Bone marrow niche trafficking of miR-126 controls the self-renewal of leukemia stem cells in chronic myelogenous leukemia

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Leukemia stem cells (LSCs) in individuals with chronic myelogenous leukemia (CML) (hereafter referred to as CML LSCs) are responsible for initiating and maintaining clonal hematopoiesis. These cells persist in the bone marrow (BM) despite effective inhibition of BCR–ABL kinase activity by tyrosine kinase inhibitors (TKIs). Here we show that although the microRNA (miRNA) miR-126 supported the quiescence, self-renewal and engraftment capacity of CML LSCs, miR-126 levels were lower in CML LSCs than in long-term hematopoietic stem cells (LT-HSCs) from healthy individuals. Downregulation of miR-126 levels in CML LSCs was due to phosphorylation of Sprouty-related EVH1-domain-containing 1 (SPRED1) by BCR–ABL, which led to inhibition of the RAN–exportin-5–RCC1 complex that mediates miRNA maturation. Endothelial cells (ECs) in the BM supply miR-126 to CML LSCs to support quiescence and leukemia growth, as shown using mouse models of CML in which Mir126a (encoding miR-126) was conditionally knocked out in ECs and/or LSCs. Inhibition of BCR–ABL by TKI treatment caused an undesired increase in endogenous miR-126 levels, which enhanced LSC quiescence and persistence. Mir126a knockout in LSCs and/or ECs, or treatment with a miR-126 inhibitor that targets miR-126 expression in both LSCs and ECs, enhanced the in vivo anti-leukemic effects of TKI treatment and strongly diminished LSC leukemia-initiating capacity, providing a new strategy for the elimination of LSCs in individuals with CML.

Chronic myelogenous leukemia is a clonal myeloproliferative disorder characterized at the cytogenetic level by the translocation of chromosomes 9q34 and 22q11 (ref. 1). This translocation creates a fusion gene, BCR–ABL1, that encodes a constitutively activated tyrosine kinase (BCR–ABL) responsible for transforming healthy (hereafter referred to as ‘normal’) HSCs into LSCs. LSCs are characterized by growth-factor-independent proliferation and enhanced survival, which results in uncontrolled myeloproliferation that eventually evolves into fatal blast crisis if left untreated. CML LSCs are at the apex of malignant clonal hematopoiesis and initiate and maintain the growth of leukemia cells. In CML, LSC activity is restricted to the LT-HSC-enriched Lin−CD34+CD38−CD90+ cell population in humans and to the Lin−Sca-1−c-Kit−Flt3−CD150+CD48− cell population in mice2. CML LSCs are thought to reside in a leukemia niche that may be anatomically and functionally different from that of normal HSCs.

Currently, oral TKIs are used as the first-line treatment to induce long-term disease remission in patients with CML. Although most of the patients treated with TKI monotherapy achieve major clinical and molecular responses, cells from the original BCR–ABL1 clone frequently persist, likely due to the failure of these agents to eliminate CML LSCs3, and the discontinuation of treatment frequently results in disease relapse. Thus, the identification of mechanisms that support CML LSC persistence is clinically relevant, as it may enable the design of novel therapeutic strategies.
of new targeting strategies aimed at complete disease elimination, allowing for discontinuation of life-long TKI therapy.

miR-126-3p (hereafter referred to as miR-126) is highly expressed in normal HSCs and hematopoietic progenitor cells (HPCs) and restrains cell-cycle progression during hematopoiesis. We and others have shown that increased miR-126 levels are associated with an increased frequency of quiescent LSCs and a worse outcome in individuals with acute myeloid leukemia (AML)5–8. Here we show that miR-126 biogenesis in CML LSCs is downregulated through a BCR–ABL-dependent mechanism, a finding that is seemingly inconsistent with a pro-leukemic role for miR-126. However, miR-126 is also highly expressed in ECs9. Anatomical and functional connections between the endothelium and normal HSCs regulate normal hematopoiesis. We hypothesized that miR-126 may mediate a functional interplay between ECs and LSCs in the leukemia BM niche that regulates CML progression. Consistent with this hypothesis, we found that ECs supply miR-126 to CML LSCs to modulate their quiescence and self-renewal.

RESULTS

Higher miR-126 levels are associated with human and mouse CML LSCs

miR-126 has been shown to contribute to leukemogenesis in individuals with acute leukemia6,8,11. To evaluate miR-126 expression in subpopulations of cells from individuals with CML, we sorted immunophenotypically defined subsets of HPCs (Lin−CD34+(CD34+)) and Lin−CD34+CD38+ (CD38+), HSCs (Lin−CD34+CD38− (CD38−) and Lin−CD34+CD38−CD90+ (CD90+)) and LT-HSCs (Lin−CD34+CD38−CD90− (CD90−)) from peripheral blood (PB) and BM samples of healthy donors (n = 12) and newly diagnosed patients with chronic-phase (CP) CML (n = 12). LT-HSCs from both healthy donors and individuals with CML showed the highest expression of miR-126 (Fig. 1a,b). Similar results were obtained in wild-type (WT) B6 and inducible SCL/TAxBCR–ABL transgenic B6 mice, a well-established mouse model of CML12 (hereafter referred to as CML mice). We isolated Lin−Sca-1−c-Kit− (L−S−K−), Lin−Sca-1−c-Kit+(L−S−K+) (which includes common myeloid progenitor (CMP), granulocyte–macrophage progenitor (GMP) and megakaryocyte–erythrocyte progenitor (MEP) cells), Lin−Sca-1+c-Kit+ (LSK) and LSK Flt3−CD150−CD48− (LT-HSC) cells from the BM of WT mice and CML mice after induction of BCR–ABL expression by tetracycline withdrawal (Supplementary Fig. 1a). As in the human samples, LT-HSCs from healthy and CML mice showed the highest expression of miR-126 (Fig. 1c,d).

To test the effects of miR-126 on the quiescence of LSCs from subjects with CML, we knocked down MIR126 or Mir126a expression in human CML Lin−CD34+CD38− cells (HSCs) and mouse CML LT-HSCs, respectively, using GFP-expressing miRZip lentiviral vectors that also encode a miR126-specific (for human HSCs) or Mir126a-specific (for mouse LT-HSCs) short hairpin RNA (shMIR126 or shMir126a, respectively; hereafter collectively referred to as miR-126 KD) or a miR-126-encoding precursor (for overexpression; hereafter referred to as miR-126 OE). After transduction, GFP+ cells were selected and cultured for 72 h. miR-126 KD increased cell cycling and apoptosis, decreased the number of colony-forming cells (CFCs) and decreased the CFC-replicating efficiency of both human CML HSCs (Fig. 1e–i) and mouse CML LT-HSCs (Fig. 1j–m); conversely, miR-126 OE decreased cell cycling and apoptosis and increased CFC-replicating efficiency. We validated these results in vivo, first by showing that the quiescent Hoechst−Pyronin− (G0) fraction of CML LT-HSCs from induced SCL/TAxBCR–ABL mice (CD45.2) expressed significantly (P = 0.0019) higher miR-126 levels than the proliferating Hoechst+Pyronin+ and Hoechst+Pyronin+ (G1-, G2-, S- and M-phase) fractions of CML LT-HSCs (Fig. 1n). The association of miR-126 with LSC activity was then demonstrated by showing that quiescent CML LT-HSCs had a significantly higher rate of long-term engraftment and leukemogenic capacity than did proliferating CML LT-HSCs after transplantation into CD45.1 congenic recipient mice (Fig. 1o,p).

BCR–ABL downregulates miR-126 expression in CML cells

Although miR-126 has similar patterns of expression and function in CML as in normal hematopoiesis, we noted that human CML Lin−CD34+CD38− (HSCs) and Lin−CD34+CD38−CD90− (LT-HSCs) cells had significantly lower miR-126 levels than their normal counterparts (Fig. 2a,b); similar differences were also observed in mouse samples (Fig. 2c). Consistent with this finding, CML HSCs and LT-HSCs expressed higher levels of phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2) and SPRED1, two validated targets of miR-126 (refs. 4,7,9,13,14), as compared to their normal counterparts (Supplementary Fig. 1b,c).

This differential expression of miR-126 observed led us to postulate that BCR–ABL itself might be involved in lowering miR-126 levels in CML cells. To test this hypothesis, we transduced LSK cells from the BM of healthy mice with retroviral BCR–ABL–expressing or control vectors, selected GFP+ cells and cultured them for 72 h. We found that induction of BCR–ABL expression (Fig. 2d) resulted in decreased miR-126 expression (Fig. 2e) and increased PIK3R2 and SPRED1 expression, as compared to the control cells (Supplementary Fig. 1d,e). This was associated with an increase in cell cycling (Fig. 2f) and cell growth (Fig. 2g). To further validate this finding, we sorted LT-HSCs from non-induced BCR–ABL–transgenic mice and cultured them with or without tetracycline to repress or induce BCR–ABL expression, respectively. After tetracycline withdrawal and induction of BCR–ABL expression (Fig. 2h), we observed reduced miR-126 levels (Fig. 2i) as well as increased cell cycling (Fig. 2j) and cell growth (Fig. 2k), as compared to non-induced controls. Conversely, inhibition of BCR–ABL activity by treatment with nilotinib (NIL), a first-line TKI for CML treatment, led to increased miR-126 expression (Fig. 2l–n), decreased PIK3R2 and SPRED1 levels (Supplementary Fig. 1f,g) and an increased fraction of quiescent cells in human CML HSCs (Fig. 2o) but not in normal HSCs (Supplementary Fig. 1h), as compared to cells treated with vehicle alone. NIL treatment also resulted in increased miR-126 expression in human BCR–ABL+ K562 cells (Supplementary Fig. 1i).

