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MicroRNA-126–mediated control of cell fate in B-cell myeloid progenitors as a potential alternative to transcriptional factors

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Lineage specification is thought to be largely regulated at the level of transcription, where lineage-specific transcription factors drive specific cell fates. MicroRNAs (miR), vital to many cell functions, act posttranscriptionally to decrease the expression of target mRNAs. MLL-AF4 acute lymphocytic leukemia exhibits both myeloid and B-cell surface markers, suggesting that the transformed cells are B-cell myeloid progenitor cells. Through gain- and loss-of-function experiments, we demonstrated that microRNA 126 (miR-126) drives B-cell myeloid biphenotypic leukemia differentiation toward B cells without changing expression of E2A immunoglobulin enhancer-binding factor E12/E47 (E2A), early B-cell factor 1 (EBF1), or paired box protein 5, which are critical transcription factors in B-lymphopoiesis. Similar induction of B-cell differentiation by miR-126 was observed in normal hematopoietic cells in vitro and in vivo in uncommitted murine c-Kit$^+$Sca1$^+$Lineage$^-$ cells, with insulin regulatory subunit-1 acting as a target of miR-126. Importantly, in EBF1-deficient hematopoietic progenitor cells, which fail to differentiate into B cells, miR-126 significantly up-regulated B220, and induced the expression of B-cell genes, including recombination activating genes-1/2 and CD79a/b. These data suggest that miR-126 can at least partly rescue B-cell development independently of EBF1. These experiments show that miR-126 regulates myeloid vs. B-cell fate through an alternative machinery, establishing the critical role of miRNAs in the lineage specification of multipotent mammalian cells.

cell fate decision | lymphopoiesis

Lineage specification is critical in mammalian development, as well as in adult tissue maintenance. In mammals, this developmental hierarchy has been most extensively studied in the hematopoietic system, where well-characterized cell-surface markers allow the purification of distinct cell populations. Lineage specification has been thought to be largely regulated at the level of transcription, where lineage-specific transcriptional factors drive specific cell fates (1–4). Early B-cell factor 1 (EBF1) specifies B-cell differentiation (5), and GATA-3 drives Th2 lineage commitment of CD4 T cells (6). However, regulation of differentiation at the transcriptional level alone does not appear to explain all hematopoietic cell-fate decisions, suggesting the presence of other as-yet-unknown mechanisms for establishing cell fate. Ectopic expression of c-enhancer binding protein-α (c/EBPα) or knock-out of paired box protein 5 (PAX5) in B cells are both capable of reprogramming B cells to macrophages; however, down-regulation of c/EBPα or ectopic expression of PAX5 or E2A immunoglobulin enhancer-binding factor E12/E47 (E2A), both critical transcription factors for B-cell differentiation, fail to reprogram myeloid-committed cells to B cells (7). Therefore, we hypothesized that the developmental fate of mammalian multipotent cells may be guided, at least in part, by a different mechanism of gene regulation, namely, microRNAs (miRNAs).

miRNAs are recently discovered class of small, noncoding RNAs that are 18–24 nt long and that down-regulate target genes at the posttranscriptional level. The majority of miRNA genes are transcribed by RNA polymerase II into long primary (pri) miRNA transcripts, processed by the nuclear nuclease, Drosha, into ∼60-bp hairpins, termed precursor (pre) miRNAs, and further cleaved in the cytosol by the Dicer nuclease into mature miRNAs. Mature miRNAs are then incorporated into the multiprotein, RNA-induced silencing complex, exerting post-transcriptional repression of target mRNAs, either by inducing mRNA cleavage, mRNA degradation, or by blocking mRNA translation (8, 9).

Each miRNA is thought to have several target mRNAs, and computational predictions suggest that more than a third of all human genes are targets of miRNAs (10, 11). In animals, miRNAs control many developmental and physiological processes. In *Caenorhabditis elegans*, abnormal expression of certain miRNAs leads to developmental arrest (12). Many studies have revealed specific changes in miRNA expression profiles that correlate with particular human tumor phenotypes (13, 14). In the hematopoietic system, miR-181a down-regulates several phosphatases that regulate the sensitivity of T cells to antigens, and overexpression of miR-181 in hematopoietic stem/progenitor cells significantly increases B-cell production. In addition, overexpression of miR-150 leads to a block in B-cell formation at the proB-to-preB cell transition step by down-regulating c-myb, among other targets (15–18).

Down-regulation of specific miRNAs in certain cancers implies that some miRNAs may act as tumor suppressors. For example, let-7 family members directly down-regulate *Ras* and other protooncogenes. Reduced expression of let-7 family members has been previously characterized in lung cancer (19, 20). On the other hand, increased expression of miR-17–92 and miR-155 often occur in B-cell lymphomas (21), implying that these miRNAs can act as oncogenes (22, 23). Thus, miRNAs are capable of acting as either oncogenes or tumor suppressors.

