

ORIGINAL ARTICLE

# Human natural killer cell microRNA: differential expression of *MIR181A1B1* and *MIR181A2B2* genes encoding identical mature microRNAs

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Natural killer (NK) and T lymphocytes share many properties, yet only NK cells respond rapidly to infection and cancer without pre-activation. We found that few microRNAs (miRNAs) differed significantly between human NK and T cells. Among those miRNAs, miR-181a and miR-181b levels rose during NK cell differentiation. Prior studies indicate that miR-181a and miR-181b are critical for human NK cell development and are co-transcribed from genes on chromosome 1 (*MIR181A1B1*) and on chromosome 9 (*MIR181A2B2*). We mapped human *MIR181A1B1* and *MIR181A2B2* transcription start sites to 78.3 kb and 34.0 kb upstream of the mature miRNAs, generating predominantly unspliced transcripts of 80–127 kb and ~60 kb, respectively. Unlike mouse thymocytes, human T cells expressed both *MIR181A1B1* and *MIR181A2B2*. We tested the hypothesis that NK cells differentially transcribe the two genes during development and in response to immune regulatory cytokines. During NK-cell differentiation, *MIR181A2B2* expression rose markedly and exceeded that of *MIR181A1B1*. TGF- $\beta$  treatment increased NK-cell *MIR181A2B2* transcription, whereas IL-2, IL-15 and IL-12/IL-18 treatments upregulated *MIR181A1B1*. The *MIR181A2B2* promoter was strongly transactivated by SMAD3 and SMAD4 transcription factors, suggesting that TGF- $\beta$  signaling upregulates *MIR181A2B2* expression, at least in part, through SMAD-dependent promoter activation.

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## INTRODUCTION

Natural killer (NK) cell deficiency is associated with increased susceptibility to infections and cancer.<sup>1,2</sup> In contrast to T cells, NK cells rapidly kill infected and transformed cells without pre-activation.<sup>1,2</sup> They contain preformed cytotoxic granules that enclose perforin and various granzymes.<sup>1</sup> Upon activation, NK cells rapidly exocytose these granules that kill target cells. NK cells also secrete various chemokines and cytokines, including IFN- $\gamma$ , that directly affect infected and transformed cells and promote adaptive immune responses.<sup>1,2</sup>

Human NK cells are classified into two major subsets based on their CD56 surface expression. Most circulating blood NK cells are CD56<sup>dim</sup>, whereas 5–15% are CD56<sup>bright</sup>.<sup>1</sup> CD56<sup>bright</sup> NK cells are poorly cytotoxic, but secrete high levels of cytokines and chemokines in response to inflammatory cytokines, such as IL-12 and IL-18.<sup>1</sup> Although they respond poorly to inflammatory cytokines, CD56<sup>dim</sup> NK cells kill target cells and secrete chemokines and cytokines in response to antibody-coated cells and tumor cells.<sup>1,3</sup> CD56<sup>bright</sup> NK cells develop into CD56<sup>dim</sup> NK cells, and CD56<sup>dim</sup> NK cells continue to mature into subsets distinguished by CD94 expression, with CD56<sup>dim</sup>CD94<sup>hi</sup> cells being less mature than CD56<sup>dim</sup>CD94<sup>low</sup> cells.<sup>1,4</sup>

microRNAs (miRNA) constitute a class of ~22 nucleotide (nt) non-coding RNAs that bind to complementary sequences in the 3'-untranslated region of target mRNAs, decreasing mRNA stability and translation.<sup>5,6</sup> miRNAs are differentially expressed in hematopoietic tissue in which they regulate immune cell development,

activation, function and selection.<sup>5,6</sup> The human NK cell miRNA transcriptome has been characterized,<sup>7,8</sup> but to our knowledge, no one has further studied the miRNAs that are preferentially expressed in NK cells compared with the developmentally similar T cells. Although both NK cells and T cells are known to be heterogeneous, we thought that comparison of these two lymphocyte subsets would be a useful starting point.

NK cells share many developmental pathways and gene expression programs with T cells, but NK cells differ from most T cells in their gene-expression programs, their ability to kill tumor cells and virus-infected cells in the absence of specific immunization, and their state of 'constant readiness'.<sup>2,9–11</sup> Global miRNA processing defects affect NK-cell maturation, function and survival.<sup>12,13</sup> We found that miR-181a and miR-181b, which are important for T-cell development,<sup>14</sup> also affect NK-cell maturation and activity, at least in part, by regulating the Notch signaling pathway.<sup>15</sup>

miRNA genes are transcribed into large primary miRNAs (pri-miRNAs), which are cropped to hairpin-containing 60–110 nt precursor miRNAs (pre-miRNAs) and exported to the cytoplasm, where they are further processed to dsRNA. Then either the 5p or 3p strand of the dsRNA is retained in the silencing complex and regulates gene expression.<sup>5,6</sup> The 5p/3p ratio in expressed miRNAs varies considerably with tissue and developmental stage and both strands may be expressed at significant levels in the same cell.<sup>16,17</sup> Mature 5p miR-181a and miR-181b are both encoded by the human chromosome 1 *MIR181A1B1* gene. Interestingly, identical mature miR-181a and miR-181b also are encoded by the

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chromosome 9 *MIR181A2B2* gene. Even though the mature 5p forms are identical, the miR-181a-1 and miR-181a-2 3p strands are moderately expressed and have different seed sequences (<http://www.mirbase.org/>). Moreover, the pri-miRNAs and pre-miRNAs encoded by *MIR181A1B1* and *MIR181A2B2* are distinct. Liu *et al.*<sup>18</sup> presented evidence that pre-miR-181 transcripts have direct biological activities in addition to being precursors of mature miRNAs. Chen proposed that both pre-miRNA and pri-miRNA regulate gene expression.<sup>19</sup> Therefore, although the predominant mature 5p forms of miR-181a and miR-181b encoded at human chromosomes 1 and 9 are identical, the *MIR181A1B1* and *MIR181A2B2* loci might have distinct biological functions.

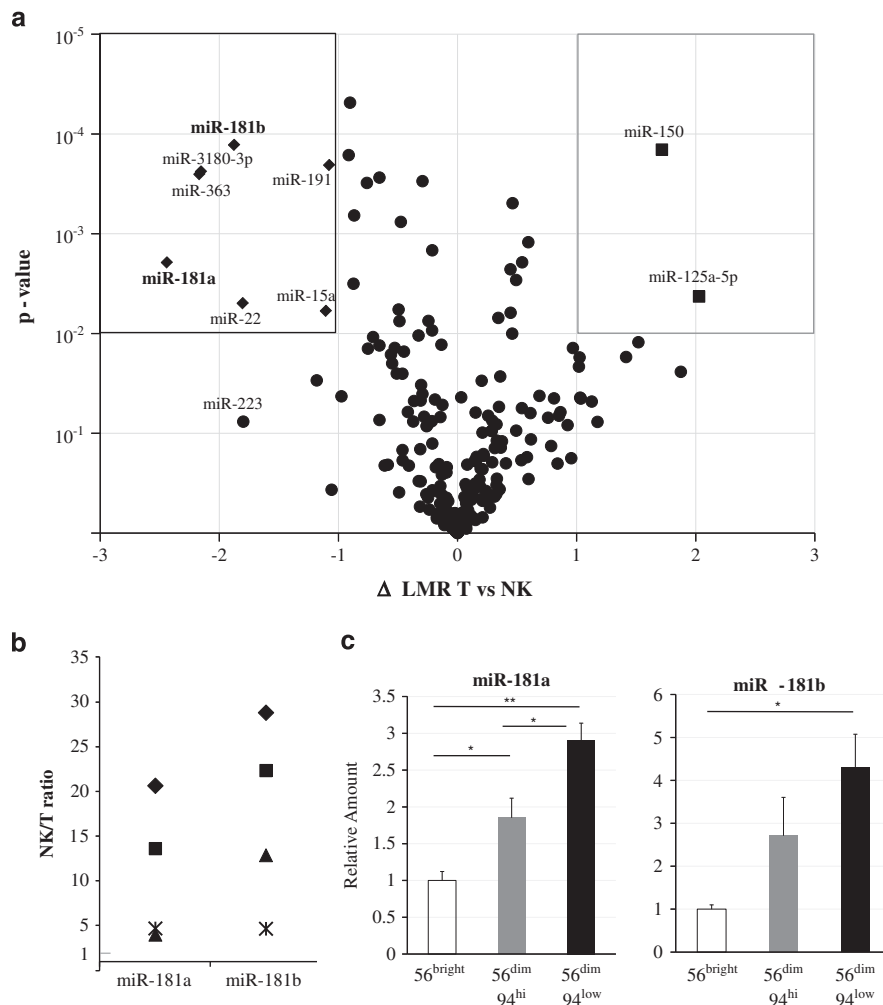
We compared miRNA expression by human blood NK and T lymphocytes. Overall, the NK- and T-cell miRNA transcriptomes were very similar, but a few miRNAs differed between these lymphocyte types. Focusing on two miRNAs preferentially expressed in NK cells, we explored how miRNA levels changed. Furthermore, we identified the *MIR181A1B1* and *MIR181A2B2*

transcription start sites (TSS) and promoters and we investigated how these loci were differentially regulated during human NK-cell development and in response to immune regulatory cytokines.