BCR–ABL deregulates miR-126 biogenesis

Whereas CML cells harbored lower levels of mature miR-126 than their normal counterparts, we noted that BCR–ABL+ cells had higher levels of primary (pri-) and precursor (pre-) miR-126 than their normal counterparts (Fig. 3a,b). This result led us to hypothesize that BCR–ABL might interfere with miR-126 biogenesis. Nucleus-to-cytoplasm shuttling and maturation of miRNAs are mediated by a protein complex comprising the GTP-binding RAS-related nuclear protein RAN, exportin-5 (XPO5; also known as EXP5) and regulator of chromosome condensation 1 (RCC1)15. After activation via tyrosine phosphorylation, SPRED1 functions as a negative regulator of RAS superfamily proteins16. We therefore postulated that the BCR–ABL tyrosine kinase can phosphorylate SPRED1, allowing SPRED1 to bind RAN, and that this binding interferes with RAN-, EXP5- and RCC1-mediated shuttling and maturation of miR-126.
Using immunofluorescence (IF), immunoprecipitation (IP) and in vitro kinase activity assays to analyze BCR–ABL+ primary CD34+ cells and/or K562 cells, we showed that SPRED1 colocalized with BCR–ABL to the cytoplasm (Fig. 3c), was directly phosphorylated by BCR–ABL (Fig. 3d), and formed an intranuclear or perinuclear protein complex with RAN (Fig. 3e,f). NIL treatment reversed these effects, resulting in SPRED1 dephosphorylation (Fig. 3d, left), decreased binding and colocalization of SPRED1 with RAN (Fig. 3f), increased formation of the RAN–EXP5–RCC1 complex (Fig. 3g,h), decreased pri- and pre-miR-126 levels, and increased mature miR-126 levels (Fig. 3i). In cells that were washed to remove NIL, the binding of SPRED1 with RAN was restored, the binding of RAN with EXP5–RCC1 was decreased (Fig. 3i), pri-miR-126 levels were increased (Fig. 3k) and mature miR-126 levels were reduced (Fig. 3l). Northern blot analysis confirmed that the ratio of pri- and pre-miR-126 levels to mature miR-126 levels decreased after exposure of BCR–ABL+ cells to NIL and increased after washing off NIL (Fig. 3m). SPRED1 knockdown (KD) by short interfering RNA (siRNA) in BCR–ABL+ primary CD34+ and K562 cells enhanced formation of the RAN–EXP5–RCC1 complex (Fig. 3n) and resulted in decreased pri- and pre-miR-126 levels (Fig. 3o) and increased mature miR-126 levels (Fig. 3p). Conversely, RAN KD by siRNA resulted in increased pri- and pre-miR-126 levels and reduced mature miR-126 levels (Fig. 3q–s). These results indicated that, in CML cells, BCR–ABL-induced SPRED1 phosphorylation interfered with RAN–EXP5–RCC1-mediated miR-126 biogenesis and lowered mature miR-126 levels. Given that miR-126 suppresses SPRED1 expression, this BCR–ABL-mediated reduction in miR-126 biogenesis may cause a further increase in SPRED1 levels, such that higher SPRED1 levels result in lower mature miR-126 levels (Supplementary Fig. 1) and that the endogenous levels of miR-126 are therefore controlled by its own target (SPRED1) in BCR–ABL+ cells.

Of note, BCR–ABL-dependent deregulation of miRNAs is unlikely to be restricted to downregulation of miR-126. In fact, by comparing
miR-126 is one of the most abundantly expressed miRNAs in ECs and is involved in angiogenesis. Consistent with this, we found that, in both normal and CML mice, BM ECs (CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>+</sup>) expressed the highest levels of miR-126 as compared to LT-HSCs and other BM stromal cell populations, including osteoblasts (OBs; CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>-</sup>CD166<sup>+</sup>Sca-1<sup>-</sup>) and mesenchymal stem cells (MSCs; CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>-</sup>CD166<sup>-</sup>Sca-1<sup>-</sup>)<sup>20</sup> (Fig. 4a,b). We therefore hypothesized that ECs supply miR-126 to CML LT-HSCs.

To test this hypothesis, we sorted ECs from the endosteal and central marrow of SCLtTA×BCR–ABL mice and transduced these cells with lentiviral vectors expressing GFP and either shMir126a or a control shRNA (shControl) (Fig. 4c). LT-HSCs from induced SCLtTA×BCR–ABL mice were then cocultured with GFP<sup>+</sup>shControl- or shMir126a-expressing ECs or were cultured without ECs (Fig. 4d). We found that, in both normal and CML mice, BM ECs (CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>+</sup>) expressed the highest levels of miR-126 as compared to LT-HSCs and other BM stromal cell populations, including osteoblasts (OBs; CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>-</sup>CD166<sup>+</sup>Sca-1<sup>-</sup>) and mesenchymal stem cells (MSCs; CD45<sup>-</sup>Ter119<sup>+</sup>CD31<sup>-</sup>CD166<sup>-</sup>Sca-1<sup>-</sup>)<sup>20</sup> (Fig. 4a,b). We therefore hypothesized that ECs supply miR-126 to CML LT-HSCs.

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To test the functional role of EC-supplied miR-126 in leukemia cell growth, we generated CML or normal mice carrying loxP-flanked (floxed) Mir126a alleles (Mir126a<sup>lox/lox</sup>) to enable conditional knockout (c-KO) of Mir126a expression in LT-HSCs, ECs or both. To generate these mice, we crossed Mir126a<sup>lox/lox</sup> with Mx1–Cre (in which the Cre recombinase is expressed in hematopoietic cells) or Tie2–Cre (in which Cre is expressed in ECs) mice, following by crossing with SCLITAXB–ABL transgenic mice. These crosses led to the generation of the following strains: Mir126a<sup>lox/lox</sup>;Mx1–Cre mice, SCLITAXB–ABL:Mir126a<sup>lox/lox</sup>;Mx1–Cre mice, Mir126a<sup>lox/lox</sup>WT; Tie2–Cre mice and Mir126a<sup>lox/lox</sup>;Tie2–Cre mice. After injecting Mir126a<sup>lox/lox</sup>;Mx1–Cre (hereafter referred to as Mx1<sup>+</sup>) mice with polyinosine–polycytosine (p,pC) to delete Mir126a in normal HSCs, we observed no significant changes in WBC counts in PB (data not shown) or BM mononuclear cell subpopulations (including LT-HSCs) (Fig. 5a,b) after 16 weeks of follow-up, as compared to control (Mir126a<sup>lox/lox</sup> (no expression of Mx1–Cre); hereafter referred to as Mx1<sup>−</sup>) mice. Using SCLITAXB–ABL Mx1<sup>−</sup> mice, we were able to target Mir126a deletion in CML LSCs by withdrawing tetracycline to induce BCR–ABL expression and injecting p,pC to induce Cre expression. In these mice, miR-126 levels in BM cells were reduced by 60%, CML development was delayed (P = 0.047), and survival was increased (P = 0.04), as compared to similarly treated SCLITAXB–ABL Mx1<sup>+</sup> control mice (Fig. 5c–e). To confirm that these results are not attributable to 'leaky' miR-126 downregulation in the non-hematopoietic compartment, we sorted CD45.2 CML LT-HSCs from BCR–ABL-induced and p,pC-injected SCLITAXB–ABL Mx1<sup>+</sup> or Mx1<sup>−</sup> mice and transplanted these cells into CD45.1 congenic recipient mice (Fig. 5f). Recipients transplanted with Mx1<sup>+</sup> (Mir126a-KO) CML LT-HSCs showed a trend for decreased CML development and increased survival, as compared to recipient mice transplanted with Mx1<sup>−</sup> control CML LT-HSCs (Fig. 5g–j).

To assess the contribution of EC-derived miR-126 in leukemia, we sorted LT-HSCs from BCR–ABL-induced CD45.1/CD45.2 SCLITAXB–ABL mice (used to track donor cells, and generated by crossing CD45.2 SCLITAXB–ABL B6 mice with CD45.1 B6 mice)
and transplanted these cells into CD45.2 congenic Mir126a$_{\text{flox/flox}}$, Tie2–Cre$^-$ (hereafter referred to as ‘het Tie2$^-$’ mice; homozygous expression of Mir126a in ECs), Mir126a$_{\text{flox/flox}}$, Tie2–Cre$^-$ (hereafter referred to as ‘het Tie2$^-$’ mice; heterozygous expression of Mir126a in ECs), or Mir126a$_{\text{flox/flox}}$, Tie2–Cre$^-$ (hereafter referred to as ‘hom Tie2$^+$’ mice; no expression of Mir126a in ECs) recipient mice (Fig 5k). Both het and hom Tie2$^+$ recipient mice showed reduced CML cell engraftment, delayed CML development and significantly increased survival, as compared to Tie2$^-$ recipients at 16 weeks after transplantation ($P = 0.009$ and $P = 0.0003$ for survival of het Tie2$^+$ and hom Tie2$^+$ mice, respectively; Fig 5l-o). A miR-126 dosage effect was evident, as 70% of hom Tie2$^+$ mice versus 10% of het Tie2$^+$ mice were alive at 28 weeks after transplantation (Fig 5l-o). Of note, no significant differences in donor cell output from transplanted normal
Endothelial cells in the niche supply miR-126 to normal and CML LT-HSCs. (a) Gating strategy for the isolation of ECs (CD45−Ter119−CD31+), osteoblasts (OBs; CD45−Ter119−CD31−CD166+Sca-1−) and mesenchymal stem cells (MSCs; CD45−Ter119−CD31−CD166−Sca-1+). These experiments were repeated five times independently, with similar results. (b) miR-126 expression, as assessed by qPCR, in LT-HSCs, OBs and MSCs from normal and CML mice (n = 5 mice per group). (c) miR-126 expression in ECs from the BM of CML mice that were transduced with lentiviruses expressing anti-miR-126 (126 KD) or a control (Ctrl) or in CML LT-HSCs that were cultured alone (none) or cocultured with the ECs that had been transduced with lentiviruses expressing a control (Ctrl EC) or anti-miR-126 (KD EC) (n = 4 biologically independent samples per group). (d-g) Cell cycle (d), apoptosis (e) and cell growth (f) analysis, and the percentage of Kit+CD150−CD48−LSK cells (*P < 0.001, n.s. n.s., * by two-tailed, paired Student’s t-test. (h) miR-126 expression, as assessed by qPCR, in suspended (Susp) and EC-attached (Att) subfractions of CML LT-HSCs (n = 6 independent experiments). (i-k) WBC counts (i), CML donor cell engraftment in PB at the indicated times (j) and survival (k) after recipient mice were transplanted with CML LT-HSCs that had been cultured alone (none) or cocultured with Ctrl or KD ECs for 96 h (1,000 cells/mouse; n = 8 mice per group). The log-rank test was used to assess significant differences between survival curves. (l,m) Representative flow cytometry plots of EC staining (l) and frequency of Sca-1− cells (m) in endosteal or central ECs from normal and CML mice (n = 4 independent samples per group). (n) miR-126 expression, as assessed by qPCR, in endosteal or central ECs (n = 4 independent samples) and in Sca-1− or Sca-1+ ECs (n = 4 independent samples) from normal and CML mice. (p) miR-126 expression, as assessed by qPCR, in endosteal and central LT-HSCs from normal and CML mice (n = 6 independent samples). (q-s) Representative flow cytometry plots of the proliferation marker Ki67 and DAPI staining of endosteal or central LT-HSCs from normal and CML mice (q) and cell cycle analysis of endosteal or central LT-HSCs from normal (r) or CML (s) mice (n = 3 mice per group). Throughout, results shown represent mean ± s.e.m. *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant; by two-tailed, paired Student’s t-test.

