Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A

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MicroRNA-125b (miR-125b) is up-regulated in patients with leukemia. Overexpression of miR-125b alone in mice causes a very aggressive, transplantable myeloid leukemia. Before leukemia, these mice do not display elevation of white blood cells in the spleen or bone marrow; rather, the hematopoietic compartment shows lineage-skewing, with myeloid cell numbers dramatically increased and B-cell numbers severely diminished. miR-125b exerts this effect by up-regulating the number of common myeloid progenitors while inhibiting development of pre-B cells. We applied a miR-125b sponge loss of function system in vivo to show that miR-125b physiologically regulates hematopoietic development. Investigating the mechanism by which miR-125b regulates hematopoiesis, we found that, among a panel of candidate targets, the mRNA for Lin28A, an induced pluripotent stem cell gene, was most repressed by miR-125b in mouse hematopoietic stem and progenitor cells. Overexpressing Lin28A in the mouse hematopoietic system mimicked the phenotype observed on inhibiting miR-125b function, leading to a decrease in hematopoietic output. Relevant to the miR-125b overexpression phenotype, we also found that knockdown of Lin28A led to hematopoietic lineage-skewing, with increased myeloid and decreased B-cell numbers. Thus, the miR-125b target Lin28A is an important regulator of hematopoiesis and a primary target of miR-125b in the hematopoietic system.

cancer | myelogenous | lymphocyte

MicroRNA-125b (miR-125b) is up-regulated in a range of human leukemias, including acute myeloid leukemia (1, 2), chronic myeloid leukemia (2), acute megakaryocytic leukemia (3), childhood acute lymphoblastic leukemia with the ETV6/Runx1 fusion protein (4), and Philadelphia chromosome-positive B-cell precursor childhood acute lymphoblastic leukemia (2). Indeed, overexpression of miR-125b alone in the bone marrow of mice is sufficient to induce leukemia (2, 5, 6). Recent in vitro work has also uncovered a role for miR-125b in the development of plasma cells (7) and effector T cells (8), suggesting that miR-125b regulates immune cell development in addition to promoting leukemia.

Lin28A is an important regulator of early embryogenesis in mice and humans (9). Levels of Lin28A are enriched in ES cells and decrease as these cells differentiate (9). Ectopic expression of Lin28A along with three other genes (Oct4, Sox2, and Nanog) causes dedifferentiation of mature human cells into induced pluripotent stem cells (10). This shows the power of Lin28A to endow cells with pluripotent qualities. Still, it remains to be seen whether Lin28A also plays developmental roles in biological systems arising from adult stem cells, such as hematopoiesis.

In this study, we examine the role of miR-125b in inducing cancer and show that overexpression of this microRNA induces a preleukemic state before aggressive frank leukemia is evident. This preleukemic state is characterized by overproduction of myeloid cells and their precursors as well as inhibition of B-cell development. We then show that the most affected target gene of miR-125b is Lin28A and that Lin28A down-regulation can mimic the preleukemic state induced by miR-125b. Furthermore, down-regulation of miR-125b has profound effects on normal hematopoiesis, and Lin28A overexpression mimics those effects.

Results

miR-125b Ectopic Expression Favors Myeloid Differentiation and Causes a Highly Invasive Myeloid Leukemia. Previously, we showed that overexpression of miR-125b in bone marrow-transplanted recipient mice causes a myeloid leukemia 4–6 mo after bone marrow reconstitution (5). Here, we found that the neoplastic myeloid cells infiltrate nonhematopoietic organs, including the brain, and overwhelm the periphery (Fig. 1 and Figs. S1 and S2). Mice overexpressing miR-125a also developed an aggressive leukemia that was shown by enlarged spleen and cancer infiltration into the liver and kidneys (Fig. S3). These mice died by 6 mo postreconstitution. Thus, dysregulated expression of miR-125b or its paralogue leads to an aggressive leukemia that efficiently invades nonhematopoietic organs.

To understand the initial events that precede the frank leukemia, we examined the spleen and bone marrow at 7 wk after reconstitution, a time well before the onset of cancer as indicated by similar white blood cell counts in the spleen (~100 million cells for both MG and 125b spleens) and bone marrow of control MG (murine stem cell virus-GFP retro-vector) and MG-125b animals (Fig. 2*A* and Fig. S4 *A* and *B*). At this time, MG-125b animals showed dramatic increases in all myeloid lineages, including granulocytes, macrophages, and dendritic cells, with T-cell numbers being similar to those in the MG mice (Fig. 2*A* and Fig. S4 *C*–*E*). However, B-cell numbers were significantly decreased in these miR-125b–overexpressing animals (Fig. 2*A* and Fig. S4*F*). Whether miR-125b physiologically regulates B-cell development will require additional studies. Thus, miR-125b overexpression at the precancerous stage causes a lineage-skewing of the hematopoietic compartment with myeloid cell numbers elevated and B cells diminished.

