The deregulated expression of miR-125b in acute myeloid leukemia is dependent on the transcription factor C/EBPα

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MicroRNAs (miRNA) are small non-coding RNAs of 21 nucleotides (nt) that regulate the expression of several genes. Transcribed as primary miRNAs are processed in the nucleus into 70-80 nt, hairpin-shaped precursors, called pre-miRNAs. They are then exported into the cytoplasm and further processed into mature miRNAs (21 nt), and incorporated into the RNA-induced silencing complex.

Figure 1. miR-125b is upregulated in AML patient samples. (a) qRT-PCR for miR-125 was carried out using bone marrow cells derived from AML patient samples (n=10 and healthy donors CD34- cells). Values were normalized to U6 and further to the expression level of three healthy donors. (b) qRT-PCR for miR-125b was carried out using AML-derived cell lines, values are expressed as fold increase over Jurkat cells. Values were normalized to U6. (c) qRT-PCR for miR-125 was carried out using PBC cells derived from T-ALL patient samples (n=7 and healthy donors CD4+ cells). Values were normalized to U6 and further to the expression level of three healthy donors. (d) qRT-PCR for miR-125b was carried out using T-ALL-derived cell lines, values are expressed as fold increase over CD4+ healthy donor-derived cells. Values were normalized to U6. (e) pri-miR-125b-1 and two transcripts were determined by qRT-PCR in the indicated cells. (f) Western blot analysis of Notch1 expression in whole cell extract in the indicated cell lines. Tubulin is shown as a loading control. (g) DND41 cell line were transfected with either miR-ctr or mature miR-125b, and mature miR-125b expression was analyzed by qRT-PCR (left). miR-ctr and miR-125b transfected cells were analyzed by western blot with the indicated antibodies. (h) Left panel, T-ALL (Jurkat) and AML (HL-60)-derived cell lines were infected with either miR-ctr/miR-125b or MiRZIP-ctr/MiRZIP125b, and Notch1 mRNA expression was analyzed. Right panel, miR-125b expression was evaluated in the samples shown in the left panel. All results were expressed as means ± s.d., and P-values are indicated.

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AMLs, thus in addition to work as a tumor suppressor C/EBPα appears to be required for the development of at least some AML subtypes.\textsuperscript{8–10} We previously showed that the manifestation of Hailey–Hailey disease, a rare skin disorder, was in part dependent on Notch1 downmodulation mediated by miR-125b upregulation.\textsuperscript{11,12} Notably, although the involvement of Notch signaling as an oncogene in T-cell acute lymphoblastic leukemia (T-ALL) is well characterized, Notch signaling acts as a tumor suppressor in myeloid malignancies. Moreover, although T-ALL cells express Notch1 receptor, its expression is silenced in AML (Lobry \textit{et al.}\textsuperscript{13} and references therein). It has been previously shown that miR-125b is overexpressed in AML; thus, we investigated whether

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\caption{miR-125b is a direct target of C/EBPα. (a) Schematic representation of examined putative C/EBPα-binding sites in the promoter regions of miR-125b. (b) C/EBPα protein expression assessed by western blotting in the indicated cell lines. (c) HEK293T cells were transfected with miR-125b-luciferase responsive promoter construct, 0.25 μg/well in 24-well dishes, and treated with the indicated amount of C/EBPα; cells were harvested 24 h after transfection for luciferase assay. All conditions were tested in triplicate samples and s.d. is indicated. (d) Chromatin derived from HL-60-C/EBPα-positive cells was immunoprecipitated with anti- C/EBPα or IgG antibodies. Recovered DNA was PCR amplified with primers specific for C/EBPα-binding amplificon. Shown is the fold change in binding affinity of the anti-C/EBPα antibody normalized to IgG. Immunoprecipitation was performed three times using different chromatin samples, and the occupancy was calculated by using the ChIP-qPCR Human IGX1A Negative Control Assay (Qiagen, Milano, Italy) as a negative control. (e) miR-125b and C/EBPα mRNA expression was analyzed at 0, 12, 24 and 48 h post RA treatment of HL-60 cells. (f) miR-125b and C/EBPα mRNA expression in CD34+ versus CD34− human primary cells. (g) miR-125b expression of C/EBPα-silenced HL-60 cells, as assessed by qRT-PCR. (h) A scheme showing transcriptional regulation and function of miR-125b in AML. miR-125b is induced by both WT and mutant C/EBPα. High expression of miR-125b leads to differentiation block, proliferation and transformation. In parallel miR-125b decreases Notch1 protein level, which has also been implicated in suppression of AML cell growth.}
\end{figure}
miR-125b overexpression might account for the differential Notch1 expression between T-ALL and AML. We compared miR-125b expression pattern in both primary AML and T-ALL leukemia as well as in AML and T-ALL-derived cell lines (Figures 1a–d). Both the human primary and AML cell lines samples demonstrated significant upregulation of miR-125b expression. Conversely, both primary and T-ALL cell lines failed to show significant enrichment of this miRNA.

