Supplemental Information

Attenuation of miR-126 Activity Expands HSC In Vivo without Exhaustion

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INVENTORY OF SUPPLEMENTAL INFORMATION

Supplemental Figures and Legends
Supplemental Results
Supplemental Experimental Procedures

Figure S1. Generation of tools for stable miR-126 gain- and loss-of-function in human cells.
This data is directly relevant to Figure 1 because it provides biological validation of the lentivirus tools that are used for enforced expression and knockdown of miR-126 in human and murine HSC.

Figure S2. miR-126 modulation affects colony formation and lineage differentiation of human and murine hematopoietic progenitors in vitro.
This data provides further support for the clonogenic data and lineage differentiation presented in Figure 2.

Figure S3. miR-126 KD in murine HSPC in vivo
This data provides further support to Fig. 3, where knockdown of miR-126 increases long-term or serial engraftment. Furthermore, this data shows that the effects are similar in strain swap transplantation experiments and provide evidence that the in vivo effects of miR-126 KD were not due to the outgrowth of a single aberrant clone.

Figure S4. miR-126 overexpression in murine primary HSPC.
This data provides important support for Figure 4, where miR-126 enforced expression leads to a progressive loss of HSC.

Figure S5. Identification of the PI3K/AKT signaling pathway as a main target of miR-126 in K562 cells.
This data provides SILAC proteomic analysis of K562 cells after 126/KD generating another unbiased approach to predict the pathways perturbed by miR-126 in Figure 5. In addition, we present further western blot validation of the PI3K/AKT/GSK3 pathway.

Figure S6. Stem cell factor (SCF) activates PI3K/AKT signaling in CD34+ huCB cells.
This data supports our hypothesis that SCF signals through PI3K/AKT in Figure 6.
Figure S1 – Related to Figure 1
Figure S2 – Related to Figure 2
Figure S3 – Related to Figure 3
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Figure S5 – Related to Figure 5
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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Generation of tools for stable miR-126 gain- and loss-of-function in human cells.

(A) miR-126 activity in K562 cells as compared to the indicated CD34+ huCB subpopulations. Cells were transduced with a miR-126 reporter BdLV or a control BdLV, and miRNA activity was calculated as previously described (Gentner et al., 2010). Results are shown as mean ± sem of n=3 independent experiments.

(B) Expression of miR-126 in K562 cells transduced with increasing doses of the 126KD (red) and 126OE LV (blue) relative to CTRL LV transduced cells. miR-16 was used as a normalizer. The average vector copy number (VCN) per genome is indicated on the x axis. Other miRNAs investigated, such as let7a and miR-142, were unchanged upon 126KD or 126OE (data not shown). Results are shown as mean ± sem of n=3 technical replicates.

(C) Representative western blots for PI3K p85β and SPRED1, two previously validated direct miR-126 targets (Fish et al., 2008; Guo et al., 2008; Wang et al., 2008), in K562 cells transduced with increasing doses of 126KD, 126OE or CTRL LV. VCN of the transduced cell samples is indicated below each Western blot lane and on the x-axis of the bar graphs. Densitometric quantification after normalization to GAPDH is shown for PI3K p85β (bottom left: results are shown as mean ± sem of n=3 to 10 replicates) and SPRED1 (bottom right).

(D) Relative expression of PIK3R2, the gene encoding for PI3K p85β, was measured by qPCR. Data was normalized to the housekeeping gene β2 microglobulin. Data is expressed relative to CTRL LV transduced K562 cells (mean of CTRL samples was set to 1). Results are shown as mean ± sem of n=6 replicates for CTRL and 126KD cells, n=3 for 126OE.

(E) Correlation of fluorescence reporter expression and miR-126 knockdown. The green curve
shows GFP mean fluorescence intensity (MFI) in relation to 126KD LV VCN, the percentages in green denote the corresponding fraction of GFP+ cells. Note that GFP MFI sharply increases above VCN 8. In parallel to the increase in GFP expression, the miR-126 natural target gene PIK3R2 is de-repressed both at the protein level (solid red line) and at the mRNA level (dotted red line). Note the wider dynamic range of the qPCR assay with respect to the Western blot.

(F) Efficient transduction of CD34+ huCB cells with 126KD LV. Left: representative FACS plots show >90% transduction for both the CTRL and the 126KD LV. Note that the MFI of the 126KD vector is lower and more spread with respect to the CTRL vector. The MFI^low cells still exhibit some degree of miR-126 activity and may thus represent a partial 126KD, while the MFI^high cells, which express the fluorescence marker to similar levels as the CTRL LV transduced cells, are enriched for full 126KD. Right: Average VCN per genome. Results are shown as mean ± sem of n=4 independent experiments.

**Figure S2.** miR-126 modulation affects colony formation and lineage differentiation of human and murine hematopoietic progenitors *in vitro*.

(A) Clonogenic progenitor assay of CD34+ huCB cells transduced with the CTRL or 126KD LV. Cells were plated on day 3 of culture. Note that, at the time of plating, the transduced cells were just starting to express the 126KD targets. Thus, we expected to see no difference in progenitor cell numbers between CTRL and 126KD cells at this time point. The almost identical number of colonies that we observed in the 2 groups suggest that 126KD did not impede differentiation of committed progenitors. Results are shown as mean ± sem of n=5 independent experiments. E, erythrocyte; GM, granulocyte/macrophage; mix, mixed granulocyte / erythrocyte / macrophage / megakaryocyte colonies.
(B) Clonogenic progenitor assays of FACS-purified CD34<sup>+</sup> huCB subpopulations after 7 day culture in TSF<sub>half</sub> medium (see Experimental Procedures). Shown is the number of E, GM and mixed colonies for CTRL (white bars) and 126KD LV transduced cells (red bars) originated from plating CD34<sup>+</sup>CD38<sup>-</sup>CD133<sup>+</sup> (left), CD34<sup>+</sup>CD38<sup>low</sup>CD133<sup>+</sup> (middle) or CD34<sup>-</sup>CD38<sup>-</sup>CD133<sup>+</sup> (right) cells. Results are shown as mean ± sem of n=4 replicates and representative of 4 independent experiments.

