miR-9 enhances the transactivation of nuclear factor of activated T cells by targeting KPNB1 and DYRK1B

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Nuclear factor of activated T cells (NFAT) was initially characterized as an inducer of IL-2 production in T lymphocytes. It represents a family of transcription factors comprising four well-characterized isoforms, NFATc1, NFATc2, NFATc3, and NFATc4 (22, 29). NFAT is regulated by Ca2+/calmodulin-dependent serine phosphatase calcineurin. In resting cells, NFAT is phosphorylated and resides in the cytoplasm as a transcriptionally inactive form. Upon stimulation, it is dephosphorylated by calcineurin, translocates to the nucleus, and becomes a transcriptionally active form that induces expression of NFAT-responsive genes (9). When Ca2+ entry is prevented or calcineurin activity is inhibited, NFAT is rephosphorylated by NFAT kinases, such as casein kinase 1 and glycogen synthase kinase 3, leading to its rapid export from the nucleus and, thus, termination of NFAT-dependent gene expression (20). The NFAT pathway is also modulated by translocation in response to PMA-ionomycin. Karyopherin-α1 (KPNB1, a nucleocytoplasmic transporter) and dual-specificity tyrosine phosphorylation-regulated kinase 1B (DYRK1B) were identified as direct targets of miR-9. Functionally, miR-9 promoted IL-2 production in stimulated human lymphocyte Jurkat T cells. Collectively, our data reveal a novel role for miR-9 in regulation of the NFAT pathway by targeting KPNB1 and DYRK1B.

NFAT; miR-9; Jurkat T cells; IL-2

MicroRNAs (miRNAs) are single-stranded ~22- to 25-nucleotide-long RNA molecules that negatively regulate gene expression by targeting the 3′-untranslated region (3′-UTR) of mRNAs (10, 16). Recently, it was reported that miRNAs are involved in regulation of the NFAT signaling pathway. For example, miRNA-184 (miR-184) inhibits the NFAT pathway by directly targeting NFATc2 in CD4+ T cells (35). miR-568 inhibits activation and function of CD4+ T cells and Treg cells by targeting NFAT5 (18). NFATc4 is a direct target of miR-133 in cardiac hypertrophy (17). miR-199b activates NFAT by targeting DYRK1A, a member of the dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) family (4). In mouse and human heart failure, miR-199b expression is up-regulated, leading to the loss of DYRK1A and, consequently, the increase in calcineurin-responsive gene expression. On the other hand, NFAT also regulates miRNA expression. For example, NFATc3 increases miR-23a expression in cardiomyocytes (19) and miR-140 in osteoarthritis chondrocytes (31).

Recently, two independent research groups reported that miR-9 enhances IL-2 production in activated human CD4+ T cells by repressing B-lymphocyte-induced maturation protein-1 (BLIMP-1) (27, 32). We previously identified eight miRNAs that robustly modulate NFAT activity. Among them, miR-9 positively regulates NFAT reporter activity in PMA-ionomycin-stimulated human embryonic kidney (HEK 293A) cells (12). However, the mechanism whereby miR-9 regulates NFAT signaling and the biological relevance of miR-9 are unclear. In this study we have identified a novel mechanism of miR-9-induced IL-2 production via activation of the NFAT pathway.

MATERIALS AND METHODS

Cell culture. Human Jurkat T, HEK 293A, HEK 293T, and HeLa cells were purchased from American Type Culture Collection (Manassas, VA). HEK 293A, HEK 293T, and HeLa cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Jurkat T cells were cultured in RPMI 1640 medium with 10% FBS. Jurkat T cells were transfected with 50 nM miRNA mimic or miRNA inhibitor using Lipofectamine 2000 (Invitrogen). At 6 h after transfection, the cells were cultured in fresh medium for 2 days and then left untreated or

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stimulated with PMA (10 ng/ml; Sigma) and ionomycin (1 μM; Sigma).

