miR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia

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Children with trisomy 21/Down syndrome (DS) are at high risk to develop acute megakaryoblastic leukemia (DS-AMKL) and the related transient leukemia (DS-TL). The factors on human chromosome 21 (Hsa21) that confer this predisposing effect, especially in synergy with consistently mutated transcription factor *GATA1* (*GATA1s*), remain poorly understood. Here, we investigated the role of Hsa21-encoded *miR-125b-2*, a microRNA (miRNA) overexpressed in DS-AMKL/TL, in hematopoiesis and leukemogenesis. We identified a function of *miR-125b-2* in increasing proliferation and self-renewal of human and mouse megakaryocytic progenitors (MPs) and megakaryocytic/erythroid progenitors (MEPs). *miR-125b-2* overexpression did not affect megakaryocytic and erythroid differentiation, but severely perturbed myeloid differentiation. The propoliferative effect of *miR-125b-2* on MEPs accentuated the *Gata1s* mutation, whereas growth of DS-AMKL/TL cells was impaired upon *miR-125b* repression, suggesting synergism during leukemic transformation in *GATA1s*-mutated DS-AMKL/TL. Integrative transcriptome analysis of hematopoietic cells upon modulation of *miR-125b* expression levels uncovered a set of *miR-125b* target genes, including *DICER1* and *ST18* as direct targets. Gene Set Enrichment Analysis revealed that this target gene set is down-regulated in DS-AMKL patients highly expressing *miR-125b*. Thus, we propose *miR-125b-2* as a positive regulator of megakaryopoiesis and an oncomiR involved in the pathogenesis of trisomy 21-associated megakaryoblastic leukemia.

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Numerical and structural aberrations involving human chromosome 21 (Hsa21) are the most common cytogenetic abnormalities found in childhood acute myeloid leukemia (AML). However, genes that confer oncogenic properties of Hsa21 remain incompletely understood (for review, see Malinge et al. 2009). Acute megakaryoblastic leukemia (AMKL) in children with trisomy 21 (Down syndrome [DS]; i.e., DS-AMKL) and the related antecedent, transient leukemia (DS-TL, also known as transient myeloproliferative disorder [TMD]) provide a unique context in which to address this issue (Hasle 2001; Klusmann et al. 2008). Five percent to 10% of all DS infants exhibit

DS-TL, which resolves spontaneously in the majority of cases, but shows progression to DS-AMKL in 20%–30% of DS-TL patients (Klusmann et al. 2008). Acquired mutations in the hematopoietic transcription factor *GATA1*—encoded on chromosome X—are consistently present in the affected cells of children with DS-AMKL and DS-TL, leading to expression of a shorter *GATA1* variant (*GATA1s*) truncated at its N terminus (Wechsler et al. 2002; Mundschau et al. 2003). However, neither mice nor humans with germline mutations leading to expression of *GATA1s* develop leukemia or myeloproliferative disease in the absence of an extra copy of Hsa21 (Li et al. 2005; Hollanda et al. 2006). The requirement for trisomy 21 in cellular transformation in DS-AMKL, therefore, appears stringent.

MicroRNAs (miRNAs) are 21- to 23-nucleotide (nt) noncoding RNAs that play crucial roles in fundamental biologic processes, including proliferation, differentiation,

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and apoptosis, by negatively regulating gene expression (for review, see Carthew and Sontheimer 2009). miRNAs are transcribed as primary miRNAs (pri-miRNAs) and processed in the nucleus by the RNase III endonuclease DROSHA to liberate ~70-nt stem loops, the precursor miRNA (pre-miRNA). The pre-miRNAs are then exported from nucleus to cytoplasm by Exportin 5/RanGTP, where further cleavage by the second RNase III enzyme DICER releases the mature miRNAs. miRNAs bind to their target mRNAs by direct base-pairing, and either repress gene translation or induce mRNA cleavage or degradation (Carthew and Sontheimer 2009). In the hematopoietic system, several miRNAs, including *miR-150*, *miR-223*, and *miR-181*, regulate lineage decisions and cellular maturation (Chen et al. 2004; Xiao et al. 2007; Johnnidis et al. 2008).

Deregulation of miRNA expression has been linked to the initiation, progression, and metastasis of human malignancies (He et al. 2005; Lu et al. 2005). Human and mouse cancers are characterized by a global reduction of mature miRNA levels compared with normal tissues (Lu et al. 2005; Kumar et al. 2007). *miR-125b-2* resides along with four other known miRNAs (*miR-99a*, *let-7c*, *miR-155*, *miR-802*) on Hsa21 (Fig. 1A; Landgraf et al. 2007). Recent reports suggested that *miR-125b* might act as an oncogene as well as a tumor suppressor, depending on the cellular context. In prostate cancer cells, high expression levels of *miR-125b* stimulate androgen-independent growth that is mediated partially by down-regulation of *Bak1* (Shi et al. 2007), whereas in breast cancer, high expression levels of *miR-125b* mediate down-regulation of *ERBB2* (HER2) and *ERBB3* (HER3), thereby suppressing tumor growth (Scott et al. 2007). The homolog *miR-125b-1* is involved in translocations found in precursor B-cell acute lymphoblastic leukemia (pre-B ALL) and myelodysplastic syndrome (MDS) (Sonoki et al. 2005; Bousquet et al. 2008). However, the role of Hsa21-encoded *miR-125b-2* in leukemogenesis has not been defined.

In this study, we investigated the role of Hsa21-encoded *miR-125b-2* in hematopoiesis and leukemogenesis. Using a genetic approach, we demonstrated that, in both murine and human contexts, overexpression of *miR-125b-2* led to specific hyperproliferation and enhanced self-renewal capacity of megakaryocytic progenitors (MPs) and megakaryocytic/erythroid progenitors (MEPs), without affecting their normal differentiation. This effect was aggravated further in cooperation with the oncogenic *Gata1s* mutation. Integrative transcriptome analysis, together with experimental validation, revealed target genes of *miR-125b* in the hematopoietic system, including *DICER1* and *ST18* as direct targets. We showed that *miR-125b* was highly expressed in DS-AMKL blasts, whereas the identified target genes of *miR-125b* were down-regulated. Thus, our study supports a role of *miR-125b-2* in the regulation of megakaryopoiesis and in the pathogenesis of trisomy 21-associated megakaryoblastic leukemia, in cooperation with GATA1s. We provide evidence that *miR-125b-2* exerts its oncogenic potential by blocking post-transcriptional miRNA processing through repression of *DICER1* expression and by inhibiting tumor suppressors, such as *ST18*.

