MicroRNA-155 promotes G-CSF-induced mobilization of murine hematopoietic stem and progenitor cells via propagation of CXCL12 signaling

Leukemia (2017) **31,** 1247–1250; doi:10.1038/leu.2017.50

Under homeostatic conditions, low levels of hematopoietic stem and progenitor cells (HSPC) constitutively egress out of the bone marrow (BM) (Supplementary Reference 1). HSPC egress is markedly enhanced in response to exogenous stimulation, termed mobilization, mimicking their behavior under stress and alarm situations such as inflammation. Repeated administration of G-CSF and chemotherapy are commonly used for clinical mobilization protocols to harvest HSPC from the peripheral blood. G-CSF-induced mobilization is currently the most widely applied, potent and safest clinical procedure; however, its mechanism of action is very complex.[1] Yet, it is pivotal to decipher this complexity as some donor patients mobilize poorly in response to G-CSF, resulting in low HSPC yields that are insufficient for transplantation. Recent studies have determined that the CXCL12–CXCR4-signaling axis plays a major role in the regulation of G-CSF-induced mobilization, and release from BM retention (Supplementary Reference 3), synergizing with proteolytic enzymes, cytokines and lipids (Supplementary Reference 4). MicroRNAs are small non-coding RNAs that regulate numerous key cellular hematopoietic processes including proliferation, differentiation and apoptosis (Supplementary Reference 7). In normal and malignant hematopoiesis, miR-155 was found to be a major regulator of physiological and pathological processes. miR-155 participates in many hematopoietic regulatory functions such as differentiation and lineage commitment, host immunity, autoimmunity, inflammation and viral infection. Aberrant expression of miR-155 plays a role in oncogenesis (Supplementary Reference 9), contributing to development of leukemia and myeloproliferative disorders.[2–8] In the present study, we explored the role of miR-155 in G-CSF-induced mobilization as it was not determined and likewise the possible contribution of microRNAs to this process was unclear. Detailed information about all experimental methods is described in Supplementary Methods.

To examine the role of miR-155 in G-CSF-induced HSPC mobilization, we first performed a quantitative reverse transcription PCR analysis for miR-155 expression in mature and immature murine hematopoietic BM populations. Following G-CSF treatment, miR-155 levels were significantly elevated in HSPC and in Mac-1$^+$Gr-1$^+$ cells, whereas miR-155 levels were significantly decreased in BM lymphoid populations (Figure 1a) that are in line with the suppression of lymphopoiesis following G-CSF administration (Supplementary Reference 10). These findings suggested that during G-CSF administration, regulation of miR-155 levels dictates the developmental program in distinct hematopoietic populations. Our results were supported by previous findings showing that miR-155 is among few microRNAs that are upregulated in Macaque CD34$^+$ HSPC during mobilization (Supplementary Reference 11). To evaluate the contribution of miR-155 to G-CSF-induced HSPC mobilization, we utilized miR-155 knockout (KO) mice, and examined peripheral blood (PB) WBC counts in wild type (WT) and miR-155 KO mice. No significant differences were observed during steady state (Supplementary Figure S1a), however reduced PB WBC numbers were observed in