Plasmid construction. The primary miR-9-1 (509 bp long) was PCR-amplified from human genomic DNA with the primers 5'-CAG CTC GAG GAT ACT CGA GTA TCT CGA ACA CTG GTA TCT-3' (forward) and 5'-CAC GGA TCT GTA GAT ATA CGT GAT ACT CGA GTA TCT CGA ACT CAAC ACA ACG TGAT TT-3' (reverse), cloned into the downstream of the enhanced green fluorescent protein (EGFP) stop codon in the pENTR/CMV-EGFP vector through Xhol- EcoRI sites, and named pENTR/CMV-EGFP-miR-9. The insert was confirmed by DNA sequencing with the EGFP sequencing primer 5'-CAT GCT GGT CCT GCT GGA GTT CGT G-3'. A ubiquitin C (UbC) promoter-driven miR-9 overexpression vector was constructed by replacement of the CMV-EGFP fragment in the pENTR/CMV-EGFP-miR-9 vector with the UbC promoter, as we described previously (12). A lentiviral vector expressing miR-9 was obtained by release of the primary miR-9 fragment from the pENTR/CMV-EGFP-miR-9 vector by Xhol-EcoRI and insertion into a modified pLVX-CMV-EGFP vector after the stop codon of the EGFP reporter, resulting in pLVX-CMV-miR-9. pLVX-CMV-c-Myc-NFATc2 and pACGFP-NFATc1, used for NFAT dephosphorylation and nuclear translocation study, were constructed as previously described (12). For construction of pACGFP-NFATc2, the NFATc2 fragment from pLVX-CMV-c-Myc-NFATc2 was subcloned into the pACGFP plasmid. The SV40 promoter-driven expression of Renilla luciferase (pRLuc-SV40) was modified from pmirGLO (Promega) plasmid by removal of the fragment between Mun1 and BglII and as used as a control vector for normalization. Plasmid vectors containing shRNA targeted to KPNB1 (sh-KPNB1), DYRK1B (sh-DYRK1B), and nonspecific control (sh-Con) were constructed based on pLVX-H6. The shRNA sequences were synthesized as follows: 5'-ACC GCC AGT GTA GTT CGA GAT ACT CGA GTA TCT CGA ACA ACT ACA CTG GTA TCT TT-3' (sh-KPNB1), 5'-ACC GCC AGA GGA TCT ACC AGT ATA TCT CGA GAT ATA CTG GTA GAT CCT CTG TT-3' (sh-DYRK1B), and 5'-ACC GCC TAA GTT GTA TTA GTC GCC CTC CTC GAG GAG GCC GAC TTA ACC TTA GTT TT-3' (sh-Con). Lentivirus package and infection. High-titer lentiviruses were packaged in HEK 293T cells by cotransfection of a lentiviral vector and Lentivirus packaging kit (Clontech) containing polyethylenimine (PEI) DNA transfection reagent (Polysciences, Washington, PA). Systematic (Promega) or lentivirus was used to infect Jurkat T cells, and the cells were observed under an inverted fluorescence microscope (Olympus) for AcGFP and DAPI. The overlapped images were examined to determine distribution of NFAT between the cytosol and nucleus. Each treatment, images were collected from at least five different fields.