(C) Effect of 126KD on HSPC and MEP during erythroid lineage commitment. Lin<sup>-</sup> huCB derived HSPC (CD34<sup>+</sup>CD38<sup>-</sup>) and MEP (CD34<sup>+</sup>CD38<sup>-</sup>CD135<sup>-</sup>CD45RA<sup>-</sup>) were transduced with CTRL and 126KD LV to investigate the effect on erythroid differentiation in serum-free liquid culture conditions (see Supplementary Experimental procedures). Five days after plating in erythrocyte differentiation promoting conditions, cells were stained for CD71, an early erythroid differentiation marker. Data was normalized to CTRL for statistical analysis. Results are shown as mean ± sem of n=4 independent experiments (HSPC) and n=2 independent experiments (MEP). The absence of an effect on transduced MEP indicates the differentiation of an upstream progenitor is affected.

(D) Effect of 126KD on HSPC and MEP during megakaryocytic lineage commitment. Similar to (C), we assessed CD42b expression 7 days after plating sorted CD34<sup>-</sup>CD38<sup>-</sup> Lin<sup>-</sup> huCB cells in megakaryocyte differentiation promoting conditions. Data was normalized to CTRL for statistical analysis. Results are shown as mean ± sem of n=4 independent experiments (HSPC) and n=2 independent experiments (MEP). We found that 126KD accelerated HSPC differentiation down both erythroid and megakaryocytic lineages, as significantly more cells expressed the differentiation markers CD71 (erythroid) and CD42b (megakaryocyte) early in the respective conditions. However, this effect was established upstream of the MEP stage, as
126KD in MEPs had no effect on marker expression. In support of this notion, no differences in expression of CD71 or CD42b were seen at later time points in HSPC cultures, although the 126KD cells exhausted earlier than CTRL LV transduced cells in erythrocye culture conditions, possibly due to accelerated differentiation (data not shown).

(E-F) Effect of 126OE on HSPC during erythroid lineage commitment. Using the same conditions as in (C), we assessed expression of the erythrocyte markers CD71 and GlyA in 126OE or CTRL LV transduced CD34^+CD38^- huCB cells. A greater than 2-fold reduction in expression of erythroid markers was found from 5 (E) to 29 (F) days after plating in erythroid differentiation promoting conditions, indicating 126OE blocks early and late erythroid commitment. Data was normalized to CTRL for statistical analysis. Results are shown as mean ± sem of n=3 independent experiments.

(G) Effect of 126OE on growth of CD34^+CD38^- huCB cells in erythrocyte promoting culture conditions. Results are shown as mean ± sem of n=3 independent experiments. A 3.8-fold reduction in total cell output upon 126OE was observed. However, constitutive expression of miR-126 had no effect on megakaryocytic differentiation (data not shown), suggesting that high levels of miR-126 in erythroid progenitors may stimulate differentiation toward megakaryocytes. This data matches our colony data from early cytokine culture where 126OE decreased overall colony number and 126KD increased overall number, with the most significant differences being in the erythroid and mixed colonies (see Figure 2c-e).

(H) Effect of 126OE on development of erythroid human graft in the xenograft environment. Cells isolated from the mouse BM 16 weeks after xenotransplantation of CTRL or 126OE LV transduced CD34^+CD38^- huCB cells were stained for GlyA. Left: representative FACS histograms, gated on OFP^+ cells. Right: bar graph to summarize data. Results are shown as mean
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± sem of n=5 mice. A significant reduction in erythroid output from long term (16 week) lin− CB CD34+CD38− xenografts is evident, confirming reduced erythropoiesis upon 126OE in an in vivo environment. Together this data implies that miR-126 bioactivity restrains erythroid progenitor differentiation.

(I) Cell cycle analysis of highly transduced murine Lin−Sca+Kit+ (LSK) cells. LSK cells were sorted from mouse BM and transduced with 126OE or 126KD LV. Representative FACS plots showing Ki67 and Hoechst profile are shown. Bottom right: Fraction of quiescent (left, G0) and dividing (right; S-G2-M) cells upon miR-126KD (red) or 126OE (blue). Data are normalized to CTRL LV transduced cells and shown as mean ± sem of n=2 independent experiments.

(J) Clonogenic progenitor assays of LSK cells transduced with CTRL, 126OE or 126KD LV. LSK cells were sorted from mouse BM, transduced as in (I) and plated in methylcellulose colony forming assays. The number of different colony types is depicted for primary and secondary plating. GM, granulocyte/macrophage; E, erythrocyte; mix: mixed granulocyte/erythrocyte/macrophage/megakaryocyte colonies. Results are shown as mean ± sem of n=8 from 2 independent experiments.

(K) The same LSK cells as in (I) were subjected to Annexin V staining to assess apoptosis induction upon 126OE or 126KD. Results are shown as mean ± sem, n=2 independent experiments.

* p < 0.05, ** p < 0.01.

Figure S3. miR-126KD in murine HSPC in vivo

(A) Murine HSPC were transduced with 126KD LV (CD45.2) and CTRL LV (CD45.1) and competitively transplanted into CD45.1/CD45.2 double positive recipient mice. Monocyte (top)
and T cell (bottom) chimerism (%CD45.2$^+$ cells) from the mice described in Figure 3e is shown. CD45.1/CD45.2 double positive recipient cells were excluded from the analysis, allowing to assess T cell reconstitution by donor cells during the early phases of engraftment. The arrow indicates when a serial BM transplant into the same congenic recipient strain was performed. Results are shown as mean ± sem n=9 mice.

(B) Peripheral blood granulocyte, B and T cell CD45.2$^+$ chimerism (left plots) and BM chimerism (GFP$^+$CD45.2: red; GFP$^+$CD45.1: white, right bar graphs) after primary (left) and secondary (right) transplantation are shown from an independent competitive BM transplant in which CD45.2$^+$ and CD45.1$^+$ Lin$^ -$ BM HSPC were transduced with 126KD and CTRL LV, respectively, and competitively engrafted into CD45.1 recipients. Results are shown as mean ± sem, n=5 mice.

