A single copy of large tumor suppressor 1 or large tumor suppressor 2 is sufficient for normal hematopoiesis

Zhi-Gang Li[1], Xue-Mei Fu[2], Cheng-Yan Chai[1], Fang-Fang Sun[1], Fei-Fei Xiao[1], Yong-Xiu Huang[1], Kai Yao[3], Jie-Ping Chen[1], Yu Hou[1]

[1]Department of Hematology, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 430008, China;
[2]Medical Research Center, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 430008, China;
[3]College of Life Sciences and Health, Wuhan University of Science and Technology, Wuhan, Hubei 430081, China.

Abstract

Background: Hematopoietic stem cells (HSCs) have the ability to differentiate into all subsets of blood cells and self-renew. Large tumor suppressor 1 (*LATS1*) and large tumor suppressor 2 (*LATS2*) kinases are essential for cell cycle regulation, organism fitness, genome integrity, and cancer prevention. Here, we investigated whether *Lats1* and *Lats2* are critical for the maintenance of the self-renewal and quiescence capacities of HSCs in mice.

Methods: Quantitative reverse transcription-polymerase chain reaction was used to determine the expression levels of *Lats1* and *Lats2* in subsets of progenitor cells and mature bone marrow cells. A clustered regularly interspaced short palindromic repeats system was used to generate *Lats1* or *Lats2* knockout mice. Complete blood cell counts were used to compare the absolute number of white blood cells, lymphocytes, monocytes, neutrophils, and platelets between *Lats1* or *Lats2* heterozygotes and littermates. Flow cytometry was used to assess the size of hematopoietic progenitor cells (HPCs) and HSC pools in *Lats1* or *Lats2* heterozygotes and littermates. The comparison between the two groups was analyzed using Student's *t* test.

Results: *Lats1* and *Lats2* were widely expressed in hematopoietic cells with higher expression levels in primitive hematopoietic cells than in mature cells. *Lats1* or *Lats2* knockout mice were generated, with the homozygotes showing embryonic lethality. The size of the HPC and HSC pools in *Lats1* (HPC: wild-type [WT] *vs.* heterozygote, $220{,}426.77 \pm 54{,}384.796$ *vs.* $221{,}149.4 \pm 42{,}688.29$, $P = 0.988$; HSC: WT *vs.* heterozygote, 2498.932 ± 347.856 *vs.* 3249.763 ± 370.412, $P = 0.105$) *or Lats2* (HPC: WT *vs.* heterozygote, $425{,}540.52 \pm 99{,}721.86$ *vs.* $467{,}127.8 \pm 89{,}574.48$, $P = 0.527$; HSC: WT *vs.* heterozygote, 4760.545 ± 1518.01 *vs.* 5327.437 ± 873.297, $P = 0.502$) heterozygotes were not impaired. Moreover, the depletion of *Lats1* or *Lats2* did not affect the overall survival of the heterozygotes (*Lats1*: $P = 0.654$; *Lats2*: $P = 0.152$).

Conclusion: These results indicate that a single allele of *Lats1* or *Lats2* may be sufficient for normal hematopoiesis.

Keywords: Hippo pathway; Hematopoietic stem cells; Stem cell self-renewal

Introduction

Hematopoietic stem cells (HSCs) are able to self-renew and differentiate into all subsets of blood cells, which is essential for maintaining hematopoietic homeostasis. At any point of time, most HSCs are found in a resting state,[1] which is essential for their life-long hematopoiesis and the preservation of their self-renewal capacity.[2] Investigating the molecular mechanisms of the regulation of HSC quiescence could improve our understanding of the molecular mechanisms underlying tissue regeneration and provide an insight into how these are disorganized under stress conditions. The quiescent state of the HSCs is precisely regulated by both extrinsic signals and intrinsic molecular mechanisms. Many cell-cycle regulators are critical in the transcriptional regulation of hematopoiesis, the regulation of oxidative stress, and chromatin modification, which have been previously reported to be regulated by intrinsic mechanisms.[3,4]

The Hippo tumor suppressor pathway is a major regulator of the critical balance between proliferation, progenitor cell renewal, and differentiation during embryogenesis.[5] Large tumor suppressor 1 (*LATS1*) and large tumor suppressor 2 (*LATS2*) are key mediators of Hippo signaling, which negatively regulate the activity of transcriptional co-activator with PDZ-binding motif

Access this article online

Quick Response Code:

Website: www.cmj.org

DOI: 10.1097/CM9.0000000000000934

Zhi-Gang Li and Xue-Mei Fu contributed equally to this work.