## RESULTS

miR-181a and miR-181b are differentially expressed in NK cells and T cells and rise during NK-cell development

miRNA processing machinery is important for NK-cell maturation, survival and function.<sup>12,13</sup> We compared the NK cell miRNA transcriptome with that of extensively investigated T cells using a microarray platform. RNA extracted from NK- and T-enriched lymphocyte populations from three healthy subjects were analyzed on a miRCURY locked-nucleic-acid array. Variations between T and NK samples were calculated as the difference between  $\text{Log}_2$  median ratios and were plotted against their statistical significance, shown as a 'volcano' plot (Figure 1a).



**Figure 1.** Differential miRNA expression. **(a)** miRNA expression in NK cells and T cells. The difference in  $\text{Log}_2$  median ratios ( $\Delta\text{LMR}$ ) between NK and T-cell groups is shown on the x axis against the significance of the difference ( $-\text{Log}_{10}$   $P$ -value of two-tailed  $t$ -test, corrected with Bonferroni's *post hoc* test) on the y axis. miRNAs with  $\text{log}_2$  changes of  $>1$  (higher in T cells) or  $<-1$  (higher in NK cells) as well as high statistical significance ( $P < 0.01$ ) are displayed as squares when higher in T cells (upper right) and diamonds when higher in NK cells (upper left). Note that two diamonds largely overlap. Also indicated is miR-223. **(b)** RT-qPCR data were normalized to RNU-24 levels and calculated as NK-to-T-cell ratio (1 = no difference) for the miRNAs listed on the x axis. NK- and T-cell RNA were analyzed from the three subjects studied above (represented as  $\blacklozenge$ ,  $\blacksquare$  and  $\blacktriangle$ ) and from a fourth subject (represented as  $\ast$ ). For each miRNA, T and NK cells differed significantly ( $P < 0.05$ ). **(c)** Differential miR-181a and miR-181b expression in NK-cell development. The indicated NK subsets were sorted to  $>95\%$  purity, and the miRNA, indicated in each panel, was quantified by RT-qPCR with RNU-24 as a standard and normalized to the level in  $\text{CD56}^{\text{bright}}$  NK cells. Differences between developmental stages are indicated:  $\ast P < 0.05$ ,  $\ast\ast P < 0.01$ . Data shown are Avg and s.e.m. of cells from three subjects, each sorted in a separate experiment.

miRNA expression overlapped considerably among T and NK cells, and < 4% of the miRNAs analyzed were both more than twofold different between T and NK cells and significant at a  $P$ -value of < 0.01 (Figure 1a boxed areas). miRNA transcriptome similarity reflects T and NK cell shared developmental pathways and functions.<sup>9–11</sup> Among the miRNAs that are preferentially expressed in NK cells, we chose to further study miR-181a and miR-181b, which are known to regulate NK-cell development.<sup>15</sup> Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) confirmed that miR-181a, miR-181b and other selected miRNAs were expressed at higher levels in NK cells than in T cells (Figure 1b, Supplementary Figure S1A). Although the ratios varied between subjects, miR-181a and miR-181b were always higher (up to 30-fold) in NK cells than in T cells (Figure 1b).

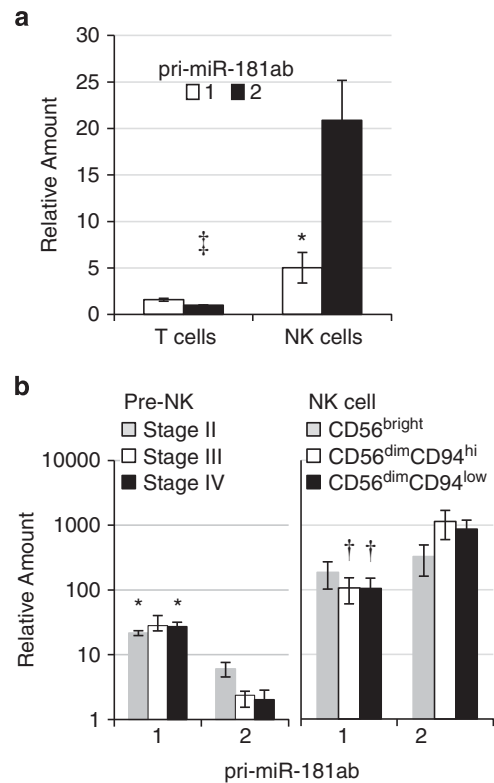
NK cells are heterogeneous, comprising functional and maturational subsets.<sup>1,4</sup> Using flow cytometry, we sorted NK cells into three groups according to their level of maturation: CD56<sup>bright</sup>, CD56<sup>dim</sup>CD94<sup>hi</sup> and CD56<sup>dim</sup>CD94<sup>low</sup>. We then examined the amount of miR-181a and miR-181b (Figure 1c) and other NK-enriched miRNAs (Supplementary Figure S1B) in each NK subset using RT-qPCR. miR-181a and miR-181b were lowest in the least mature CD56<sup>bright</sup> NK cells and highest in the most mature CD56<sup>dim</sup>CD94<sup>low</sup> NK cells (Figure 1c). The rise of miR-181a and miR-181b during late NK-cell maturation extends our previous finding that levels of these miRNAs increased during development from CD34<sup>+</sup> progenitor cells into CD56<sup>+</sup> NK cells.<sup>15</sup> Thus, miR-181a/b levels rise during the development from primitive hematopoietic precursors to late-stage NK cells.

NK and T cells differentially regulate the *MIR181A1B1* and *MIR181A2B2* loci

Because miR-181a and miR-181b control NK-cell development,<sup>15</sup> we asked how these miRNAs are themselves regulated. *MIR181a* and *MIR181b* are closely linked and are co-transcribed in the same pri-miRNA. miR-181 regulation is complex because human miR-181a and miR-181b are both encoded at the *MIR181A1B1* locus on chromosome 1 and the *MIR181A2B2* locus on chromosome 9. Mouse thymocytes express *Mir181ab-1*, but not *Mir181ab-2*.<sup>20</sup> To explore the use of *MIR181ab* loci in humans, we isolated blood T and NK cells and quantified pri-miR-181ab transcripts using two distinct RT-qPCR techniques, which gave virtually identical results. Unlike mouse thymocytes, freshly isolated human blood T cells expressed pri-miR-181ab-1 and pri-miR-181ab-2 at similar levels, with pri-miR-181ab-1 being ~1.6-fold more abundant (Figure 2a). In contrast to both mouse thymocytes and human T cells, human NK cells expressed chromosome 9 *MIR181A2B2* approximately fourfold higher than chromosome 1 *MIR181A1B1* (Figure 2a). Compared with T cells, NK cells expressed > 20-fold more pri-miR-181ab-2 from chromosome 9 and the pri-miR-181ab-1/-2 ratio was significantly different in T cells vs NK cells (Figure 2a). These results show differential pri-miR-181 regulation in human blood T and NK cells, and that elevated miR-181a and miR-181b levels in NK cells is due to a moderate upregulation of the chromosome 1 *MIR181A1B1* locus and marked upregulation of the chromosome 9 *MIR181A2B2* locus.