(C) Polyclonal in vivo expansion of miR-126 KD HSC. BM cells from the secondary transplanted mice shown in Figure 3e/f were sorted into CD45.1$^+$ CTRL- and CD45.2$^+$ 126KD LV transduced cells. DNA was extracted and processed for LV integration site analysis by linear amplification mediated (LAM)-PCR as previously described (Biffi et al., 2011). Each lane showed the restriction digest by Tsp509I of PCR amplified products from either the CTRL or the 126KD transduced cells of each individual mouse. The common band among all samples represents a PCR product amplified from the vector backbone and serves as internal control. M, DNA size markers. The appearance of a smear on the digest indicates highly polyclonal composition of both CTRL and 126KD donor-derived cell populations in all mice.

(D) Chimerism and transduction levels in the mice chosen as BM donors for the limiting dilution analysis shown in Figure 3i. CD45.1 mice were reconstituted with CD45.2$^+$ BM HSPC transduced with either the 126KD (red bars) or the CTRL LV (white bars). Shown is the
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Peripheral blood chimerism for CD45.2\(^+\) donor cells (upper graph) and their transduction levels (% GFP\(^+\), bottom graph) at sixteen weeks after transplant. Results represent the mean ± sem of n=3 mice per group.

(E) Bone marrow analysis of the mice shown in panel D, performed at sixteen weeks after the primary transplant. Shown is the contribution of GFP\(^+\)CD45.2\(^+\) cells to the indicated BM populations in the CTRL group (left, white bars) and in the 126KD group (right, red bars). Results represent the mean ± sem of n=3 mice per group. Please note that – in agreement with previous experiments shown in **Figure 3e/f** and **S3**, 126KD chimerism was significantly increased in HSC and decreased in Kit\(^-\)Sca\(^+\)Lin\(^-\) progenitors, while chimerism of CTRL vector transduced cells was not significantly different between these populations.

(F) Linear regression analysis of data presented in **Figure 3i**. Circles (red: 126KD; black: CTRL) represent the percentage of non-engrafted mice for each cell dose, and dotted lines represent the 95% confidence interval (red: 126KD; black: CTRL).

**Figure S4. miR-126 overexpression in murine primary HSPC.**

(A-B) Ectopic miR-126 expression levels in hematopoietic cells *in vivo* after transduction with CTRL (white) or 126OE LV (blue). Murine HSPC were transduced with the SFFV (A) or the EF1\(\alpha\) promoter-driven 126OE LV (B), and competitively transplanted. Six to 8 weeks later, the expression levels of miR-126, let-7a and miR-16 in peripheral blood cells were assessed by qPCR. In (A), qPCR analysis was performed on total nucleated PB cells, and the expression level of miR-126 was corrected by a factor of 2.5, since FACS analysis performed at that time demonstrated that 40% of the cells expressed SFFV-126OE while there was no miR-126
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eexpression in unmanipulated peripheral blood. In (B), EF1α-126OE expressing cells were sorted. Data was normalized to let-7a. Results are shown as mean ± sem of n=3 technical replicates.

(C) Congenic Ptprca and Ptprcb murine Lin- BM cells were transduced with EF1α-126OE or EF1α-CTRL LV, respectively, and competitively transplanted. Left: contribution of EF1α-CTRL CD45.2⁺NGFR⁺ cells (black) and CD45.1⁺EF1α-126OE NGFR⁺ (blue) cells to the granulocyte and B cell lineage over time. Results are shown as mean ± sem of n=3 mice of a representative experiment. Right: quantification of the persistence of 126OE- versus CTRL LV expressing cells at 25 weeks post-transplant as compared to 3 weeks. Every dot represents one mouse (n=11 mice for EF1α-CTRL and n=15 mice for EF1α-126OE, horizontal line indicates the mean ± sem).

(D) Analysis of early engraftment kinetics by 126OE transduced HSPC. Mice were transplanted with 126OE- (blue line) or CTRL LV (black line) transduced CD45.2⁺ HSPC. Serial complete blood counts (CBC) were performed at indicated timepoints. The parameters shown are the total white blood cell count (WBC), absolute neutrophil count (ANC: %CD11b⁺ cells x WBC/100), absolute B lymphocyte count (ABC: %CD19⁺ cells x WBC/100), platelet count (PLT) and hemoglobin levels (HGB). Results are shown as mean ± sem of n=8 126OE mice and n=7 CTRL mice.

(E) Part of the mice transplanted for the study of engraftment kinetics (see panel D), was used for short-term BM homing studies. CD45.2 HSPC transduced with either 126OE or CTRL LV were transplanted into CD45.1 recipients. At day 3 after transplant, homing to the BM was assessed as the percentage of transplanted cells (CD45.2) transduced with either CTRL or 126OE LV (OFP⁺). Results are shown as mean ± sem for n=8 126OE mice and n=7 CTRL mice.

(F) Bone marrow analysis of the mice shown in panel E, performed at sixteen weeks after the
primary transplant. Shown is the contribution of OFP^+CD45.2^+ cells to the indicated BM populations in the CTRL group (left, white bars) and in the 126OE group (right, blue bars). Results represent the mean ± sem of n=7 mice per group. Please note that – in agreement with previous experiments shown in Figure 4G, 126OE chimerism was significantly reduced in HSC and in LSK progenitors, while chimerism of CTRL vector transduced cells was not significantly different between these populations. * p < 0.05, ** p < 0.01.

Figure S5. Identification of the PI3K/AKT signaling pathway as a main target of miR-126 in K562 cells.

(A) Chart showing the distribution of the 2,273 proteins quantified in the SILAC experiment. Proteins with no statistically significant difference in expression (p>0.05) upon 126OE are shown in grey, while proteins with a significant difference (p<0.05) are highlighted in blue.

(B) Canonical pathways significantly regulated in the K562 cell proteome upon 126OE. The chart represents the 15 canonical pathways (out of 217 interrogated) that were significantly affected by 126OE in our dataset. Statistical significance was evaluated according to Benjamini and Hochberg (B-H) test. PI3K/AKT signaling was among the most significantly regulated pathways (p=0.0024 after B-H correction).

