Experimental Hematology

Experimental Hematology 2014;42:909–918

MicroRNA-125 family members exert a similar role in the regulation of murine hematopoiesis

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(Received 12 June 2014; accepted 13 June 2014)

MicroRNAs (miRNAs) are crucial for proper functioning of hematopoietic stem and progenitor cells (HSPCs). Members of the miRNA-125 family (consisting of miR-125a, miR-125b1, and miR-125b2) are known to confer a proliferative advantage on cells upon overexpression, to decrease the rate of apoptosis by targeting proapoptotic genes, and to promote differentiation toward the myeloid lineage in mice. However, many distinct biological effects of the three miR-125 species have been reported as well. In the current study, we set out to assess whether the three miRNA-125s that carry identical seed sequences could be functionally different. Our data show that overexpression of each of the three miR-125 family members preserves HSPCs in a primitive state in vitro, results in a competitive advantage upon serial transplantation, and promotes skewing toward the myeloid lineage. All miR-125 family members decreased the pool of phenotypically defined $Lin^{-}Sca^{+}Kit^{+}CD48^{-}CD150^{+}$ long-term hematopoietic stem cells, simultaneously increasing the self-renewal activity upon secondary transplantation. The downregulation of miR-125s in hematopoietic stem cells abolishes these effects and impairs long-term contribution to blood cell production. The introduction of a point mutation within the miRNA-125 seed sequence abolishes all abovementioned effects and leads to the restoration of normal hematopoiesis. Our results show that all miR-125 family members are similar in function, they likely operate in a seed-sequence-dependent manner, and they induce a highly comparable hematopoietic phenotype.

The limited lifespan of differentiated blood cells requires their constant replenishment by hematopoietic stem cells (HSC). A defined pool of HSCs ensures a daily blood cell production exceeding 10^{12} blood cells [1]. These rare and unique cells are able to avoid exhaustion by balancing self-renewal and differentiation. Stem cell homeostasis is regulated by multiple mechanisms that affect gene expression patterns. One of these mechanisms for fine-tuning gene expression networks is regulated by microRNAs (miRNAs). These are small, noncoding RNAs that can pair with the 3′ untranslated region (UTR) of their target mRNA, leading to its destabilization, which results in degradation or translational repression [2]. More than 80% of miRNA targets are recognized by the seed sequence, a 6–8 nucleotide, highly conservative sequence, although seed-sequence-independent targets are also known. Alternative target recognition sites operate via extensive pairing of the 3′ end, or the central 11–12 contiguous nucleotides of the miRNA, with the 3′ UTR of the target transcript [3,4]. The seed sequence determines the classification of different miRNAs into families. Importantly, a single miRNA can potentially target hundreds of transcripts, generating an extensive network of interactions [5].

It has been shown that miRNAs are crucial for the functioning of hematopoietic stem and progenitor cells (HSPCs) [6]. In the absence of Dicer, which cleaves precursor miRNA transcript to mature miRNA [7], HSPCs exhibited impaired function and an increased rate of apoptosis. Further studies focused on the identification of miRNAs that are relevant for primitive hematopoietic cells [6,8–10]. Members of the miRNA-125 family (consisting of miR-125a, miR-125b1, and miR-125b2) have been shown to be highly expressed

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Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2014.06.010.

0301-472X/$ - see front matter.
http://dx.doi.org/10.1016/j.exphem.2014.06.010

in Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) hematopoietic cells, although their expression decreased upon differentiation [9,10]. The microRNA-125 family members 125a, 125b1, and 125b2 are located on three different chromosomes; in the murine genome these are chromosomes 17, 9, and 16, respectively. All family members are most likely expressed in clusters with other miRNAs [9,10].

Although all miR-125 family members have been shown to provide a proliferative advantage on cells upon their overexpression [6,8,10], decrease the rate of apoptosis by downregulating proapoptotic genes [6,9,11] and promote differentiation toward the myeloid lineage in mice [6,8,10], multiple different biological effects of the three miR-125 species have been reported as well. Enhanced self-renewal of HSPCs has been reported [6,9], but, in other studies, including our own, an increase in differentiation was observed [10,12]. Similarly, differences in granulocyte-colony stimulating factor (G-CSF) responsiveness have been noticed for miR-125b1 and miR-125b2 in vitro [11]. Additionally, the extent of differentiation skewing toward the myeloid or lymphoid lineage upon overexpression of miR-125 family members has been disputed [6,8,10]. Aside from its effect on normal hematopoiesis, upregulation of miR-125a and miR-125b expression has been linked to myeloproliferative disorders, acute myeloid leukemia [13,14], and childhood T-cell lymphoblastic lymphoma [15], while downregulation of miR-125b has been observed in aggressive and indolent chronic lymphocytic leukemias [16] in human patients. Therefore, members of the miR-125 family may play a role in the etiology of myeloid and lymphoid human malignancies [13–16].

Although the contribution of miR-125 family members in blood cell production is now well recognized, it remains unclear whether the variable effects described above result from intrinsic differences in effects caused by distinct miR family members, or rather reflect differences of the various experimental models that were used. In the current study, we set out to assess whether these miRNAs, which carry identical seed sequences, could be functionally different. To this end, we compared all miR-125 family members in various in vitro and in vivo tests. Our results unequivocally show that all miR-125 family members are similar in function and induce a highly comparable hematopoietic phenotypes that are seed sequence dependent. Moreover, a single nucleotide mutation of the seed sequence restores hemostasis and documents the seed-sequence-dependent action of miR-125 family members. Downregulation of miR-125s in hematopoietic stem cells (HSCs) leads to loss of long-term repopulating ability.

Methods

Mice

Female C57BL/6 (B6, CD45.2) mice were purchased from Harlan and housed under clean conventional conditions. Female C57BL/6.SJL (B6.SJL, CD45.1) mice were bred at the Central Animal Facility of the University of Groningen (Groningen, The Netherlands). All animal experiments were approved by the Groningen University Animal Care Committee.

Retroviral vectors

The miR-125a vector was constructed as previously described [10]. MiR-125b1 and miR-125b2 were amplified from genomic DNA from B6 mice (125b1 forward primer: 5′-TTGT GAAGGGGAGAGGTGTAGGG-3′, reverse primer: 5′-CGAG TGGCAGCTCCTGGC-3′; miR-125b2 forward primer: 5′-CTC GAGAGCTGTCCGTTTACCTGGAAGAAG-3′, reverse primer: 5′-CAATTGGCGCCTATGCAGAATCTATGCC-3′) (Biolegio, The Netherlands) using XhoI+EcoRI restriction site cloned into MXW pPGK- IRES-EGFP for miR-125b1 and XhoI+MunI restriction site for miR-125b2.

