Modulation by miR-29b of intestinal epithelium homoeostasis through the repression of menin translation

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Menin regulates distinct cellular functions by regulating gene transcription through its interaction with partner transcription factors, but the exact mechanisms that control menin levels remain largely unknown. In the present study we report that Men1 mRNA, encoding menin, is a novel target of miR-29b and that miR-29b and miR-29b/Men1 mRNA association regulates menin expression post-transcriptionally in rat intestinal epithelial cells (IECs). Overexpression of a miR-29b precursor lowered the levels of Men1 mRNA modestly, but reduced new synthesis of menin robustly; conversely, antagonism of miR-29b enhanced menin protein synthesis and steady-state levels. The repressive effect of miR-29b on menin expression was mediated through a single binding site in the coding region of Men1 mRNA, because point mutation of this site prevented miR-29b-induced repression of menin translation. Increasing cellular polyamines due to overexpression of ornithine decarboxylase (ODC) enhanced menin translation by reducing miR-29b, whereas polyamine depletion by inhibiting ODC increased it, thus suppressing menin expression. Moreover, an increase in menin abundance in an miR-29b-silenced population of IECs led to increased sensitivity to apoptosis, which was prevented by silencing menin. These findings indicate that miR-29b represses translation of Men1 mRNA, in turn affecting intestinal epithelial homoeostasis by altering IEC apoptosis.

Key words: intestinal epithelial cell, intestinal epithelium homoeostasis, menin, microRNA, post-transcriptional regulation.

INTRODUCTION

Menin, the product of the MEN1 gene in humans, is a scaffold protein that participates in many aspects of cellular functions through control of gene expression and cell signalling [1,2]. The MEN1 gene is mutated in patients with multiple endocrine neoplasia type 1 (MEN1) syndrome [3], and homozygous loss of Men1 in mice leads to embryonic lethality, with defects in multiple organs [4]. Menin is ubiquitously expressed in various tissues, but its function is cell-type and tissue-specific, sometimes playing opposing roles in different organs, e.g. it acts as a tumour suppressor in endocrine organs, yet it is necessary for leukaemic transformation [5,6]. Menin probably possesses these dichotomous functions because it regulates gene expression in opposite directions through association with a multitude of binding partner proteins that have diverse functions [1,7]. As a repressor, menin interacts with the AP-1 transcription factor JunD and inhibits its transcriptional activity [8], whereas it can also function as an activator through its interaction with the trithorax group proteins (Drosophila sp.) and the mixed-lineage leukaemia (human) histone methyltransferase complex, thus enhancing histone tail modification and the transcription of target genes [6,9]. Although the exact function of menin in the gut mucosa is essentially unknown, it has been reported that MEN1 gene mutation is responsible for duodenal gastrinomas [10,11] and that menin overexpression prevents JunD-mediated activation of gastrin gene expression [12]. The gut hormone somatostatin stimulates menin expression in human AGS adenocarcinoma and mouse STC neuroendocrine cells [13].

In response to stressful environments, intestinal epithelial cells (IECs) elicit rapid changes in gene expression patterns to regulate their survival, adapt to stress and maintain epithelial homoeostasis [14]. In addition to the stimulus-altered gene transcription, changes in post-transcriptional regulation also potently affect the steady-state levels of many transcripts and the levels of the encoded proteins [14,15]. Post-transcriptional processes, in particular altered mRNA stability and translation, are primarily controlled by the interaction of specific mRNA sequences (cis elements) with specific trans-acting factors such as RNA-binding proteins (RBPs) and miRNAs [15–18]. The miRNAs are a class of small non-coding RNAs that post-transcriptionally repress the expression of target genes and regulate a variety of cellular processes [16,17,19]. Generally, miRNAs act by binding to the 3′-UTRs of target mRNAs, destabilizing them and/or inhibiting their translation [19–21]. High-throughput and functional studies show that miRNAs play important roles in many aspects of cellular physiology and pathological processes, such as inflammation and tumorigenesis [19,20]. Recently, miRNAs have also emerged as master regulators of gut epithelial homoeostasis [14,22,23], and several intestinal epithelial tissue-specific miRNAs, including
miR-222 [22], miR-322/503 [24,25], miR-211/155 [26], miR-195 [21] and miR-122b [27], have been shown to modulate IEC proliferation, apoptosis and cell-to-cell interaction.