(C) PI3K/AKT pathway chart summarizing our findings on proteins regulated in K562 cells upon 126OE. Thirteen proteins involved in the pathway were significantly regulated by 126OE in the SILAC experiment as highlighted (orange indicates up regulation, while green indicates down regulation). PI3Kp85b and AKT2 down regulation upon 126OE were confirmed by western blot (see Figure S1D and Figure S5D) while SILAC quantification did not reach
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significance. Purple shadow indicates that the transcript encoding the indicated protein is a putative miR-126 direct target according to previously published data literature data and the miRecords database (http://mirecords.biolead.org).

(D) Expression of AKT isoforms in K562 cells upon 126KD and 126OE. Top: representative western blots of K562 cells transduced with different amounts of the indicated vectors. Only AKT2 was significantly modulated by miR-126. Bottom left: bar graph indicating the quantification of the AKT2 protein band normalized to GAPDH and relative to the average of CTRL LV transduced cells. Results are shown as mean ± sem of n=2 to 5 independent experiments. VCN is indicated on the x-axis. Bottom right: bar graph showing AKT2 mRNA levels in 126KD cells as assessed by qPCR, normalized to the average of CTRL LV transduced cells. Results are shown as mean ± sem of n=2 independent experiments.

(E) Phosphorylation of AKT at Ser473 was evaluated in K562 cells transduced with different doses of 126KD LV in comparison to CTRL LV transduced cells. Left: representative western blot. Right: densitometric quantification of the P-AKTSer473 band normalized to GAPDH. VCNs are indicated on the x-axis. Results are shown as mean ± sem of n=2 independent experiments per VCN.

**Figure S6. Stem cell factor (SCF) activates PI3K/AKT signaling in CD34+ huCB cells.**

CD34⁺ huCB cells were starved for 3h in cytokine free medium and then stimulated with the indicated cytokine alone or the TSF6 combination (see Experimental procedures). Phosphoflow analysis of Phospho-AKT (Ser473) was performed 5 minutes after stimulation. Histograms show the MFI of the stimulated sample (grey filled area), non-stimulated control
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(black line) and a cytokine stimulated, Wortmannin treated sample (silver line). Percentages refer to the stimulated cells falling into the positive gate, the number below denotes the MFI of Phospho-AKT (Ser473) positive cells.
SUPPLEMENTAL RESULTS

Development of Lentiviral Tools for Stable miR-126 Gain- and Loss-of-Function Studies

In order to study the function of miR-126 in HSPC, we developed lentiviral vectors for stable overexpression (gain-of-function approach) or antagonism of miR-126 (loss-of-function or knock-down approach). Both vectors exploit the strong spleen focus forming virus (SFFV) promoter and couple miR-126 up- or down-modulation with the expression of a transcriptionally linked (co-transcribed) fluorescence reporter. In the over-expressing vector (126OE vector) (Amendola et al., 2009), the pri-mir-126 sequence is cloned within an intron upstream of the reporter. In the "knockdown" or "sponge" vector (126KD) (Gentner et al., 2008) the reporter transcript contains 8 tandem copies of an imperfectly complementary miR-126 target sequence within the 3' untranslated region (UTR) (Fig. 1c). If not otherwise mentioned, vectors containing the empty intron sequence or scrambled target sequences not complementary to any known miRNA served as controls for 126OE or 126KD, respectively.

Vectors were first tested in K562 cells that naturally express miR-126 to a similar extent as CD34+CD38- HSPC (Fig. S1a). Transduction with the 126OE vector led to a clear dose-dependent increase (up to 8-fold) in miR-126 levels, whereas the 126KD vector led to 2-fold decrease in miR-126 levels (Fig. S1b), likely due to increased target-mediated turnover. This is not, however, the main mechanism of action of the KD vector, which inhibits miRNA activity by expressing excess miRNA targets that compete with the endogenous ones. Neither vector detectably perturbed the expression of unrelated miRNAs (Fig. S4A and data not shown). We then determined the expression of previously reported miR-126 targets including sprouty-related EVH1 domain containing 1 (SPRED1) and phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) (Fish et al., 2008; Guo et al., 2008; Wang et al., 2008) by Western blot and qPCR.
PIK3R2 but not SPRED1 was responsive to 126OE or 126KD in K562 cells, revealing a vector dose-dependent, 1.5- to 2-fold down- or up-regulation, respectively, as compared to CTRL vector-transduced cells, both at the protein and mRNA level. PIK3R2 up-regulation by increasing doses of 126KD was paralleled by a disproportionate increase in the expression of the fluorescence reporter as compared to the lower doses. This surge in fluorescence is expected because the reporter transcript carries miR-126 target sequences in the 3' UTR and its expression is subjected to miR-126 suppression until the target or "sponge" sequence reaches sufficient concentration to saturate miR-126 activity. Thus, the reporter expression level provides a surrogate marker to identify the cells in which miR-126 has been knocked down most effectively (Gentner et al., 2008). Knock-down of miR-126 was clearly detectable in the bulk population at ≥ 8 average vector copies per cell, a realistic goal that can be achieved in primary hematopoietic cells using an optimized transduction protocol (Santoni de Sio and Naldini, 2009; Biffi et al., 2011). These data show that stable 126OE and 126KD are feasible in a cell line resembling human HSPC in terms of miR-126 expression level, without evidence of deregulation of miRNA processing or other types of toxicity.

Using an optimized transduction protocol, we consistently achieved >90% transduction of CD34+ lin- CB cells in cytokine-supplemented serum-free medium and up to 10 average vector integrations per cell. The degree of miR-126 up- or down-modulation was functionally relevant, as the expression levels of the previously validated target PIK3R2 were specifically and significantly decreased upon overexpression, and increased upon knockdown.

**miR-126 modulation alters megakaryocyte/erythrocyte differentiation**

In suspension cultures designed to promote erythrocyte and megakaryocyte differentiation, we
noted increased erythroid and megakaryocytic differentiation of CD34^+CD38^- lin- CB cells upon 126KD and a strong inhibition of erythropoiesis, upon 126OE (Fig. S2c-h). Interestingly, the effects on erythroid and megakaryocyte differentiation must occur in a primitive cell type, since 126KD in flow purified MEP populations displayed no alterations in differentiation potential.