Figure 1. Hsa21-encoded *miR-125b* is up-regulated in AMKL patient samples. (A) Schematic diagram showing the location of *miR-125b-2* and four other miRNAs (*miR-99a*, *let-7c*, *miR-155*, and *miR-802*) on human Hsa21 (<http://www.ensembl.org>). (B) The expression level of *miR-125b* was analyzed by qRT-PCR in sorted leukemic blasts from patients with DS-AMKL ($n = 5$), DS-TL ($n = 4$), non-DS-AMKL ($n = 3$), and AML FAB M5 (AML M5; $n = 2$), and in CD34⁺-HSPCs ($n = 2$) and megakaryocytes (BM-Meg; $n = 1$) from healthy donors. Data are presented as means $\pm$ standard error of the mean (SEM) normalized to CD34⁺-HSPCs. (*) $P_{U\text{-Test}} < 0.05$.

Results

miR-125b is up-regulated in DS-AMKL and DS-TL

To interrogate a potential role for Hsa21-encoded *miR-125b-2* in trisomy 21-associated megakaryoblastic leukemia (Fig. 1A), we first measured expression levels of *miR-125b* in sorted leukemic blasts from patients with DS-AMKL ($n = 5$), DS-TL ($n = 4$), non-DS-AMKL ($n = 3$), and AML FAB M5 ($n = 2$), and in CD34⁺-HSPCs (hematopoietic stem and progenitor cells) ($n = 2$) and megakaryocytes ($n = 1$) from healthy donors. All DS-AMKL and DS-TL patients harbored a *GATA1s* mutation, whereas none of the non-DS-AMKL patients did (data not shown). Cytogenetic data were available for DS-AMKL ($n = 3$) and non-DS-AMKL ($n = 2$) patients (Supplemental Table S1). The expression of *miR-125b* was markedly elevated in DS-AMKL (26.4-fold), DS-TL (18.5-fold), and non-DS-AMKL (8.9-fold) compared with normal CD34⁺-HSPCs (Fig. 1B). The up-regulation of *miR-125b* is not a general feature of AML, as expression was reduced (threefold) in AML FAB M5 in comparison with CD34⁺-HSPCs. High levels of *miR-125b* are not associated with megakaryocytic

differentiation, as the expression level of *miR-125b* in megakaryocytic cells was similar to that in CD34⁺-HSPCs. Expression of *miR-125b* was threefold higher in DS-AMKL than non-DS-AMKL.

Overexpression of miR-125b-2 increases proliferation and self-renewal of MPs

Overexpression of *miR-125b* in AMKL, and specifically in DS-AMKL and DS-TL, suggests a potential role of Hsa21-encoded *miR-125b-2* in AMKL pathogenesis. To test the consequences of *miR-125b-2* overexpression on megakaryocyte development, we transduced mouse MPs from fetal livers (FLs) of embryonic day 12.5 (E12.5) embryos with retroviruses (MSCV-based) containing the genomic region of *miR-125b-2* or control empty vectors [*LPIG* containing only the *miR-30* backbone [*LMPIG*] (Dickins et al. 2005), or *MSCV-Puro*]. We chose to transduce fetal progenitors because we showed previously that progenitor cells at this stage of development are the likely cells of origin for transformation in DS-AMKL (Li et al. 2005). The level of mature *miR-125b* attained in transduced cells was comparable with that seen in the DS-AMKL cell line (CMK), as confirmed by Northern blot (Supplemental Fig. S1A). Following transduction, we performed megakaryocytic colony-forming assays. Retroviral overexpression of *miR-125b-2* markedly accelerated the proliferation of MPs, as demonstrated by increased sizes and numbers of the megakaryocyte colony-forming units (CFU-MKs) (Fig. 2A–C; Supplemental Fig. S1B). Megakaryocytic differentiation proceeded normally in the presence of *miR-125b-2* overexpression, as indicated by numerous acetylcholine esterase (AChE)-positive cells and large megakaryocytes with proplatelet formation within CFU-MK colonies (Fig. 2A; Supplemental Fig. S1B). To confirm that the observed effect is specific for *miR-125b-2* and does not reflect a nonspecific response to excessive overexpression of short RNAs, we generated *miR-125b-2* mutants by changing 3 nt in its seed region (Supplemental Fig. S1C). FL MPs transduced with *miR-125b-2* mutants failed to exhibit an increase in the number and size of CFU-MKs (Fig. 2B,C), thus excluding the possibility of a nonspecific response of FL MPs.

Stem cells possess the capacity for unlimited self-renewal and asymmetric cell division, which distinguishes them from progenitor cells. To investigate the possibility that *miR-125b-2* confers a self-renewal potential to MPs, we performed serial replating assays in methocellulose-based colony-forming assays in the presence of TPO (thrombopoietin) only. Whereas we did not observe CFU-MKs in empty vector-transduced cells in the first replating, *miR-125b-2*-transduced MPs possessed replating capacity throughout the time of investigation (Fig. 2D). Concomitantly, *miR-125b-2*-transduced MPs sustained and proliferated in liquid culture in the presence of only TPO for >30 d, whereas the empty vector control MPs died out after 18 d (Supplemental Fig. S1D). FL MPs transduced with *miR-125b-2* mutants did not show an enhanced replating efficiency (data not shown).

Thus, *miR-125b-2* not only increases the proliferation of MPs, it also enhances their ability to self-renew.

As these effects might be species-specific, we tested whether overexpression exerts similar effects in human hematopoietic cells. We retrovirally overexpressed *miR-125b-2* in CD34⁺-HSPCs. Similar to findings in murine cells, forced expression of *miR-125b-2* markedly increased the formation of larger CFU-MKs without blocking megakaryocytic differentiation (Fig. 2E,F).

To further assess consequences of *miR-125b-2* overexpression on megakaryocytic development, we transduced K562 cells with *miR-125b-2*. K562 cells, which do not express *miR-125b* (Supplemental Fig. S2A), are multipotent and differentiate along the megakaryocytic lineage upon induction with chemical compounds or genetic alteration (Tetteroo et al. 1984). Forced expression of *miR-125b-2* (~7.8-fold higher levels relative to endogenous *miR-125b* expression of CMK cells) (Supplemental Fig. S2A) led to increased CD61 and reduced glycophorin A (GlyA) expression, indicating the initiation of megakaryocytic differentiation (Fig. 2G). The mRNA expression of megakaryocytic markers was increased, whereas the expression of erythroid markers was decreased (Supplemental Fig. S2B). The proliferation rate of *miR-125b-2*-transduced K562 cells was not markedly altered (data not shown).