Target prediction and verification. The TargetScan algorithm (http://www.targetscan.org) was applied to predict targets of miRNAs, which were verified using a 3'-UTR reporter assay. Among 1,237 predicted targets, 4 genes, including KPNB1 (28), DYRK1B (5), DYRK2 (8), and casein kinase 1α (CSNK1A1) (7), were selected for verification, because they are negative regulators of the NFAT pathway. The 3'-UTRs of these predicted target genes were PCR-amplified from human genomic DNA (Promega) and inserted into a modified pGL3 control vector (Promega) through EcoRI-XbaI restriction sites, resulting in the four 3'-UTR reporter constructs, pFLuc-3UTR-KPNB1, pFLuc-3UTR-DYRK1B, pFLuc-3UTR-DYRK2, and pFLuc-3UTR-CSNK1A1. The primer pairs used for PCR amplification are as follows: 5'-GAG AAT TCA TTT GGA TGA TTA CAC GCT GAT CTG GTA TCT TT-3' for KPNB1, 5'-GAG AAT TCA TTT GGA TGA TTA CAC GCT GAT CTG GTA TCT TT-3' for DYRK1B, 5'-GAG AAT TCA TTT GGA TGA TTA CAC GCT GAT CTG GTA TCT TT-3' for DYRK2, and 5'-GAG AAT TCA TTT GGA TGA TTA CAC GCT GAT CTG GTA TCT TT-3' for CSNK1A1. For construction of a mutated 3'-UTR reporter vector, five residues in the seed sequence of the predicted miR-9 recognition region were mutated by overlap PCR. The 3'-UTR reporter assays were carried out in HEK 293A cells seeded onto 24-well plates. When the cells reached 80–90% confluence, they were transfected with 50 ng of 3'-UTR reporter vector, 500 ng of pENTR/CMV-EGFP-miR-9, and 1 ng of pSV40-RLuc using PEI transfection reagent; pENTR/CMV-EGFP without a miRNA insertion was used as a negative control. At 2 days after transfection, the cells were harvested for assay of dual-luciferase activities.

RNA extraction and quantitative real-time RT-PCR. Total RNAs were extracted with RNAiso Plus (TaKaRa Biotechnology) and quantitated using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). DNase-treated total RNAs (1 μg) were reverse-transcribed with oligo(dT)18 plus random hexamer primers (Promega) using Maloney's murine leukemia virus reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with gene-specific primers and iQ SYBR Green Supermix (Bio-Rad) on the StepOnePlus real-time PCR system (Applied Biosystems). The mRNA expression level of each gene was normalized to that of β-actin and calculated using the comparative threshold (2^(-ΔΔCt)) method (21).

The mature miRNA expression level was determined using the S-Poly(T) miRNA assay kit (www.mirNLAB.com), as described previously (13). The relative expression level of a miRNA was normalized to SNORD44 and calculated using the 2^(-ΔΔCt) method.

Western blotting. Cells were collected and dissolved in RIPA mammalian protein extraction lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, and 0.1% SDS) and then boiled for 10 min. Protein concentrations were determined by using the BCA Protein Assay kit (Pierce) according to the manufacturer's instructions. Equal amounts of protein were loaded onto a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked in 5% milk and previ-
150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 1 mM EDTA] supplemented with protease inhibitor cocktail (Roche). The same amount of protein samples was fractionated by SDS-PAGE and electroblotted to a nitrocellulose membrane. After the membrane was blocked with 5% fat-free milk in Tris-buffered saline-Tween 20 [20 mM Tris·HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20], the membrane was incubated with the corresponding primary antibodies and then with horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution). Then the membrane was developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA) and exposed to X-ray film. The primary antibodies were as follows: rabbit polyclonal anti-KPNB1 (1:1,000 dilution).

![Fig. 1. MicroRNA-9 (miR-9) enhances activity of nuclear factor of activated T cells (NFAT) on stimulation. A: human embryonic kidney (HEK 293A) cells were cotransfected with firefly luciferase reporter (pNFAT-FLuc, pNF-κB-FLuc, or pAP1-FLuc), together with Renilla luciferase normalization vector (pSV40-RLuc) and miR-9 overexpression vector (pENTR/CMV-miR-9) or miR-Con control vector (pENTR/CMV-EGFP). At 2 days after transfection, cells were treated with the corresponding stimulation (sti) reagents (miR-Con sti and miR-9 sti) or left untreated as controls. Firefly luciferase activities were normalized to Renilla luciferase activities. Results are expressed as ratios of the relative luciferase activities to activity of the miR-Con vector under unstimulated conditions. Values are means ± SD (n = 4). **P < 0.01 vs. miR-Con sti. B: relative expression levels of miR-9 in human Jurkat T cells during PMA-ionomycin (Io) stimulation. Jurkat T cells were stimulated with PMA (10 ng/ml) and ionomycin (1 μM) for 0–24 h. Expression of miR-9 was determined by quantitative RT-PCR and normalized to SNORD47. Relative miR-9 expression was calculated compared with unstimulated control (0 h). Values are means ± SD (n = 3). *P < 0.05, **P < 0.01 vs. 0 h. C: relative expression of miR-9 in Jurkat T cells treated with cyclosporin A (CsA, 20 or 100 nM) and stimulated 30 min later with PMA-ionomycin. Expression of miR-9 was measured 12 h later by quantitative RT-PCR. Values are means ± SD (n = 3). **P < 0.01 vs. untreated control.