**In vitro effects of miR-126 modulation are conserved in murine cells**

To assess whether the regulatory function of miR-126 was conserved across species, we performed liquid culture and colony forming assays upon 126KD or 126OE in sorted lineage depleted (Lin^-) Kit^+ murine HSPC. Cell cycle analysis gated on highly transduced Sca1^+ cells showed an increase in S/G2/M and a decrease in G0 upon 126KD, with an opposing behavior upon 126OE (Fig. S2i). Colony forming potential was significantly increased after re-plating of 126KD cells, and reduced upon 126OE (Fig. S2j). Of note, Annexin V staining showed negligible levels of apoptosis in all treatment groups (Fig. S2k).

**126OE Phenotype is not Dependent on Artificially High Expression Levels**

To examine the effect of miR-126 over-expression on the fate of murine HSCs *in vivo*, we performed competitive murine transplantation experiments. Lin^- BM cells from congenic mice (Ptpc^a and Ptpc^b, respectively) were transduced with a 126OE vector or with a CTRL vector, and injected in a 1:1 ratio into myeloablated recipients. qPCR analysis performed on peripheral blood cells from mice engrafted with HSPC transduced with the 126OE vector based on the SFFV promoter (see main text and Fig.4e-h) demonstrated that OFP marker positive cells ectopically expressed miR-126 to similar levels as miR-16, one of the most abundant
endogenous miRNAs (Fig. S4a). To exclude the possibility that the observed phenotype was dependent on toxicity due to very high levels of miR expression, we repeated the competitive transplantation experiments with ectopically expressing miR-126 from the weaker EF1α promoter. This vector resulted in a moderate level of overexpression of miR-126 in the peripheral blood of mice, to levels similar to the ubiquitously expressed miRNA let-7a (Fig. S4b). Importantly, all aspects of the 126OE phenotype (initial burst followed by progressive reduction in 126OE cells) were faithfully reproduced (Fig. S4c).

**Impact of 126OE on the early engraftment phase**

Serial complete blood counts (CBC) performed every 3-4 days during the engraftment phase revealed a significant advantage of mice transplanted with 126OE cells in platelet and leukocyte recovery which was, however, limited to a short time window (Fig. S4d). The duration of severe neutropenia (ANC<500/μl) and thrombocytopenia (<5x10⁴/μl) was significantly shortened in mice transplanted with 126OE cells (Fig. S4d). The differences in engraftment kinetics were not due to a difference in homing capacity of 126OE cells, since mice had, in comparison to the control groups, equal or slightly reduced grafts in the BM at 3 days post infusion (Fig. S4e). These experiments suggest that, initially after transplantation, miR-126 overexpression expands specific progenitor cell subsets, which is reflected in an increased multi-lineage output during the first weeks after transplant.

**SILAC Analysis Reveals PI3K/AKT Signaling is Target of miR-126**

As the effect of miRNA modulation of gene expression may be stronger at the protein level and
to provide another unbiased approach, we performed a quantitative analysis on the proteome of K562 cells stably transduced with 126OE or CTRL vectors using the SILAC (Stable Isotope Labeling of Amino acids in Culture) technique. We were able to reliably quantify 2,273 protein groups, and 510 of them showed a significant change (p<0.05) between 126OE and CTRL samples (Fig. S5a). We then interrogated this dataset using unsupervised Ingenuity Pathway Analysis (IPA) and found that the PI3K/AKT signaling pathway was among the most significantly affected canonical pathways upon 126OE (Fig. S5b,c), validating the bioinformatic analysis of the mRNA arrays. Taken together, our data pinpoints PI3K/AKT signaling as the foremost target of miR-126 in hematopoietic cells.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse strains

B6.SJL-Ptprca (CD45.1) and C57BL/6NTac-Ptprcb (CD45.2) mouse strains were purchased from Charles Rivers Laboratories (Milan, Italy) and maintained in specific-pathogen-free (SPF) conditions. CD45.1 and CD45.2 mice were also crossed to generate CD45.1-CD45.2 double positive mice to be used as recipients of competitive bone marrow transplants. All the procedures involving animals were designed and performed with the approval of the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (IACUC #324) and communicated to the Ministry of Health and local authorities according to Italian law.

Lentiviral Constructs and Cord Blood Sample Preparation

Lentiviral vector platforms for ectopic miRNA expression and stable knockdown were described previously (Gentner et al., 2008; Amendola et al., 2009). For ectopic expression, the 717 bp genomic fragment containing human pri-mir-126 was cloned into the intron of LV.EF1α.intron.ΔLNGFR (Amendola et al., 2009). In a second step, the EF1α promoter was substituted by the spleen focus forming/enhancer promoter (Gentner et al., 2008), and the ΔLNGFR marker gene was replaced by the Orange Fluorescent Protein mOrange2 (OFP, (Shaner et al., 2008). For stable knockdown, 8 tandem copies of an imperfectly complementary miR-126 target sequence (5' gcattattactgatccacggtacga 3') were synthesized as described (Gentner et al., 2008) and cloned into the LV.SFFV.GFP or LV.SFFV.intron.OFP backbones. Third generation lentiviral vector particles pseudotyped with VSV-G were generated as described (Follenzi et al., 2000; Guenechea et al., 2000). CB samples were obtained from normal full term deliveries after informed consent, according to procedures approved by the institutional...
review boards of the University Health Network and Trillium Hospital, or the San Raffaele Institute (TIGET01). Alternatively, CB cells were bought from commercial sources (“Poietics” CD34⁺ cord blood cells – Lonza). Mononuclear cells were obtained by density gradient centrifugation on Ficoll. Stem and progenitor cells were enriched either by lineage depletion using the StemSep Human Progenitor Cell Enrichment Kit according to the manufacturer's protocol (StemCell Technologies) or by positive selection using anti-CD34-tagged magnetic beads and a VarioMacs magnet (Miltenyi Biotec). The lin⁻ cells (50-75% CD34⁺) or the CD34 enriched cells (purity >90%) were stored at -150°C until use.