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The *myeloid/lymphoid leukemia* (*MLL*) gene is located at 11q23, a site frequently involved in chromosomal translocations that occur in aggressive human lymphoid and myeloid leukemias. MLL-AF4 acute lymphoblastic leukemia (ALL) is associated with steroid resistance, has a poor prognosis (24, 25), and is associated with "lineage fragility." MLL-AF4 ALL often expresses both B-cell and monomyelocytic surface antigens; hence, it is often described as "biphenotypic" leukemia. This characteristic suggests that early hematopoietic progenitors are transformed in MLL-AF4 ALL.

A recent survey of miRNAs in ALL showed that miRNA expression patterns differ among ALL subtypes (13). We analyzed publicly available raw data (www.broad.mit.edu/mpr/publications/projects/microRNA/ALL.gct) and discovered that many miRNAs were down-regulated in ALL with *MLL* rearrangements, compared with ALL that do not harbor *MLL* rearrangements (26). Importantly, some miRNAs that have been reported to be tumor suppressors were down-regulated to considerable degrees, raising the question whether these miRNAs are involved in the biology of *MLL*-rearranged ALL, especially in regard to its lineage fragility.

Here, we focused on miR-126, which is down-regulated in *MLL*-rearranged ALL compared with other types of ALL. Through gain- and loss-of-function experiments, we showed that miR-126 positively regulated B-cell fate without affecting expression of EBF1, E2A, and PAX5 by targeting insulin regulatory subunit-1 (IRS-1). Most importantly, miR-126 could partly rescued failed B-cell development in EBF1-deficient hematopoietic progenitor cells (HPCs). Our results elucidate a unique mechanism involved in cell fate, which can partially rescue B lymphopoiesis in EBF1 deficiency.

Results

miR-126 Is Down-Regulated in MLL-AF4 ALL, Compared with Other Types of ALL. We analyzed publicly available raw data (www.broad.mit.edu/mpr/publications/projects/microRNA/ALL.gct) and found that in *MLL*-rearranged ALL, many miRNAs were down-regulated, compared with other types of ALL (13, 26). Of the 10 miRNAs that showed the most dramatic down-regulation, we chose to further analyze miR-126, which has been reported to have tumor-suppressive activity in lung cancer (27) (Fig. 1*A*). We also analyzed other previously published raw data (28) and found that miR-126 is gradually down-regulated during B-cell differentiation (Fig. 1*B*). This result was confirmed by real-time PCR analysis of miR-126 expression in $CD43^+B220^+$, $CD43^-B220^+$, and IgM^+B220^+ mouse bone marrow (BM) cells, which correspond to proB, preB, and mature B cells, respectively (Fig. 1*C*). Therefore, we hypothesized that miR-126 is a tumor suppressive miRNA and potential regulator of B-cell development.

miR-126 Shifts the Balance of B-Cell/Monomyeloid Differentiation Toward B Cells in MLL-AF4 ALL Cells. To explore the role of miR-126 in hematopoietic cells, we designed a retroviral vector that expresses the miRNA gene together with GFP. The vector was transduced, via retroviral infection, into SEM cells established from an MLL-AF4 ALL patient. In agreement with the observations from other *MLL*-rearranged ALL cell lines, SEM cells endogenously express mature miR-126 at a low level (29).

To examine the functions of miR-126, we overexpressed it in SEM cells. The expression level of mature miR-126 was more than 600-times higher in miR-126–transduced cells than in control cells (29).

SEM cells were transduced with retrovirus vectors expressing either let-7b, miR-126, miR-128b, or no miRNA (negative control). The transduced cells were sorted for those expressing GFP (a marker gene on all of the retroviral vectors) and cultured in RPMI containing 10% (vol/vol) FCS. At 8 wk posttransduction, a significant up-regulation of CD20 (~16%) and CD19 (mean fluorescence intensity, ~600) was observed in SEM cells expressing miR-126, but control cells or cells expressing let-7b or miR-128b showed ~1–2% $CD20^+$ cells and a mean-fluorescence intensity of CD19 expression of 350–450 (Fig. 2). Furthermore, suppression of miR-126 promoted the differentiation of SEM cells into myeloid cells, inducing the down-regulation of CD19 and up-regulation of CD15 (Fig. S1). Accordingly, gain- and loss-of-function experiments in a cell line derived from an MLL-AF4 ALL patient suggested that miR-126 drives B-cell myeloid biphenotypic leukemia differentiation toward B cells, at the expense of myeloid cells.

miR-126 Shifts the Balance of B-Cell/Monomyeloid Differentiation Toward B Cells Without Up-Regulating Transcription Factors Critical for B-Cell Development. To confirm that miR-126 affects B-cell development beyond regulating the expression of CD19, CD20, and CD15, we performed a comprehensive analysis of the mRNA transcripts that were up-regulated or down-regulated in SEM cells that expressed miR-126. Using Agilent gene-expression arrays, we identified a set of B-cell genes and a set of monomyeloid genes, as defined by IPA software (Ingenuity Systems). B-cell genes in $miR\text{-}126^+$ SEM cells were significantly up-regulated compared with those in control SEM cells, but the monomyelocyte genes were not (Fig. 3 *A* and *B*, and Dataset S1). These results suggest that miR-126–expressing SEM cells up-regulated not only CD20 and CD19 but also the global expression of other B-cell genes in SEM cells. We concluded that miR-126 shifted the balance of B-cell/monomyeloid differentiation toward B cells in MLL-AF4 ALL cells. Interestingly, PAX5, EBF1, and E2A, critical transcription factors in B-lymphopoiesis, were not up-regulated. Instead, E2A was slightly down-regulated in miR-126–expressing cells (Fig. 3*C*). Expressions of non–B-cell genes targeted by E2A, EBF1, or PAX5 were not altered by the transduction of miR-126 (Figs. S2 and S3, and Dataset S2). This finding suggests that neither transcriptional nor functional activity of PAX5, EBF1, and

