miR-99 regulates normal and malignant hematopoietic stem cell self-renewal
Khalaj, Mona; Woolthuis, Carolien; Hu, Wenhao; Durham, Benjamin H.; Chu, S. Haihua; Qamar, Sarah; Armstrong, Scott A.; Park, Christopher Y.

Published in:
Journal of Experimental Medicine

DOI:
10.1084/jem.20161595

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
miR-99 regulates normal and malignant hematopoietic stem cell self-renewal

Mona Khalaj,2,3 Carolien M. Wootlhuys,2 Wenhuo Hu,2 Benjamin H. Durham,2 S. Haihua Chu,4 Sarah Qamar,2,3 Scott A. Armstrong,4 and Christopher Y. Park1

C.M. Wootlhuys’s present address is Dept. of Hematology, Cancer Research Center Groningen, University Medical Center Groningen, University of Groningen, Groningen, Netherlands.

INTRODUCTION

Acute myeloid leukemia (AML) is composed of functionally heterogeneous cells including leukemic stem cells (LSCs), which exhibit the ability to self-renew and propagate disease (Kreso and Dick, 2014). Because LSCs and normal hematopoietic stem cells (HSCs) display shared functional properties, it is not surprising that they are regulated by similar molecular pathways (Yilmaz and Morrison, 2008). The clinical importance of these observations is highlighted by the finding that AML transcriptomes enriched for HSC and LSC signatures are associated with worse prognoses (Gentles et al., 2010; Eppert et al., 2011; Metzeler et al., 2013). Thus, better understanding the mechanisms that regulate HSC function is likely to improve our understanding of not only HSCs, but also LSC function. Although several studies have identified numerous protein–coding genes that regulate HSCs and LSCs (Yilmaz and Morrison, 2008), it has become increasingly clear that noncoding RNAs also play prominent functional roles in these stem cell populations (Marcucci et al., 2011; Ciccone and Calin, 2015).

MicroRNAs (miRNAs) are small, non–protein–coding RNAs that regulate gene expression predominantly by binding to the 3′ UTR of mRNAs and promoting degradation of transcripts or inhibiting translation (Ha and Kim, 2014). These noncoding elements coordinate expression of targets from multiple signaling pathways, making them potential HSC and LSC regulators. miRNAs demonstrated to support HSC function have typically been studied because of their selective expression in HSCs. For example, miRNAs expressed at the highest levels in HSCs compared with committed progenitors, such as miR-125, miR-146a, and miR-29a/b1, promote HSC self-renewal (Guo et al., 2010; Ooi et al., 2010; Gerrits et al., 2012; Zhao et al., 2013; Hu et al., 2015), whereas others, such as miR-126, the miR-212/132 complex, and miR-193b, suppress HSC function (Lechman et al., 2012; Haetscher et al., 2015; Mehta et al., 2015). Moreover, the role of some of these miRNAs in myeloid leukemogenesis has been demonstrated, as high expression of miR-125 and miR-29a can induce myeloid leukemia (Bousquet et al., 2008, 2012; Han et al., 2010; Klusmann et al., 2010; O’Connell et al., 2010). Furthermore, individual miRNAs, such as miR-126, miR-196, and miR-21, as well as the polycistronic miR-17–92 cluster, promote LSC self-renewal (Wong et al., 2010; Velu et al., 2014; Lechman et al., 2016). Together, these
studies indicate that miRNAs are important regulators of normal and malignant stem cells.

Among the most highly expressed miRNAs in HSCs are members of the miR-99 family, a broadly conserved family that exhibits decreased expression upon differentiation (Ooi et al., 2010; Gerrits et al., 2012). One member, miR-99b, was recently identified as a potential LSC regulator, as it was enriched in AML patients whose transcriptomes exhibited a high "stem cell core enrichment" score (Metzeler et al., 2013). Another member, miR-99a, was the most significantly enriched miRNA in experimentally defined patient LSC populations (Lechman et al., 2016). Although these data suggest a role for miR-99 family members in both HSCs and LSCs, to date, a functional role for miR-99 has not been established. In fact, one study reported that miR-99b overexpression did not cause a significant change in HSC long-term repopulating capacity (Guo et al., 2010). Despite the lack of evidence of miR-99 regulation of HSCs, another group showed that enforced expression of miR-100, a miR-99 family member, inhibited differentiation of AML cells in vitro, suggesting a potential role for the miR-99 family in AML (Zheng et al., 2012); however, studies have yet to be performed to confirm this function in primary AML blasts or in a leukemia model in vivo. Because all miR-99 family members are expressed at high levels in HSCs and LSCs, we sought to determine the role of miR-99 in their maintenance. We used a loss-of-function approach to assess miR-99 function, because it is less prone to experimental artifacts (Concepcion et al., 2012). Using this strategy, we demonstrate that miR-99 is a critical regulator of both HSC and LSC self-renewal, primarily by inhibiting differentiation.

RESULTS

miR-99 supports hematopoietic stem cell clonogenic capacity

To identify miRNAs that regulate HSC function, we compared miRNA gene expression levels in mouse hematopoietic stem and progenitor cell (HSPC) populations (Chao et al., 2008). Remarkably, we found that all three members of the highly conserved miR-99 family are expressed at significantly higher levels in mouse HSCs compared with more differentiated populations (Fig. 1 A–C), suggesting they might play a role in maintaining HSC function.

