The balance between self-renewal and differentiation is crucial to ensure the homeostasis of the hematopoietic system, and is a hallmark of hematopoietic stem cells. However, the underlying molecular pathways, including the role of micro-RNA, are not completely understood. To assess the contribution of micro-RNA, we performed micro-RNA profiling of hematopoietic stem cells and their immediate downstream progeny multi-potent progenitors from wild-type control and Pbx1-conditional knockout mice, whose stem cells display a profound self-renewal defect. Unsupervised hierarchical cluster analysis separated stem cells from multi-potent progenitors, suggesting that micro-RNA might regulate the first transition step in the adult hematopoietic development. Notably, Pbx1-deficient and wild-type cells clustered separately, linking micro-RNAs to self-renewal impairment. Differential expression analysis of micro-RNA in the physiological stem cell-to-multi-potent progenitor transition and in Pbx1-deficient stem cells compared to control stem cells revealed miR-127-3p as the most differentially expressed. Furthermore, miR-127-3p was strongly stem cell-specific, being quickly down-regulated upon differentiation and not re-expressed further downstream in the bone marrow hematopoietic hierarchy. Inhibition of miR-127-3p function in Lineage-negative cells, achieved through a lentiviral-sponge vector, led to severe stem cell depletion, as assessed with serial transplantation assays. miR-127-3p-sponged stem cells displayed accelerated differentiation, which was uncoupled from proliferation, accounting for the observed stem cell reduction. miR-127-3p overexpression in Lineage-negative cells did not alter stem cell pool size, but gave rise to lymphopenia, likely due to lack of miR-127-3p physiological downregulation beyond the stem cell stage. Thus, tight regulation of miR-127-3p is crucial to preserve the self-renewing stem cell pool and homeostasis of the hematopoietic system.
miR-127 controls HSC maintenance

Methods

Mice

Tie2Cre·Pbx1+/− and Mx1Cre·Pbx1−/− mice have been described. Briefly, Tie2Cre·Pbx1−/− and Mx1Cre·Pbx1−/− mice were bred with Pbx1+/f mice to obtain Tie2Cre·Pbx1−/− or MxCre·Pbx1−/− Pbx1-cKO and their littermate controls.

miRNA profile and bioinformatic analysis

RNA was extracted with MirVana isolation kit (Ambion, ThermoFisher Scientific) from 1.4-2x10^6 HSC and 2-6x10^3 Flk2-MPPs (Table 1) sorted from the BM of Polyinosinic-polycytidylic acid (poly(I:C)) (InvivoGen)-treated Tie2Cre+.Pbx1+/− and Mx1Cre+.Pbx1+/f mice and their littermate controls. miRNA profiling was performed using the Megaplex TaqMan-based real-time stem-loop PCR method and subjected to normalization, filtering and analysis (Figure 1A). A global analysis through unsupervised hierarchical clustering indicated a clear distinction between HSC and Flk2-MPP at the level of miRNA expression (Figure 1B), suggesting that miRNA regulate the first transition step in adult hematopoietic development. Within each group, Pbx1-deficient and control cells clustered separately, linking miRNA to self-renewal impairment. Differential expression analysis indicated that 71 miRNA are differentially expressed (DE) during the physiological HSC-to-MPP transition (Figure 1C, right, and Online Supplementary Table S1). A similar analysis on Pbx1-deficient HSC revealed 48 miRNA DE compared to control HSC (Figure 1C, left), half of which (n=25) are concordantly DE in the HSC-to-MPP list (Online Supplementary Table S2), in accordance with the hypothesis that miRNA are involved in HSC self-renewal. This result is similar to that obtained by analyzing mRNA, with Pbx1-deficient HSC exhibiting a transcriptional profile typical of their immediate downstream progeny.

Results

Pbx1-cKO mouse as a model to identify candidate miRNA regulating hematopoietic stem cell self-renewal

Hematopoietic stem cells and Flk2-MPP were prospectively isolated from the BM of five Pbx1-cKO (Mx1Cre·Pbx1−/−) and four control (Mx1Cre·Pbx1+/f) individual mice. The expression level of 376 different miRNA was obtained using a multiplexed Taq-Man-based real-time stem-loop PCR method and subjected to normalization, filtering and analysis (Figure 1A). A global analysis through unsupervised hierarchical clustering indicated a clear distinction between HSC and Flk2-MPP at the level of miRNA expression (Figure 1B), suggesting that miRNA regulate the first transition step in adult hematopoietic development. Within each group, Pbx1-deficient and control cells clustered separately, linking miRNA to self-renewal impairment. Differential expression analysis indicated that 71 miRNA are differentially expressed (DE) during the physiological HSC-to-MPP transition (Figure 1C, right, and Online Supplementary Table S1). A similar analysis on Pbx1-deficient HSC revealed 48 miRNA DE compared to control HSC (Figure 1C, left), half of which (n=25) are concordantly DE in the HSC-to-MPP list (Online Supplementary Table S2), in accordance with the hypothesis that miRNA are involved in HSC self-renewal. This result is similar to that obtained by analyzing mRNA, with Pbx1-deficient HSC exhibiting a transcriptional profile typical of their immediate downstream progeny.

