MicroRNA fingerprints during human megakaryocytopeniosis


*Departments of Molecular Virology, Immunology, and Human Genetics and Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210; 1Department of Experimental and Clinical Pharmacology, University of Catania, I-95125 Catania, Italy; and 5Department of Blood and Marrow Transplantation, University of Texas, M. D. Anderson Cancer Center, Houston, TX 77030

Contributed by Carlo M. Croce, January 25, 2006

MicroRNAs (miRNAs) are a highly conserved class of noncoding RNAs with important regulatory functions in proliferation, apoptosis, development, and differentiation. To discover novel regulatory pathways during megakaryocytic differentiation, we performed microRNA expression profiling of in vitro-differentiated megakaryocytes derived from CD34+ hematopoietic progenitors. The main finding was down-regulation of miR-10a, miR-126, miR-106, miR-10b, miR-17 and miR-20. Hypothetically, the down-regulation of microRNAs unblocks target genes involved in differentiation. We confirmed in vitro and in vivo that miR-130a targets the transcription factor MAFB, which is involved in the activation of the GPIX promoter, a key protein for platelet physiology. In addition, we found that miR-10a expression in differentiated megakaryocytes is inverse to that of HOXA1, and we showed that HOXA1 is a direct target of miR-10a. Finally, we compared the microRNA expression of megakaryoblastic leukemia cell lines with that of in vitro differentiated megakaryocytes and CD34+ progenitors. This analysis revealed up-regulation of miR-101, miR-126, miR-99a, miR-135, and miR-20. Our current profile of microRNAs during megakaryocytopeniosis and suggest a regulatory role of microRNAs in this process by targeting megakaryocytic transcription factors.

leukemia | hematopoiesis

miRNAs are a small noncoding family of 19- to 25-nt RNAs that regulate gene expression by targeting mRNAs in a sequence specific manner, inducing translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and their targets (1, 2). Many miRNAs are conserved in sequence between distantly related organisms, suggesting that these molecules participate in essential processes. Indeed, miRNAs are involved in the regulation of gene expression during development (3), cell proliferation (4), apoptosis (5), glucose metabolism (6), stress resistance (7), and cancer (8–11).

There is also strong evidence that miRNAs play a role in mammalian hematopoiesis. In mice, miR-181, miR-223, and miR-142 are differentially expressed in hematopoietic tissues, and their expression is regulated during hematopoiesis and lineage commitment (12). The ectopic expression of miR-181 in murine hematopoietic progenitor cells led to proliferation in the B cell compartment (12). Systematic miRNA gene profiling in cells of the murine hematopoietic system revealed different miRNA expression patterns in the hematopoietic system compared with neuronal tissues and identified individual miRNA expression changes that occur during cell differentiation (13). A recent study has identified down modulation of miR-221 and miR-222 in human erythropoietic cultures of CD34+ cord blood progenitor cells (14). These miRNAs were found to target the oncogene c-KIT. Further functional studies indicated that the decline of these two miRNAs in erythropoietic cultures unblocks KIT protein production at the translational level leading to expansion of early erythroid cells (14). In line with the hypothesis of miRNAs regulating cell differentiation, miR-223 was found to be a key member of a regulatory circuit involving CREB and NFIA, which control granulocytic differentiation in all-trans retinoic acid-treated acute promyelocytic leukemia cell lines (15).

miRNAs have also been found deregulated in hematopoietic malignancies. Indeed, the first report linking miRNAs and cancer involved the deletion and down-regulation of the miR-15a and miR-16-1 cluster, located at chromosome 13q14.3, a commonly deleted region in chronic lymphocytic leukemia (8). High expression of miR-155 and host gene BIC also was reported in B cell lymphomas (16). More recently it was shown that the miR-17-92 cluster, which is located in a genomic region of amplification in lymphomas, is overexpressed in human B cell lymphomas and the enforced expression of this cluster acted in concert with c-MYC expression to accelerate tumor development in a mouse B cell lymphoma model (10). These observations indicate that miRNAs are important regulators of hematopoiesis and can be involved in malignant transformation.