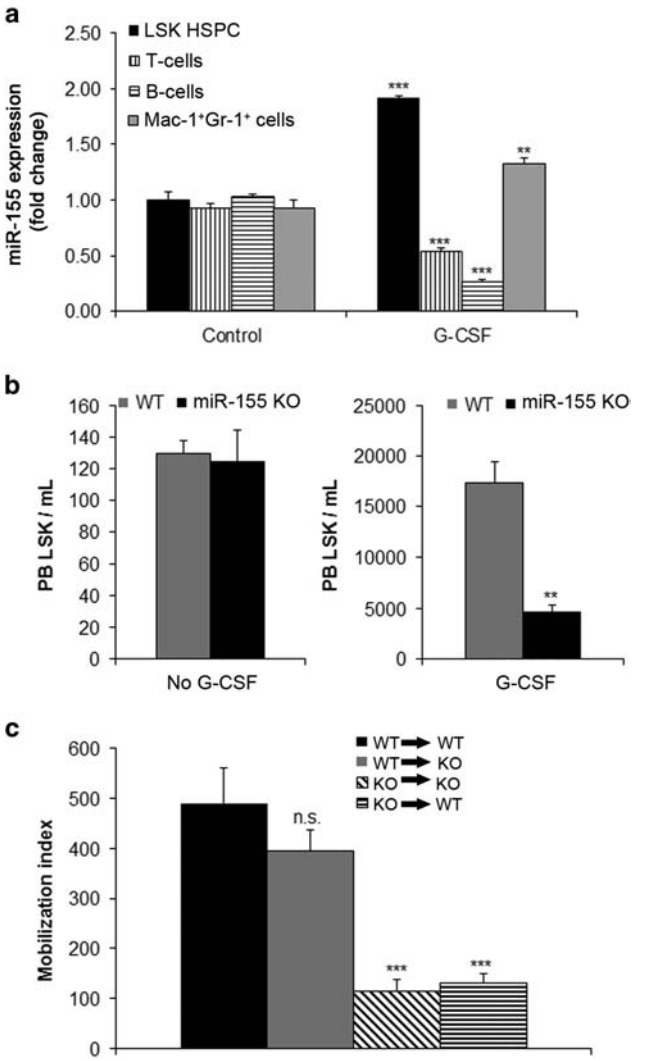

Figure 1. HSPC elevate and require miR-155 for successful G-CSF-induced mobilization. (**a**) Relative miR-155 RNA expression levels in different types of hematopoietic BM populations following vehicle (control, $n=6$) or G-CSF ($n=6$) treatments, as determined by quantitative reverse transcription PCR. (**b**) PB lineage$^-$/Sca-1$^+$/c-Kit$^+$ (LSK) HSPC counts from WT ($n=5$) or miR-155 KO ($n=5$) with or without G-CSF treatment. (**c**) Mobilization index (fold change) of PB HSPC counts from G-CSF-treated mice ($n=4$) relative to control mice ($n=4$), for each type of chimeric model. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.005$.

miR-155 KO mice following G-CSF (Supplementary Figure S1b). The same phenomenon was observed when we examined committed progenitors (Supplementary Figures S1c and S1d) and immature HSPC numbers in PB following G-CSF stimulation as well as following the treatment with AMD 3100 (Plerixafor),

Accepted article preview online 8 February 2017; advance online publication, 24 February 2017