Lentiviral constructs

Design and production of lentiviral vectors for stable ectopic miRNA-overexpression (OE vector) or functional downregulation (DR vector) were as previously described. Briefly, both vectors exploit the spleen focus forming virus (SFFV) promoter and couple miR-127-3p up- or downregulation with the expression of a co-transcribed fluorescence reporter protein (mOrange fluorescent protein, OFF, for OE vector and destabilized Green Fluorescence Protein, dGFP, for DR vector respectively). For the OE vector, a 274bp fragment containing murine pre-miR-127 was PCR-amplified and cloned into the Xhol and Mulu sites inside the EF1a intron of lentiviral transfer plasmid #1394 (SFFV/EFintron.OFF; described by Lechman et al.). In the DR or ‘spponge’ vector, four tandem copies of an imperfectly complementary miR-127-3p target sequence were synthesized as described by Gentner et al. and cloned into the 3’ untranslated region (Xbal-Xmal sites) of the #1081scrT (LV.SFFV.dGFP) lentiviral backbone. Third-generation lentiviral vector particles pseudotyped with VSV-G were generated as described.

Ethics

The study was approved by the Humanitas Clinical and Research Center - IRCCS Institutional Ethical Committee (prot. n. CE Humanitas, as per Ministerial Decree 127/14 of 8/2/2013).

Statistical analysis

Data are represented as mean±Standard Error (SE) when n>3. The significance of differences was determined by two-tailed Mann-Whitney unpaired test unless otherwise stated. *P<0.05 was considered statistically significant (ns: not significant; *P<0.05; **P<0.005; ***P<0.0005; ****P<0.0001). Statistical analyses were performed with GraphPad Prism (GraphPad Software). Additional methods are presented in the Online Supplementary Methods.

are constantly required to replenish differentiated blood cells characterized by high turnover or to respond to peripheral injuries such as bleeding, a portion of HSC must awake from their dormant state and re-activate proliferation and differentiation programs. Thanks to their ability to correctly balance self-renewal and multi-potent differentiation the HSC pool size is maintained. However, the molecular pathways underlying this regulation are not completely understood, including the role of micro-RNA (miRNA). These are evolutionary conserved small non-coding RNA (ncRNA) that regulate miRNA stability and translation at the post-transcriptional level through non-perfect binding to target sequences.

To study the potential role for miRNA in HSC self-renewal, we took advantage of the Pbx1 conditional knockout (Pbx1-cKO) mouse model. Pbx1 is a homeodomain transcription factor that positively regulates HSC quiescence. Its absence in post-natal HSC causes an excessive proliferation that ultimately leads to their exhaustion, indicating a profound self-renewal defect, and a premature myeloid differentiation at the expense of lymphoid differentiation. Therefore, the study of Pbx1-deficient HSC provides the opportunity to identify miRNA involved in the maintenance of HSC identity.

We employed Pbx1-cKO mice (and controls) to perform miRNA profiling of HSC and their immediate downstream progeny multi-potent progenitors (MPP) characterized by the absence of the Flk2 marker on their cell surface (Flk2-MPP, previously known as short-term HSC), representing one of the very first differentiation steps from HSC, with similar multi-potent differentiation capacity but reduced self-renewal. This approach allowed the identification of an HSC-specific miRNA, miR-127-3p. By modulating its activity through lentiviral vectors we demonstrate that miR-127 acts as a novel brake on differentiation that HSC employ to maintain their pool.

Differential expression (DE) analysis was performed by Statistical Analysis of Microarray (SAM) (FDR with q-value <0.1).
Importantly, the expression of most of the DE miRNA changed more than 2-fold. Of note, the most up-regulated miRNA in the normal HSC-to-MPP transition is miR-221 (Online Supplementary Figure S1). This represents a positive control of our analysis, since miR-221 has been predicted to be an important regulator of HSC maturation due to its ability to repress cKit.15 We compared the lists of DE miRNA with those of DE transcripts obtained by microarray.6 None of the DE miRNA were different between mutant HSC or Flk2-MPP and corresponding controls were located within a transcript detected in the microarray, indicating that their different level of expression is not the result of the level of expression of a host gene. Among the 23 over-lapping miRNA (Figure 1C), we applied stringent criteria to select a few candidates for subsequent studies. We considered only miRNA that: 1) were DE also by applying a different normalization method; 2) had a fold difference higher than five; 3) had anti-correlated predicted targets (PT) among previously described DE miRNA in Pbx1-deficient HSC;4 4) were DE also with independent real-time PCR in wild-type (wt) samples. Only three miRNA candidates fulfilled each condition, all evolutionarily conserved and all down-regulated (miR-127-3p, miR-411-5p, miR-34b-3p). Among these, the most DE both in Pbx1-deficient HSC and in the normal HSC-to-MPP transition is miR-127-3p (Online Supplementary Table S3). This miRNA was therefore chosen as a candidate HSC self-renewal regulator.

