expression of these genes exhibited diurnal rhythmicity in rat jejunum, peaking between HALO 11-17 in antiphase to *mir-16*.

Conclusions—This is the first report of circadian rhythmicity of specific microRNAs in rat jejunum. Our data provide a link between anti-proliferative *mir-16* and the intestinal proliferation rhythm and point to *mir-16* as an important regulator of proliferation in jejunal crypts. This function may be essential to match proliferation and absorptive capacity with nutrient availability.

Keywords

microRNA; diurnal; proliferation; enterocyte

INTRODUCTION

Circadian rhythms (24-h oscillations) play a key role in the regulation of numerous physiological functions. Circadian rhythmicity of up to 10% of gene transcripts and an even greater fraction of proteins indicate the involvement of both transcriptional and translational pathways.[1–5] Regulation at both the transcriptional and post-transcriptional level suggests a role for microRNAs in this process. MicroRNAs are non-coding RNAs able to silence numerous genes simultaneously. Bioinformatics analysis suggests that up to 30% of mammalian gene transcripts are regulated by microRNAs, short non-coding RNAs.[6–9] microRNAs suppress protein expression following recognition of complementary sequences on the 3′UTR (untranslated region) of target genes, either by inducing mRNA cleavage (which manifests as changes in mRNA levels) or inhibiting translation (manifesting as changes in protein levels).[10–12] Presence of the target sequence for each microRNA on multiple genes permits simultaneous regulation of protein expression from numerous genes by a single microRNA.[6,13,14] The postulated role of microRNAs in “fine-tuning” gene expression suggests they also contribute to coordinating the circadian rhythmicity of many genes and proteins.[15–18]

The intestine displays profound rhythmicity of morphology, resulting in peak absorptive function (e.g. for glucose) coinciding with maximal nutrient delivery to the bowel.[19,20] The number of enterocytes per villus also exhibits a diurnal rhythmicity, with an increase about the time of maximal nutrient availability.[21] Similar rhythmicity has been reported in human gastrointestinal mucosa.[22,23] The exact pathways coordinating rhythmicity in proliferation are presently unknown.

We hypothesize that microRNAs are integral components for mediating circadian rhythms in intestinal proliferation, morphology, and function. To investigate this, we profiled microRNAs in the intestine of *ad libitum* fed rats using oligonucleotide arrays. The anti-proliferative microRNA mir-16 was expressed in both crypt and villus enterocytes but exhibited circadian rhythmicity only in the crypts. The cell cycle regulators Ccnd1, Ccnd2, Ccnd3, Ccne1, and Cdk6 also exhibited circadian rhythmicity but in antiphase to *mir-16*. An anti-proliferative role for *mir-16* was supported by its ability to inhibit proliferation and decrease expression of genes involved in cell cycle regulation when over-expressed in rat IEC-6 cells. These studies point to *mir-16* as a potentially important microRNA in regulating circadian rhythms in the intestine.

METHODS

Animal studies

All animal study protocols were prospectively approved by the Harvard Medical Area Standing Committee on Animals.

Sprague-Dawley rats (50 males, 7 weeks old) were purchased from Harlan World (Indianapolis, IN) and acclimatized to a 12:12-h light: dark photoperiod for 5 days with *ad libitum* access to food and water. Time is designated as Hours After Light Onset (HALO), with HALO 0 at 7 am (lights on). Rats were injected with BrdU (5-bromo-2-deoxyuridine, 50mg/kg; Sigma, St Louis, MO) 1 h before harvest to label DNA as an index of S-phase. Rats were killed at 3-h intervals over 24 h (n=6–7 per time) and jejunum harvested for microRNA microarrays, RNA and protein determination, and morphological analysis (harvest protocol detailed in Supplementary Material).

Microarrays and validation by real-time PCR

Total RNA from jejunum was extracted using the mirVana kit (Ambion; Austin, TX) and profiled on in situ hybridization arrays (Exiqon, Woburn, MA) against a reference sample consisting of RNA pooled from HALO 0 rats. Dye swaps were incorporated in the arrays to correct for any dye bias. Data were subjected to Lowess normalization and log transformed.

Expression profiles of selected microRNAs were confirmed by real-time PCR. Specific microRNAs were selected from total extracted RNA by reverse transcription using the stem-loop hybridization based microRNA reverse transcription kit and microRNA-specific primers (Taqman microRNA reverse transcription kit and Taqman microRNA assays, Applied Biosystems, Foster City, CA). microRNA expression was quantified in triplicate using the Taqman microRNA PCR primers and Taqman gene expression mastermix (Applied Biosystems). Reverse transcription and PCR were performed simultaneously on all samples to minimize differences introduced by variable reaction efficiency.

mir-16 overexpression vector

The human *mir-16* gene was amplified from human genomic DNA by PCR and inserted into the MluI/ClaI sites of the tetracycline-inducible TRIPZ shRNAmir expression vector (Open Biosystems, Huntsville, AL) using restriction sites incorporated into the primers (Supplemental Table 1). A non-silencing TRIPZ inducible shRNAmir vector was used as a control (Open Biosystems). Vectors were sequenced to ensure fidelity of the microRNA sequence and insertion. Details of cell transfection are available in Supplementary Material.

Proliferation and cell counting

IEC-6 cells were seeded in 96-well plates at a density of 1000 cells per well in triplicate. Proliferation indices were measured 48 h later using the CellTiter96 Aqueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, WI). Cell growth rates were confirmed by cell counting in trypsinized, 48-h cultures seeded in triplicate at 10^4 cells/ml in 6-well dishes. All experiments were performed thrice.

Cell cycle changes and apoptosis

For cell cycle analysis, trypsinized cells were counted and fixed overnight in 70% ethanol at −20°C. Fixed cells were collected by centrifugation at 1200rpm for 10 min at 4°C, suspended in propidium iodide (BD Biosciences, San Jose, CA) for 30mins at 37°C in darkness, and analyzed by flow cytometry (BD FACScan, BD, Franklin Lakes, NJ). Data were analyzed by ModFit (Verity, Topsham, ME). To determine apoptosis and viability,

trypsinized cells were counted and stained with Annexin V-FITC (BD) and Sytox Blue (Invitrogen), respectively, and analyzed by flow cytometry (10,000 events per sample). Data were analyzed using Diva (BD).

RNA extraction, mRNA reverse transcription and real-time PCR

mRNA levels of *Ccnd1*, *Ccnd2*, *Ccnd3*, *Ccne1*, *Cdk4* and *Cdk6* were quantified by real-time PCR as previously described[24] (detailed in Supplementary Material) and expressed relative to *B*-actin. All genes had Cts within the same range, between Ct 22 and 27. Primers were custom-ordered from Invitrogen (Supplemental Table 2), with the exception of *Ccnd1* mRNA which was measured using the Taqman primer-probe and gene expression Master Mix (Applied Biosystems).