To determine if miR-99 regulates HSCs, we first performed overexpression studies. Consistent with previous findings (Guo et al., 2010; Emmrich et al., 2014), enforced expression of miR-99 in HSCs did not significantly alter colony formation (Fig. S1 A). We hypothesized that the absence of a phenotype in the context of overexpression may indicate miR-99 is present in vast excess relative to its target genes and that loss-of-function studies might better reveal its biological function. Thus, we used a lentiviral vector expressing an antisense miR-99 oligonucleotide (anti–miR-99) to knock down miR-99 in Lin–c–Kit+Sca-1+CD34+CD150+ HSCs (Fig. S1 B). This approach allows for simultaneous knockdown (KD) of multiple miR-99 family members, helping address the potential redundant roles of miR-99 family members (Fig. 1 D). Using luciferase reporters containing 3’ UTRs of known miR-99 targets, SMARCA5 and HS2ST3 (Sun et al., 2011; Yang et al., 2015), we confirmed that anti–miR-99 relieves inhibition of miR-99 targets in a dose-dependent manner (Fig. S1, C and D). miR-99 KD in HSCs did not significantly alter the number of colonies in the first plating in methylcellulose. However, more CFU-M colonies were formed at the expense of CFU-GM and BFU-E colonies (Fig. 1 E). Upon secondary plating, miR-99 KD also led to reduced colony size and decreased serial replating capacity (Fig. 1, F and G). miR-99 KD also resulted in increased relative numbers of CFU-M colonies in secondary platings (Fig. S1, E and G), supporting a monocytic differentiation bias in miR-99 KD HSPCs. Comparable reductions in colony number were observed using a second anti–miR-99 construct (vector 2; Fig. S1 F). The decrease in HSC colony formation was accompanied by accelerated myelopoiesis, as evidenced by an increased proportion of Gr1+Mac1+ cells upon miR-99 KD (Fig. 1 H). In addition, total GFP+ miR-99 KD cells exhibited increased apoptosis at the end of the first plating (Fig. S1 G). Consistent with these observations, liquid culture of miR-99 KD HSCs showed a reduced absolute number of HSCs 8 d after transduction (Fig. 1 I). Collectively, these observations demonstrate that miR-99 helps maintain HSPCs in vitro by suppressing their differentiation.

miR-99 regulates HSC long-term reconstitution capacity

Because miR-99 is required to preserve HSC clonogenic capacity in vitro, we sought to test whether miR-99 maintains HSCs in vivo. Transplant of anti–miR-99–transduced HSCs demonstrated a significant reduction in long-term reconstitution capacity compared with scramble (Scr) controls (Fig. 2 A). The difference in engraftment was not caused by defects in homing, as HSCs expressing anti–miR-99 or the Scr control had similar numbers of GFP+ HSCs in the BM 24 h post-transplant (Fig. S2A). In agreement with the in vitro experiments, the peripheral blood (PB) of mice harboring miR-99 KD HSCs showed a significant increase in the proportion of donor-derived Mac1+ myeloid cells (Fig. 2 B). Further analysis of the PB showed that all lineages, including B cells (B220+), T cells (CD3+), granulocytes (Gr1+ Mac1+), and monocytes (Gr1neg Mac1+), exhibited reduced GFP+ chimerism, with the reduction being more prominent in the lymphoid lineage (Fig. S2, B–E). Analysis of the BM in miR-99 KD stable grafts revealed reduced GFP chimerism, consistent with the reduced donor chimerism observed in the PB (Fig. S2 F). Mice transplanted with miR-99 KD HSCs also displayed decreased absolute numbers of GFP+ HSCs (Fig. 2 C) and multipotent progenitors (Fig. 2, D and E). Together, these data demonstrate that miR-99 maintains HSC reconstitution potential.

We next sought to determine the mechanistic basis for the alterations in miR-99 KD HSC function. Analysis of the
miR-99 is highly expressed in hematopoietic stem and progenitors and suppresses myeloid differentiation in vitro. (A–C) Normalized expression levels of miR-99b, miR-99a, and miR-100 as determined by quantitative RT-PCR using miRNA TaqMan probes in mouse hematopoietic cell populations: hematopoietic stem cell (HSC), multipotent progenitor (MPP) Flk−, MPP Flk+, common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte-macrophage progenitor (GMP), and megakaryocyte-erythroid progenitor (MEP) cells. Expression was normalized against mmu-mir-16. Error bars denote SEM. Representative data from five independent experiments are shown. (D) miR-99 is down-regulated 48 h post-transduction of HSCs with the lentiviral anti–miR-99 vector as shown by quantitative RT-PCR. Expression was normalized against U6 (Student’s t test; n = 3). Representative data from two independent experiments are shown. (E) Comparable number of colonies form after miR-99 KD in first plating, with an increase in the number of CFU macrophage (CFU-M) colonies. 100 GFP+ HSC cells were cultured in methylcellulose. The colonies were scored after 7 d. Data represent mean percentage ± SEM (Student’s t test; n = 3) and are representative of three independent experiments. (F) Smaller colonies were observed after second plating of GFP+ cells derived from miR-99 KD HSCs. Representative data of three independent experiments are shown. (G) Colony-forming capacity of HSCs is reduced after miR-99 KD in a second plating: 15,000 GFP+ cells were replated 7 d after the first plating. Colony types were scored after 7 to 10 d. Data represent mean ± SEM (Student’s t test; n = 3) and are representative of three independent experiments. (H) miR-99 KD in HSCs induces granulocytic differentiation in methylcellulose colony assays. 7 d after plating, colonies were analyzed for expression of myeloid differentiation markers by flow cytometry. Mean percentage ± SEM (Student’s t test; n = 2). Representative data of three independent experiments are shown. (I) Flow cytometry analysis of LSK cells transduced with anti–miR-99 or Scr vectors and maintained in liquid culture for 8 d reveals a decrease in the absolute number of GFP+Lin−Sca-1−c-Kit−CD150+ HSCs. FSCw denotes forward scatter-width. The data shown are gated on LSK cells. Data represent mean count ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 2. 