Discovering the patterns and sequence of miRNA expression during hematopoietic differentiation may provide insights about the functional roles of these tiny noncoding genes in normal and malignant hematopoiesis.

In the present study, we investigate the miRNA gene expression in human megakaryocyte cultures from bone marrow CD34+ progenitors and in acute megakaryoblastic leukemia cell lines. The results of this analysis indicate that several miRNAs are down-regulated during normal megakaryocytic differentiation. We demonstrate that these miRNAs target genes involved in megakaryocytopeniosis, whereas others are overexpressed in cancer cells.

Results and Discussion

miRNA Expression During in Vitro Megakaryocytic Differentiation of CD34+ Progenitors. Using a combination of a specific megakaryocytic growth factor (thrombopoietin) and nonspecific cytokines (stem cell factor and IL-3), we were able to generate in vitro pure, abundant megakaryocyte progeny from CD34+ bone marrow progenitors suitable for microarray studies (Fig. 4, which is published as supporting information on the PNAS web site). Total RNA was obtained for miRNA chip analysis from three different CD34 progenitors at baseline and at days 10, 12, 14, and 16 of culture with cytokines. We initially compared the expression of miRNA between the CD34+ progenitors and the pooled CD34+ differentiated megakaryocytes at all points during the differentiation process. We identified 19 miRNAs (Table 1) that are sharply down-regulated during megakaryocytic differentiation. There were no statistically significant miRNAs up-
the megakaryocytic differentiation process from CD34
target cluster. The multiplicity of miRNAs predicted to
be its regulator. For example, these transcription factors has more than one miRNA predicted
regulated in differentiated megakaryocytes. Moreover each of
miR-106
be the target of
and
with well known function in megakaryocytopoiesis,
megakaryocytic differentiation. Among the transcription factors
system are predicted to target genes with important roles in
hypothesis, miRNAs that are sharply down-regulated in our
unblock target genes involved in differentiation. In line with this
megakaryocytopoiesis, we hypothesized that these miRNAs may
obtained by miRNA chip analysis (Fig. 1).

All differentially expressed miRNAs have q value <0.01 (false-positive rate).
* t test P < 0.05.
† These miRNAs were identified by PAM as predictors of a megakaryocytic class with the lowest misclassification error. All, except miR-143,
are down-regulated during megakaryocytic differentiation.
‡ miRNA precursor sequence that not contain the mature miRNA, therefore no putative target is shown.