Protein extraction and Western blotting

Protein expression of Ccnd1, Ccnd2, Ccnd3, Ccne1, Cdk4 and Cdk6 was measured in total lysates from jejunal mucosal scrapings or IEC-6 cell lysates as previously described, and detailed in Supplementary Material.[19]

Analysis of morphologic parameters and BrdU labeling

Sections of jejunum were fixed overnight in 10% formalin, then orientated and embedded in paraffin blocks, cut at 7μm thickness, mounted and stained with haematoxylin and eosin. Crypt depth, villus height, villus width, crypt enterocyte width, villus enterocyte width, and number of enterocytes per crypt were measured by a blinded observer under light microscopy (Olympus BX50; Center Valley, PA) at 100x or 400x magnification. Only samples displaying a single layer of enterocytes and villi with a visible central lacteal were included in the analysis (5–10 measurements per rat). For measurement of rhythmicity of proliferation, blocks of jejunum were cut at 7μm and sections incubated with anti-BrdU primary antibody (Sigma), biotinylated secondary antibody, and visualized using the avidin-biotin-peroxidase complex method with diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with haematoxylin and eosin to facilitate counting of BrdU-negative nuclei.

Laser capture microdissection

Sections of jejunum from rats killed at HALO 6 and HALO 18, the respective circadian peak and trough of *mir-16* expression, were embedded in OCT compound over dry ice and isopentane. Sections (9μm) were cut from the fresh frozen specimens and stained with Histogene staining solution (Molecular Devices, Sunnyvale, CA). Crypts (all cells in the lower half), villi (all cells in the top half), or smooth muscle were isolated by laser capture microdissection (Veritas Microdissection System, Molecular Devices). Total RNA was extracted from each section (RNAqueous RNA extraction kit, Ambion, Austin, TX) and subjected to microRNA reverse transcription and real-time PCR as described above for quantification of mir-16 expression in each fraction.

Statistical analysis

Data are presented as means ± SE. Graphical analysis was performed using GraphPad Prism (San Diego, CA). microRNAs exhibiting a 2-fold or greater difference between any two timepoints were selected for further analysis, and a false discovery rate (q-value) of <0.05 was considered significant. Circadian rhythmicity of microRNAs, gene and protein expression and morphological changes in rat tissue was determined by cross-sectional analysis and assuming a 24-h period as described previously, using the Cosinor procedure which is freely available online. [25–27] The acrophase (time of peak expression), mesor (rhythm-adjusted mean), amplitude of rhythmicity, and significance of fit to a 24-h period

(as indicated by $p<0.05$) for each gene was abstracted from the program. ANOVA (analysis of variance) with post-hoc Tukey's multiple comparisons test was used to identify significant differences across the 3 intestinal fractions at each timepoint. T-tests were used to compare the effects of *mir-16* overexpression with control cells in the in vitro experiments.

RESULTS

1. microRNAs exhibit diurnal rhythmicity in rat intestine

Of 238 microRNAs tested on in situ hybridization arrays, 13 microRNAs exhibited ≥ 2-fold difference between peak and trough values (range 2.0- to 3.4-fold; $q<0.05$), 8 of which are conserved among human, mouse and rat and were therefore selected for further evaluation. Real-time PCR (qPCR) confirmed circadian rhythmicity for *mir-16*, *mir-20a* and *mir-141* as determined by the cosinor procedure, with a 24-hour periodicity. Peak expression of these three microRNAs occurred between HALO 4 and 6, corresponding to the lights-on fasting period (Supplementary Table 3, Figure 1A–C). Two of these are reportedly involved in proliferation: *mir-20a* is pro-proliferative and *mir-16* is anti-proliferative.[28–34] Intestinal villus height and cell number have been shown to peak in anticipation of maximal nutrient intake in previous studies.[35] Because anti-proliferative *mir-16* began to wane late in the light phase, when intestinal proliferation has been shown to increase, we selected this microRNA for further study and designed experiments to ascertain its role in the rhythm of intestinal proliferation.

To compare *mir-16* expression levels in crypt, villus and smooth muscle, these cell types were isolated by laser capture microdissection at HALO 6 and 18, the respective *mir-16* peak and nadir. At HALO 18, expression was not significantly different across all three cell types (Fig. 1D; $p=0.97$). However, *mir-16* expression was 3.2-fold higher in crypts at HALO 6 vs. HALO 18 (Fig. 1D; $p=0.003$) while it was not detectably different in villi or smooth muscle. Thus, *mir-16* rhythmicity appears restricted to crypts, the proliferative compartment of the intestinal mucosa.

2. *mir-16* suppresses proliferation in enterocytes by inducing G1 arrest

To determine the effect of *mir-16* on enterocyte proliferation, *mir-16* was overexpressed in rat IEC-6 cells, a cell line derived from intestinal crypts. Stable transfection of IEC-6 cells with the *mir-16* expression vector led to a 2.1-fold increase in *mir-16* expression vs. the control ($p=0.03$, Figure 2A). This modest difference, comparable to the peak/trough difference observed in *mir-16* expression on a diurnal basis, had a profound effect on cell proliferation. At 48 h after plating, the proliferation rate was decreased 76% vs. control cells as measured by the MTS assay (4.2-fold difference; $p=0.006$, Fig 2B) and by 80% as measured by cell counts (5.2-fold difference; $p=0.02$, Fig 2C). Over-expression of *mir-16* also led a significantly larger fraction of cells in G1 compared to control as revealed by flow cytometry (65% vs. 24%, $p<0.001$, Fig 2D). This result indicates that proliferation was curbed by arresting enterocytes in G1 rather than the reported effect of *mir-16* on apoptosis.[31, 36] The lack of increase in apoptosis in IEC-6 cells over-expressing *mir-16* ($p=0.63$, Figure 2E) substantiates this conclusion. These results point to an effect of *mir-16* on the cell cycle in enterocytes, specifically regulators of the G1/S transition.

3. *mir-16* suppresses key G1/S regulators in enterocytes

To identify specific *mir-16* targets involved in reducing proliferation in enterocytes, the microRNA target prediction algorithm Targetscan was interrogated for the presence of *mir-16* binding sequences in the 3′UTRs of G1/S regulatory genes.[37] Potential *mir-16* targets in both rat and human included *Cyclin D1* (*Ccnd1*), *cyclin D2* (*Ccnd2*), *cyclin D3*

(*Ccnd3*), *cyclin E* (*Ccne1*) and *cyclin-dependent kinase 6* (*Cdk6*). These are all known to regulate the G1/S transition and were therefore examined for responsiveness to *mir-16*. *Cyclin-dependent kinase 4 (Cdk4)*, a G1 regulator lacking a *mir-16* target site in its mRNA 3′UTR, was included as a negative control.