Among many species, miR-29b is highly conserved and it targets several mRNAs encoding the proteins implicated in many cellular functions [14,28]. It regulates DNA methylation-related reprogramming events by targeting dnm3a and dnm3b [29], and promotes fibrosis by altering expression of collagen isoforms [30,31]. In addition, miR-29b modulates cell proliferation and apoptosis in different cell types [14,32] and plays a role in the development of abdominal aortic aneurysms in the mouse [33]. The abnormal expression of miR-29b is associated with tumorigenesis and cancer progression [34], and miR-29b is shown to alter the tumour microenvironment to repress metastasis [35]. Our recent study showed that mucosal atrophy in the small intestine, induced by fasting or polyamine depletion, is associated with increased expression of miR-29b, whereas miR-29b silencing in mice stimulates mucosal growth in the small intestine [36]. Our efforts to identify miR-29b target mRNAs implicated in these processes revealed that miR-29b interacted with the 3′-UTR of the mRNA encoding cyclin-dependent kinase 2 (CDK2) and repressed Cdk2 mRNA translation [14,36]. In the present study, we report that miR-29b interacts with Men1 mRNA via its coding region (CR) and represses menin translation in normal IECs. Interestingly, cellular polyamines, the physiological regulators of gut mucosal growth [37–39], increase menin levels in IECs by reducing miR-29b. Moreover, the miR-29b-mediated reduction in menin abundance altered the sensitivity of IECs to apoptosis, thus contributing to the maintenance of intestinal epithelium homeostasis.

EXPERIMENTAL

Chemicals and cell culture

Tissue culture medium and dialysed FBS were from Invitrogen and biochemicals from Sigma. The antibodies recognizing menin, p53, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were from Cell Signaling and Santa Cruz Biotechnology. The secondary antibody conjugated to horseradish peroxidase was from Jackson Immunoresearch. The antibodies recognizing p53, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were from Cell Signaling and Santa Cruz Biotechnology. The secondary antibody conjugated to horseradish peroxidase was from Jackson Immunoresearch. The antibodies recognizing menin or GAPDH; after incubation with secondary antibodies, the blots were incubated with primary antibodies recognizing menin or GAPDH; after incubation with secondary antibodies, immunocomplexes were developed using chemiluminescence.

RT and quantitative real-time PCR analyses

Total RNA was isolated using RNeasy Mini Kit (Qiagen), and used in reverse transcription (RT) and PCR amplification reactions as described [41]. The levels of Gapdh PCR product were assessed to monitor the evenness in RNA input in RT–PCR samples. Quantitative real-time PCR (qPCR) analysis was performed using 7500-Fast Real-Time PCR Systems with specific primers, probes and software (Applied Biosystems). For miRNA studies, the levels of miR-29b were also quantified by qPCR using a TaqMan MicroRNA assay (Applied Biosystems); levels of small nuclear RNA (snRNA) U6 were measured as an endogenous control.

Western blotting analysis

Whole-cell lysates were prepared using 2% SDS, sonicated and centrifuged (10000 g) at 4°C for 15 min. The supernatants were boiled for 5 min and size fractionated by SDS/PAGE (10% acrylamide). After transferring proteins on to nitrocellulose filters, the blots were incubated with primary antibodies recognizing menin or GAPDH; after incubation with secondary antibodies, immunocomplexes were developed using chemiluminescence.