LT-HSCs (CD45.1) were observed in the PB and BM from CD45.2 het Tie2+ recipients relative to that from het Tie2+ recipients after 16 weeks of follow-up (data not shown).

Next, to assess the functional effect of concurrent Mir126a KO in both LT-HSCs and ECs, CML LT-HSCs from BCR–ABL-induced and plpC-injected SclTα×BCR–ABL Mx1+ or Mx1− mice were transplanted into het Tie2+ or het Tie2− recipient mice, respectively (Fig. 5p). Het Tie2+ mice transplanted with Mx1+ CML LT-HSCs showed significantly delayed CML development (P = 0.007) and prolonged survival (P = 0.002) as compared with het Tie2− mice transplanted with Mx1− CML LT-HSCs (Fig. 5q,r). At day 200 after transplantation, 83% of the mice with Mir126a KO in both ECs and

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**Figure 4** Endothelial cells in the niche supply miR-126 to normal and CML LT-HSCs. (a) Gating strategy for the isolation of ECs (CD45−Ter119−CD31+), osteoblasts (OBs; CD45−Ter119−CD31−CD166+Sca-1−) and mesenchymal stem cells (MSCs; CD45−Ter119−CD31−CD166−Sca-1+). These experiments were repeated five times independently, with similar results. (b) miR-126 expression, as assessed by qPCR, in LT-HSCs, OBs and MSCs from normal and CML mice (n = 5 mice per group). (c) miR-126 expression in ECs from the BM of CML mice that were transduced with lentiviruses expressing anti-miR-126 (126 KD) or a control (Ctrl) or in CML LT-HSCs that were cultured alone (none) or cocultured with the ECs that had been transduced with lentiviruses expressing a control (Ctrl EC) or anti-miR-126 (KD EC) (n = 4 biologically independent samples per group). (d-g) Cell cycle (d), apoptosis (e) and cell growth (f) analysis, and the percentage of Kit+CD150−CD48−LSK cells (n = 4) (g), in CML LT-HSCs that were cultured alone or with control or KD ECs (n = 4 biologically independent samples per group). (h) miR-126 expression, as assessed by qPCR, in suspended (Susp) and EC-attached (Att) subfractions of CML LT-HSCs (n = 6 independent experiments). (i-k) WBC counts (i), CML donor cell engraftment in PB at the indicated times (j) and survival (k) after recipient mice were transplanted with CML LT-HSCs that had been cultured alone (none) or cocultured with Ctrl or KD ECs for 96 h (1,000 cells/mouse; n = 8 mice per group). The log-rank test was used to assess significant differences between survival curves. (l,m) Representative flow cytometry plots of EC staining (l) and frequency of Sca-1− cells (m) in endosteal or central ECs from normal and CML mice (n = 4 independent samples per group). (n) miR-126 expression, as assessed by qPCR, in endosteal or central ECs (n = 4 independent samples) and in Sca-1− or Sca-1+ ECs (n = 4 independent samples) from normal and CML mice. (p) miR-126 expression, as assessed by qPCR, in endosteal and central LT-HSCs from normal and CML mice (n = 6 independent samples). (q-s) Representative flow cytometry plots of the proliferation marker Ki67 and DAPI staining of endosteal or central LT-HSCs from normal and CML mice (q) and cell cycle analysis of endosteal or central LT-HSCs from normal (r) or CML (s) mice (n = 3 mice per group). Throughout, results shown represent mean ± s.e.m. *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant; by two-tailed, paired Student’s t-test.
LT-HSCs, but none of the controls with intact mir126a in both ECs and LT-HSCs, were alive. Taken together, these results support a functional role of EC-derived miR-126 in sustaining leukemia in the CML LSC niche.

In assessing the relevance of these results to humans, we first showed that human umbilical vein ECs (HUVECs) expressed significantly higher levels of miR-126 than human CML CD34+ subpopulations (P < 0.0001; Supplementary Fig. 2a). We next transduced the HUVECs with a lentiviral vector encoding shMIR126 (to knock down miR-126 expression) or shControl (Supplementary Fig. 2b,c) and then cocultured CML Lin–CD34+CD38– cells (HSCs) with the shMIR126-expressing or control HUVECs for 96 h. CML HSCs cocultured for 96 h with control HUVECs had significantly higher miR-126 expression, as assessed by qPCR, in donor CML LT-HSCs (n = 3 independent samples) (d), WBC counts (e) and survival (f) of these mice subjected to tetracycline withdrawal and plpC injection (n = 9 mice per group). (f) Experimental design. CD45.2 CML LT-HSCs (400 cells/mouse) from BCR–ABL–induced and plpC-injected SCLTAxBCR–ABL Mx1+ or Mx1− mice were transplanted into CD45.1 congenic recipient mice (n = 10 mice per group). (g–j) miR-126 expression, as assessed by qPCR, in donor CML LT-HSCs (n = 3 independent samples) (g), WBC counts (h), CML cell engraftment in PB (i) and survival (j) of recipient mice from f (n = 10 mice per group). (k) Experimental design. CD45.1/CD45.2 CML LT-HSCs (400 cells/mouse) from induced CML mice were transplanted into CD45.2 congenic Mx1+ (n = 14), Mx1− (n = 10) and hom Tie2+ (n = 8) recipient mice. (i–o) miR-126 expression, as assessed by qPCR, in ECs sorted from the recipient mice from k (n = 3 independent samples) (I), WBC counts (m), CML cell engraftment in PB (n) and survival (o) of recipient mice from k. (p) Experimental design. CD45.2 CML LT-HSCs (400 cells/mouse) from BCR–ABL–induced and plpC-injected SCLTAxBCR–ABL Mx1+ or Mx1− mice were transplanted into CD45.2 het Tie2+ or het Tie2– recipient mice (n = 12 per group), respectively. (q,r) WBC counts (q) and survival (r) of the recipient mice from p. Throughout, results shown represent mean ± s.e.m. *P ≤ 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001; n.s., not significant; by a two-tailed, unpaired Student's t-test (a–d,g–i,m,n,q) or the log-rank test (e,j,r).
shMIR126-expressing HUVECs, or cultured alone. Next, we either cocultured cocultured with EVs derived from shMIR126-expressing HUVECs derived EVs showed significantly increased miR-126 levels (P < 0.001; Supplementary Fig. 3o) and CD45*CD34+ (P = 0.0008; Supplementary Fig. 3p) BM engraftment after 16 weeks, as compared mice that received cells that were cocultured with EVs derived from shMIR126-expressing HUVECs. qPCR analysis confirmed that the engrafted cells in both groups of mice were BCR–ABL (data not shown). We also sorted CML LT-HSCs from induced CML LT-HSCs and cocultured them with EVs derived from control or shMIR126-expressing HUVECs for 96 h and then transplanted the cells into CD45.1 recipient mice. LT-HSCs that were cocultured with control-HUVEC-derived EVs showed increased miR-126 expression (P = 0.009; Supplementary Fig. 3q), enhanced CML progression (P = 0.002), an increased engraftment rate (P = 0.06) and reduced survival (P = 0.04), as compared to LT-HSCs cocultured with EVs derived from shMIR126-expressing HUVECs (Supplementary Fig. 3r–t). Taken together, these observations suggested that EV-mediated trafficking was responsible for the transfer of miR-126 from ECs to LT-HSCs.

Although we could not completely exclude the possibility that decreased CML LT-HSC quiescence and engraftment capacity might have resulted from functional changes in ECs induced by Mil126a KO, rather than from a decrease in miR-126 trafficking from ECs to LT-HSCs, Kuo et al. previously showed that Mil126a-KO mice have no substantial changes in BM EC structure9. Moreover, we did not observe detectable changes in the morphology and growth rate of shMIR126-expressing HUVECs or Mil126a-KD mouse ECs as compared to controls (data not shown).

Knockdown of miR-126 expression enhances TKI-mediated targeting of CML LSCs

Given that BCR–ABL activity reduces endogenous levels of miR-126 in LT-HSCs and that pharmacologic inhibition of BCR–ABL by NIL treatment increased miR-126 levels and the frequency of quiescent LT-HSCs (Fig. 2d–o), we postulated that miR-126 downregulation may enhance the anti-leukemic activity of TKI treatment and eliminate CML LSCs. To test this hypothesis, we subjected human CML HSCs to MIR126 KD or OE by transduction of GFP-expressing lentiviruses (Supplementary Fig. 4a). We selected GFP+ cells and cultured them for 96 h in the presence of NIL. Combined MIR126 KD and NIL treatment resulted in increased cell cycling and apoptosis (Supplementary Fig. 4b,c) and decreased cell growth, CFC and CFC-replating efficiency (Supplementary Fig. 4d–f), as compared to only NIL-treated control cells. Conversely, combined MIR126 OE and NIL treatment resulted in decreased cell cycling and apoptosis and increased CFC and CFC-replating efficiency (Supplementary Fig. 4b,c,e,f) relative to only NIL-treated control cells. We obtained similar results for CML LT-HSCs from SCLiTAXBCR–ABL mice (Supplementary Fig. 4g–k).

To further test the concept that knockdown of miR-126 expression can enhance the anti-leukemic activity of TKI treatment, we transduced human CML HSCs with lentiviral vectors encoding shMIR126 or shControl; we selected GFP+ cells and treated them with NIL for 96 h and then transplanted them into irradiated NSG-SGM3 mice. Recipient mice that received NIL-treated shMIR126-expressing cells showed reduced engraftment of human CD45+ cells in PB at 4 weeks after transplantation (Fig. 6a) and in BM at 16 weeks after transplantation (Fig. 6b and Supplementary Fig. 5a), as compared to the
controls. BCR–ABL1 levels were reduced in BM cells from NSG-SGM3 mice that were transplanted with shMIR126-expressing CML cells as compared to mice that were transplanted with control CML cells (Supplementary Fig. 5b).

