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Cutting Edge: MicroRNA-181 Promotes Human NK Cell Development by Regulating Notch Signaling

Frank Cichocki,* Martin Felices,* Valarie McCullar,* Steven R. Presnell,† Ahmad Al-Attar,‡ Charles T. Lutz,‡,§ and Jeffrey S. Miller*

MicroRNAs (miRs) have recently been identified as important regulators of gene expression at the posttranscriptional level. Although it has clearly been established that miRs influence the ontogeny of several immune cell lineages, the role of individual miRs during NK cell development has not been described. In this study, we show that miR-181 expression levels have a profound impact on the development of human NK cells from CD34+ hematopoietic progenitor cells and IFN-γ production in primary CD56+ NK cells. We also demonstrate that nemo-like kinase (NLK), an inhibitor of Notch signaling, is a target of miR-181 in NK cells, and knockdown of NLK mirrors the developmental effect of miR-181 overexpression. We conclude that miR-181 promotes NK cell development, at least in part, through the suppression of NLK, providing an important link between miRs and Notch signaling.

**Materials and Methods**

* NK cell and CD34+ hematopoietic progenitor cell isolation

The use of all human tissue was approved by the Committee on the Use of Human Subjects in research at the University of Minnesota (Minneapolis, MN), and informed consent was obtained in accordance with the Declaration of Helsinki. NK cells were magnetically isolated from peripheral blood through negative selection (StemCell Technologies, Vancouver, BC, Canada). CD34+ hematopoietic progenitor cells (HPCs) were isolated from umbilical cord blood by double-column positive selection using anti-CD34 microbeads (Miltenyi Biotec, Auburn, CA).

**Retroviral transductions and in vitro NK cell cultures**

Scramble miR, full-length precursor miR-181a/b, miR-181a antisense knockdown, miR-181b antisense knockdown (System Biosciences, Mountain View, CA), small interfering RNA (siRNA) scramble, and nemo-like kinase (NLK) siRNA (Open Biosystems, Huntsville, AL) lentiviral vectors were used to transduce purified CD34+ cells. GFP+ cells were isolated by FACS and cultured for 28 d on the EL08-1D2 stromal line (8). The culture media and cytokines for NK cell differentiation are published (9). miR vectors were also used to transduce peripheral blood NK cells and NK92 cells. GFP+ cells were isolated by FACS. Peripheral NK cells were cultured for 14 d in the presence of 10 ng/ml IL-15 (R&D Systems, Minneapolis, MN). NK92 cells were cultured in alpha medium containing 12.5% FCS, 12.5% horse serum (HyClone Laboratories, Logan, UT), 0.1 mM 2-ME, 100 U/ml penicillin, 100 U/ml streptomycin (Invitrogen, Carlsbad, CA), and 500 U/ml recombinant human IL-2 (Chiron, Emeryville, CA).

**Functional assay for IFN-γ production in NK cells**

Transduced peripheral blood NK cells were treated overnight with or without 1 μg/ml IL-12 and 10 μg/ml IL-18. Cells were then analyzed for the expression of GFP, CD56, and IFN-γ.

**Flow cytometry and Abs used**

Cell sorting was performed on the FACSARia (BD Biosciences, Franklin Lakes, NJ), and phenotypic analysis was performed on the FACSCalibur (BD Biosciences, Mountain View, CA). The Abs used in this study were allophycocyanin-conjugated NCAM13.2 (CD56) and CD34, PE-conjugated CD56, NKG2A, and B220 (BD Biosciences) or LSR II (BD Biosciences) using CellQuest Pro Software (BD Biosciences). The Abs used in this study were allophycocyanin-conjugated NCAM13.2 (CD56) and CD34, PE-conjugated CD56, NKG2A, and CD16, allophycocyanin-Cy7–conjugated CD117, FITC-conjugated CD94.
For miR quantitative RT-PCR (qRT-PCR), cDNA was synthesized with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA), and primer/probe sets for miR-181a, miR-181b, and RNU-24 were purchased from Applied Biosystems. qRT-PCR for Hes5 and NLK were performed with the SYBR Green reagent (Applied Biosystems). All primer/probe sets were purchased from Applied Biosystems except for NLK. NLK forward primer was 5'-CAGCCCATATTTCCATCACC-3', and the NLK reverse primer was 5'-GACAACACAAAGGCCTCAGA-3'.