To investigate the developmental stage at which miR-125b overexpression promotes myelopoiesis and compromises B-cell development, we analyzed hematopoietic stem and progenitor cell (HSPC) numbers in the bone marrow at 7 wk after bone marrow transplantation. In these mice, the hematopoietic stem cell (HSC) numbers were similar in MG-125b animals compared with MG controls (Fig. S4 *G* and *H*). Progenitors directly downstream of HSCs, however, including multipotent progenitors (MPPs), common myeloid progenitors and granulocyte

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Conflict of interest statement: D.B. is a Director of Regulus, a company devoted to commercialization of antimicroRNA therapies.

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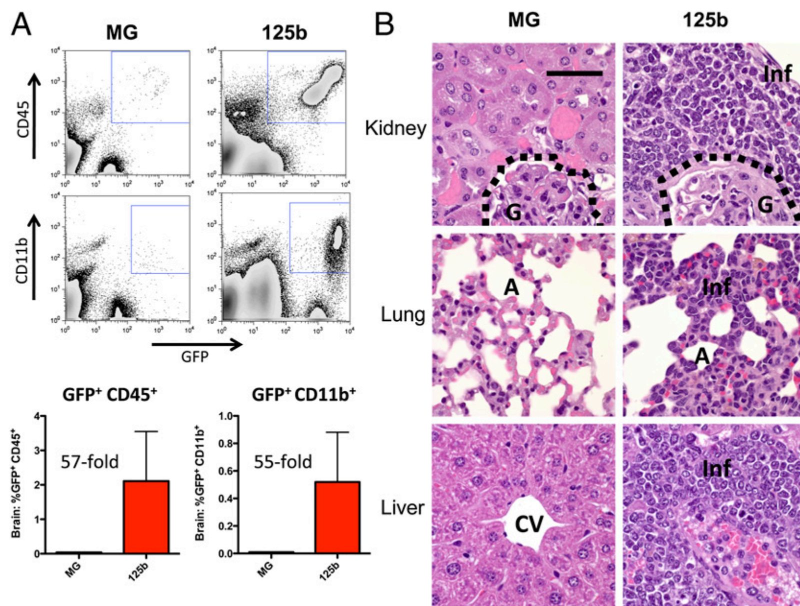

Fig. 1. miR-125b overexpression causes an aggressive invasive myeloid leukemia. (*A*) Infiltration of GFP$^+$ CD45$^+$ and GFP$^+$ CD11b$^+$ cells into the brain. (*Upper*) Representative flow cytometric plots are shown. (*Lower*) Average percent GFP$^+$ CD45$^+$ and GFP$^+$ CD11b$^+$ in the brain are shown from three MG and two MG-125b mice. (*B*) Leukemic cell infiltration into nonhematopoietic organs. Sections from the kidney, lung, and liver were stained with H&E. The normal structures of the MG-125b mouse kidney, lung, and liver are effaced by a dramatic infiltrate of leukemic cells. A representative image for each tissue is shown. The brain, kidney, lung, and liver were harvested from animals 5 mo after bone marrow reconstitution. During the time of harvest, the average percent GFP$^+$ cells in the spleens of MG and MG-125b mice were 49 ± 9.7% and 91 ± 2.9%, respectively. All plots shown depict the mean with SEM. All data are representative of two independent experiments. Inf, infiltrate; G, glomerulus; A, alveolar space; CV, central vein.

macrophage progenitors (CMPs/GMPs), myeloid–erythroid progenitors (MEPs), and common lymphoid progenitors (CLPs), were all significantly increased (Fig. 2*B*). Of these progenitors, CMPs/GMPs were the most drastically augmented, being elevated 8.4-fold in MG-125b animals compared with controls (Fig. 2*B*). Notably, pre–B-cell numbers were severely decreased (Fig. 2*B*), providing a developmental basis for the decreased B-cell numbers that we observed in MG-125b animals. This finding also indicates that miR-125b overexpression causes a developmental block between the CLP and pre–B-cell stages. In summary, we show here that, before the onset of leukemic disease, miR-125b skews hematopoietic differentiation to the myeloid lineage likely by increasing CMP/GMP and blocking B-cell development.

Physiological Regulation of Hematopoiesis by miR-125b. Although overexpression studies are relevant to the role of miR-125b as an oncomiR, we developed a loss of function system to assess the potential physiological role of miR-125b in hematopoiesis. We generated a sponge decoy to competitively inhibit miR-125b binding to its natural targets (11–14) (Fig. S5*A*). Of note, the sponge does not decrease miR-125b expression, but rather, it serves as a decoy to compete miR-125b away from endogenous targets. Indeed, the sponge decoy (MG sponge) was capable of significantly derepressing a luciferase reporter vector containing an artificial 3′ UTR with two perfect miR-125b complementary sequences (Fig. S5*B*). Showing the effectiveness of this approach, a UTR-less reporter vector was not derepressed by the sponge (Fig. S5*C*). To determine the hematopoietic effect of miR-125b loss of function, recipient mice were reconstituted with equal numbers of sorted GFP$^+$ MG and MG sponge-transduced bone marrow cells (Fig. S5*D*). The MG sponge mice at 1 mo postreconstitution had significantly fewer white blood cells (CD45$^+$), myeloid cells (CD45$^+$ CD11b$^+$), pre-erythrocytes (CD45$^+$ Ter119$^+$), and granulocytes (CD45$^+$ Gr1$^+$) compared with the MG controls (Fig. 3). The blood was also noticeably thinner in the sponge mice compared with controls, indicating that mature erythrocytes were decreased as well. The sponge data indicate an important physiological role for miR-125b in hematopoietic development, whereas the overexpression data show that excess levels lead to cancer.