**Erythrocyte and megakaryocyte differentiation conditions**

For erythrocyte differentiation, following transduction cells were expanded in StemPro34 (Gibco) supplemented with 2 mM L-glutamine, 100 U/mL P/S and G-CSF (1 ng/mL), IL-3 (2 ng/mL), SCF (20 ng/mL) and erythropoietin (1 unit/mL). For megakaryocyte differentiation, after transduction cells were plated in StemPro34 supplemented with L-glutamine, P/S and Flt3-L (2.5 ng/mL), IL-6 (50 ng/mL), SCF (2.5 ng/mL) and TPO (100 ng/mL) for 7 days, followed by 7 day replating in the same medium with different cytokines concentrations: Flt3-L (50 ng/mL), IL-6 (50 ng/mL), SCF (50 ng/mL) and TPO (100 ng/mL).

**Colony forming cell assay**

Human clonogenic assays were plated at day 3, 7 or 14 of culture in a methylcellulose-based medium (MethoCult H4434, StemCell Technologies). The following cell numbers were plated: day 3, 0.8x10³/ml; day 7, 2x10³/ml; day 14, 10x10³/ml. Two weeks after plating, colonies were
counted under an inverted microscope and identified according to morphological criteria.

Mouse clonogenic assays were performed in M3434 Methocult (StemCell Technologies) by plating $1 \times 10^3$/ml lineage depleted progenitors. Colonies were counted and identified according to morphological criteria after 1 week of culture, plucked and brought into single cell suspension and replated in fresh methylcellulose medium as a constant fraction of the resuspension volume roughly corresponding to $5 \times 10^4$ cells.

**Cell sorting**

After thawing, human lin- CB cells were resuspended at $10^7$ cells / mL and stained with surface markers in PBS with 2% FCS. Cells were sorted on the BD FACS Aria or on a MoFlo sorter (DAKO), consistently yielding >95% purity. The following anti-human antibodies were used for sorting: CD133-Biotin (Miltenyi) and PE-labeled streptavidin (Biolegend), CD38-APC (clone HB7, BD Bioscience), CD34-APC7 (BD, custom made), CD38-PC7 (BD, Cat. #335790), CD90-PE (BD, Cat. # 555596), CD45RA-FITC (BD, Cat. # 55488), CD49f-PC5 (BD, Cat. # 551129), CD10-APC (BD, Cat. # 340923), CD135-PE (BD, Cat. # 558996) and CD7-PC5 (Coulter, Cat. # IM3613U).

**Ki67 and Hoechst flow cytometry**

Cells were stained for surface markers, washed and fixed using BD Cytofix buffer (Cat. #554655), washed and permeabilized with BD Perm 2 (Cat. # 347692), washed and stained with PE- or FITC- or PerCP-Cy5.5 conjugated Ki67 antibody (BD) and finally resuspended in BD Cytofix buffer with Hoechst at 1 µg/mL. The cells were then analyzed on a BD LSRII machine with UV laser.
**Quantitative PCR (miRNA, mRNA, VCN)**

Vector copy number analysis was carried out as described previously (Gentner et al., 2008). Genomic DNA was extracted from cells after a minimum of 14 days in culture after LV transduction in order to get rid of non-integrated vector forms (Follenzi and Naldini, 2002). All reactions were carried in out in triplicate in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA).

miRNA expression was analyzed as described (Gentner et al., 2008). Briefly, small RNAs were extracted using miRNeasy mini kit (Qiagen) and miRNA expression levels were determined by the Applied Biosystems Taqman® microRNA Assay system. Reactions were carried out in triplicate in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA). miRNA expression was normalized to miR-16 or RNU48.

For gene expression analysis, total RNA was isolated from cells in culture using either the RNEasy mini or micro kit (Qiagen) according to the manufacturer's instructions. Retro transcription of total RNA was performed using Invitrogen Superscript III First Strand Synthesis™ System for RT-PCR, by loading a maximum of 500 ng of total RNA per reaction. Taqman® gene expression analysis was performed using the following primer/probe sets (Applied Biosystems): *PIK3R2* (Hs00178181_m1), *AKT2* (Hs01086102_m1), *AKT1* (Hs00920503_m1). The following housekeeping genes were used for normalization: *Beta2 microglobulin* (*B2M*) (Hs00984230_m1), *Phospho-glycerate kinase 1* (*PGK-1*) (Hs99999906_m1), *TATA box-binding protein* (*TBP*) (Hs00427620_m1). Reactions were carried out in triplicate in an ABI Prism 7900 by using a standard amplification protocol, according to manufacturer’s instructions. Gene expression was calculated by the $2^{-\Delta Ct}$ or by the $2^{-\Delta\Delta Ct}$ method,
with calibrator samples indicated for each experiment.

Western Blot and SILAC

Total cellular proteins were extracted with RIPA buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors: 1 mM PMSF, 10 mM NaF, 1 mM Na₃VO₄, CompleteMini™ and PhosStop™ (Roche). Samples were resuspended in the lysis solution and incubated at 4°C for 30 min. Cell lysates were cleared by centrifugation at 10,000 × g for 10 min at 4°C, and the supernatants were collected and assayed for protein concentration using Lowry assay based method (DC, BioRad). 40-50 micrograms of proteins were run on SDS-PAGE under reducing conditions. For immunoblotting, proteins were transferred to nylon membranes (Hybond+, Amersham), incubated with the specific antibody followed by peroxidase-conjugated secondary antibodies (ECL Mouse IgG or ECL Rabbit IgG; GE Healthcare) and detected using chemiluminescent reagents (Luminata Forte, Millipore / ECL prime, Amersham) and exposure to autoradiography films.

The following antibodies were used: mouse monoclonal anti-PI3K p85β (Abcam ab28356, 1:1000), rabbit polyclonal anti-PI3K p85α (Millipore, 1:1000), rabbit polyclonal anti-phospho Akt Ser473 (Cell Signaling #9271, 1:500) or rabbit monoclonal anti-phospho AKT Ser473 (Cell Signaling #4060), rabbit monoclonal anti-Akt2 (Cell Signaling #2964, 1:1000), mouse monoclonal anti-Akt1 (Cell Signaling #2967, 1:500), mouse monoclonal anti-SPRED1 (Abcam ab64740, 1:500) and mouse anti-GAPDH (1:10,000, Sigma). Secondary HRP-conjugated anti-mouse or anti-rabbit IgG (Sigma) were utilized and bands were visualized by Western Blotting Analysis System (GE Healthcare) on Amersham Hyperfilm™ (GE Healthcare). Densitometric
analysis of scanned films was performed using ImageJ software (http://rsb.info.nih.gov/ij). Fold-change was calculated as the ratio between the optical density of the investigated band and the corresponding loading control band (GAPDH).