The seed sequence mutant T4C miR-125a 5p has been obtained by site-directed mutagenesis using a Zero Blunt TOPO PCR Cloning Kit (Life Technologies, Naarden, The Netherlands) PCR-TOPO-miR-125a vector with primers containing a point mutation in the 5p arm of the miR-125a stem loop (forward primer: 5′-CTCTGGGTCCCCGAGACCCTTTAACCTG-3′, reverse primer: 5′-CAGGTTAAAGGGTCTCGGGGACCCAGAG-3′) (Integrated DNA Technologies, Brussels, Belgium) and Phusion polymerase as suggested by manufacturer (New England Biolabs, Hitchin, UK). The resulting PCR product was treated with DpnI and used for bacteria transformation. The mutated miR-125a stem loop was cut out from the PCR vector using XhoI + EcoRI and subcloned into the XhoI + EcoRI site of the MXW-pPGK-IRES-EGFP vector, which is a murine stem cell virus–based vector with constitutively active PolII type promoter [17].

The miR-125a antagomir (ZIP) was subcloned from a commercially available vector (SBI, Mountain View, CA) into MXW pPGK- IRES-EGFP vector using XhoI + MfeI restriction sites. The MXW pPGK-IRES-EGFP vector was provided by Dr. Chang-Zheng Chen (Stanford University School of Medicine, Stanford, CA).

Retroviral overexpression of miR-125a, miR-125b1, miR-125b2 or miR-125a antagomir

Primary bone marrow (BM) cells were isolated from donor mice (CD45.1+) 4 days after IP injection of 150 mg/kg 5-fluorouracil (Pharmachemie Haarlem, Haarlem, The Netherlands), and cultured in StemSpan (StemCell Technologies, Vancouver, Canada) supplemented with 10% FCS, 300 ng/mL recombinant mouse SCF (rmSCF) (Peprotech, Hamburg, Germany), 20 ng/mL rmIL11 (R&D Systems, Minneapolis, MN), 1 ng/mL Flt3 ligand (Amgen, Thousand Oaks, CA), penicillin, and streptomycin (Life Technologies). Virus was produced by transfecting Phoenix ecotropic packaging cells (National Gene Vector Biorepository [NGVB], Indianapolis, IN) with 1–2 μg of plasmid DNA (MXW empty vector, MXW-miR-125a, MXW-miR- 125b1, MXW-miR-125b2, or MXW- ZIP) and 3–6 μL of Fugene HD (Roche, Basel, Switzerland). Virus-containing supernatant harvested 48 and 72 hours later was used to transduce 4–7.5 $\times$ 10^5 BM cells per 3.5-cm well. Three independent transductions were performed per condition, per experiment. Five days after the first transduction, viable (propidium iodide-negative) green fluorescent protein (GFP)$^+$ cells were sorted and tested in in vitro assays and RNA gene expression studies. Nonsorted cells were tested in an

in vivo BM transplantation setting. In experiments with the antagomir miR-125a we followed the sorting strategy for stem cells and the transduction protocol described previously [18].

Quantitative polymerase chain reaction validation

Quantitative PCR analyses were performed as previously described [10]. To quantify miR-125b1 and miR-125b2 abundance, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit with specific TaqMan probes to discriminate between precursor-miR-125b1 and precursor-miR-125b2 transcripts (Applied Biosystems, Hitchin, UK).

Cobblestone-area forming cell assays

The cobblestone-area forming cell (CAFC) assay was performed as previously described [19–21].

Primary bone marrow transplantation

Bone marrow transplantations were performed as previously described [10]. As a source of donor cells, B6.SJL mice were used, and lethally (9 Gy) irradiated B6 mice served as recipients in both transplantation experiments. In the first experiment, mice were transplanted with 10^7 and, in the second, 7.5×10^6 whole bone marrow cells. In the experiments with the ZIP- or mutT4C-sorted long-term (LT)-HSC ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^+$), cells that were transduced and nonresorted for GFP+ were transplanted into lethally (9 Gy) irradiated B6 mice simultaneously with $5–20 \times 10^5$ radioprotective B6 cells. Gene transfer efficiencies were determined at 3 to 5 days post-transduction in an aliquot of cells left in the culture, which were further expanded and FACS-sorted to test overexpression levels.

Secondary bone marrow transplantation

Bone marrow cells from primary miR-125a, miR-125b1, and miR-125b2 and empty vector mice were used for secondary transplantations. Transplanted cells were competed with freshly isolated wild type $CD45.2^+$ BM cells in lethally irradiated recipients (CD45.2) at 4:1 (test to freshly isolated) ratios. For each of the conditions, nine secondary mice (three secondary recipients per

