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JunD enhances miR-29b levels transcriptionally and posttranscriptionally to inhibit proliferation of intestinal epithelial cells

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MICRORNAs (miRNAs) REGULATE gene expression by triggering translational repression and/or degradation of target mRNAs in a sequence-specific manner (19, 25, 30) and have recently emerged as master regulators of gut epithelium homeostasis (2, 46, 48). The levels of miRNAs in the gut epithelium are commonly altered in patients with mucosal atrophy/hyperplasia, cancers, inflammation, and barrier dysfunctions (25, 36, 47, 49, 52), but the exact mechanisms that control miRNA expression patterns in intestinal epithelial cells (IECs) have not been fully investigated. The complex process of miRNA biosynthesis begins with the transcription of primary (pri-) miRNAs (27, 29, 35) by RNA polymerase II, their cleavage into precursor (pre-) miRNAs by the nuclear RNase Drosha, and exported to the cytoplasm where RNase Dicer further processes pre-miRNAs into 22-nt double-stranded RNA duplexes (3, 7, 16, 38). One strand of the resulting duplex is selectively loaded into the RNA-induced silencing complex, which acts as the effector regulating target mRNAs (17, 19). Several transcription factors, such as p53, SMAD, MYC, and NF-kB, are shown to direct RNA polymerase II-dependent transcription of pri-miRNAs and modulate the expression levels of miRNAs (4, 27, 29, 35). In mammalian miRNA biogenesis, the Drosha microprocessor complex consists of Drosha, DiGeorge syndrome critical region gene 8, and the DEAD box RNA helicases p68 (DDX5) and DDX17, two helicases required for the maturation of many miRNAs (9, 16).

JunD, a basic region leucine zipper DNA-binding protein, is a member of the Jun family of proteins that are primary components of the activating protein-1 (AP-1) transcription factor (12). Jun proteins can form AP-1 homodimers or heterodimers among themselves or with members of the related Fos or activating factor/cAMP response element binding protein families, and directly bind to their target promoters at specific DNA elements, such as TGGTCA (classical AP-1 site) and TGACGTC (14, 33). All three Jun proteins (c-Jun, JunB, and JunD) are similar in DNA-binding affinity, but their expression patterns vary in response to stress and during cell proliferation and transformation (1, 14). c-Jun and JunB behave as immediate-early response genes and enhance the G1-to-S-phase transition upon mitogenic stimulation, while JunD can inhibit cell proliferation (12, 20, 24). JunD also regulates apoptosis and protects against oxidative stress by modulating genes involved in the antioxidant defense and hydrogen peroxide production (10, 18). Mice lacking JunD exhibit shortened life span (18), multiple defects in the reproductive system (39), accelerated age-related endothelial dysfunction (32), chronic kidney disease (34), and increased incidence of aggressive cancers (39, 40). Our laboratory’s previous studies showed that JunD inhibits intestinal mucosal growth and is crucial for maintaining the gut epithelium homeostasis by modulating the transcription of genes encoding p21 (20), cyclin-dependent kinase 4 (CDK4) (44), and tight junction proteins (5) through dimerization with activating transcription factor-2 (45). JunD expression in IECs is tightly regulated by cellular polyamines at the posttranscriptional level through the interaction of RNA-binding proteins HuR (Hu-antigen R)
and AUFI (AU-binding factor 1) with the 3′-untranslated region of the JunD mRNA (54).

Both JunD and miRNAs regulate intestinal epithelial renewal and apoptosis (20, 47), and select miRNAs, including miR-29b, miR-222, and miR-503, are known to have abnormal expression in mice with small intestinal mucosal atrophy (6, 46, 47) and in human malignancies (37, 51). Previous studies have revealed that transcription factors that repress cell proliferation, such as p53 and SMAD, activate miRNA expression (8, 38), whereas the transcription factor MYC, which stimulates cell division, inhibits miRNA expression (27, 29). For example, p53 transactivates genes encoding miR-34a and miR-34b (35), but MYC overexpression induces transcriptional suppression of miR-29b1/miR-29a promoter (27). In light of the fact that JunD acts as a growth repressor in IECs (10, 20, 44), we sought to investigate if JunD also regulates miRNA expression. Our results show that JunD activates transcription of the miR-29b gene via interaction with AP-1 sites within the miR-29b1 promoter, and it also regulates miR-29b biogenesis by altering Drosha/DDX5 association. We also report that cellular polyamines downregulate miR-29b expression by lowing JunD levels, and that miR-29b silencing prevented the JunD-induced repression of IEC proliferation, incorporating the importance of JunD-mediated miR-29b activation in gut epithelium homeostasis.