Lin28A Is a Primary Target of miR-125b in the Hematopoietic System. To identify miR-125b targets that might regulate hematopoietic development, we performed an unbiased screen using Targetscan (15–17) to isolate putative miR-125b targets with $P_{\text{Conserved Targeting}} > 0.8$ ($P > 0.8$ that the putative microRNA site is evolutionarily maintained because of selective microRNA targeting rather than chance) (17). Application of this screen yielded 192 genes. Gene ontology analyses indicate that these genes are functionally enriched for biological processes that include transcriptional regulation, vasculature development, proteolysis, and apoptosis (David Functional Annotation Bioinformatics, $P < 0.05$). We focused on the processes that were proapoptotic or involved with stem cell regulation, because these processes have been correlated with leukemic development. This focus yielded four candidate genes (Bak1, Trp53inp1, BMF, and Lin28A) that, independently, have been previously validated as miR-125b targets by other groups (2, 18–20). We examined and confirmed by quantitative PCR that miR-125b represses these genes, including Lin28A, in 5-fluorouracil (5-FU)–treated bone marrow hematopoietic cells enriched for HSPCs (Fig. 4*A*). As evidence of these genes being miR-125b direct targets, we found that the 3′ UTR luciferase reporter of all of these genes, but not the negative control Picalm, was significantly repressed by miR-125b overexpression (Fig. 4*B*). Notably, endogenous expression of Lin28A, Trp53inp1, and BMF was derepressed by the miR-125b sponge decoy in HSPC-enriched bone marrow (Fig. S6*A*). The miR-125b sponge decoy also derepressed fivefold a luciferase reporter containing the mouse Lin28A 3′ UTR (Fig. 4*C*), which contains a 9-nt miR-125b seed sequence (Fig. S6*B*). Showing that the miR-125b:Lin28A interaction exhibits species conservation between mouse and human hematopoietic cells, human myeloid K562 cells transduced with MG-puromycin retro-vector (MGP), MGP-125b-1, MGP-125b-2, or a seed mutant of miR-125b exhibited seed-dependent repression of Lin28A by both miR-125b species at both the RNA and protein levels (Fig. 4*D* and Fig. S6 *C–E*). Of note, the expression of the Lin28B isoform was not repressed by miR-125b (Fig. S6 *F* and *G*). In summary, miR-125b directly represses Lin28A in hematopoietic cells, an interaction conserved between mouse and human. Previously, upregulation of Lin28A and Lin28B has been associated with cancer development (21). Thus, we examined the expression of these genes in leukemic samples overexpressing miR-125b but found that Lin28A and Lin28B expression was similar in the cancer samples and control cells (Fig. S7).

