Published in final edited form as:

Science. 2016 December 02; 354(6316): 1156–1160. doi:10.1126/science.aaf5530.

Self-renewal of a purified *Tie2*⁺ hematopoietic stem cell population relies on mitochondrial clearance

Kyoko Ito^{1,2,3}, Raphaël Turcotte⁴, Jinhua Cui^{1,5,*}, Samuel E. Zimmerman^{1,6,*}, Sandra Pinho^{1,5,*}, Toshihide Mizoguchi^{1,5}, Fumio Arai⁷, Judith M. Runnels⁴, Clemens Alt⁴, Julie Teruya-Feldstein⁸, Jessica C. Mar^{1,6,9}, Rajat Singh^{2,10,11}, Toshio Suda^{7,12}, Charles P. Lin⁴, Paul S. Frenette^{1,2,3,5}, and Keisuke Ito^{1,2,3,5,11,†}

¹Ruth L. and David S. Gottesman Institute for Stem Cell and Regenerative Medicine Research, Albert Einstein College of Medicine, Bronx, NY 10461, USA

²Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA

³Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA

⁴Center for Systems Biology, Advanced Microscopy Program, Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA

⁵Departments of Cell Biology and Stem Cell Institute, Albert Einstein College of Medicine, Bronx, NY 10461, USA

⁶Department of Systems and Computational Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

⁷Department of Cell Differentiation, The Sakaguchi Laboratory of Developmental Biology, School of Medicine, Keio University, Japan

⁸Department of Pathology, Sloan Kettering Institute, Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA

⁹Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY 10461, USA

¹⁰Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

¹¹Diabetes Research Center, Albert Einstein College of Medicine, Bronx, NY 10461, USA

¹²Cancer Science Institute of Singapore, National University of Singapore, Singapore

†Corresponding author. keisuke.ito@einstein.yu.edu.

*These authors contributed equally to this work.

We declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/354/6316/1156/suppl/DC1

Materials and Methods

Supplementary Text

Figs. S1 to S14

References (14–59)

Movies S1 and S2

Abstract

A single hematopoietic stem cell (HSC) is capable of reconstituting hematopoiesis and maintaining homeostasis by balancing self-renewal and cell differentiation. The mechanisms of HSC division balance, however, are not yet defined. Here we demonstrate, by characterizing at the single-cell level a purified and minimally heterogeneous murine *Tie2*⁺ HSC population, that these top hierarchical HSCs preferentially undergo symmetric divisions. The induction of mitophagy, a quality control process in mitochondria, plays an essential role in self-renewing expansion of *Tie2*⁺ HSCs. Activation of the PPAR (peroxisome proliferator-activated receptor)-fatty acid oxidation pathway promotes expansion of *Tie2*⁺ HSCs through enhanced Parkin recruitment in mitochondria. These metabolic pathways are conserved in human TIE2⁺ HSCs. Our data thus identify mitophagy as a key mechanism of HSC expansion and suggest potential methods of cell-fate manipulation through metabolic pathways.

Precise mechanisms enable efficient and regulated hematopoietic stem cell (HSC) renewal versus differentiation, and these processes are coordinated. HSCs must withstand various stresses to maintain hematopoiesis for the duration of their life cycles. The repair or clearance of damage is critical to precisely controlling their cell fates and maintaining stemness upon division, especially during HSC expansion through symmetric self-renewing division. HSC exhaustion can result from defective cellular metabolism and/or impaired autophagy (1–6), a lysosomal degradation pathway that breaks down damaged or unwanted proteins and/or organelles (7). The FOXO3A (forkhead box O3a)-driven proautophagy program, for example, protects HSCs from metabolic stress (5). In a similar process in mammary epithelial stem-like cells, older mitochondria are pushed into daughter cells fated to differentiate by asymmetric division (AD), thus maintaining high-quality stem cell homeostasis (8). Despite extensive study of HSC renewal and differentiation, however, much remains unclear regarding the mechanisms of division balance due to the heterogeneity of HSC-enriched fractions.