MATERIALS AND METHODS

Chemicals and cell culture. Tissue culture medium and dialyzed fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA) and biochemicals were from Sigma (St. Louis, MO). The antibodies recognizing JunD, Drosa, DDX5, and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA) and BD Biosciences, and the secondary antibody conjugated to horseradish peroxidase was from Sigma. Pre-miR miRNA precursor and anti-miR miRNA inhibitor of miR-29b were purchased from Ambion (Austin, TX). 1,2-Difluoro-methylborinic acid (DFMO) was purchased from Genzyme (Cambridge, MA). The IEC-6 cell line (derived from normal rat intestinal crypt cells) was purchased from American Type Culture Collection (ATCC) at passage 13 and was maintained in T-150 flasks in Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated FBS. Passages 15–20 were used in experiments, and there were no significant changes of biological function and characterization of IEC-6 cells at passages 15–20 (2, 46). Stable ornithine decarboxylase (ODC)-transfected IEC-6 cells (ODC-IECs) were developed as described in our laboratory’s previous studies (22) and expressed a more stable ODC variant with full enzyme activity. They were maintained in Eagle’s minimum essential medium with 5% heat-inactivated FBS, as described previously (21). Caco-2 cells (a human colon carcinoma cell line) were also purchased from ATCC and cultured similarly to the IEC-6 cells. Passages 18–23 were used for the experiments, as described previously (20).

Plasmid construction. Recombinant adenoviral plasmids containing human JunD (AdJunD) were constructed by using the Adeno-X Expression System, according to the protocol provided by the manufacturer (Clontech). Briefly, the full-length cDNA of human wild-type JunD was cloned into the pShuttle by digesting the BamHI/HindIII and ligating the resultant fragments into the XbaI site of the pShuttle vector (52). pAdeno-JunD was constructed by digesting the pShuttle construct with PI-SceI/Cl-Ig and ligating the resultant fragment into the PI-SceI/Cl-Ig sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting human embryonic kidney-293 cells using LipofectAMINE Plus reagent (Gibco-Bethesda Res Laboratory, Gaithersburg, MD). Titers of the adenoviral stock were determined by standard plaque-forming assay. Recombinant adenoviruses were screened for the expression of the introduced gene by Western blot analysis using anti-JunD antibody. pAdeno-X, which was the recombinant replication-incompetent adenovirus carrying no JunD cDNA insert (Adnull), was grown and purified as described above and served as a control adenovirus. Cells were infected with AdJunD or Adnull, and expression of JunD was assayed 48 h after the infection.

The full-length miR-29b1 promoter (positions from −1530 to +165) luciferase (Luc) reporter construct was kindly provided by Dr. Justin L. Mott (Mayo Clinic, Rochester, MI) and described previously (27). The 5′-deletion mutation and internal deletion mutation of the miR-29b1 promoter were generated using QuickChange site-directed mutagenesis kit and performed according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Mutations of AP-1 binding site within the miR-29b1 promoter were verified by DNA sequencing. Transient transfection was performed with LipofectAMINE Reagent from Invitrogen. The promoter constructs were transfected into cells along with pRL-null, a Renilla Luc control reporter vector from Promega (Madison, WI), to monitor transfection efficiencies. The transfected cells were lysed for assays of promoter activity using the Dual Luciferase Reporter Assay System (Promega). The Luc activity from individual constructs was normalized by Renilla-driven Luc activity in every experiment. All of the primer sequences for generating these constructs and sequences deleted from the miR-29b1 promoter are provided in Table 1.

RT-PCR and real-time quantitative PCR analysis. Total RNA was isolated by using RNasy mini kit (Qiagen, Valencia, CA) and used in reverse transcription and PCR amplification reactions as described (23, 54). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product were assessed to monitor the evenness in RNA input in RT-PCR samples. Real-time quantitative PCR analysis was performed using 7500-Fast Real-Time PCR Systems with specific primers, probes, and software (Applied Biosystems, Foster City, CA). The levels of master and primary (pri-) miR-29b were quantified by quantitative PCR by using Taqman miRNA assay; small nuclear RNA U6 was used as endogenous control.