another agent used for clinical mobilization (Figure 1b, Supplementary Figures S3k and S3l). In addition, WBC count in BM and Spleen were similar during steady state and under G-CSF stimulation (Supplementary Figures S3a and S3c), whereas miR-155 KO mice had reduced lineage$^-$/Sca-1$^+$/c-Kit$^+$ in the BM and spleen during steady state (Supplementary Figures S3b and S3d). These observations suggest that miR-155 KO mice display normal levels of PB HSPC at steady state, but reduced PB HSPC levels following AMD 3100 and G-CSF-induced mobilization. miR-155 KO and WT mice were injected once with lipopolysaccharide (150 μg per mouse) and monitored for the next 5 days. All the miR-155 KO mice died while WT mice survived suggesting that miR-155 KO mice were less tolerant to lipopolysaccharide-induced stress (data not shown). SHIP-1, a downstream target of miR-155 is critical during sub-lethal radiation induced myelosuppression (Supplementary References 12 and 13) as its inhibition resulted in enhanced recovery of RBC, neutrophils and platelets in irradiated myelosuppressed hosts. We found that miR-155 KO mice were less tolerant to 5-Fluoruracil chemotherapy-induced myelosuppression (Supplementary Figure S5c). Wang *et al.*[9] have recently reported that miR-155 expression in BM endothelial cells regulated inflammation in the BM, affecting the development of myeloproliferative disorders. In addition, we recently showed how BM endothelial cells regulate mature and immature leukocyte trafficking.[10] Thus, we investigated whether miR-155 upregulation in HSPC during G-CSF-induced mobilization is a consequence of hematopoietic self-intrinsic activity or derived from the BM stromal and/or endothelial microenvironment. Examining the cellular *in vitro* migration capacity toward CXCL12 gradients in Transwells, using BM cells isolated from G-CSF-treated mice, we observed no difference in migration capacity of BM WBC count taken from WT or miR-155 KO mice (Supplementary Figure S1e), but noticed reduced migration capacity of immature BM HSPC isolated from miR-155 KO mice (Supplementary Figure S1f). Previously, miR-155-deficient dendritic cells were found to cause less severe graft versus host disease through reduced migration and defective inflammasome activation.[11] To further support our findings *in vivo*, we transplanted lethally irradiated WT mice with BM cells both from WT or miR-155 KO mice, and after established chimerism, subjected them to G-CSF mobilizing treatment (Supplementary Figure S1g). WT or miR-155 KO mice transplanted with miR-155 KO BM displayed reduced HSPC mobilization (Figure 1c), while miR-155 KO mice transplanted with WT BM cells displayed normal G-CSF-induced mobilization (Figure 1c). These findings indicated that hematopoietic predominant self-intrinsic mechanisms regulated by miR-155 promote G-CSF-induced mobilization and can overcome stromal/endothelial miR-155 defects. G-CSF treatment increased the number of PDGFβR$^+$ mesenchymal stem and progenitor cells and colony forming fibroblasts in miR-155 KO mice, whereas reduced the same in WT mice (Supplementary Figures 3f, g and h). Recently, inhibition of a downstream target of miR-155, SHIP-1, has been shown to induce G-CSF production mediating HSC and mesenchymal stem cell expansion (Supplementary References 12, 14). These results suggest that although cell motility and cytokine response is impaired in miR-155 KO mice, their HSPC and mesenchymal stem cell expand better under G-CSF stimulation. An activated monocyte–macrophage population that expresses α-smooth muscle actin (αSMA) has been implicated in retaining and preserving the primitive HSPC in the BM niche (Supplementary Reference 15). We found that G-CSF treatment reduced numbers of BM αSMA$^+$ macrophages in WT mice, while increasing them in miR-155 KO mice (Supplementary Figure 3e) that might contribute toward reduced HSPC egress from the BM. Our migration results indicated that G-CSF-treated miR-155-deficient HSPC failed to properly respond to CXCL12 apparently due to inhibition of their cellular migratory machinery. We have applied the ImageStream analysis to assess HSPC cellular activation by comparing the ratio of changes between their 'resting state' displaying rounded cellular morphology and equal actin distribution (Figure 2a, i) to their 'activated state' displaying elongated cellular morphology with cellular extensions and actin polarization to leading edges (Figure 2a, ii). Comparing BM HSPC, taken from G-CSF-treated WT or miR-155 KO mice, in terms of their 'activation' in response to