**miR-99 inhibition impairs HSC reconstitution capacity in vivo by inducing differentiation and increased cell cycling.** (A) GFP+ chimerism of mice transplanted with miR-99 KD HSCs. HSCs were transduced with anti–miR-99 or scramble (Scr) control vectors, and 48 h later, 5,000 GFP+ cells were transplanted into lethally irradiated recipients along with 300,000 cells from Sca-1–depleted helper BM. Peripheral blood GFP chimerism was analyzed every 4 wk. Data represent mean percentages ± SEM (Student’s t test; n = 11 for Scr and n = 13 for miR-99 KD mice) and are representative of two independent experiments. Post tx, post-transplantation. (B) Flow cytometry analysis of the peripheral blood every 4 wk after transplantation of HSCs transduced with anti–miR-99 or Scr vectors. Data represent mean percentages ± SEM (Student’s t test; n = 11 for Scr and n = 13 for miR-99 KD mice) and are representative of two independent experiments. (C–E) Absolute number of GFP+Lin−c-Kit+Sca-1−CD48−CD150+ HSCs (C), CD48+CD150+ MPPa’s (multipotent progenitors a; (D), and CD48−CD150neg  MPPb’s (multipotent progenitors b; E) in bilateral long bones and hips 16 wk post-transplantation of HSCs. Data represent mean count ± SEM (Student’s t test; n = 4) and are representative of two independent experiments. (F) Ki-67/DAPI staining of donor-derived GFP+Lin−c-Kit+ HSPCs 6 mo post-transplant of miR-99 KD or Scr HSCs. Data represent mean percentage ± SEM (Student’s t test; n = 7 for Scr and n = 8 for miR-99 KD) and are representative of two independent experiments. (G) RNA-seq analysis of LSK cells FACS sorted from BM of mice transplanted with miR-99 KD or scramble control HSCs 3 mo after transplant (n = 2). (H and I) Gene set enrichment analysis for differentially expressed genes in stably engrafted miR-99 KD versus Scr LSK cells. FDR, false discovery rate; NES, normalized enrichment score. (J) Annexin V staining of total GFP+ cells in the BM 6 mo post-transplant. Data represent mean percentage ± SEM (Student’s t test; n = 7 for Scr and n = 8 for miR-99 KD) and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
BM revealed that miR-99 KD induced a significant increase in the percentage of cycling lineage-negative Sca1-positive c-Kit-negative (LSK) cells and progenitor cells (Fig. 2 F). To investigate the molecular alterations induced with miR-99 inhibition, we performed RNA sequencing (RNA-seq) on GFP+ miR-99 KD or Scr LSK cells 3 mo after stable engraftment into lethally irradiated recipients (Fig. 2 G). Gene set enrichment analysis (GSEA) revealed depletion of genes constituting previously defined HSC signatures, including c-Kit, Mpl, Hoxa9, Meis1, Tie1, and Angpt1 (Ivanova et al., 2002; Subramanian et al., 2005; Jaatinen et al., 2006). In addition, miR-99 inhibition resulted in enrichment for genes associated with more differentiated progenitors, further confirming miR-99 maintains HSCs in an undifferentiated state (Fig. 2, H and I; Ivanova et al., 2002). Although total GFP+ miR-99 KD BM cells displayed increased apoptosis (Fig. 2 J), there were no differences in apoptosis in Scr control versus miR-99 KD LSK cells or progenitors (Fig. S2, G and H). These findings suggest that at least at the level of LSK and committed progenitors, the major effect of miR-99 KD is to induce differentiation, leading to their depletion, rather than apoptosis. Overall, these results demonstrate that miR-99 maintains mouse HSCs in a quiescent and undifferentiated state.

miR-99 maintains LSCs in MLL-AF9+ acute myeloid leukemia

To investigate whether miR-99 may play a role in malignant hematopoiesis, we analyzed the miRNA-sequeencing data from 153 AML patients from The Cancer Genome Atlas (TCGA) database. Our analysis revealed that expression of miR-99 family members inversely correlates with AML differentiation. Genes enriched in miR-99 KD mice displayed increased apoptosis (Fig. S2 I; Ivanova et al., 2006). We used anti–miR-99 vector 2 to perform these studies because it knocked down miR-99a to levels comparable to the anti–miR-99 vector 1 but was a more efficient inhibitor of miR-99b expression in human AML cells (Fig. S2 J and not depicted). To generate leukemia, LSK cells were transduced concomitantly with an MLL-AF9–expressing vector (tandem dimer Tomato [tdTom]–positive MIT retrovirus) and the retroviral anti–miR-99 vector (GFP+ LMN vector; DiCicco et al., 2005; Chen et al., 2013; Fig. S2 J). Efficient KD of both miR-99a and miR-99b by anti–miR-99 was confirmed by quantitative RT-PCR (Fig. S2, K and L). Transduced cells were transplanted into sublethally irradiated animals. Although miR-99 KD mice displayed reduced splenic involvement, evidenced by decreased c-Kit+ cells and fewer LSCs (Fig. S3, A and B), these primary recipients did not exhibit differences in overall survival (Fig. S3 C). Colony-formation assays using leukemic blasts from the BM of primary recipients revealed significant reduction in colony-forming ability upon miR-99 KD, demonstrating that miR-99 regulates MLL-AF9+ AML maintenance (Fig. 3, B and C).

To assess the role of miR-99 in the maintenance of established post-transformed AML, we secondarily transplanted leukemic blasts from the BM of primary recipients. Mice transplanted with miR-99 KD blasts exhibited a significant improvement in survival (median survival 48 d in Scr vs. 92 d in miR-99 KD recipients; Fig. 3 D). miR-99 family members have been shown to be highly expressed in LSC-enriched populations from patient AML samples (Metzeler et al., 2013; Lechman et al., 2016). Thus, we asked whether the improved survival is mediated through depletion of the LSC compartment. As leukemic granulocyte–macrophage progenitors (L-GMPs) are the LSCs in the MLL-AF9 model of AML (Krivtsov et al., 2006; Somervaille and Cleary, 2006; Stubbs et al., 2008), we analyzed the BM of secondary recipients at the time of sacrifice for alterations in L-GMP frequency. This analysis revealed a reduction in the percentage of L-GMPs in mice transplanted with miR-99 KD blasts (Fig. 3, E and F). To further evaluate the effect of miR-99 KD on LSC function, we performed limiting dilution assays using MLL-AF9 blasts from primary recipients. Leukemia-initiating cell frequency was significantly reduced after transduction with anti–miR-99 (Fig. 3 G). Collectively, these data demonstrate that miR-99 is a critical positive regulator of LSC self-renewal.

miR-99 maintains LSCs self-renewal by inhibiting differentiation

We next asked whether the decrease in L-GMP induced by miR-99 KD was caused by alterations in differentiation and/or cell cycling of these cells, similar to the phenotype observed in LSK cells. Consistent with this hypothesis, L-GMPs from animals transplanted with miR-99 KD MLL-AF9 AML displayed an increased proportion of cycling cells (Fig. 4 A). This phenotype was associated with a dramatic increase in the number of peripheral blood white blood cells (WBCs; Fig. 4 B). Because secondary transplantation of miR-99 KD blasts resulted in improved survival, we hypothesized that the WBCs were composed of more differentiated cells as opposed to blasts, a phenomenon that has been described with AML differentiation–inducing agents used in the clinic, such as ATRA and IDH1 inhibitors (Nowak et al., 2009). As expected, Wright–Giemsa staining of PB smears revealed that WBCs from miR-99 KD mice displayed greater differentiation (Fig. 4 C). Moreover, miR-99 KD mice displayed a significant increase in the absolute number of c-Kit+ cells in the BM, compatible with induction of differentiation (Fig. 4 D).