Western blot analysis. Whole cell lysates were prepared using 2% SDS, sonicated, and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatants were boiled for 5 min and size-fractionated by SDS-PAGE (7.5% acrylamide). After proteins were transferred onto nitrocellulose filters, the blots were incubated with primary antibodies recognizing JunD, Drosa, or DDX5; following incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

Chromatin immunoprecipitation assays. Forty-eight h after infection with AdJunD or Adnull, cells were fixed with 1% formaldehyde.
to crosslink chromatin. Chromatin immunoprecipitation (ChIP) analysis was performed using the Active-Motif ChIP-IT kit (Carlsbad, CA), following the manufacturer’s recommendations with minor modification, as described previously (44). Briefly, cells were suspended in lysis buffer and gently dounced on ice with 10 strokes to aid in nuclei release. After centrifugation, the nuclear pellet was suspended in digestion buffer, and the chromatin was sheared with Enzymatic Shearing Cocktail. The sheared DNA samples were centrifuged, and the supernatants were collected and precleared with Protein-G beads. The precleared DNA samples were then incubated with the anti-JunD or control IgG antibodies overnight with constant rotation. The immunocomplexes were captured by addition of Protein-G beads, and the immunoprecipitated DNA was collected from the beads using ChIP elution buffer. DNA-protein cross-links were reversed and deproteinized, and DNA was recovered and amplified by PCR. Primers to amplify the proximal region of the miR-29b1 promoter containing two AP-1 binding sites were 5'-TCGCAGAGGATTAGACAGAG-3' and 5'-AGGGATTTCACAACGTTCAT-3'. Primers to amplify the proximal region of the GAPDH promoter (a negative control) were 5'-TACTAGCGGTTTTACGGGCG-3' and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'.

Statistics. Results are expressed as the means ± SE from three to six samples. The significance of the difference between means was determined by ANOVA; P values < 0.05 were considered significant.

RESULTS

JunD specifically stimulates miR-29b expression. JunD acts as a repressor of gut epithelial renewal (20, 44), but the precise mechanisms by which JunD inhibits IEC proliferation remain not fully understood. To determine the possibility that JunD-induced inhibition of intestinal mucosal growth is mediated through given miRNAs, we first examined the effect of overexpressing the wild-type *Jund* gene on the expression levels of different miRNAs in IEC-6 cells. Transient infection with the AdJunD increased JunD protein (by 6-fold at 48 h after infection) compared with JunD levels in the populations of control cells and cells infected with the Adnull (Fig. 1A).

Increased JunD specifically stimulated miR-29b expression, as...
indicated by an increase in the levels of mature miR-29b (Fig. 1Ba), although it inhibited miR-503 expression (Fig. 1Bc). JunD overexpression did not alter the expression levels of other miRNAs, including miR-222 (Fig. 1Bb), miR-34a, miR-34c, miR-195, miR-133a, miR-203, miR-221, miR-495, miR-542, and miR-675 (data not shown). The expression levels of this selected group of miRNAs were examined in this study because their basal levels are relatively high in the intestinal epithelium, and their expression patterns changed significantly during growth inhibition of the intestinal mucosa, as reported in our laboratory’s previous miRNA expression profiling studies after a period of fasting or polyamine depletion (46, 47).

The results presented in Fig. 1Ca further show that JunD activated miR-29b expression at the transcription level, since there was a significant increase in the levels of pri-miR-29b1 in cells overexpressing JunD. On the other hand, increased JunD did not affect the levels of pri-miR-29b2 (Fig. 1Cb), pri-miR-222 (Fig. 1Cc), or pri-miR-503 (Fig. 1Cd).

Second, we used small interfering RNA (siRNA) targeting the Jund mRNA (siJunD) to examine the influence of JunD silencing on miR-29b expression. The siJunD nucleotides were designed to inhibit JunD expression with high specificity, efficacy, and low toxicity, as reported previously (44). With >95% cells transfected (data not shown), the levels of JunD protein were decreased by >85% at 48 h after transfecting siJunD in IEC-6 cells (Fig. 2A), although there were no changes in levels of c-Jun and MYC proteins in siJunD-transfected cells (data not shown). As expected, JunD silencing inhibited miR-29b expression, as demonstrated by a decrease in the levels of mature miR-29b (Fig. 2Ba) and pri-miR-29b1 (Fig. 2Ca), but it induced miR-503 expression (Fig. 2Bc). JunD silencing did not affect miR-222 expression (Fig. 2Bb andCc) and also failed to alter the levels of pri-miR-29b2 and pri-miR-503. These results indicate that JunD specifically activates miR-29b1 transcription, but inhibits miR-503 expression through a distinct mechanism.