**miR-127-3p is an HSC-specific miRNA**

As further proof that miR-127-3p downregulation correlates with loss of self-renewal, we confirmed its DE in phenotypically-defined HSC from a different previously described Pbx1-cKO mouse model (Tie2Cre+Pbx1 -/-) (Figure 2A).

We then prospectively isolated HSC and Flk2-MPP from wt mice and compared the expression level of miR-127-3p to that of other miRNA previously associated to HSC, such as miR-99b, miR-125, let-7, miR-221 and miR-126.11,15-19 We found that miR-127-3p expression in HSC is not particularly high, being similar to that of other miRNA already reported to play a role in HSC biology (Figure 2B). We confirmed the recently reported downregulation of miR-99b in Flk2-MPP10 (Figure 2C).

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**Table 1. Analyzed and/or sorted cell populations.**

| Cell population | Acronyms or abbreviation | Phenotype |
|-----------------|---------------------------|-----------|
| Stem and progenitor cells | LKS | Lin/c-Kit^+/Sca1^- |
| Hematopoietic Stem Cells | HSC | LKS/CD34+/Flk2^- for miRNA profiling; LKS/CD48^-/CD150^-/Flk2^- for analysis of transplanted mice; Sca1^-/CD48^-/CD150^-/EPCR^- for in vitro differentiation experiments |
| Multi-Potent Progenitors (at different stages of maturation) | MPP | LKS/CD34+/Flk2^- for miRNA profiling; LKS/CD127-/CD34+/Flk2^- or Flk2^+/EPCR^- for all other analysis |
| Lineage restricted progenitors | CLP | Lin/c-Kit^-/+ Sca1^- |
| Common Lymphoid Progenitors | CMP | Lin/c-Kit^-/+ Sca1^-/CD48^-/CD150^- |
| Granulocyte/Macrophage Progenitors | MEP | Lin/c-Kit^-/+ Sca1^-/CD41^- |
| Megakaryocyte/Erythrocyte Progenitors | Pre-MegE | Lin/c-Kit^-/+ Sca1^-/CD150^-/CD41^-/CD16^-/32^-/CD105^- |
| Erythrocyte Precursors (at different stages of maturation) | Pre-E | Lin/c-Kit^-/+ Sca1^-/CD16^-/32^-/CD105^-/CD150^- |
| Erythrocyte Precursors | Mkp | Lin/c-Kit^-/+ Sca1^-/CD16^-/32^-/CD105^-/CD150^- |
| From R1 stage to R4 | Ter119^-/CD44^- (R1); Ter119^-/CD44^+ (R2) | |
| Myeloid precursors and mature cells | PMN | CD11b^-/Ly6G^- |
| Poly-Morpho-Nucleated Leukocytes | CD11b^-/CD31^-/Ly6C^- |
| Monoblasts | CD11b^-/CD31^-/Ly6C^- |
| Pro-Monoocyte | CD11b^-/CD31^-/Ly6C^- |
| Monocyte | CD11b^-/CD31^-/Ly6C^- |
| Lymphoid precursors and mature cells | Pro-B | NK1.1^-/CD3^-/CD45^-/B220^- |
| Pre-B | NK1.1^-/CD3^-/CD45^-/B220^-/IgM |
| Immature B cells | NK1.1^-/CD3^-/CD45^-/B220^-/IgM |
| Mature B cells | NK1.1^-/CD3^-/CD45^-/B220^-/IgM |
| Natural Killer | NK1.1^-/CD3^-/CD45^-/B220^-/IgM |
| T cells | CD3^-/NK1.1^- |

Modified from Liu et al. Monoclonal antibodies are listed in Online Supplementary Table S4.
However, miR-127-3p showed the highest downregulation (>100-fold; miR-99b 3-fold) in the first step of hematopoietic differentiation, whereas the majority of the other miRNA are still expressed at the progenitor level and have only a modest reduction compared to HSC. We then used real-time PCR to investigate whether miR-127-3p is expressed in other hematopoietic cell subpopulations other than HSC within the BM in steady-state conditions. This issue was addressed by analyzing miR-127-3p expression from purified populations including different lineage committed progenitors, megakaryocyte and erythrocyte precursors, myeloid precursors and mature cells, and various lymphoid subsets (Table 1 and Figure 2D), since expression in only one subgroup would not be detectable in pooled populations. Impressively, miR-127-3p was highly HSC-specific, being quickly down-regulated in the earliest step of hematopoietic differentiation, where loss of self-renewal occurs. Furthermore, we confirmed its expression in human HSC-enriched CD34+ cells from different sources (Figure 2E).