To investigate the molecular mechanisms underlying miR-99 KD–induced AML differentiation, we performed RNA-seq on L-GMPs sorted from secondary transplant recipients (Fig. 4 E). GSEA revealed that miR-99 KD induces genes up-regulated in normal hematopoietic progenitors compared with HSCs, including Irf8 and Met (Ivanova et al., 2008).
et al., 2002), and this finding was validated by quantitative RT-PCR (Fig. 4, F and G). To identify an expression signature that enriches for leukemia differentiation genes, we used publicly available RNA-seq data to generate a gene set that is up-regulated in normal GMPs compared with MLL-AF9 L-GMPs ($P_{adj} < 0.05$; Krivtsov et al., 2006; Table S1). Remarkably, L-GMPs from miR-99 KD mice exhibited enrichment for this gene set, confirming that miR-99 suppresses...
Figure 4. *miR*-99 KD depletes LSCs in the MLL-AF9 mouse model of AML by inducing differentiation. (A) Representative flow cytometry graph and the corresponding bar graph for the cell cycle staining performed on L-GMPs FACS sorted from the BM of secondary MLL-AF9 transplantation recipients. Data represent mean percentage ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. (B) White blood cell (WBC) count of the secondary transplantation recipients. Data represent mean count ± SEM (Student’s t test; n = 6) and are representative of three independent experiments. (C) Wright–Giemsa staining of the peripheral blood from secondary transplant recipients, and summary of cytological features based on a 200-cell manual count per condition (bars, 25 µm). (D) *miR*-99 KD increases the absolute number of normal c-Kit+/GFP−/tdTom+ cells in the BM. Data represent mean count ± SEM (Student’s t test; n = 5 for Scr and n = 3 for *miR*-99 KD) and are representative of three independent experiments. (E) Heat map of RNA-seq data generated from L-GMPs derived from the BM of secondary transplantation recipients with or without *miR*-99 KD at the time of death.
differentiation in LSCs (Fig. 4 H). Together, these data indicate that miR-99 maintains LSCs in an MLL-AF9 mouse model of AML in the undifferentiated and quiescent state.

### miR-99 suppresses differentiation in human AML

Given that the mature sequences of all miR-99 family members are identical in mouse and human, we hypothesized miR-99 might exert similar biological effects in both mouse and human. To directly assess the roles of miR-99 in human hematopoiesis, we first evaluated miR-99 expression in different HSPC populations from normal human BM, and we found that miR-99 family members display a trend in expression similar to that seen in mice, with the highest expression levels in HSPCs (Fig. 5 A). To determine whether miR-99 maintains human HSPCs, we knocked down miR-99 in CD34+ umbilical cord blood cells and performed methylcellulose colony assays. miR-99 KD resulted in decreased colony formation, similar to studies using mouse HSPCs (Fig. 5 B).

To determine whether miR-99 also regulates human AML blast function, we knocked down miR-99 in the MonoMac6 AML cell line, which harbors an MLL-AF9 rearrangement (Super et al., 1995). miR-99 KD increased CD14+ and CD15+ myelomonocytic differentiation (Fig. 5 C). Evaluation of Wright–Giemsa–stained cytospins confirmed maturation of blasts with reduced nuclear to cytoplasmic ratios and increased cytoplasmic granules (Fig. 5 D). These observations demonstrate miR-99 suppresses differentiation in MonoMac6 cells. Similar to the effects observed in normal mouse HSPCs, miR-99 KD also induced apoptosis (Fig. S3 E).

To test whether miR-99 is required for the maintenance of human AML in vivo, we xenotransplanted miR-99 KD and Scr MonoMac6 cells (Fig. 5 E). After 4 wk, miR-99 KD leukemic cells showed a significant reduction in engraftment levels as well as increased CD14+ monocytic differentiation (Fig. 5 F and G). To investigate the effect of miR-99 KD on patient primary AML blasts, we performed in vitro differentiation assays. miR-99 KD induced CD14+ monocytic differentiation in two of three samples (Fig. 5 H). Collectively, these results replicate our findings in the MLL-AF9 mouse model of AML and demonstrate that miR-99 similarly suppresses differentiation in human AML blasts.