In order to investigate whether the deregulation of miR-125b expression occurs either at the transcriptional or processing level, primary miR-125b expression levels were analyzed in both Jurkat and HL-60 cell lines, as well as in primary AML samples (Figure 1e, Supplementary Figure S1, Supplementary Table 1 and Supplementary Figure S7). Specifically, quantitative reverse transcriptase PCR was performed to compare the levels of primary and mature miRNA. The primary miRNA levels of the miR-125b were found to parallel mature miR-125b expression in both cell lines examined (Figures 1b and e). In most primary AMLs, we found that miR-125b expression was transcriptionally upregulated (Supplementary Figure S1). Nevertheless, we observe that in some samples primary miRNAs basal transcription efficiency was associated with a low abundance of the mature miRNA (Figure 1a, AMLs 8, 9 and 10). Thus, these observations indicated that in this cellular context there is generally a high rate of primary miR-125b transcription, although an altered processing efficiency might determine the level of mature miRNAs.

We found an inverse correlation of miR-125b expression and Notch1 protein levels in both T-ALL and AML cell lines, as well as in primary AML samples (Figures 1b, d and f and Supplementary Figure S2). We observed higher level of miR-125b expression in AML when compared with T-ALL samples (Supplementary Figure S3). Importantly, the Notch1 target genes, Hes-1 and Deltex1, were significantly higher in T-ALL when compared with AML (Supplementary Figure S3). Recently, we found that NOTCH1 is a target of miR-125b,12 thus, we analyzed the potential involvement of miR-125b in regulating the differential expression of Notch1 between T-ALL and AML cells. We analyzed Notch1 protein expression after overexpression of either miR-125b or AntagomiR-125b in T-ALL and AML cell lines, respectively.

DND41 cells, but not Jurkat and HL-60 cells, are highly transfectable. To overcome these limitations, DND41 cells were analyzed by transient transfection and both Jurkat and HL-60 cells were transduced by lentiviral infection. We found that deregulated miR-125b expression impaired Notch1 levels in DND41 (Figure 1q), and although with a lower effect also in Jurkat and HL-60 cell lines (Figure 1h). Together, these results suggest that deregulation of miR-125b expression has a critical role in the differential expression of Notch1 between T-ALL and AML. However, ME-1 cells devoid of miR-125b expression have undetectable level of Notch1 expression (Figures 1b and f). Additionally, in the T-ALL derived cell line, Molt3, miR-125b enforced expression did not affect Notch1 expression (data not shown); thus, it is likely that other mechanisms alone or synergistically with the miR-125b are involved in Notch1 downmodulation in AML14 or alternatively an unknown mechanism antagonizes the repressive activity of miR-125b on the 3’ untranslated region of Notch1 in a cell context-specific manner.

To explore the mechanism regulating miR-125b expression, we first characterized the miR-125b promoter region using the Genomatix MatInspector software package (Genomatix Software GmbH, Munich, Germany), focusing on those transcription factors that have been shown to have a role in either T-ALL or AML. A scan of 2 kb of genomic sequence located upstream of the predicted pre-miR-125b start site identified putative Hes-1, GATA3 and one C/EBPα consensus binding sites (Figure 2a), suggesting the involvement of those factors in the regulation of miR-125b expression. Thus, protein extracts from AML and T-ALL-derived cell lines were first analyzed for expression of those factors. Interestingly, C/EBPα expression was correlated with miR-125b expression in the cell lines examined (Figures 1b and 2b). We next examined the role of these transcription factors in the regulation of miR-125b expression by generating a miR-125b promoter construct and testing it in a luciferase reporter assay. As shown in the Figure 2c, we found the induction of miR-125b promoter activity by C/EBPα transfection in a dose-dependent fashion, but neither by HES-1 nor GATA3 (data not shown), indicating that C/EBPα might be a transcriptional regulator of miR-125b expression. C/EBPα is a key myeloid transcription factor, frequently mutated in AML, but none of the described mutations result in the full loss of its function.10 Recently, it has been shown that C/EBPα-dependent activity has an important role in AML etiology.11 Next, we investigated whether C/EBPα directly regulates miR-125b promoter. To test whether C/EBPα binds directly to the miR-125b promoter, we performed chromatin immunoprecipitation experiments in both HL-60 cells and primary AML samples. The chromatin fragments were immunoprecipitated with an anti-C/EBPα antibody. The DNA fragments were analyzed with specific primers for the indicated regions of the miR-125b regulatory region (Figure 2d and Supplementary Figure S4). We were able to observe an enrichment of DNA from the predicted C/EBPα-binding sites when compared with the immunoglobulin control (Figure 2d). Additionally, we observed an increased recruitment of C/EBPα onto the miR-125b promoter in AML primary samples highly expressing miR-125b primary transcript (Supplementary Figures S1 and S4).