**Inhibition of miR-127-3p function severely impairs HSC self-renewal**

To investigate the role of miR-127-3p in HSC we transplanted lineage-negative (Lin-) cells from CD45.2+ wt mice into lethally irradiated syngeneic CD45.1+ recipients, after permanently inhibiting binding to its targets through a lentiviral sponge vector carrying the reporter protein dGFP, as previously described for other miRNA. Since miR-127-3p targets in HSC are not known, we assessed effective downregulation of miR-127-3p activity on K562 cells. Upregulation of XBP-1 and BLIMP-1, two miR-127-3p targets, indicated that the sponge vector worked as expected (Online Supplementary Figure S2).

Engraftment and multipotent reconstitution by Lin-transduced cells was then monitored through periodic blood sampling of transplanted mice and at necropsy several weeks after transplant. The BM of some of the reconstituted mice was transplanted into secondary recipients to assess if, in the absence of miR-127-3p activity, HSC maintain their self-renewal ability (Figure 3A).

In primary recipients, survival curves were similar for all
transplanted mice (Online Supplementary Figure S2B). Similarly, the level of engraftment by sponge-transduced HSC (named 127DR for miR-127-3p functional downregulation), measured as the proportion of cells expressing the donor marker CD45.2 over time, was comparable to that of HSC transduced with a control vector carrying only dGFP (named EV for empty vector) (Figure 3B) or with untransduced cells (data not shown), with the exception of one mouse out of eleven. Sponge and control vectors express dGFP under the SFFV promoter, which is highly active in HSC, progenitors and myeloid cells, particularly monocytes. Due to its rapid turnover, dGFP is only detectable when expressed at very high levels. Therefore, even though the SFFV promoter has good and modest activity in B and T lymphocytes, respectively, the dGFP marker is hardly detectable in these lineages.22 For this reason, we followed by FACS analysis dGFP expression in monocytes; this also served as an indirect measure of dGFP expression in HSC since, due to their high turnover, myeloid cells are continuously generated from HSC. Virtually all monocytes were GFP+, indicating a very efficient transduction of long-term reconstituting cells (Figure 3C). Importantly, GFP-negative or low B and T lymphocytes isolated from the spleen of transplanted mice contained vector sequences (Online Supplementary Figure S2C), indicating that they derived from transduced cells. We therefore safely monitored the presence of transduced cells in vivo through the donor marker CD45.2, since we could not rely on dGFP expression in all cells.

The kinetics of peripheral myeloid, B- and T-cell reconstitution from donor cells was similar in mice transplanted with 127DR- or EV-transduced HSC, as well as the proportion and number of the different cell types (Figure 3D), suggesting that suppression of miR-127-3p activity did not affect multi-lineage reconstitution. However, FACS analysis of the BM at necropsy revealed a significant depletion of donor-derived phenotypically defined HSC, which were GFP+ (Figure 3E), without affecting their output of multi-potent progenitors and their lymphoid and myeloid differentiation potential, as confirmed on BM and spleen.
None of the transplanted animals displayed extramedullary hematopoiesis (data not shown), indicating that the observed HSC reduction was not due to abnormal egress from the BM.

To evaluate whether there was a decrease in functional HSC independently of the immunophenotype used to define them, secondary transplants were performed by injecting a high dose of total BM cells from two individual primary recipients in lethally irradiated CD45.1+ secondary recipient mice. The number of transplanted donor BM cells was sufficient for allowing recovery after irradiation; however, most of the mice transplanted with cells derived from 127DR BM succumbed 4-6 weeks after transplant, soon after the challenge of the first blood withdrawal, suggesting a severe HSC defect (Figure 4A). Four weeks after transplant, donor myeloid chimerism was very high in all mice, without significant differences among the groups of mice transplanted with 127DR or EV transduced BM (Figure 4B), indicating that the early mortality of 127DR BM-transplanted mice was likely not due to homing or engraftment impairment. However, sponging miR127-3p severely compromised myeloid cell and platelet produc-
in secondary recipients (Figure 4C and D), in accordance with a stem cell defect.

Two out of eleven mice transplanted with 127DR cells survived throughout the experiment, with very different levels of donor chimerism (Online Supplementary Figure S3A). Several weeks after transplant, we analyzed the most primitive hematopoietic compartment within the BM of the two secondary sponge recipients that were still alive. This analysis revealed that HSC were either absent (Figure 4E) or belonged to the recipient (Online Supplementary Figure S3B). LKS multipotent progenitors were donor-derived GFP+, indicating that HSC had originally engrafted at the time of the secondary transplant and were able to generate differentiated progeny throughout time; however, they failed to self-renew. Therefore, miR-127-3p activity is crucial for the maintenance of phenotypically and functionally defined HSC.

miR-127-3p prevents premature differentiation

To discern the biological mechanism at the basis of HSC loss, we tested if miR-127-3p is involved in regulating HSC quiescence, metabolic properties, survival, or prevention of premature differentiation, all necessary to preserve self-renewal. All these features were initially analyzed on Lin– cells isolated ex vivo from transplanted mice, as opposed to newly transduced cells, in order to avoid potential influences of cell culture.