JunD activates miR-29b transcription via an AP-1 site within its promoter. To define the mechanism by which JunD enhances miR-29b transcription, the miR-29b1 promoter fragment was cloned from genomic DNA. As shown in Fig. 3A, it contained several potential AP-1 sites. To map the JunD-responsive region in the miR-29b1 promoter, different reporter constructs were prepared containing 5′-deletion or internal deletion of AP-1 binding sites (Table 1). As shown in Fig. 3B, the elements that contained a 1,530-bp region of the miR-29b1 promoter and the AP1-binding sites within this region were required for basal and regulatory miR-29b expression, as the 5′-deletion mutation or internal deletion mutation of AP-1-binding sites from the miR-29b1 promoter gradually decreased basal levels of the reporter gene activity. The results presented

![Fig. 2. JunD silencing lowers miR-29b levels. A: representative JunD immunoblots 48 h after transfecting cells with small interfering RNA (siRNA) targeting Jund mRNA (siJunD) or control siRNA (C-siRNA). B: changes in the levels of mature miRNAs in cells described in A. Values are means ± SE of data from three separate experiments. *P < 0.05 compared with control or cells transfected with C-siRNA. C: levels of pri-miRNAs in cells described in A.](http://ajpcell.physiology.org/DownloadedFrom)
Cf further show that ectopic expression of the *Jund* gene increased the miR-29b1 promoter activity, and that this activation was mediated through AP-1 binding sites within its promoter region. The miR-29b1 promoter activity increased significantly after JunD overexpression when cells were transfected with either the F-Luc construct (full-length promoter with four AP-1 sites), Mut-1 (−924-bp promoter fragment containing two AP-1 sites), or Mut-2 (−910-bp promoter fragment containing only one AP-1 site). However, this stimulation completely disappeared when cells were transfected with the Mut-3 (−900-bp promoter fragment lacking AP-1 site). Moreover, repression of miR-29b1 promoter activity by JunD silencing was weakened or completely abolished when AP-1 sites were deleted partially or completely from the promoter (Fig. 3D). When cells were transfected with the Mut-3, there was no difference in the levels of miR-29b1 promoter activity between control and JunD-silenced cells.

ChIP analysis was used to further examine the in vivo association of JunD with the miR-29b1 promoter. Nuclear fractions were immunoprecipitated using a specific anti-JunD antibody (Ab); IgG was used as a negative control. Cross-linked chromatin isolated from cells infected with either AdJunD or Adnull was IP by using an anti-JunD Ab, and the associated chromosomal DNA fragments were amplified by PCR using miR-29b1 promoter-specific primers and *Gapdh* promoter-specific primers, as described in MATERIALS AND METHODS. The expected size of the PCR product was 294 bp. Chromosomal DNA input was subjected to the same procedures and served as a positive control.

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antibody in cells infected with AdJunD or Adnull, and the associated DNA was purified. Using specific PCR primers, a 294-bp PCR product was obtained that matched the sequence of the region of miR-29b1 promoter from −1431 to −1137 (containing two potential AP-1 sites) relative to the transcriptional start site. JunD was found to bind the miR-29b1 promoter in vivo, as shown using an anti-JunD antibody in cells overexpressing JunD (Fig. 3E, top, lane 5). This association was relative specific for JunD, since no PCR product was detectable in AdJunD-infected cells when using a nonspecific antibody (IgG, Fig. 3E, top, lane 7) or when using primers to an unrelated promoter, such as the Gapdh promoter (Fig. 3E, bottom, lane 5). JunD also bound to the Cdk4 promoter (data not shown), as reported previously (44), which served as a positive control in this study. These results indicate that JunD activates miR-29b transcription by interacting with AP-1-binding sites within the miR-29b1 promoter.