Overexpression of miR-125b-2 increases proliferation and self-renewal of MEPs

In comparison with non-DS-AMKL, DS-AMKL and DS-TL represent a unique entity in that their blast cells frequently exhibit features of cells in the erythroid lineage. Since megakaryocytic and erythroid cells arise from a common precursor (MEP), DS-AMKL/TL may be originated from MEPs. To analyze a possible effect of *miR-125b-2* on MEPs and the erythroid lineage, we performed serial replating assays of wild-type FL progenitors in the presence of TPO along with EPO (erythropoietin), SCF (stem cell factor), IL-3 (interleukin-3), and IL-6 (interleukin-6), which support erythroid burst-forming units (BFU-E) and other CFUs of FL cells. In the third replating, whereas *miR-125b-2*-transduced FL progenitors mainly formed CFU-MKs (66.4 ± 2.8%; or better described as CFU-MK/Es, since many of these CFU-MK colonies also contained hemoglobinized erythroid cells) (Fig. 2H,I), a fraction of all colonies were BFU-Es (17.5 ± 4.2%) (Fig. 2H,I). In contrast, the empty vector-transduced control cells failed to form CFU-MKs or BFU-Es. They exclusively formed CFUs of other lineages (CFU-other), mainly CFUs containing mast cells (CFU-mast) (Fig. 2H,I). Concomitant with this observation, by fluorescence-activated cell sorting (FACS) analysis, we found ~39% of all cells from the third replating plate were immunophenotypically CD41⁺CD71⁺ (compared with ~0.3% from the control plate), and ~30% were CD41⁺CD71⁺ (erythroid precursors; control: ~0.3%) (Fig. 2J).

The observed BFU-Es might have originated from unipotent erythroid progenitors and not from MEPs. However, we did not observe individual BFU-Es in the first and second rounds of replating. Instead, we observed

Figure 2. *miR-125b-2* overexpression induced proliferation and differentiation of MPs and MEPs. (A) Microscopic (left, middle) and macroscopic (right) images of AChE-stained and unstained (Phase) CFU-MKs from *miR-125b-2*-transduced and empty vector-transduced (*LMP1G* or *MSCV-puro*) murine FL cells. The inset of the bottom middle panel shows proplatelet formation. Pictures here are representative images from $n = 3$ independent experiments. (B,C) Megakaryocytic colony-forming assay of *miR-125b-2*-transduced, mutated *miR-125b-2*-transduced (mut1), and empty vector-transduced (*LMP1G*; control) wild-type FL MPs using methocellulose-based assays in the presence of 20 ng/mL TPO. (B) Diagram and statistics showing the number of CFU-MKs per 10^4 plated cells. Data of replicates from one of two independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$. (C) Diagram and statistics showing the number of cells per CFU-MK. Data from replicates from one representative experiment are shown as means $\pm$ SD. (*) $P < 0.05$. (D) Diagram and statistics showing the number of CFU-MKs (per 10^4 plated cells) in serial replating of *miR-125b-2*-transduced and empty vector-transduced (*LMP1G*; control) wild-type FL progenitors using methocellulose-based assays. Data from replicates from one of two independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$. (E) Diagram and statistics showing the number of CFU-MKs per 10^4 *miR-125b-2*-transduced and empty vector-transduced (*MIGR1*; control) human CD34⁺-HSPCs. Data from $n = 2$ independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$. (F) Representative images of immunohistochemically stained (CD41) and hematoxylin and eosin-stained (HE) colony-forming assays as shown in E. (G) CD61 and GlyA expression in *miR-125b-2*-transduced and empty vector-transduced K562 cells. The representative FACS profiles from one of two independent experiments are shown. (Blue line) Empty vector (*LMP1G*; control); (red line) *miR-125b-2*. (H) Representative microscopic images showing unstained (Phase) CFUs (CFU-Mast, BFU-E, and CFU-MK/E) from *miR-125b-2*-transduced and empty vector-transduced (*MSCV-puro*; control) murine wild-type FL cells from the third replating using methocellulose-based assays in the presence of TPO, SCF, EPO, IL-3, and IL-6. (I,J) Diagrams showing the relative distribution of CFUs (I) and the percentages of CD71⁺CD41⁻ and CD71⁺CD41⁺ cells (J) as assessed by flow cytometry in the third replating of *miR-125b-2*-transduced and empty vector-transduced (*MSCV-puro*) murine wild-type FL cells as shown in H.

many large CFU-MKs with hemoglobinized erythroid cells (data not shown). Thus, these data argue that *miR-125b-2* can expand early hematopoietic progenitor cells (i.e., MEPs) with the potential to differentiate along the megakaryocytic and erythroid lineages without affecting their differentiation.

Overexpression of *miR-125b-2* cooperates with *Gata1s* mutation

GATA1s mutations are consistently present in leukemic blasts of children with DS-AMKL and DS-TL. In the

knock-in mouse model, *Gata1s* increases the proliferation of FL MPs in vitro and in vivo (Li et al. 2005). To test a potential synergistic function of *Gata1s* and *miR-125b-2*, we transduced *Gata1s* mutant FL cells with *miR-125b-2* or the empty vector control and performed serial replating assays. Consistent with previous results (Li et al. 2005), we observed an increase in the number and size of CFU-MKs from *Gata1s* mutant FL progenitors (empty vector-transduced) and an enhanced replating efficiency in comparison with their wild-type counterparts (Fig. 3A; Supplemental Fig. S3). Strikingly, *miR-125b-2*-transduced *Gata1s* FL progenitors frequently

Figure 3. *miR-125b-2* overexpression synergizes with *Gata1s* mutation, whereas *miR-125b* repression inhibits DS megakaryocytic leukemia cell growth. (A) Diagram and statistics showing the number of CFU-MKs (per 10⁴ plated cells) in serial replating of *miR-125b-2*-transduced and empty vector-transduced (*MSCV-puro*; control) *Gata1s* FL cells (*G1s*-FLCs) using methocellulose-based assays in the presence of TPO, SCF, EPO, IL-3, and IL-6. Data from replicates in one representative experiment are presented as means $\pm$ SD. (*) $P < 0.05$. (B) Macroscopic images of the colony-forming assays from the third replating as shown in A and C. (C) Flow cytometric analysis of the third replating of *miR-125b-2*-transduced and empty vector-transduced (control) murine *Gata1s* FL cells. The diagram shows percentages of CD71⁺CD41⁻ and CD71⁺CD41⁺ cells. (D) Cell counts of M-07 ($n = 4$), CMK ($n = 4$), and K562 cell lines ($n = 4$), as well as CD34⁺-HSPCs ($n = 3$) and

primary DS-TL blasts ($n = 3$) 48 h after transfection with anti-*miR-125b*, in relation to the nonsilencing oligonucleotide-transfected cells as a control. Data from independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$. (E) Cell cycle analysis using 7-AAD and BrdU of CMK cells 48 h after transfection with anti-*miR-125b* or nonsilencing oligonucleotides. The diagram shows the percentage of cells in G0/G1, S, and G2/M phase. Data from $n = 2$ independent experiments are shown as means $\pm$ SD.