*MIR181A1B1* and *MIR181A2B2* expression during NK-cell development and activation

We wished to trace the activity of the two *MIR181ab* loci during NK-cell development. Freud *et al.* have divided pre-NK cell development into four stages, starting with CD34<sup>+</sup> primitive hematopoietic precursor cells and progressing through more mature stages that lose CD34 expression and eventually gain low-level CD56 expression.<sup>21</sup> We isolated Stage II–IV cells in sufficient numbers for analysis from the umbilical cord blood of three infants, allowing us to trace pri-miR-181 levels from CD34<sup>+</sup> precursors to CD56<sup>low</sup>–CD94<sup>+</sup> pre-NK cells. Chromosome 1



**Figure 2.** *MIR181A1B1* and *MIR181A2B2* are regulated independently. (a) Differential regulation in NK cells and T cells. RNA was assayed by RT-qPCR, and quantification cycle ( $C_q$ ) values were corrected with those of 18S rRNA or GAPDH, as described in Materials and Methods. The two RT-qPCR methods produced essentially identical relative amounts. Corrected  $C_q$  values were compared by  $t$ -test: pri-miR-181ab-1 vs pri-miR-181ab-2 for NK cells ( $*P < 0.03$ ); T vs NK cell for pri-miR-181ab-2 ( $\ddagger$ ,  $t < 0.02$ ). The pri-miR-181ab-1/-2 ratio was significantly different in T cells vs NK cells ( $t < 0.02$ ). For graphic presentation, corrected  $C_q$  values were converted to numerical values and normalized to the level of T-cell pri-miR-181ab-2. Avg and s.e.m. of three donors are presented. (b) Differential regulation during NK-cell development. Umbilical cord blood lymphocytes were sorted into Stages II–IV and adult blood lymphocytes were sorted into CD56<sup>bright</sup>, CD56<sup>dim</sup>CD94<sup>hi</sup> and CD56<sup>dim</sup>CD94<sup>low</sup> subsets. RNA was extracted and analyzed for pri-miR-181ab-1 and pri-miR-181ab-2 levels by RT-qPCR. Shown are Avg and SEM pri-miR-181 RNA levels relative to GAPDH RNA. All values were multiplied by 10 000 for display purposes.  $*P < 0.05$ ,  $^{\ddagger}P < 0.01$  for pri-miR-181ab-1 vs pri-miR-181ab-2 differences.

pri-miR-181ab-1 levels did not significantly change during Stages II–IV of pre-NK-cell development (Figure 2b). In contrast, chromosome 9 pri-miR-181ab-2 transcripts were low in Stage II cells and fell to very low levels during successive pre-NK-cell developmental stages. We then compared *MIR181ab* transcripts during mature NK-cell development. To facilitate comparison with the pre-NK cells, results are presented on the same scale relative to GAPDH RNA levels. In adult subjects, the level of chromosome 1 pri-miR-181ab-1 transcript was substantially higher in CD56<sup>bright</sup> NK cells, compared with pre-NK cells (Figure 2b). Chromosome 1 pri-miR-181ab-1 levels fell slightly during maturation into CD56<sup>dim</sup> NK cells. Chromosome 9 pri-miR-181ab-2 levels were ~100-fold higher in CD56<sup>bright</sup> NK cells than in pre-NK cells (Figure 2b). The pri-miR-181ab-2 transcript appeared to rise during adult NK-cell maturation and was significantly higher than the pri-miR-181ab-1 transcript in both CD56<sup>dim</sup>CD94<sup>hi</sup> and CD56<sup>dim</sup>CD94<sup>low</sup> NK cells (Figure 2b). Although the pre-NK-cell subsets isolated from cord blood and the NK-cell developmental stages

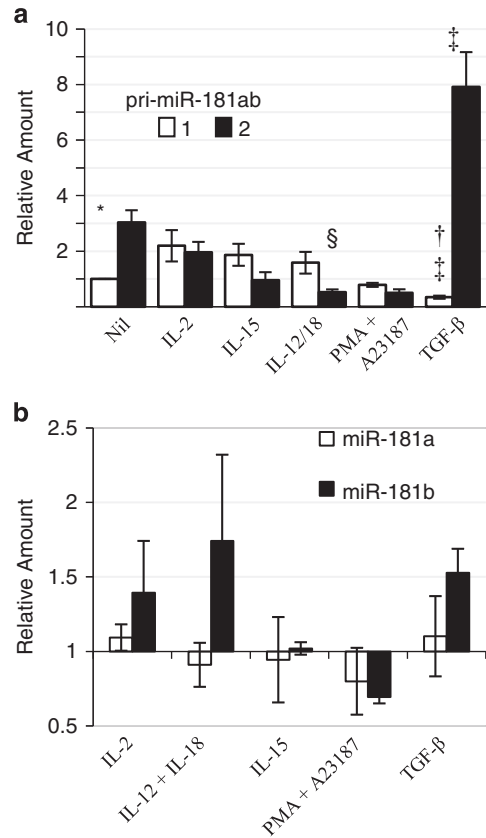
isolated from adult blood are not directly comparable, our results suggest that the *MIR181A2B2* locus is strongly activated as cells mature from pre-NK to CD56<sup>bright</sup>.

We next tested how immune regulatory cytokines affect the two *MIR181ab* loci. NK cells expressed relatively more of the chromosome 9 pri-miR-181ab-2 than the chromosome 1 transcript after overnight culture in culture medium alone. IL-2, IL-15 and IL-12/18 treatment tended to increase the chromosome 1 pri-miR-181ab-1 and tended to decrease the chromosome 9 pri-miR-181ab-2 so that the ratios were significantly different than those in culture medium alone (Figure 3a). In contrast, TGF- $\beta$  decreased the chromosome 1 transcript threefold and increased the chromosome 9 transcript 2.6-fold (Figure 3a). TGF- $\beta$  and the other stimuli did not significantly alter mature miR-181a and miR-181b levels, reflecting the opposing changes in pri-miR-181ab-1 and -2 levels (Figure 3b). Our results show that NK cells differentially regulate pri-miR-181ab in response to cytokines. Because pri-miRNA is upstream of Drosha and other RNA processing events, we propose that pri-miR-181 levels reflect differences in *MIR181A1B1* and *MIR181A2B2* transcription.

#### Mapping *MIR181A1B1* and *MIR181A2B2* transcriptional units

The *MIR181A1B1* and *MIR181A2B2* transcriptional units and their promoters have not been established, so we first performed RT-qPCR at intervals upstream and downstream of the mature miR-181 sequences to measure the extent of the *MIR181ab* transcriptional units. Figure 4 shows that RT-qPCR signals were relatively constant for several kb upstream and downstream of the mature miR-181a/b sequences on both chromosomes 1 and 9 in human NK cells. Variations in RT-qPCR signal strength may be due to unequal RNA degradation rates, differences in priming efficiency or RNA secondary structure formation affecting reverse transcription efficiency. Some unspliced pri-miRNAs are present at higher levels than their spliced counterparts<sup>22</sup> and the relative constancy of pri-miR-181 levels across the transcriptional units suggests that the major forms are unspliced transcripts. Levels of pri-miR-181ab-1 gradually fell 67-fold in the interval 2–49 kb downstream of the mature miRNA sequences (Figure 4a). This gradual decline suggests the absence of a single dominant RNA polymerase II termination site in *MIR181A1B1*. In contrast, pri-miR-181ab-2 levels fell >50-fold somewhere in the interval 21.5–29 kb downstream of the mature miR-181a/b-2-coding sequences (Figure 4c), consistent with a dominant *MIR181A2B2* RNA polymerase II termination site.