Analysis of newly translated protein

Nascent menin was detected by the Click-IT® protein analysis detection kit (Life Technologies) and performed following the manufacturer’s manual with minor modification [36]. Briefly, cells were incubated in methionine-free medium and then exposed to L-azidohomoalaine (AHA). Cell lysates were mixed with reaction buffer containing biotin/alkyne reagent and CuSO4, for 20 min, and the biotin/alkyne–azide-modified protein complex was pulled down using paramagnetic streptavidin-conjugated Dynabeads (Invitrogen). The pull-down material was resolved using 10% SDS/PAGE and analysed by Western immunoblotting analysis using antibodies against menin or GAPDH.

Polysome analysis was performed as described [41]. Briefly, cells at approximately 70% confluence were incubated for 15 min in 0.1 mg/ml of cycloheximide (CHX), lifted by scraping in 1 ml of a polysome extraction 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 masses. The eluted fractions were prepared with a fraction collector (Brandel), and their quality was monitored at 254 nm using a UV-6 detector (ISCO). After the RNA in each fraction had been extracted, the level of each individual mRNA was quantified by qPCR in each of the fractions.

Plasmid construction

The chimaeric firefly luciferase reporter construct of the Men1 CR, 5′-UTR or 3′-UTR was generated as described [36]. The full-length Men1 CR, 5′-UTR or 3′-UTR was amplified and subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) to generate the pmirGLO-Luc-menin-CR, pmirGLO-Luc-menin-5′-UTR and pmirGLO-Luc-menin-3′-UTR; cloning was confirmed by DNA sequencing and enzyme digestion. Transient transfections were performed using the Lipofectamine reagent (Invitrogen) as recommended by the manufacturer. The luciferase reporter constructs were transfected into cells along with phRL-null, a Renilla luciferase control reporter vector from Promega, to monitor transfection efficiencies as described previously [38]. Luciferase activity was measured using the Dual-Luciferase Assay System (Promega), and the level of pmirGLO-Luc-menin-CR, menin-5′- or menin-3′-UTR luciferase activity was normalized to Renilla luciferase activity, and further compared with the levels of luciferase mRNA in every experiment. All the primer sequences for generating these constructs are provided in Supplementary Table S1. The vector expressing GFP-tagged menin protein was from OriGene.
Biotin-labelled miR-29b pull-down assays

Binding of miR-29b to target mRNAs was examined by biotin-labelled miR-29b as described [24,36]. Briefly, biotin-labelled miR-29b was transfected into cells and 24 h later whole-cell lysates were collected, mixed with streptavidin–Dynabeads, and incubated at 4 °C with rotation overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to RT–PCR followed by qPCR analysis. Input RNA was extracted and served as a control.

Determination of apoptosis

Apoptosis was induced by treatment with tumour necrosis factor-α (TNF-α) in combination with CHX as described previously [24]. After various experimental treatments, cells were photographed with a Nikon inverted microscope before fixation. Annexin-V staining of apoptosis was carried out using a commercial apoptosis kit (Clontech Laboratories) and performed according to the manufacturer’s protocol. Briefly, cells were rinsed with 1× binding buffer, and resuspended in 200 μl of 1× binding buffer. Annexin-V (5 μl) was added on the slide and then incubated at room temperature for 10 min in the dark. Annexin-V-stained cells were visualized and photographed under a fluorescence microscope using a dual filter set for FITC and rhodamine, and the percentage of apoptotic cells was determined.

Statistics

Values are the means ± S.E.M. from three to six samples. Immunoblotting results were repeated three times. The significance of the difference between the means was determined using ANOVA. The level of significance was determined using Duncan’s multiple-range test [42].