To test whether miR-126 downregulation in ECs also enhanced the sensitivity of CML HSCs to TKI treatment, we cultured human CML HSCs alone or with HUVECs in which MIR126 expression was knocked down or not, and with or without NIL treatment, for 72 h. CML HSCs cocultured with shMIR126-expressing HUVECs had significantly increased apoptosis and decreased cell growth and CFCs, as compared to CML HSCs cocultured with control HUVECs with or without NIL treatment (Supplementary Fig. 5c–e). Of note, CML HSCs cocultured with EVs derived from shMIR126-expressing HUVECs also showed increased apoptosis and decreased cell growth as compared to CML HSCs cocultured with control HUVEC-derived EVs, with or without NIL treatment (Supplementary Fig. 5f). Next we sorted CML LT-HSCs from induced CD45.1/CD45.2 SCLtTA×BCR–ABL mice and transplanted them into CD45.2 het Tie2+ (heterozygous Mir126a-KO allele in ECs) or Tie2− (WT Mir126a allele in ECs) mice. After confirming CML development, we treated the mice with NIL (50 mg per kg body weight (mg/kg), daily by oral gavage) or vehicle for 3 weeks. Vehicle-treated het Tie2+ mice showed delayed CML development (P = 0.02) and increased survival (P = 0.001) as compared to vehicle-treated Tie2− mice (Fig. 6c–e). Moreover, NIL-treated het Tie2+ mice showed significantly reduced WBC counts in PB (P = 0.03) and increased survival (P = 0.02) as compared to NIL-treated het Tie2− mice (Fig. 6c–e), and all of the NIL-treated het Tie2− mice were alive at day 150 after transplantation (Fig. 6e). Taken together, these results support the hypothesis that miR-126 downregulation in ECs and CML HSCs enhances the anti-leukemic activity of TKI treatment.
To sort out the mechanistic basis by which knock down of miR-126 expression enhances TKI activity, we showed that, consistent with previous studies23,24, NIL treatment enhanced activation of the MAPK–ERK signaling pathway and increased expression of the apoptosis regulator BCL-2, thereby promoting survival in CML CD34+ cells (Supplementary Fig. 5h). Thus, we reasoned that NIL treatment might enhance MAPK–ERK activation through upregulation of miR-126 and consequent downregulation of SPRED1 (Supplementary Fig. 5i), a reported inhibitor of the MAPK–ERK pathway. Indeed, knockdown of miR-126 expression in CML CD34+ cells resulted in SPRED1 upregulation and decreased phospho-ERK (p-ERK) and BCL-2 levels (Supplementary Fig. 5j,k), whereas SPRED1 KD increased p-ERK and BCL-2 levels and rescued NIL-induced apoptosis (Supplementary Fig. 5l–o). Moreover, NIL-induced apoptosis was enhanced by treatment with the MAPK pathway inhibitor PD0325901 or by BCL2 KD (Supplementary Fig. 5p–s). These results support a model in which NIL-induced upregulation of miR-126 expression decreases SPRED1 expression, which results in spurious activation of the MAPK–ERK pathway and ultimately in an increase in BCL-2 levels. Knockdown of miR-126 expression counteracted these effects, thereby increasing the anti-leukemic activity of NIL.

Effective in vitro and in vivo uptake and gene silencing effects of the CpG–miR-126 inhibitor

In view of the enhancing effects of miR-126 KD on the anti-leukemic activity of NIL, we reasoned that miR-126 could represent a therapeutic target for eliminating LSC. Although miRNAs can be targeted with oligonucleotide therapeutics (ONTs), it remains challenging to achieve efficient and cell-selective delivery of ONTs in vivo. Thus, we designed a novel miR-126 inhibitor by linking a Mir126a-specific antisense oligodeoxynucleotide (ODN) to a cytosine–guanine dinucleotide ( CpG) ODN, a ligand for the intracellular protein Toll-like receptor 9 (TLR9). To allow for systemic administration, we chemically modified the CpG–miR-126 inhibitor (CpG–miR-126i) to resist serum nuclease by using phosphothioation and 2′ O-methyl-modified nucleotides in the CpG ODN and anti-miR-126 moieties, respectively. We compared the specificity and efficiency of CpG–mir126i uptake with a nanoparticle (NP) delivery method previously reported by our group6. We incubated K562 cells with fluorescently labeled CpG–miR-126i–Cy3 (CpG), human CD45–specific antibody (Ab)- or transferrin (TF)-conjugated NPs containing miR-126i–Cy3 (Ab-NP or TF-NP), or naked miR-126i–Cy3 (control), in the absence of any reagents routinely used for nucleic acid transfection. Flow cytometric analysis at 4 h and 24 h after treatment (Supplementary Fig. 6a,b) showed that CpG–mir126i–Cy3 was taken up by 99% of the K562 cells at both 4 h and 24 h, as compared to 24% and 30% of the cells incubated with Ab-NP and 74% and 88% of the cells incubated with TF-NP at 4 h and 24 h, respectively. K562 cells did not take up naked miR-126i (control) (Supplementary Fig. 6b). Efficient miR-126 downregulation by CpG–miR-126i–Cy3 in K562 cells was shown at 24 h (Supplementary Fig. 6c). We further showed by flow cytometry that, even without routinely used transfection reagents, the CpG–miR-126i–Cy3 was internalized by HUVECs, as well as by human normal and CML Lin−CD34+CD38− cells at 4 h (Supplementary Fig. 6d–f); >95% cells were positive for CpG–miR-126i–Cy3 uptake in all three cell types. CpG–miR-126i–Cy3 uptake led to efficient miR-126 downregulation in HUVECs and HSCs (Supplementary Fig. 6g–i). We also observed increased cell cycling in both CpG–miR-126i-treated normal and CML HSCs, as compared to CpG–scrambled RNA (scrRNA)-treated controls (Supplementary Fig. 6j,k).

Next we evaluated CpG–miR-126i–Cy3 uptake in mouse LT-HSCs and ECs in vitro and in vivo. Following in vitro exposure to CpG–miR-126i–Cy3, efficient uptake at 4 h and Mir126a downregulation at 24 h were shown by flow cytometry and qPCR, respectively (Supplementary Fig. 6l,m). We also observed increased cell cycling in normal and CML BM LT-HSCs that were treated with CpG–miR-126i (Supplementary Fig. 6n). We treated normal and CML mice with one dose (5 mg/kg, by intravenous (i.v.) injection) of CpG–miR-126i–Cy3. At 16 h after treatment, efficient in vivo uptake was demonstrated by flow cytometry in both LT-HSCs (56 ± 5%) and ECs (62 ± 3%) isolated from femurs (Supplementary Fig. 6o). After CpG–miR-126i treatment (5 mg/kg/d, i.v., daily) for 3 d, we sorted LT-HSCs and ECs and found substantial downregulation of miR-126 expression (Supplementary Fig. 6p,q).

CpG–miR-126i enhances in vivo targeting of CML LSCs in combination with TKI treatment

We next tested the effects of CpG–miR-126i in healthy mice to ensure that the compound did not have hematologic toxicity. We treated WT B6 mice with CpG–scrRNA or CpG–miR-126i (inhibitor; 5 mg/kg/d, i.v.) for 3 weeks, after which we collected their BM cells and transplanted them into recipient mice (3 × 10^5 BM cells/mouse). As compared with CpG–scrRNA-treated control mice, inhibitor-treated mice showed increased numbers of red blood cells (RBCs) but no significant differences in the numbers of WBCs or platelets (PLTs) in the PB (Supplementary Fig. 7a–c) or in the numbers of mononuclear cells, LT-HSCs or ECs in the BM (Supplementary Fig. 7d–f). These findings are consistent with the observation that miR-126 downregulation in normal HSCs increases hematopoietic output1. In the recipient mice that received BM cells from donor mice treated with CpG–scrRNA or inhibitor, we observed no significant differences in donor cell engraftment in the PB, BM or spleen (Supplementary Fig. 7g,h), or in donor LT-HSC numbers in the BM (Supplementary Fig. 7i), at 16 weeks after transplantation. These data demonstrated that the inhibitor lacked preclinical toxicity for normal hematopoiesis.

We then tested the effects of the inhibitor alone or in combination with NIL on human and mouse CML LT-HSCs in vivo. First, we transplanted human CD34+ cells from patients with chronic-phase CML into NSG-SGM3 mice. At 6 weeks after transplantation, the mice were divided into four groups and treated with CpG–scrRNA (5 mg/kg, i.v., four times a week), inhibitor (5 mg/kg, i.v., four times a week), CpG–scrRNA + NIL (50 mg/kg, daily by oral gavage) or inhibitor + NIL for 3 weeks, followed by assessment of human cell engraftment in PB, BM and spleen. We observed significantly reduced human CD45+ cell, CD45+CD34+CD38− HSC and CD45+CD34+CD38−CD90+ LT-HSC engraftment in the BM of mice that were treated with inhibitor + NIL, as compared to mice that were treated with CpG–scrRNA alone, inhibitor alone or scrRNA + NIL (Fig. 6f–h and Supplementary Fig. 7j). qPCR analyses confirmed that the engrafted human CD45+ cells were BCR–ABL1 positive (Supplementary Fig. 7k).

Next we transplanted BM cells from SCLtTA×BCR–ABL mice (CD45.2) into congenic B6 mice (CD45.1). After confirming of CML development at 4 weeks after transplantation, mice were treated as described above with CpG–scrRNA, inhibitor, CpG–scrRNA + NIL or inhibitor + NIL for 3 weeks. Because EC-derived miR-126 has a key role in LSC maintenance, we sorted BM ECs from treated mice and confirmed that there were significantly lower miR-126 levels in total ECs, Sca-1+ ECs and Sca-1− ECs from inhibitor-treated mice, as compared to CpG–scrRNA-treated mice (Supplementary Fig. 7l,m). Mice that received the combination of inhibitor + NIL had a significant
reduction in the percentage of CD45.2+ CML cells in the PB, spleen and BM (Fig. 6i–k), and a significant reduction in the numbers of CML LSK cells and LT-HSCs in the spleen and BM, as compared to all of the other groups (Fig. 6l–o). We followed a cohort of mice for survival studies after 3 weeks of treatment and found that all of the mice that were treated with CpG–scrRNA alone died of leukemia within 60 d after the discontinuation of treatment, whereas 50% of the mice that were treated with inhibitor alone or with scrRNA + NIL, and 90% of the mice that were treated with the combination of inhibitor + NIL, survived ($P = 0.0012$; Fig. 6p).