The proportion of cycling and quiescent HSC was similar in 127DR- and EV-transduced BM (Figure 5A), as well as the proportion of apoptotic cells (Figure 5B), indicating that miR127-3p downregulation does not alter HSC cell cycle or apoptosis. Since oxidative stress has been described to negatively affect HSC function, we measured reactive oxygen species (ROS) production in response to cytokine stimulation, as described. ROS production induced upon short in vitro cytokine exposure was similar in HSC within Lin– cells isolated from 127DR or EV recipients (Figure 5C), suggesting that miR-127-3p downregulation does not lead to an increase in oxidative stress. However, after only one day of culture, a reduced expression level (measured by MFI) of the stem-cell associated cKit marker was observed in 127DR cells (Figure 5D), suggesting that miR-127-3p downregulation leads to accelerated differentiation. Lin– cells isolated from 127DR or EV recipients were also subjected to a standard colony assay (Figure 5E). 127DR stem and progenitor cells generated a significantly lower proportion of immature colonies compared to mature ones, in accordance with the hypothesis that they are more prone to differentiate. We confirmed this phenotype using freshly isolated HSC cultured in vitro. HSC were sorted from wt mice using the EPCR marker, transduced and analyzed at different time points. Single-cell colony assay performed early after transduction revealed that, despite the fact that the total number of colonies was comparable in the 127DR and EV groups, sponged cells generated fewer CFU-GEMM colonies, which originate from the most immature cells (Figure 5F). FACS analysis did not show any differences in the two groups when the conventional CD48, CD150 staining was performed (Online Supplementary Figure S4A); however, at day 6, the proportion of HSC expressing EPCR was reduced (Figure 5G and Online Supplementary Figure S4B). Moreover, a higher proportion of cells displayed early signs of differen-
tiation in cytospin preparation (Figure 5H). The proportion of cKit+ within dGFP+ cells was very high in both experimental groups at day 6. However, at day 9, it dropped significantly in cultures derived from sponged HSC, while this decrease was less dramatic in the EV group. Accordingly, cKit expression level was significantly lower at day 9 in the 127DR group (Figure 5I). Finally, the expression of genes with myeloid expression pattern (Gene Expression Commons\(^26\)) was higher in sponged cells from day 9 cultures compared to cells transduced with EV (Figure 5J).

Overall, these data suggest that a faster differentiation

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**Figure 5.** miR-127-3p prevents premature differentiation. (A) Cell cycle status of 127DR- and empty vector (EV)-transduced hematopoietic stem cell (HSC) was determined by FACS analysis with Ki67/Sytox staining on the bone marrow (BM) of primary recipients (n= 4 and 5, respectively). (B-D) FACS analysis of 127DR- and EV-transduced Lin– cells isolated ex vivo from the BM of primary recipients (n= 5 and 6). (B) Analysis of early apoptosis (AnnexinV'PI') in 127DR- and EV-transduced HSC identified by FACS within the Lin– cell population. (C) Reactive oxygen species (ROS) production in 127DR- and EV-transduced HSC in response to short (24 hours) cytokine stimulation in culture with StemSpan medium supplemented with SCF, TPO, Flt-3 Ligand and IL-3, measured by FACS after incubation with cellROX reagent. Vertical axis shows the ratio of the mean fluorescence intensity (MFI) at day (d) 1 versus mean fluorescence intensity (MFI) immediately after Lin– isolation (d0); (D) Early differentiation of ex vivo isolated 127DR and EV-transduced Lin– cells measured as reduction of cKit expression after one day of culture as in (C) (MFI ratio, d0/d1); (E) Colony forming cell (CFC) assay performed on ex vivo isolated 127DR- and EV-transduced Lin– cells (n=6). Colonies were scored as immature (CFU-GM and CFU-GEMM) and mature (CFU-M and CFU-G) at d9. Vertical axis shows the ratio between immature and mature colonies; (F) CFC assay performed on wild-type (wt) HSC two days after lentiviral transduction (d3 of culture, n=4 individual mice). (Left) Total number of colonies; (right) number of each different type of colonies; (G) FACS analysis for endothelial protein C receptor (EPCR) expression on GFP+cKit+CD48–CD150+ cells (n=4). (H) Representative cytospin preparations stained with May-Grunwald and Giemsa of 127DR- and EV-transduced HSC at d6 of culture. (I) FACS analysis for cKit expression at d6 and d9 (n=4); (J) Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of the expression of the indicated genes in transduced HSC at d5 of culture relative to d9 non-transduced cells (n=3).
to the next maturation step is the underlying cause of the observed HSC loss in vivo when miR-127-3p is not active.