Overexpression of *mir-16* significantly decreased protein levels of Ccnd1, Ccnd2, Ccnd3, Ccne1 and Cdk6 in IEC-6 cells compared to the non-silencing control (levels 0.3–0.5 that of controls, $p<0.05$, Table 1, Supplemental Figure 1). *mir-16* appeared to affect translation of *Ccnd1*, *Ccnd3* and *Ccne1* rather than mRNA cleavage because mRNA levels did not change detectably (p=0.660, 0.151 and 0.181 respectively, Table 1). In contrast, reduction of *Ccnd2* and *Cdk6* mRNAs by 75% (p=0.002) and 58% (p=0.001), respectively (Table 1) indicated that *mir-16* overexpression primarily affected transcription and/or mRNA stability of these regulators. Our data point to one or more of these G1/S proteins as *mir-16*-regulated mediators on cell cycle progression. As expected, neither Cdk4 mRNA (p=0.591) or protein (p=0.223) levels were altered detectably by *mir-16* overexpression (Table 1). These results confirm that Cdk4 is not a *mir-16* target and indicate that *mir-16* overexpression does not exert non-specific effects on cell cycle proteins.

4. G1/S regulatory proteins targeted by *mir-16* peak in antiphase to *mir-16* expression in jejunum

Diurnal rhythmicity in intestinal proliferation is likely to be mediated by an underlying diurnal rhythmicity in cell cycle proteins.[21,38] Moreover, involvement of *mir-16* in the jejunal mucosa cell cycle via suppression of these proteins as suggested by the IEC-6 studies would likely be evidenced by a corresponding displacement of their rhythms from *mir-16*. To these ends, we examined the temporal protein expression patterns for the 5 *mir-16* targets as well as Cdk4 in jejunum. All six proteins exhibited diurnal rhythmicity with a 24-hour period, with acrophases (expression peaks) falling between HALO 11 and HALO 17 ($p<0.05$, Figure 3A–E; Table 2, Supplemental Figure 2) and nadirs between HALO 3 and 6. These temporal patterns would be expected for targets suppressed by *mir-16* with its peak expression at HALO 6. *Ccnd2*, *Ccnd3* and *Cdk4* displayed rhythmicity at the transcriptional level (p=0.011, 0.00018, and 0.00015, respectively; Figure 4B–C, Table 2). *Ccnd1* and *Ccne1* mRNAs exhibited temporal changes but these did not qualify as significant circadian rhythms, in keeping with the lack of response at an mRNA level with mir-16 overexpression in vitro. In contrast, *Cdk6* did not display diurnal rhythmicity of transcription *in vivo* (p=0.77, Figure 4F) despite its transcriptional responsiveness to *mir-16* overexpression in IEC-6 cells.

5. Diurnal rhythmicity in DNA synthesis and morphology in rat jejunum

To define the relationship of proliferation to the cyclin expression rhythm, we assessed the temporal patterns of DNA synthesis (S-phase) and crypt-villus morphology. The number of cells in S-phase, as measured by BrdU labeling, peaked at HALO 5 ($p<0.001$, Supplemental Figure 3A). Crypt cell number peaked several hours later at HALO 12 (p=0.001, Supplemental Figure 3B), followed by crypt depth and villus height at HALO 13 and HALO 14, respectively (p=0.005 and 0.043, Supplemental Figure 3C and D). Enterocyte number per 100μm of villus increased modestly in anticipation of nutrient arrival but significant rhythmicity was not achieved (p=0.099, Supplemental Figure 3E). Cell width exhibited circadian rhythmicity in crypts with a peak at HALO 15 (p=0.033, Supplemental Figure 3F) but not in villi (p=0.217). Overall these data demonstrate that a combination of cell proliferation and hypertrophy produced the observed changes in crypt and villus morphology (Table 3).

DISCUSSION

This study is the first to profile microRNA expression in rat jejunum as well as to establish rhythmic expression of specific microRNAs. In particular, our data supports a role for the anti-proliferative microRNA *mir-16* in the intestinal proliferation rhythm. In support of this, we have shown that *mir-16* expression peaks at HALO 6, coincident with the troughs in villus height and in crypt depth and cell number. *mir-16* rhythmicity was also restricted to intestinal crypts, the primary site of proliferation. The anti-proliferative effect of mir16 was confirmed in vitro, where *mir-16* inhibited proliferation of IEC-6 enterocytes, and suppressed expression of 5 key G1/S regulators—Ccnd1, Ccnd2, Ccnd3, Ccne1 and Cdk6. Finally, protein abundances of all five G1/S regulators presumably targeted by *mir-16* as well as the non-target Cdk4 exhibit diurnal rhythmicity in rat jejunum in antiphase to *mir-16*. These coordinated responses point to *mir-16* as an important regulator of proliferation in jejunal crypts. This function may be essential to coordinate intestinal circadian rhythms, serving to optimally match proliferation and absorptive capacity with nutrient availability.

Circadian rhythmicity of microRNA expression has been shown to regulate cell behavior and gene expression. In the suprachiasmatic nucleus, rhythmic expression of *mir-219* and *mir-132* mediate photic entrainment of circadian clock activity.[16] Similarly, depletion of *mir-122* in liver disrupted the circadian rhythmicity of numerous transcripts regulating metabolism.[18,39] In the retina, 12 microRNAs display circadian rhythmicity of which two – *mir-96* and *mir-182* – were shown to mediate rhythmic expression of the *Adcy6* (adenylate cyclase type 6) gene.[17] Here we highlight another potential role for microRNAs as regulators of intestinal circadian rhythms. Interestingly, the 1.8- to 3.2-fold amplitude changes we observed in intestinal microRNAs are consistent with the 1.25- to 3-fold changes observed in the retina.[15–17] Three microRNAs, *mir-16*, *mir-20a* and *mir-141* were shown to exhibit circadian rhythmicity in this study, however the limited amount of tissue obtained from laser capture microdissection restricted us to the examination of only *mir-16* expression at HALO 6 and 18. Further studies are necessary to determine the rhythmicity of the remaining microRNAs in the individual intestinal fractions at circadian timepoints, particularly for mir-20a which is known to have a pro-proliferative function and may therefore contribute to the regulation of rhythmicity of intestinal proliferation.