JunD also enhances formation of the Drosha/DDX5 microprocessor complexes. To determine the involvement of JunD in the regulation of miR-29b maturation, we studied the levels of Drosha and DDX5 expression and their association after altering JunD expression levels. As shown in Fig. 4A, neither JunD silencing nor JunD overexpression altered the total levels of Drosha and DDX5 proteins, indicating that JunD does not regulate the expression of Drosha and DDX5 in IEC-6 cells. To examine Drosha/DDX5 association, immunoprecipitation of Drosha from whole cell lysates was followed by detection of Drosha, DDX5, and JunD in the immunoprecipitation materials by Western blot analysis. We found that Drosha/DDX5 association is JunD inducible, since levels of Drosha/DDX5 complexes decreased significantly in JunD-silenced population of cells, but increased in cells overexpressing JunD when measured using the antibody against Drosha, DDX5, or JunD (Fig. 4B). To examine whether JunD-induced Drosha/DDX5 association is involved in the regulation of miR-29b maturation, cellular abundances of Drosha and DDX5 were downregulated by specific siRNAs. Drosha silencing by transfection with siRNA targeting Drosha (Fig. 5A) decreased the levels of mature miR-29b (Fig. 5Ba), although pri-miR-29b1 content did not change significantly. A similar inhibitory effect on miR-29b processing was also observed when DDX5 expression was inhibited by siRNA targeting DDX5 (Fig. 5, C and D). Importantly, the induction of mature miR-29b by JunD was almost completely abolished after silencing Drosha or DDX5 (Fig. 5Ea). In contrast, the stimulation of pri-miR-29b1 by JunD was not altered by silencing Drosha or DDX5 (Fig. 5Eb). These results indicate an essential role of Drosha/DDX5 microprocessor complexes in the JunD-regulated expression of miR-29b, suggesting that JunD upregulates miR-29b at least partially by enhancing Drosha/DDX5 association.

Polyamines regulate miR-29b expression by altering JunD. The natural polyamines (spermidine, spermine, and their precursor putrescine) are organic cations found in all eukaryotic cells and are absolutely required for normal gut mucosal growth (28, 43). To examine if polyamines modulate miR-29b expression through their ability to lower JunD, two sets of experiments were performed. First, we examined the changes in miR-29b expression levels following an increase in endogenous JunD abundance by depleting cellular polyamines. As previously reported (20, 44), exposure to 5 mM DFMO (a specific inhibitor of polyamine biosynthesis) for 4 days completely inhibited ODC enzyme activity and almost totally depleted cellular polyamines in IEC-6 cells. The levels of putrescine and spermidine were undetectable on day 4 after treatment with DFMO, and spermine decreased marginally (data not shown). Polyamine depletion by DFMO increased the steady-state levels of JunD protein (Fig. 6A), which was associated with the stimulation of miR-29b expression. Levels of mature miR-29b (Fig. 6Ba) and pri-miR-29b1 (Fig. 6Ca) increased significantly in polyamine-deficient cells compared with those observed in control cells. In the absence of cellular polyamines, JunD silencing prevented the activation of miR-29b expression, as indicated by a decrease in the levels of mature miR-29b and pri-miR-29b1. Polyamine depletion also caused the transactivation of miR-222 (Fig. 6, Bb and Cb), but it inhibited miR-503 expression (Fig. 6, Bc and Cc). Neither mature miR-222 nor pri-miR-222 level in polyamine-deficient cells was affected by JunD silencing, suggesting that polyamine depletion promotes miR-222 expression through a process that is independent of induced JunD. Moreover, JunD...
silencing restored mature miR-503 to normal level in polyamine-deficient cells without effect on pri-miR-503 content.

Second, we examined change in miR-29b expression after decreased levels of endogenous JunD by increasing cellular polyamines through the stable transfection of the ODC gene in IEC-6 cells. Two clonal populations of IECs stably expressing ODC (ODC-IEC) were used in this study. As reported previously (22, 53), ODC-IEC cells exhibited very high levels of ODC protein (by ~10-fold) and >50-fold increase in ODC enzyme activity, which was associated with a significant increase in the levels of cellular putrescine (by ~12-fold), spermidine (by ~2-fold), and spermine (by ~25%). The results in Fig. 6D show that increasing the levels of cellular polyamines due to ectopic overexpression of the ODC gene decreased cellular JunD abundance, along with a reduction in miR-29b levels (Fig. 6E, left). The inhibitory effects of ODC overexpression on JunD and subsequent miR-29b expression were not simply due to clonal variation, since two different clonal populations, ODC-IEC-C1 and ODC-IEC-C2, showed similar responses. Furthermore, ectopic JunD overexpression in ODC-IEC cells by transient infection with the AdJunD prevented the decreased expression of miR-29b. In fact, the levels of miR-29b in ODC-IEC cells after JunD overexpression were much higher than those observed in control cells (Fig. 6E, right). These results indicate that cellular polyamines down-regulate miR-29b expression by reducing JunD.