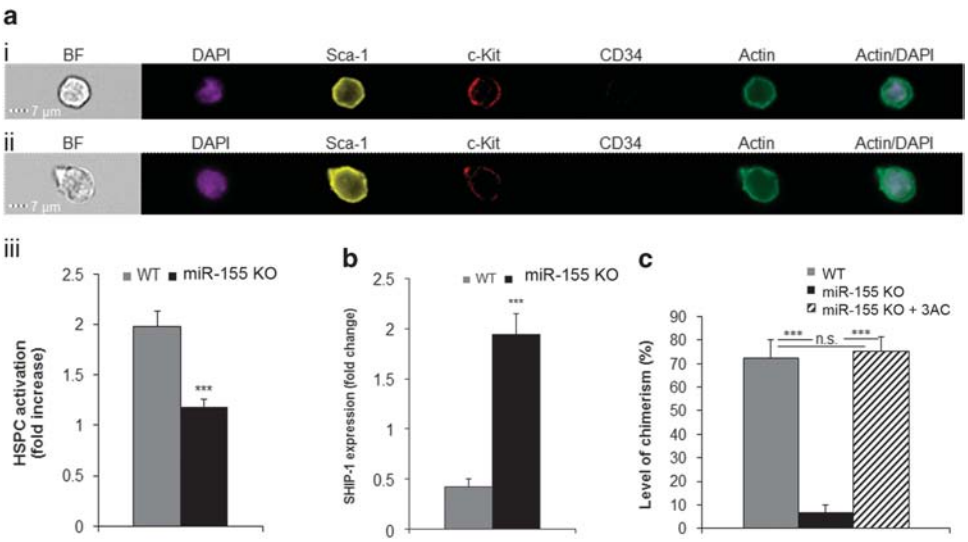

Figure 2. miR-155 promotes HSPC activation in response to CXCL12 via downregulation of SHIP-1. (**a**) Representative ImageStream images showing (i) non-activated- and (ii) activated lineage-depleted BM CD34$^-$/Sca-1$^+$/c-Kit$^+$ HSPC. (iii) Statistically analyzed ImageStream data indicating the activation levels from G-CSF-treated BM HSPC from WT ($n=8$) and miR-155 KO ($n=8$) mice shown as fold increase in activated cells in response to CXCL12 (125 ng/ml) stimulation relatively to control. (**b**) SHIP-1-fold change was determined as the relative MFI signal change from G-CSF-treated BM HSPC to control BM HSPC, taken from WT ($n=5$) or miR-155 KO ($n=5$) mice. SHIP-1 levels were similar in WT and in miR-155 KO during steady state. (**c**) Frequency of chimerism indicating for LTR–HSC activity in lethally irradiated mice transplanted with G-CSF-mobilized whole blood from WT ($n=9$), miR-155 KO ($n=9$) or miR-155 KO mice+3AC treatment ($n=9$). ***$P<0.005$.