Several observations from our studies merit further discussion. First, a modest increase of *mir-16* in IEC-6 cells, similar to the diurnal change in jejunum, almost completely arrested growth in these cells. *mir-16* has been suggested to act as a tumour suppressor gene in prostate: *mir16* is frequently downregulated in advanced prostate cancer and *mir16* knockdown in prostate cancer cells promotes proliferation and invasiveness.[40] Similarly, *mir-16* expression is reduced in squamous cell carcinomas and adenocarcinomas of the lung, and *mir-16* overexpression in lung cancer cell lines induces cell cycle arrest.[34] Our findings reveal that the anti-proliferative function of *mir-16* serves an important physiological role in normal tissues. We note that, in contrast to its lack of effect on IEC-6 cell apoptosis, *mir-16* was shown to increase apoptosis in leukaemic cell lines, gastric cancer cells and prostate cancer via downregulation of pro-survival protein BCL2 (B-cell lymphoma 2).[31,36,40,41] This apparent discrepancy in our observations, may in fact be due to different properties of BCL2 pathways in the small intestine; while Bcl2 is expressed in enterocytes, it may perform different functions in this tissue. Indeed, ablation of Bcl2 in mice increases the apoptosis rate in the colon but not the small intestine.[42,43]

Second, in IEC-6 enterocytes *mir-16* suppressed levels of several cell cycle proteins involved in the G1/S transition concomitantly with G1 arrest. In normal cell cycle progression, D-type cyclins (Ccnd1, Ccnd2 and Ccnd3) complex with cyclin-dependent

kinases (Cdk4 and Cdk6) during G1 to phosphorylate and thereby inactivate the retinoblastoma protein pRb, in turn activating cell cycle proteins (including Ccne1 and its complex Cdk2) vital for entering S-phase.[44,45] Upregulation of *mir-16* expression suppressed expression of Ccnd1, Ccnd2, Ccnd3, Ccne1 and Cdk6 *in vitro*, thereby corroborating existing evidence that small changes in microRNA expression alter cellular phenotypes by downregulating multiple components of single pathways.[13,46,47] *In vivo*, we found that G1 proteins Ccnd1 and Ccnd2 peaked at HALO 12, while the remaining D-type cyclin family member Ccnd3 peaked later at HALO 17. These findings are consistent with reported differences in the relative timing of D cyclins in various cell types, as well as differential regulation and a degree of functional redundancy.[48,49] We were unable to definitively corroborate rhythms of mir-16 in the crypt with rhythms of cell cycle proteins in the crypt due to the small amount of tissue obtained from laser capture microdissection, however previous studies have demonstrated that in the intestine the D-type cyclins and cyclin-dependent kinases are most strongly expressed in intestinal crypts.[50] Our study showed peak S-phase at HALO 5, indicating a G1/S duration of approximately 12 to 17 hours, in agreement with previous studies showing a long G1/S and short G2/M period in the small intestine.[51–53] The 63% change in cell labeling we observed at HALO 6 vs. HALO 15 is also similar to the 30–60% increase at HALO 3 in murine jejunum reported by Scheving et al.[38,54] The rhythmicity in proliferation translated to rhythmicity in morphological parameters in the jejunum. The large number of crypts and villi across the length of the intestine suggests that these small changes are likely to result in a large change in absorptive surface area over the diurnal period. Examination of these morphological parameters in the terminal ileum and corroboration of these measurements with mir-16 expression in the ileum may reveal new insights into the regulation of mir-16.

Our data show that mir-16 is able to affect translation of Ccnd1, Ccnd3 and Ccne1 without affecting mRNA expression, corroborating previous data showing microRNAs are able to suppress protein levels independent of mRNA expression.[46] This was also demonstrated by our data in vivo; Ccnd1 and Ccne1 showed rhythmicity only at the protein level. This is in keeping with previous data showing that almost half of the proteins demonstrating circadian rhythmicity in the mouse liver lack a corresponding cycling transcript.[5] Together with our findings this suggests the possibility that the rhythmic protein expression in jejunum in our study may be produced solely by miRNAs, whether by *mir-16* alone or in combination with others. Cell-type specificity of *mir-16* rhythmicity, such as seen in the intestinal crypts in our study, would then lead to consequent rhythmicity of target proteins. Cell cycle proteins are known to have a relatively short half-life[55], which is likely to facilitate regulation of these proteins by rhythmicity in microRNA expression and allow increased responsiveness to other stimuli that may accelerate or arrest the cell cycle.

Regulation of gene expression by microRNAs is a complex process, with the potential for each to target many related or unrelated genes and for responsive genes to be regulated by multiple microRNAs. In the case of the cell cycle, microRNAs *let-7a*, *mir-34a*, *mir-192* and *mir-215* have been shown, like *mir-16*, to arrest cells in G1, while *mir-106b* and *mir-221* accelerate G1/S progression by suppressing the cyclin-dependent kinase inhibitors p21 and p27, respectively.[56–61] Factors other than microRNAs are also clearly important in cuing the intestinal proliferation rhythm. For instance, clock gene Period 2 (Per2) regulates proliferation in peripheral tissues via cell cycle genes c-*Myc*, *Cyclin A*, *Mdm-2* and *Gadd45α*, as well as the *mir-16* target *Ccnd1*.[62] Ultimately, proliferation rhythms likely result from combined inputs of circadian clock components, other transcription factors and rhythmic microRNAs. The ability of non-microRNA transcriptional regulators such as clock genes to regulate rhythmicity of proliferation may explain rhythmicity in Cdk4, a cell cycle gene not regulated by *mir-16*, and the lack of transcriptional rhythmicity in *Cdk6* in vivo despite responsiveness to *mir-16* overexpression in vitro. Generation of knockout mice

lacking *mir-16* will be invaluable in defining its functions and dissecting these regulatory pathways.

Finally, a broader implication can be drawn from our study. The behavior of *mir-16* reveals another potential route for linking proliferation to nutrient availability, which cues the intestinal rhythms. Rhythmic *mir-16* expression in crypt cells could be initiated by luminal nutrients directly or via neuro-hormonal pathways. In either case, proliferation may be a key early component to expand the mucosal surface area in the anticipatory diurnal increases in absorptive capacities for glucose, peptides, and other nutrients.[19,63]

In summary, we show for the first time rhythmicity of microRNA expression in the intestine, and anti-proliferative effects of the diurnally expressed *mir-16* in untransformed enterocytes *in vitro*. We hypothesize that rhythmicity of *mir-16* in jejunum may act to mediate the rhythmicity in intestinal proliferation and coordinate the proliferative response with nutrient availability to optimize intestinal absorption and function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge the excellent technical assistance of Jan Rounds and Roger Lis in the experimental procedures.

Grant support: NIH - 5 R01 DK047326 (SWA), ADA - 7-05-RA-121 (DBR), Harvard Clinical Nutrition Research Center - P30-DK040561 (AT), Nutricia Research Foundation (AB) and Berkeley Fellowship (ATS).