![Fig. 2. miR-9 promotes NFAT dephosphorylation. A: HEK 293T cells were cotransfected with c-Myc-tagged NFATc2 overexpression vector (pLVX/CMV-c-Myc-NFATc2) and miR-9, miR-124, miR-15b, miR-375, or miR-150 overexpression vector. The same backbone vector without miRNA sequences (miR-Con) was used as control. At 2 days after transfection, cells were treated with 1 μM ionomycin and 1 mM CaCl2 (Io/Ca2⁺) for 15 or 60 min, and total proteins were extracted for Western blotting with c-Myc antibody. B: statistical analysis for effect of miRNAs on the ratio of phosphorylated to dephosphorylated c-Myc-NFATc2 expression at 15 and 60 min. Summary data (means ± SD, n = 3) were quantitated and compared at each time point. *P < 0.05, **P < 0.01 vs. miR-Con. C: HEK 293T cells were cotransfected with pLVX/CMV-c-Myc-NFATc2 and miR-9 overexpression vector (miR-Con) or control vector (miR-Con). At 2 days after transfection, cells were treated with 1 μM ionomycin and 1 mM CaCl2 for 0, 7.5, 15, 30, and 60 min, and total proteins were extracted for Western blotting with c-Myc antibody. D: statistical analysis for effect of miR-9 on the ratio of phosphorylated to dephosphorylated c-Myc-NFATc2 expression compared with miR-Con at each time point. Values are means ± SD (n = 3). *P < 0.05, **P < 0.01 vs. miR-Con.
activation; Abcam), rabbit polyclonal anti-DYRK1B (1:500 dilution; Proteintech Group), mouse monoclonal anti-c-Myc (1:3,000 dilution; Sigma), and mouse monoclonal anti-β-tubulin (1:5,000 dilution; Sigma).

IL-2 ELISA. Jurkat T cells (5 × 10^5) were cultured on 60-mm dishes and transfected with miR-9 mimic or control using Lipofectamine 2000 (Invitrogen). After 2 days of transfection, cells were suspended in FBS-free RPMI 1640 medium, seeded on a 24-well plate (2.5 × 10^3/well), and stimulated with PMA-ionomycin for different time intervals. Cells treated with the same amount of solvent were also included as an unstimulated control. After 6 h of stimulation, cells were centrifuged at 1,000 g for 10 min for collection of cell pellets and supernatants.

IL-2 level in supernatants was measured by ELISA using the human IL-2 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. Plates were read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA).

**Statistical analyses.** Each experiment was repeated at least three times. Values are means ± SD. When only two groups were compared, statistical analysis was performed using a standard two-sample Student’s t-test. Statistical significance was determined using a two-tailed distribution assumption. Otherwise, statistical significance was determined using one- or two-way ANOVA, depending on the number of variables.

**RESULTS**

**miR-9 enhances NFAT activity upon stimulation.** To identify potential miRNAs involved in regulation of the NFAT signaling pathway, we previously performed a cell-based high-throughput screening using a human primary miRNA expression library and NFAT luciferase reporter system (12). Eight miRNAs were found to modulate NFAT activity with at least a twofold change. In this study we further evaluated the effects of miR-9 on activation of NFAT, as well as NF-κB and AP1. All three transcription factors translocate from the cytoplasm to the nucleus upon stimulation. As shown in Fig. 1A, miR-9 overexpression caused the largest (2.3-fold) increase in activity of the NFAT reporter, confirming our previous findings (12). Moreover, miR-9 increased AP1 activity by 1.4-fold but had little effect on NF-κB activity.