### Table 1. miRNAs down-regulated during in vitro CD34⁺ megakaryocytic differentiation

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Chromosomal location</th>
<th>t test*</th>
<th>Fold change</th>
<th>Putative targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-010a†</td>
<td>17q21</td>
<td>-9.10</td>
<td>50.00</td>
<td>HOXA1, HOXA3, HOXD10, CRK, FLT1</td>
</tr>
<tr>
<td>hsa-miR-126†</td>
<td>9q34</td>
<td>-2.73</td>
<td>8.33</td>
<td>CRK, EVI2, HOXA9, MAFB, CMAF</td>
</tr>
<tr>
<td>hsa-miR-106‡</td>
<td>xq26.2</td>
<td>-2.63</td>
<td>2.86</td>
<td>TAL1, FLT1, SKI, RUNX1, FOG2, FL, PDGFR, CRK</td>
</tr>
<tr>
<td>hsa-miR-010b†</td>
<td>2q31</td>
<td>-2.17</td>
<td>11.11</td>
<td>HOXA1, HOXA3, HOXD10, ETS-1, CRK, FLT1</td>
</tr>
<tr>
<td>hsa-miR-130a†</td>
<td>11q12</td>
<td>-2.08</td>
<td>4.76</td>
<td>MAFB, MYB, FOG2, CBFB, PDGFR, SDFR1, CXCL12</td>
</tr>
<tr>
<td>hsa-miR-130a-prec†</td>
<td>11q12</td>
<td>-2.07</td>
<td>7.69</td>
<td>NA‡</td>
</tr>
<tr>
<td>hsa-miR-124a</td>
<td>8q23</td>
<td>-1.81</td>
<td>2.78</td>
<td>TAL1, SKI, FLT1, FOG2, ETS-1, CBFB, RAF1, MYB</td>
</tr>
<tr>
<td>hsa-miR-032-prec†</td>
<td>9q31</td>
<td>-1.76</td>
<td>3.57</td>
<td>NA‡</td>
</tr>
<tr>
<td>hsa-miR-101</td>
<td>1p31.3</td>
<td>-1.75</td>
<td>3.33</td>
<td>TAL1, CXCL12, MEIS1, MEIS2, ETS-1 RUNX1, MYB</td>
</tr>
<tr>
<td>hsa-miR-30c</td>
<td>6q13</td>
<td>-1.71</td>
<td>2.56</td>
<td>CBFB, MAFG, HOXA1, SBF1, NCO2, ERG</td>
</tr>
<tr>
<td>hsa-miR-213†</td>
<td>1q31.3</td>
<td>-1.69</td>
<td>2.38</td>
<td>MAX-SATB2</td>
</tr>
<tr>
<td>hsa-miR-132-prec</td>
<td>17p13</td>
<td>-1.67</td>
<td>4.17</td>
<td>NA‡</td>
</tr>
<tr>
<td>hsa-miR-150‡</td>
<td>19q13.3</td>
<td>-1.63</td>
<td>5.26</td>
<td>MYB, SDFR1</td>
</tr>
<tr>
<td>hsa-miR-020</td>
<td>13q31</td>
<td>-1.62</td>
<td>2.17</td>
<td>TAL1, SKI, RUNX-1, FLT1, CRK, FOG2, RARB</td>
</tr>
<tr>
<td>hsa-miR-339</td>
<td>7p22</td>
<td>-1.60</td>
<td>3.03</td>
<td>HOXA1, ET, VEG, GATA2, FLT1, RAF1B, JUNB, MEIS2</td>
</tr>
<tr>
<td>hsa-let-7d</td>
<td>9q22</td>
<td>-1.58</td>
<td>2.94</td>
<td>HOXA1, HOXA9, MEIS2, ITGB3, PLDN</td>
</tr>
<tr>
<td>hsa-let-7f</td>
<td>9q11</td>
<td>-1.56</td>
<td>2.17</td>
<td>HOXA1, HOXD1, ITGB3, RUNX1, PDGFR</td>
</tr>
<tr>
<td>hsa-miR-181c</td>
<td>19p13</td>
<td>-1.55</td>
<td>2.50</td>
<td>RUNX-1, KIT, HOXA1, MEIS2, ETS-1 ETVE, PDGFR</td>
</tr>
<tr>
<td>hsa-miR-181b</td>
<td>1q31.3</td>
<td>-1.53</td>
<td>2.13</td>
<td>RUNX-1, KIT, ITGA3, HOXA1, MEIS2, ETS-1, SDFR1,</td>
</tr>
<tr>
<td>hsa-miR-017</td>
<td>13q31</td>
<td>-1.38</td>
<td>1.82</td>
<td>TAL1, SKI, FLT1, RUNX1, CRK, FOG1, ETS-1, MEIS1</td>
</tr>
</tbody>
</table>

regulated during megakaryocytic differentiation. Using predictive
analysis of microarray (PAM) we identified 8 microRNAs that predicted megakaryocytic differentiation with no misclassifi-
cation error: miR-10a, miR-10b, miR-30c, miR-106, miR-126, miR-130a, miR-132, and miR-143 (Table 3, which is published as
supporting information on the PNAS web site). All of these
miRNAs, except miR-143, are included in the 17 miRNAs
identified by significance analysis of microarray. Northern blots
and real-time PCR for several miRNAs confirmed the results
obtained by miRNA chip analysis (Fig. 1).