formed larger CFU-MKs than *Gata1s*-only CFU-MKs throughout the serial replating experiment (Supplemental Fig. S3). In addition, the number of CFU-MKs from *miR-125b-2*-transduced *Gata1s* FL progenitors increased constantly in each replating and was significantly higher in the third replating in comparison with the empty vector-transduced *Gata1s* cells (Fig. 3A,B), suggesting a synergy of *Gata1s* and *miR-125b-2* to further enhance proliferation and self-renewal of FL MPs. Consistent with this, we also observed increased proportions of CD41⁺ and CD71⁺ cells in replating plates with *miR-125b-2*-transduced *Gata1s* FL progenitors in comparison with empty vector-transduced control cells (Fig. 3C). In addition, we did not observe BFU-E colonies emerging from replating assays from *miR-125b-2*-transduced *Gata1s* FL progenitors in comparison with *miR-125b-2*-transduced wild-type FL progenitors (data not shown). This is expected, since *Gata1s* mutation impairs fetal erythropoiesis, as we demonstrated previously in *Gata1s* knock-in mice (Li et al. 2005).

Down-regulation of *miR-125b* in DS megakaryocytic leukemia cells inhibits their proliferation

To test dependency of proliferation of megakaryoblastic leukemia cells on continuous expression of *miR-125b*, we transfected CMK (DS-AMKL) and M-07 (non-DS-AMKL) cell lines with chemically modified oligonucleotides complementary to the sequence of *miR-125b* (anti-*miR-125b*). *miR-125b* is highly expressed in both cell lines, as confirmed by Northern blot and quantitative RT-PCR (qRT-PCR), whereas expression of *miR-125b* is nearly undetectable in the presence of anti-*miR-125b* (Supplemental Figs. S2A, S4A). The number of viable cells was reduced upon transfection of anti-*miR-125b* in comparison with cells transfected with a nonsilencing control

oligonucleotide (Fig. 3D). In contrast, K562 cells, which do not express *miR-125b*, did not show a similar response (Fig. 3D; Supplemental Fig. S2A). The effect was caused mainly by reduced proliferation, as indicated by cell cycle analysis with less BrdU⁺ cells in S phase (Fig. 3E; Supplemental Fig. S4B,C). To investigate this effect in primary human cells, we transfected DS-TL leukemic blasts and CD34⁺-HSPCs with anti-*miR-125b*. Similar to the cell lines, DS-TL leukemic blasts ceased to proliferate (Fig. 3D); in contrast, the number of CD34⁺-HSPCs, which express only low levels of *miR-125b* in comparison with DS-TL leukemic blasts (Fig. 1B), was not altered significantly (Fig. 3D).

In conclusion, proliferation of DS-AMKL cell lines and primary DS-TL leukemic blasts is dependent on expression of *miR-125b*, and the hyperproliferative phenotype can be reverted upon down-regulation of *miR-125b*.

miR-125b-2 perturbs myeloid differentiation of HSPCs

miR-125b-2 overexpression increases the proliferation of MEPs and is required for growth of DS-AMKL cell lines, as well as primary DS-TL blasts. To investigate a broader role of *miR-125b-2* in hematopoiesis, we transduced HSPC-enriched mononuclear cells from the bone marrow (BM) of adult mice. The number of CFUs of the *miR-125b-2*-transduced BM cells exceeded the number of the control CFUs (Fig. 4A). Three major types of CFUs were present in the *miR-125b-2*-transduced BM cells: two types of large CFUs (type 1A and 1B), and one type of medium-sized, compact clusters (type 2) (Fig. 4B), as described previously for MLL-AF9-transduced myeloid cells (Johnson et al. 2003). We picked individual CFUs from each type and analyzed their cell composition by cytopsin and flow cytometry (FACS). Type 1A CFUs contained mainly Mac-1^{high} macrophages and type 1B

Figure 4. Overexpression of *miR-125b-2* perturbs myeloid differentiation of HSPCs. (A) Number of CFUs per 10^4 plated *miR-125b-2*-transduced and empty vector-transduced (*LMP1G*) murine BM cells. Data of replicates from one of three independent experiments are presented as means $\pm$ SD. (*) $P < 0.05$. (B) Microscopic images of colony-forming assays show representative CFUs from one of $n = 3$ independent experiments of *miR-125b-2*-transduced murine BM cells. The bottom panel shows May-Grünwald-Giemsa (MGG)-stained cytopins and the immunophenotype of the respective colonies. Type 1A CFUs contained *Mac-1*^{high} macrophages and type 1B CFUs contained mast cells (CFU-others). Type 2 CFUs contained a mixture of granulocytes, macrophages, and immature myeloblasts (CFU-L). (C) Diagram showing the number of CFUs (per 10^4 plated cells) in serial replating of *miR-125b-2*-transduced and empty vector-transduced (*MSCV-puro*; control) BM cells using methocellulose-based assays. Error bars represent means $\pm$ SD of replicates in one representative experiment. (D) Number of CFUs per 3×10^3 plated *miR-125b-2*-transduced and empty vector-transduced (*MIGR1*; control) human CD34⁺-HSPCs using collagen-based colony-forming assays. Data from $n = 3$ independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$. (E) Representative images of hematoxylin and eosin (HE)-stained colony-forming assays from *miR-125b-2*-transduced and empty vector-transduced (*MIGR1*; control) human CD34⁺-HSPCs as shown in D. The left panel shows microscopic images and the right panel shows the colony-forming assay without magnification. (F,G) Colony-forming assays of *miR-125b-2*-transduced, mutated *miR-125b-2*-transduced (mut1), and empty vector-transduced (*LMP1G*; control) human CD34⁺-HSPCs using methocellulose-based assays. (F) Diagram and statistics showing the number of CFUs per 5×10^3 plated cells. (G) Diagram and statistics showing the number of cells per CFU. Replicates from one representative experiment are shown as means $\pm$ SD. (*) $P < 0.05$. (H–J) Cell counts (in relation to day 0; =100%) (H), differential WBC counts (I), and May-Grünwald-Giemsa-stained cytopins (day 9) (J) of *miR-125b-2*-transduced and empty vector-transduced (control) human CD34⁺-HSPCs cultured in granulocytic differentiation medium. Data from $n = 3$ (H) and $n = 2$ (I) independent experiments are shown as means $\pm$ SD. (*) $P < 0.05$; (**) $P < 0.01$.

CFUs contained mast cells (CFU-other) (Fig. 4A,B). The vast majority of the CFUs (type 2) displayed a unique colony morphology and contained a mixture of granulocytes, macrophages, and immature myeloblasts (CFU-L) (Fig. 4A,B). Cells in these colonies were largely *Mac-1*^{low/mid}, whereas 10% of cells also expressed *Gr-1*. The cells stained negative for other markers, such as *Ter-119*, *Sca-1*, *c-kit*, *CD3*, and *B220* (data not shown).