** Forced miR-127-3p expression does not affect hematopoietic stem cell maintenance**

We next asked whether increased miR-127-3p levels affect HSC function in vivo. Lentiviral-mediated miR-127 overexpression was performed in wt CD45.2+ Lin- cells, followed by transplantation in CD45.1+ recipient mice (Online Supplementary Figure S5A). miR-127-overexpression (127OE) vector carried the Orange Fluorescent Protein (OFP) as reporter gene and the corresponding empty vector (EV), used as control. We obtained very high transduction efficiency with both vectors (data not shown). Expression analysis through real-time PCR before and after transduction confirmed that the progeny of infected cells expressed miR-127-3p, reaching levels similar to those of miR-16, which is present in all hematopoietic cells (Online Supplementary Figure S5B). Periodic PB analysis revealed that 127OE Lin cells were able to engraft and undergo multi-lineage differentiation, similar to Lin- cells transduced with the EV (data not shown). However, several weeks after transplant, peripheral B- and T-cell counts were significantly lower in mice transplanted with miR-127 over-expressing cells, compared to mice transplanted with the EV (Figure 6A). These differences were present also in BM (Figure 6B) and spleen (data not shown), and are consistent with the reported suppression of germinal center regulators by human miR-127,21,27 likely reflecting the lack of miR-127 downregulation from the MPP stage onward rather than upregulation in HSC. In mice transplanted with 127OE cells, the level of miR-127-3p expression in the spleen several weeks after transplantation was
miR-127 controls HSC maintenance

In this report we demonstrate that miR-127-3p is an important novel regulator of the balance between self-renewal and differentiation in HSC. The miR-127 gene is encoded in a large evolutionary conserved miRNA gene cluster located within an imprint-domain (14q32 in human, 12q3 in mouse), the Dlk1-Gtl2 locus. On the maternally imprinted allele, this locus encodes multiple long ncRNA, snoRNA and a mega-cluster of miRNA organized in two groups, among which the miR-127/miR-136 cluster composed of 7 miRNA genes. RNA-seq data showed that the entire locus, which is regulated by a common cis-element, is specifically transcribed in CD49blo long-term HSC compared to all other miR-127/miR-136 cluster encoded multiple long ncRNA, snoRNA and a mega-cluster of miRNA genes. The entire locus has been inactivated in the maternally-derived allele. Due to embryonic lethality, the consequences on hematopoiesis were studied using fetal liver stem and progenitor cells. The phenotype observed presented features similar to what we observed in mice transplanted with 127DRIcLin cells, such as reduction of HSC despite normal progenitor and lineage cell counts, and defective long-term reconstitution capacity. Altered PI3K-mTOR pathway affecting mitochondrial metabolism explained this phenotype, although other molecular mechanisms cannot be excluded. Our data on ROS production and apoptosis do not support the hypothesis that miR-127-3p is involved in regulating oxidative stress in HSC, and indeed miR-127-3p was not one of the miRNA for which a target within the PI3K-mTOR pathway was confirmed. A mouse model with specific deletion of miR-127 was generated; however, the effects on hematopoiesis have not been investigated.

miR-127 is aberrantly expressed in the context of several solid tumors, potentially acting as either an oncogene (oncomiR) or as a tumor suppressor. Within hematopoietic malignancies, miR-127 was found up-regulated, together with other miRNA included within the 14q32 domain, in the acute promyelocytic leukemia (APML) due to PML-RARA translocation. It would be worth investigating whether miR-127 aberrant expression contributes to the differentiation block in APML myeloid progenitors. Although miR-127-3p overexpression did not lead to myelo- or lymphoproliferation, we cannot exclude that, in the presence of other genetic abnormalities, miR-127-3p may act as an oncomiR.

A number of miR-127-3p targets have been found in cancer cells, but they are either not expressed in hematopoietic progenitors, or their expression does not change in the HSC-to-MPP transition. Our search for predicted miR-127-3p targets within DE mRNA in the HSC-to-MPP transition and in Pbx1-deficient HSC revealed two potential targets, Gp1bb and Nek2. The physiological downregulation of miR-127-3p observed in the transition from HSC to MPP coincides with the loss of self-renewal ability that is known to occur during this differentiation step. Moreover, miR-127-3p is absent in HSC obtained from Pbx1-cKO mice, which display a profound self-renewal defect. This stimulates future studies to investigate if Pbx1, together with its homeobox partners, directly regulates miR-127 expression, or if miR-127 OE can rescue part of the severe Pbx1-cKO phenotype. Our data strongly suggest that HSC must maintain proper levels of miR-127-3p to preserve their long-term self-renewal capacity. We also found that miR-29a, miR-99 and miR-126a are expressed in HSC but also...
present in Flk2-MPP although at lower levels (Figure 2B and C and Online Supplementary Table S1). However: 1) none of them was DE in HSC from our self-renewal defective mouse model; or 2) the downregulation was less than 3-fold. Among other miRNA whose function in the hematopoietic system has been previously established, miR-29a and miR-126 were shown to play a role in HSC self-renewal and to be differentially expressed in HSC compared to lineage-committed progenitors, but still highly expressed at the MPP stage, whereas miR-99 was recently found to be DE in the HSC-to-MPP transition. Interestingly, these miRNA regulate HSC pool maintenance through different biological mechanisms. miR-126 restrains HSC cell cycle entry, while miR-99 and miR-29a limit HSC maturation, similar to miR-127. However, the accelerated differentiation displayed by miR-127DR cells was not associated to increased apoptosis or altered cell cycle. This might explain why the effect of miR-127DR was only observed with secondary transplants, since HSC depletion was likely due to a gradual exit of HSC from the self-renewing pool. Likewise, it is not surprising that the accelerated differentiation did not result in an increased differentiated progeny in vivo, since: a) differentiation was not associated with increased proliferation; and b) miR-127 is not normally expressed beyond the HSC stage, therefore an effect of miR-127DR in more mature cell population is not expected. In conclusion, we identified miR-127 as novel player for preserving the HSC pool. Our discovery has potential implications for several translational aspects of experimental hematology, including hematopoietic malignancies, BM transplantation, and regenerative medicine.