RESULTS

Men1 mRNA is a novel target of miR-29b in IECs

The rat Men1 mRNA is a potential target for miR-29b, because there is a predicted binding site for miR-29b within the CR of the Men1 mRNA (Figure 1A), using standard online software (RNA22). Sequences that match miR-29b seed regions are not found in 5′-UTR or 3′-UTR of rat Men1. To determine the role of miR-29b in the regulation of menin expression, we examined the association of miR-29b with the Men1 mRNA by RNA pull-down assays using biotin-labelled miR-29b (Dharmacon; see Figure 1B, a). At 24 h after transfection, miR-29b levels increased significantly, but levels of the housekeeping non-coding RNA U6 did not (Figure 1B, b and c). The Men1 mRNA was enriched in the materials pulled down by biotin-miR-29b but not in materials from cells transfected with control scrambled RNA (Figure 1C). The enrichment of the cdk2 PCR product was also examined and served as a positive control, because the cdk2 mRNA is a known target for miR-29b [36]. The association of miR-29b with Men1 mRNA was specific, because increasing the levels of biotin-miR-29b did not increase its interaction with myc mRNA. These results strongly suggest that Men1 mRNA is a novel target of miR-29b in IECs.

Interaction of miR-29b with Men1 mRNA represses menin translation

To examine the functional consequences of the miR-29b/Men1 mRNA association, we first investigated whether increasing the levels of miR-29b through transfection with its precursor (pre-miR-29b) repressed menin expression. As shown in Figure 2A, a, levels of miR-29b increased by transfection with pre-miR-29b decreased menin protein levels (Figure 2A, b). To determine whether miR-29b inhibited menin expression by repressing its translation, we examined the changes in menin protein synthesis after ectopic overexpression of miR-29b; we found that the levels of newly synthesized menin protein decreased significantly in cells transfected with pre-miR-29b compared with cells transfected with the scrambled oligomer (Figure 2A, c). Inhibition of menin protein synthesis by miR-29b induction was specific, because there was no change in nascent GAPDH synthesis after transfection with pre-miR-29b. To further define the roles of miR-29b in the regulation of Men1 mRNA translation, we examined the relative distribution of Men1 mRNA in individual fractions from polyribosome gradients, after ectopic overexpression of pre-miR-29b as reported previously [22,36]. In the present study, mRNAs in fractions 1–3 were considered to be untranslated, because they were not associated with components of the translation machinery nor did they co-sediment with ribosome subunits (monosomes); transcripts in fractions 4–6 were bound to single ribosomes or formed polysomes of low molecular mass and were considered to be translated at low-to-moderate levels.
Figure 2  Ectopic overexpression of miR-29b inhibits menin translation

(A) Levels of miR-29b (a), total menin protein (b) and new synthesis of menin protein (c) 48 h after transfection of pre-miR-29b (50 nM). Whole-cell lysates were prepared for Western blotting; equal loading was monitored by assessing β-actin levels. For measuring new synthesis of menin protein, cells were exposed to AHA, and then 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. Values are means ± S.E.M. from three separate experiments. *P < 0.05 compared with scramble. (B) Distributions of Men1 (left) and Gapdh (right) mRNAs in each gradient fraction of the polysomal profile after ectopic miR-29b overexpression. After fractionation through sucrose gradients, total RNA was isolated from different fractions; the levels of Men1 and Gapdh mRNAs were measured by qPCR analysis and plotted as a percentage of the total Men1 mRNA and Gapdh mRNA levels in the samples. (C) Levels of reporter activities after ectopic overexpression of miR-29b. Top: schematic diagram of plasmids of different chimaeric firefly luciferase menin reporters. Bottom: levels of activity of luciferase reporters containing the menin 5′-UTR, CR or 3′-UTR. At 24 h after transfection with pre-miR-29b, cells were transfected with different menin luciferase reporter plasmids. Levels of firefly and Renilla luciferase activities were assayed 24 later. Results were normalized to the Renilla luciferase activities and expressed as the means ± S.E.M. of data from three separate experiments. *P < 0.05 compared with cells transfected with scrambled RNA. (D) Effect of point mutation of specific miR-29b-binding site (schematic diagram) in Men1 CR on luciferase reporter activities after ectopic overexpression of miR-29b. *P < 0.05 compared with cells transfected with control scrambled oligomer.