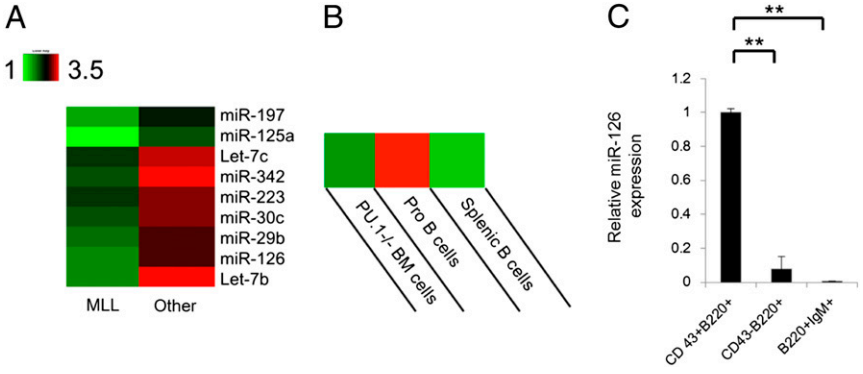

Fig. 1. Expression of miR-126 in acute lymphocytic leukemia and mouse hematopoietic cells. (*A*) The miRNAs that are most highly down-regulated in *MLL*-rearranged ALL compared with other types of ALL. These data were previously published and were reanalyzed here and presented as a heat map. (*B*) Expression of miR-126 in $PU.1^{-/-}$ BM cells, BM proB cells, and splenic B cells. These data were previously published and were reanalyzed here and presented as a heat map. (*C*) miRNA expression normalized by U6 expression in B-cell precursors detected by quantitative RT-PCR. B-cell precursor cells at various stages of differentiation were isolated from BM ($n = 3$) by FACS. ProB cells, $B220^+CD43^+IgM^-$; PreB cells, $B220^+CD43^-IgM^-$; immature B cells, $B220^+CD43^-IgM^+$. $^{**}P < 0.05$.

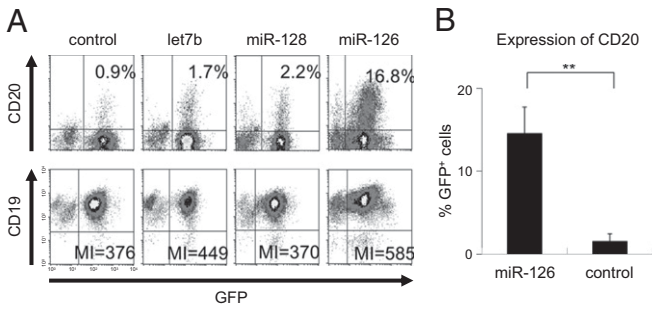

Fig. 2. Induction of B-cell differentiation by miR-126. (*A*) Representative flow plots of sorted SEM cells transduced with control, let-7b, miR-128b, or miR-126 vectors. The pregated live cells were analyzed. MI, mean intensity. (*B*) Percentages of $CD20^+$ cells among miR-126 or control vector-transduced SEM cells ($n = 3$).

E2A are enhanced by miR-126. Therefore, the shift toward B-cell lineage might be independent of PAX5, EBF1, and E2A.

miR-126 Regulates Lineage Fate in Lymphoid-Myeloid Progenitor Cells. To further analyze the B-cell differentiation caused by miR-126, we studied the effects of miR-126 transduction in a bilineage primary cell culture system. Mouse Lin^- HPCs isolated from fetal liver (FL), when cultured on thymic stromal (TSt)-4 cells, differentiate into monomyelocytic cells and B cells in vitro. This system allowed us to quantify the proportion of B cells and monomyelocytic cells derived from HPCs (30) following the perturbation of miR-126 expression. We transduced Lin^- cells with a retroviral construct harboring miR-126 or a control miRNA, and cultured them on TSt-4 cells. Then B cells were enumerated by flow cytometric analysis of the $CD19^+$ population. On day 5, in contrast to the control Lin^- cells, miR-126–expressing cells yielded an average fourfold enrichment of B cells (Fig. 4 *A* and *B*), and the proportion of $mac1^+$ monomyelocytic cells showed a reciprocal reduction (Fig. 4*B*). This result indicates that miR-126 shifts the balance of B-cell/monomyeloid differentiation toward B cells in normal HPCs.