We 'walked upstream' until the RT-qPCR signal precipitously declined, consistent with a dominant TSS. Chromosome 1 pri-miR-181ab-1 RT-qPCR signal dropped >10 000-fold, ~78 kb upstream of the miR-181a/b-1-coding sequences (Figure 4a). Likewise, ~35 kb upstream of the mature miR-181a/b-2 sequences, RT-qPCR signal dropped ~100-fold (Figure 4c). Overlapping with the putative *MIR181A2B2* transcriptional unit on chromosome 9, the *NR6A1* gene is expressed at low level in many adult somatic tissues,<sup>23</sup> and is transcribed antisense relative to *MIR181A2B2*. Thus, the relatively small 100-fold drop-off upstream of the putative *MIR181A2B2* TSS is likely due to background NR6A1 RNA rather than weak *MIR181A2B2* promoter strength. Interestingly, the putative promoter sites indicated by our analysis are consistent with bioinformatic data. For both *MIR181ab* loci, the putative promoters localized with sites of DNase I hypersensitivity, FAIRE clusters,<sup>24</sup> chromHMM hot spots<sup>25</sup> and chromatin immunoprecipitation of transcription factors, acetylated histone H3K27, and trimethylated histone H3K4, all of which are associated with TSS (data not shown). No other high-likelihood promoter elements were located in the interval between the putative TSS and the miR-181a/b coding sequences (data not shown). We more finely mapped the region just upstream of the mature miR-181a sequence on chromosome 1 because another group had cloned

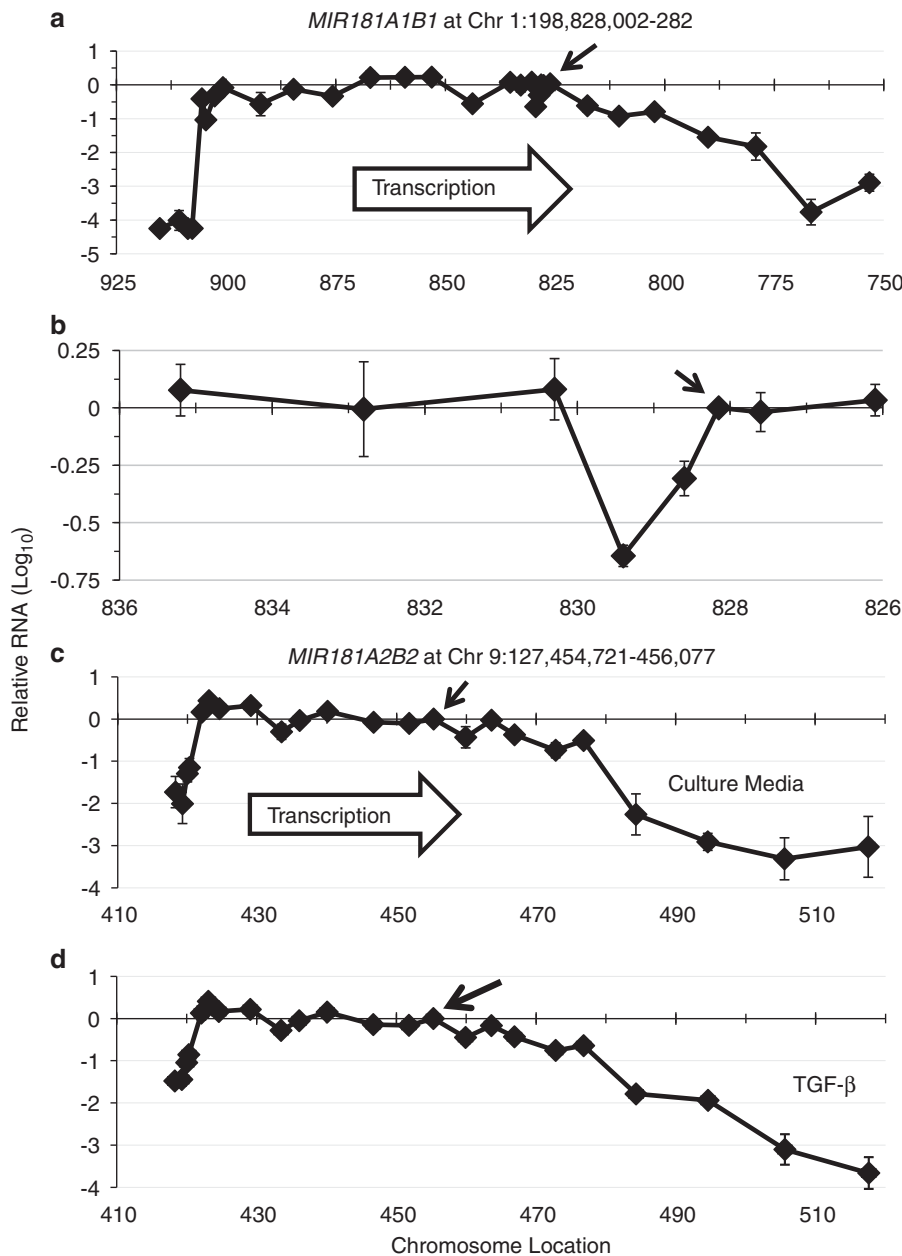


**Figure 3.** Primary miR-181ab-1 and miR-181ab-2 levels change in response to immune regulatory cytokines. Blood NK cells were cultured overnight in the presence of media alone (Nil) or the indicated stimulants. Avg and s.e.m. of three donors are presented. **(a)**  $C_q$  values were corrected with those of 18S rRNA. Statistical significance is indicated: pri-miR-181ab-1 and -2 differ under the indicated conditions (\* $P < 0.05$ ; † $P < 0.01$ ); the pri-miR level differs between media alone and the indicated stimulation (‡ $P < 0.05$ ; § $P < 0.01$ ). Compared with media alone, the ratio of pri-miR-181ab-1 vs -2 was significantly different for all stimulations (IL-12/IL-18,  $P < 0.05$ ; all others,  $P < 0.01$ ). For graphic presentation, corrected  $C_q$  values were converted to numerical values and normalized to the level of pri-miR-181ab-1 (Nil). **(b)** miRNA levels were assessed by RT-qPCR and were normalized to RNU-24 and to the unstimulated controls.

a putative promoter based on the assumption that 'the core promoter of miRNA is known to locate close to its pre-miRNA'.<sup>26</sup> RT-qPCR signal was 2.0–4.4-fold lower immediately upstream of the pre-miR-181a sequence, but this was within the range of variation that we saw elsewhere in the transcript (Figure 4b). Although we cannot exclude the possibility that a weak promoter lies immediately upstream of the pre-miR-181a-1 sequence, our data indicate that the major *MIR181A1B1* promoter lies much further upstream.

We tested whether TGF- $\beta$  stimulation induced transcription at alternative sites. Across the ~60 kb pri-miR-181ab-2 transcript, levels were 3–4-fold higher after TGF- $\beta$  stimulation than after incubation of NK cells in culture media alone (Figures 4c and d and data not shown). In TGF- $\beta$ -treated NK cells, there was a precipitous decrease in pri-miR-181ab-2 level precisely where the TSS was located in unstimulated NK cells (Figures 4c and d). Therefore, TGF- $\beta$  appears to be acting at the same chromosome 9 promoter that drives constitutive pri-miR-181ab-2 expression in NK cells. Likewise, *MIR181A1B1* appeared to use the same





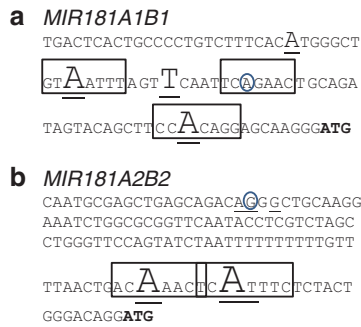
**Figure 4.** Primary miR-181ab-1 and -2 transcript mapping. Purified NK cells of three subjects were cultured overnight with culture media (**a–c**) or with TGF- $\beta$ . (**d**) Following reverse transcription, qPCR was performed using primers with sequences homologous to genomic DNA at the location shown by figure data points. For each subject, determination of relative RNA levels compared with the ‘middle primers’ located between the miR-181a and miR-181b-coding sequences (arrows) were calculated as: fold difference =  $2^{(Cq \text{ middle primer} - Cq \text{ specific primer})}$ . When no amplification was detected, the  $C_q$  was set to 40.0. The Avg and s.e.m. are presented on a  $\text{log}_{10}$  scale; some error bars are too small to be seen. The numbers in each graph indicate genomic position. In **a**, for example, ‘825’ indicates Chromosome 1:198 825 000. In **c**, ‘450’ represents Chromosome 9:127 450 000. (**a**, **b**) *MIR181A1B1*. (**b**) An expanded view of Chromosome 1:198 826 100-198 835 200, which highlights the region near pre-miR-181a. (**c**, **d**) *MIR181A2B2*. The amount of pri-miR-181ab-2 RNA in NK cells incubated in culture media (**c**) or with TGF- $\beta$  (20 ng  $\text{ml}^{-1}$ , **d**).

chromosome 1 TSS in both resting and TGF- $\beta$ -stimulated NK cells (Figure 4a and data not shown). Upon TGF- $\beta$  treatment, the chromosome 1 pri-miR-181ab-1 RNA level slightly fell across the transcript (data not shown).