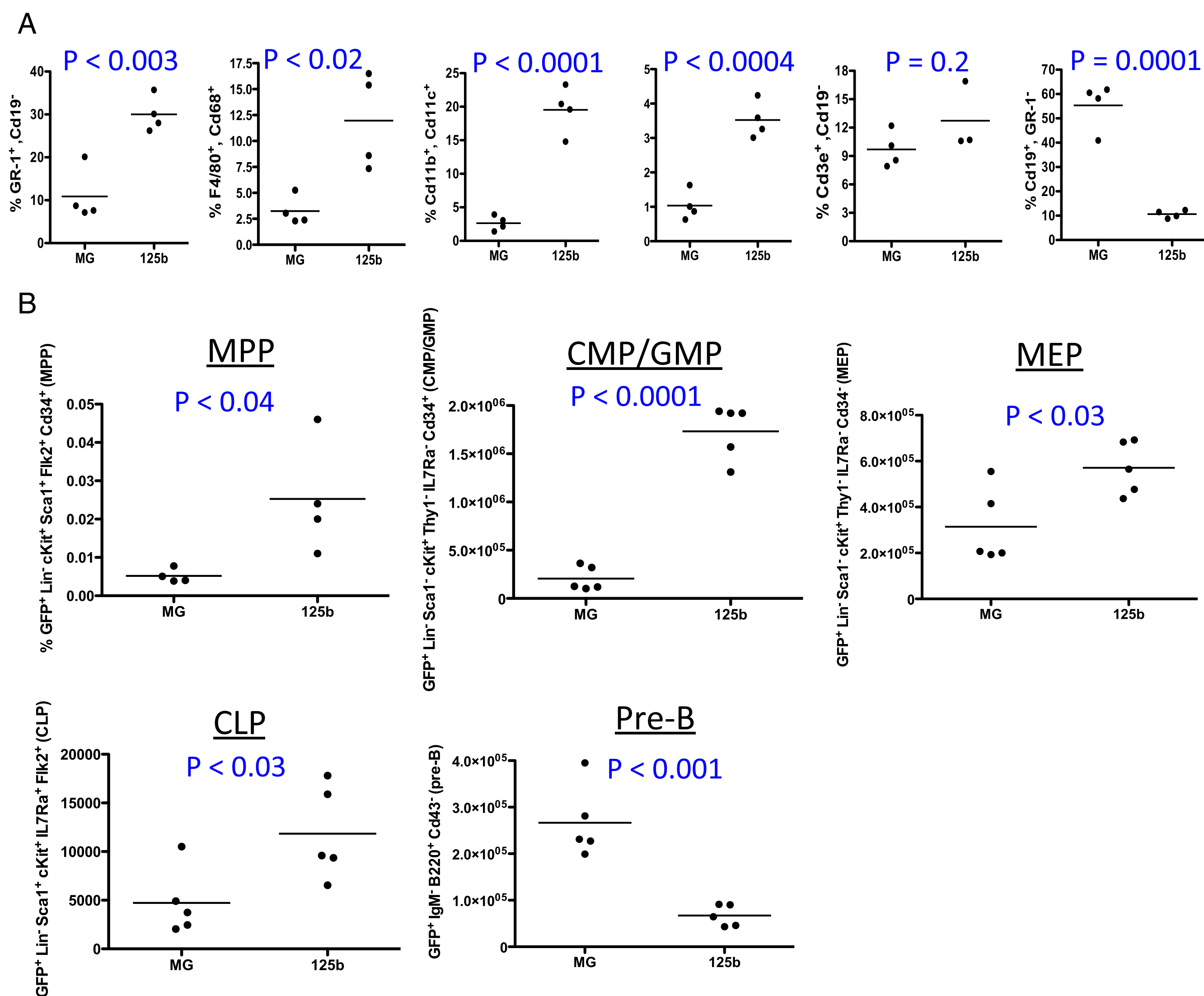

Fig. 2. miR-125b overexpression causes a skewing of the hematopoietic system at 7 wk postreconstitution of bone marrow. (*A*) Flow cytometric analyses of MG and MG-125b spleens were used to quantify the percent of granulocytes (GR-1$^+$ CD19$^-$), macrophages (F4/80$^+$ CD68$^+$), dendritic cells (CD11b$^+$ CD11c$^+$), T cells (CD3ε$^+$ CD19$^-$), and B cells (CD19$^+$ GR-1$^-$). Total leukocyte counts in the spleen were similar between MG and MG-125b mice. (*B*) The percent of MPPs and total numbers of CMPs/GMPs, MEPs, CLPs, and pre-B cells in the bone marrow were measured by flow cytometry. Horizontal lines represent the means, and each dot represents an individual mouse. Data are representative of two to three independent experiments with four to five mice per group.

Lin28A Regulates Aspects of Hematopoiesis That Mirror Those Controlled by miR-125b. The regulation of Lin28A by miR-125b is highly conserved across species. The *Caenorhabditis elegans* homolog of miR-125b Lin-4 also represses the RNA binding protein Lin28A. The Lin-4:Lin28A cascade has been shown to be critical for proper worm development. However, a potential critical developmental function of miR-125b:Lin28A signaling in mammals has not yet been explored. In addition, Lin28A is known to be involved in maintaining pluripotency of ES cells, but its role in other biological events, such as hematopoiesis, has not been fully characterized. Thus, we were interested in determining whether miR-125b–mediated repression of Lin28A has a role in the hematopoietic system. We bypassed the repressive effect exerted by endogenous miR-125b by overexpressing Lin28A lacking its 3′ UTR. We used the murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP (MIG) vector system (22), which allowed the coexpression of Lin28A and GFP from the same vector. A Western blot was performed to confirm expression of Lin28A from the MIG-Lin28A vector in transfected 293T cells (Fig. S8*A*). Next, we performed bone marrow transplant experiments with HSPC-enriched bone marrow cells transduced with either MIG or MIG-Lin28A. We achieved over 80% transduction efficiency as measure by GFP-positive cells (Fig. S8*B*). Similar to mice reconstituted with the miR-125b sponge decoy (Fig. 3), animals with Lin28A overexpression had significant decreases in total white blood cells (CD45$^+$), total myeloid cells (CD45$^+$ CD11b$^+$), granulocytes (CD45$^+$ Gr1$^+$), and erythrocytes (Ter119$^+$ CD11b$^-$) (Fig. 5). Also similar to the sponge mice, the blood was noticeably thinner from MIG-Lin28A animals compared with MIG controls, likely because of the diminished erythrocyte numbers that we observed. Of importance, whereas all of the controls remained healthy, the Lin28A overexpressing mice with the thinnest blood (2 of 15) died at 5 wk postreconstitution, likely as a consequence of impaired hematopoietic development. Thus, overexpressing Lin28A in the hematopoietic system mirrored the phenotypes observed on miR-125b inhibition (Fig. 3).