In searching for a potential HSC marker, we observed high *Tie2* levels in the CD34⁺ HSC-enriched compartment (fig. S1A). We therefore established a *Tie2*-green fluorescent protein (GFP) reporter line (*Tie2*-reporter mice or *Tie2* Tg) to validate *Tie2*-expression as an HSC marker (fig. S1, B and C). *Tie2*-GFP was specifically enriched in the HSC fraction but was reduced during differentiation, in parallel with mRNA levels (fig. S1, D to G). *Tie2*-GFP⁺ and -GFP[−] fractions in CD34⁺CD150⁺CD48^{low/−}CD135[−]KSL cells were purified from *Tie2* reporter mice (hereafter, *Tie2*-GFP⁺ or *Tie2*-GFP[−] HSCs), and *Tie2* levels were detected in *Tie2*-GFP⁺ HSCs but not in *Tie2*-GFP[−] HSCs (fig. S1H). The cell cycle kinetics (9) revealed that cell cycling was slightly slower in *Tie2*-GFP⁺ versus *Tie2*-GFP[−] HSCs (fig. S1I). A higher proportion of phenotypic *Tie2*-GFP⁺ HSCs than *Tie2*-GFP[−] HSCs was closely associated with arteriolar structure (fig. S1J) and expressed endothelial and HSC markers (10, 11) (fig. S1, K to M).

In addition, the *Tie2*-GFP⁺ fraction displayed a highly significant (5.75-fold) increase in long-term culture-initiating cell (LTC-IC) frequency relative to the *Tie2*-GFP[−] fraction (Fig. 1A). Single-cell transplantation showed that a high percentage of single *Tie2*-GFP⁺ cells (68.0%) exhibited reconstitution capacity without lineage bias and maintained high donor

chimerism upon secondary transplantation. Donor contribution was observed in all recipients transplanted with three *Tie2*-GFP⁺ HSCs, whereas 40% showed reconstitution capacity with three *Tie2*-GFP⁻ HSCs (Fig. 1B and fig. S2, A to D), demonstrating that *Tie2*-GFP identifies an HSC fraction with minimal heterogeneity. To accurately supply the bone marrow with individual HSCs, we employed multiphoton microscopy guidance (local transplantation) (fig. S2E) to deliver single *Tie2*-GFP⁺ HSCs near an opening to the marrow of live mice, and we then tracked these cells during subsequent homing to locations some distance from the delivery site (Fig. 1C and movies S1 and S2). Recipients showed reconstitution from locally transplanted single (100%) and multiple *Tie2*-GFP⁺ HSCs (fig. S2F). A single *Tie2*-GFP⁺ HSC can exhibit reconstitution capacity in a recipient mouse for more than 6 months, once it is located in the bone marrow.

We also tested, in vivo and in vitro, whether *Tie2* acts as an HSC marker under stress conditions. Of the polyinosinic:polycytidylic acid (pIpC, inducing the interferon response)–treated single *Tie2*-GFP⁺ HSCs, 65.0% showed repopulation capacity at levels comparable to those seen in control mice (fig. S3, A to D). But whereas the *Tie2*-GFP⁺/*Tie2*-Ab⁺ fraction exhibited high reconstitution capacity, neither the *Tie2*-GFP⁻/*Tie2*-Ab⁺ fraction nor any of the 12 single *Tie2*-GFP⁺ KSL cells showed high reconstitution. The majority of the re-sorted *Tie2*-GFP⁺ cells (70.6% after 3-day culture; 85.7% after 7-day culture) exhibited multilineage reconstitution, though *Tie2*-GFP⁻ cells did not. None of 90 *Tie2*-GFP⁻ HSCs provided donor-derived *Tie2*-GFP⁺ HSCs in recipient mice. These data suggest that *Tie2* serves as a useful marker that can prospectively identify HSCs (figs. S2G and S3, E to L).