To confirm and more precisely map the TSS locations, we carried out 5’RACE on RNA from freshly isolated blood NK cells using RT and PCR primers homologous to regions just downstream of the putative *MIR181ab* TSS (Supplementary Table 1, Figure 4). For *MIR181A1B1*, we identified several TSS near where a previously cloned cDNA had been mapped (Figure 5). These TSS are located 78.3 kb upstream of the mature miR-181a/b-coding

sequences. For *MIR181A2B2* (Figure 5), two major TSS localized to consensus sites for Initiators, core promoter elements that are often required for efficient gene transcription and are found in miRNA gene promoters.<sup>27,28</sup> We identified additional weaker *MIR181A2B2* TSS, one of which corresponds to the 5’ end of a previously cloned cDNA. These TSS are located 34.0 kb upstream of the mature miR-181a/b-coding sequences.

Previous work suggested the identification of a *MIR181A1B1* promoter just proximal of the pre-miR-181a sequence. Therefore, we performed 5’RACE using primers homologous to regions between or immediately upstream of the mature miR-181a/b-1



**Figure 5.** *MIR181A1B1* and *MIR181A2B2* TSS. RNA was harvested from the NK cells of two (*MIR181A2B2*) or three (*MIR181A1B1*) subjects and 5'RACE was performed. Underlined nt denotes the 5' end of RACE clones, with major TSS indicated by larger font size. Boxes enclose potential Initiator elements (consensus sequence, YYANWYY). Transcription direction is left to right. (a) Chr1:198 906 606–519 depicting *MIR181A1B1* TSS. The number of 5'RACE clones found (underlined letters from left to right) were 2 (A), 16 (A), 9 (T), 15 (A). The circled 'A' shows the 5' end of the previously reported NR\_040073 transcript. (b) Chr9:127 420 695–824 depicting *MIR181A2B2* TSS. The number of 5'RACE clones found (underlined letters from left to right) were 1 (A), 1 (G), 1 (G), 11 (A), 17 (A), and 1 (T). The underlined and circled 'G' refers to the beginning of the previously reported NR\_038975 transcript, which coincides with the start of a 5'RACE clone. For each sequence, an 'ATG' that was modified to 'TTG' in the luciferase reporter plasmids is shown as bold letters.

sequences using two different strategies (primers in Supplementary Table 1). Yet, no TSS were identified near the pre-miR-181a-coding sequence (data not shown). We conclude that the major *MIR181A1B1* TSS in human NK cells is located 78.3 kb upstream of the miR-181a/b-1 coding sequences.

*MIR181A1B1* and *MIR181A2B2* promoters

Next, we tested promoter function by cloning regions surrounding the *MIR181A1B1* and *MIR181A2B2* TSS. Sequences were placed into a Luciferase reporter vector and tested for promoter activity in NK and T-cell lines by transient transfection. Both full-length promoters (containing ~900 nt of *MIR181A1B1* and *MIR181A2B2* sequence, constructs 181-1 I and 181-2 I) produced strong transcriptional activity in YT NK cells and in other NK and T cell lines (Figures 6a and c). To further characterize the promoters, we created a series of 5' truncations (constructs II–V) and tested them for activity in YT NK cells. For both promoters, constructs I–III directed similar transcriptional activity, suggesting that the minimal promoter was contained within 201 and 301 nt regions upstream of the *MIR181A1B1* and *MIR181A2B2* major TSS, respectively. Further deletion of 5' sequences decreased transcriptional activity, suggesting that the regions 51–201 nt and 150–301 nt upstream of the major TSS for the *MIR181A1B1* and *MIR181A2B2* genes, respectively, contained important transcription factor-binding sites (Figures 6a and b).

Because *MIR181A2B2* transcription was activated by TGF-β (Figure 3a), we asked if TGF-β acted on the promoter. TGF-β activation is often mediated through SMAD3 and SMAD4, which after ligand binding are recruited by the TGF-β receptor, are phosphorylated, and then enter the nucleus and bind to specific promoter sites.<sup>29</sup> We therefore tested whether 181-1 and 181-2 promoters were transactivated by SMAD3 and SMAD4 transcription factor expression plasmids. The 181-2 promoter-reporter Luciferase construct was strongly transactivated by SMAD3 and SMAD4 transcription factors, with relatively weaker activation of the 181-1 promoter-reporter Luciferase construct (Figure 6d).

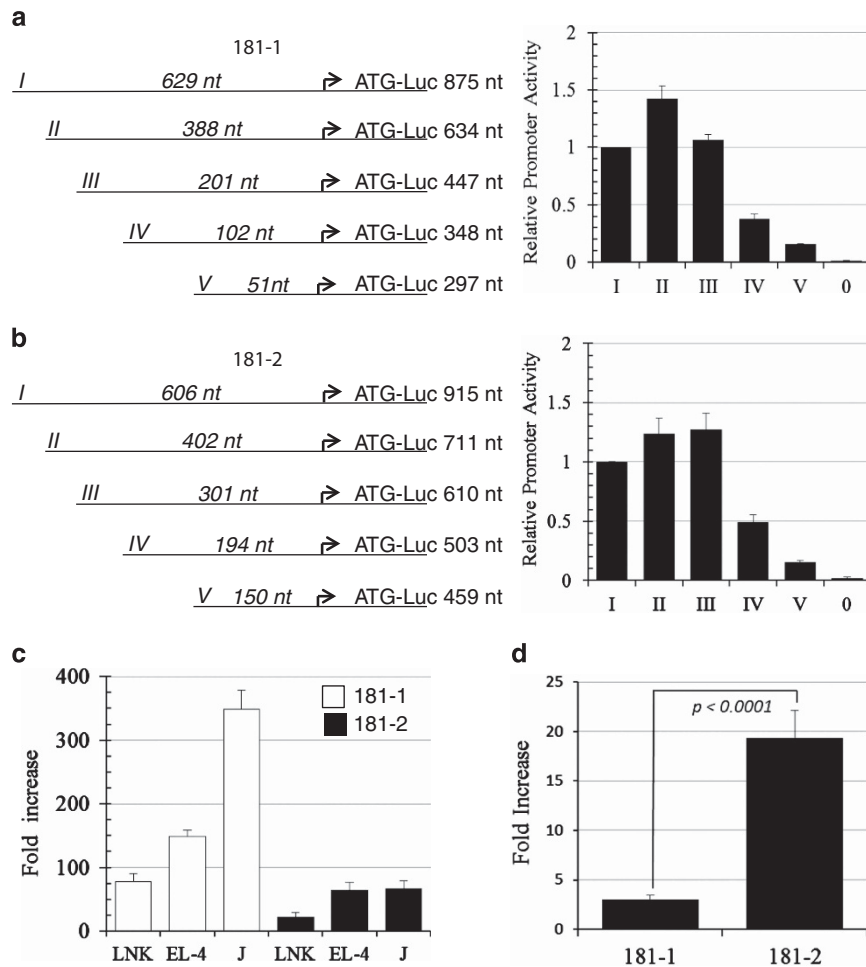
SMAD3/4 transactivated the 181-2 promoter 6.6-fold more than the 181-1 promoter (Figure 6d). This finding is consistent with the hypothesis that TGF-β signaling upregulates *MIR181A2B2* expression, at least in part, through SMAD-dependent promoter activation.