### An shRNA screen identifies functional miR-99 targets

We next sought to identify miR-99 targets that mediate its effects on Lin−Sca-1−c-Kit+ (LSKs) cells by performing an shRNA library screen against candidate miR-99 targets to test their ability rescue miR-99 KD-induced HSPC clonogenic defects; such shRNAs would be predicted to be enriched upon serial replating of miR-99 KD LSK cells. To design a list of genes for inclusion in the shRNA library, we acutely knocked down miR-99 in LSK cells followed by RNA-seq after 48 h to determine which of the predicted target genes are likely direct targets of miR-99 (Fig. 6 A). Consistent with induction of differentiation, genes up-regulated after miR-99 KD included cytokine and Toll-like receptor signaling pathways (Miranda and Johnson, 2007; Fig. 6 B). Furthermore, GSEA revealed significant induction of the NF-κB pathway and chemokine signaling (Fig. S3 F). We also observed genes induced with differentiation of HSCs into committed progenitors including Cdl4, Cdl86, I17R, and Ccl5 (Fig. 6 C; Jaatinen et al., 2006). To include all potential genes mediating the phenotype, we compiled miR-99 predicted target genes from multiple algorithms and generated a list of 344 miR-99 targets (Table S2 A). 4,179 genes showed increased expression in the miR-99 KD experimental group (log2 fold change >0.2; Table S2 B), including 81 predicted target genes (Fig. S3 G and Table S2 C). We generated shRNAs against 45 candidate genes based on their miRNA-binding scores and also included shRNAs against two genes (Tet2 and Tgif1) previously shown to increase colony formation when knocked down as positive controls (Moran-Crusio et al., 2011; Yan et al., 2013). LSK cells were cotransduced with the shRNA library and anti–miR-99 vectors and then FACs sorted into methylcellulose and replated after 10 d. Genomic DNA (gDNA) was extracted from the resulting colonies to assess retroviral integrants (Fig. 6 E). The relative representation (enrichment score) of each shRNA was calculated immediately after transduction (T0) and at the end of the secondary plating for each replicate. Enrichment scores after secondary plating were normalized to T0 enrichment scores (i.e., the normalized enrichment score). Average normalized enrichment scores from three biological replicates were used to rank the genes, and genes predicted to be targeted by miR-99 in both mouse and human were prioritized for functional validation. As expected, the positive controls were among the most highly enriched shRNAs, validating the robustness of the screen (Fig. 6 F). Among the top enriched target genes shared between mouse and human were a number of genes including the ribonucleoprotein Raver2, bone morphogenetic protein receptor type II (Bmpr2), and the transcription factor Hoxa1 (Fig. 6 F and Table S2 G).

### miR-99 inhibits differentiation by targeting HOXA1

Among the top enriched genes identified in our shRNA screen, we prioritized Hoxa1 for functional validation, because all four shRNAs designed against this gene exhibited enrichment (Fig. 6 G). In addition, Hoxa1 has been shown to exhibit an inverse expression pattern to miR-99 in several experiments. (G) TaqMan quantitative RT-PCR analysis of Met and Irf8 in miR-99 KD and Scr L-GMPs sorted from secondary recipients. Expression data are normalized to Actb (Student’s t test; n = 3) and are representative of two independent experiments. (H) GSEA of miR-99 KD and scramble control L-GMPs reveals induction of genes present in a signature up-regulated in normal GMPs compared with L-GMP (Table S1). **, P < 0.01; ***, P < 0.001.

(n = 2). (F) GSEA of differentially expressed genes in miR-99 KD and scramble control L-GMPs shows induction of a more differentiated signature.
Figure 5. miR-99 functionally suppresses human AML differentiation. (A) Normalized expression levels of miR-99a, miR-99b and miR-100 by quantitative PCR using miRNA TaqMan probes in human HSPCs, including hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs). Expression was normalized to sno-R2. Data represent mean ± SEM and are representative of five independent experiments. (B) The colony-forming capacity of CD34+ human cord blood cells is reduced after miR-99 KD. CD34+ cells were transduced with lentiviral anti-miR-99 or scramble control. GFP+ cells were isolated and cultured in complete methylcellulose, and the colonies were scored after 14 d. Data represent mean ± SEM (Student's t test; n = 3) and are representative of two independent experiments. (C) Flow cytometric evaluation of myeloid differentiation marker expression on MonoMac6 AML cells 5 d after transduction with anti–miR-99 or scramble control. Data represent mean ± SEM (Student's t test; n = 3) and are representative of three independent experiments. (D) Wright–Giemsa stains of cytospin preparations of MonoMac6 cells 8 d post-transduction with lentiviral anti-miR-99 or Scr reveals induction of differentiation upon miR-99 KD (bars, 25 µm). (E) Overview of the xenotransplantation experiment performed on MonoMac6 AMLs. Cells were transduced with anti-miR-99 or Scr. After 48 h, GFP+ cells were sorted, and 800,000 cells were transplanted into sublethally irradiated NSGs. BM was analyzed 4 wk after the transplant. (F) miR-99 KD reduces the of GFP+ engraftment of MonoMac6 cells in the BM of the recipient animals 4 wk post-transplantation. Data represent mean percentage ± SEM (Student's t test; n = 4) and are representative of two independent experiments. (G) Representative histogram and aggregated data from flow cytometric evaluation of CD14 expression on GFP+ xenografted cells in the BM of the recipient animals 4 wk post-transplantation. Data represent mean percentage ± SEM (Student's t test; n = 4) and are representative of two independent experiments. (H) Flow cytometry analysis of three AML patient samples after miR-99 KD. Patient samples were transduced with anti–miR-99 or scramble control and analyzed for the expression of differentiation markers 5–8 d later. Data represent mean percentage ± SEM (Student's t test; n = 2) and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 6. **Forward genetic screen identifies miR-99 target genes.** (A) Heat map shows genes differentially expressed between scramble control and miR-99 KD LSK cells. RNA-Seq was performed on LSK cells 48 h after transduction with miR-99 KD or scramble control lentivirus. Infected cells were FACS sorted based on GFP expression (n = 3 for control and n = 2 for miR-99 KD). (B) Functional annotation of genes up-regulated in LSK cells after miR-99 KD.
HOX1 is shown to be a conserved target of the miR-99 family using luciferase assays (Chen et al., 2014; Wang et al., 2015). Moreover, HOX1 is shown to be a conserved target of the miR-99 family in mouse and human (Fig. S3 J).

To investigate whether HOX1 mediates the effects of miR-99 on differentiation, we overexpressed HOX1 in MonoMac6 cells, where we previously demonstrated miR-99 normally inhibits differentiation. Confirming miR-99 targeting of HOX1 in the hematopoietic context, miR-99 overexpression reduced HOX1 mRNA expression (Fig. 7 A), whereas miR-99 KD increased HOX1 mRNA expression (Fig. 7 B). Although Hox family members are generally thought to promote self-renewal (Argiropoulos and Humphries, 2007), enforced expression of HOX1 (Fig. S3 K) induced myeloid differentiation in MonoMac6 cells as evidenced by increased expression of CD14 and CD15 by flow cytometry (Fig. 7 C, D). Wright–Giemsa staining confirmed increased differentiation of leukemic blasts demonstrated by acquisition of cytological features consistent with myelomonocytic maturation (Fig. 7 E). As expected, enforced expression of HOX1 fully mimicked the miR-99 KD phenotype, as the increased differentiation was also associated with reduced cell growth and induction of apoptosis (Figs. 7 F, G). Together, these data demonstrate that miR-99 suppresses differentiation in MonoMac6 cells by targeting HOX1.