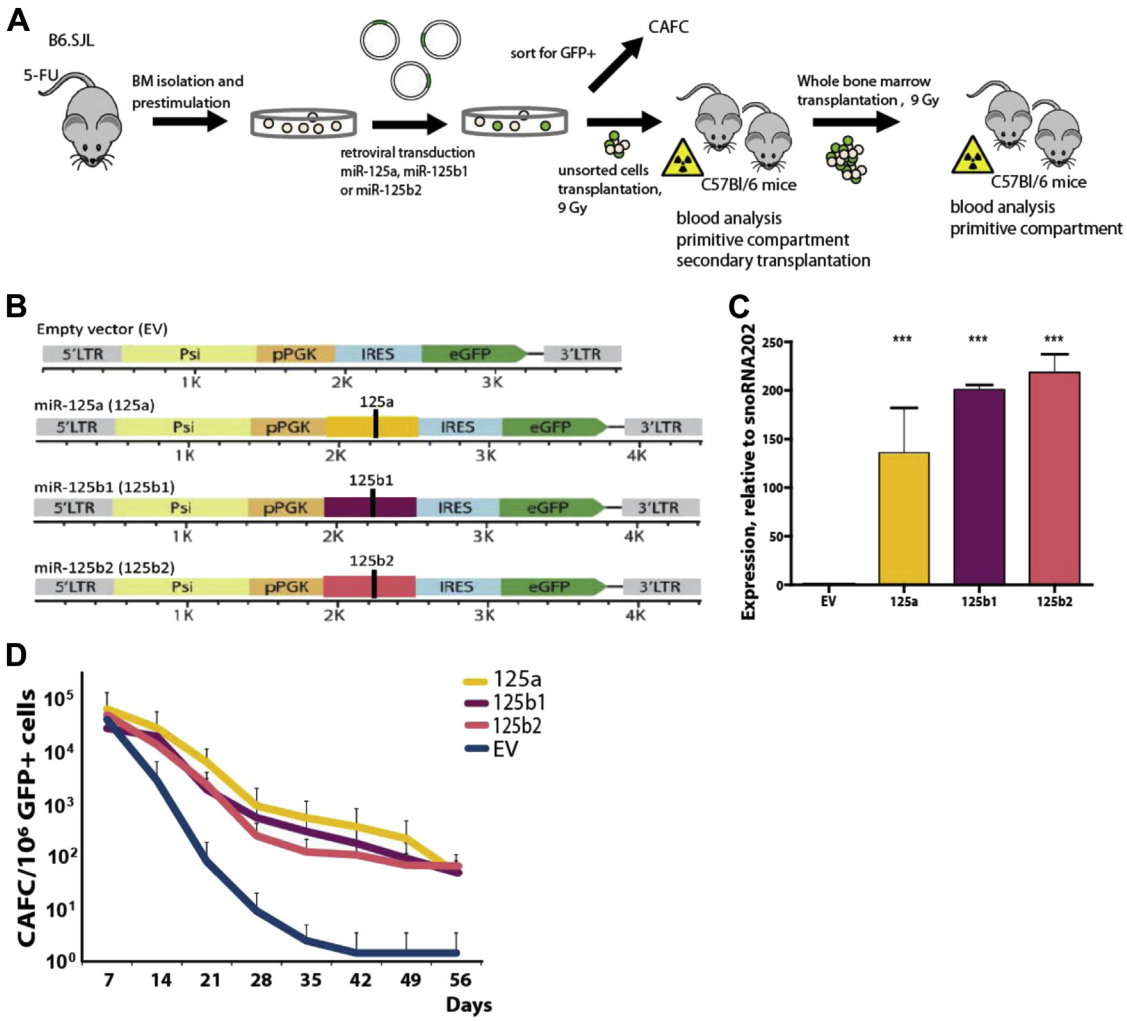

Figure 1. Overexpression of miR-125 family members inhibits differentiation of HSPCs. (**A**) Experimental setup. (**B**) Schematic representation of retroviral vectors used to overexpress miR-125 family members. (**C**) Quantitative reverse transcription PCR results showing the expression levels of miR-125a, miR-125b1, and miR-125b2 relative to the endogenous control snoRNA202, measured in post 5-FU GFP^+ bone marrow cells. Shown is the mean + SEM. (**D**) CAFC data showing the frequency of HSPCs (in three independent experiments). The differences between groups were evaluated by unpaired *t* test. $*p < 0.01$; $**p < 0.01$; $***p < 0.0001$. (Color illustration of figure appears online.)

primary mouse) received transplants with a total of 5×10^6 cells each. Blood analyses were performed every 4–6 weeks posttransplantation.

Results

Ectopic expression of miR-125a, miR-125b1, and miR-125b2 retains HSPCs in a primitive state

To overexpress all three members of the miR-125 family in murine hematopoietic cells, we have cloned miR-125a, -b1, and -b2 into retroviral vectors in their natural genomic context (as 250–500 base pair long genomic sequence) (Fig. 1A and B). Bone marrow cells of 5-FU-treated mice were transduced with the various vectors. Transduced GFP^+ cells were used to initiate in vitro assays and to analyze the overexpression level by quantitative PCR with primers recognizing mature miRNA sequences (Fig. 1C). To discriminate between identical mature miR-125b1 and miR-125b2, we confirmed their respective overexpression using primers recognizing the precursor form of miRNA-125b1 and miR-125b2. Levels of miR-125a, miR-125b1, and miR-125b2 were markedly increased (fold increases of 140, 200, and 210, respectively) compared with the empty vector control (all differences compared with the empty vector were statistically significant).

We used GFP^+ cells to initiate CAFC assays. In this assay, early appearing cobblestones (day 7) are considered to represent differentiated cells, and late appearing cobblestones (day 35 and later) are proportional to the number of more immature or primitive cells [19–22]. No differences between the control and miRNA-overexpressing cells were observed for day-7 CAFC activity. However, at day 35, an approximately hundredfold increase in the frequency of CAFCs was observed in all conditions where miRs were overexpressed. No statistically significant differences were found between the three studied miRNAs (Fig. 1D). Furthermore, all miR-125 family members similarly prolonged the CAFC activity until day 60, which was not observed for nonmanipulated cells.

Taken together, BM cells overexpressing individual members of the miR-125 family showed strongly increased frequency of HSPCs in in vitro assay. This effect was similar in all family members. These results suggest that ectopic expression of miR-125 family members retains cells in a more immature stage.

Overexpression of miR-125 family members in hematopoietic stem and progenitor cells confers an in vivo proliferative advantage and sporadically leads to myeloproliferative disorder

Parallel with in vitro assays, miRNA-overexpressing cells were transplanted into lethally irradiated recipients to assay their long-term competitive repopulation potential in vivo (Fig. 1A). At 10 weeks posttransplantation, blood cell counts