Because we found mainly down-regulation of miRNAs during
megakaryocytogenesis, we hypothesized that these miRNAs may
unblock target genes involved in differentiation. In line with this
hypothesis, miRNAs that are sharply down-regulated in our
system are predicted to target genes with important roles in
megakaryocytic differentiation. Among the transcription factors
with well known function in megakaryocytogenesis, RUNX-1
(17), Fli-1 (18), FLTI (19), ETV6 (20), TALI (21), ETSI (22),
and CRK (23) are putative targets for several miRNAs down-
regulated in differentiated megakaryocytes. Moreover each of
these transcription factors has more than one miRNA predicted
to be its regulator. For example, RUNX1 (AML1) is predicted
to be the target of miR-106, miR-181b, miR-101, let7d, and
the miR-17–92 cluster. The multiplicity of miRNAs predicted to
target AML1 suggests a combinatorial model of regulation.

We then looked at the temporal expression of miRNAs during
the megakaryocytic differentiation process from CD34⁺
progenitors. We focused on miRNAs that have been described in
hematopoietic tissues, such as miR-223, miR-181, miR-155, miR-
142, miR-15a, miR-16, miR-106, and the cluster of miR-17–92
(Fig. 1; see also Fig. 5, which is published as supporting
information on the PNAS web site). We found sequential changes in
the expression of miR-223: Initially, miR-223 is down-regulated
during megakaryocytic differentiation, but after 14 days in
culture, its expression returns to levels comparable with that of
CD34 progenitors (Fig. 1C). The miR-15a and miR-16-1 cluster
also follows the same pattern of expression as miR-223 (Fig. 1D),
whereas miR-181b, miR-155, miR-106a, miR-17, and miR-20
were down-regulated during differentiation (Fig. 6, which is
published as supporting information on the PNAS web site). The
temporal variation of the expression of miR-223 and miR-15a/
miR-16-1 suggests a stage-specific function.

**MAFB Transcription Factor is a Target of miR-130a.** By using three
target prediction algorithms [TARGETSCAN (http://genes.mit.edu/
targetscan), MIRANDA (www.microrna.org/miranda_new.html),
and PICTAR (pictar.bio.nyu.edu)] we identified that miR-130a
is predicted to target MAFB, a transcription factor that is up-
regulated during megakaryocytic differentiation and induces the
GPIb gene, in synergy with GATA1, SP1, and ETS-1 (24). To
investigate this putative interaction, first, we examined MAFB
protein and mRNA levels in CD34⁺ progenitors at baseline and
after cytokine stimulation (Fig. 2A). We found that the MAFB
protein is up-regulated during in vitro megakaryocytic differ-
centiation. Although the mRNA levels for MAFB by PCR increase with
differentiation, this increase does not correlate well with the
intensity of its protein expression. The inverse pattern of expression of MAFB and miR-130a suggested in vivo interaction that was
further investigated.

To demonstrate a direct interaction between the 3’ UTRs of
MAFB with miR-130a, we inserted the 3’ UTR region predicted
to interact with this miRNA into a luciferase vector. This
experiment revealed a repression of ~60% of luciferase activity
compared with control vector (Fig. 2B). As an additional control
experiment, we used a mutated target miRNA sequence for
MAFB lacking five of the complementary bases. As expected, the
mutations completely abolished the interaction between miR-
130a and its target 3’UTRs (Fig. 2B).

We also determined the in vivo consequences of overexpress-
ing miR-130a on MAFB expression. The pre-miR-130a and a
negative control were transfected by electroporation into K562
cells, which naturally express MAFB and lack miR-130a. Trans-
fecation of the pre-miR-130a, but not the control, resulted in a
decrease in the protein levels at 48 h (Fig. 2C). Northern blotting
confirmed successful ectopic expression of miR-130a in K562 cells (Fig. 7, which is published as supporting information on the PNAS web site).