To quantify the frequency of leukemia-initiating cells (LICs) after treatment, BM cells from mice with leukemia that were treated with CpG–scrRNA, inhibitor, CpG–scrRNA + NIL or inhibitor + NIL for 3 weeks were transplanted in limiting dilution assays into secondary congenic CD45.1 recipient mice. Treatment with the combination of inhibitor + NIL resulted in a significantly higher level of depletion of LICs, as assessed by leukemia development in secondary recipient mice after 16 weeks of follow up, as compared to mice that were treated with CpG–scrRNA alone, inhibitor alone or CpG–scrRNA + NIL (Fig. 6q). None of the secondary recipients that received BM cells from the mice treated with the combination of inhibitor + NIL developed leukemia. These results indicated that, as compared with NIL treatment only, treatment with a combination of NIL and the CpG–miR-126i enhanced the eradication of CML LSCs that were capable of engraftment in secondary recipients.

**DISCUSSION**

We report here that miR-126 expression levels in both human and mouse CML cells follow the hierarchy of hematopoietic differentiation, with more primitive hematopoietic stem cells or progenitors expressing higher levels of miR-126 than mature cells. Moreover, quiescent CML LT-HSCs had higher levels of miR-126 and a higher leukemia engraftment capacity than proliferating CML LT-HSCs, consistent with findings reported for normal hematopoiesis8. Unexpectedly, we found that miR-126 levels were significantly lower in CML LT-HSCs than in their normal counterparts, consistent with previous findings showing that a lower frequency of long-term repopulating cells are observed within CML LT-HSCs as compared to normal LT-HSCs2. Indeed, we demonstrated that BCR–ABL expression lowered mature miR-126 levels and increased pri- and pre-miR-126 levels, whereas pharmacological inhibition of BCR–ABL activity by a TKI increased mature miR-126 levels and decreased pri- and pre-miR-126 levels. Taken together, these data support a role for BCR–ABL in altering the biogenesis of endogenous miR-126.

To our knowledge, BCR–ABL-dependent downregulation of miR-126 in CML cells has not been previously reported. SPRED1, a validated miR-126 target4,7,9,13,14, is a tyrosine kinase substrate known to inhibit growth-factor-mediated activation of RAS protein family members and, in turn, the RAS–MAPK–ERK pathway15. Tyrosine residue phosphorylation is required for SPRED1 inhibition of RAS–MAPK–ERK activation16. We show here that SPRED1 is a substrate for BCR–ABL and that BCR–ABL-induced SPRED1 phosphorylation is critical for miR-126 biogenesis in CML. We found that BCR–ABL–phosphorylated SPRED1 binds with RAN, a RAS family member, disrupts the RAN–EXP5–RCC1 complex, which is involved in pre-miRNA nucleus-to-cytoplasm shuttling, increases nuclear levels of pri- and pre-miR-126 and decreases cytoplasmic levels of mature miR-126. Conversely, BCR–ABL inhibition disrupted the binding of SPRED1 with RAN, enhanced formation of the RAN–EXP5–RCC1 complex, increased mature miR-126 levels and decreased pri- and pre-miR-126 levels (Supplementary Fig. 1j).

Because miR-126 is necessary for normal and clonal HSC quiescence and continuous downregulation of miR-126 can cause clonal exhaustion1,8, we reasoned that this autoregulatory loop must be circumvented to maintain a reservoir of quiescent CML LSCs. Previous reports have suggested that BM ECs participate in the regulation of normal hematopoiesis10,25. Here we showed that among cell populations in the leukemic BM niche, ECs expressed the highest miR-126 levels and supplied miR-126 to CML cells, likely through EV trafficking. Furthermore, consistent with previous reports showing that Sca-1+ ECs are associated with quiescent normal HSCs and that Sca-1+ ECs are associated with proliferating normal HSC in the marrow19, we found that endosteal Sca-1+ ECs express higher levels of miR-126 than central marrow Sca-1+ ECs and are associated with a larger fraction of quiescent BCR–ABL+ LT-HSCs, which also express higher miR-126 levels than proliferating BCR–ABL+ LT-HSCs. Our data support a functional interplay between ECs and HSCs in CML, which results in a nonrandom BM distribution of the quiescent CML LSC fraction that is more likely to be found proximal to the high-level miR-126-expressing ECs from the endosteal marrow than to the low-level miR-126-expressing ECs from the central marrow. The functional relevance of the exchange of miR-126 between ECs and LT-HSCs to leukemia growth was demonstrated by showing a decreased engraftment of CML LSCs and improved survival in recipient mice with a Mir126a KO in the endothelial compartment that were transplanted with BCR–ABL+ LT-HSCs.

Our results may be clinically relevant to patients with CML who are treated with a TKI. Persistence of CML LSCs during TKI treatment is an active area of investigation, as these agents are remarkably potent against cycling cells but fail to eliminate quiescent CML LSCs3,26. Using primary human CML cells and mouse models of CML, we showed that the resistance of CML LSCs to TKI treatment is likely mediated by decreased levels of phosphorylated SPRED1 due to BCR–ABL inhibition, leading to increased endogenous miR-126 levels, which pushes LSCs into a relatively treatment-refractory quiescent state. Furthermore, miR-126 upregulation in TKI-treated CML CD34+ cells resulted in decreased SPRED1 levels, activation of the MAPK–ERK pathway and increased cell survival24,27. Accordingly, knockdown of miR-126 expression in CML cells and/or ECs enhanced the anti-leukemic activity of TKI treatment by counteracting the undesired TKI-induced miR-126 upregulation. In vivo, all NIL-treated CML mice with a genetic Mir126a KD in ECs survived, demonstrating the therapeutic potential of targeting miR-126 in CML.

For clinical translation of this concept, we designed a novel CpG–miR-126 ODN inhibitor that could efficiently be taken up by both hematopoietic and nonhematopoietic cells in the BM niche. We have previously shown that the uptake of CpG–ODN conjugated molecules depends on endocytosis by scavenger family dextran-sulfate-sensitive receptors (SRs)28,29, which are expressed on the surface of normal and malignant myeloid cells30,31. Following SR-mediated internalization, CpG-conjugates bind to endosomal TLR9, triggering their cytoplasmic release28. SRs and TLR9 are both expressed on hematopoietic cells and ECs30,31, and likely facilitate the efficient intracellular delivery of CpG–miR-126i and its subsequent endosomal release and pharmacologic activity. We found that CpG–miR-126i was efficiently taken up by both LT-HSCs and ECs in vitro and in vivo, downregulated miR-126 expression and reduced LT-HSC quiescence and frequency. Combination treatment with CpG–miR-126i and a TKI in CML mice resulted in increased survival, as compared to survival after treatment with either agent alone; moreover, no leukemia development in secondary recipients transplanted with BM cells from combination-treated mice was observed, suggesting that combination
treatment resulted in the elimination of CML LSCs. In support of the possibility of clinical translation of this treatment strategy, we observed no hematological toxicity in normal mice that were treated with CpG–miR-126.

In summary, we report that BCR–ABL-mediated SPRED1 phosphorylation downregulates miR-126 biogenesis in CML LSCs, such that CML LSC quiescence and leukemogenic capacity rely on trafficking of miR-126 from ECs to LSCs in the BM niche. On the basis of the proof-of-concept findings reported here, showing that in vivo treatment of CML mice with a newly developed CpG–miR-126 inhibitor enhances TKI activity in vivo and results in LSC elimination, this CpG–miR-126 inhibitor is now being translated to the clinic for the treatment of patients with CML.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

ACKNOWLEDGMENTS

We acknowledge the support of the Animal Resources Center, Analytical Cytometry, Pathology (Liquid Tumor), Bioinformatics, Electron Microscopy, Light Microscopy, Integrative Genomics and DNA/RNA Cores at City of Hope (COH) Comprehensive Cancer Center, which is supported by the National Cancer Institute (NCI) of the US National Institutes of Health (NIH) under award number P30CA33572. We thank C.J. Kuo (Stanford University) for the M126Δmin/mice and are grateful to the COH Comprehensive Cancer Center, the Glasgow Experimental Cancer Medicine Centre and the SPIRIT trials, together with the patients and their physicians, for providing primary patient material for this study. This work was supported in part by NCI grants CA205247 (Y.-H.K.), CA110301 (G.M.), CA201184 (G.M.), CA213131 (M.K.), CA180861 (G.M.), CA158350 (G.M.), CA163800 (P.D.), and CA213131 (M.K.), the Gehr Family Foundation (G.M.), the George Hoag Family Foundation (G.M.), Cancer Research UK grant C11074/A11008 (T.L.H.) and The Howat Foundation (T.L.H.).

AUTHOR CONTRIBUTIONS

B.Z. and L.X.T.N. designed and conducted experiments, analyzed data and wrote the manuscript; L.L., D.Z., B.K., H. Wu, F.P., Y.-L.S., C.B., H. Wang, T.M. and E.T. conducted experiments; A.L., D.S.S., H.A. and A.S.S. provided samples and reviewed the patients’ data; P.S. and M.K. designed the CpG–miR-126 inhibitor and reviewed data and the manuscript; L.H., C.-C.C., A.D., V.P., Y.-C.Y., D.P. and N.C. analyzed data; C.I.K. provided the miR-126 c-KO mouse model; R.B. provided the B6 SCITαx-BCR-ABL mouse model of CML; M.C., T.L.H. and S.J.F. provided patient samples, designed experiments and reviewed the manuscript; M.K. and Y.-H.K. designed experiments, analyzed data and reviewed the manuscript; G.M. designed experiments, analyzed data and provided the manuscript and administered administrative support.

COMPETING INTERESTS

The authors declare no competing interests.

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ONLINE METHODS

Human samples. PB and BM samples from healthy individuals (‘normal’) were obtained from donors at the City of Hope National Medical Center (COHNMC). CP CML samples were obtained from patients who had not received prior TKI treatment from the COHNMC, from the Glasgow Experimental Cancer Medicine Centre and from UK SPIRIT2 clinical trial patients (http://spirit-cml.org). All CML samples used in this study are P210 BCR–ABL positive, as confirmed by FISH analysis and qPCR. Mononuclear cells were isolated using Ficoll separation. CD34+ cells were then isolated using a positive magnetic bead selection protocol (Miltenyi Biotec, Germany). All of the patients with CML and the healthy donors signed an informed consent form. Sample acquisition was approved by the Institutional Review Boards at the COHNMC and the Greater Glasgow and Clyde NHS Trust, in accordance with an assurance filed with and approved by the Department of Health and Human Services, and met all requirements of the Declaration of Helsinki.