Fractions 7–10 comprised the mRNAs that were associated with polysomes of high molecular mass and were thus considered to be actively translated. Although increasing the levels of miR-29b did not affect global polysomal profiles as described in our previous studies [36], the association of Men1 mRNA with actively translating fractions (fractions 7–9) decreased dramatically, shifting to low-translating fractions (fractions 4–5; Figure 2B, left). In contrast, housekeeping Gapdh mRNA distributed similarly in both groups (Figure 2B, right).

Secondly, we determined whether this inhibitory effect was mediated through the Men1 CR, 3′-UTR or 5′-UTR. Fractions of the Men1 5′-UTR, CR or 3′-UTR were sub-cloned into the pmirGLO dual-luciferase miRNA target expression vector to generate pmirGLO-menin-5′-UTR, pmirGLO-menin-CR and pmirGLO-menin-3′-UTR reporter constructs (Figure 2C, top). Overexpression of miR-29b by transfection with pre-miR-29b selectively decreased the levels of menin-CR luciferase reporter activity (Figure 2C, bottom), but failed to inhibit the activity of menin-5′-UTR or menin-3′-UTR reporter activity, indicating that miR-29b represses Men1 mRNA translation through interaction with the Men1 CR rather than its 5′- or 3′-UTR. To further characterize the specific binding site of miR-29b in the Men1 CR, point mutation of the predicted site located at the Men1 CR was performed, in which two nucleotides were mutated (Figure 2D, top). Interestingly, menin repression by miR-29b was completely prevented when this specific binding site was mutated from the Men1 CR. Taken together, these results indicate that miR-29b interacts with Men1 mRNA predominantly via the specific binding site at the CR and represses Men1 mRNA translation.

Third, we examined the influence of decreasing the level of miR-29b by transfecting the corresponding antisense oligomer (antagomir) targeting miR-29b (anti-miR-29b) on menin expression. Transfection with anti-miR-29b decreased the levels of miR-29b by approximately 80% (Figure 3A) but it increased...
the level of menin protein (Figure 3B). The results presented in Figure 3C show further that silencing miR-29b induced the synthesis of new menin protein. These results indicate that decreasing the levels of miR-29b enhances menin translation by reducing formation of the miR-29b/Men1 mRNA complex. In addition, we examined the effect of miR-29b on Men1 mRNA levels and found that overexpression of miR-29b also decreased Men1 mRNA (by about 35%), but it did not affect the stability of Men1 mRNA (see Supplementary Figure S1). Further studies showed that miR-29b overexpression specifically increased cellular levels of the transcription factor p53 without effect on other transcription factors, including ATF2, c-Myc and JunD (see Supplementary Figure S2). Moreover, ectopic overexpression of the p53 gene repressed Men1 gene transcription as indicated by decreases in Men1 promoter luciferase reporter gene activity and Men1 mRNA (see Supplementary Figure S3), suggesting that miR-29b also indirectly represses MEN1 gene transcription through a process involving p53.

Polyamines regulate menin expression by altering miR-29b levels

The natural polyamines spermidine, spermine and their precursor putrescine are organic cations found in all eukaryotic cells and are implicated in many aspects of cellular physiology, including the regulation of mRNA biogenesis [43,44]. To test the possibility that polyamines regulate menin expression through miR-29b, we first determined the effect of increasing the levels of cellular polyamines on miR-29b and menin expression. Two clonal populations of IECs stably expressing ODC, a key enzyme for polyamine biosynthesis, were used in the present study. As reported previously [39,40], ODC-IEC cells exhibited very high levels of ODC protein and more than a 50-fold increase in ODC enzyme activity. Consistently, the levels of putrescine, spermidine and spermine in ODC-IEC cells were increased by approximately 12-fold, 2-fold and 25%, respectively, when compared with cells transfected with the control vector lacking ODC cDNA as reported previously [39]. The results in Figure 4(A) show that increasing the levels of cellular polyamines due to ectopic overexpression of the ODC gene decreased the levels of miR-29b, but it increased miR-503 as reported previously (results not shown) and failed to alter the levels of U6 RNA (see Supplementary Figure S4). The decreased levels of miR-29b were associated with an induction of menin translation, as shown by an increase in the levels of menin protein (Figure 4B, a), and activity of the menin-CR luciferase reporter gene (Figure 4B, b) in ODC-IEC cells compared with those observed in cells infected with the control vector. Restoration of miR-29b expression in ODC-IEC cells by transfection with pre-miR-29b (Figure 4C) prevented increased menin expression (Figure 4D). The stimulatory effects of ODC overexpression on menin expression were not simply due to clonal variation, because two different clonal populations, ODC-IEC-C1 and ODC-IEC-C2, showed similar responses. These results indicate that activation of menin expression by increasing cellular polyamines results primarily from a reduction in miR-29b content.