**DISCUSSION**

We showed that human blood NK cells and T cells share largely overlapping miRNA transcriptomes with <4% of the miRNAs significantly differing between the two lymphocyte types. It is likely that a few more miRNAs would have reached statistical significance had more subjects been analyzed and it is likely that expression of other miRNAs differs between specific T-cell subsets and NK cells as a whole or specific NK-cell subsets. Even with this consideration, a relatively small number of miRNAs differed between NK cells and T cells, which was anticipated because NK cells and T cells share developmental precursors, receptors and functions.<sup>9,10</sup> Because of the importance of miR-181 in NK-cell development and activation,<sup>15</sup> we investigated how miR-181a and miR-181b are regulated. miR-181a and miR-181b expression rose in concert with NK maturation from the least mature CD56<sup>bright</sup> NK cells through CD56<sup>dim</sup>CD94<sup>hi</sup> cells to the most mature CD56<sup>dim</sup>CD94<sup>low</sup> NK cells. Combined with our prior results, this shows that miR-181a and miR-181b expression levels rise from primitive hematopoietic progenitor cells through the most mature NK cells.<sup>15</sup> Other members of the miR-181 family, miR-181c and miR-181d, were below the limit of microarray detection in both NK cells and T cells. miR-181a and miR-181b are differentially expressed in hematopoietic cells and help to commit primitive cells to lineage differentiation.<sup>30</sup> Specifically, we showed that miR-181a and miR-181b are required for efficient NK development from primitive progenitor cells.<sup>15</sup> Notch signaling is needed for proper NK-cell development and is suppressed by Nemo-like kinase, which in turn is regulated by miR-181a and miR-181b.<sup>15,31</sup>

Mature miR-181a and miR-181b each are encoded by loci on chromosomes 1 and 9. Gene knockout of the *MIR181A1B1* locus in mice abolished mature miR-181a and miR-181b in the thymus, whereas knockout of *MIR181A2B2* did not change mature miR-181a and miR-181b levels.<sup>20</sup> This indicated that the *MIR181A2B2* locus is not significantly expressed by mouse thymocytes. Examining pri-miR-181ab by two different RT-qPCR strategies, we found that the chromosome 1 *MIR181A1B1* locus was expressed at levels similar to those of the chromosome 9 *MIR181A2B2* locus in human blood T cells. Therefore, the *MIR181ab* loci appear to be regulated much differently in mouse and human T lymphocytes. In human blood NK cells, pri-miR-181ab-2 levels were higher than pri-miR-181ab-1 levels and both were higher than in T cells. This finding is consistent with our microarray and RT-qPCR data showing that mature miR-181a and miR-181b levels were significantly higher in NK cells than in T cells. Treatment with immune regulatory cytokines showed that the two pri-miR-181ab loci were independently regulated in NK cells. In resting NK cells, pri-miR-181ab-2 was more highly expressed than pri-miR-181ab-1. However, the ratio was reversed after culture with several cytokines known to stimulate NK cells: IL-2, IL-15 and IL-12/IL-18. Interestingly, the immunosuppressive cytokine, TGF-β, accentuated the ratio by suppressing pri-miR-181ab-1 expression while boosting pri-miR-181ab-2 expression (summarized in Figure 7). TGF-β and other TGF family members increase miR-181 expression in a variety of cancer cell lines and contribute to metastasis.<sup>32,33</sup> Taylor et al.<sup>33</sup> showed that TGF-β treatment upregulated pre-miR-181a1 slightly and pre-miR-181a2 extensively in the MDA-MB-231 human breast cancer cell line.

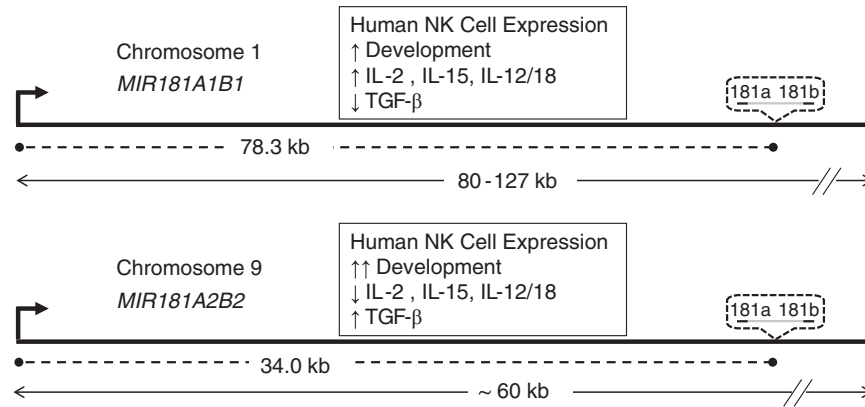
We had previously shown that miR-181a and miR-181b levels rise as cells develop through pre-NK cell stages into CD56<sup>+</sup> NK cells.<sup>15</sup> In the current work, we extend this finding to show that miR-181a and miR-181b levels continue to rise during NK-cell



**Figure 6.** Characterizing the *MIR181A1B1* and *MIR181A2B2* promoters. **(a, b)** Shown are schematic views of promoter truncation experiments. The arrow indicates the major TSS. Full length (I) and truncation fragments (II–V) of the 181-1 **(a)** and 181-2 **(b)** promoter were transfected into YT-HY NK cells and promoter activity was measured as described in Materials and Methods. The length of the promoter fragment 5' of the major TSS and the length of the total promoter fragment are shown. pXPG-181-1-I-Luc or pXPG-181-2-I-Luc produced luciferase values that were 70-fold and 45-fold above empty vector background. Values represent averages from tests of 2–4 different plasmid preparations over at least two different experiments. '0' indicates background level produced by the empty pXPG-Luc basic vector. **(c)** *MIR181* promoters are active in T and NK-cell lines (LNK, EL4, and Jurkat). The white and black bars indicate the 181-1 and 181-2 promoters, respectively. Fold increase refers to normalized luciferase activity compared with the empty pXPG-Luc basic vector. The experiment was done three times with two different plasmid preps each time; all Avg were significant above those of the empty vector ( $*P < 0.002$ ). **(d)** SMAD3/4 strongly transactivate the 181-2 promoter. HepG2 cells were transfected with pXPG-181-1-I-Luc and pXPG-181-2-I-Luc reporter plasmids, along with expression constructs for SMAD3 and 4, or empty vector. Fold increase refers to transactivation activity of SMAD3/4 divided by transactivation activity of empty vector. Shown is Avg and s.e.m. of five experiments.

differentiation, from CD56<sup>bright</sup> NK cells through more mature CD56<sup>dim</sup>CD94<sup>hi</sup> and CD56<sup>dim</sup>CD94<sup>low</sup> stages. The relatively smooth rise of mature miR-181a/b levels belies the marked changes in *MIR181A2B2* gene expression. The chromosome 9 gene was expressed at very low levels in pre-NK-cell development and then rose markedly by the time cells entered the CD56<sup>bright</sup> NK cell stage. During pre-NK-cell development, pri-miR-181ab1 was higher than pri-miR-181ab2. Although pri-miR-181ab1 levels rose as cells transitioned from Stage IV pre-NK cells into CD56<sup>bright</sup> NK cells, pri-miR-181ab2 levels rose even more, and were statistically higher than pri-miR-181ab1 levels in CD56<sup>dim</sup> mature NK cells. *MIR181* gene expression reflects the marked changes in phenotype that cells undergo as they transition from pre-NK cells into mature CD56<sup>+</sup> NK cells. miR-181a and miR-181b help cause this change by regulating the expression of nemo-like kinase and Notch signaling.<sup>15,31</sup> It will be of interest to investigate which transcription factors are responsible for the marked increase in *MIR181A2B2* gene expression.