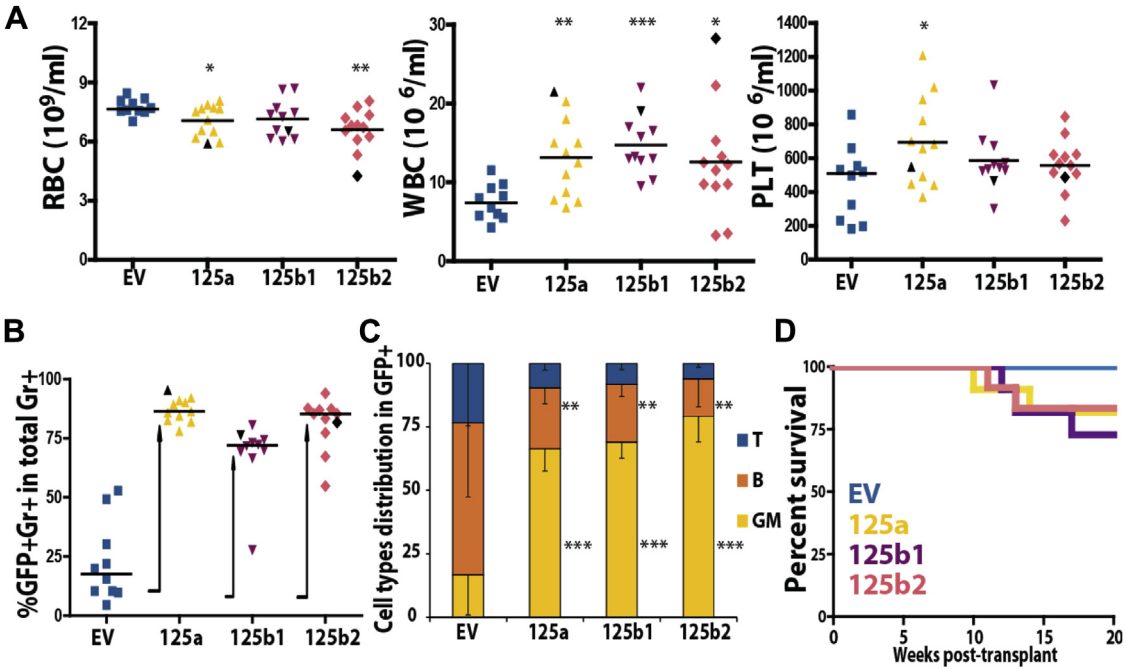

Figure 2. Sustained overexpression of miR-125 family members perturbs hematopoiesis. (**A**) RBC, WBC, and PLT 10 weeks after transplantation (n = 8–12 mice/group). The differences between groups were evaluated by unpaired t test. *$p < 0.01$; **$p < 0.01$; ***$p < 0.0001$. (**B**) Chimerism levels were measured at 10 weeks after transplantation by analyzing GFP^+ cells in the total fraction of granulocytes. The arrows indicate the growth of chimerism during first 10 weeks posttransplantation. (**C**) Cell type distribution in GFP^+ cells in the blood as assessed by FACS (n = 8–12 mice/group). Shown is the mean - SD. (**D**) Survival of mice (n = 8–12 mice/group). Closed black symbols indicate moribund miR-125 mice. B = B lymphocytes; GM = granulocytes/macrophages; PLT = platelets; RBC = red blood cells; T = T lymphocytes; WBC = white blood cells. (Color illustration of figure appears online.)

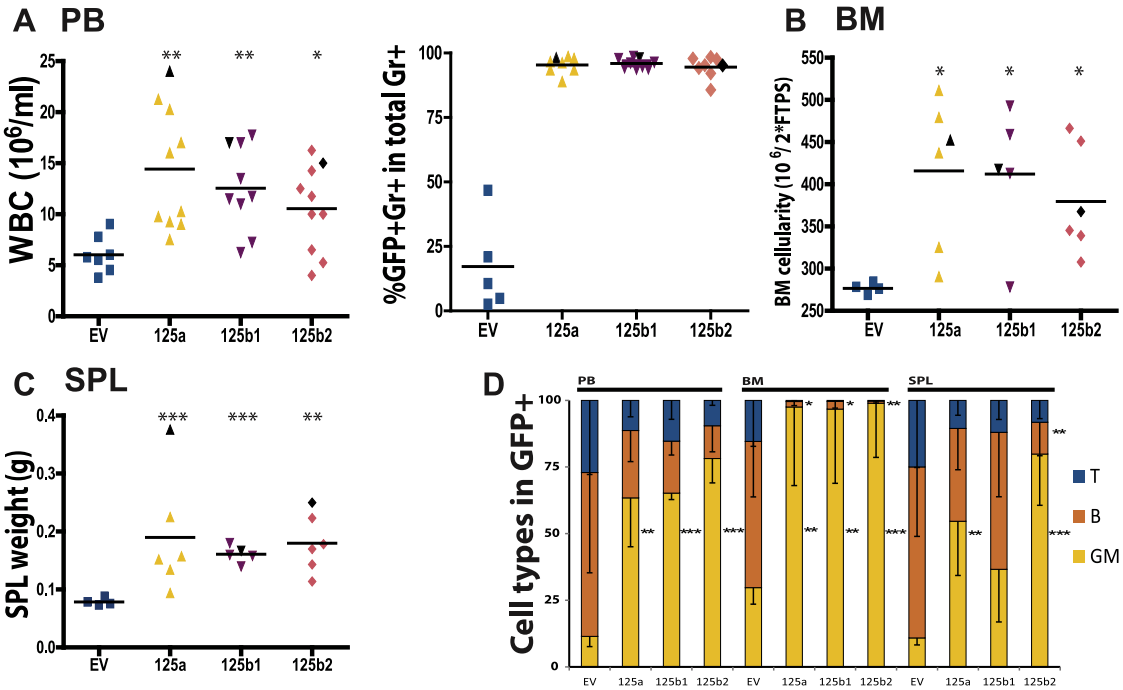

Figure 3. Overexpression of miR-125 family members causes splenomegaly and promotes myeloid differentiation. (**A**) WBC and chimerism levels in the blood 16 weeks posttransplantation. (**B**) Bone marrow cellularity (representing two femurs, tibias, pelvic bones, sternum, and the spine). (**C**) Spleen weight 16 weeks upon transplantation in primary recipients (n = 5–9 mice/group). (**D**) Cell type distribution in GFPþ$^{+}$ in the blood, bone marrow, and the spleen as assessed by FACS (n = 5–9 mice/group). Shown is the mean - SD. The differences between groups were evaluated by unpaired t test. *$p < 0.01$; **$p < 0.01$; ***$p < 0.0001$. Closed black symbols indicate moribund miR-125 mice. B = B lymphocytes; GM = granulocytes/macrophages; PLT = platelets; T = T lymphocytes; WBC = white blood cells. (Color illustration of figure appears online.)