Next, we used Lin28A loss of function studies to examine whether Lin28A physiologically regulates hematopoiesis. Also,

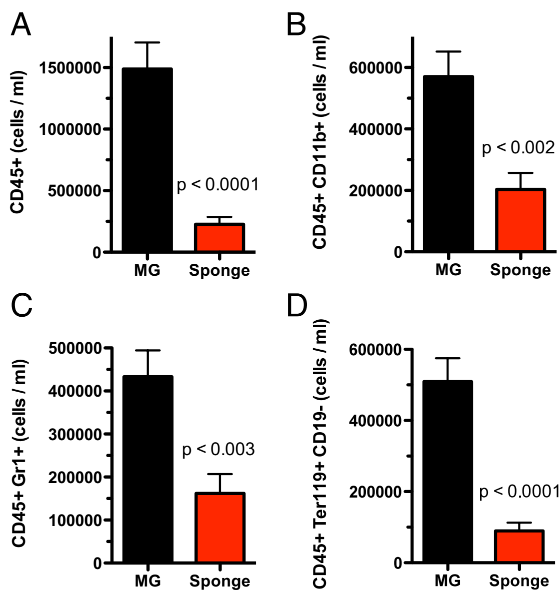

Fig. 3. Inhibiting miR-125b function decreases hematopoietic output. Equal numbers of sorted GFP+ MG- and MG-Sponge–transduced progenitor-enriched bone marrow cells (BMCs) were injected into recipient animals. (*A*) Total numbers of white blood cells, (*B*) myeloid cells, (*C*) granulocytes, and (*D*) pre-erythroid cells were measured by flow cytometry. Data represent mean number of cells per milliliter of blood (SEM shown). *P* values were calculated using unpaired Student *t* test. All results are representative of two independent experiments with 9–10 animals per group.

loss of function assays allowed us to determine whether inhibiting Lin28A function would mimic specific aspects of hematopoietic development observed on overexpressing miR-125b. Thus, we transduced HSPC-enriched bone marrow with Lin28A shRNA and achieved ~70% transduction with a twofold knockdown of Lin28A as assessed by quantitative PCR (Fig. 6 *A* and *B*), less than the level of repression exerted by miR-125b overexpression (Fig. 4*A*). Strikingly, mice reconstituted with shLin28A exhibited similar but less dramatic features of hematopoietic development to those features caused by overexpression of miR-125b at the precancerous stage: expansion of myeloid cells ($CD45^+$ $CD11b^+$, $CD45^+$ $Gr1^+$) and a decrease of B cells ($CD19^+$ $CD11b^-$) (Fig. 6 *C*–*E*). The Lin28A knockdown mimicked the alteration of the hematopoietic system that we observed in miR-125b overexpressers (Fig. 2*A*), namely increased myeloid cells and decreased B cells (Fig. 6 *C*–*E*). Thus, we show that Lin28A is a primary target of miR-125b in hematopoiesis and that its levels influence development of the hematopoietic system.

Discussion

We show here that (*i*) miR-125b overexpression causes a highly invasive myeloid leukemia; (*ii*) in the preleukemic stage of disease, miR-125b induces a skewing of the hematopoietic system favoring myeloid development; (*iii*) before terminal leukemia development, miR-125b drastically increases CMP/GMP numbers; (*iv*) in addition to the pathological role of miR-125b, it also physiologically regulates hematopoiesis as shown by down-regulating it with a sponge construct; (*v*) Lin28A is a bona fide primary target of miR-125b in hematopoietic cells; and (*vi*) Lin28A overexpression and knockdown mimic important aspects of miR-125b loss of function and gain of function, respectively. We conclude that miR-125b physiologically regulates hematopoiesis and constitutive overexpression of miR-125b in our experimental system, leading to uncontrolled generation of myeloid progenitors and mature myeloid cells that subsequently causes a myeloid leukemia. We also conclude that the miR-125b primary target Lin28A is an important regulator of hematopoietic development.