To clarify which progenitor populations of Lin^- cells were affected by miR-126, we fractionated Lin^- cells into three groups: $Lin^-Flt3^+c\text{-}Kit^+Sca1^+IL\text{-}7R^-$ cells, $Lin^-c\text{-}Kit^{low}Sca1^{low}IL\text{-}7R^+$ cells, and $Lin^-c\text{-}Kit^+Sca1^-IL\text{-}7R^-$ cells. Although $Lin^-c\text{-}Kit^{low}Sca1^{low}IL\text{-}7R^+$ cells were slightly affected by miR-126, the most dramatic effect of miR-126 was on $Lin^-Flt3^+c\text{-}Kit^+Sca1^+IL\text{-}7R^-$ cells. Forced expression of miR-126 resulted in a statistically significant increase in $CD19^+$ cells in $Lin^-Flt3^+c\text{-}Kit^+Sca1^+IL\text{-}7R^-$ cells (Fig. 4*C* and Fig. S4). Next, we determined whether miR-126 had reprogrammed the myeloid-committed cells into B cells. To address this theory, we transduced miR-126 into $Lin^-c\text{-}Kit^+Sca1^-IL\text{-}7R^-$ cells, the majority of which were committed to the monomyelocyte lineage. miR-126 did not increase the proportion of $Lin^-c\text{-}kit^+Sca1^-IL\text{-}7R^-$ cells that were positive for CD19, indicating that miR-126 cannot reprogram monomyelocyte-committed cells (Fig. 4*C* and Fig. S4). Considering that $Lin^-c\text{-}Kit^{low}Sca1^{low}IL\text{-}7R^+$ cells are lymphoid-restricted progenitor cells, which still have potential to differentiate into myeloid cells although much less so than $Lin^-Flt3^+c\text{-}Kit^+Sca1^+IL\text{-}7R^-$ cells (7), these experiments suggest that miR-126 primarily regulates lymphoid versus myeloid lineage commitment in the multipotent cell population, and does not regulate the expansion of lymphoid- or myeloid-restricted progenitor cells.

miR-126 Increases B Cells in Vivo. Having established a functionally important role for miR-126 in an in vitro model of B-cell differentiation, we next examined the function of miR-126 in vivo. The competitive transplantation assays were performed in the Ptprc congenic mouse model, transducing $Ptprc^b$ (CD45.2) or $Ptprc^a$ (CD45.1) lin^- BM hematopoietic stem and progenitor cells, respectively, with either the miR126 or the control vector. The data were published in ref. 31.

Using flow cytometry, we characterized BM cells according to their expression of cell surface markers for B cells (CD19), T cells (CD3), or monomyeloid cells (Mac1). Remarkably, compared with control cells, the BM cells expressing miR-126 exhibited a significant expansion of $CD19^+$ B cells and reduction of $CD3^+$ T cells and $mac\text{-}1^+$ monomyeloid cells in the peripheral blood 4 wk after BM transplantation ($CD19^+$ cell frequency, $45.5 \pm 9.9\%$ vs. $70.7 \pm 05.4\%$; $P < 0.05$; $CD3^+$ cell frequency, $13.3 \pm 5.8\%$ vs. $5.5 \pm 2.0\%$; $P < 0.05$; $mac1^+$ cell frequency, $40.8 \pm 8.5\%$ vs. $23.1 \pm 6.1\%$; $P < 0.05$) (Fig. 5).

IRS-1 Is a Functional Target of miR-126 During B-Cell Expansion. The experiments described above establish an important role for miR-126 in B-cell development of HPCs. We next sought to determine the mRNA target of miR-126 that would explain its effect on B-lymphopoiesis. We initially focused on targets that were commonly predicted across multiple sequence-based prediction algorithms (10, 32–34). We chose *IRS-1* as a candidate because its gene expression was reduced in Lin^- FL cells overexpressing miR-126 (Fig. 6 *A* and *B* and Fig. S5). We focused on IRS-1 because it has known functions in cell proliferation and differentiation processes; higher expression of IRS-1 is associated with proliferation, and lower levels are associated with differentiation (35). First, we cloned the *IRS-1* 3′ UTR into a luciferase reporter vector and found that miR-126 repressed reporter activity by more than twofold, consistent with the predicted targeting of IRS-1 by miR-126 (34). This experiment confirmed that miR-126 negatively regulates the mRNA expression level of IRS-1 directly through its 3′ UTR (29). Then, we addressed whether the repression of IRS-1 could explain the B-cell differentiation observed with miR-126 overexpression using the in vitro mouse FL cell coculture system with TSt-4 cells. We tested whether complementation of IRS-1 by exogenous cDNA