We determined whether polyamine depletion repressed menin expression by increasing miR-29b. As reported in our previous studies [38,40], inhibition of ODC activity by treatment with α-difluoromethylomithine (DFMO) for 4 days almost totally depleted polyamines in IEC-6 cells. Putrescine and spermidine were undetectable by 4 days of continuous treatment with DFMO, and spermine had decreased by ∼ 60% (results not shown). As shown in Figure 5A, polyamine depletion by DFMO increased the levels of miR-29b, although it reduced miR-503 levels (results not shown). Supplementation with putrescine reversed the DFMO-triggered changes in the levels of miR-29b, as did spermidine supplementation (results not shown). The induction of miR-29b in polyamine-deficient cells was associated with a decrease in the levels of menin protein (Figure 4B, a) and activity of the menin-CR luciferase reporter gene (Figure 4B, b), which was completely prevented by addition of exogenous putrescine. These results show that polyamine depletion inhibits menin expression by inducing miR-29b abundance.

Induction of menin by miR-29b silencing enhances the susceptibility of IECs to apoptosis

To investigate the biological significance of miR-29b-modulated menin expression, we examined the role of increased levels of menin by miR-29b silencing in the control of intestinal epithelial homeostasis via measurement of changes in apoptosis. Increased levels of endogenous menin resulting from anti-miR-29b transfection (see Figure 3) did not directly induce apoptosis (Figure 6A, a and b, left). There were no apparent differences in cell viability between miR-29b-antagonized populations and control cells, including no morphological features of apoptosis and no detectable levels of active caspase-3, regardless of whether or not treated with the miR-29b antagonist. To determine whether increased menin via antagonism of miR-29b altered the susceptibility of IECs to apoptosis, cells were exposed to treatment with TNF-α/CHX. When control cells were exposed to TNF-α/CHX for 4 h, we observed morphological features characteristic of programmed cell death, elevated annexin-V staining, a classic indicator of apoptotic cells (Figures 6A, a, right, and 6B, left), and increased levels of active caspase-3 (Figure 6C). Increasing menin by miR-29b silencing increased the susceptibility of IECs to TNF-α/CHX-induced apoptosis, as indicated by increases in the percentages of apoptotic cells (Figures 6A, a and 6B) and in the levels of active caspase-3 protein (Figure 6C). This stimulatory effect remained intact when cells were transfected with control siRNA (Figure 6A, c), but it was lost when menin expression was silenced by siRNA targeting Men1 mRNA (siMen). The percentages of apoptotic cells (Figures 6A, a and 6B, right) and the levels of active caspase-3 protein (Figure 6C, right) in miR-322-silenced cells transfected with siMen were lower than those seen in miR-29b-antagonized cells transfected with control siRNA after exposure to TNF-α/CHX. In contrast, decreased levels of endogenous menin by miR-29b overexpression through transfection with pre-miR-29b protected IECs against TNF-α/CHX-induced apoptosis; this protective effect was significantly decreased by ectopic overexpression of the MEN1 gene in cells overexpressing...
mRNA (Figure 7). These results indicate that menin is a pro-apoptotic factor in IECs and that increased levels of miR-29b protect cells against apoptosis by decreasing menin.