In FL MPs/MEPs, enforced *miR-125b-2* expression led to increased self-renewal with enhanced replating efficiency. To investigate the self-renewal capacity in *miR-125b-2*-transduced BM HSPCs, we performed serial

replating assays. In contrast to MPs/MEPs, the number of CFUs from *miR-125b-2*-transduced BM HSPCs decreased significantly after the first replating (Fig. 4C). These data indicate that overexpression of *miR-125b-2* specifically increases the self-renewal capacity of FL MPs and MEPs, but not the adult BM HSPCs.

To test if high levels of *miR-125b-2* perturb human myelopoiesis, we retrovirally overexpressed *miR-125b-2* in CD34⁺-HSPCs. In colony-forming assays, we observed a marked increase in the number and size of CFUs upon *miR-125b-2* transduction in comparison with empty vector- and mutated *miR-125b-2*-transduced control cells

(Fig. 4D–G). Colonies exhibited unique, abnormal morphology, and contained a mixture of immature cells (CFU-L) (Fig. 4E).

During granulocytic differentiation, immature myeloblasts differentiate through the stages of promyelocytes, myelocytes, and metamyelocytes to become granulocytes in the presence of granulocyte colony-stimulating factor (G-CSF). As expected, when we cultured empty vector-transduced CD34⁺-HSPCs in granulocytic differentiation medium, we observed a gradual reduction of immature promyelocytes and an increase of more mature granulocytic cells (myelocytes, metamyelocytes, and granulocytes) with time in culture. In contrast, forced expression of *miR-125b-2* led to a 2.2-fold increase of the absolute number of cells in the culture (Fig. 4H), accompanied by an increase of the proportion of promyelocytes, whereas mature neutrophils and macrophages were greatly underrepresented, as assessed by differential white blood cell (WBC) counts (Fig. 4I,J). Only basophils were seen in the culture (Fig. 4I). Consistent with these data, we observed an increase of CD33⁺ promyelocytes upon forced *miR-125b-2* expression, whereas the mature granulocytes in the control were CD33[−] (Supplemental Fig. S5A). Consistent with those data obtained in murine BM-HSPCs, the replating efficiency was not increased in human CD34⁺-HSPCs (Supplemental Fig. S5B). Neither the myeloid differentiation block nor the increased proliferation of CD34⁺-HSPCs could be observed upon forced expression of *miR-125b-2*, mutated in the seed region, validating the specificity of the observed effects of *miR-125b-2* (Supplemental Fig. S5C–F).

These data indicate that *miR-125b-2* overexpression in HSPCs enhances their proliferation and blocks myeloid differentiation at the promyelocyte stage without enhancing replating efficiency.

Identification of *miR-125b* target genes in hematopoietic cells by integrative bioinformatic analysis

To identify target mRNAs of *miR-125b* in the hematopoietic system, we performed integrative bioinformatic analysis. We performed global microarray gene expression profiling of *miR-125b-2*-overexpressing HSPCs (*miR-125b-2* vs. empty vector), as well as *miR-125b* knock-down AMKL cell lines (anti-*miR-125b* vs. control). We reasoned that target genes of *miR-125b* would carry a *miR-125b*-binding site in the 3'UTR (untranslated region) (predicted by TargetScan) (Lewis et al. 2005; Grimson et al. 2007), and would be inversely regulated after overexpression or repression of *miR-125b*. Using dChip software (Li and Wong 2001), we identified 56 probes, representing 53 genes, among the putative *miR-125b* target gene list (as predicted by TargetScan). These genes were up-regulated in the AMKL cell lines upon *miR-125b* knockdown and down-regulated in HSPC upon *miR-125b-2* overexpression (Fig. 5A; Supplemental Fig. S6). The most represented Gene Ontology categories of the 53 target genes, as indicated by the DAVID bioinformatics database (Dennis et al. 2003), include RNA

Figure 5. Integrative bioinformatic analysis identified *miR-125b* target genes in hematopoietic cells. (A) Venn-diagram showing the number of probes (Affymetrix HG U133 plus 2.0) for genes predicted as target genes by TargetScan 4.1, up-regulated >1.2-fold upon transfection of anti-*miR-125b* in M-07 ($n = 2$) and CMK ($n = 2$) cells in comparison with the nonsilencing control ($n = 2$, respectively), and down-regulated >1.2-fold upon transduction of HSPCs with *miR-125b-2* in comparison with empty vector-transduced control cells (*MIGR1*). (B) Dual-luciferase reporter assay of HEK293T cells cotransfected with Luc-DICER1-ORF, Luc-DICER1-3'UTR, or Luc-ST18-3'UTR, together with *MIGR1-miR-125b-2* or *MIGR1-mut*. Relative light units (RLUs) are presented as means $\pm$ SD of $n = 3$ independent experiments in relation to the control (=100%). (*) $P < 0.05$. (C) Number of CFUs per 5×10^3 plated *miR-125b-2*-transduced, mutated *miR-125b-2*-transduced, *shST18*-transduced, *shDICER1*-transduced, and empty vector-transduced (*LMP1G*; control) human CD34⁺-HSPCs. Replicates from one representative experiment are shown as means $\pm$ SD. (*) $P < 0.05$. (D) Colony-forming assays of *shDICER1*-transduced and empty vector-transduced (*LMP1G*; control) murine FL cells. (Left) Microscopic AChE-stained images showing representative CFUs. (Right) Diagram showing the number of AChE-positive cells per CFU. Cell counts of five randomly picked colonies from one of three independent experiments are shown as means $\pm$ SD.

binding, cell communication, intracellular signaling cascade, and regulation of biological processes (Supplemental Table S2). The microarray data were confirmed for the candidate target genes *DICER1* and *ST18* (suppression of tumorigenicity 18) by qRT-PCR (Supplemental Fig. S7A). The knockdown of *DICER1* upon overexpression

of *miR-125b-2* in K562 cells was validated further by Western blot (Supplemental Fig. S7B).

We next sought to verify a direct regulation of these two target genes by *miR-125b-2* using a luciferase reporter assay. *ST18* has one predicted target site in the 3'UTR, and *DICER1* has one site in the 3'UTR and one conserved site (17 genomes) within the ORF, as described previously (Forman et al. 2008). We subcloned the 3'UTRs and the ORF containing the *miR-125b*-binding site of these transcripts into the luciferase-based reporter vector (*pMIR-REPORT*) and cotransfected the reporter constructs in the HEK293T cells with *pMIGR1-mut* (mutated *miR-125b-2*) or *pMIGR1-miR-125b-2*. *miR-125b-2* expression repressed the relative luciferase activity of reporter constructs containing *ST18* 3'UTR, *DICER1* 3'UTR, and *DICER1* ORF (Fig. 5B) in comparison with the mutated *miR-125b-2*. This shows that both *ST18* and *DICER1* are direct targets of *miR-125b-2*.