Mouse studies. Inducible transgenic SCLtTA×BCR–ABL mice in the FVB/N background2–12 were backcrossed to the B6 background (CD45.2) for ten generations. Transgenic BCR–ABL mice were maintained on tetracycline-containing water at 0.5 g/liter. Withdrawal of tetracycline results in expression of BCR–ABL and generation of a CML-like disease in these mice2–12. Unless otherwise indicated, BCR–ABL expression was induced for 2–3 weeks by tetracycline withdrawal in 6- to 8-week-old male and female mice, and then BM cells (from both males and females) were collected for experiments. SCLtTA×BCR–ABL mice (CD45.2, B6) were also bred with CD45.1 B6 mice to produce CD45.1/CD45.2 SCLtTA×BCR–ABL mice as donors. Mir126Δfox/fox mice (B6, from Dr. C. J. Kuo, Stanford University) were crossed with Mx1−Cre, Tie2−Cre (both from The Jackson Laboratory) and SCLtTA×BCR–ABL mice (all B6) to obtain the following strains: Mir126Δfox/fox;Mx1−Cre (referred to as Mx1− mice), SCLtTA×BCR–ABL;Mir126Δfox/fox;Mx1−Cre (referred to as SCLtTA×BCR–ABL;Mx1− mice), Mir126Δfox/fox;Tie2−Cre (referred to as Aftie2− mice) and Mir126Δfox/fox;Tie2−Cre (referred to as Frohie2− mice). Recipient mice in the CD45.1 B6 background (from Charles River) were used to allow tracking of donor CD45.2 cells after transplantation. Recipients were 6- to 8-week-old male and female mice and were irradiated at 900 cGy within 24 h before transplantation. The number of mice for each study group was chosen based on the expected endpoint variation (i.e., engraftment rate and latency period of leukemia) and the availability of mice of different strains. Mice of the same gender and age were randomly divided into groups. Investigators were blinded to mouse genotype while performing treatment or monitoring engraftment or survival. Mouse care and experimental procedures were performed in accordance with federal guidelines and protocols and were approved by the Institutional Animal Care and Use Committee at the City of Hope.

Engraftment of human cells in immunodeficient mice. GFP+ cells (2 × 105 cells/mouse) selected from CML Lin−CD34+CD38− cells transduced with mir-126-5p CMV−scrRNA (50 mg/kg, daily by oral gavage), or inhibitor + NIL for 21 d. After 3 weeks of treatment, mice were euthanized, and BM cells from the right femur and spleen cells were analyzed for CML cell output. BM cells from the left femur of the treated mice were pooled, and 4 × 106, 2 × 106, 1 × 106 and 5 × 105 cells/mouse (6 mice/dose × 4 doses × 4 conditions = 96 mice) were transplanted into irradiated (900 cGy) recipient mice (CD45.1). WBC counts and CML cell engraftment were monitored every 4 weeks. The mice were euthanized at 16 weeks, followed by assessment of donor CML cell engraftment in PB, BM and spleen. The fraction of mice showing evidence of CML development at 16 weeks after secondary transplantation was determined, and the frequency of LICs was calculated using L-Calc software. Another cohort of mice was followed for survival up to 60 d after discontinuation of treatment. To determine the in vivo effect of the CpG−miR-126i on normal hematopoiesis, normal mice were treated with CpG−scrRNA or inhibitor for 3 weeks, followed by assessment of WBC, RBC and PLT counts in PB and BM subpopulations in the BM.

Flow cytometry analyses. Human Lin−CD34+CD38− committed progenitors, Lin−CD34+CD38− and Lin−CD34+CD38− primitive progenitors, and Lin−CD34+CD38− CD90+ stem cells were obtained by flow-cytometry-based sorting. Antibodies to the following human proteins were used: human biotinylated lineage antibodies against CD2 (clone RPA-2.10, cat 553325, BD Biosciences), CD7 (124–D1–14, 13-0079-80, ebioscience), CD10 (2C-ALLA–1529943, ThermoFisher), CD11b (C67F154, 13019682, ThermoFisher), CD19 (eBio1D3 (13), 13-0193-82, Thermofisher), CD33 (HIM3–4, MAI–19522, Thermofisher), CD235a (HI2–GA-R2), 13–9987–82, ThermoFisher); human antibodies against adenocarcinoma (PE–Cy7, 581, 567010; FITC, 581, 5585821; APC, 581, 5585842; all from BD Biosciences), CD38 (PE, 5676, 347787, BD Biosciences), CD45 (FITC, 2D11–1–495–94–22, PerCP–Cy5.5, 2D11–4946–22, Thermofisher), CD31 (3E6–B5, 576, 556027; FITC, 581, 556026, BD Biosciences), CD63 (PE–Cy7, 5656824, BD Biosciences), CD9 (FITC, H19a, 312104, BioLegend), and CD48 (APC, 581, 561958, BD Biosciences). These antibodies were used to analyze human cells in PB, BM and spleen. For analysis of stem and progenitor cells, c-kit+ cells were selected using anti-mouse CD117 microbeads or Lin− cells were selected using Lineage depletion microbeads (both from Miltenyi Biotec, San Diego, CA). The following mouse antibodies were used: mouse biotinylated lineage antibodies (all from ebioscience) against: CD3 (clone 17A2, cat 13-0031-85), CD4 (GK1.5, 13-0041-85), CD8 (53-6.7, 13-0083-85), B220 (RA3-6B2, 13-0452-85), CD19 (eBio1D3 (13), 13-0193-85), IgM (eBi121–15F9, 13-5790-85), B1 (BCE8C–62, 13-5931-85), CD11b (M1/70, 13-0112-85, NK1.1 (PK136, 13-5941-85), Ter119 (clone Tering-119, cat 13-5921–85), Flt3 (A2F10, 13–1351–85); mouse antibodies against Flt3 (PE, A2F10, 12-1351–82, ebioscience), Sca-1 (PE–Cy7, 2D5, 25-5981–82, ebioscience), CD117 (APC–eflu780, ACK2, 47–1172–82, ebioscience), CD16–CD32 (PE–Cy7, 2.4G2, 560829, BD Biosciences), CD34 (Alexa Fluor 647, RAM34, 560230, BD Biosciences), CD150 (PerCP–Cy5.5, TC12–1512–82, ImmunoLogic), CD48 (APC, HM48–1, 13–0481–82, ebioscience; Pacific blue, HM48–1, 13–0418, BioLegend), CD45 (PE–Cy7, A20, 25–0453–82, PerCP–Cy5.5, A20, 25–0453–80, both from ebioscience), CD45.2 (FITC, 104, 11–0454–85; efluor450, 104, 48–0454–82, both from ebioscience), CD45.1 (PE–Cy7, 30–F11, 25–0451–82, ebioscience), Ter119 (APC–eflu780, TRB1–19, 50-162–15, ebioscience), CD31 (APC, 390, 13–0311–82, PE, 390, 12–0311–82, both from ebioscience), CD166 (PE, FAB1172R, R&D Systems). Other antibodies include anti-streptavidin (PE, 12–4317–87; FITC, 11–4317–87; PerCP–Cy5.5, 45–4317–80, ebioscience) and...
Annexin V (PE, 559763, BD Biosciences). Myeloid progenitors were identified as Lin–Sca-1–c−kit+CD34+Fc−γRII/IIIb+ (CMP), Lin–Sca-1–c−kit+CD34+Fc−γRII/IIIb+ (GMP), or Lin–Sca-1–c−kit+CD34+Fc−γRII/IIIb+ (MEP)1,2. Stem and progenitor populations were identified as LSK cells (Lin−Sca-1−c−kit+) and long-term hematopoietic stem cells (LT-HSCs; LSK Flt3−CD150−CD48+1,2,3,8). Endothelial cells were identified as CD45−Ter119−CD31+. All analyses were performed on a LSRII flow cytometer (BD Biosciences), and sorting was performed on ARIAIII or ARIA SORP instruments (BD Biosciences).

Cell culture. Human HPCs (Lin−CD34+ and HSCs (Lin−CD34+CD38−) were cultured in Stemspan serum-free medium II (SEF II, StemCell Technologies), supplemented with low concentrations of growth factors (GFs) similar to those present in long-term BM culture stroma-conditioned medium (granulocyte–macrophage colony-stimulating factor (GM-CSF) 200 pg/ml, leukemia inhibitory factor (LIF) 50 pg/ml, granulocyte-colony-stimulating factor (G-CSF) 1 ng/ml, stem cell factor (SCF) 200 pg/ml, macrophage inflammatory protein (MIP)-1α 200 pg/ml, and interleukin (IL)-6 1 ng/ml)2,9. Mouse BM LT-HSCs were cultured in SEF II supplemented with 10 ng/ml SCF and 10 ng/ml thrombopoietin (TPO). HUVECs and K562 cells were recently purchased from Lonza and ATCC, respectively. We confirmed that HUVEC cells were positive for human CD31 expression by flow cytometry and that K562 cells were positive for BCR–ABL1 expression by qPCR. These two cell lines were tested for mycoplasma contamination, and both were negative. HUVEC cells were cultured in complete EGM-2 medium (Lonza), and mouse BM ECs were cultured in complete mouse endothelial cell medium (CellBiosciences). K562 cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Life Technologies). All cells were cultured at 37 °C with 5% CO2 and high humidity.

Differential ultracentrifugation. Conditioned medium was made by centrifuging the culture medium containing FBS for 8–10 h at 100,000g and 4 °C to remove the FBS-derived EV contamination, and the supernatant was collected (leaving the FBS-derived EVs per protein pellet). The cells were cultured with the conditioned medium for 2 d. EVs were isolated by differential ultracentrifugation21,22,40. Briefly, the supernatant was collected and centrifuged for 5 min at 500g and 4 °C, the supernatant was then collected and centrifuged for 10 min at 2,000g and 4 °C, and the resulting supernatant was then collected and centrifuged for 15 min at 10,000g and 4 °C. It was important that none of the pellets consisting of cells and cell debris were collected. The supernatant was then transferred to a new tube and centrifuged for 60 min at 100,000g and 4 °C. The EV pellet was washed once with PBS and centrifuged again for 60 min at 100,000g and 4 °C to obtain the final EV pellet. The EV pellet was dissolved in 50 μl of PBS and stored at 4 °C for up to 1 week.