**DISCUSSION**

Menin interacts with its various partner proteins and is implicated in the regulation of gene transcription and cell signalling, thereby impacting on distinct cellular functions [1,2,7], but the exact mechanisms that control menin abundance are not well characterized. Recently, several studies revealed that miR-24 and miR-421 down-regulate menin expression in the endocrine pancreas [45], neuroblastoma [46] and parathyroid adenomatous tissue [47], although there are no studies available showing the role of miRNAs in regulating menin expression in normal IECs. In the present study, we identify miR-29b as a translational repressor of menin in IECs and provide insight into the control of menin expression at the post-transcriptional level in the intestinal epithelium. Interestingly, miR-29b-mediated repression of Men1 mRNA translation is highly regulated by polyamines, and increasing the levels of cellular polyamines stimulates menin expression by reducing miR-29b. The results of the present study also indicate that control of Men1 mRNA translation by miR-29b is relevant for maintaining intestinal epithelial homeostasis via alteration of the susceptibility of IECs to apoptosis. These findings advance our knowledge of the molecular mechanisms underlying the regulation of menin expression in IECs, and indicate that association of miR-29b with Men1 mRNA controls its translation and therefore modulates menin-dependent signalling pathways and cellular functions.

Unlike the more frequent finding that miRNAs exert their regulatory actions through interactions with the 3′-UTRs of target transcripts [17,19], the miR-29b-binding site in the Men1 CR appeared to be the predominant site through which miR-29b repressed menin translation. The results of the present study show further that the 1983–2005 site of the Men1 CR was primarily used by miR-29b for its inhibitory effect, because menin repression by miR-29b was completely prevented when this specific binding site was mutated from the Men1 CR. Although the CR is not the typical region of miRNA action, there are earlier examples of CR hosting miRNAs, e.g. miR-519 was shown to repress translation...
Figure 6  Increasing endogenous menin by miR-29b silencing enhances apoptotic cell death

(A) TNF-α/CHX-induced apoptosis after various treatments; 48 h after cells were transfected with anti-miR-29b alone or co-transfected with anti-miR-29b and siRNA targeting Men1 mRNA (siMen), apoptosis was measured following 4 h of treatment with TNF-α/CHX. (a) Cells transfected with control oligomer; (b) cells transfected with anti-miR-29b; (c) cells transfected with anti-miR-29b and control siRNA (C-siRNA); and (d) cells transfected with anti-miR-29b and siMen. Original magnification ×150. (B) Percentages of apoptotic cells after different treatments as described in (A). Values are means ± S.E.M. from three experiments. *P < 0.05 compared with untreated cells; +P < 0.05 compared with control cells exposed to TNF-α/CHX; #P < 0.05 compared with miR-29b-silenced cells exposed to TNF-α/CHX. (C) Levels of caspase-3 and menin in cells described in (A). Whole-cell lysates were harvested, and the levels of procaspase-3, caspase-3 and menin were assessed by Western blot analysis. β-Actin immunoblotting was performed as an internal control for equal loading.

Figure 7  Decreasing menin by miR-29b overexpression protects cells against apoptosis

(A) TNF-α/CHX-induced apoptosis after various treatments. Cells were transfected with pre-miR-29b or scramble; 48 h after transfection, apoptosis was measured following 4 h of treatment with TNF-α/CHX. (a) cells transfected with scramble; (b) cells transfected with pre-miR-29b alone; (c) cells co-transfected with pre-miR-29b and control vector; and (d) cells co-transfected with pre-miR-29b and menin expression vector (MEN1 O/E). Original magnification ×150. (B) Percentages of apoptotic cells after different treatments as described in (A). Values are means ± S.E.M. from three experiments. *P < 0.05 compared with untreated cells; +P < 0.05 compared with control cells exposed to TNF-α/CHX; #P < 0.05 compared with cells co-transfected with pre-miR-29b and control vector, and then exposed to TNF-α/CHX. (C) Changes in levels of caspase-3 and menin in cells described in (A).
of the RBP HuR by directly interacting with the HuR CR [44] and miR-503-induced repression of CUGBP1 translation is also mediated through CUGBP1 CR, rather than the respective 3′-UTRs [25]. By contrast, miR-24 and miR-412 interact with and repress menin expression by associating with the Men1 3′-UTR [45–47].