Furthermore, we wondered if NFAT signaling activation in human T lymphocytes was accompanied by increased expression of miR-9. Thus, in Jurkat T cells stimulated with PMA-ionomycin and treated with cyclosporin A (CsA), we analyzed endogenous miR-9 expression. As shown in Fig. 1B, expression of miR-9 was significantly increased upon PMA-ionomycin stimulation. However, CsA treatment suppressed almost entirely the increase in miR-9 expression upon stimulation (Fig. 1C), indicating that miR-9 upregulation is associated with NFAT activation. All these results suggest that miR-9 may be involved in activation of the NFAT signaling pathway in human T cells.

**miR-9 promotes NFAT dephosphorylation.** Since dephosphorylation of NFAT is the early step in activation of the NFAT signaling pathway, we determined whether miR-9 could promote the calcineurin-dependent dephosphorylation of NFAT in response to ionomycin stimulation. Among the four well-studied NFAT isoforms, NFATc2 is often selected for the dephosphorylation assay, since it has a lower molecular weight than other NFAT members and can be easily detected by Western blotting (28). We cotransfected c-Myc-tagged NFATc2 overexpression plasmid (pLVX/CMV-c-Myc-NFATc2) and each selected miRNA overexpression vector or the control vector into HEK 293T cells and then monitored the electrophoretic mobility of the c-Myc-NFATc2 with anti-c-Myc antibodies after stimulation with ionomycin for 15 and 60 min. As shown in Fig. 2A, phosphorylated and dephosphorylated NFATc2 were clearly distinguishable on the basis of their electrophoretic mobility on SDS-PAGE. Upon stimulation for 15 and 60 min, miR-9 significantly decreased the ratio of phosphorylated NFATc2 compared with vector control (Fig. 2A and B). Moreover, miR-150 also inhibited phosphorylation of NFATc2. In contrast, miR-15b markedly increased the ratio...
of phosphorylated NFATc2, and miR-124 showed a significant increase in NFATc2 phosphorylation upon 60 min of stimulation. However, miR-375 had little effect on phosphorylation of NFATc2 (Fig. 2, A and B). These results are consistent with our previous report that NFAT pathway reporter activities are upregulated by miR-150 or miR-9, downregulated by miR-124 or miR-15b, and unaffected by miR-375 (12).

To confirm the impact of miR-9 on NFAT phosphorylation, we further analyzed the calcium-independent dephosphorylation of NFATc2 in response to ionomycin stimulation for 0–60 min. As shown in Fig. 2, C and D, miR-9 overexpression markedly decreased the ratio of phosphorylated to dephosphorylated NFATc2 upon 15 min of stimulation. These results further demonstrate that miR-9 can promote dephosphorylation of NFAT.

miR-9 facilitates NFAT nuclear translocation. As translocation of dephosphorylated NFAT from the cytoplasm to the nucleus is the next step in its binding to the target promoter, we examined the effect of miR-9 overexpression on NFAT nuclear translocation. We cotransfected UbC promoter-driven miR-9 or its control and CMV promoter-driven AcGFP-NFATc2 or AcGFP-NFATc1 expression vectors into HeLa cells and monitored the distribution of AcGFP-NFATc2 and AcGFP-NFATc1 fusion protein directly using an inverted fluorescence microscope. We used the different promoters to avoid the promoter competition and, thus, achieve a higher fluorescent signal for AcGFP-NFATc2 and AcGFP-NFATc1. Images of cells treated at 0–30 min and statistical analysis are shown in Fig. 3, A and C. In the resting cells without PMA-ionomycin stimulation, almost all AcGFP-NFATc2 and AcGFP-NFATc1 fusion proteins remained in the cytoplasm in each group. After 15 min of stimulation with PMA-ionomycin, miR-9 overexpression led to a higher ratio of cells with AcGFP-NFATc2 (87%) and AcGFP-NFATc1 (~92%) proteins translocated from the cytoplasm into the nucleus compared with the control group (63% and ~65%). After 30 min of stimulation, almost all AcGFP-NFATc2 and AcGFP-NFATc1 proteins were translocated into the nucleus in miR-9-treated and control cells. These results further suggest that miR-9 not only promotes NFAT dephosphorylation but also accelerates the subsequent nuclear translocation of NFATc2 and NFATc1.