**MiR-10a Correlates with HOXB Gene Expression.** It has been reported that in mouse embryos, miR-10a, miR-10b, and miR-196 are expressed in HOX-like patterns (25) and closely follow their “host” HOX cluster during evolution (26). These data suggest common regulatory elements across paralog clusters. **MiR-10a** is located at chromosome 17q21 within the cluster of the HOX genes (Fig. 8, which is published as supporting information on the PNAS web site) and **miR-10b** is located at chromosome 2q31 within the HOXD gene cluster. To determine whether the miR-10a expression pattern correlates with the expression of HOX genes, we performed RT-PCR for HOXB4 and HOXB5, which are the genes located 5’ and 3’, respectively, to miR-10a in the HOX cluster. As shown in Fig. 9, which is published as supporting information on the PNAS web site, HOXB4 and HOXB5 expression paralleled that of miR-10a, suggesting a common regulatory mechanism.

**MiR-10a Down-Regulates HOXA1.** We determined by miRNA array and Northern blot that miR-10a is sharply down-regulated during megakaryocytic differentiation. Interestingly, we found several HOX genes as putative targets for miR-10a (Table 1). We thus investigated whether miR-10a could target a HOX gene. We performed real-time PCR for the predicted HOX targets of miR-10: HOXA1, HOXA3, and HOXD10. After normalization with 18S RNA, we found that HOXA1 mRNA is up-regulated 7-fold during megakaryocytic differentiation compared with CD34 progenitors (Figs. 3A). HOXA1 protein levels were also up-regulated during megakaryocytic differentiation (Fig. 3B). These results are in sharp contrast with the down-regulation of miR-10a in megakaryocytic differentiation, suggesting that miR-10a could be an inhibitor of HOXA1 expression. To demonstrate a direct interaction of miR-10a and the 3’ UTR sequence of the HOXA1 gene, we carried out a luciferase reporter assay as described in Materials and Methods. When the miRNA precursor miR-10a was introduced in the MEG01 cells along with the reporter plasmid containing the 3’ UTR sequence of HOXA1, a 50% reduction in luciferase activity was observed (Fig. 3C). The degree of complementarity between miR-10a and the HOXA1 3’ UTR is shown in Fig. 3D, as predicted by PICTAR (http://pictar.bio.nyu.edu).

To confirm in vivo these findings, we transfected K562 cells with the pre-miR-10a precursor by using nucleoporation and measured HOXA1 mRNA expression by RT-PCR and HOXA1 protein levels by Western blotting. Successful ectopic expression of miR-10a was documented by Northern blot (Fig. 3E). A significant reduction at the mRNA and protein levels for HOXA1 was found for K562 cells transfected with the miR-10a precursor but not with the negative control (Fig. 3 F and G). These data indicate that miR-10a targets HOXA1 in vitro and in vivo.

It has been reported that miR-196 induces cleavage of HOXB8 mRNA, pointing to a posttranscriptional restriction mechanism of HOX gene expression (27). Contrary to the miR-196–HOXB8 interaction, where an almost perfect complementarity exists, the degree of pairing between miR-10a and the human HOXA1 3’
target complexes to cytoplasmic processing bodies or is a primary event (29).

miRNA Profiling in Acute Megakaryoblastic Leukemia (AMKL) Cell Lines. After the identification of the miRNA expression profile of CD34⁺ cells during megakaryocytic differentiation, we then investigated miRNA expression in AMKL cell lines with the goal to identify differentially expressed miRNAs that could have a pathogenic role in megakaryoblastic leukemia. We initially compared miRNA expression in four AMKL cell lines with that of in vitro CD34⁺-differentiated megakaryocytes. Using significance analysis of microarray, we identified 10 miRNAs up-regulated in AMKL cell lines compared with that of CD34 in vitro-differentiated megakaryocytes (Table 2). These miRNAs are as follows (in order of the fold increase with respect to differentiated megakaryocytes): miR-101, miR-126, miR-99a, miR-99-prec, miR-106, miR-339, miR-99b, miR-149, miR-33, and miR-135. Results were validated by RT-PCR as shown in Fig. 10, which is published as supporting information on the PNAS web site. Using PAM, we compared miRNA expression in CD34⁺ cells with in vitro differentiated megakaryocytes and AMKL cell lines (Table 4, which is published as supporting information on the PNAS web site). Interestingly, we found five miRNAs involved in the megakaryocytic differentiation signature (miR-101, miR-126, miR-106, miR-20, and miR-135) that were up-regulated in the leukemic cell lines (Table 5, which is published as supporting information on the PNAS web site). Whether this profile represents merely a differentiation state of the cells or has a truly pathogenic role remains to be elucidated. Supporting the second hypothesis, miR-106, miR-135, and miR-20 are predicted to target RUNX1, which is one of the genes most commonly associated with leukemia (30). Moreover, mutations of RUNX1 have been described in familial thrombocytopenias with a propensity to develop acute myeloid leukemia (31).