Single-cell gene expression assays showed that a substantial number of genes—including HSC factors and markers (e.g., *Tie2*, *Hif1a*, *Mpl*, and *Foxo3a*), as well as *Pml* (*promyelocytic leukemia*) and its downstream signaling pathways, which regulate fatty acid oxidation (FAO) via *Cpt1a* (*carnitine palmitoyltransferase 1a*), *Ppard* (*peroxisome proliferator-activated receptor-delta*), and *Acox1* (*acyl-coenzyme A oxidase I*)—were markedly higher in the *Tie2*-GFP⁺ fraction than the *Tie2*-GFP⁻ fraction (Fig. 1D). Hierarchical clustering, HSC signature, and signatures for both PPAR signaling and fatty acid metabolism demonstrate that *Tie2*-GFP⁺ HSCs are molecularly farther away from both KSL cells and *Tie2*-GFP⁻ HSCs (fig. S4). The functions of the PPAR-FAO pathway in *Tie2*-GFP⁺ HSCs were then assessed. Impaired reconstitution ability of single *Pml*^{-/-} *Tie2*-GFP⁺ HSCs could be rescued by GW501516, a PPAR δ -specific agonist (fig. S5, A to C). Thus the PPAR-FAO pathway plays critical roles in directly regulating the self-renewal of the top hierarchical HSCs.

Mitochondria are central metabolic organelles responsible for the final steps of FAO, which cleaves two carbons every cycle to form acetyl-coenzyme A. Once the PPAR-FAO pathway is activated, fatty acids have been imported into the mitochondrial matrix (12). Our gene expression assays revealed higher expression of mitochondrial autophagy (mitophagy)–related genes, including *Parkin* (*Park2*), *Pink1* (*PTEN-induced putative kinase 1*), *Optineurin*, *Tom 7*, *Map1lc3a* (*Lc3*), and *p62/Sqstm1*, in *Tie2*-GFP⁺ HSCs at steady-state conditions. Most of these genes were further up-regulated by activation of the PPAR-FAO pathway (Fig. 2A and fig. S5, D to F). To assess whether mitophagy-related genes are regulated by the PPAR-FAO pathway at the transcriptional level, *Pink1*-luciferase reporters

were used. A PPAR δ agonist transactivated *Pink1* reporters, which were reduced by FAO inhibition or mutation of FOXO-response elements. *Foxo3a* was up-regulated in HSCs upon treatment with a PPAR δ agonist, and this process was attenuated by the ablation of *Ppard* or *Cpt2*, which are FAO-related genes. These data suggest that the PPARFAO pathway transcriptionally regulates PINK1, in part through FOXO3a (fig. S6).

We then sought to determine whether PPAR δ -induced mitophagy correlates with a specific metabolic state or profile. Cellular FAO activity was specifically enhanced after PPAR δ agonist treatment and was accompanied by Pink1, whereas glucose uptake or levels of pyruvate and tricarboxylic acid cycle metabolites were not altered (fig. S7, A to D). PPAR δ agonists also enhanced mitophagy, with the increased colocalization of Tom20 (translocase of outer membrane 20) with LAMP1 (lysosomal-associated membrane protein 1). Flux analyses were then performed to quantify mitochondrial turnover in lysosomes (the organelles where mitophagy occurs) by evaluating the accumulation of Tom20 in the presence of leupeptin, an inhibitor of lysosomal proteolysis. Pharmacological PPAR-FAO activation enhanced the net mitochondrial flux toward lysosomal degradation. These data strongly suggest that the PPAR-FAO pathway activates mitophagy (Fig. 2, B and C).

Other findings supported the enhancement of mitophagy by a PPAR agonist, including increased colocalization of Parkin with pyruvate dehydrogenase (PDH), a matrix mitochondrial protein. Mitophagy enhancement was again confirmed in GW501516-treated HSCs by the increased colocalization of Parkin with both Tom20 and the autophagosome marker LC3 (figs. S7E and S8, A to D). To explore another mitophagy indicator, mitochondrial DNA (mtDNA) nucleoids were quantified after mitochondrial damage with oligomycin and antimycin A. GW501516-treated HSCs were nearly devoid of mtDNA and exhibited increased net mtDNA flux, whereas mtDNA was still partially retained in control HSCs (Fig. 2D). Thus, damaged mitochondria are cleared more quickly due to enhanced mitophagy activation in *Tie2*-GFP⁺ HSCs treated with a PPAR agonist.