KPNB1 and DYRK1B are direct targets of miR-9. To understand the possible molecular mechanism of miR-9-mediated NFAT activation, we applied the TargetScan program to predict gene targets of miR-9. Among >1,000 of the predicted targets, we chose only those molecules relevant to the NFAT signaling pathway. The combination of these analyses resulted in four potential targets, KPNB1, DYRK1B, DYRK2, and CSNK1A1. Then we performed the 3’-UTR reporter dual-luciferase assay to experimentally determine the interaction between miR-9 and these predicted targets. As shown in Fig. 4,
miR-9 enhances NFAT activity and promotes IL-2 production in Jurkat T cells. To confirm that miR-9-mediated NFAT transactivation occurs in Jurkat T cells, several key experiments were performed. 1) We examined the effect of miR-9 overexpression on NFAT reporter activity. Similar to our observation in HEK 293A cells, overexpression of miR-9 increased NFAT reporter activity ~2.6-fold in stimulated Jurkat T cells compared with the miRNA control group (Fig. 5A). 2) We validated KPNB1 and Dyrk1b as the targets of miR-9 in Jurkat T cells. Western blotting showed that KPNB1 and Dyrk1b protein levels were decreased ~39% and 42%, respectively, in miR-9-overexpressed Jurkat T cells compared with the control group (Fig. 5, B and C). 3) We examined the effect of miR-9 overexpression on IL-2, the downstream target of the NFAT transcription factor. We stimulated the Jurkat T cells that stably overexpressed miR-9 or their controls with PMA-ionomycin and analyzed the mRNA level of endogenous IL-2 by quantitative RT-PCR. As shown in Fig. 5D, expression of IL-2 mRNA was significantly higher in miR-9-overexpressed Jurkat T cells (infected with lentiviral-expressing vector) than in the control group without or with stimulation for 2 h. In addition, we transfected Jurkat T cells with chemically synthesized miR-9 mimics or the mimic control. At 2 days after transfection, cells were stimulated with ionomycin-PMA for 2 h. Similarly, we observed that IL-2 mRNA expression was upregulated nearly twofold in the miR-9-overexpressed cells compared with mimic control cells (Fig. 5E). Figure 5F shows that IL-2 protein level was ~16% higher in the medium of miR-9 mimic-transfected Jurkat T cells than in the mimic control cells upon 4 h of stimulation as measured by ELISA.
These data suggest that miR-9 promotes IL-2 production by affecting the NFAT pathway activity in human T cells.

To confirm that regulation of miR-9 on NFAT pathway activation is achieved by targeting KPNB1 and DYRK1B, we carried out the following experiments. First, to confirm the role of the two target genes of miR-9, NFAT luciferase reporter gene assay was performed by transfection with KPNB1 or DYRK1B interfering vector (sh-KPNB1 or sh-DYRK1B). As shown in Fig. 6A, NFAT luciferase activity under PMA-ionomycin stimulation was significantly increased by knockdown of KPNB1 or DYRK1B compared with the stimulated control group. Furthermore, a rescue experiment was performed to confirm that miR-9 inhibition-mediated NFAT activity repression could be rescued by silencing KPNB1 or DYRK1B. As shown in Fig. 6B, NFAT activity was decreased by miR-9 inhibition (anti-miR-9 together with sh-Con) under stimulation. Transfection in combination with sh-KPNB1 or sh-DYRK1B significantly upregulated the NFAT activity repressed by miR-9 inhibitor. These results further confirm that the positive role of miR-9 on NFAT signaling activity can be fulfilled by targeting KPNB1 and DYRK1B.