Correspondence to: Dr. Yu Hou, Department of Hematology, Southwest Hospital, Third Military Medical University (Army Medical University), Chongqing 400038, China
E-Mail: houyuxn@vip.126.com

Chinese Medical Journal 2020;133(16)

Received: 16-03-2019 **Edited by:** Peng Lyu

(*TAZ*) and the oncogenic transcriptional co-activators Yes-associated protein (*YAP*).[6,7] The *LATS1* and *LATS2* kinases are critical for cell cycle regulation, genome integrity, cancer prevention, and organism fitness.[8] The depletion of *Lats2* before E12.5 is embryonic lethal, and has been suggested as the result of accumulating genomic instability, mitotic defects, and abnormal proliferation.[9] However, a small number of *Lats1* knockout mice are viable, although they suffer from various developmental defects and oncogene-induced sarcomas.[10] The dysregulation of *LATS1* or *LATS2* is sufficient to promote tumorigenesis in mice due to the hyperactivation of *YAP/TAZ*.[11,12] Moreover, the loss of *LATS2* is associated with malignant mesothelioma.[13,14] Although the majority of the studies agree with the tumor suppressor model of the Hippo pathway, the functions of the Hippo pathway in cancer biology indicate that *YAP* may play a tumor suppressive role in hematologic malignancies.[15,16] In summary, these results indicate that *LATS1* and *LATS2* function as tumor suppressors and stem cell regulators.

While Hippo signaling and *LATS1* and *LATS2* have been extensively studied in many tissues, the role of *LATS1* and *LATS2* in the hematopoietic system remains unclear. In this study, we investigated the role of the Hippo pathway in hematopoiesis by generating *Lats1 or Lats2* knockout mice using a clustered regularly interspaced short palindromic repeats (CRISPR) system. We demonstrate that a single allele of *Lats1* or *Lats2* is sufficient for normal hematopoiesis.

Methods

Ethical approval

All animal experiments and methods were performed with the approval of the Animal Committee of the Institute of Zoology, Third Military Medical University (No. AMU-WEC20191324).

Animals

C57BL/6 wild-type mice were obtained from the Third Military Medical University Center for Animal Experiment (Chongqing, China; License No. SCXK[Yu]2017-0002). Heterozygotes of *Lats1* or *Lats2* were obtained from Cyagen Biosciences (Suzhou, China; License No. SCXK [Su]2018-0003). All data were generated from mice at 8 to 12 weeks of age.

Plasmid constructs

LentiCRISPRv2-small guide RNA (sgRNAs) were constructed as described previously.[17,18] Briefly, sgRNAs against *Lats1* or *Lats2* were designed on the CRISPR design website (http://crispor.tefor.net/crispor.py). SgRNAs were synthesized with a *Bsm*BI (Fermentas, Waltham, MA, USA) sticky-end, and the lentiCRISPRv2 plasmid was digested with the same enzyme. The sgRNAs were denatured for 10 min at 95°C and annealed by slowly cooling down to room temperature, then ligated into the sgRNA expression plasmid. *In vitro* transcription plasmids for CRISPR-associated protein 9 (*Cas9*) and sgRNAs were obtained from Addgene (Watertown, MA, USA; #44758 and #51132).

Generation of Lats1 and Lats2 knockout mice and genotyping

Cas9 messenger RNA (mRNA) was generated from a linearized *Cas9* expression plasmid using the mMACHINE T7 kit (Fisher Scientific, Goteborg, Sweden), according to the manufacturer's instructions. SgRNAs were transcribed from linearized sgRNA expression plasmids using the MEGAshortscript T7 kit (Fisher Scientific). The mRNAs obtained were purified using a commercial transcription Clean-Up Kit (Fisher Scientific) and stored at −80°C for microinjection. Zygotes were obtained from C57BL/6J mice. *Cas9* mRNA (150 ng/mL) and sgRNAs (100 ng/mL) were mixed together and microinjected into the zygotes in M2 culture medium (Sigma, St. Louis, MO, USA). The zygotes were then transplanted into pseudopregnant surrogate mice.