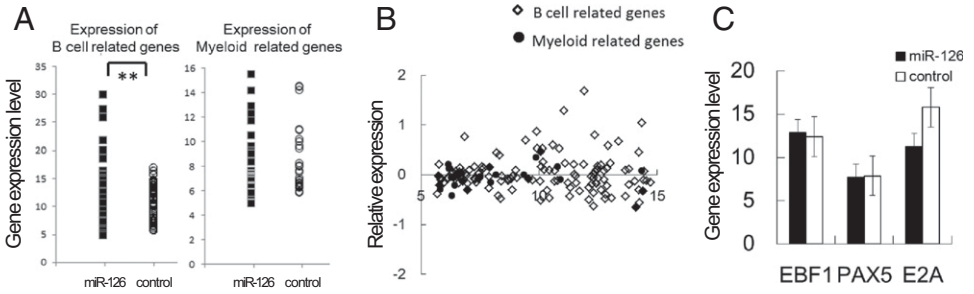

Fig. 3. miR-126 induces B-cell differentiation in MLL-AF4 ALL cells without up-regulating EBF1, PAX5, or E2A. (*A*) B-cell–related genes were significantly up-regulated in miR-126–transduced MLL-AF4 ALL cells compared with control-transduced MLL-AF4 ALL cells (*Left*), but monomyelocyte related genes were not (*Right*). The *y* axis shows log-transformed data. (*B*) Intensity scatter plot comparing miRNA profiles in miR-126–transduced and control-transduced MLL-AF4 ALL cells. The *x* axis shows log-transformed data, and the *y* axis shows the log ratio. (*C*) Intensity of E2A, PAX5, or EBF mRNA expression in miR-126– or control-transduced SEM cells. The *y* axis shows log-transformed data.

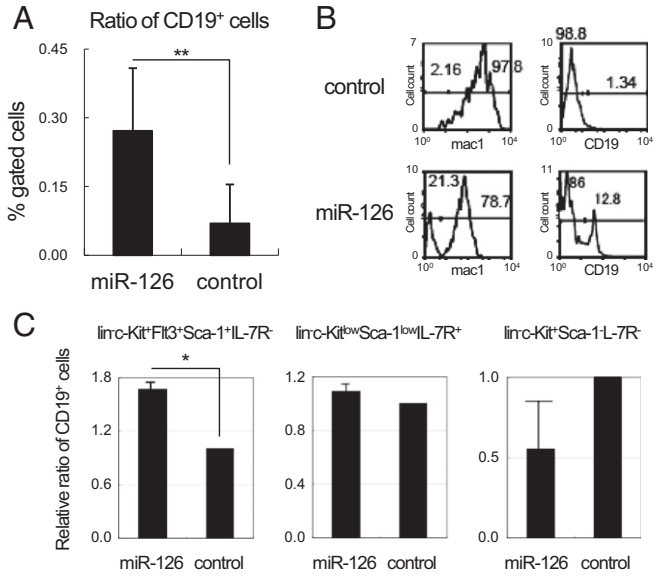

Fig. 4. miR-126 increases the proportion of B cells among progenitor FL cells cocultured with TSt-4 cells. Hematopoietic progenitors derived from mouse FL cells were sorted into Lin^- cells and transduced with control and miR-126–expressing viral constructions. The cells were then cocultured with TSt-4 cells to differentiate them into B cells or monocytes and analyzed after 5 d of differentiation using flow cytometric analysis with the lineage markers CD19 (B cells) and mac1 (monocytes). (*A*) Effects of the expression constructs on the percentage of $CD19^+$ cells. Error bars represent SD ($n = 3$). $^{**}P < 0.03$. (*B*) Representative flow histogram of control vector- and miR-126–transduced Lin^- FL cells. Expression of CD19 and mac1 were determined. These plots pregated on GFP^+ live cells. (*C*) Hematopoietic progenitors derived from mouse FL cells were sorted into $Lin^-Flt3^+c\text{-}Kit^+Sca1^+IL\text{-}7R^-$ cells, $Lin^-c\text{-}Kit^{low}Sca1^{low}IL\text{-}7R^+$ cells, and $Lin^-c\text{-}Kit^+Sca1^-IL\text{-}7R^-$ cells and transduced with control and miR-126-expressing viral constructs. Then, the cells were cocultured with TSt-4 cells to differentiate them into B cells or monocytes and analyzed by flow cytometry after 5 d of differentiation for the lineage markers CD19 (B cells). Effects of the expression constructs on the percentage of $CD19^+$ cells. The *y* axis represents the $CD19^+$ miR-126 transduced cells relative to the control. The error bars indicate SD ($n = 3$). $^*P < 0.03$.

that is not affected by miR-126 could reverse the observed increase in B-cell differentiation. Murine stem cell virus-based constructs containing the *IRS-1* coding sequence were generated with human nerve growth factor receptor (NGFR). Then, the IRS-1 coding sequence or a control vector was cotransduced with miR-126 into Lin^- cells to see whether IRS-1 expression could rescue the miR-126 phenotype. As shown in Fig. 6 *C* and *D*, $CD19^+$ B cells accounted for 2.34% of the 11.8% of Lin^- FL cells transduced with IRS-1 and miR-126, and 36.2% of the 56% cells transduced with control vector and miR-126. A total of 20% of IRS-1- and miR-126–transduced Lin^- FL cells and 70% of control vector- and miR-126–transduced cells were $CD19^+$, indicating that there were significantly fewer $CD19^+$ B cells among IRS-1–transduced Lin^- FL cells than among control cells (Fig. 6*D*). The opposite results were obtained for $mac1^+$ monomyelocytic cells (Fig. 6 *C* and *D*). These findings indicate that the influence of miR-126 on B-cell differentiation was abrogated by the introduction of IRS-1 lacking its 3′ UTR in murine FL cells, suggesting that the target genes of miR-126 in this system include *IRS-1*.