Hematopoiesis-specific *Ppard* conditional knockout mice were then generated. *Ppard*-deleted HSCs exhibited neither enhanced Tom20 and Parkin colocalization nor increased mitochondrial net flux after PPAR δ agonist treatment, although these agonists stimulated mitophagy in wild-type HSCs, and a mitochondrial uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), induced mitophagy in both genotyped HSCs (fig. S8E). Conditional heterozygous deletion of *Cpt2* partially but significantly attenuated the enhanced Parkin recruitment in mitochondria induced by a PPAR agonist (fig. S8F). Taken together, these direct genetic approaches reveal that PPAR δ agonists stimulate mitophagy in a PPAR-FAO-dependent manner.

To test whether mitophagy is critical for *Tie2*-GFP⁺ HSC expansion, *Parkin* and *Pink1* were first silenced by RNA interference-mediated knockdown. siPark2 decreased net mitochondrial flux (a hallmark of mitophagy) in GW501516-treated HSCs, as indicated by decreased colocalization of Tom20 and PDH with LAMP1, whereas cell death, proliferation rate, FAO activity, and *Cdk6* levels were not altered (Fig. 3A and figs. S7A and S9). *Parkin*-knockdown in GW501516-treated HSCs led to accumulating signals of Tom20 and PDH, as well as reduced colocalization of LAMP1 with LC3 (fig. S10, A to C). mtDNA was retained

in *Parkin*-silenced HSCs, and these cells were refractory to PPAR agonist treatment (Fig. 3B and fig. S10D). These data suggest that Parkin has an essential role in PPAR-FAO-induced mitophagy.

Because Pink1 is known to be essential for mitophagy and Pink1 levels were increased by PPAR-FAO activation, we next assessed the effect of *Pink1* knockdown on the process. *Pink1* silencing reduced Tom20 colocalization with both LAMP1 and Parkin and decreased net flux of mitochondria in GW501516-treated HSCs, accompanied by accumulating Tom20 signals. mtDNA was also retained in *Pink1*-silenced HSCs (Fig. 3, B and C, and fig. S10, D to G). Consequently, Parkin and Pink1 play important roles in PPARFAO-induced mitophagy in HSCs.

We then explored potential clinical applications of pharmacological FAO modulation in human hematopoiesis. *TIE2* expression is enriched in the HSC fraction, and TIE2-positivity enhances LTC-IC frequency and in vivo repopulation capacity in human bone marrow (fig. S11, A to G). GW501516 enhances LTC-IC frequency with mitophagy activation, whereas low doses of an FAO inhibitor reduce its capacity. Fatty acid metabolism can therefore control self-renewal capacity and the cell fates of human HSCs (Fig. 3D and fig. S11, H to J).

Finally, we explored the effects of FAO-induced mitophagy on *Tie2*-GFP⁺ HSC expansion. In vivo paired daughter cell (PDC) assays can determine the division patterns of HSCs retrospectively, by assessing the reconstitution capacity of each daughter cell (3, 13). In vivo PDC assays revealed that *Tie2*-GFP⁺ HSCs preferentially undergo symmetric division (SD) (in 20 out of 27 divisions, or 74.1%, both daughter cells showed a reconstitution capacity) rather than AD or symmetric commitment [(SC), both daughter cells exhibited no reconstitution capacity], whereas *Tie2*-GFP⁻ HSCs undergo either AD or SC (Fig. 4, A to C, and fig. S12). To further test the restoration capacity of hematopoiesis by single *Tie2*-GFP⁺ HSCs, we performed transplantation assays under high-stress conditions, as are found in single-cell transplantation without supporting cells. The survival ratio after single *Tie2*-GFP⁺ HSC transplantation was 85.7%, whereas no recipients of single *Tie2*-GFP⁻ HSCs survived (Fig. 4D and fig. S13, A and B). Functional HSCs are present in the *Tie2*-GFP⁻ fraction, but they fail to adequately respond to the demand for hematopoietic recovery under high-stress conditions without the enhanced symmetric expansion provided by *Tie2*-GFP⁺ HSCs.