**DISCUSSION**

In this study we demonstrate that miR-9 is upregulated in Jurkat T cells in response to PMA-ionomycin stimulation and activates the NFAT signaling pathway, as supported by increases in NFAT dephosphorylation and nuclear translocation and induction of the NFAT downstream target gene IL-2. Moreover, miR-9-mediated NFAT transactivation is proven by posttranscriptional repression of two inhibitors, KPNB1 and DYRK1B, by targeting their 3'-UTRs. The model shown in Fig. 7 delineates our findings on the role of miR-9 in NFAT signaling.

miR-9 was initially identified as a brain-specific miRNA. It is implicated in mammalian neuronal development and function. Downregulation of miR-9 is observed in Alzheimer’s disease and Huntington’s disease, suggesting its role in neurodegeneration (11, 26). miR-9 also plays an important role in the control of insulin secretion in β-cells (25) and is positively associated with malignancy of human cancers, including breast cancer and Hodgkin’s lymphoma (14, 15). miR-9 is an LPS-responsive miRNA in primary human neutrophils and monocytes (1, 6), as well as CD4+ cells (33). Its overexpression enhances IL-2 production in activated human CD4+ T cells (32), indicating a role for miR-9 in the immune response. However, the molecular mechanism of miR-9-mediated IL-2 production remains unclear.

Recently, using a miRNA expression library and NFAT-responsive luciferase reporter, we performed a high-throughput screening of potential miRNAs involved in regulation of the NFAT signaling pathway. Of ~300 miRNAs, we identified 8 that modulate NFAT activity (12). Of these, miR-9 upregulates NFAT activity in the stimulated condition. In this study we provide additional evidence to support the notion that miR-9 positively regulates NFAT pathway activity. 1) miR-9-induced activation of the NFAT pathway luciferase reporter was observed, not only in HEK 293A cells, but also in Jurkat T cells. 2) Overexpression of miR-9 promoted NFAT dephosphorylation and accelerated its subsequent nuclear translocation, two key steps involved in Ca2+-induced NFAT signaling. 3) miR-9...
increased production of the NFAT downstream target gene of IL-2 in PMA-ionomycin-stimulated Jurkat T cells.

Recent studies showed that miR-9 can increase production of IL-2. Since the IL-2 promoter is transcriptionally regulated by NFAT, NF-κB, and AP1 through different mechanisms, we evaluated the effects of miR-9 on activation of NFAT, as well as NF-κB and AP1. Our data indicate, for the first time, that miR-9-induced IL-2 production is more likely to be dependent on activation of the NFAT pathway. Furthermore, we showed increased expression of miR-9 in Jurkat T cells upon PMA-ionomycin stimulation. Tsitsiou and Lindsay (33) reported induction of miR-9 in LPS-induced bovine CD4^+ cells. Recently, Thiele et al. (32) showed that treatment with CsA, an inhibitor of calcineurin, almost entirely suppressed the T-cell receptor-induced increase in miR-9 expression in human CD4^+ T cells (32). In this study we further confirmed that upregulation of miR-9 in human Jurkat T cells stimulated by PMA-ionomycin could be blocked by CsA treatment. Thus our current study, together with existing data in the literature, may imply a positive-feedback loop between miR-9 and the NFAT signaling pathway. However, further investigation is needed to reveal the mechanism involved.

The miR-9-mediated NFAT transactivation could be achieved at different steps, including promotion of Ca^{2+} mobilization, increased calmodulin and calcineurin activation, acceleration of calcineurin docking onto NFAT, inhibition of NFAT kinases, and an increase in the NFAT nuclear import rate (34). To identify the targets linking miR-9 and the NFAT pathway, we used bioinformatics software to predict miR-9 targets, yielding >1,000 of the predicted targets. Among these targets, KPNB1, DYRK1B, DYRK2, and CSNK1A1 are inhibitors of the NFAT pathway. Through 3’-UTR dual-luciferase reporter assay and endogenous protein expression analyses, we confirmed that KPNB1 and DYRK1B are two new targets of miR-9, involved in the regulatory network between miR-9 and NFAT signaling.