To investigate whether HOX1 mediates miR-99 function in the stem cell compartment, colony-forming assays were performed on Lin−c−Kit+Sca-1+ cells in which miR-99 and HOX1 were simultaneously suppressed. HOX1 KD efficiency was confirmed by quantitative RT-PCR (Fig. 7 H). Consistent with our shRNA library screen results, HOX1 KD in LSK cells reduced the rescue in colonies formed upon secondary plating of miR-99 KD cells (Fig. 7 I). Furthermore, simultaneous KD of HOX1 increased the size of colonies that were smaller with miR-99 KD alone (Fig. 7 J). Together, these data confirm HOX1's central role in mediating miR-99 KD phenotypes in normal hematopoiesis.

To investigate the role of HOX1 in MLL-AF9 AML, we transduced blasts from primary MLL-AF9 miR-99 KD AMLs with the more potent HOX1 shRNA virus (shHOX1-#2; Fig. 7 H). The infected cells (GFP+GFP+ mCherry+) were transplanted into sublethally irradiated mice (Fig. 7 K). miR-99 KD resulted in improved survival as expected, whereas simultaneous KD of HOX1 rescued this phenotype and shortened survival (Fig. 7 L). To test whether HOX1 KD affects LSC function, we analyzed the BM of the leukemia-engrafted animals at the time of death. miR-99 KD resulted in decreased L-GMPs, whereas simultaneous KD of HOX1 reversed this effect (Fig. 7 M). Analysis of L-GMPs from secondary recipients confirmed the expected changes in HOX1 mRNA expression, with miR-99 KD resulting in higher levels of HOX1 and HOX1 KD reversing this effect (Fig. 7 N). Collectively, these data demonstrate that miR-99 mediates its role in MLL-AF9 AML LSCs by suppressing HOX1.

**DISCUSSION**

Using a combination of in vitro and in vivo approaches, our studies demonstrate that miR-99 is a critical regulator of self-renewal in both HSC and LSCs. miR-99 maintains stem cell function by suppressing differentiation, and this role is conserved in both mouse and human hematopoiesis. Although gene expression studies including our own had suggested that miR-99 might serve a functional role in HSCs and LSCs, prior studies using overexpression approaches failed to demonstrate a function for miR-99 in HSC regulation. However, our loss-of-function approach reveals a significant effect of miR-99 on HSC and LSC self-renewal. These findings suggest targeting miR-99 is a promising therapeutic strategy in contexts where depletion of one or both populations is desirable, such as eradication of preleukemic HSCs and LSCs in AML as well as HSC differentiation therapies for diseases that arise in HSCs, such as CML and MDS.

miRNAs exert their biological effects by inhibiting multiple downstream target genes (Lewis et al., 2005; Bartel,
Figure 7. **HOXA1 mediates miR-99 function in normal and malignant hematopoiesis.** (A) TaqMan quantitative RT-PCR for HOXA1, BMPR2, and RAVER2 expression upon miR-99 overexpression in MonoMac6 AML cells 48 h post-transduction. Expression was normalized to ACTB (Student’s t test; n = 3). Representative data from two independent experiments are shown. (B) TaqMan quantitative RT-PCR for HOXA1, BMPR2, and RAVER2 expression upon miR-99 KD in MonoMac6 AML cells 48 h post-transduction. Expression was normalized to ACTB (Student’s t test; n = 3). Representative data from two independent experiments are shown.

**Legend:**
- Scr: Control
- miR-99 OE: miR-99 overexpression
- miR-99 KD: miR-99 knockdown

**Graphs:**
- **A:** Relative gene expression
- **B:** Relative gene expression
- **C:** %CD15+ within GFP+
- **D:** %CD4+ within GFP+
- **E:** Representative data from two independent experiments
- **F:** Absolute number of GFP+ cells (X10²)
- **G:** %Annexin V+
- **H:** Relative HOXA1 expression
- **I:** Number of colonies /15000 GFP+ cells plated
- **J:** Representative data from two independent experiments
- **K:** Schematic diagram
- **L:** Percent survival
- **M:** %LGMP in GFP+ mCherry+ cells
- **N:** Relative HOXA1 expression normalized to Scr
two independent experiments are shown. (C and D) Flow cytometry analysis of MonoMac6 cells 4 d after transduction with HOXA1-overexpressing virus. miR-99 KD induces the myeloid differentiation markers CD15 (C) and CD14 (D). Data represent mean percentage ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. (E) Wright–Giemsa staining of MonoMac6 cells 4 d post-transduction with miR-99 KD (bars, 25 μm). (F) Growth curve for MonoMac6 cells as a function of time after transduction. Data represent mean count ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. (G) Representative flow cytometry graph and the corresponding diagram depicting Annexin V apoptosis assay with HOXA1 overexpression in MonoMac6 cells. Data represent mean percentage ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. (H) TaqMan quantitative RT-PCR for Hoxa1 expression 48 h after shHoxa1 transduction of LSKs. Expression was normalized to Actb (Student’s t test; n = 3). Representative data from two independent experiments are shown. (I) Hoxa1 KD partially rescues colonies reduced upon miR-99 KD. LSK cells were infected with anti-miR-99 (GFP+) and shHoxa1 (mCherry+) viruses. 2 d post-transduction, the resulting GFP+ mCherry+ cells were sorted into Methocult M3434 and replated after 7 d. Shown are the results from secondary colonies, which were scored 10 d after plating. Data represent mean count ± SEM (Student’s t test; n = 3) and are representative data of two independent experiments. (J) Simultaneous KD of Hoxa1 in miR-99 KD LSKs increases the size of miR-99 KD colonies. Shown are representative data from secondary plateings. (K) Schematic for Hoxa1 KD LSC rescue experiments. miR-99 KD MLL-AF9 (GFP+ tdTom+) BM blasts from primary recipients were transduced with shHoxa1 (mCherry+), and the resulting GFP+ tdTom+ mCherry+ cells were transplanted into sublethally irradiated mice. (L) Kaplan–Meier curves of mice transplanted with MLL-AF9 blasts. 100,000 BM blasts from primary recipients of the indicated genotypes were transplanted into sublethally irradiated secondary recipients (Mantel–Cox test; n = 8 per condition). Representative data from two independent experiments are shown. (M) Flow cytometry analysis of L-GMPs from the BM of secondary recipients. Cells were pregated on GFP+ tdTom+ mCherry+ CD16/32+ cells. Data represent mean percentage ± SEM (Student’s t test; n = 3 for Scr and shHoxa1#1 and n = 4 for miR-99 KD and shHoxa1#2) and are representative of two independent experiments. (N) TaqMan quantitative RT-PCR for Hoxa1 expression in L-GMPs sorted from secondary recipients at the time of death. Expression was normalized to Actb. Data represent mean ± SEM (Student’s t test; n = 3) and are representative of two independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
ing down miR-99 would still be able to release inhibition of miR-99 targets and induce phenotypes. Supporting this possibility, studies have established the importance of miRNA/target mRNA stoichiometry in determining the physiological consequences of miRNA expression, as the presence of fewer predicted target transcripts allows miRNAs to down-regulate their target genes more efficiently (Arvey et al., 2010). Therefore, bioinformatic approaches and the presence of fewer predicted target transcripts allows miR-99 regulation of HSC and LSC self-renewal | Khalaj et al.