RNAi-mediated knockdown of miR-125b target genes DICER1 and ST18 recapitulates the hyperproliferative effect of miR-125b-2

We investigated further whether the phenotypes elicited upon overexpression or reduction of *miR-125b* levels might be reproduced by modulating expression of its target genes. The association of reduced *DICER1* and *ST18* expression levels and cancer has been described previously (Jandrig et al. 2004; Karube et al. 2005; Kumar et al. 2009). We transduced human CD34⁺-HSPCs with a retrovirus (*LMPiG*) expressing shRNAs directed to *DICER1* (*shDICER1*) or *ST18* (*shST18*), and confirmed knockdown by qRT-PCR (data not shown). Repression of *DICER1* or *ST18* increases the number of CFUs by 1.4-fold (Fig. 5C). The results were validated using two (*ST18*) and three (*DICER1*) different shRNAs, excluding the possibility of off-target effects (Supplemental Fig. S7C). In accordance with these data, when we repressed *Dicer1* expression in mouse FL cells with a MSCV-based retrovirus expressing shRNA directed to *Dicer1*, we observed hyperproliferative, AChE-positive CFU-MKs in the colony-forming assay (Fig. 5D; Supplemental Fig. S7D).

Thus, we show by integrative analysis of the transcriptome of hematopoietic cells that *miR-125b-2* may exert its propoliferative effect on hematopoietic progenitor cells by repression of *DICER1* expression and by inhibiting the tumor suppressor *ST18*.

miR-125b target genes are repressed in DS-AMKL and DS-TL

To determine if the above-identified target genes of *miR-125b* are down-regulated in primary leukemic blasts highly expressing *miR-125b*, we analyzed the global gene expression profiles of patient samples for which both *miR-125b* expression data and microarray gene expression data were available. Pearson's correlation within the Gene Set Enrichment Analysis (GSEA) (Subramanian et al. 2005) was used to determine the degree of linear relationship between *miR-125b* expression and the expression of the identified *miR-125b* target gene set. We confirmed the

negative relationship, and therefore down-regulation of the curated target genes set of *miR-125b* in samples highly expressing *miR-125b* (Fig. 6A). To validate our findings, we performed GSEA using an independent, previously published data set that includes 23 patients with the DS-AMKL and 37 patients with non-DS-AMKL (Bourquin et al. 2006). Similarly, we observed negative enrichment of the curated *miR-125b* target gene set in patients with DS-AMKL in comparison with those with non-DS-AMKL (Fig. 6B). Of note was the fact that the genes *DICER1* and *ST18* were among the leading edge subsets (Fig. 6A,B).

Thus, our data show that *miR-125b* is highly expressed in DS-AMKL, whereas its putative target genes are down-regulated in DS-AMKL samples. These findings support a potential role of *miR-125b-2* in the pathogenesis of DS-AMKL.

Discussion

We demonstrated that an Hsa21-encoded miRNA, *miR-125b-2*, plays a pivotal role in megakaryopoiesis by increasing proliferation and self-renewal of MPs and MEPs without affecting their differentiation. Our functional genetic studies further indicate that *miR-125b-2* overexpression perturbs myeloid differentiation. In authentic human DS-AMKL, *miR-125b-2* is highly overexpressed. In line with the observed impairment of proliferation of the DS-AMKL cell line and primary DS-TL blasts upon knockdown of *miR-125b*, and the demonstration of synergism between *miR-125b-2* overexpression and *Gata1s* mutation in FL progenitors, our data indicate that *miR-125b-2* is a potential oncomiR involved in the pathogenesis of trisomy 21-associated megakaryoblastic leukemia. Using an integrative bioinformatic approach, we analyzed the transcriptome after experimental alteration of *miR-125b* expression levels, and defined a set of target genes of *miR-125b* in hematopoietic cells that were down-regulated in DS-AMKL patient samples. The multidisciplinary approach we took underscores the validity of our findings and their biological relevance.

Interestingly, human Hsa21 carries several genes—including *RUNX1*, *ERG*, *ETS2*, and *GABPA*—that encode transcription factors serving as key regulators of megakaryocyte development (Elagib et al. 2003; Rainis et al. 2005; Pang et al. 2006). It was shown recently that overexpression of *ERG* and *ETS2* promoted megakaryopoiesis and synergized with *GATA1* mutations (*Gata1s* or *Gata1* knockdown) to immortalize fetal progenitors in vitro (Salek-Ardakani et al. 2009; Stankiewicz and Crispino 2009). However, none of these genes is overexpressed in DS-AMKL and DS-TL samples in comparison with non-DS-AMKL samples (Bourquin et al. 2006). Thus, it is uncertain whether the in vitro synergy between *ERG* or *ETS2* overexpression and *GATA1s* mutation is directly relevant to the pathogenesis of DS-TL and DS-AMKL in vivo. Here we show that *miR-125b-2* is another “megakaryocyte gene” on Hsa21 encoding for a different class of developmental regulators: miRNAs. In contrast to *ERG* and *ETS2*, *miR-125b* is indeed highly overexpressed in DS-AMKL and DS-TL blasts, whereas its expression is

Figure 6. *miR-125b* target genes are down-regulated in DS-AMKL. (A) GSEA of the identified *miR-125b* target gene set in leukemic blasts from patients with DS-AMKL (DS-M7, $n = 3$), DS-TL ($n = 3$), non-DS-AMKL (M7, $n = 2$), and AML FAB M5 (M5, $n = 2$). Pearson's correlation was used to determine the degree of linear relationship between *miR-125b* expression and the expression of the identified *miR-125b* target gene set. (Top) Heat map of the leading-edge subset including *DICER1* and *ST18* (arrows), with the *miR-125b* expression below. Samples are ordered according to their *miR-125b* expression. Normalized enrichment score (NES) = -1.63 ; false discovery rate (FDR) $q = 0.007$; $P = 0.007$. (B) GSEA of the identified *miR-125b* target gene set in leukemic blasts from patients with DS-AMKL (DS-M7, $n = 23$) and non-DS-AMKL (M7, $n = 37$) from a previously published data set (Bourquin et al. 2006). (Top) Heat map of the leading-edge subset including *DICER1* and *ST18* (arrows). (Bottom) Enrichment plot and statistics. NES = -1.50 ; FDR $q = 0.042$; $P = 0.042$.