Conclusions
In this study, we have found down-regulation of miRNAs during megakaryocytopenia. Hypothetically the down-regulation of miRNAs unblocks target genes involved in differentiation. In line with this hypothesis, miRNAs that are sharply down-regulated in our system are predicted to target genes with important roles in megakaryocytic differentiation. Thus, we have shown that miR-130a targets MAFB and miR-10a modulates HOXA1. The fact that we found several differentially expressed miRNAs during differentiation and leukemia that are predicted to target HOXA1 suggests a function for HOXA1 in megakaryocytogenesis. Loss and gain studies will ultimately be needed to define the role of HOXA1 in this differentiation process. Our findings delineate the expression of miR-

Table 2. microRNAs up-regulated in acute megakaryoblastic cell lines compared with in vitro-differentiated megakaryocytes

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Chromosomal location</th>
<th>t-test score</th>
<th>Fold change</th>
<th>Putative targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-mir-101</td>
<td>1p31.3</td>
<td>6.14</td>
<td>11.85</td>
<td>MEIS2, RUNX1, ETS-1, C-MYB, FOS, RARB, NFE2L2</td>
</tr>
<tr>
<td>hsa-mir-126</td>
<td>9q34</td>
<td>4.91</td>
<td>11.97</td>
<td>V-CRK</td>
</tr>
<tr>
<td>hsa-mir-099a</td>
<td>21q21</td>
<td>3.30</td>
<td>6.83</td>
<td>HOXA1, EIF2C, FOX1</td>
</tr>
<tr>
<td>hsa-mir-099b-prec</td>
<td>21q21</td>
<td>2.85</td>
<td>7.59</td>
<td>NA</td>
</tr>
<tr>
<td>hsa-mir-106</td>
<td>xq26.2</td>
<td>2.79</td>
<td>3.33</td>
<td>FLT1, SKI, EZF1, NCOA3, PDGFRα, CRK</td>
</tr>
<tr>
<td>hsa-mir-339</td>
<td>7p22</td>
<td>2.58</td>
<td>3.36</td>
<td>HOXA1, FLT1, PTP4A1, RAP1B</td>
</tr>
<tr>
<td>hsa-mir-099b</td>
<td>19q13</td>
<td>2.46</td>
<td>4.19</td>
<td>HOXA1, MYCBP2</td>
</tr>
<tr>
<td>hsa-mir-149</td>
<td>2q37</td>
<td>2.29</td>
<td>3.53</td>
<td>RAP1A, MAF, PDGFRα, SP1, NFIB</td>
</tr>
<tr>
<td>hsa-mir-033</td>
<td>2q13</td>
<td>2.27</td>
<td>3.23</td>
<td>PDGFRα, HIF1A, MEIS2</td>
</tr>
<tr>
<td>hsa-mir-135</td>
<td>3p21</td>
<td>2.12</td>
<td>3.97</td>
<td>SP1, HIF1A, SP3, HNRPA1, HOXA10, RUNX1</td>
</tr>
</tbody>
</table>

All the miRNAs have a q value <0.01 (false-discovery rate). The same miRNAs, except hsa-mir-339 and hsa-mir-149, were found by using PAM to predict a megakaryoblastic leukemia class with no misclassification error.
miRNAs in megakaryocytic differentiation and suggest a role for miRNA modulation of this lineage by targeting megakaryocytic transcription factors. Furthermore, in megakaryoblastic leukemia cell lines, we have found inverse expression of miRNAs involved in normal megakaryocytic differentiation. These data provide a starting point for future studies of miRNAs in megakaryocytogenesis and leukemia.