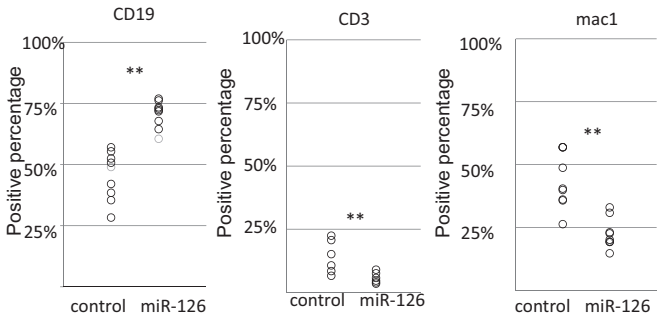

Fig. 5. miR-126 induces B-cell expansion in vivo. The competitive transplantation assays were performed in the Ptprc congenic mouse model, transducing $Ptprc^b$ (CD45.2) or $Ptprc^a$ (CD45.1) lin^- BM hematopoietic stem and progenitor cells, respectively, with either the miR126 or the control vector. The peripheral blood of recipient mice were analyzed 4 wk post-transplantation, when the hematopoietic system had largely recovered in the hosts. The data were published previously (31). Each dot represents data from one recipient mouse. $^{**}P < 0.01$.

miR-126 Can Rescue Failed B-Cell Differentiation in EBF1-Deficient HPCs. Mice lacking EBF1 fail to express most B-cell genes, including Cd79a and Cd79b, and do not undergo Igh V-to-DJ recombination in the BM. Neither E2A nor PAX5, which are critical transcriptional factors, can rescue the failure in EBF1-deficient HPCs. Ectopic expression of EBF1 is able to rescue B-lymphopoiesis in multipotent progenitors blocked at earlier stages of development because of targeted deletion of key lymphoid transcription factors such as E2A, PU.1, and PAX5. Taken together, these data indicate that EBF1 is an essential specification factor for the B-cell lineage. Accordingly, we tested whether failure of B-cell development because of EBF1 deficiency, can be rescued by miR-126. miR-126 induces B-cell differentiation in MLL-AF4 ALLs without affecting EBF1, PAX5, and E2A, suggesting that it may potentially induce B-cell differentiation by an alternative mechanism to transcriptional factors. We established EBF1-deficient HPCs expressing low amounts of B220 but not CD19. Retrovirus-mediated expression of miR-126 in these cells markedly up-regulated B220, but not CD19 (Fig. 7*A*). CD19 expression is contingent on EBF1. In the BM, B220 is up-regulated during the maturation of prepro B cells into mature B cells (36). Moreover, expression of B-cell genes, such as PAX5, CD79a/b, and recombination activating gene (RAG)1/2, is up-regulated (Fig. 7*B*). Although RAG1/2 was up-regulated by miR-126, V-DJ rearrangements were not induced by miR-126. miR-126 affected not only gene expression profiles, but the growth of EBF1-deficient HPCs. Although the proportion of control vector-carrying EBF1-deficient HPCs gradually decreased during 28-d culture, the proportion of EBF1-deficient HPCs carrying miR-126 increased (Fig. 7*C*). This finding suggested miR-126 promoted the proliferation or survival of EBF1-deficient HPCs. Next, we calculated the doubling time of them. The doubling time of control EBF1-deficient HPCs (26.6 ± 1.5 h) was comparable to that of the original EBF1-deficient HPCs (27.0 ± 2.0 h). In contrast, the doubling time of miR-126-transduced EBF1-deficient HPCs (21.4 ± 1.0 h) was significantly shorter than that of control cells. This result indicates that miR-126 enhanced the proliferation of EBF1-deficient HPCs. These results suggest that miR-126 can partly rescue failed B-cell lineage development and specification because of EBF1 deficiency.

Discussion

We found that miR-126 was down-regulated in MLL-AF4 ALL, a cancer with an immature B-cell phenotype, compared with other types of ALL. Furthermore, miR-126 expression strikingly decreased during successive stages of B-cell maturation in the BM, suggesting that this miRNA may participate in early B lymphopoiesis, and deregulation of its expression is involved in leukemogenesis. Indeed, inducing the re-expression of miR-126 promoted B-cell development in MLL-AF4 ALL, the mouse FL, and BM hematopoietic cells. miR-126, which was ectopically expressed in preB and immature B cells, did not affect B-cell