low in CD34⁺-HSPCs, normal megakaryocytes, and AML FAB M5. Its overexpression appears closely associated with AMKL (especially with DS-AMKL/TL), and not with AML in general or with megakaryocytic differentiation. In fact, by miRNA profiling, it was shown that *miR-125b* was expressed in human MEPs, but was down-regulated during megakaryocyte differentiation (Lu et al. 2008). Consistent with this, we observed dramatic expansion of MEPs in response to *miR-125b-2* overexpression, indicating a potential role of this miRNA in controlling proliferation of MEPs. More importantly, we also observed synergy between overexpression of *miR-125b-2* and *Gata1s* mutation to further increase proliferation and self-renewal of fetal MPs and MEPs (presumed cells of origin for DS-AMKL/TL), indicating potentially direct involvement of this miRNA in the pathogenesis of DS-AMKL/TL. Currently, factors leading to overexpression of *miR-125b*, particularly in DS-AMKL/TL blasts, remain to be defined. It will also be of great interest to determine whether *miR-125b* is overexpressed in human DS FL cells in the absence of *GATA1s* mutation, as FLs of healthy fetuses with DS have already contained hematopoietic progenitors preferentially differentiating toward megakaryocytic and erythroid lineages (Chou et al. 2008; Tunstall-Pedoe et al. 2008).

In mouse mesenchymal stem cells, exogenous *miR-125b* expression reduced proliferation and mediated differentiation (Mizuno et al. 2008), indicating a cell context-dependent role of *miR-125b*, inducing a proliferation program in one cell type and repressing a proproliferative

program in another. The cell context-dependent difference of the effect of *miR-125b* is underscored by previous studies in prostate cancer cell lines. Despite the observation that down-regulation of *miR-125b* in AMKL cell lines and prostate cancer cell lines results in a proliferation arrest (Lee et al. 2005), the mechanism differs between cell types. The stimulation of androgen-independent growth of prostate cancer cells by *miR-125b* is at least partially mediated by down-regulation of *BAK1*, and thereby inhibition of apoptosis (Shi et al. 2007). For AMKL cell lines, we failed to detect repression of *BAK1* (data not shown). Also, the recently defined target tumor suppressor *p53* is not among our list of target genes (Le et al. 2009). This argues for a differential gene expression program involved in crucial biological processes regulated by *miR-125b*.

Previous studies showed that both human and mouse cancers are characterized by a global reduction of mature miRNA levels compared with normal tissues (Lu et al. 2005; Kumar et al. 2007). We identified and experimentally validated *DICER1* as a direct target of *miR-125b*. Our results indicate a regulatory negative feedback loop between *DICER1* and *miR-125b-2*. Production of mature *miR-125b* by *DICER1* results in repression of *DICER1* expression levels and, consequently, impaired overall miRNA processing (Fig. 7). Therefore, we propose a miRNA-mediated mechanism for low *DICER1* expression levels, and provide an explanation for the observed failure to express mature miRNAs in many cancers. In concert with those data, we observed a global reduction of

Figure 7. Proposed model for the role of Hsa21-encoded *miR-125b-2* in leukemogenesis. DICER1 and *miR-125b-2* form a regulatory negative feedback loop. Production of mature *miR-125b-2* by DICER1 results in repression of *DICER1* expression levels and, subsequently, impaired overall miRNA processing. This miRNA-mediated mechanism for low *DICER1* expression levels leads to a failure to express mature miRNAs essential for terminal hematopoietic differentiation. These pro-oncogenic effects of *miR-125b-2* can be enhanced further by the repression of tumor suppressors such as *ST18*.

miRNA expression in DS-AMKL samples highly expressing *miR-125b* (K Böhmer and D Reinhardt, unpubl.). Recently, *DICER1* post-transcriptional regulation that affects overall miRNA expression has been shown for *let-7* (Tokumaru et al. 2008). *Let-7c* resides proximal of *miR-125b-2* on Hsa21, implicating that both miRNAs may coordinately regulate *DICER1* expression. The oncogenic properties of this function of *miR-125b-2* are underscored by recently published observations that impaired miRNA biogenesis and *Dicer1* haploinsufficiency accelerates oncogenic transformation and oncogenesis of human cells and mouse models through the deregulation of target oncogenes (Kumar et al. 2007, 2009). Our study suggests a mechanism of tumor propagation and regulation of differentiation by miRNAs. Similar to embryonic stem cells (Viswanathan et al. 2008), post-transcriptional blockage of miRNA processing could perturb miRNA-mediated differentiation of HSPCs and promote development of leukemia.

These pro-oncogenic effects of *miR-125b-2* may be further enhanced by repression of tumor suppressors such as *ST18*. *ST18*, which is encoded in a frequently imbalanced region of chromosome 8q11, was first identified as a breast cancer tumor suppressor gene (Jandrig et al. 2004). This zinc finger DNA-binding protein acts as a transcriptional regulator. In breast cancer cell lines and in ~80% of primary breast tumors, *ST18* mRNA is down-regulated, partially by epigenetic mechanisms, such as hypermethylation of the promoter region. Ectopic expression of *ST18* in MCF-7 breast cancer cells inhibits colony formation as well as the formation of tumors in a xenograft mouse model (Jandrig et al. 2004). Fibroblasts overexpressing *ST18* exhibit increased apoptosis and in-

creased expression of *tumor necrosis factor-α* (*TNF-α*) and *TNF*-induced proapoptotic and proinflammatory genes (Yang et al. 2008).

In conclusion, we showed that *miR-125b-2* is an oncogene potentially involved in the pathogenesis of trisomy 21-associated leukemia. Our finding raises the possibility that up-regulation of this miRNA—either in response to, or together with, modest (1.5-fold) overexpression of other Hsa21-encoded “megakaryocyte genes” such as *ERG*, *ETS2*, *RUNX1*, or *GABPA*—may confer the leukemia-predisposing effects of Hsa21 to the megakaryocytic lineage by inducing proliferation and self-renewal of MPs and MEPs, while blocking differentiation of other lineages. Consistent with the finding of translocations involving the *miR-125b-2* homolog *miR-125b-1* in pre-B-ALL and MDS (Sonoki et al. 2005; Bousquet et al. 2008), our findings suggest a cell type-specific role of *miR-125b* in hematopoietic cells. We propose that *miR-125b-2* exerts its effect by at least two different mechanisms: inhibition of post-transcriptional miRNA processing by repressing *DICER1*, and down-regulation of tumor suppressor genes such as *ST18*.