Abbreviations

Adcy6	adenylate cyclase type 6
BCL2	B-cell lymphoma 2
BrdU	5-bromo-2-deoxyuridine
Ccnd1	cyclin D1
Ccnd2	cyclin D2
Ccnd3	cyclin D3
Ccne1	cyclin E1
Cdk4	cyclin-dependent kinase 4
Cdk6	cyclin-dependent kinase 6
HALO	hours after light onset
3′UTR	3′ untranslated region

References

1. McDonald MJ, Rosbash M. Microarray analysis and organization of circadian gene expression in Drosophila. Cell. 2001; 107:567–578. [PubMed: 11733057]
2. Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol. 2002; 12:540–550. [PubMed: 11937022]

3. Claridge-Chang A, Wijnen H, Naef F, Boothroyd C, Rajewsky N, Young MW. Circadian regulation of gene expression systems in the Drosophila head. Neuron. 2001; 32:657–671. [PubMed: 11719206]
4. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell. 2002; 109:307–320. [PubMed: 12015981]
5. Reddy AB, Karp NA, Maywood ES, Sage EA, Deery M, O'Neill JS, Wong GK, Chesham J, Odell M, Lilley KS, Kyriacou CP, Hastings MH. Circadian orchestration of the hepatic proteome. Curr Biol. 2006; 16:1107–1115. [PubMed: 16753565]
6. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005; 120:15–20. [PubMed: 15652477]
7. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. 2001; 294:853–858. [PubMed: 11679670]
8. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science. 2001; 294:858–862. [PubMed: 11679671]
9. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science. 2001; 294:862–864. [PubMed: 11679672]
10. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993; 75:843–854. [PubMed: 8252621]
11. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell. 1993; 75:855–862. [PubMed: 8252622]
12. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005; 433:769–773. [PubMed: 15685193]
13. Farh KK, Grimson A, Jan C, Lewis BP, Johnston WK, Lim LP, Burge CB, Bartel DP. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. Science. 2005; 310:1817–1821. [PubMed: 16308420]
14. Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. Cell. 2005; 123:1133–1146. [PubMed: 16337999]
15. Yang M, Lee JE, Padgett RW, Edery I. Circadian regulation of a limited set of conserved microRNAs in Drosophila. BMC genomics. 2008; 9:83. [PubMed: 18284684]
16. Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, Nakazawa T, Shimizu K, Okamura H, Impey S, Obrietan K. microRNA modulation of circadian-clock period and entrainment. Neuron. 2007; 54:813–829. [PubMed: 17553428]
17. Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. J Biol Chem. 2007; 282:25053–25066. [PubMed: 17597072]
18. Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F, Ruskeepaa AL, Oresic M, Esau CC, Zdobnov EM, Schibler U. Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev. 2009; 23:1313–1326. [PubMed: 19487572]
19. Balakrishnan A, Stearns AT, Rounds J, Irani J, Giuffrida M, Rhoads DB, Ashley SW, Tavakkolizadeh A. Diurnal rhythmicity in glucose uptake is mediated by temporal periodicity in the expression of the sodium-glucose cotransporter (SGLT1). Surgery. 2008; 143:813–818. [PubMed: 18549898]
20. Tavakkolizadeh A, Berger UV, Shen KR, Levitsky LL, Zinner MJ, Hediger MA, Ashley SW, Whang EE, Rhoads DB. Diurnal rhythmicity in intestinal SGLT-1 function, V(max), and mRNA expression topography. Am J Physiol Gastrointest Liver Physiol. 2001; 280:G209–215. [PubMed: 11208542]
21. Scheving LE, Burns ER, Pauly JE, Tsai TH. Circadian variation in cell division of the mouse alimentary tract, bone marrow and corneal epithelium. The Anatomical record. 1978; 191:479–486. [PubMed: 697058]

22. Marra G, Anti M, Percesepe A, Armelao F, Ficarelli R, Coco C, Rinelli A, Vecchio FM, D'Arcangelo E. Circadian variations of epithelial cell proliferation in human rectal crypts. Gastroenterology. 1994; 106:982–987. [PubMed: 8144003]
23. Buchi KN, Moore JG, Hrushesky WJ, Sothern RB, Rubin NH. Circadian rhythm of cellular proliferation in the human rectal mucosa. Gastroenterology. 1991; 101:410–415. [PubMed: 2065918]
24. Balakrishnan A, Stearns AT, Rhoads DB, Ashley SW, Tavakkolizadeh A. Defining the transcriptional regulation of the intestinal sodium-glucose cotransporter using RNA-interference mediated gene silencing. Surgery. 2008; 144:168–173. [PubMed: 18656622]
25. Stearns AT, Balakrishnan A, Rhoads DB, Ashley SW, Tavakkolizadeh A. Diurnal rhythmicity in the transcription of jejunal drug transporters. J Pharmacol Sci. 2008; 108:144–148. [PubMed: 18787306]
26. Nelson W, Tong YL, Lee JK, Halberg F. Methods for cosinor-rhythmometry. Chronobiologia. 1979; 6:305–323. [PubMed: 548245]
27. http://www.circadian.org.
28. Xiao C, Srinivasan L, Calado DP, Patterson HC, Zhang B, Wang J, Henderson JM, Kutok JL, Rajewsky K. Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. Nature immunology. 2008; 9:405–414. [PubMed: 18327259]
29. Ventura A, Young AG, Winslow MM, Lintault L, Meissner A, Erkeland SJ, Newman J, Bronson RT, Crowley D, Stone JR, Jaenisch R, Sharp PA, Jacks T. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell. 2008; 132:875–886. [PubMed: 18329372]
30. Inomata M, Tagawa H, Guo YM, Kameoka Y, Takahashi N, Sawada K. MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell lymphoma subtypes. Blood. 2009; 113:396–402. [PubMed: 18941111]
31. Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:13944–13949. [PubMed: 16166262]
32. Linsley PS, Schelter J, Burchard J, Kibukawa M, Martin MM, Bartz SR, Johnson JM, Cummins JM, Raymond CK, Dai H, Chau N, Cleary M, Jackson AL, Carleton M, Lim L. Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. Molecular and cellular biology. 2007; 27:2240–2252. [PubMed: 17242205]
33. Liu Q, Fu H, Sun F, Zhang H, Tie Y, Zhu J, Xing R, Sun Z, Zheng X. miR-16 family induces cell cycle arrest by regulating multiple cell cycle genes. Nucleic acids research. 2008; 36:5391–5404. [PubMed: 18701644]
34. Bandi N, Zbinden S, Gugger M, Arnold M, Kocher V, Hasan L, Kappeler A, Brunner T, Vassella E. miR-15a and miR-16 are implicated in cell cycle regulation in a Rb-dependent manner and are frequently deleted or down-regulated in non-small cell lung cancer. Cancer research. 2009; 69:5553–5559. [PubMed: 19549910]
35. Stevenson NR, Day SE, Sitren H. Circadian rhythmicity in rat intestinal villus length and cell number. International journal of chronobiology. 1979; 6:1–12. [PubMed: 437929]
36. Xia L, Zhang D, Du R, Pan Y, Zhao L, Sun S, Hong L, Liu J, Fan D. miR-15b and miR-16 modulate multidrug resistance by targeting BCL2 in human gastric cancer cells. International journal of cancer. 2008; 123:372–379.
37. http://www.targetscan.org.
38. Scheving LE, Tsai TH, Scheving LA. Chronobiology of the intestinal tract of the mouse. The American journal of anatomy. 1983; 168:433–465. [PubMed: 6320626]
39. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005; 438:685–689. [PubMed: 16258535]
40. Bonci D, Coppola V, Musumeci M, Addario A, Giuffrida R, Memeo L, D'Urso L, Pagliuca A, Biffoni M, Labbaye C, Bartucci M, Muto G, Peschle C, De Maria R. The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nature medicine. 2008; 14:1271–1277.