JunD-mediated miR-29b expression plays a role in regulating IEC proliferation. We also investigated the biological functions of miR-29b activation by JunD and demonstrated that JunD represses IEC proliferation by increasing miR-29b expression. Increasing the levels of cellular miR-29b by JunD overexpression resulted in growth arrest in G1 phase (Fig. 7, A and B) and inhibited cell growth in IEC-6 cells (Fig. 7C). Populations in the S and G2/M phases decreased in AdJunD-infected cells, along with increases in G1 cells, similar to growth arrest in cells overexpressing miR-29b (Fig. 7D). Furthermore, in cells overexpressing JunD, silencing miR-29b by transfection with the anti-miR-29b oligonucleotides (anti-miR-29b) (data not shown) rescued the JunD-induced growth inhibition, since changes in cell cycle distribution and cell growth

Fig. 5. Silencing Drosha or DDX5 prevents JunD-induced activation of miR-29b processing. A: representative immunoblots of Drosha 48 h after transfection with siRNA targeting Drosha (siDrosha) or with C-siRNA. B: levels of mature miR-29b (a) and pri-miR-29b1 (b) in cells described in A. Values are means ± SE of data from three separate experiments. *P < 0.05 compared with control or C-siRNA. C: immunoblots of DDX5 48 h after transfecting cells with siRNA targeting DDX5 (siDDX5) or C-siRNA. D: levels of mature miR-29b (a) and pri-miR-29b1 (b) in cells described in C. E: inhibition of Drosha or DDX5 by transfection with specific siRNAs decreased the levels of mature miR-29b (a) but failed to alter pri-miR-29b1 content (b) in cells overexpressing JunD. *P < 0.05 compares with control. +P < 0.05 compared with cells infected with AdJunD alone or AdJunD-infected cells transfected with C-siRNA.
induced by JunD overexpression almost returned to those observed in controls when AdJunD-infected cells were transfected with anti-miR-29b (Fig. 7, A–C, right). These results indicate that JunD represses IEC proliferation primarily by activating miR-29b expression, suggesting that JunD-regulated miR-29b activation plays an important role in maintaining the gut epithelium homeostasis.

**DISCUSSION**

Our laboratory’s previous studies demonstrated that inhibition of intestinal mucosal growth associates with an increase in the levels of both JunD and miR-29b (20, 47), but the exact relationship between induced JunD and activation of miR-29b expression remains unknown. In this study, we report that the
miR-29b ACTIVATION BY JunD

C821

gene encoding the pri-miR-29b1 is a direct transcriptional target of JunD. Increasing JunD ectopically or following poly
amine depletion resulted in transcriptional activation of the miR-29b1 gene, whereas JunD silencing repressed miR-29b expression. Positive regulation of miR-29b1 transcription by JunD is mediated via AP-1 sites located within the miR-29b1 promoter, since this stimulation disappeared completely when AP-1 sites were mutated from the miR-29b1 promoter. JunD also enhances miR-29b biogenesis by promoting Drosha/ DDX5 association that is essential for miRNA processing in the nucleus (9). Moreover, inhibition of miR-29b expression by its specific antagonist prevented JunD-induced inhibition of IEC proliferation. These findings highlight a novel role of JunD in the regulation of miR-29b expression, although some indirect evidence exists.

Our results indicate that there are four functional AP-1 binding sites within the miR-29b1 promoter. Two pieces of evidence from the present studies demonstrate that these four AP-1 sites are crucial for JunD-mediated activation of miR-29b1 promoter. First, 5’-deletion of two AP-1 sequences from the distal region of miR-29b1 promoter partially prevented JunD-induced promoter activation in AdJunD-infected cells and significantly blocked the repression of promoter activity in JunD-silenced population of cells. Further internal deletion mutation of the remaining two AP-1 sites from the proximal region of miR-29b1 promoter completely abolished both JunD-mediated promoter activation and repression of promoter activity induced by JunD silencing. Second, ChIP analysis revealed that JunD physically interacted with and bound to the miR-29b1 promoter in vivo. These findings are consistent with results from our laboratory’s previous studies (5, 44, 45) that have demonstrated that the transcriptional activity of JunD is mediated primarily through its specific antagomir prevented JunD-induced inhibition of miR-29b activation in patients with gut mucosal atrophy.
On the other hand, MYC, hedgehog, and NF-κB suppress the miR-29b1 transcription by interacting different binding sites within the miR-29b1 promoter (27).