The results of the present study also show that polyamines regulate menin translation by altering the level of miR-29b. Polyamines have been recognized for many years as key molecules that control multiple signalling pathways and have distinct cellular functions [37,43]. The levels of cellular polyamines are highly regulated and depend on the dynamic balance of polyamine biosynthesis, degradation and transport. We have recently demonstrated that decreasing cellular polyamines increased miR-29b, whereas miR-29b expression level decreased in cells containing high levels of polyamines [22,36]. Although the exact mechanism by which polyamines regulate miR-29b biogenesis remains unknown, our current studies show that decreased levels of miR-29b, by increasing cellular polyamines due to ODC gene overexpression, were associated with an increase in menin levels, which was prevented by ectopic overexpression of miR-29b. In contrast, DFCO-mediated polyamine depletion increased miR-29b levels, thus suppressing menin translation. Polyamines were previously shown to influence multiple distinct signalling pathways leading to alterations in gene transcription [39,48], post-transcriptional events [40,49], protein phosphorylation [38,50,51], and protein trafficking including the nuclear import of transcription factors such as nuclear factor κB (NF-κB) [52], Smads [53] and JunD [49,54]. Polyamines also modulate the cytoplasmic abundance of RBPs, such as HuR and CUGBP1, that are intimately involved in post-transcriptional events [22,40]. It is unknown at the present time whether polyamine-altered cytoplasmic abundances of HuR and CUGBP1 also play a role in miR-29b-induced repression of menin translation.

The data obtained in the present study also indicate that the miR-29b-modulated menin translation is of biological significance, playing a role in maintaining homeostasis of the intestinal epithelium by regulating IEC apoptosis. The epithelium of the intestinal mucosa is a rapidly self-renewing tissue and its homeostasis is preserved through strict regulation of cell proliferation, migration, differentiation and apoptosis [14,37]. IECs continuously replicate within the intestinal crypts, and this process is counterbalanced by apoptosis that occurs in the crypt area, where it maintains a critical balance in cell numbers between newly divided and surviving cells, and at the luminal surface of the intestinal mucosa, where differentiated cells are lost. Increasing endogenous menin by silencing miR-29b heightened the sensitivity of IECs to TNF-α/CHX-induced apoptosis, and this effect was abolished by silencing menin. In contrast, decreasing menin by miR-29b overexpression protected IECs against apoptosis, which was almost completely prevented by ectopic overexpression of the Men1 gene. Our previous studies and studies from other laboratories [55] have demonstrated that NF-κB [52], Akt kinase [50], ATF-2 [56], XIAP [57], MEK1 [58], CUGBP1 [25] and Smurf2 [24] are involved in the control of IEC apoptosis, and these signals are highly regulated at multiple levels by numerous intracellular and extracellular factors including RBPs and miRNAs. These studies provide additional evidence that the miR-29b-modulated menin is a new member in this family of regulators of intestinal epithelial homeostasis. In summary, our results indicate that miR-29b suppresses Men1 mRNA translation through interaction with the Men1 CR rather than its 5′-UTR and 3′-UTR, and that polyamines increase menin expression by reducing miR-29b levels. As miR-29b-regulated menin translation alters the susceptibility of IECs to apoptosis, our findings suggest that control of cellular menin abundance by miR-29b is crucial for maintaining intestinal epithelial homeostasis in physiological and pathological conditions.

AUTHOR CONTRIBUTION
Miao Ouyang and Weijie Su contributed equally to this work.

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