41. Guo CJ, Pan Q, Li DG, Sun H, Liu BW. miR-15b and miR-16 are implicated in activation of the rat hepatic stellate cell: An essential role for apoptosis. Journal of hepatology. 2009; 50:766–778. [PubMed: 19232449]
42. Nakayama K, Nakayama K, Negishi I, Kuida K, Sawa H, Loh DY. Targeted disruption of Bcl-2 alpha beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91:3700–3704. [PubMed: 8170972]
43. Nakayama K, Nakayama K, Negishi I, Kuida K, Shinkai Y, Louie MC, Fields LE, Lucas PJ, Stewart V, Alt FW, et al. Disappearance of the lymphoid system in Bcl-2 homozygous mutant chimeric mice. Science. 1993; 261:1584–1588. [PubMed: 8372353]
44. Deshpande A, Sicinski P, Hinds PW. Cyclins and cdks in development and cancer: a perspective. Oncogene. 2005; 24:2909–2915. [PubMed: 15838524]
45. Murray AW. Recycling the cell cycle: cyclins revisited. Cell. 2004; 116:221–234. [PubMed: 14744433]
46. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature. 2008; 455:64–71. [PubMed: 18668037]
47. Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, Kosaka N, Kawamata M, Kelnar K, Bader AG, Brown D, Ochiya T. Systemic Delivery of Synthetic MicroRNA-16 Inhibits the Growth of Metastatic Prostate Tumors via Downregulation of Multiple Cell-cycle Genes. Mol Ther. 2009
48. Ko TC, Sheng HM, Reisman D, Thompson EA, Beauchamp RD. Transforming growth factor-beta 1 inhibits cyclin D1 expression in intestinal epithelial cells. Oncogene. 1995; 10:177–184. [PubMed: 7824270]
49. Ko TC, Pan F, Sheng H, Brown DB, Thompson EA, Beauchamp RD. Cyclin D3 is essential for intestinal epithelial cell proliferation. World journal of surgery. 2002; 26:812–818. [PubMed: 11948368]
50. Guo J, Longshore S, Nair R, Warner BW. Retinoblastoma protein (pRb), but not p107 or p130, is required for maintenance of enterocyte quiescence and differentiation in small intestine. J Biol Chem. 2009; 284:134–140. [PubMed: 18981186]
51. Lesher S. Compensatory reactions in intestinal crypt cells after 300 Roentgens of cobalt-60 gamma irradiation. Radiat Res. 1967; 32:510–519. [PubMed: 6065246]
52. Cohen AM, Philips FS, Sternberg SS. Studies on the cytotoxicity of bleomycin in the small intestine of the mouse. Cancer Res. 1972; 32:1293–1300. [PubMed: 4113145]
53. DuBois RN, Shao J, Tsujii M, Sheng H, Beauchamp RD. G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. Cancer research. 1996; 56:733–737. [PubMed: 8631005]
54. Scheving LE, Tsai TH, Scheving LA, Feuers RJ. The potential of using the natural rhythmicity of cell proliferation in improving cancer chemotherapy in rodents. Annals of the New York Academy of Sciences. 1991; 618:182–227. [PubMed: 2006787]
55. Guo Y, Stacey DW, Hitomi M. Post-transcriptional regulation of cyclin D1 expression during G2 phase. Oncogene. 2002; 21:7545–7556. [PubMed: 12386817]
56. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D, Slack FJ. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer research. 2007; 67:7713–7722. [PubMed: 17699775]
57. Tazawa H, Tsuchiya N, Izumiya M, Nakagama H. Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104:15472–15477. [PubMed: 17875987]
58. Braun CJ, Zhang X, Savelyeva I, Wolff S, Moll UM, Schepeler T, Orntoft TF, Andersen CL, Dobbelstein M. p53-Responsive micrornas 192 and 215 are capable of inducing cell cycle arrest. Cancer research. 2008; 68:10094–10104. [PubMed: 19074875]
59. Georges SA, Biery MC, Kim SY, Schelter JM, Guo J, Chang AN, Jackson AL, Carleton MO, Linsley PS, Cleary MA, Chau BN. Coordinated regulation of cell cycle transcripts by p53-

Inducible microRNAs, miR-192 and miR-215. Cancer research. 2008; 68:10105–10112. [PubMed: 19074876]

60. Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, Schelter JM, Kobayashi SV, Lim L, Burchard J, Jackson AL, Linsley PS, Cleary MA. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol. 2008; 28:2167–2174. [PubMed: 18212054]
61. Gillies JK, Lorimer IA. Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. Cell cycle (Georgetown, Tex). 2007; 6:2005–2009.
62. Lee CC. Tumor suppression by the mammalian Period genes. Cancer Causes Control. 2006; 17:525–530. [PubMed: 16596306]
63. Pan X, Terada T, Irie M, Saito H, Inui K. Diurnal rhythm of H+-peptide cotransporter in rat small intestine. Am J Physiol Gastrointest Liver Physiol. 2002; 283:G57–64. [PubMed: 12065292]

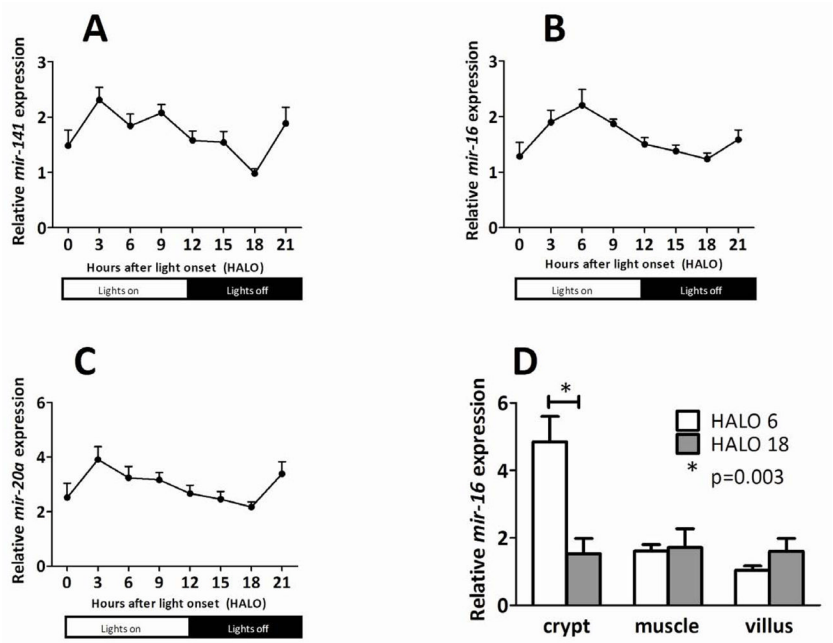

Figure 1.