As expected, PPAR-FAO activation increased the number of *Tie2*-GFP⁺ cells in vitro with an increased rate of SD (fig. S13, C to G). *Tie2*-GFP⁺ HSC numbers were increased in comparison with the original number of transplanted donor cells in recipients and were further increased by PPAR δ agonist treatment (about a fourfold increase). Furthermore, PPAR δ agonist treatment expanded *Tie2*-GFP⁺ HSCs under physiological stress, as induced by pIpC (Fig. 4E and figs. S3B and S13H).

However, silencing *Park2* or *Pink1* not only abrogated the expansion of *Tie2*-GFP⁺ cells in vitro but also inhibited their maintenance (fig. S13I). Most single *Parkin*-silenced *Tie2*-GFP⁺ cells failed to show reconstitution capacity (92.3%), whereas the majority of control *Tie2*-

GFP⁺ cells (66.7%) exhibited this capacity (Fig. 4F and fig. S13J). *Pink1*^{-/-} HSCs failed to adequately respond to the demand for hematopoietic recovery under high-stress conditions, even though a PPAR δ agonist was administered (fig. S13K). In conclusion, *Tie2*-GFP⁺ HSCs can maintain stem cell potential during cell cycling due to high mitochondrial clearance through mitophagy and up-regulation of *Parkin* and *Pink1* (fig. S14). Our data from this highly purified HSC fraction identifies mitochondrial clearance by induction of mitophagosome formation as a key mechanism in maintaining stemness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all members of the Ito lab and Einstein Stem Cell Institute for their comments on HSC self-renewal and M. Wolfgang, A. Carracedo, H. You, and the Einstein Flow Cytometry and Analytical Imaging core facilities (grant P30CA013330) for help and materials. This work was supported by NIH (grants R01DK98263 and R01DK100689 to Ke.I.) and NYSTEM (New York State Stem Cell Science single-cell-core, grant C029154 to Ke.I.), Harvard Stem Cell Institute (to C.P.L.), NIH and Ellison Medical Foundation (to R.S.), NIH and Leukemia Lymphoma Society (to P.S.F.), and Japan Society for the Promotion of Science (to T.S.).

REFERENCES AND NOTES

1. Ito K, Suda T. Nat. Rev. Mol. Cell Biol. 2014; 15:243–256. [PubMed: 24651542]
2. Shyh-Chang N, Daley GQ, Cantley LC. Development. 2013; 140:2535–2547. [PubMed: 23715547]
3. Ito K, et al. Nat. Med. 2012; 18:1350–1358. [PubMed: 22902876]
4. Mohrin M, et al. Science. 2015; 347:1374–1377. [PubMed: 25792330]
5. Warr MR, et al. Nature. 2013; 494:323–327. [PubMed: 23389440]
6. Mortensen M, et al. Proc. Natl. Acad. Sci. U.S.A. 2010; 107:832–837. [PubMed: 20080761]
7. Galluzzi L, Pietrocola F, Levine B, Kroemer G. Cell. 2014; 159:1263–1276. [PubMed: 25480292]
8. Katajisto P, et al. Science. 2015; 348:340–343. [PubMed: 25837514]
9. Arai F, et al. Cell. 2004; 118:149–161. [PubMed: 15260986]
10. Acar M, et al. Nature. 2015; 526:126–130. [PubMed: 26416744]
11. Kunisaki Y, et al. Nature. 2013; 502:637–643. [PubMed: 24107994]
12. Kunau WH, Dommes V, Schulz H. Prog. Lipid Res. 1995; 34:267–342. [PubMed: 8685242]
13. Yamamoto R, et al. Cell. 2013; 154:1112–1126. [PubMed: 23993099]