In this study, we observed that miR-125b overexpression induces an aggressive myeloid leukemia that is highly invasive. Of note, 2 of 14 mice analyzed in two cohorts exhibited significantly increased lymphoid cells as well, supporting the studies by Enomoto et al. (2), Bousquet et al. (6), and Ooi et al. (23) that show that miR-125b is capable of causing leukemias involving lymphoid cell types. Interestingly, the work by Enomoto et al. (2) confirms the remarkable potency of miR-125b as an oncomiR by showing that B cell-restricted ectopic expression of miR-125b can drive lymphoid leukemia. In our system, miR-125b and GFP are expressed from the same transcript. Interestingly, the GFP intensity in the $CD11b^+$ myeloid cells of the MG-125b mice was much higher than the intensity of $CD19^+$ B cells (geometric mean fluorescence of 85.2 ± 7.3 versus 15.7 ± 0.8, respectively), suggesting that miR-125b is expressed more highly in the former

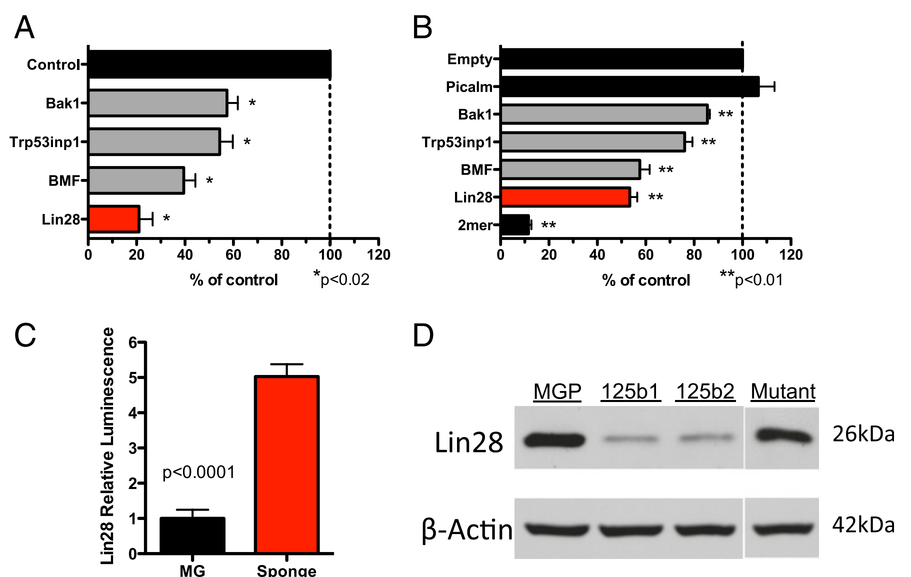

Fig. 4. miR-125b represses Lin28A expression in mouse and human hematopoietic cells. (*A*) Relative expression of miR-125b candidate targets in miR-125b–overexpressing progenitor-enriched BMCs compared with MG control. Bone marrow cells from 5-FU–treated mice were infected with miR-125b–overexpressing vector, and RNA was subsequently harvested for expression level analysis. The relative expression level was measured by quantitative PCR. (*B*) 3′ UTR reporter analyses. The 3′ UTR of the indicated genes was cloned into a luciferase reporter and cotransfected with a miR-125b expression vector. Relative luminescence of the luciferase reporters was measured and normalized to an empty vector control. The Picalm 3′ UTR, which contains no miR-125b putative binding sites, serves as a negative control. The 2mer positive control, which consists of two adjacent miR-125b antisense sites, is also shown. (*C*) Relative luminescence of the Lin28A 3′ UTR reporter cotransfected with either MG or miR-125b sponge into 293T cells. (*D*) Protein expression of Lin28A transduced with either MGP empty vector or MGP expressing miR-125b-1, miR-125b-2, or a miR-125b seed mutant in K562 cells. Protein levels were obtained by Western blot, and β-actin was included as a loading control. All data shown represent mean with SEM of two to three independent experiments. $*P < 0.02$, $**P < 0.01$.

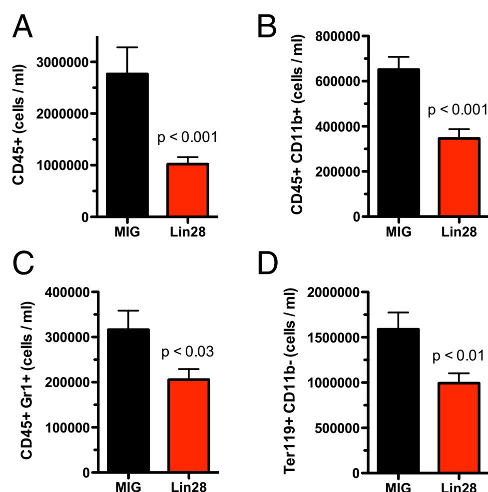

Fig. 5. Lin28A overexpression inhibits hematopoiesis. Recipients were transplanted with progenitor-enriched BMCs transduced with MIG-Lin28A or MIG. Five weeks postreconstitution, the number of (*A*) white blood cells, (*B*) myeloid cells, (*C*) granulocytes, and (*D*) erythrocytes per milliliter in the blood were measured by flow cytometry. We did not observe an increase in B cells in the MIG-Lin28A animals. Data are pooled from two independent groups of MIG-Lin28A animals, each with seven to eight animals, and compared with MIG (six animals). Data shown graphically represent the mean and SEM.

lineages. This finding might explain the predominance of myeloid leukemia observed in our system. Of importance, miR-125b has been found to be overexpressed in a variety of human leukemias, including acute myeloid leukemia (1, 2), chronic myeloid leukemia (2), acute megakaryocytic leukemia (3), and childhood acute lymphoblastic leukemia (2, 4), suggesting that deregulated miR-125b expression in different contexts can lead to distinct types of cancer.