revealed a moderate decrease in red blood cell (RBC) numbers in a proportion of the mice (in the case of miR-125a and −125b2 overexpression) and a strong increase in the number of white blood cells (WBCs) in essentially all mice transplanted with cells overexpressing any of the miR-125 family members (Fig. 2A). Platelet counts were increased in mice reconstituted with cells overexpressing miR-125a (Fig. 2A). Chimerism levels were determined by quantifying the percentage of GFP^{+} granulocytes in the total fraction of granulocytes (defined as Gr-1^{+}). Although, within a period of 10 weeks, the chimerism levels of the control mice remained stable at ∼15%, chimerism in mice transplanted with cells overexpressing miR-125a, -b1, or -b2 increased from 8% to 90%, 4.5% to 75%, and 5% to 90%, respectively. This suggests that ectopic expression of any miR-125 family member provides a strong competitive advantage over nontransduced cells (Fig. 2B). Analysis by FACS revealed that cells overexpressing miR-125a, -b1, or -b2 preferentially differentiate into the myeloid lineage (granulocytes and macrophages, defined as Gr-1^{+} or Mac-1^{+}) at the expense of B lymphocytes (defined as B220^{+}), compared with the control (Fig. 2C). Whereas the percentage of GFP^{+} T cells (CD3+) was decreased, the absolute numbers of GFP^{+} T cells produced by HSPCs overexpressing miR-125 family members were increased compared with the control (Supplementary Figs. E1A, E1B, and E1C, online only, available at www.exphem.org). A similar trend was observed for B cells and granulocytes; this pattern is consistent with previous studies [10]. In one out of two independent transplantation experiments, 7 out of 42 mice developed myeloproliferative neoplasms (MPNs), leading to lethality between 10 and 16 weeks posttransplantation (2 mice, miR-125a; 3 mice, miR-125b1; 2 mice, miR-125b2) (Fig. 2D). In the second experiment, we observed similarly high chimerism levels and mainly myeloid cells in GFP^{+} compartment, but no lethality (up to 28 weeks posttransplantation). Compared with our previous study, we have observed a somewhat higher mortality rate in our in vivo experiments. As the transduction efficiencies in these experiments were modest (8%, 4.5%, and 5% for miR-125a, -b1 and -b2, respectively), most cells will not have integrated more than a single copy vector (Supplementary Fig. E2, online only, available at www.exphem.org). Therefore, we do not consider that insertional mutagenesis contributed to myeloproliferative disease.

To obtain more insight into the type of hematologic malignancy that developed in mice transplanted with miR-125-overexpressing cells, we performed hematologic analysis on six moribund mice (in one case, pathology was not possible). We observed increased WBC counts (Fig. 3A), increased bone marrow cellularity (Fig. 3B), splenomegaly (Fig. 3C), pale bones, and a large number of blast-like cells

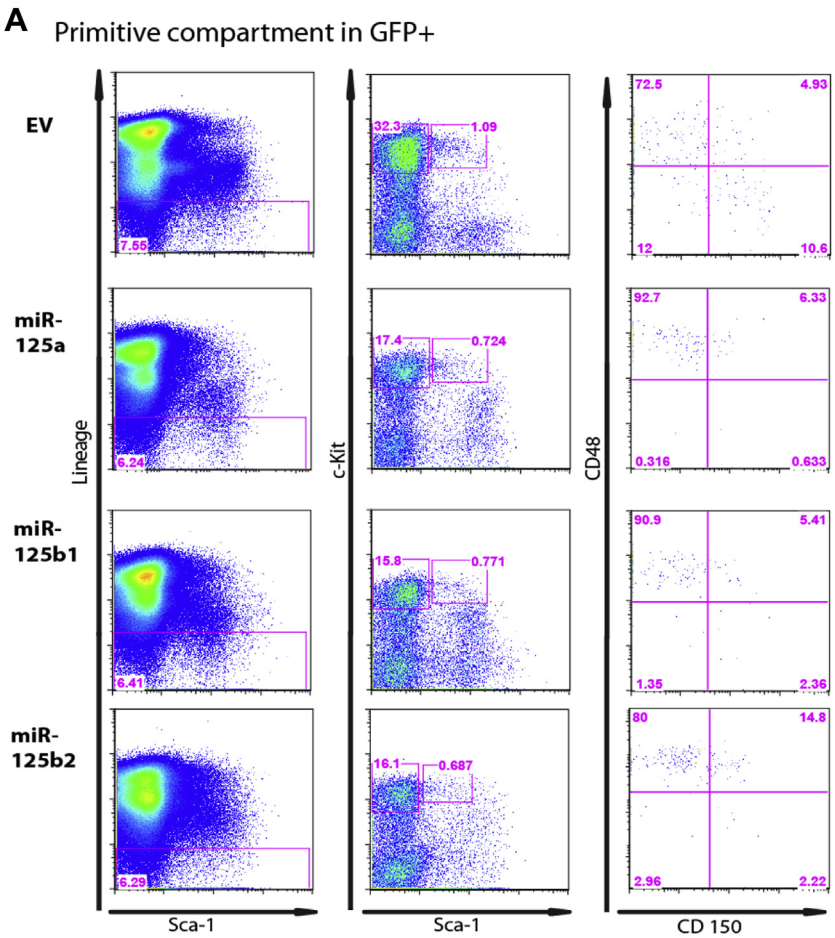

Figure 4. Influence of ectopic miR-125 family members' expression on the primitive compartment. (**A**) Representative FACS plots of the GFP$^+$ BM compartment 16 weeks post–primary transplantations.

in the bone marrow. These are similar features, as we have reported in our previous paper [10]. Importantly, these phenotypes were similar for all miR-125 family members and resembled characteristics of previously observed MPNs [10]. Detailed FACS analysis of peripheral blood, BM, and spleens revealed the dominance of GFP$^+$ cells of myeloid origin (Gr-1$^+$ and/or Mac-1$^+$) (Fig. 3D) and infiltration of those cells in liver and lungs.

To summarize, the data demonstrate that, upon transplantation in mice, stem and progenitor cells in which miR-125 family members are overexpressed show a competitive advantage, are myeloid skewed, and induce a MPN-like phenotype. Although almost all mice transplanted with miR-125a-, miR-125b1-, and miR-125b2-overexpressing cells display features of MPNs, only 15% of the cases were lethal.

The competitive advantage provided by miR-125s is stable over time, but coincides with loss of phenotypically defined hematopoietic stem cells

To quantify the number of HSPCs in the BM of healthy miR-125-overexpressing mice, we performed immunophenotypic analyses of the primitive BM compartment. We observed a reduced frequency and absolute number of GFP$^+$ progenitors (LSK cells) (Fig. 4C) and LT-HSCs (defined as Lin$^-$Sca-1$^+$c-Kit$^+$CD48$^-$CD150$^+$ cells) (Fig. 4B) in the bone marrow of miR-125a-, miR-125b1-, and miR-125b2-overexpressing mice compared with their control counterparts 4 months post transplantation (Fig. 4A and B). At the same time, we noticed an increased frequency of Lin$^-$Sca-1^{mid}c-Kit^{mid} cells (Fig. 4A). When calculating the total number of LSK or LT-HSC cells, we have taken into consideration the increased bone marrow cellularity, as well as higher chimerism levels in miR-125 mice. Phenotypically defined LT-HSCs were also absent from spleens or peripheral blood in mice transplanted with cells overexpressing miR-125 family members.