We located TSS of the pri-miR-181ab loci by 'walking' upstream of the miR-181 mature sequences with a series of RT-qPCR primers. Although others have used Drosha knockdown or knockout to block pri-miRNA processing and thereby increase detection of pri-miRNAs,<sup>34</sup> we analyzed human NK cells without changing Drosha expression. This avoided artifacts that might be associated with the manipulation of miRNA processing. miRNA can be the products of spliced or unspliced primary transcripts.<sup>6,22</sup> For both *MIR181A1B1* and *MIR181A2B2*, pri-miRNA levels were relatively constant over many kb. This strongly suggests that the dominant forms of pri-miR-181ab1 and pri-miR-181ab2 are unspliced transcripts, as has been reported for other pri-miRNAs.<sup>22</sup> We confirmed and precisely mapped the *MIR181A1B1* and *MIR181A2B2* TSS using 5' RACE on freshly isolated NK cells with normal intact miRNA processing. DNA regions just upstream of the TSS cloned into luciferase reporter vectors demonstrated strong promoter activity in both NK cells and in T cells, consistent with these regions being promoters for the transcriptional units in



**Figure 7.** Schematic diagram showing the distances (dashed lines) between human *MIR181A1B1* and *MIR181A2B2* TSS (bent arrows) and mature miRNA-coding sequences (dotted bubbles) and length (double arrow lines) of the pri-miRNAs. Distances are not drawn to scale. Summarized is human NK cell differential regulation of *MIR181A1B1* and *MIR181A2B2* gene expression in response to developmental signals and immune regulatory cytokines (boxes).

these cell types (Figures 6a and c). In co-transfection experiments, the *MIR181A2B2* promoter was strongly transactivated by SMAD3 and SMAD4 transcription factors, suggesting that TGF-β treatment upregulated *MIR181A2B2* transcription by direct or indirect SMAD-dependent activation of the *MIR181A2B2* promoter.

Other investigators started with the assumption that miRNA core promoter elements are located close to the presumptive 110 nt pre-miR-181a-1 DNA sequence. A 600 nt segment immediately upstream of the pre-miR-181a-1 DNA sequence had significant promoter activity in squamous cell carcinoma cells and in embryonic kidney cells and bound C/EBPα isoforms *in vitro*.<sup>26,35</sup> However, these authors did not provide any independent evidence that pri-miR-181ab-1 transcription initiated in this region. We cannot rule out the possibility that this site or other sites can be used as alternative promoters in NK cells or other cells, but our data indicate that the major promoters in NK cells are 78.3 and 34.0 kb upstream of the mature miR-181a/b sequences on chromosomes 1 and 9, respectively (shown schematically in Figure 7). It is remarkable that 22-23 nt gene products are produced from such large primary transcripts, 80-127 kb for pri-miR-181ab-1 and ~60 kb for pri-miR-181ab-2. However, large sizes have been reported for other pri-miRNAs.<sup>28</sup>

Several bioinformatic algorithms have been used to predict *MIR* TSS.<sup>28</sup> For example, a recent described algorithm predicted five potential TSS for *MIR181A2B2*, distanced 7.8, 33.0, 33.5 and 34.0 kb upstream of the miR-181a-coding sequence<sup>36</sup> and an earlier algorithm predicted a TSS 33.7 kb upstream of the coding sequence.<sup>37</sup> Several of these predicted sites approximate the 34.0 kb distance that we identified by 'chromosome walking' and 5'RACE. In contrast, forecast of the *MIR181A1B1* TSS was less successful. One algorithm<sup>36</sup> listed possible TSS at 2.4, 28.6, 44.8, 47.1 and 47.4 kb upstream of the miR-181a-coding sequence, all very different than the TSS that we identified 78.3 kb upstream of the coding sequence; the other algorithm did not predict a *MIR181A1B1* TSS.<sup>37</sup> Our results show that although bioinformatic information can be quite useful, it does not replace experimental verification. In our view, this requires 'chromosome walking' to approximate the TSS, which is then precisely located with 5'RACE or comparable technique. Confirmation of the same TSS under different stimulatory or developmental conditions further strengthens its identification.

Given that the chromosome 1 and 9 sites produce identical 23 nt miR-181a and miR-181b mature 5p species, is there biological significance to the differential regulation of *MIR181A1B1* and *MIR181A2B2*? There are at least two ways in which pri-miR-181ab-1 and -2 might have different effects. First, the 5p/3p

ratio in expressed miRNAs varies considerably with tissue and developmental stage and both strands may be expressed at significant levels in the same cell.<sup>16,17</sup> RNA-processing machinery favors the 5p form of both miR-181a and miR-181b, but a significant amount of the 3p strand is selected from both pre-miR-181a-1 and pre-miR-181a-2 in NK cells (data not shown). Unlike the 5p strands, the 3p strands differ between miR-181a-1 and miR-181a-2, including the nt 2-8 seed sequences that dictate target mRNA selection: 5' ACCA(UC/CU)GACCGUUGA(U/C)UGU ACC. Second, there is increasing evidence that regions outside the mature miRNA 22-23 nt sequence regulate mRNA.<sup>19</sup> Mutation and swapping miR-181a-1 and miR-181c sequences suggested that loop nt and other pre-miRNA sequences influence miRNA biological action unrelated to mature miRNA production.<sup>18</sup> Interestingly, knockout of the mouse *MIR181A2B2* locus, but not of *MIR181A1B1* locus, affected the rate of embryonic stem cell self renewal, despite expression of *MIR181A1B1*, *MIR181A2B2* and *MIR181CD* loci in WT cells.<sup>19</sup> Therefore, the differential expression of pri-miR-181ab-1 and -2 may influence cell physiology other than simply by dictating levels of the predominant 5p form of the mature miRNA.

We have demonstrated differential control of two genes that both encode mature 5p forms of miR-181a and miR-181b. Given that differential gene expression is found in both mice and humans, this begs the question of what use the dual gene system has for cells, including lymphocytes. Future research should investigate gene regulatory roles of pri-miRNA and pre-miRNA. In addition, we plan to investigate how *MIR181A1B1* and *MIR181A1B1* promoters are controlled by developmental signals and immune regulatory cytokines.

## MATERIALS AND METHODS

### Cell isolation and stimulation

Tissues and informed consent were obtained in accordance with the guidelines of the Human Subjects Institution Review Board. NK cells and T cells were isolated from heparinized adult blood by negative selection using RosetteSep Human NK Cell Enrichment and Human T Cell Enrichment cocktails, respectively (Stemcell Technologies, Vancouver, British Columbia, Canada), as per manufacturer's instructions. The purity of the isolated NK and T-cell populations were consistently >90% and >98%, respectively. Umbilical cord blood was obtained from the Memorial Blood Bank (Minneapolis, MN, USA), Placental Blood Program of the New York Blood Center (New York, NY, USA), St Louis Cord Blood Bank (St Louis, MO, USA), or local obstetric units. Mononuclear cells were isolated by density gradient centrifugation, and CD34<sup>+</sup> hematopoietic cells were purified using the MACS magnetic bead selection system (Miltenyi Biotech,



Bergisch Gladbach, Germany). Primary NK cells were cultured in NK complete medium (RPMI 1640, 10% fetal bovine serum, amino acids, antibiotics and other additives, Lonza Life Sciences, Ann Arbor, MI, USA and HyClone, South Logan, UT, USA).

For flow cytometry analysis and cell sorting of adult blood, fluorescently-labeled antibodies to the following antigens were used, CD3 (UCHT1), CD16 (3G8) CD56 (HCD56) and CD94 (DX22; BioLegend, San Diego, CA, USA); for cord blood, CD34 (581), CD117 (104D2), CD94 (HP-3D9), CD3 (UCHT1), and CD56 (NCAM16.2) (BD Biosciences, Franklin Lakes, NJ, USA). NK cells ( $1 \times 10^6$ ) were incubated in 1 ml of NK complete medium for 24 h at 37 °C in 5% CO<sub>2</sub> with IL-2 (100 U ml<sup>-1</sup>, Biological Resources Branch, National Cancer Institute, Frederick, MD, USA), IL-12 (10 U ml<sup>-1</sup>, Peprotech, Rocky Hill, NJ, USA) plus IL-18 (100 ng ml<sup>-1</sup>, R&D Systems, Minneapolis, MN, USA), IL-15 (100 ng ml<sup>-1</sup>, BioLegend), TGF-β (10 ng ml<sup>-1</sup>, R&D Systems), or PMA (10 ng ml<sup>-1</sup>, Enzo, Farmingdale, NY, USA) plus the calcium ionophore, A23187 (250 ng ml<sup>-1</sup>, Sigma-Aldrich, St Louis, MO, USA).