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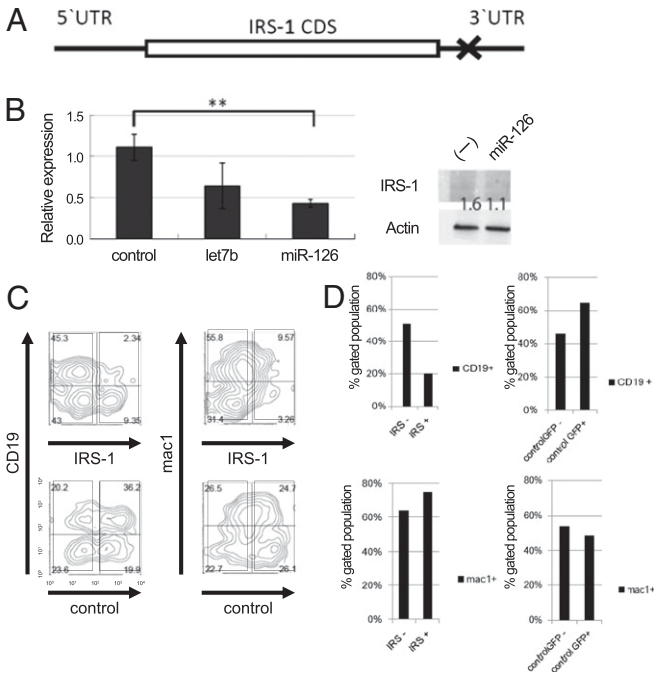

Fig. 6. IRS-1 is a functional target of miR-126 during B-cell expansion. (*A*) Location of the predicted (Pictar) miR-126 target sequence (X) in the 3′ UTR of the human IRS-1 mRNA. (*B*) Relative expression of IRS-1 mRNA normalized to U1A expression by quantitative PCR analysis of Lin$^-$ FL cells overexpressing miR-126, let-7, or control vector (n = 3, *Left*). $^{**}P < 0.05$. Western blot analysis of IRS-1 was performed with total protein extracts of miR-126–overexpressing or control Lin$^-$ FL cells (*Right*). The relative intensity of each band (indicated below the bands) was determined using Multigauge software and normalized to the GAPDH loading control. (*C* and *D*) Ectopic expression of IRS-1 in miR-126-expressing Lin$^-$ FL cells decreases B-cell numbers. (*C*) Representative plots for CD19 and mac1 expression in IRS-1$^+$/IRS-1$^-$ miR-126$^+$ and controlGFP$^+$/controlGFP$^-$ miR-126$^+$ Lin$^-$ FL cells. (*D*) Percentage of CD19$^+$ or mac1$^+$ cells in pregated IRS1$^+$, IRS1$^-$, control$^+$, or control$^-$ cells. Square indicates each pregated cells.

maturation, suggesting that its effect was limited to early B-cell development. Its effect was most dramatically observed in Lin$^-$c-Kit$^+$Sca1$^+$ uncommitted progenitor cells in the FL, suggesting miR-126 is involved in cell fate regulation. Notably, even though the expression of key transcription factors in B-cell development; E2A, EBF, and PAX5, were unchanged in miR-126–expressing cells, miR-126 plays a critical role in regulating the differentiation of B cells in leukemia, in which the deregulation of differentiation because of dysfunction of transcription factors is supposed to be involved in leukemogenesis (37). Moreover, expression of miR-126 in EBF1-deficient HPCs partly rescued B-lymphopoiesis, leading to the up-regulation of several B-cell genes and enhanced proliferation. Importantly, CD79a, which is critically regulated by EBF1, was up-regulated by miR-126 in EBF1-deficient cells. The stepwise expression and function of several factors is involved in cell fate determination. EBF1 can rescue B-cell development in E2A-, PU.1-, and PAX5-deficient hematopoietic stem cells. Conversely, these transcriptional factors cannot rescue B-cell development, indicating that EBF1 controls the minimal essential system for B-cell development. Our finding that miR-126 partly rescued B-cell development in EBF1-deficient HPCs suggests that miR-126, which is not a transcriptional factor, has critical roles in B-cell development. However, miR-126 is dispensable for B-cell development, because miR-126 deficiency does not cause any defects in B-lymphopoiesis. Taken together, our observations lead us to conclude that miR-126–mediated B-cell differentiation is at least partly independent of canonical assembly of a transcriptional factor regulatory network. miR-126 has the potential to compensate for the deregulation of cell fate caused by dysfunction of transcription factors in leukemia and is critically involved in B-cell lineage specification. These results challenge the view that miRNAs merely play fine-tuning roles in establishing lineage fate (8).

Surveying the predicted targets of miR-126, we found several genes that play important roles in myeloid development, including *IRS-1*, *v-crk sarcoma virus CT10 oncogene homolog* (*CRK*), and *homeobox A9* (*HOXA9*). Two highly conserved 8-nt sites in the 3′ UTR of IRS-1 mRNA, one conserved 7-nt site in the CRK 3′ UTR, and one conserved 7-nt site in the HOXA9 3′ UTR are complementary to the miR-126 "seed" region. Among these targets, we demonstrated that miR-126 targets IRS-1 during B-cell differentiation. IRS-1 is the main docking protein of both type 1 insulin-like growth factor I receptor and the insulin receptor. IRS-1 is a principal substrate of the insulin receptor tyrosine kinase. IRS-1 undergoes multisite tyrosine phosphorylation and mediates insulin signaling by associating with various signaling molecules containing Src homology 2 domains (38). Overexpression of *IRS-1* inhibits differentiation and promotes transformation of hematopoietic cells into a tumor-forming cell line (35). Although the function of IRS-1 in B-cell development has yet to be determined, it is reasonable that IRS-1, an inhibitor of differentiation, is down-regulated by miR-126 during B-cell differentiation.