KPNB1 is a member of the karyopherin/importin-β family of nuclear transport factors that mediate the nuclear import of proteins through binding of nuclear localization signals (30). Previous studies have shown that KPNB1, together with four additional proteins and a large noncoding RNA, NRON, directly mediates nucleocytoplasmic transport of NFAT transcription factors (36). Knockdown of KPNB1 activates NFAT activity, while overexpression of KPNB1 has the opposite effect. DYRKs constitute an evolutionarily conserved family of kinases, the activity of which is dependent on phosphorylation of a conserved tyrosine residue in the activation loop of the catalytic domain (3). The DYRK family has multiple members that can be divided into two distinct subgroups on the basis of their localization: class 1 DYRKs, including DYRK1A and DYRK1B, possess a nuclear localization signal and are localized predominantly in the nucleus; class 2 DYRKs (DYRK2, DYRK3, and DYRK4) are localized exclusively in the cytoplasm (2). Early studies indicate that DYRK1 functions as a NFAT export kinase, whereas DYRK2 phosphorylates NFAT in the cytoplasm and functions as a maintenance kinase (8). Our results show that overexpression of miR-9 leads to loss of KPNB1 and DYRK1B and, consequently, activation of NFAT activity, which sensitizes calcineurin-responsive gene expression, providing a new connection between miR-9 and IL-2 production through the NFAT pathway. Furthermore, overexpression of miR-9 elevated nuclear NFATs and IL-2 production, even in the absence of PMA-ionomycin stimulation. However, it had no significant influence on basal NFAT activity in the luciferase assay. On the one hand, it is likely due to the low sensitivity of the reporter vector, for there is more than one NFAT binding site within the IL-2 promoter. On the other hand, it may imply that, rather than directly modulating the transcriptional activity of NFAT, miR-9 likely regulates subcellular localization of NFAT.

Recently, two independent research groups reported that miR-9 enhances IL-2 production through repression of BLIMP-1, which directly suppresses IL-2 production by binding to the promoter region of IL-2 and its activator Fos in activated CD4^+ T cells (27, 32). Thiele et al. (32) also showed that CsA treatment could not entirely block the increase in IL-2 secretion caused by miR-9 overexpression compared with its vector control under the same condition. It implied that miR-9-mediated regulation of IL-2 production might be not only an NFAT-dependent approach. However, all these results demonstrate that miR-9 is an important posttranscriptional regulator closely related to IL-2 production in human T cells and suggest that it is a potential target in immune suppression.

Our study reveals a novel role for miR-9 in promotion of NFAT activity in human T cells by targeting KPNB1 and DYRK1B, providing new evidence for the physiological role of miR-9 in the immune response. We propose that PMA-ionomycin activates NFAT signaling and induces miR-9 expression, which in turn depresses two NFAT inhibitors, KPNB1 and DYRK1B, and further amplifies NFAT signaling and the subsequent expression of NFAT-responsive genes. This study broadens our knowledge of the posttranscriptional mechanisms that control NFAT activity and IL-2 production and leads to a better understanding of immune function in T cells.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.Z., Y.W., Z.W., K.K., X.P., W.P., and J.Q. analyzed the data; Y.Z., Y.W., K.K., X.P., L.L., and D.G. interpreted the results of the experiments; Y.Z. and Y.W. prepared the figures; Y.Z. drafted the manuscript; Y.Z., Y.W., Z.W., K.K., X.P., W.P., J.Q., L.L., J.U.R., and D.G. approved the final version of the manuscript; Y.Z., Z.W., and X.P. performed the experiments; J.U.R. and D.G. edited and revised the manuscript; D.G. developed the concept and designed the research.

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