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References

1. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631-644.
2. Wilson A, Laurenti E, Trumpp A. Balancing dormant and self-renewing hematopoietic stem cells. Curr Opin Genet Dev. 2009;19(5):461-468.
3. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Bio. 2014;15(8):509-524.
4. Picara E, Murphy MJ, Lin M, Cleary ML. Pbx1 regulates self-renewal of long-term hematopoietic stem cells by maintaining their quiescence. Cell Stem Cell. 2008;2(5):484-496.
5. Picara E, Crisafulli L, Lin C, et al. Pbx1 restrains myeloid maturation while preserving lymphoid potential in hematopoietic progenitors. J Cell Sci. 2018;126(Pt14):3101-3119.
6. Koss M, Bolze A, Brendolan A, et al. Congenital asplenia in mice and humans with mutations in a Pbx/Nkx2-5/P15 module. Dev Cell. 2012;22(2):913-926.
7. Deo A, Carlsson J, Lindlof A. How to choose a normalization strategy for miRNA quantitative real-time (qPCR) arrays. J Bionif Comput Biol. 2011;9(6):795-812.
8. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 2001;98(9):5116-5121.
9. Amendola M, Passerini L, Fucci G, Gentner B, Bacchetta R, Naldini L. Regulated and multiple miRNA and siRNA delivery into primary cells by a lentiviral platform. Mol Ther. 2009;17(6):1059-1052.
10. Gentner B, Schira G, Giustacchini A, et al. Stable knockdown of microRNA in vivo by lentiviral vectors. Nat Methods. 2009;6(1):63-66.
11. Lechman ER, Gentner B, van Galen P, et al. Attenuation of miR-126 activity expands HSC in vivo without exhaustion. Cell Stem Cell. 2012;11(6):799-811.
12. Follenzl A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-I pol sequences. Nat Genet. 2000;25(2):1217-1222.
13. Guenechea G, Gan OL, Inamitsu T, et al. Transduction of human CD34+ CD38- bone marrow and cord blood-derived SCID-repopulating cells with third-genera- tion lentiviral vectors. Mol Ther. 2000;1(6):566-573.
14. Chen C, Rudzun DA, Broomer AJ, et al. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 2005;33(20):e179.
15. Felli N, Fontana L, Pelosi E, et al. MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via a receptor down-modulation. Proc Natl Acad Sci U S A. 2005;102(50):18081-18086.
16. Gernits A, Walasek MA, Olthof S, et al. Genetic screen identifies microRNA cluster 99b/let-7/e125a as a regulator of primitive hematopoietic cells. Blood. 2012;119(2):577-587.
17. Guo S, Lu J, Schlanger R, et al. MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci U S A. 2010;107(2):14229-14234.
18. O’Connell RM, Chaudhuri AA, Rao DS, Gibson WS, Balazs AB, Baltimore D. MicroRNAs enriched in hematopoietic stem cells differentially regulate long-term hematopoietic output. Proc Natl Acad Sci U S A. 2010;107(32):14235-14240.
19. Ooi AG, Sahoo D, Adorno M, Wang Y, Weissman IL, Park CY. MicroRNA-125b expands hematopoietic stem cell enriches for the lymphoid-balanced and lymphoid-biased subsets. Proc Natl Acad Sci U S A. 2010;107(50):21505-21510.
20. Kajal M, Woolhuis CM, Hu W, et al. miR-99 regulates normal and malignant hematopoietic stem cell self-renewal. J Exp Med. 2017;214(8):2453-2470.
21. Leucci E, Onnis A, Cocco M, et al. B-cell differentiation in EBV-positive Burkitt lymphoma is impaired at posttranscriptional level by miRNA-altered expression. Int J Cancer. 2010;126(6):1516-1526.
22. Zanoni E, Fucci P, Saini M, et al. A role for miR-155 in enabling tumor-infiltrating innate immune cells to mount effective antitumor responses in mice. Blood. 2015;126(2):243-252.
23. Ito K, Hirao A, Ariai E, et al. Regulation of oxidative stress by ATM is required for self-renewal of hematopoietic stem cells. Nature. 2004;431(7011):997-1002.
24. Kent DG, Copley MR, Benz C, et al. Prospective isolation and molecular charac- terization of hematopoietic stem cells with durable self-renewal potential. Blood. 2009;113(5):6434-6438.
25. Kent DG, Dykstra BJ, Eaves CJ. Observation and Assessment of Single Long-Term Reconstituting Hematopoietic Stem Cells from Adult Mouse Bone Marrow. Curr Protoc Stem Cell Biol. 2016;50:4.A.1-2.A.4.24.
26. Seita J, Sahoo D, Rossi DJ, et al. Gene Expression Commons: an open platform for absolute gene expression profiling. F1000. 2012;7(7):e4021.
27. Chen J, Wang M, Guo M, Xie Y, Cong YS. miR-127 regulates cell proliferation and senescence by targeting BCL6. F1000. 2015;8(11):e2005.
28. Qian F, He XC, Paulson A, et al. The Dlk1-Gtl2 Locus Preserves LT-HSC Function by...
Inhibiting the PI3K-mTOR Pathway to Restrict Mitochondrial Metabolism. Cell Stem Cell. 2016;18(2):214-228.