Fig. 1. *Tie2*-GFP marks a distinct HSC subset

(A) LTC-IC frequencies of *Tie2*-GFP⁺ and *Tie2*-GFP⁻ HSCs were determined in a limiting-dilution assay (LDA). (B) Percentages of successful long-term reconstitution. (C) In vivo imaging of single *Tie2*-GFP⁺ HSCs immediately after local delivery in the calvarium. (Left) Top (upper) and side (lower) views of the delivery site. The arrow indicates the opening to the bone marrow. (Right) In vivo imaging of the same cell, 24 hours after local delivery (en face maximum intensity projection). (D) Single-cell gene expression analysis using the BioMark System array (Fluidigm) (see materials and methods).

Fig. 2. The PPAR-FAO pathway activates mitochondrial autophagy in HSCs
(A) *Parkin* and *Pink1* levels in GW501516-treated (GW) *Tie2*-GFP⁺ HSCs. **(B and C)** PPAR δ activators, GW501516 or L-165041 (L165), enhance colocalization of Tom20 with LAMP1. Treatment with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used as a control for mitophagy induction [(B), left]. Mitochondrial net flux was calculated by Tom20-accumulation in the presence of leupeptin (Leup) [(B), right]. Representative immunofluorescence (IF) images of colocalized Tom20 with LAMP1 [4',6-diamidino-2-phenylindole (DAPI), blue; Tom20, red; LAMP1, green; Merge, yellow] in PPAR δ -agonist-treated HSCs. White dashed boxes represent the magnified images shown at right. Scale bars, 2 μ m (C). **(D)** Representative images of GW501516-treated HSCs immunostained to label mtDNA (left) and quantified for mitophagy [12 hours oligomycin and antimycin A] (right top) and net mtDNA flux (right bottom). In (A), (B), and (D), error bars represent mean $\pm$ SEM (at least 10 cells analyzed per each condition per each experiment).

Fig. 3. Parkin is important for PPAR-FAO-induced mitophagy in HSCs

(A) Relative Tom20 colocalization (left) and mitochondria net flux (right) in GW501516-treated HSCs in the presence of siPark2. (B) Quantification of mitophagy based on the remaining mtDNA staining 12 hours after treatment with oligomycin and antimycin A. (C) *Pink1*-silencing reduces PPAR δ -enhanced mitophagy. Relative Tom20 colocalization (top left), net mitochondria flux (top right), and representative IF images depicting LAMP1 and Tom20 colocalization in GW501516-treated HSCs in the presence of siPink1 (bottom). (D) GW501516 enhances colocalization of TOM20 with PARKIN (left) and increases mitochondrial net flux (right). Scale bars in (C) and (D), 2 μ m.

Fig. 4. Self-renewing expansion of *Tie2*⁺ HSC through mitophagy

(A) Representative pictures of *Tie2*-GFP positivity, with bright-field images (BF) collected during symmetric division. (B) Representative repopulation kinetics of SD during the first and second bone marrow transplantation (BMT). PB-MNCs, peripheral blood mononuclear cells. (C) Division patterns of *Tie2*-GFP⁺ HSCs (left) and *Tie2*-GFP⁻ HSCs (right), in terms of reconstitution capacity. SD, symmetric division; AD, asymmetric division; SC, symmetric commitment. (D) Single *Tie2*-GFP⁺ HSC transplantation without supporting cells. Survival of recipient mice was examined by plotting Kaplan-Meier survival curves (also see fig. S13B). (E) PPAR δ activation induces in vivo expansion of *Tie2*-GFP⁺ HSCs. The number of transplanted *Tie2*-GFP⁺ HSCs (donor cells) is denoted by the dashed line marked "Original" (also see fig. S13H). (F) Frequency of successful hematopoietic reconstitution of shParkin-silenced *Tie2*-GFP⁺ HSCs (also see fig. S13J).