The results reported here also provide evidence of the role of JunD in the regulation of miR-29b biogenesis through modulation of Drosha microprocessor complex. JunD was found to physically interact with Drosha and DDX5 in IEC-6 cells, and this association was markedly decreased by JunD silencing and increased by JunD overexpression, although altering JunD levels did not affect the total Drosha or DDX5 abundance. The molecular mechanisms underlying this enhanced interaction were not elucidated in our analysis. Furthermore, silencing Drosha or DDX5 attenuated miR-29b maturation, as indicated by a decrease in the levels of mature miR-29b without changing pri-miR-29b1 content in cells overexpressing JunD. Whether the enhanced association of Drosha and DDX5 promoted the processing of other miRNAs was not investigated in analysis and is focused in our ongoing studies.

Consistent with the present findings, several recent studies have shown that tumor suppressor networks play an important role in miRNA biogenesis by modulating the Drosha microprocessor complex (7, 16, 38). Tumor suppressors, such as p53, SMAD, and BRCA1 (breast cancer 1), are shown to enhance the posttranscriptional maturation of different miRNAs with growth-suppressive function, including miR-16-1, miR-143, miR-145, miR-21, let-7a-1, and miR-34a (8, 16, 38), but their roles in the regulation of miR-29b biogenesis remain to be elucidated. It is still unknown at present whether JunD associates with p53, SMAD, or/and BRCA1 in IECs, and if these potential interactions affect JunD-regulated miR-29b maturation.

The data obtained in the present study further demonstrate that cellular polyamines regulate miR-29b expression by altering JunD. Polyamines have been recognized as biological regulators of normal gut mucosal growth, and their cellular concentrations are highly regulated and depend on the dynamic balance among polyamine biosynthesis, degradation, and transport (28, 43). The supply of polyamines to the dividing cells in the crypts is essential for normal intestinal mucosal growth, whereas polyamine depletion inhibits epithelial renewal predominantly by increasing JunD (20, 44). As reported in our laboratory’s previous studies (20, 31, 47), decreasing cellular polyamines by DFMO increased both JunD and miR-29b, but expression levels of JunD and miR-29b decreased in cells containing high levels of polyamines. Our results further shows that JunD silencing prevented the transactivation of miR-29b in polyamine-deficient cells, and that ectopically expressed JunD restored miR-29b expression in stable ODC-IEC cells. Although the full mechanisms by which polyamines downregulate JunD expression are not fully understood, our laboratory’s previous studies show that polyamine depletion stabilizes JunD mRNA by modulating the competitive binding of its 3′-untranslated region to the RNA-binding proteins HuR and AUFI (54).

Finally, the JunD-regulated miR-29b activation is of biological significance because it plays an important role in the regulation of IEC proliferation. The epithelium of the intestinal mucosa is a rapidly self-renewing tissue, and its homeostasis is preserved through strict regulation of cell proliferation and apoptosis (48, 50). IECs continuously replicate within the intestinal crypts, and this process is counterbalanced by apoptotic death that occurs in the crypt area, where it maintains a critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the intestinal mucosa, where differentiated cells are lost. As shown, ectopic overexpression of JunD resulted in G1 phase growth arrest in IEC-6 cells, which was abolished by silencing miR-29b, showing the essential role of induced miR-29b in the repression of cell proliferation by JunD. In summary, our results indicate that JunD activates transcription of the miR-29b gene through interaction with the AP-1 sites within the miR-29b1 promoter, but it induces miR-29b maturation by enhancing Drosha/DDX5 association. Since JunD-regulated miR-29b activation represses IEC proliferation, our findings strongly suggest that regulation of miR-29b expression by JunD is crucial for maintaining intestinal epithelial homeostasis under physiological and various pathological conditions.

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miR-29b ACTIVATION BY JunD

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