To functionally determine the presence of HSCs in the bone marrow of primary recipients of miR-125 overexpressing cells, we performed secondary BM transplantations. We assessed the in vivo repopulating ability of GFP$^+$ cells isolated from the BM of primary recipients, compared with freshly isolated BM cells in a competitive transplantation setting.

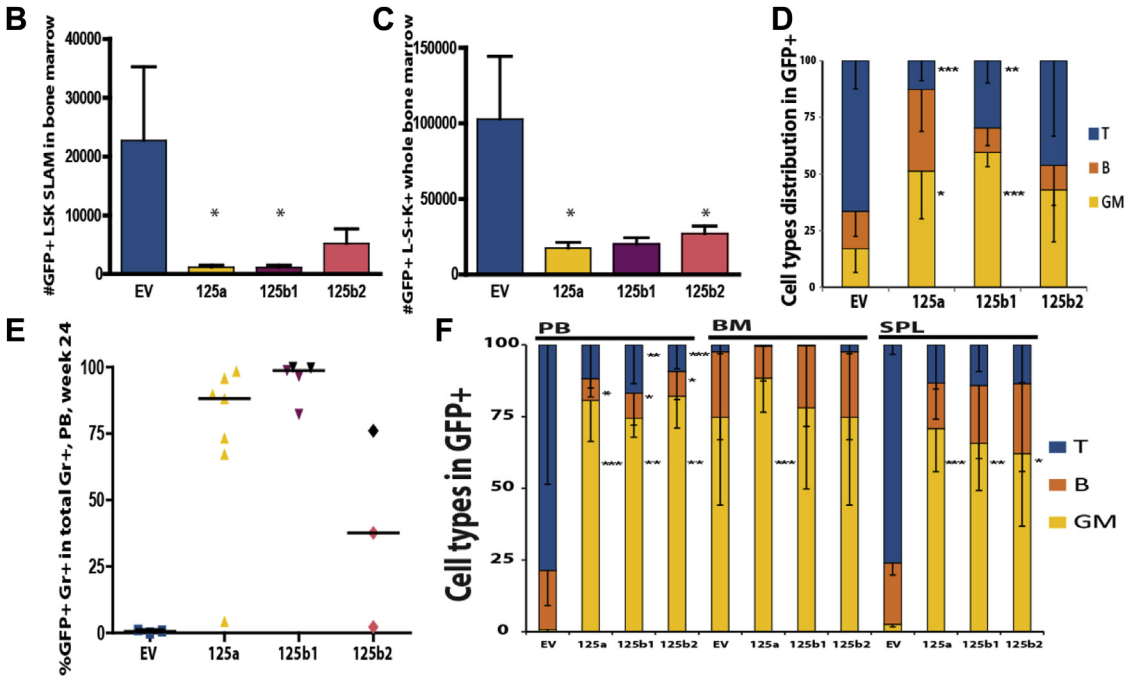

Figure 4. (*continued*) (**B**) Quantification of the total cell number of LSK SLAM and (**C**) LSK cells in total BM at 16 weeks posttransplantation. (**D**) Cell type distribution in blood of empty vector or miR-125 family members upon secondary transplantation tested by FACS 10 weeks posttransplantation (n = 3–7 mice/group). (**E**) Chimerism levels 24 weeks upon secondary transplantation. (**F**) Cell type distribution in GFP^+ in the blood, BM, and spleen of empty vector and miR-125 family members 24 weeks upon secondary transplantation as assessed by FACS. Shown is the mean - SEM (n = 3–7 mice/group). Analyses were performed using FlowJo (TreeStar), followed by the quantification of the number of LSK SLAM (n = 3–7 mice/group). Differences between groups were evaluated by unpaired t test. $*p < 0.01$; $**p < 0.01$; $***p < 0.0001$. B = B lymphocytes; GM = granulocytes/macrophages; Gr^+ = granulocytes; *LSK* SLAM = $Lin^-Sca^+Kit^+CD48^-CD150^+$; T = T lymphocytes. (Color illustration of figure appears online.)

We observed clear differences in chimerism levels among miR-reconstituted and control mice 10 weeks and 24 weeks after transplantation. Three cases of MPNs developed, resembling the features we have described in our primary recipients (Fig. 4E) [10]. Owing to the unexpectedly high engraftment levels upon miRNA-125 overexpression in secondary transplanted mice, an exact LT-HSC frequency estimate was not possible. Nevertheless, the high chimerism levels in Gr-1^+ cells at later time points (up to 24 weeks posttransplantation) indicate presence of functional LT-HSCs in the bone marrow of mice transplanted with miR-125 family members.

Collectively, our data illustrate that miR-125a, miR-125b1, and miR-125b2 affect hematopoiesis similarly. All three miRNAs conferred a competitive advantage to long-term engrafting hematopoietic cells, but these cells cannot be identified by the expression of commonly used cell surface markers for LT-HSCs.

The role of the miR-125 seed sequence

To better understand the molecular mechanism by which miRNA-125 family members are able to modulate hematopoiesis, we tested the extent to which the observed phenotype was seed sequence dependent. To address this question, we determined whether a single nucleotide mutation within the seed sequence would affect the miRNA-125 induced-phenotype in vitro and in vivo. Since our previous experiments showed no essential differences between the various miR-125 family members, we performed these studies with miR-125a only. We introduced a T4C mutation (i.e., the fourth nucleotide within the seed sequence has been mutated from T to C) in the seed sequence of miR-125a (Fig. 5A). Next, we transduced highly purified LT-HSCs with a vector carrying mutated T4C-miR-125a 5p and compared this with LT-HSCs expressing wild type miR-125a. Overexpression and processing of the mutant T4C miR-125a 5p and miR-125a in LT-HSCs were confirmed by quantitative PCR (Fig. 5F). When we analyzed blood 16 weeks posttransplantation, cells overexpressing mutant miR-125a did not show clear competitive advantage, in contrast to wild type miR-125a overexpressing LT-HSCs (Fig. 5D). Furthermore, donor cells clearly showed skewing toward the myeloid lineage in mice transplanted with LT-HSCs overexpressing wild type miR-125a, as well as a compromised frequency of B lymphocytes, which was absent in mice transplanted with cells overexpressing either mutant T4C miR-125a 5p or the empty vector control (Fig. 5E).