29. Saito Y, Liang G, Egger G, et al. Specific activation of microRNA-127 with down-regulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006;9(6):435-445.

30. Lin SP, Youngson N, Takada S, et al. Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. Nat Genet. 2003;35(1):97-102.

31. Zhou Y, Cheunsuchon P, Nakayama Y, et al. Activation of paternally expressed genes and perinatal death caused by deletion of the Gtl2 gene. Development. 2010;137(16):2649-2652.

32. Ito M, Sferruzzi-Perri AN, Edwards CA, et al. A trans-homologue interaction between reciprocally imprinted miR-127 and Rtl1 regulates placenta development. Development. 2015;142(14):2425-2430.

33. Jiang H, Hua D, Zhang J, et al. MicroRNA-127-3p promotes glioblastoma cell migration and invasion by targeting the tumor-suppressor gene SEPT7. Oncol Rep. 2014;31(5):2261-2269.

34. Shi L, Wang Y, Lu Z, et al. miR-127 promotes EMT and stem-like traits in lung cancer through a feed-forward regulatory loop. Oncogene. 2017;36(12):1631-1643.

35. Wang Y, Kong D. Knockdown of IncRNA MEG3 inhibits viability, migration, and invasion and promotes apoptosis by sponging miR-127 in osteosarcoma cell. Cell Biochem. 2018;119(1):669-679.

36. Bi L, Yang Q, Yuan J, et al. MicroRNA-127-3p acts as a tumor suppressor in epithelial ovarian cancer by regulating the BAG5 gene. Oncol Rep. 2016;36(2):2563-2570.

37. Gao X, Wang X, Cai K, et al. MicroRNA-127 is a tumor suppressor in human esophageal squamous cell carcinoma through the regulation of oncogene FMNL3. Eur J Pharmacol. 2016;791:603-610.

38. Herr I, Sahr H, Zhao Z, et al. MiR-127 and miR-376a act as tumor suppressors by in vivo targeting of COA1 and FDN1 in giant cell tumor of bone. Cancer Lett. 2017;409:49-55.

39. Wang D, Tang L, Wu H, Wang K, Gu D. MiR-127-3p inhibits cell growth and invasiveness by targeting ITGA6 in human osteosarcoma. IUBMB Life. 2018;70(5):411-419.

40. Yu Y, Liu L, Ma R, Gong H, Xu P, Wang C. MicroRNA-127 is aberrantly downregulated and acted as a functional tumor suppressor in human pancreatic cancer. Tumor Biol. 2016;37(10):14249-14257.

41. Zhang J, Hou W, Chai M, et al. MicroRNA-127-3p inhibits proliferation and invasion by targeting SETDB8 in human osteosarcoma cells. Biochem Biophys Res Commun. 2016;469(4):1006-1011.

42. Dixon-McIver A, East P, Mein CA, et al. Distinctive patterns of microRNA expression associated with karyotype in acute myeloid leukaemia. PloS One. 2008;3(5):e2141.

43. Ma H, Lin Y, Zhao ZA, et al. MicroRNA-127 Promotes Mesendoderm Differentiation of Mouse Embryonic Stem Cells by Targeting Left-Right Determination Factor 2. J Biol Chem. 2016;291(23):12126-12135.

44. Zhai L, Wu R, Han W, Zhang Y, Zhu D. miR-127 enhances myogenic cell differentiation by targeting SFRP3. Cell Death Dis. 2017;8(5):e2707.

45. Han YC, Park CY, Bhagat G, et al. microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med. 2010;207(3):475-489.

46. Liu J, Zhang J, Ginzburg Y, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. Blood. 2013;121(8):e43-49.