Temporal pattern of microRNAs showing a 2-fold or greater change between any two timepoints (Fig 1A–C). Total RNA was extracted from the intestinal mucosa of rats harvested at the indicated times, run on microRNA microarrays and microRNAs showing a 2-fold or greater change between any two timepoints validated by real-time PCR. *mir-16* expression in enterocyte fractions (Fig 1D). Laser capture microdissection and real-time PCR were used to determine *mir-16* expression in fractions of crypt, villus and smooth muscle in cryofixed sections of jejunum.

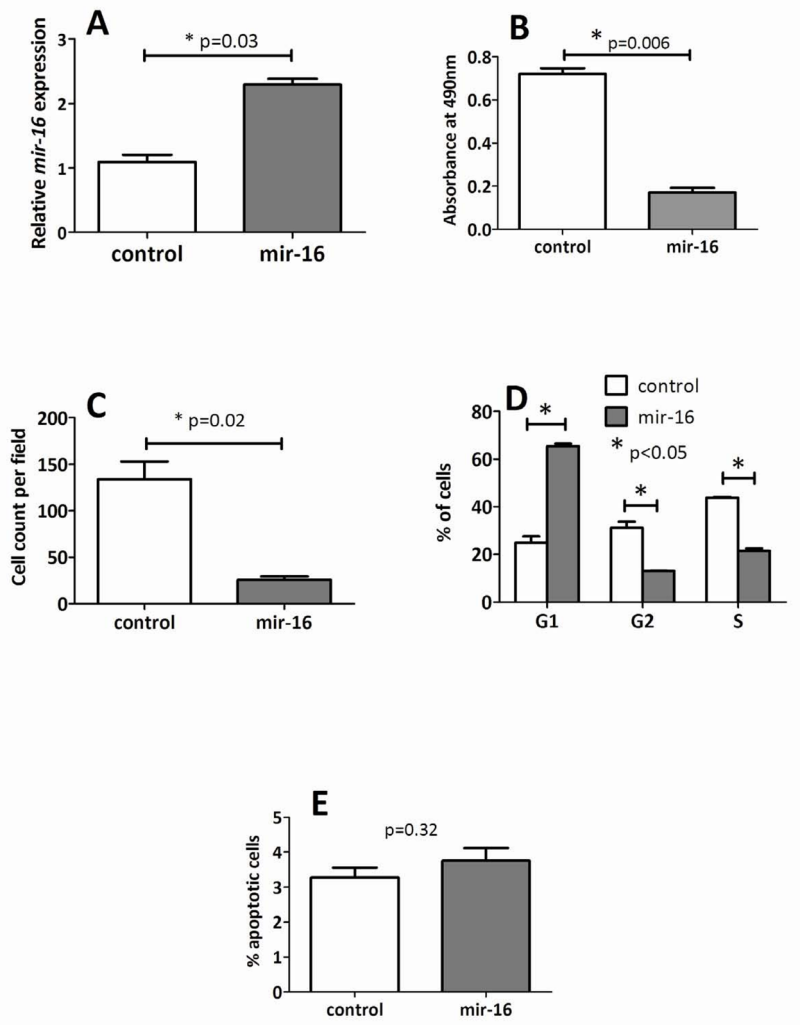

Figure 2.

Effects of *mir-16* on enterocyte phenotype *in vitro*. *mir-16* (or a scrambled control) was stably overexpressed in IEC-6 cells as described in Materials and Methods. All assays were performed 48 hours after plating. *mir-16* expression was quantified by real-time PCR following RNA extraction and reverse transcription (Fig 2A). Proliferation and cell viability were determined using the MTS assay and cell counting respectively (Fig 2B and C). For analysis of cell cycle, cells were fixed in 70% ethanol and stained with propidium iodide and subjected to flow cytometry as described in Materials and Methods (Fig 2D). For determination of apoptosis, cells were stained with Annexin V-FITC and Sytox Blue and analyzed by flow cytometry (Fig 2E).

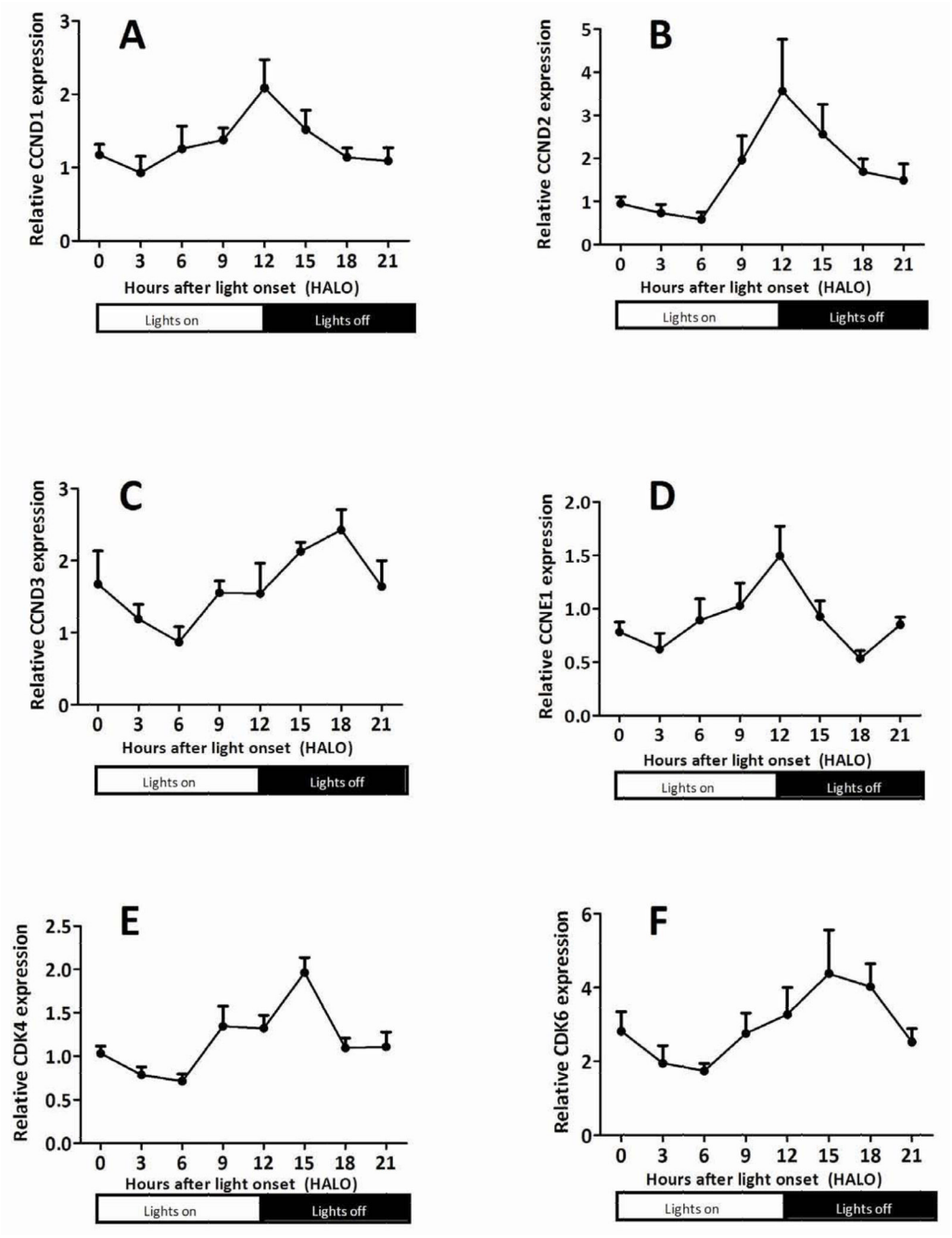

Figure 3.