Interestingly, the work by Ooi et al. (23) showed that recipient mice reconstituted with miR-125b overexpression display elevated HSC numbers. Although we did not observe an increase in HSC numbers, our study does not exclude that miR-125b regulates HSC development; the discrepancy likely reflects differences in experimental models used between the two studies. The work by Ooi et al. (23) overexpressed miR-125b in purified HSCs using a lentiviral system, whereas we ectopically expressed the microRNA in unsorted progenitor-enriched bone marrow hematopoietic cells using a retroviral system. It is possible that our retroviral system may not adequately overexpress miR-125b in HSCs to observe a phenotype in these cells. Nonetheless, we show here that miR-125b overexpression is capable of inducing an aggressive myeloid leukemia and increases levels of MPPs, CMPs/GMPs, MEPs, and CLPs independent of HSC numbers. In the future, it will be interesting to investigate whether miR-125b overexpression drives tumorigenesis in myeloid progenitors and/or mature myeloid cells.

We show here that miR-125b is an important regulator of hematopoiesis both physiologically and pathologically. Searching for a mechanism by which miR-125b exerts its effects on the hematopoietic system, we found that the direct target of miR-125b, Lin28A, is also a regulator of hematopoiesis. The miR-125b seed sequence within 3′ UTR of Lin28A has been previously identified and shown to be functionally important for miR-125b repression (20). In our study, manipulating Lin28A expression levels in mice mimicked the hematopoietic phenotypes of those mice with modulated miR-125b levels. Of note, shRNA-mediated knockdown of Lin28A was less effective than miR-125b–mediated knockdown, indicating that better specific knockdown of Lin28A might more precisely mimic the miR-125b overexpression phenotype. Although we show Lin28A as a miR-125b target important for hematopoiesis, we do not exclude that other miR-125b targets might play a role as well. Indeed, the work by Le et al. (24) has recently showed that miR-125b regulates a panel of genes in the p53 pathway. Potentially, Lin28A knockdown by miR-125b may help set the stage for myeloid leukemia. We did not, however, observe obvious signs of leukemia in the shLin28A mice at 3 mo after bone marrow transplantation, suggesting that either cancer will occur later or repression of other targets, such as the proapoptotic genes Bak1, Trp53inp1 and BMF, may collaborate with Lin28A inhibition during miR-125b–mediated cancer.

In summary, this work links two molecules shown in different contexts to be important in development, miR-125b and Lin28A, and indicates that their interaction serves as a fundamental regulator of hematopoietic physiology and pathology. Indeed, the interplay between miR-125b and Lin28A is one that might be toggled therapeutically to ameliorate hematopoietic disorders such as leukemia and bone marrow failure.

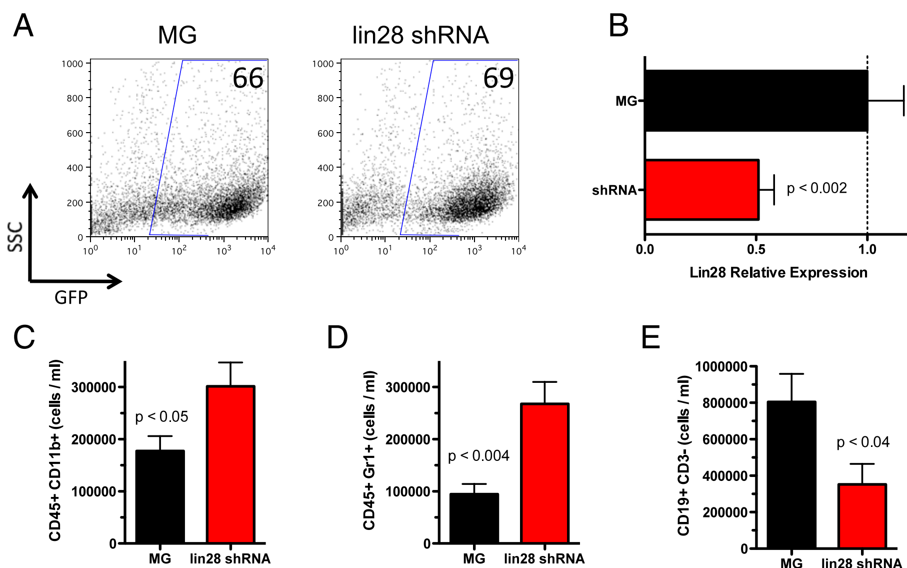

Fig. 6. Inhibition of Lin28A increases the number of myeloid cells but decreases B cells. (*A*) Flow cytometry of progenitor-enriched bone marrow transduced with MG-Lin28A shRNA or MG control vector was performed, and the percent GFP^+ cells is indicated. (*B*) Lin28A mRNA expression was obtained by quantitative PCR on the samples shown in *A*, and the results were normalized to L32. Representative of two independent experiments with four samples per group. (*C*) Total myeloid, (*D*) granulocytes, and (*E*) B cells were measured from blood 5 wk postreconstitution of bone marrow with seven to eight animals per group. Data shown represent mean and SEM. *P* values are indicated.