For quantitative proteomics, K562 cells transduced with the 126OE vector or the CTRL vector were analyzed using Stable Isotope Labeling of Amino acids in Culture (SILAC) method (Ong et al., 2002). Briefly, cells were cultured in DMEM medium deficient in arginine and lysine (from Invitrogen) that was supplemented with stable isotope-encoded arginine and lysine (Sigma-Aldrich). For “heavy” labeling we used l-[13C6, 15N4]arginine (Arg10) and l-[13C6, 15N2]lysine (Lys8) and for the “light” condition l-[12C6, 14N4]arginine (Arg0) and l-[12C6, 14N2]lysine (Lys0) were used. In each SILAC condition, medium was supplemented with 10% dialyzed fetal calf serum (10KDa cutoff) and 1% (10 mg/ml) streptomycin/(10,000 units/ml) penicillin, and 1% l-glutamine (200 mm in 0.85% NaCl; all from Invitrogen). General cell culture conditions were 37 °C, 5% CO2, and humidified atmosphere. After 2 weeks in culture (corresponding to > 6 cell doublings), cell cultures were mixed at an equal ratio and proteins were extracted, separated into 4 fractions (cytoplasm, nucleus, membrane and cytoskeleton, according to Qproteome fractionation kit, Qiagen) and run on SDS-PAGE. Single bands were cut from gel, reduced, alkylated and digested with trypsin as described (Shevchenko et al., 1996). The resulting peptide mixture was separated in multiple run of nanoscale C18 reverse-phase liquid chromatography (Proxeon) coupled online to an LTQ-Orbitrap mass spectrometer (Thermo). Raw MS spectra processing and protein quantification was performed using freeware MaxQuant software (Cox, NatBt 2008) and statistical analysis on overrepresented pathways was conducted using Ingenuity Pathway Analysis (Ingenuity).
**Analysis of Chimerism**

Chimerism of congenic donor cells was expressed as percentage of cells expressing the fluorescence protein transduction marker (destabilized GFP (dGFP) or OFP). Only for the peripheral blood of mice carrying dGFP LVs, the chimerism was expressed as percentage of CD45.1 or CD45.2 cells, due to the sub-optimal expression of dGFP in some of the sub-populations of interest.

**Microarray**

RNA from transduced human CB cells was extracted using Trizol (Invitrogen) and gene expression assayed on HT-12_v4 microarrays (Illumina). Quantile normalization was performed and probes were filtered by detection p-value ($\leq 0.1$) (GeneSpring GX, Agilent). Next, to remove uninformative probes, those that did not exceed a threshold of 7.8 in all replicates of any one condition were eliminated, leaving 15812 probes for analysis.

**Pathway and Network Analysis**

The gene expression data were analyzed using GSEA (Subramanian et al., 2005) with parameters set to 2000 gene-set permutations and gene-sets size between 15 and 500. Genes were ranked using the fold change (logFC) of the logarithm to base 2 of the expression values (GSEA parameter ‘Diff of classes’) between the 126KD or 126OE versus the CTRL samples. The gene-sets included in the GSEA analyses were obtained from KEGG, MsigDB-c2, NCI, Biocarta, IOB, Netpath, HumanCyc, Reactome and the Gene Ontology (GO) databases, updated September 2011 (http://baderlab.org/GeneSets). An enrichment map (version 1.2 of Enrichment
Map software (Merico et al., 2010)) was generated using enriched gene-sets with a nominal p-value <0.001, FDR<1% and the overlap coefficient set to 0.5. To identify gene-sets that may be significant due to variability in the CTRLs, GSEA analysis was repeated on 126KD vs. 126OE CTRLs (three CTRLs each, total six) and all nine possible permutations thereof. The most frequently appearing gene-sets observed in these comparisons were removed from the enrichment results (frequency greater than the median of the sum of the GSEA nominal enrichment scores (NES) for each gene-set across all permutations).

**Correlation of the miR-126 predicted targets and the miR-126 modulated pathways**

Four databases were used to create a list of miR-126 predicted targets (DIANA microT, picTar, TargetScan from the miRbase website (http://www.mirbase.org)) and miRanda from the microCosm website (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/). The list was compared to enriched gene-sets in the enrichment map and overlap was scored using Fisher’s Exact Test p-value. Overlaps with p-value smaller than 0.05 were visualized.

**Flow cytometry**

Routine flow cytometry was performed using BD LSRII and BD Canto cytometers. Data was analyzed with FlowJo software (Tree Star, Inc.) or FCS Express software (DeNovo Software). The following antibodies against human surface markers were used:

| Antigen | Conjugated | Company | Catalogue number |
|---------|------------|---------|-----------------|
| CD10    | APC        | BD      | 340923          |
| CD11b   | APC7       | BD      | 557754          |
| Antibody | Color | Vendor | Catalog Number |
|----------|-------|--------|---------------|
| CD133    | APC   | Miltenyi | 130-090-826   |
| CD133    | PE    | Miltenyi | 130-080-801   |
| CD133    | Biotin | Miltenyi | 130-090-664   |
| CD135    | PE    | BD Ph   | 558996        |
| CD14     | PC7   | Coulter | 22331         |
| CD15     | V450  | BD      | 642917        |
| CD19     | PC7   | BD      | 557835        |
| CD33     | PC5   | Coulter | 2647          |
| CD33     | APC   | BD      | 340474        |
| CD34     | PC7   | BD      | 348811        |
| CD34     | APC   | BD      | 340441        |
| CD34     | APC7  | BD      | not available |
| CD36     | PE    | BD      | 555455        |
| CD38     | PC7   | BD      | 335790        |
| CD38     | APC   | BD      | 345807        |
| CD41     | PC7   | Coulter | 6607115       |
| CD42b    | APC   | BD      | 551061        |
| CD45     | V450  | BD      | 560367        |
| CD45     | PC5   | Coulter | 2653          |
| CD45RA   | FITC  | BD      | 555488        |
| CD49f    | PC5   | BD Ph   | 551129        |
| CD7      | PC5   | Coulter | IM3613U       |
| CD71     | APC   | BD      | 551374        |
| CD90     | PE    | BD      | 555596        |
| GlyA     | PC7   | Coulter | PNA71564      |
| GlyA     | PE    | Coulter | 2211          |
| NGFR     | PE    | BD      | 557196        |
| NGFR     | Alexa647 | BD | 560326    |