We also analyzed the effect of depletion of miR-125s in LT-HSCs by transducing highly purified LT-HSCs with a vector carrying an antagomir for miR-125a (Fig. 5A). In transduced cells, we confirmed decreased levels of the mature form of miR-125a, as measured by quantitative PCR

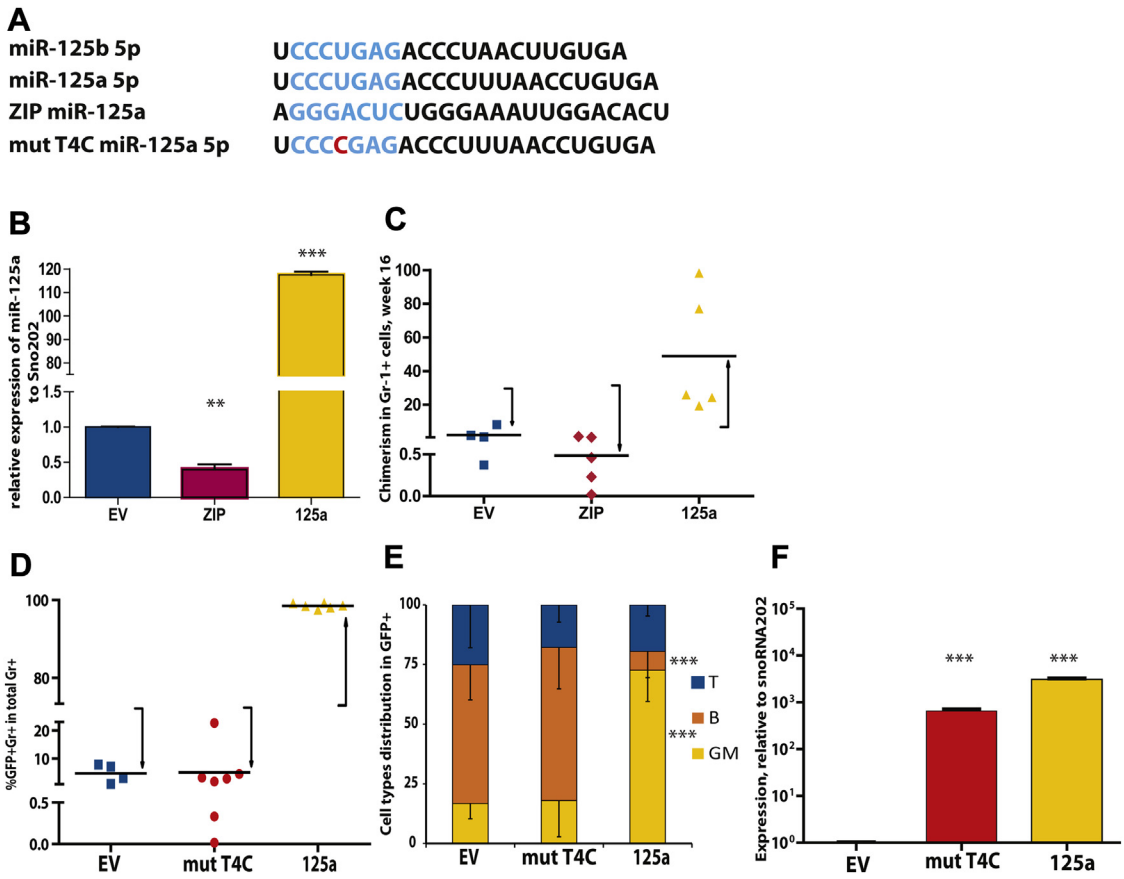

Figure 5. Downregulation of miR-125a abolishes competitive advantage, and a single nucleotide mutation within the seed sequence restores normal hematopoiesis. (**A**) The mature sequence of miR-125b 5p, miR-125a 5p, and the sequence of ZIP-miR-125a. The seed sequence of miR-125a 5p (depicted in blue) has been mutated T4C (marked in red). (**B**) Quantitative reverse transcription PCR results showing the expression levels of miR-125a 5p, in samples in which miR-125a or ZIP-miR-125a 5p is overexpressed, relative to the endogenous control snoRNA202, measured in GFP^+ cells, sorted 4 days after transduction of LT-HSCs. Mir-125 b 5p expression level was below the detection level. Shown is the mean + SEM. (**C**) Chimerism levels were measured at 16 weeks after transplantation by analyzing $GFP^+Gr\text{-}1^+$ cells in total $Gr\text{-}1^+$ fraction (n = 4–5 mice/group). The arrows indicate changes in chimerism during the first 16 weeks posttransplantation. (**D**) Chimerism levels in miR-125a, mutT4C miR-125a 5p, and controls were measured at 16 weeks after transplantation by analyzing $Gr\text{-}1^+$ GFP^+ cells in total population of granulocytes (n = 4–11 mice/group). The arrows indicate changes of chimerism during first 16 weeks posttransplantation. (**E**) Cell-type distribution in the blood as assessed by FACS (n = 4–11 mice/group). Shown is the mean - SD. (**F**) Quantitative reverse transcription PCR results showing the expression levels of miR-125a 5p and mutT4C miR-125a 5p relative to the endogenous control snoRNA202. Shown is the mean + SEM. B=B lymphocytes; GM = granulocytes/macrophages; T = T lymphocytes. (Color illustration of figure appears online.)

(Fig. 5B). Since mature miR-125s share over 50% sequence similarity, we also tested the endogenous expression level of miR-125b. This miRNA was below the detection level. As expected, cells overexpressing miR-125a clearly showed a proliferative advantage over nontransduced cells. Conversely, cells in which miR-125 was downregulated or mutated made no significant contribution to long-term engraftment (Fig. 5C). Observed chimerism levels were low in control mice; however, mice transplanted with ZIP miR-125a 5p-transduced LT-HSCs were given 17 times more cells compared with controls (8,500 transduced GFP^+ LT-HSC vs. 500 GFP^+ LT-HSC). Moreover, 2 million fresh bone marrow competitor cells were used, which also conferred low contribution of control cells to blood cell production. Nevertheless, three out of four control mice were positive for transplanted GFP^+ HSCs as assessed by the chimerism levels >0.5% GFP^+ $Gr\text{-}1^+$ in the total fraction of $Gr\text{-}1^+$ cells, whereas only two out of five ZIP miR-125a 5p transplanted mice were positive (Fig. 5C).

Taken together, our data clearly demonstrate that miR-125 is indispensable for hematopoiesis. The downregulation of miR-125s leads to the loss of competitive advantage of LT-HSCs. The phenotype induced by miR-125 family members depends on the seed sequence. Even a single nucleotide change within the seven-nucleotide-long seed sequence is sufficient to abolish the aberrant effects in hematopoiesis caused by wild type miR-125a overexpression.