Mononuclear cells or purified NK cells were incubated with human IgG (Sigma-Aldrich) to block Fc receptors, and then stained with antibody to CD3, CD16 and CD56. Stained samples were read on an LSR-II cytometer (BD Biosciences) and data were analyzed using FlowJo software (Treestar, Ashland, OR, USA). NK-enriched samples were sorted into CD3<sup>+</sup>CD56<sup>bright</sup>CD16<sup>-</sup>, CD3<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup>CD94<sup>hi</sup> and CD3<sup>+</sup>CD56<sup>dim</sup>CD16<sup>+</sup>CD94<sup>low</sup> populations using MoFlo Legacy High-Speed Cell Sorter (Beckman Coulter, Brea, CA, USA). For cord blood, antibody-stained NK cell precursors were sorted using a FACS Aria (BD Biosciences) based on their developmental stage: Stage I (CD3<sup>+</sup>CD34<sup>+</sup>CD117<sup>-</sup>CD56<sup>-</sup>CD94<sup>-</sup>), Stage II (CD3<sup>+</sup>CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>-</sup>CD94<sup>-</sup>), Stage III (CD3<sup>+</sup>CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>-</sup>CD94<sup>-</sup>) and Stage IV (CD3<sup>+</sup>CD34<sup>+</sup>CD117<sup>+</sup>CD56<sup>low</sup>-CD94<sup>-</sup>). The numbers of Stage I cells recovered were insufficient for miRNA analysis.

#### miRNA analysis

RNA was extracted from NK- and T-enriched lymphocyte populations from three subjects using standard TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and analyzed on a miRCURY locked nucleic-acid microarray (Version 11.0; Exiqon; Vedbaek, Denmark) consisting of control probes, mismatch probes and 1769 capture probes complementary to human, mouse, rat and viral sequences from the miRBase v.14.0 release. miRNA levels were calculated based on normalized Hy3 labeling signals (log<sub>2</sub> transformed) from all hybridizations using image analysis software. Only miRNAs with six valid measurements were analyzed. Microarray data were deposited with GEO, Accession # GSE61215. Mature miRNA levels were quantified in RT-qPCR using the appropriate TaqMan primer/probe sets and the TaqMan PCR Mastermix (Applied Biosystems, Foster City, CA, USA) in a modification of the manufacturer's instructions. In brief, RNA (25 ng) was reverse transcribed in a 10 μl reaction containing 1 × RT buffer, dNTPs (each at 0.375 mM), 5 U μl<sup>-1</sup> MultiScribe reverse transcriptase, 50 nM stem-loop RT primers and 0.38 U μl<sup>-1</sup> RNase inhibitor (Applied Biosystems) at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min. Real-time PCR amplifications were performed in triplicate in 10 μl volumes. Quantitative miRNA expression data were acquired and analyzed on Applied Biosystems 7000 and 7500 instruments, using RNU-24 small nucleolar RNA as an endogenous control. Control and target miRNA standard curves were generated in each experiment.

#### pri-miR-181 assay

RNA was harvested with TRIzol, DNase I-treated (1 U μg<sup>-1</sup>, Promega, Madison, WI, USA), and reverse transcribed with random octamers and oligo(dT) primers (RNA to cDNA High Capacity Kit, Invitrogen) at 37 °C for 60 min. pri-miR-181 was assayed with two methods, which produced virtually identical results. In the first method, qPCR was carried out using primers (Supplementary Table 1) at 500 nM in 1 × SsoAdvanced SYBR Green Mix (Bio-Rad, Hercules, CA, USA) on a CFX96 real-time qPCR instrument (Bio-Rad), using GAPDH mRNA as an endogenous control, with initial melting at 95 °C (3 min), then 95 °C (5 s), 64.5 °C (30 s) for 40 cycles. The second method used *hsa-mir-181a-1* and *-2* TaqMan Pri-miRNA Assays (Life Technologies, Carlsbad, CA, USA) including proprietary primers and TaqMan probes, as per the manufacturer's instructions, using 18S rRNA as an endogenous control. Thermal cycling conditions were: initial melting at 95 °C (10 min), then 95 °C (15 s), 60 °C (60 s) for 40 cycles.

#### TSS mapping

TSS were identified using the RLM-RACE (Ambion, Waltham, MA, USA) kit followed by cloning 5' rapid amplification of cDNA ends (5'RACE) products

into the pCR4-TOPO vector. In brief, DNase I-treated RNA was phenol:chloroform:isoamyl alcohol (25:24:1, Invitrogen) extracted and treated with calf alkaline phosphatase (Ambion). Reverse transcription of the RNA adapter-tagged sample was primed using gene-specific primers (sequences in Supplementary Table S1) using Superscript III (Invitrogen) at 50 °C, following manufacturer's instructions. A first PCR was carried as described above for 32 cycles using the 'outer' Ambion primer with the 1st nested gene-specific primer (Supplementary Table 1). A second (nested) PCR was carried out as above using the 'inner' Ambion primer with the 2nd nested gene-specific primer, at one of several annealing/extension temperatures (54–68 °C, 60 s) for 40 cycles. Products were purified using a Qiagen spin column and cloned into the pCR4-TOPO vector using the TOPO-TA cloning kit (Invitrogen). Sequencing was carried out by the Northwestern University sequencing facility (Chicago, IL, USA).

#### Promoter-luciferase constructs

Regions 879 and 915 nt in length located at 78.3 and 34.0 kb upstream of the 181-a/b-1 and 181-a/b-2 sequences, respectively, were cloned into the Kpn I/Hind III and Kpn I/NcoI sites of the pXPG-luciferase vector.<sup>38</sup> ATGs near the TSS were mutated to TTG (indicated in Figure 5) to eliminate potential translation start sites upstream of the luciferase sequence. Inserts were confirmed by DNA sequencing. Promoter regions were 5' truncated using PCR producing pXPG-181-1-Luc constructs of 634 nt, 447 nt, 348 nt and 297 nt (constructs II–V). Similarly, pXPG-181-2-Luc constructs of 711 nt, 610 nt, 503 nt and 459 nt were produced (constructs II–V, Supplementary Table 1).

YT-HY, Jurkat and LNK were cultured and transfected with plasmid mini-preps (Qiagen, Venlo, The Netherlands) using DEAE-dextran or electroporation, as previously described.<sup>39,40</sup> EL-4 (a kind gift from F Marti) was cultured and transfected in the same manner as Jurkat,<sup>40</sup> but using 950 uF and 280 V electroporation settings. HepG2 (a kind gift of B Spear) was cultured in 1:1 Ham's F-12: DMEM (Sigma, St Louis, MO, USA) supplemented with 10% Cosmic Calf Serum (HyClone), glutamine (2 mg ml<sup>-1</sup>, Invitrogen), and insulin (4 ug ml<sup>-1</sup>, Invitrogen). HepG2 cells (~2 × 10<sup>6</sup>) in individual 35-mm wells were transfected using Lipofectamine and Plus reagent (Invitrogen) with 1.0 μg of pXPG-181-1-Luc or pXPG-181-2-Luc promoter plasmid, 5 ng of control SV40-renilla plasmid (Promega) and 250 ng of CS2-Myc-SMAD3 plus 250 ng CS2-HA-SMAD4 expression constructs (a kind gift of T Woodruff) or 500 ng of an empty vector in place of the SMAD expression constructs. Manufacturer's instructions were followed, except that cells received fresh media at 3 and 24 h after transfection. We determined that SMAD3/4 variably decreased control renilla activity, and so we report SMAD3/4-induced MIR181 promoter activity without normalization. When results were normalized to renilla luciferase activity, SMAD3/4 caused a 12.0-fold increase in pXPG-181-1-Luc promoter activity, compared with that of pXPG-181-1-Luc (data not shown). Preferential action of SMAD3/4 on the MIR181A2B2 promoter was confirmed in transfections carried out without renilla plasmid control (data not shown).

#### Statistics and analysis

The student *t*-test with and without Bonferroni correction was used to evaluate microarray data and other data, respectively. ANOVA test with Bonferroni correction was used to analyze miRNA responses to cytokine stimulation. F-statistics indicated that luciferase data showed equivalent variances after logarithmic transformation, and these were tested using paired, two-tailed *t*-tests. The *P*-values are listed in the figures and figure legends. All error bars represent s.e.m. Chromosome sites were accessed on the UCSC Genome Browser (<https://genome.ucsc.edu/>), based on assembly Feb. 2009 (GRCh37/hg19).

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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