The myeloid cell lines provide an important in vitro model system for studying the cellular and molecular events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage. Both HL-60 and NB4 pro-myelocytic leukemia cell lines have the potential to differentiate toward granulocytic lineage by exposure to retinoic acid. Thus, to explore further the role of C/EBPα in the induction of miR-125b expression, we compared C/EBPα and miR-125b expression after retinoic acid treatment (Figure 2e). Treatment with retinoic acid (1 μM) strongly decreased both C/EBPα protein and miRNA expression (Figure 2e and Supplementary Figure S5a), in parallel with induction of granulocytic differentiation (Supplementary Figure S5). Notably, in both cell lines, HL-60 and NB4, the downregulation of C/EBPα expression by retinoic acid parallels that of miR-125b (Figure 2e and Supplementary Figure S6). Interestingly a similar parallel expression was observed in CD34+ and CD34+ primary cells (Figure 2f). Finally, small interfering RNA against C/EBPα in HL-60 abolished the basal level of miR-125b expression (Figure 2g), further supporting our finding that miR-125b is a direct target of C/EBPα.

In summary, several studies have made important advances in elucidating the contribution of both C/EBPα and miR-125b into the molecular mechanisms of AML development. Our study implicates the transcription factor C/EBPα as a critical determinant of miR-125b expression in AML, supporting a model whereby C/EBPα functions to enhance miR-125b expression to regulate a group of genes whose deregulation leads to acute myeloid transformation.

CONFICT OF INTEREST
The authors declare no conflict of interest.

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A novel recurrent EP300–ZNF384 gene fusion in B-cell precursor acute lymphoblastic leukemia

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In pediatric patients with B-cell precursor acute lymphoblastic leukemia (BCP-ALL), approximately three-quarters harbor well-characterized, clinically relevant chromosomal alterations, including hyperdiploidy, hypodiploidy, t(12;21) ETV6/RUNX1, t(1;19) E2A/PBX1, t(9;22) BCR/ABL1 and the rearrangement of MLL at 11q23, and they can facilitate diagnosis, risk stratification and targeted therapy. 1,2 In the remaining patients, however, major pathogenic or driver gene abnormalities and their association with the clinical outcome have yet to be fully clarified. As recent advanced genomic studies using next-generation sequencing have identified a number of novel fusion genes and stratified a high-risk subtype in BCP-ALL, 3–5 unknown genetic alterations that constitute characteristic subgroups may still exist in the remaining patients. We therefore intended to investigate unknown fusion genes in BCP-ALL by using next-generation sequencing.

As a consequence of whole transcriptome sequencing performed on complementary DNA from 55 selected samples of pediatric BCP-ALL patients without conventional genetic abnormalities (Supplementary Information), an EP300–ZNF384 fusion gene was identified in two patients (Cases 1 and 2) as a repeatable and plausible candidate fusion gene (Figure 1a). The 3’72-bp fragment of the EP300–ZNF384 fusion cDNA was amplified by RT-PCR using a pair of specific primers, and Sanger sequencing of the PCR products revealed a sequence of the products identical to that assembled from complementary DNA from 55 selected samples of BCP-ALL patients. As recent advanced genomic studies using next-generation sequencing have identified a number of novel fusion genes and stratified a high-risk subtype in BCP-ALL, 3–5 unknown genetic alterations that constitute characteristic subgroups may still exist in the remaining patients. We therefore intended to investigate unknown fusion genes in BCP-ALL by using next-generation sequencing.

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