Materials and Methods

Cell Lines and Human CD34+ Cells. The human chronic myeloid leukemia blast crisis cell lines K-562 and MEG-01 were obtained from American Type Culture Collection and maintained in RPMI medium 1640 (Gibco) containing 10% FBS with penicillin-gentamycin at 37°C with 5% CO2. The human megakaryoblastic leukemia cells UT-7 and CMK and the chronic myeloid leukemia in blast crisis LAMA were obtained from DSMZ (Braunschweig, Germany). All cells were maintained in RPMI medium 1640 with 20% FBS and antibiotics, except UT-7, which is factor-dependent and was cultured in MEM-alpha with 20% FBS and 5 ng/ml granulocyte-macrophage colony-stimulating factor. Fresh and frozen human bone marrow CD34+ cells were obtained from Stemcell Technologies (Vancouver, BC, Canada). FACS analysis for CD34 antigen revealed a purity >98%.

Human Progenitor CD34+ Cell Cultures. Human bone marrow CD34+ cells were grown in STEM media (Stemcell Technologies), which includes Isocove-modified Dulbecco’s medium supplemented with human transferrin, insulin, bovine serum albumin, human low-density lipoprotein, and glutamine, in the presence of 100 ng/ml human recombinant thrombopoietin (TPO) for the first 4 days, followed by a combination of 100 ng/ml TPO, IL3, and stem cell factor (cytokine mixture CC-200, Stemcell Technologies). The initial cell density was 100,000 cells per ml; three times a week, the cell density was adjusted to 100,000 to 200,000 cells per ml. To increase the purity of the cells for microarray analysis, cell sorting was performed at day 10 of culture. Cells were incubated on ice for 45 min with anti-human CD34+, anti-human CD41+, anti-human CD61+, and their respective isotypes. After washing twice with PBS 3% FBS, cells were sorted by using a FACS Aria sorting machine in bulk in two separate populations; CD34+ CD61+ and CD34+ CD61+ cells for culture and RNA extraction. The purity of the sorted populations was >95%.

Megakaryocytes Characterization. Cytospin preparations of CD34+ progenitors in culture were performed and stained with May–Grunwald Giemsa at different time points during the megakaryocytic differentiation induction. For FACS analysis, the primary antibodies used were as follows: CD41A, CD61A, CD42B, and CD34 with their respective isotypes (BD Pharmingen). Cytometric studies were performed as described in ref. 32 by using a FACScalibur (BD Biosciences) and CELLQUEST software (BD Biosciences).

RNA Extraction, Northern Blotting, and miRNA Microarray Experiments. Procedures were performed as described in detail in ref. 33. Raw data were normalized and analyzed in GENESPRING 7.2 software (zcomSilicon Genetics, Redwood City, CA). Expression data were median-centered by using both the GENESPRING normalization option and the global median normalization of the BIOCONDUCTOR package (www.bioconductor.org) with similar results. Statistical comparisons were done by using the GENESPRING ANOVA tool, predictive analysis of microarray (PAM), and the significance analysis of microarray (SAM) software (http://www-stat.stanford.edu/~tibs/SAM/index.html).

RT-PCR and Real-Time PCR. Total RNA isolated with TRIzol reagent (Invitrogen) was processed after DNase treatment (Am-
This work was supported by National Institutes of Health Program Project Grants P01CA76259, P01CA16058, and P01CA81534 (to C.M.C.) and P01CA16672 (to M.A.). The Leukemia Clinical Research Foundation supports C.D.B. Both G.A.C. and R.A. are supported by Kimmel Foundation grant awards, and G.A.C. is supported by a Chronic Lymphocytic Leukemia Global Research Foundation grant.