Materials and methods

Patient samples

BM specimens from patients with non-DS-AMKL, DS-AMKL, DS-TL, or AML FAB M5, or from healthy donors were obtained from different children, and were provided by the collaborative AML “Berlin–Frankfurt–Münster” Study Group (AML-BFM-SG, Münster, Germany). The study group performed a central review of the diagnosis, classification, and clinical follow-up of the patients. Mobilized CD34⁺-HSPCs from donors were positively selected by immunomagnetic labeling with corresponding magnetic cell-sorting beads (Miltenyi Biotech). All investigations were approved by the institutional review board and Ethics Committee of the Medical School Hannover, and informed consent was obtained according to local laws and regulations.

Mice

All studies involving mice were approved by the Children’s Hospital Boston Institutional Animal Care and Use Committee, and were performed in accordance with the relevant protocols. *Gata1s* mice were generated as described (Li et al. 2005).

Cell culture and transduction

All cell lines (CMK, M-07, and K562) were obtained from the German National Resource Center for Biological Material (DSMZ), and were cultured under the recommended conditions. Fetal MPs and BM cells from adult mice were processed, expanded, and infected as published previously (Li et al. 2005). Culture, transduction on RetroNectin-coated (Takara) plates, and in vitro differentiation of CD34⁺-HSPC cells was performed as described (Klein et al. 2007; Skokowa et al. 2009). The mouse stem cell virus (pMSCV)-based pMSCV-puro (Clontech), pMIGR1 (Pui et al. 1999), pMSCV-Puro-IRES-GFP (pLPIG), and pMSCV-Puro-IRES-GFP containing the *miR-30* backbone (pLMPIG) vectors were used. Both pLPIG and pLMPIG retroviral vectors (Dickins et al. 2005) were generous gifts from Dr. Scott Lowe. pGIPZ vectors containing the shRNA sequence directed

to *DICER1* and *ST18* were obtained from OpenBiosystems, and were cloned into the *pLMP1G* vector. Retroviral and lentiviral supernatant was prepared using standard protocols. Detailed procedures and vector design are provided in Supplemental Material.

Flow cytometry and cell sorting

Standard protocols were followed. Detailed procedures and antibodies are provided in Supplemental Material.

Colony-forming assays

Murine FL cells were plated in the MegaCult-C collagen-based system (StemCell Technologies) with TPO (5 ng mL⁻¹) and were stained for AChE activity in accordance with the manufacturer's instructions, or in Methocult M3231 methylcellulose medium (StemCell Technologies) in the presence of TPO (20 ng mL⁻¹), or in Methocult M3434 methylcellulose medium (StemCell Technologies) in the presence of TPO (20 ng mL⁻¹). BM cells from adult mice were assayed for their colony-forming activity in Methocult M3231 methylcellulose medium (StemCell Technologies) in the presence of erythropoietin (Epo; 3 U mL⁻¹), IL-3 (10 ng mL⁻¹), IL-6 (10 ng mL⁻¹), and SCF (50 ng mL⁻¹); or in Methocult M3434 methylcellulose medium (StemCell Technologies). Puromycin (1 µg/mL) was included to select for transduced, puromycin-resistant cells. Human CD34⁺ or K562 cells were plated in the CollagenCult, MegaCult, or MethoCult #4434 colony-forming assays in accordance with the manufacturer's instructions (StemCell Technologies). For serial replating, cells from methocellulose-based colony-forming assays were harvested, washed in IMDM, counted, and seeded in the respective colony-forming assays at a density of 1 × 10⁴ to 2 × 10⁴ cells per plate.

Microarray and bioinformatics analysis

Microarray expression profiles were collected using Affymetrix Human Genome U133 Plus 2.0 chips, and were analyzed using dChip (Li and Wong 2001) and GSEA (Subramanian et al. 2005). For miRNA target gene prediction, we used the TargetScan 4.1 algorithm (Lewis et al. 2005; Grimson et al. 2007). Analysis of Gene Ontology categories was performed using DAVID bioinformatics database (Dennis et al. 2003). All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) with GEO Series accession numbers GSE19680 and GSE19681.

miRNA expression studies

miRNA expression was measured on a 7900HT Fast Real-Time PCR System using a TaqMan Human MicroRNA Array version 1.0 (Applied Biosystems) in accordance with the manufacturer's instructions. For miRNA Northern blots, total RNA was separated in 15% polyacrylamide TBE/Urea gel, transferred to GeneScreen Plus transfer membrane (PerkinElmer), and UV-cross-linked in Stratilinker (Stratagene). Anti-*miR-125b* (Ambion) was labeled by γ-³²P-ATP using the mirVana miRNA Detection Kit according to the manufacturer's instructions (Ambion) and was used as the probe. Standard procedures were followed for hybridization/washes and detection.

Transfection of 2'-O-methyl antisense oligonucleotides

2'-O-methyl antisense oligonucleotides labeled with Cy3 and directed against the mature *miR-125b* were chemically synthesized (BioSpring). Cells (1 × 10⁶) were electroporated (330 V, 10

msec) in 100 µL of medium containing 0.5 µg of 2'-O-methyl antisense oligonucleotides in a 4-mm electroporation cuvette using an EPI 2500 gene pulser (Fischer). Transfected cells were FACS-sorted after 24 h as described above.

Western blot

Total cell lysis and Western blotting were performed using standard protocols as described previously (Li et al. 2007). Western blots were probed with the following antibodies: DICER1 (Abcam) and GAPDH (Abcam). Proteins were visualized with ECL (Amersham) according to the manufacturer's protocols.

qRT-PCR

RNA for mRNA qRT-PCR was isolated using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For mRNA qRT-PCR, the QuantiTect SYBR Green PCR kit (Qiagen) was used according to the manufacturer's specifications. For each 25-µL reaction mixture, 250 ng of cDNA were used. mRNA expression was normalized against expression of human *GAPDH* (Qiagen) expression levels. RNA for miRNA qRT-PCR was isolated by use of miRvana miRNA Isolation Kit (Ambion). cDNA Synthesis was performed with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. miRNA expression was quantified using TaqMan miRNA assays for *hsa-miR-125b* and *RNU44* as the housekeeping gene (Applied Biosystems). Analysis was done on a StepOnePlus (Applied Biosystems) real-time thermal cycler.

Luciferase reporter assay

For reporter assays, the 3'UTRs or ORF fragment of the respective mRNAs containing the *miR-125b*-binding site were cloned from cDNA into *pMIR-REPORT* miRNA Expression Reporter Vector (Ambion). 293T cells (1 × 10⁴) were plated and cotransfected in 96-well plates by using FuGENE HD (Roche), according to the manufacturer's protocol, with 6.25 ng of the firefly luciferase report vector, 0.25 ng of the control vector containing renilla luciferase PGL4.7 (Promega), and 50 ng *miRNA* expression vector (*pMIGR1-miR-125b-2* and *pMIGR1-mut*). Firefly and renilla luciferase activities were measured consecutively by using dual-luciferase assays (Promega) 48 h after the transfection. All experiments were performed in duplicates.

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