Future investigations exploring the regulation of miR-126 expression are needed to understand its function; its expression is known to be greatest in highly vascularized tissues, such as the lung, heart, and kidney (39–41), and is also present in bronchial epithelium (27). miR-126 is located on chromosome 9q34.3 and is encoded within intron 5 of *epidermal growth factor like-7* (39). Recently, miR-126 was shown to function in angiogenesis, as miR-126–deficient mice are embryonic lethal because of vascular malformation (42). In the hematopoietic system, Landgraf et al. (43) reported qualitative detection of miR-126 in the CD34$^+$ pool containing hematopoietic stem cells, but they did not examine the function of miR-126 in this cell population. miR-126 has also

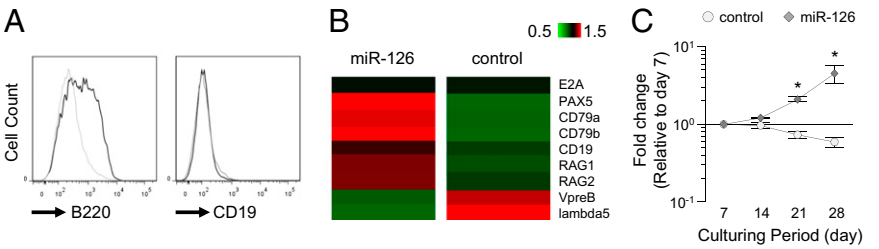

Fig. 7. miR-126 up-regulates B-cell markers and promotes cell proliferation in EBF1-deficient HPCs. (*A*) HPC lines derived from EBF1-deficient mouse FL were transduced with control and miR-126–expressing viral constructs. Cells were cocultured with TSt-4 cells in the presence of IL-7, stem cell factor, and Flt3 ligand and analyzed after 10 d of differentiation by flow cytometry for the B-cell lineage markers B220 and CD19. The black and gray lines indicate miR-126 and control vector-transduced pregated live cells, respectively. The same results were obtained three times. (*B*) cDNA analysis of B-lineage gene expression in the HPC lines derived from EBF1-deficient HPCs transduced with a viral construct expressing miR-126 (*Left*) or a control vector (*Rght*). (*C*) The proportion of miR-126–transduced EBF1-deficient HPCs in culture gradually increases, but that of control vector-transduced cells does not. The proportions of vector-carrying EBF1-deficient HPCs are shown as fold changes relative to day 7. $^*P < 0.05$.

been reported to be down-regulated during terminal megakaryocytopoiesis and up-regulated in megakaryocytic cell lines (44). We and others have found that miR-126 is down-regulated in B-cell differentiation. The regulation of miR-126 expression should be further investigated.

Finally, the observed function of miR-126 as an inducer of differentiation suggests miR-126 might be a promising therapeutic target. In acute promyelocytic leukemia, retinoic acid induces terminal differentiation of leukemic cells. This "differentiation induction" therapy has been tried in many types of tumors without much success. miR-126 may be an agent for differentiation induction therapy for ALL; thus, further studies are needed to evaluate its potential as a differentiation inducer.

Materials and Methods

Ebf1$^{-/-}$ Progenitor Cells. *Ebf1*$^{-/-}$ hematopoietic progenitor (Lin$^-$) cells were isolated from the *Ebf1*$^{-/-}$ livers of 14 d postcoitum embryos and cultured on TSt-4 stromal cells in IMDM containing 10% (vol/vol) FCS, 2-ME (5×10^{-5} M), penicillin (10 U/mL), and streptomycin (10 μg/mL) in the presence of stem cell factor, IL-7, and Flt3 ligand (10 ng/mL each), as described previously (45, 46).

Gene-Expression Analysis. RNA from cells used for microarray analysis was isolated using the RNeasy Mini Kit (Qiagen). For microarray analysis, splenocytes were cultured for 72 h with or without 10 μM IM. Gene-expression microarray analysis was performed using two-color microarray-based gene-expression analysis (Agilent Technologies) according to the manufacturer's instructions. After scanning, expression values for the genes were determined using GeneSpringGX software. All experiments were done in the duplicates.

Western Blot Analysis. FL cells transduced with either control or miR-126 vector were lysed in sample loading buffer and separated by SDS/PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was incubated with primary antibody against IRS-1 (Cell Signaling Technology), followed by peroxidase-conjugated anti-rabbit Ig (GE Healthcare).

BM Transplantation. Lin- BM cells from congenic mice (Ptprca and Ptprcb, respectively) were transduced with a 126OE vector or with a CTRL vector, and injected in a 1:1 ratio into myeloablated recipients (31).

Peripheral blood analysis was performed 4 wk after the BM transplantation. Mononuclear cells were stained with various antibodies and analyzed on a FACSCalibur flow cytometer (BD Biosciences) and using FlowJo software (Tree Star).

Antibodies. Antibodies specific for CD3, CD19, CD20, Mac1, Gr-1, Flt3, c-kit, Sca1, and IL-7Rα were purchased from eBioscience.

Primer sequences, reagents, and more detailed methods are shown in *SI Materials and Methods*.

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