Western blot analysis
Western blots for NLK protein were performed with a rabbit anti-NLK polyclonal Ab (Millipore, Billerica, MA) at a 1:5000 dilution in 3% milk w/v overnight. A donkey ECL anti-rabbit IgG, HRP-linked whole Ab (GE Healthcare, Buckinghamshire, U.K.) was used for the secondary staining at a 1:5000 dilution in 3% milk for 3 h. Densitometric analysis was performed using an Alphaimager Gel Documentation system (ProteinSimple).

Results
miR-181 levels determine the efficiency of human NK cell development
Validated scramble, miR-181a, and miR-181b knockdown and miR-181a/b overexpression lentiviral constructs were transduced separately into primary CD34+ HPCs, which were subsequently differentiated along the NK cell lineage for 28 d. Knockdown of either miR-181a or miR-181b led to an ∼3-fold decrease in the percentage of mature CD56+ NK cells. In contrast, overexpression of miR-181a/b resulted in a 2-fold increase in CD56+ NK cells at day 28 compared with scramble controls (Fig. 1A, 1B). A similar defect in NK cell development was seen when analyzing other markers of differentiated NK cells (NKG2A and CD16) in miR-181a and miR-181b knockdown cultures (Supplemental Fig. 1). These results were not due to differences in proliferation between cultures, as the total cell counts throughout development were comparable (Supplemental Fig. 2B). To confirm that the results observed in Fig. 1 were not simply due to miR-181a or miR-181b affecting the expression of the CD56 protein, we transduced primary CD56+ cells from the peripheral blood of healthy donors with each expression construct and cultured the cells for 14 d. Knockdown or overexpression of miR-181 had no impact on CD56 expression, proliferation, or survival in these cells (Supplemental Fig. 2A). Collectively, these results suggest that the expression of miR-181a and miR-181b are important for the differentiation of precursors through the NK cell lineage.

Overexpression of miR-181a/b enhances IFN-γ production in primary CD56+ NK cells
The observation that overexpression of miR-181a/b promotes NK cell development led us to hypothesize that miR-181a/b...
could also enhance NK cell function. To test this hypothesis, we transduced primary peripheral blood CD56+ NK cells with the scramble control or the miR-181a/b overexpression vector. Overexpression of miR-181a/b significantly enhanced IFN-γ production by NK cells in response to cytokine stimulation compared with scramble controls (58 ± 12% versus 13 ± 2% SEM; \( p = 0.003 \)) (Fig. 1C). In this setting, the enhanced function is analogous to the enhanced activation seen in T cells in which miR-181 targets phosphatases (10).

**miR-181a and miR-181b transcript levels increase during NK cell development**

We isolated stage 1 NK cell progenitors (CD34+CD117−CD94−CD56−), stage 2 progenitors (CD34+CD117+CD94−CD56−), stage 3 progenitors (CD34−CD117+CD94−CD56lo−), and lineage-committed CD56+ NK cells from umbilical cord blood (11). Consistent with the observation that high expression levels of miR-181a or miR-181b are associated with efficient NK cell development (Fig. 1A, 1B), we observed a steady increase in miR-181a (Fig. 2A) and miR-181b (Fig. 2B) mature transcript levels throughout normal NK cell development, providing physiologic relevance to our findings.

**FIGURE 2.** The expression levels of miR-181a and miR-181b increase throughout NK cell development. qRT-PCR analysis of miR-181a (A) and miR-181b (B) levels throughout NK cell development (n = 4). Results are shown as the mean expression values normalized against stage 1. Error bars represent SEM, and statistical significance was determined by one-way ANOVA. *\( p < 0.05 \), **\( p < 0.01 \).
NLK is an miR-181 target in NK cells

A combined database search using miRGator (http://genome. ewha.ac.kr/miRGator/miRGator.html) yielded 951 unique, annotated targets shared by miR-181a and miR-181b. We searched this list for genes with potential relevance to NK cell development and identified NLK, which has a 3’ untranslated region site with strong alignment scores for predicted miR-181a and miR-181b binding. NLK is an evolutionarily conserved protein kinase that negatively regulates Notch-dependent transcriptional activation by decreasing the formation of its ternary complex (12). Because we and others have shown that Notch signaling supports human NK cell development (13, 14), we hypothesized that miR-181a and miR-181b promote NK cell development by downregulating a negative regulator of Notch signaling. To establish that NLK is a target of miR-181 in NK cells, we knocked down miR-181a and miR-181b individually in NK92 cells and measured NLK protein expression by Western blot. Knockdown of either miR-181a or miR-181b led to a substantial increase in NLK protein levels (Fig. 3A, 3B), supporting a role for miR-181 in the negative regulation of NLK expression in NK cells. miR-181 has previously been implicated in the regulation of NLK expression in hepatic cells (15).