Methods

Cell Culture. 293T cells were cultured in complete DMEM with 10% FBS, 100 units/mL penicillin, and 100 units/mL streptomycin. K562 cells and splenocytes from mice were cultured in complete RPMI with 10% FBS, 100 units/mL penicillin, 100 units/mL streptomycin, and 50 μM 2-Mercaptoethanol.

DNA Constructs. The retroviral vectors MG, MGP, MGP-125b-1, and MGP-125b-2 have been described previously (5, 25–27). Both Lin28A shRNA and MGP-125b-mutant were cloned using an miR-155 arms and loop format, which has been previously described (5, 25–27). The Lin28A shRNA sequence was predicted using the Invitrogen BlockiT RNAi Designer. The 3′ UTRs for mouse Lin28A, Trp53inp1, Bak1, and BMF as well as the antisense 2mer were cloned downstream of luciferase in the pMIR-REPORT vector (Ambion). For the Lin28A overexpression vector, the mouse Lin28A coding sequence (Open Biosystems) was cloned downstream of the LTR and preceding the IRES-GFP of the MIG vector (22). All primers are listed in Table S1.

Retroviral Transduction and Bone Marrow Reconstitution Experiments. C57BL/6 mice were bred and housed in the Caltech Office of Laboratory Animal Resources facility. The Caltech Institutional Animal Care and Use Committee approved all experiments related to mice. Virus production and reconstitution experiments were performed as previously described (5, 25–27). K562 cells were transduced with vesicular stomatitis virus glycoprotein–pseudotyped MGP, MGP-125b-1, MGP-125b-2, and MGP-125b-Mut as described previously (5).

Cell Counting and Flow Cytometry. Cells were counted using a Coulter Counter (Beckman Coulter) or a MACSQuant flow cytometer (Miltenyi). Absolute cell counts in the blood were obtained by FACS staining equivalent volumes of blood, resuspending the stained samples in equivalent volumes of FACS buffer, and running equal volumes of each sample using the MACSQuant. For flow cytometry, cells were harvested and homogenized, and red blood cells were lysed. Cells were stained with the following fluorophore-conjugated antibodies that were purchased from Biolegend, Ebioscience, or BD Pharmingen and assayed using MACSQuant (Miltenyi) or a FACSCalibur (Becton Dickinson). All data were analyzed with FlowJo software (Treestar). Specific gating strategies are available on request.

Cell Sorting for Sponge Reconstitution. 5-FU–enriched bone marrow was transduced with retroviral vectors as previously described (5, 25–27). Before i.v. injection into mice, GFP-positive MG- and MG-Sponge–transduced samples were sorted using an iCyt cell sorter. Equal numbers of cells were then injected into lethally irradiated recipient animals.

Target Analysis. Predicted miR-125b targets in *Homo sapiens* were downloaded from Targetscan and rank-ordered by their probability of conserved targeting (P_{CT}) scores. Repeated target genes were removed from this list using a PERL script (available on request). Literature search was performed on the resulting list to identify candidate target genes. For one target (Lin28A), the 3′ UTR for the mouse version of the gene was obtained and aligned with miR-125b using TargetScan.

Luciferase Reporter Assays. Reporter assays to measure microRNA-based repression of target 3′ UTRs were performed as previously described (25–27). For derepression assays with the sponge construct, we added MG (empty vector) or MG-Sponge to the transfection mix. After 24 h, cells were lysed with Passive Lysis Buffer (Promega), and luciferase assays were performed as previously described (25–27).

RNA Preparation and Quantitation. RNA was isolated with TRIzol (Invitrogen) as per the manufacturer's instructions. We performed SYBR Green-based (Quanta Biosciences) quantitative real-time PCR with a 7300 Real-Time PCR machine (Applied Biosystems) to assay mRNA levels. A Taqman approach was used to quantify miR-125b and snoRNA-202 expressions (Applied Biosystems). All mRNA levels were normalized to L32, whereas microRNA-125b levels were normalized to snoRNA-202. Sequence-specific primers are listed in Table S1.

Immunoblotting. Total cell extracts were fractionated by electrophoresis on a 12% SDS polyacrylamide gel and electroblotted onto a Trans-Blot nitrocellulose membrane with a semidry transfer apparatus. Protein detection was performed using the following antibodies: Lin28A (A177; Cell Signaling), β-Actin (A1978; Sigma), anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology), and anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology).

Statistical Tests. Statistical tests were performed using Graphpad PRISM software or Microsoft Excel. The two-tailed Student *t* test was used to determine *P* values.

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