Nanoparticle tracking analysis. NanoSight measurements were carried out in 0.2-μm-filtered PBS. The concentration and size distribution profile of the particles isolated by differential ultracentrifugation were evaluated using a NanoSight NS300 instrument (Malvern, Worcestershire, UK) and NTA 3.2 software. Videos were recorded at camera level 15. Samples were diluted 1:100 in PBS to achieve a measured particle concentration of 5–15 × 108/ml in accordance with the manufacturer's recommendations. For each sample, three 60-s videos were recorded and analyzed in the batch-processing mode.

Electron microscopy (EM). Specimens at an optimal concentration were placed onto 300-mesh carbon–formvar-coated grids and allowed to absorb to the formvar for a minimum of 1 min. Grids were rinsed with double-distilled water and stained for contrast using 1% uranyl acetate. The samples were viewed with an FEI Tecnai T12 transmission electron microscope at 120 keV, and images were taken with a Gatan Ultrascan 2K charge-coupled device (CCD) camera.

Cytotoxicfluorometric analysis. A fraction was isolated from HUVEC-derived EVs using magnetic beads coated with anti-CD63 (10606D, ThermoFisher). Briefly, EVs isolated from HUVECs by differential ultracentrifugation were incubated with magnetic beads coated with anti-CD63 overnight. The bead-bound CD63+ EVs were selected using a DynaMag-5 magnetic separator (12303D, ThermoFisher), stained with PE–Cy7-conjugated anti-human-CD63, FITC-conjugated anti-human-CD9 and APC-conjugated anti-human-CD81 (BD Biosciences) antibodies and then analyzed by flow cytometry.

Lentiviral or retroviral transduction of human and mouse cells. GFP-expressing miRZip anti-miR-126-3p (miR-126 KD, CS940MZ-1, a custom order from System Biosciences, with EFE1A1 promoter for the miRNA and PGK1 promoter for GFP-T2A-Puro expression) and control (MZIP000-PA-1, miRZip negative control; CD813A-1, pCDH-EF1-MCS-(PGK-GFP-T2A-Puro); both from System Biosciences) lentiviruses were produced and used for transduction of human and/or mouse HSCs, HUVECs and mouse ECs. Briefly, human HPCs or HSCs were cultured overnight in SEF II supplemented with IL-3 (25 ng/ml), IL-6 (10 ng/ml), SCF (50 ng/ml), TPO (100 ng/ml) and Flt-3 ligand (100 ng/ml). Mouse BM LT-HSCs were cultured overnight in SEF II supplemented with mouse SCF (10 ng/ml) and mouse TPO (10 ng/ml). The next day, cells were resuspended in SEF II and lentiviral supernatant (multiplicity of infection (MOI) = 10–20), supplemented with the above GFs and 1× TransDux virus transduction reagent (System Biosciences), and centrifuged at 1,500g for 90 min by transduction by spinoculation. We observed 30–60% of GFP+ cells in human HSCs and 90–100% in mouse LT-HSCs transduced with miR-126 KD lentivirus (MOI = 20), and 10–30% of GFP+ cells in human HSCs and 30–50% in mouse LT-HSCs transduced with miR-126 OE lentivirus (MOI = 10) at 48 h. HUVECs and mouse BM ECs were exposed to miR-126 KD or control lentiviral supernatant (MOI = 10) with 1× TransDux virus transduction reagent, and 100% of GFP+ cells were detected at 48 h. Normal BM LSK cells were transduced with supernatants from BCR–ABL1 expressing or control retroviruses (MOI = 5) with polybrene (5 μg/ml, American Bioanalytical) by spinoculation, and 20–30% of GFP+ cells were detected at 48 h. GFP+ cells from the samples with low transduction efficiency (<80%) were selected by flow cytometry at 48 h for further studies.

Apopotosis, cell cycle, cell growth and colony-forming cell assays. After transduction performed as above, GFP+ cells selected at 48 h were exposed to NIL (2 μM and 5 μM, Novartis) for another 72 h, and analyzed using assays for cell growth, apoptosis, cell cycle, CFC and CFC replating. Human HSCs and mouse LT-HSCs were also treated with Cpg-miR-126 inhibitor or Cpg-scrRNA (500 nM), with or without NIL (5 μM), for 72 h and analyzed for cell growth, apoptosis, cell cycle and CFC. Human CML HSCs co-cultured with control or miR-126 KD HUVECs and mouse CML LT-HSCs co-cultured with control or miR-126 KD ECs for 96 h, with or without NIL (5 μM), were also analyzed for cell growth, apoptosis, cell cycle and CFC. Cell growth was measured by Lumino Glo (Promega). Apoptosis was measured by labeling cells with Annexin V–PE or FITC and APC and 4,6-diamidino-2-phenylindole (DAPI) (all from BD Biosciences, San Diego, CA) and analyzed by flow cytometry. Cell cycle was analyzed using the SuperScript III First-Strand Kit, and then qPCR was performed using K–67–Alexa Fluor 647 (B56, BD Biosciences) and DAPI labeling based on the manufacturer's protocol. Cells were also exposed to EdU (C10640, Invitrogen) for 2 h and EdU staining was analyzed according to the manufacturer's protocol. Cell proliferation was also measured by CFSE staining (Molecular probes) based on the manufacturer's protocol. For CFC, Lin−CD34−CD38− cells cultured with or without NIL (5 μM) were plated in methycellulose progenitor culture, and burst-forming unit-erythroid and colony-forming unit-granulocyte and macrophage cells were counted after 14 d. Colony-replating assays were performed by collecting and pooling colonies from primary CFC assays and plating 10,000 cells in secondary CFC assays.

qPCR analysis. To measure miRNA and mRNA expression, total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA). For miRNA expression, reverse transcription using MultiScribe Reverse Transcriptase and qPCR analysis using Taqman assays (Applied Biosystems; also Supplementary Table 1) were performed according to the manufacturer's protocol. Expression of the snoRNA was used as internal controls for expression of human and mouse miRNAs, respectively. For mRNA expression, first-strand cDNA was synthesized using the SuperScript III First-Strand Kit, and then qPCR was performed using TaqMan Gene Expression Assays (ThermoFisher). BCR–ABL1 expression in human and mouse samples was measured with primer and probe sequences for BCR–ABL1 (B3A2 or B2A2, the two most common transcripts of BCR–ABL1), as
previously described. Results are presented as a log₂-transformed ratio according to the 2⁻ΔΔCt method (ΔΔCt = ΔCt of target – ΔCt of reference).

miRNA labeling and analysis. K562, HUVEC, and normal and CML CD34⁺CD38⁻ cells were cultured and incubated with a miR-126 SmartFlare RNA probe (EMD Millipore) for 16 h. To ensure that the cell types, including K562, HUVEC and primary CML cells, were able to effectively endocytose the SmartFlare probes, we examined the uptake of probes in these cells using a SmartFlare uptake control, a scrambled control and a housekeeping 18S control (according to the manufacturer’s guidelines). Cells were then washed in 1× PBS and fixed in 4% paraformaldehyde for 3 min. Nuclei were counterstained with DAPI, and the images were analyzed using a confocal microscope (Carl Zeiss).

Immunofluorescence. K562 cells were collected and washed in PBS followed by spinning them down onto slides using the CytoSpin 4 Cytocentrifuge (600 r.p.m., 10 min). The cells were then fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.5% Triton X-100 for 15 min. Nonspecific epitopes (600 r.p.m., 10 min). The cells were then fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.5% Triton X-100 for 15 min. Nonspecific epitopes

Nuclear fractionation. The nuclear fraction of 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Polar Lipids, Alabaster, AL) was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA). smRNA sequencing was performed using Illumina HiSeq2500 at the COH Integrative Genomics Core following the manufacturer’s sample-preparation protocol (TruSeq Small RNA Sample Prep Kit, Illumina, Inc.) with some modifications. Briefly, 250 ng of total RNA was used for smRNA sequencing library construction. Total RNA was ligated to the modified 5’ adaptor (5’-TGCGGATATCCGCTGGTGCCAAGAATCCCTC-3’), 3’ adaptor (5’-GTGCCAAGGACGGCATACGAGAT-3’), and miR-126 inhibitor or scrRNA was linked using 5 U of C3 carbon chain linker, (CH2)3 (indicated by ‘x’). The constructs were also conjugated with Cy3 dye to the membrane. Briefly, total RNA was prepared using Trizol reagent (Life Technologies). 20 µg of total RNA was separated on 15% TBE–urea gel (Life Technologies) and transferred onto positively charged nylon membranes (Roche). The blots were hybridized with LNA miR-126 probes for 12 h. After washing twice with 2× saline–sodium citrate (SSC) and 0.1× SSC (containing 0.1% SDS), the blots were immunoblotted with an anti-DIG antibody (clone 1:71.256, Roche) for 1:1,000 dilution and exposed.

Small RNA (smRNA) deep-sequencing using Illumina HiSeq2500. LSK cells from non-induced and induced CML mice were sorted, and total RNA was extracted using the mirNeasy Mini Kit (Qiagen, Valencia, CA). smRNA sequencing was performed using Illumina HiSeq2500 at the COH Integrative Genomics Core following the manufacturer’s sample-preparation protocol (TruSeq Small RNA Sample Prep Kit, Illumina, Inc.) with some modifications. Briefly, 250 ng of total RNA was used for smRNA sequencing library construction. Total RNA was ligated to the modified 5’ adaptor (5’-GGGGATTCTTGTCCGAGAATCCCTCA-3’), and the product subsequently was ligated to the modified 5’ adaptor (5’-GGUAAGAUCUAAGACUCCGACGACGAAUA-3’). The final libraries were sequenced using Illumina HiSeq2500 platform in the single-read mode of 51 cycles of read1 and seven cycles of index read. Real-time analysis (RTA) 2.2.38 software was used to process the image analysis and base-calling.

Oligonucleotide design and synthesis. The partially phosphothioated ODN and miR-126 inhibitor or scrRNA was linked using 5 U of C3 carbon chain linker, (CH2)3 (indicated by ‘x’). The constructs were also conjugated with Cy3 to track the internalization in cells by flow cytometry. The sequences were as follows:

- CpG-miR-126 inhibitor: 5’-G*G*TGACATCGATCGAG*G*G*Gxxxxx

CmGmCa: mAmUmAmUmAmCmAmCmGmGmAmA-3’, where (*) indicates phosphorothioation. One nonbridging atom of oxygen on phosphate was replaced with sulfur. ‘m’ indicates the 2’-O-methyl analog of the nucleotide.