Knockdown of NLK enhances human NK cell development

A corollary of our hypothesis that miR-181a and miR-181b promote NK cell development by targeting NLK is that knockdown of NLK will enhance NK cell development. Following this reasoning, we used RNA interference technology to successfully knock down NLK expression, as determined by Western blot analysis (Fig. 4A). CD34+ HPCs were transduced with either scramble siRNA or NLK siRNA lentiviral vectors and differentiated along the NK cell lineage for 28 d. Knockdown of NLK led to an increase in NK cell development compared with scramble controls as measured by the percentage of GFPþ cells that acquired CD56 in culture (74.9 ± 7.6% versus 48.1 ± 5.4% SEM; p = 0.008) (Fig. 4B), mirroring the phenotype observed with overexpression of miR-181a or miR-181b (Fig. 1A, 1B).

Knockdown of NLK correlates with an increase in the expression of the Notch target Hes5

To answer the question of whether NLK knockdown enhances signaling through the Notch pathway, we analyzed Hes5 transcript levels in developing NK cells expressing the siRNA targeting NLK that were sorted out of culture at day 28. Compared to scramble controls, we observed ~3-fold more Hes5 transcript in NK cells harboring the small interfering NLK expression vector (Fig. 3A). These results support the conclusion that miR-181 influences NK cell development by regulating the Notch pathway.

Discussion

The development of HPCs into distinct blood lineages is controlled by a complex set of molecular events that must be coordinately regulated to facilitate proliferation, commitment, and maturation simultaneously. The recent discovery of miRs adds a new layer to this complexity, as several miRs have unique expression patterns in cells of the innate and adaptive immune systems (2). Ectopic expression of miR-181a in mouse progenitor cells leads to a substantial increase in the B cell compartment (16), and high levels of miR-181a are necessary for progression through the double-positive stage of thymocyte development (17). In this study, we show that miR-181 family members are also important for the development of NK cells. Therefore, miR-181 contributes to the development of all three lymphoid lineages.

Our data suggest that miR-181 promotes the development of human NK cells, at least in part, through downregulation of NLK, which has the effect of increasing Notch activity. We have previously shown that Notch signaling is important in NK cell development (14), but miR-181 targeting of NLK points to a cell-intrinsic effect in the modulation of Notch signaling. Cell-intrinsic modulation of Notch signaling is necessary as earlier stages of NK cell development require Notch signals, but these signals are detrimental to development at later stages. Given the availability of Notch ligands in the bone marrow compartment during development, the maturing NK cells must be capable of shutting down Notch signals in a cell-intrinsic manner. In addition, we show that expression of miR-181 also enhances IFN-γ production in NK cells, possibly through regulation of the Notch pathway. The production of IFN-γ is a tightly controlled process that appears to involve multiple miRs including miR-29 (18).

NLK also functions within the Wnt signaling pathway to antagonize canonical Wnt/β-catenin signaling (19), and pharmacological inhibition of this pathway inhibits NK cells development in vitro (20). We analyzed several genes that are validated targets of the Wnt pathway (21) in NK92 cells with knockdown or overexpression of miR-181 and observed a moderate effect on their expression levels that did not reach statistical significance in all cases (Supplemental Fig. 2C), which warrants further study.

This report demonstrates the importance of miR-181 expression during NK cell development. It is becoming increasingly clear that NK cell development is tied to a complex process of NK cell commitment, acquisition of NK cell receptors, and ultimately the acquisition of function that will be needed to protect against infection and cancer. Our data show that miR-181 directly regulates the developmental process at least in part through interaction with Notch pathways.

Disclosures

The authors have no financial conflicts of interest.

References