CXCL12 stimulation, we observed no response to CXCL12 in BM HSPC taken from miR-155 KO mice (Figure 2a, iii). Therefore, based on our chimeric models, *in vitro* migration assay and actin rearrangement data, we suggest that there is a crucial and predominant cell-intrinsic defect of hematopoietic cells in exerting reduced response to CXCL12 signaling, thus affecting HSPC migration and mobilization in miR-155 KO mice. Previous studies from our lab showed that Akt activation is necessary to promote G-CSF-induced mobilization. Here, BM HSPC from miR-155 KO did not activate Akt in response to G-CSF treatment (Supplementary Figure S2a) and cAMP activator Forskolin (data not shown). This inability of miR-155 KO HSPC to activate Akt in response to G-CSF treatment was rescued by treatment with the SHIP-1 small molecule inhibitor 3AC. SHIP-1, a validated miR-155 target, regulates Akt signaling by modulating its membranal docking domain.[12] Moreover, SHIP-1-deficient HSPC display enhanced actin polymerization and migratory response to CXCL12,[13] and SHIP-1 small molecule inhibitors can mobilize functional HSPC.[14] In line with these reports, we have noted that G-CSF treatment did not reduce SHIP-1 levels in BM HSPC from miR-155 KO mice like they did in WT mice (Figure 2b), while SHIP-1 levels were similar in WT and in miR-155 KO mice during steady state. We next aimed to restore miR-155 KO mice mobilization capacity and HSPC yield by combining G-CSF treatment with 3AC treatment. Inhibition of SHIP-1 during G-CSF stimulation in miR-155 KO mice restored HSPC mobilization (Supplementary Figures S2b, and 3i and j). HSPC mobilized from miR-155 KO mice exhibited reduced short-term and long-term engraftment (Supplementary Figure 4 and Figure 2c) as compared to the WT mice. More importantly, 3AC restored normal mobilization levels of engraftable long-term repopulating hematopoietic stem cells after long-term transplantation (Figure 2c). Our results about 3AC-mediated restored long-term engraftment are consistent with the previous study[14] where 3AC was shown to mobilize $Sca^{+}Kit^{+}Lin^{-}Flk2^{-}$ HSPC, which upon transplantation protected hosts from lethal radiation dose as compared to HSPC from the vehicle control. Therefore, combining G-CSF treatment with the inhibition of miR-155 downstream targets or administration of miR-155 mimetics based on LNA technology[15] might be a novel approach to improve the yield of adequate HSPC numbers in patients as well as in healthy donors, which obviously warrants further studies in humans. Our findings provide evidence for miR-155 participation in G-CSF-induced HSPC mobilization by regulating cellular sensitivity to CXCL12-mediated signaling that promotes activation of the migratory machinery in HSPC (summarized in Supplementary Figure S3). Furthermore, we found that miR-155 overexpressing progenitor cells showed increased proliferation and colony formation (Supplementary Figures S5a and b), and miR-155 over expression enhanced the motility of HSPC toward a gradient of CXCL12 (Supplementary Figure S5). In agreement with our results, O'Connell *et al*.[4] have shown that *in vitro*-transduced miR-155-overexpressing HSPC have increased *in vivo* reconstitution potential. Recently, naturally occurring single-nucleotide polymorphisms in the human and mouse miR-155 locus were reported to affect miR-155 expression and the immune responses by modulating its secondary structure (Supplementary Reference 16). These miR-155 single-nucleotide polymorphisms carrying mice that were suggested as natural models to study the mechanisms of immune diseases caused by abnormal expression of miR-155 in humans, can be also applied as models for motility and mobilization studies. Recently, Zhang *et al*.[15] have shown that the Stat5/miR-155 pathway promotes HSPC proliferation, survival and their leukemic transformation. According to their results, individuals having elevated G-CSFRIV levels were susceptible to aberrantly increased miR-155 levels, thus increasing the risk of *de novo* leukemia or leukemia relapse (Supplementary Reference 18). On the basis of our results, we suggest that miR-155 modulates SHIP-1 expression that subsequently affects CXCL12–CXCR4 signaling axis via Akt activation (summary model in Supplementary Figure 6). Modulation of miR-155 levels or its downstream target SHIP-1 during mobilization may increase HSPC yields for stem cell harvest, thus improve engraftment, and therefore reduce the risk of transplantation-related mortality or slow reconstitution.

CONFLICT OF INTEREST

WGK has an equity position in a private venture that may license and commercialize SHIP-1 inhibitor technology for mobilization and harvest of BM stem cells. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Ziv Porat for technical assistance with ImageStream analysis and Dr Ron Rotkopf for assistance with statistical data analysis. TL was supported by the Ministry of Science, Technology and Space, Israel and the DKFZ, Germany, grants from the Israel Science Foundation (851/13), the Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine, and EU FP7-HEALTH-2010 (CELL-PID #261387). FK was supported from the Deutsche Krebshilfe (109420, Max-Eder program); a fellowship 2010/04 by the European Hematology Association; and by the Deutsche Forschungsgemeinschaft (DFG) (SFB 1074, project A5). AR was supported by the DFG (SFB 1074, project A5) as well as the gender equality program by the DFG (SFB 1074, project Z2). WGK was supported by NIH grants R01 HL072523 and R01 HL107127, the Paige Arnold Butterfly Run, and is an Empire Scholar of the State University of New York.

AUTHOR CONTRIBUTIONS

TI designed and performed the experiments, analyzed the data and wrote the manuscript; AK performed the experiments and edited the revised version of manuscript; SG-C, OK, KG and EK-M performed the experiments; and AR and ES designed and performed the miR-155-transduced HSPC proliferation, colony formation and Transwell assay. CL, HG and FK designed and performed miR-155 qRT-PCR-related experiment, analyzed the data and helped writing the manuscript; RB, CMR, JDC and WGK synthesized 3AC and helped in designing 3AC-related experiments; EH helped and guided in the design of experiments; and TL guided and designed the research and wrote the manuscript.

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Supplementary Information accompanies this paper on the *Leukemia* website (http://www.nature.com/leu)