Discussion

In this article, we compared the regulatory role of all three miR-125 family members in the biology of HSPCs. All

miRNAs that belong to this family induced a striking increase in the number of late-appearing CAFCs in vitro and remarkably extended their activity. Furthermore, transplantation experiments demonstrated a significant competitive advantage among cells transduced with each of the miRNA-125 family members, which also promoted myeloid differentiation at the expense of the lymphoid lineage. Occasionally, deficiency in the erythroid differentiation and excessive skewing toward the myeloid lineage preceded the development of lethal MPNs. These data are in line with an earlier study in which miR-125b induced MPNs in a dose-dependent way [8].

Interestingly, we were not able to identify phenotypically defined LT-HSCs in the BM of recipient mice, although unfractionated BM from these mice had a strong competitive advantage (and must, therefore, have contained functional stem cells) in serial transplantations. This suggests that increased levels of miRNA-125a, 125b1, and 125b2 all lead to enhanced self-renewal. Increased self-renewal of cells overexpressing miR-125b or miR-125a, respectively, was reported earlier [9,23]. The observed loss of phenotypically defined LT-HSCs might result from the aberrant expression of the stem cell markers Sca-1 and/or c-Kit, which prevented their identification using FACS analysis.

The decreased frequency of phenotypically, but not functionally, defined LT-HSCs may also partially explain the discrepancies in the literature. Our functional data originating from secondary transplantation studies of cells overexpressing miR-125a, miR-125b1, or miR-125b2 do not support the concept of stem cell exhaustion, which one would expect from the FACS profiles of the BM compartment shown in Figure 4A.

Moreover, inconsistencies that have been reported in the literature regarding the effect of miR-125 family members on hematopoiesis may originate from the genomic context of each miRNA. In our previous paper [10], we have shown LT-HSC exhaustion upon secondary transplantation when the entire genomic region containing the miR-99b-let7e-125a cluster was overexpressed. It is known that the miRNA-let7 family is involved in the regulation of differentiation via a Lin28b-let7e feedback loop [24,25]. The expression level of Lin28b is inversely correlated with the expression of mature let-7 family members. High expression level of Lin28b is observed in early stages of embryonic stem cell differentiation and hematopoiesis and decreases during differentiation, whereas an opposite expression pattern is apparent for let7 miRNAs [24,25]. The previously observed effect of ectopic expression of the miRNA cluster 99b-let7e-125a may give an initial competitive advantage to HSPCs but lead to their differentiation and exhaustion upon secondary transplantation, reflecting the opposite effects induced by miR-125a and let7e [10].

Collectively, our data reinforce the important role of all three miR-125 family members in the regulation of hematopoiesis. We show that all three miRNAs confer very similar phenotypes when overexpressed, and our studies highlight the essential role of the CCCUGAG seed sequence. We did not detect meaningful biological differences between miR-125a, b1, and b2 during hematopoiesis. The effects of downregulation of mir-125 highlight the loss of competitive advantage of LT-HSCs. Although all three family members appear to exert similar effects, they are located on different chromosomes, and, therefore, their normal modes of regulation might differ. It is possible that the endogenous expression and the stability of the three mature miRNAs transcripts may be distinct in different cell types, yet control similar self-renewing and differentiation pathways [11,16,26]

Acknowledgments

We thank H Moes, G Mesander, and R-J van der Lei for expert cell sorting assistance. We thank E Verovskaya, RP van Os, and H Schepers for valuable discussions, suggestions, and assistance in the laboratory. This work was supported by grants from Netherlands Organization for Scientific Research, Eurocancer Stemcell Training (EuroCSCTraining) Network (grant no. 264361), and the Netherlands Institute for Regenerative Medicine (NIRM).

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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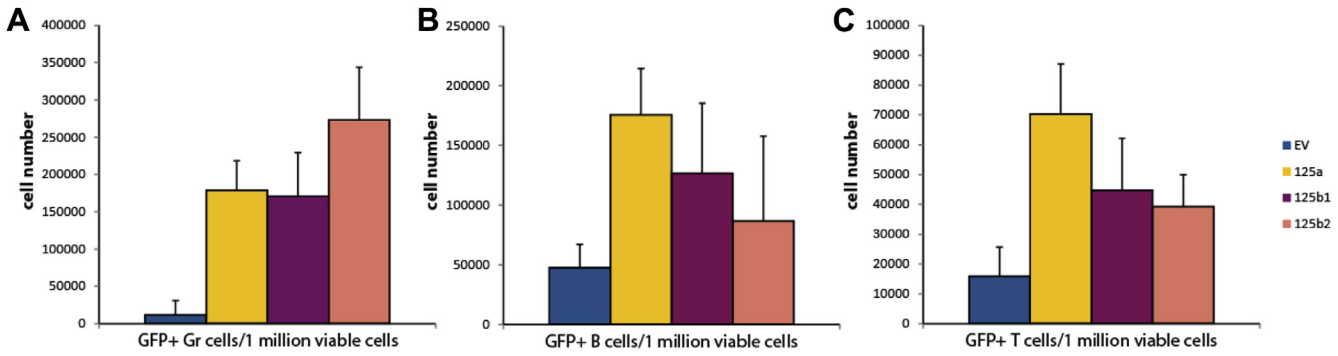
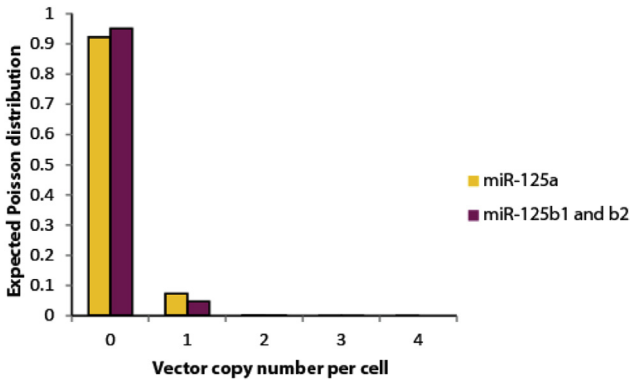


Supplementary Figure E1. The absolute cell numbers produced by GFP$^+$ cells. (**A**) Total number of Gr-1 cells (defined as Gr-1$^+$), (**B**) total number of B cells (defined as B220$^+$), and (**C**) total number of T cells (defined as CD3$^+$) produced by 1 million viable cells 10 weeks posttransplantation.



Supplementary Figure E2. The expected Poisson distribution of vector copy numbers in GFP$^+$ HSPCs. Shown is the distribution of the vector copy number per cell in post 5-FU bone marrow cells in the first experiment.