MATERIALS AND METHODS

Cell sorting

Antibody staining and enrichment procedures for HSCs and multipotent progenitor, common myeloid progenitor, common lymphoid progenitor, granulocyte-macrophage progenitor, and megakaryocyte-erythroid progenitor cell sorting and analyses were performed as previously described (Akashi et al., 2000; Christensen and Weissman, 2001; Kiel et al., 2005).

Constructs

Lentiviral vectors (Fig. S1 B) were constructed in the miR-Zip backbone (Systems Biosciences) using the following sequences for anti–miR-99 vector 1: 5′-GAACCCCGTTGATCCGATCCGGTTCCTCTGCACAGCAAGATCGGATCTACGGGTGTTTTT-T3′ and anti–miR-99 vector 2: 5′-GCACCGTGGAACCCGACCCTGAGCTTCCGTCAACCCAGGTCGGTTCTACGGGTGTTT3′. Retroviral vectors (Fig. S3 B) were constructed using previously described miR-30 shRNA expression vectors (Dickins et al., 2005). The classical shRNA sequences used in lentiviral backbones were modified into sequences compatible with miR-30 constructs. The sequence used for vector 2 is 5′-AAGTGTATGCTGGTATGTGAGGACACGCCCGTGAAACCGACCGTGAGTGAATGAGCCACAGATGTACGCAAGGTGGTCTAAGGGGTGGTGCCTACTGCCTGCGTA3′.

shRNA sequences used to knock down Hoxa1 included the following 97-mer oligo sequences: shHoxa1#1, 5′-TGGTTGGATACTGAGCGGCACCTCCACTTACAAAGTACCTTATAGAAGGACACATGTAAATGTTACTTGGTTGAAGTGGATGCGCTACTGCTCCGGA-3′; shHoxa1#2, 5′-TGGCTGTTGACATGAGCGGACACATGTAAATGTTACTTGGTTGAAGTGGATGCGCCTACTGCTCCGGA-3′.

ACTCATATAGTGAAGCCACAGATGTATGTAGGTGAATGTACTGGGTGCTACTGCTCCGGA-3′.

Methylcellulose assays

For colony replating assays, mouse LT-HSCs (c-Kit+ Sca-1+ Lin− CD150+CD34+) were transduced with lentiviral anti–miR-99. Cells were maintained in DMEM-F12 with 10% FCS, 1× penicillin/streptomycin, 1× Glutamax, 10 ng/ml mSCF, 10 ng/ml mFlt3 ligand, 10 ng/ml thrombopoietin (mTPO), 10 ng/ml mIL-3, and 10 ng/ml IL-6.

48 h after transduction, 100 GFP+ cells were plated into complete methylcellulose media containing mIL-3, mIL-6, mSCF, and mEPO (Methocult M3434; STEMCELL Technologies) for 7 d. After counting the colonies, all cells were resuspended in FACS media and analyzed for expression of cell surface markers. 20,000 GFP+ cells were used for replating assays, and the colonies were scored 7 to 10 d afterwards.

For human colony-forming assays, CD34+ cells were enriched using CD34 magnetic beads (catalog no. 130-046-702; Miltenyi Biotec). Prior to adding the CD34+ cells, lentiviral miR-Zip virus was spinofected for 2 h at 2,300 g in a 24-well plate precoated with 100 µg/ml Retronectin according to the manufacturer’s guidelines (catalog no. T100A; Clontech). The cells were then added to the plate and spun at 400 g for 10 min. Cells were maintained in StemSpan (STEMCELL Technologies) supplemented with 10% FCS, 20 ng/ml TPO, 20 ng/ml IL-3, 20 ng/ml granulocyte colony-stimulating factor, and 1× penicillin/streptomycin overnight. 1,500 GFP+ cells were plated into H4435 media (STEMCELL Technologies), which contains SCF, IL-3, IL-6, EPO, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor. The colonies were scored after 14 d in culture.

For MLL-AF9 colony-formation assays, 1,000 GFP+ tdTom+ blasts from primary recipients were plated into M3434 media (STEMCELL Technologies). 1,000 GFP+ tdTom+ cells were replated every 7 d.

AML cell line differentiation in vitro

MonoMac6 cells were obtained from ATCC and maintained according to their guidelines. MonoMac6 cells were supplemented with RPMI containing 10% FCS, 1× penicillin/streptomycin, and 1× Glutamax (Thermo Fisher Scientific). 105 cells were plated in 12-well plates and transduced with lentiviral anti–miR-99 or Hoxa1 overexpression viruses by spinofection at 1,200 g at 37°C for 1 h. Cells were washed the next day and then cultured in RPMI as described above. Expression of myeloid differentiation markers was analyzed by flow cytometry 5–8 d after infection.