The following antibodies against mouse surface markers were used:
| Antigen                      | Conjugated       | Company          | Catalogue number |
|-----------------------------|------------------|------------------|------------------|
| Lineage markers (CD11b, Ter119, CD3, B220, Gr-1) | Biotin           | BD Pharmingen    | 559971           |
| CD11b                       | APC-efluor 780   | eBioscience      | 47-0112          |
| CD11b                       | PB               | Biolegend        | 101224           |
| CD117 (c-kit)               | APC              | BD Pharmingen    | 553356           |
| CD117 (c-kit)               | APC-efluor 780   | eBioscience      | 47-1171          |
| CD117 (c-kit)               | PC7              | Biolegend        | 105814           |
| CD150                       | APC              | Biolegend        | 115910           |
| CD19                        | APC              | BD Pharmingen    | 550992           |
| CD19                        | PC7              | Biolegend        | 115519           |
| CD3                         | PB               | Biolegend        | 100214           |
| CD3                         | PE               | BD Pharmingen    | 555275           |
| CD45.1                      | APC-efluor 780   | eBioscience      | 47-0453          |
| CD45.1                      | PC7              | eBioscience      | 25-0453          |
| CD45.1                      | PB               | Biolegend        | 110721           |
| CD45.1                      | FITC             | BD Pharmingen    | 553775           |
| CD45.1                      | PE               | BD Pharmingen    | 553776           |
| CD45.2                      | PB               | Biolegend        | 109820           |
| CD45.2                      | FITC             | BD Pharmingen    | 553772           |
| CD45.2                      | PE               | Biolegend        | 109807           |
| CD48                        | PB               | Biolegend        | 103417           |
| CD48                        | Biotin           | eBioscience      | 13-0481-81       |
| Ly-6A/E                     | PC7              | BD Pharmingen    | 558162           |
| Ly-6A/E                     | PB               | Biolegend        | 122519           |
| Ly-6A/E                     | APC              | eBioscience      | 17-5981          |
| Streptavidin                | PE               | BD Pharmingen    | 554061           |
| Streptavidin                | PercP            | BD Pharmingen    | 554064           |

**In vivo nucleoside incorporation assay**

In vivo proliferation assays were performed at 3 weeks after bone marrow transplant, using 5-ethynyl-2’deoxyuridine (EdU, Invitrogen), a nucleoside analogue to thymidine used as an alternative to BrdU. EdU was dissolved in sterile 1x PBS at a concentration of 10 mg/ml. Mice
received two pulses of EdU (i.p.), 100µg each, 24 and 12 hours before sacrifice. BM was harvested and HSPCs were purified by pools of 2 or 3 mice (Lineage cell depletion kit, Miltenyi Biotec). Enriched HSPCs were processed according to manufacturer’s instructions (Click-iT® EdU Flow Cytometry Assay Kit, Invitrogen), and percentages of EdU incorporation into HSPC subsets were measured by FACS.

**Phosphoflow**

Phosphoflow cytometry was performed as published elsewhere (Krutzik et al., 2011). Briefly, CD34+ cells cultured in TSF6 (see *Viral transduction of cord blood and in vitro expansion*) were washed with fresh StemSpan medium and pelleted to eliminate conditioned medium. After 2 washes, cells were starved in cytokine-free StemSpan medium at 37°C for 3 hours. Then, cells were stimulated with TSF6 for 5, 10 or 15 minutes prior to fixation. As a negative control, cells were administered 100 nM Wortmannin (CST) 30 minutes before stimulation with TSF6. Cells were immediately fixed with paraformaldehyde (final concentration: 1.6%) for 10 minutes at room temperature. Cells were then centrifuged, washed once with PBS 1 % BSA to remove residual PFA and permeabilized with ice-cold Perm buffer III (BD Phosflow for 30 min at 4°C followed by 2 washes in order to remove traces of methanol). Antibody staining was then performed. The GFP fluorescence was augmented by antibody staining (FITC-conjugated polyclonal goat anti-GFP, Abcam ab6662). Optionally, surface staining for CD133 and CD34 was performed. Phospho-Akt Ser473 was detected by a monoclonal antibody (clone D9E; Cell Signaling) conjugated to Alexafluor 647 (#4075). Phospho-GSK3β (Ser9) was detected by a rabbit monoclonal unconjugated (clone D85E12, Cell Signaling #5558) combined with an Alexa 647 conjugated secondary antibody. Staining was performed by incubating permeabilized cells
with the phospho-specific antibody for 30 minutes on ice and at dark, diluted in PBS 1% BSA at a final concentration of 1:50. After incubation, cell were washed in PBS 1% BSA and then analyzed by flow cytometry. Since the efficiency of 126KD is directly dependent on the amount of miR-126 targets expressed per cell which varies according to the transduction level achieved by the 126KD vector, different pre-gates on GFP or OFP expression were set to stratify the cells into poorly vs. highly transduced cells. GFP/OFP gates were defined by dividing the spectrum of fluorescence marker expression achieved by each vector into quartiles and by choosing the 10% of cells with highest fluorescence marker expression. Within these pre-gates, the mean fluorescence intensity (arithmetic mean) of Phospho-Akt (Ser473) or Phospho-GSK3β (Ser9) was determined for CTRL, 126KD or 126OE vector transduced cells. As a negative control, the samples were pretreated with Wortmannin. Statistical differences between 126KD LV and CTRL LV transduced cells were revealed by a paired t-test.
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