Temporal pattern of cell cycle protein expression in rat intestine. Whole-cell protein lysates of rat intestinal mucosa, harvested at the indicated times, were analyzed by immunoblotting. Diurnal amplitudes and p-values are summarised in Table 2.

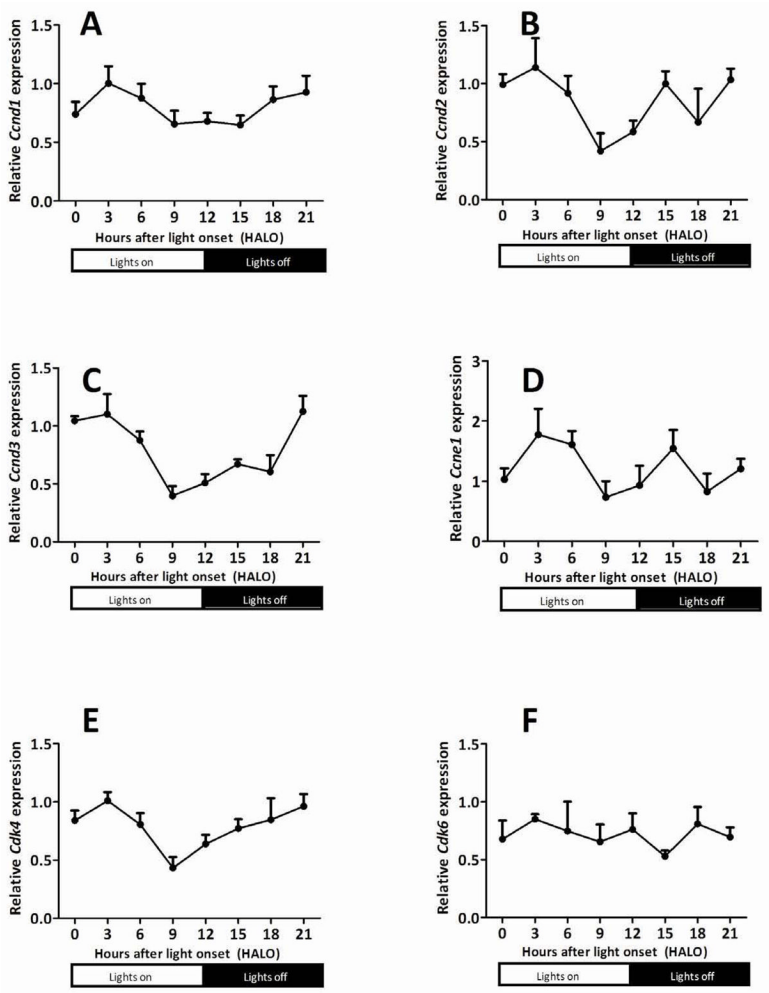

Figure 4.

Temporal pattern of mRNA levels for cell cycle genes in rat intestine. Total RNA was extracted from rat intestinal mucosa, harvested at the indicated times, were analyzed by real-time PCR. Diurnal amplitudes and p-values are summarised in Table 2.

Table 1

Expression of *mir-16* target protein and mRNA following overexpression of *mir-16* in IEC-6 cells. Levels have been normalized to that in control cells. Our data demonstrate regulation of targets *Ccnd2* and *Cdk6* at the transcriptional level *in vitro*, and *Ccnd1*, *Ccnd2* and *Ccne1* at the post-transcriptional level. *Cdk4* is not a predicted target of *mir-16* and correspondingly did not demonstrate alteration in expression following *mir-16* overexpression. All predicted *mir-16* targets were suppressed at the protein level.

Protein expression of G1/S regulators			mRNA expression of G1/S regulators		
Target	**Fold-change vs control**	**p-value**	**Target**	**Fold-change vs control**	**p-value**
Ccnd1	0.4	0.030	*Ccnd1*	0.9	0.660
Ccnd2	0.4	0.037	*Ccnd2*	0.2	0.002
Ccnd3	0.3	0.030	*Ccnd3*	1.2	0.151
Ccne1	0.5	0.030	*Ccne1*	0.8	0.181
Cdk6	0.4	0.039	*Cdk6*	0.4	0.001
Cdk4	1.2	0.223	*Cdk4*	1.0	0.591

Table 2

Expression of cell cycle G1/S regulator protein and mRNA expression at diurnal timepoints in rat jejunum. The cosinor procedure was used to determine the fit of the data to a cosinor curve with 24-hour periodicity. Our data demonstrate circadian rhythmicity of *Ccnd2*, *Ccnd3* and *Cdk4* at the transcriptional level in vivo, and Ccnd1, Ccne1 and Cdk6 at the post-transcriptional level.

Rhythmicity of protein expression				Rhythmicity of mRNA expression			
Target	**p value**	**Acrophase (HALO, h)**	**Fold-change peak/trough**	**Target**	**p value**	**Acrophase (HALO, h)**	**Fold-change peak/trough**
Ccnd1	0.009	12	2.3	*Ccnd1*	0.120		
Ccnd2	0.001	14	4.4	*Ccnd2*	0.011	0	2.7
Ccnd3	0.004	17	2.8	*Ccnd3*	0.000	0	2.8
Ccne1	0.013	11	2.8	*Ccne1*	0.330		
Cdk4	0.000	15	2.8	*Cdk4*	0.005	2	2.3
Cdk6	0.004	16	2.5	*Cdk6*	0.770		

Table 3

Diurnal rhythmicity in morphological parameters and BrDU labeling in rat jejunum. The cosinor procedure was used to determine the fit of the data to a cosinor curve with 24-hour periodicity. BrdU labeling, indicative of cell cycle S-phase, peaks at HALO 5, followed by peaks in crypt depth and villus height after 8 to 9 hours. Rhythmicity in numbers of enterocytes per crypt suggests that increases in cell numbers contributes to this rhythmicity in crypt villus morphology.

Parameter	p value	Acrophase (HALO, h)	Fold change (peak/trough)
BrdU	0.005	5	1.6
Crypt depth	0.005	13	1.3
Villus height	0.043	14	1.1
Enterocytes per crypt	0.001	12	1.2
Villus width	0.610		
Crypt width	0.080		