Mice and transplantations

LT-HSCs (c-Kit+Sca-1− Lin− CD150− CD34+) were double-FACS sorted and transduced with lentiviral anti–miR-99. The cells were kept in DMEM-F12 with 10% FCS, 10 ng/ml SCF, 10 ng/ml Flt3 ligand, 10 ng/ml TPO, 10 ng/ml IL-3, and 10 ng/ml IL-6. 48 h after transduction, GFP+ cells were
sorted and used for transplants along with 300,000 Sca-1–depleted BM helper cells. Transplantations were performed by retro-orbital injection into lethally irradiated (2 × 475 rad) C57BL/6J mice (5,000 GFP+ cells per mouse). Helper cells were Sca-1 depleted using Sca-1–Biotin (catalog no. 108104; BioLegend) antibody and streptavidin beads (catalog no. 130-048-102; Miltenyi Biotec) according to the manufacturer’s instructions. All mice were maintained under pathogen-free conditions according to a Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Animal Care and Use Committee–approved protocol.

shRNA library screen
The shRNA library against candidate miR-99 target genes was generated by designing four shRNA sequences per gene (Table S2, D and E) and cloning them into an miR–E–based shRNA expression retrovirus (Fig. S3 H; Fellmann et al., 2013), including 192 shRNAs against 47 target and control genes. 4–7 × 10^6 LSK cells were sorted from 10 to 15 C57BL/6J mice per replicate and transduced simultaneously with a lentiviral GFP+ anti–miR-99 and retroviral mCherry+ shRNA library. Cells were maintained in DMEM-F12 with M3434 Methocult media (STEMCELL Technologies) for scien-
tific advice and all members of the Park laboratory for constructive discussions. We wish to thank Dr. Scott Lowe (MSKCC) for scientific advice and all members of the Park laboratory for constructive discussions. We thank Dr. Ralph Garippa, Dr. Qing Xiang, and Sanjoy Mehta from the MSKCC RNAi core for assistance with shRNA library deep sequencing and analysis and Dr. Nicholas Socci for RNA-seq analysis.

Lentiviral transduction of patient AML samples
AML patient samples were thawed and plated in StemSpan (STEMCELL Technologies) supplemented with 10% FCS, 20 ng/ml hTPO, 20 ng/ml hIL-3, 20 ng/ml granulocyte colony-stimulating factor (hG-CSF), and 1× penicillin/streptomycin overnight. Lentiviral anti–miR-99 vector in MLL-AF9 AML. Fig. S2 shows data related to characteriza-
tion of MLL-AF9 AML primary recipients, limiting dilution transplants, nshRNA library screen results. Tables S1 and S2 are provided as Excel files. Table S1 lists genes up-reg-
ulated comparing LGMPs with normal GMPs. Table S2 shows RNA-Seq and DNA deep sequencing data regard-
ing the shRNA library experiment on primary hematopoietic stem and progenitors.

Luciferase assays
HS3ST2 and SMARCA5 3’ UTRs were amplified by PCR using human genomic DNA and the following primers: HS3ST2 forward, 5’-GCTGTCCTGAGCCGTAGATT GCTCCAGA-3’; HS3ST2 reverse, 5’-ATAGCGGCCGGCT TAAGGACAGAGAGCCA-3’; SMARCA5 forward, 5’-GCTGTCCTGAGCAGTAGTCTTATATTACTAGGT CT-3’; SMARCA5 reverse, 5’-ATAGCGGCCGGCAAG CTTATTTTATTTCAAGGGTT-3’. The resulting PCR products were then cloned in the PsiCHECK-2 vector for measurement of luciferase activity (catalog no. C8021; Promega).

Cell cycle analysis
BM cells were sorted for the indicated cell populations and subsequently fixed and permeabilized using 80% ethanol. The cells were incubated with anti–Ki67–PE antibody (catalog no. 556027; BD PharMingen) and re-
suspended in 500 µl PBS and 0.2 µg/ml DAPI before flow cytometry analysis.

Reverse transcription quantitative PCR
Total RNA was extracted from cells using TRIzol (Sigma) or an RNasey kit (QiAGEN). cDNA was generated using a First-strand cDNA Synthesis Kit (Thermo Fisher Scientific), a TaqMan MicroRNA Reverse Transcript Kit (Applied Biosystems), or Quantimir (Systems Biosciences). TaqMan probes for individual microRNAs were purchased from Applied Biosystems. β-Actin and miR-16 were used as internal housekeeping controls.

Online supplemental material
Fig. S1 shows results from luciferase reporter assays to identify miR-99 targets as well as in vitro experiments after miR-99 KD and overexpression in HSPCs. Fig. S2 includes data for in vivo transplant studies of miR-99 KD HSCs as well as studies characterizing the retroviral anti–miR-99 vector in MLL-AF9 AML. Fig. S3 shows data related to characterization of MLL-AF9 AML primary recipients, limiting dilution transplants, nshRNA library screen results. Tables S1 and S2 are provided as Excel files. Table S1 lists genes up-reg-
ulated comparing LGMPs with normal GMPs. Table S2 shows RNA-Seq and DNA deep sequencing data regard-
ing the shRNA library experiment on primary hematopoietic stem and progenitors.

ACKNOWLEDGMENTS
We wish to thank Dr. Scott Lowe (MSKCC) and Dr. Andrea Ventura (MSKCC) for scientific advice and all members of the Park laboratory for constructive discussions. We thank Dr. Ralph Garippa, Dr. Qing Xiang, and Sanjoy Mehta from the MSKCC RNAi core for assistance with shRNA library deep sequencing and analysis and Dr. Nicholas Socci for RNA-seq analysis.

This work was supported by the National Institutes of Health National Cancer Institute (R01 grant CA164120-01A1, to C.Y. Park). B.H. Durham was supported by the American Society of Hematology Senior Research Training Award for Fellows and a New York State Council on Graduate Medical Education Empire Clinical Research Investigator Program Fellowship.

The authors declare no competing financial interests.

Submitted: 22 September 2016
Revised: 18 April 2017
Accepted: 8 June 2017

REFERENCES