Transferrin- or anti-CD45.2-conjugated nanoparticle preparation. Previously we developed a transferrin (TF)-targeted neutral NP delivery system4,41. Briefly, positively charged polyethyleneimine and negatively charged miR-126i-Cy3 or scrRNA–Cy3 form a polyplex core. This core was then loaded into pre-made anionic liposomal NPs to form lipopolyplex NPs. The formulation consisted of 1,2-dioleoyl-sn-glycerol-3-phosphothanolamine (DOPE), 1,2-dimyristoyl-sn-glycerol, methoxy(polyethylene glycol) (DMG–PEG) and linoleic acid. TF or anti-human-CD45 antibody conjugated with 1,2-diaryloxyl or–glycerol-3–phosphothanolamine-N-[-maleimide(polyethylene glycol)-2000] (DSPEPEG2000 maleimide) was then post-inserted to the surface of lipopolyplex nanoparticles.
(to form TF-NP and Ab-NP, respectively). The molar ratio of lipids to TF was 2,000 as previously described\textsuperscript{6,41}, and the molar ratio of lipids to anti-CD45 antibody was optimized to 10,000.

**Statistical analysis.** Comparison between groups was performed by a two-tailed, paired or unpaired Student's \( t \)-test. The log-rank test was used to assess significant differences between survival curves. All statistical analyses were performed using Prism version 6.0 software (GraphPad Software). Sample sizes chosen are indicated in the individual figure legends and were not based on formal power calculations to detect prespecified effect sizes. All of the *in vitro* experiments were performed 3–6 times using biologically independent samples; the *in vivo* experiments were performed using 6–16 mice in each group. \( P \leq 0.05 \) was considered to be significant. Results shown represent mean ± s.e.m. *\( P \leq 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) and ****\( P < 0.0001 \).

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data availability.** miRNA deep sequencing data produced in our laboratory and analyzed in this study are available at the Gene Expression Omnibus (GEO) repository of the National Center for Biotechnology Information, under the accession number GSE107431. Supplementary Table 1, Supplementary Figures 1–7, source data for Figure 3 and Supplementary Figures 3c and 5h.k.m.p as shown in Supplementary Figures 8–10, and gating strategy for flow cytometry analysis as shown in Supplementary Figure 11, are provided with the online version of this paper. All other data sets generated during this study are available from the corresponding author on reasonable request.

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Experimental design

1. Sample size
   - Describe how sample size was determined.
   - Sample size selection was based on sample availability, sample variation, and previous experience. All of the in vitro experiments were performed using at least 3-4 samples from mouse or human individuals to ensure adequate power. The in vivo experiments were performed using 6-16 mice per group. Animal numbers were chosen based on experimental group size, mouse availability and leukemia burden variability.

2. Data exclusions
   - Describe any data exclusions.
   - No data were excluded from the analyses.

3. Replication
   - Describe whether the experimental findings were reliably reproduced.
   - All experimental findings were reliably reproduced.

4. Randomization
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - For animal studies, mice with the same gender or age were divided randomly into experimental groups to reduce variation and enhance power.

5. Blinding
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - Investigators were blinded to group allocation during data collection and/or analysis.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   - The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

The data were analyzed by Prism GraphPad 6.0. The limiting dilution leukemia initiation data were analyzed by Calc software (version 1.1). The size and distribution of Extracellular vesicles collected by NanoSight was analyzed by NTA 3.2 software. Protein levels were determined by densitometry using Image-Quant software 1.1. miRNA deep sequencing data were analyzed by Real-time analysis (RTA) 2.2.38 software.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All of the unique materials such as mouse models or plasmids are available from us or other independent investigators, or from commercial sources, which was indicated in the MS.

Human biotinylated lineage antibodies against CD2 (RPA-2.10, 555325, BD), CD7(124-101, 13-0079-80, ebioscience), CD10(C8-CALLA, 15259439, Thermo Fisher), CD11b (C67F154, 13019682, Thermo Fisher), CD19 (eBio1D3 (1D3), 13-0193-82, thermo Fisher), CD33 (HIM3-4, MA1-19522, thermo Fisher), CD235a (HiR2 (GA-R2), 13-9987-82, thermo Fisher); human antibodies against CD34 (PE-Cy7, S81, 560710; FITC, S81, 555821; APC, S81, 555824; all from BD), CD38 (PE, HI2, 560981; APC, HI2, 555462, both from BD), CD90 (PerCP-Cy5.5, eBio1D3 (1D3), 45-0909-42, Thermo Fisher), CD33 (PE, P67.6, 347787, BD), CD45 (FITC, 2D1,11-9459-42, PerCP-Cy5.5, 2D4, 45-9459-42, Thermo Fisher), CD3 (PECAM-1, PE, 390, 50-103-20, Thermo Fisher), Ki-67 (PE, B56, 556027; FITC, B56, 556026, BD), CD63 (106060, Thermo Fisher), CD63 (PE-Cy7, HSC6, 561982, BD), CD9 (FITC, H19a, 312104, Biolegend), and CD81 (APC, JS1, 561958, BD). Mouse biotinylated lineage antibodies against CD3 (17A2, 13-0031-85), CD4 (GK1.5, 13-0041-85), CD8 (53-6.7, 13-0083-85), B220 (RA3-6B2, 13-0452-85), CD19 (eBio1D3 (1D3), 13-0193-85), IgM (eB121-15F9, 13-5790-85), Gr-1 (RB6-8C5, 13-5931-85), CD11b (M1/70, 13-0112-85), NK1.1 (PK136, 13-5941-85), Ter119 (TER-119, 13-5921-85), Flt3 (A2F10, 13-1351-85) (all from ebioscience); mouse antibodies against Flt3 (PE, A2F10, 12-1351-82, ebioscience), Sca-1 (PE-Cy7, D7, 25-0591-82, ebioscience), CD117 (APC-eflu780, ACK2, 47-1172-82, ebioscience), CD16/32 (PE-Cy7, 2.4G2, 560828, BD), CD34 (Alexa Fluor 647, RAM34, 560230, BD), CD150 (PerCP-Cy5.5, TC15-12F12.2, 115922, Biolegend), CD48 (APC, HM48-1, 104518, Biolegend ), CD45.1 (PE-Cy7, A20, 25-0453-82; PerCP-Cy5.5, A20, 45-0453-80, both from ebioscience ), CD45.2 (FITC, 104, 11-0454-85; eFluor450, 104, 11-0454-82, both from ebioscience), CD45 (PE-Cy7, 30-F11, 25-0451-82, ebioscience), Ter119 (APC-eflu780, TER-119, 50-162-15, ebioscience), CD31 (APC, 390, 17-0311-82; PE, 390, 12-0311-82, both from ebioscience), CD166 (PE, FAB1172P, R&D Systems), streptavidin (PE, 13-4317-87; FITC, 11-4317-85; PE-Cy7, 30-F11, 25-0451-82, ebioscience), Annexin V (PE, 559763, BD). Antibodies for western blot include: anti-SPRED1 antibody (M23-P2G3, #ab64740, Abcam), anti-RAN antibody (C-20, #SC-1156, Santa Cruz), anti-Exportin5 antibody (D7W6W, #12565, Cell Signaling), anti-PARP antibody (#9542, Cell Signaling), anti-RCC1 antibody (F-2, #SC-376049, Santa Cruz), anti-Actin antibody (C-4, #SC-47778, Santa Cruz), anti-phospho-Tyrosine antibody (4G10, #05-321, Millipore), anti-Bcl-2 antibody (124, #15071, Cell Signaling), anti-phospho-ERK (#9101, Cell Signaling), anti-normal mouse IgG (#SC-2025, Santa Cruz), anti-normal rabbit IgG (#SC-2027, Santa Cruz), CD63 (10628D, ThermoFisher), TSG101(SAB2702167, Sigma), HSP90 (2D12, Enzo Life Sciences), Cytochrome C (sc-13156, Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (Westgove, PA).
10. Eukaryotic cell lines
   a. State the source of each eukaryotic cell line used. Human Umbilical Vein Endothelial Cells (HUVEC) and K562 cells were recently purchased from Lonza and ATCC respectively.
   b. Describe the method of cell line authentication used. The cells were purchased from Lonza and ATCC recently. We confirmed that HUVEC cells are human CD31 positive by flow cytometry and that K562 cells are BCR-ABL positive by QPCR.
   c. Report whether the cell lines were tested for mycoplasma contamination. The cell lines were tested negative for mycoplasma contamination.
   d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. The cell lines used are not listed in the database of commonly misidentified cell lines.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals
   Provide details on animals and/or animal-derived materials used in the study.

6-8 weeks old male and female CML mice in CD45.2 B6 background were induced by tetracycline withdrawal and 6-8 weeks old CD45.1 WT B6 male and female mice were used as recipients.

Policy information about studies involving human research participants

Description of human research participants
Describe the covariate-relevant population characteristics of the human research participants.

Human samples were obtained from donors at City of Hope National Medical Center (COHNMC). CML patient samples were obtained from COHNMC and Glasgow Experimental Cancer Medicine Centre and UK SPIRIT trials, and the patients had not received prior TKI treatment and are P210 BCR-ABL positive confirmed by FISH analysis or QPCR.
Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

- 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- 3. All plots are contour plots with outliers or pseudocolor plots.
- 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation. Cells were collected, washed with PBS+1% BSA, stained with different fluorescence-conjugated antibodies at 4C for 30 min, washed with PBS+1% BSA once, and then analyzed using FACS ARIA II, ARIA III, ARIA SORT.

6. Identify the instrument used for data collection. BD FACS ARIA II, BD FACS ARIA III, BD FACS SORP

7. Describe the software used to collect and analyze the flow cytometry data. BD FACS Diva was used to collect data and analyze the data and prepare figures.

8. Describe the abundance of the relevant cell populations within post-sort fractions. Cell population purity within post-sort fractions was determined by analyzing again using BD FACS ARIA II, III or SORP.

9. Describe the gating strategy used. All samples are FSC-A and SSC-A gated, followed by FSC-A/FSC-H gating to select singlet cells. Subsequent relevant gating was conducted, shown in the main figures or supplementary.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ✗