For the screening of the F0 founder of *Lats1*, polymerase chain reaction (PCR) products were generated from PCR genotyping using primers listed in Table 1, whereby the knockout allele generated a 630 bp band. The amplicons were then purified for DNA sequencing analysis. The DNA sequencing primers used are listed in Table 1. The DNA sequencing results revealed that Mouse-ID#3 was missing 2255 bases and had five-point mutations (GCAAG> TGTGC) in one strand; Mouse-ID#12 was missing 2251 bases and had one point mutation (C>A) in one strand. For the screening of the F0 founder of *Lats2*, the PCR products were generated from PCR genotyping using the primers listed in Table 1, whereby the knockout allele generated a band of 680 bp. The amplicons were then purified for DNA sequencing analysis. The DNA sequencing primers are listed in Table 1. The DNA sequencing results revealed that Mouse-ID#19 was missing 13,108 bases in one strand and Mouse-ID#22 was missing 13,130 bases in one strand.

The genotyping of adult mice tails was conducted by combined multiplex PCR using a mixture of three primers: *Lats1-F*, *Lats1-R*, and *Lats1-Wt/He-R*. The wild-type alleles produced a 421 bp band and the knockout allele generated a 630 bp band. Similarly, for the genotyping of the *Lats2* knockout mice, *Lats2-F*, *Lats2-R*, and *Lats2-Wt/He-R* were used. The wild-type alleles generated a 530 bp band and the knockout allele yielded a 680 bp band. All of the primers used are listed in Table 1.

RNA extraction and quantitative reverse transcription PCR

Total RNA was isolated using RNAiso Plus (Takara, Shiga, Japan), according to the manufacturer's instructions. Complementary DNA (cDNA) was obtained using the PrimeScript RT Reagent Kit (Takara). Then, cDNA was subjected to real-time PCR with TB Green® *Premix Ex Taq*™ II (Takara) in a real-time PCR detection system (Bio-Rad, Hercules, CA, USA). All of the primers used are listed in Table 1. Experiments were conducted in triplicate for all samples. The results were analyzed using the $2^{-\Delta\Delta Ct}$ (Ct; cycle threshold) method, and the actin, beta gene was used as a control.

Table 1: Primers used in this study.

Primers	Sequences
Lats1-F	5′-CGACAGAGAGTACAGTAAGGGCTGGA-3′
Lats1-R	5′-CTGTGGCTGCCTTAGAACTAACAATGC-3′
Lats1-Wt/He-R	5′-ACTGATTTGTCACATAGCCCAGGGT-3′
Lats1-seq	5′-GTGAGAACCACCATGCCTGGTGA-3′
Lats2-F	5′-CTGGCCCTCTGGAAGAGTGACAT-3′
Lats2-R	5′-TCGAGCCAATACAACAGAATAAAGGAC-3′
Lats2-Wt/He-R	5′-GCTACTTTCCAGAAACGCATGGTG-3′
Lats2-seq	5′-AGAGAGTACCTTCAATATGGCGAGTT-3′
Lats1-qPCR-F	5′-TCCTTTCTTGGCACAAACCC-3′
Lats1-qPCR-R	5′-CGGTCTTCTGGTCCTCGACA-3′
Lats2-qPCR-F	5′-CGGGACACCAAATTACATCGC-3′
Lats2-qPCR-R	5′-AGAATCACACCGACGCTCCAC-3′
Actin-qPCR-F	5′-ACCTTCTACAATGAGCTGCG-3′
Actin-qPCR-R	5′-CTGGATGGCTACGTACATGG-3′

F: Forward; *Lats1*: Large tumor suppressor 1; *Lats2*: Large tumor suppressor 2; qPCR: Quantitative polymerase chain reaction; R: Reverse.

Blood cell counts

Samples of peripheral blood were obtained from the tail vein of the mice and place into anticoagulant tube with ethylenediaminetetraacetic acid (EDTA). The complete blood counts were determined using a Hemavet 950FS (Drew Scientific, Dallas, FL, USA).

Flow cytometry

Single-cell suspensions were prepared from bone marrow (BM) (femurs and tibiae). Red cells were lysed with red blood cell lysis buffer (Solarbio, Beijing, China) and then incubated with the corresponding antibodies for 15 min on ice. The following biotin- or fluorochrome-conjugated antibodies obtained from Biolegend (San Diego, CA, USA) were used: anti-Gr-1 (catalogue No. 108404; dilution 1:2000), anti-TER-119 (116204; 1:1000), anti-B220 (103204; 1:1000), anti-CD19 (115504; 1:1000), anti-IgM (408903; 1:1000), IL-7R (135006; 1:2000), and CD3 (100304; 1:2000) for lineage markers; streptavidin-phycoerythrin-cyanine-5 (PE-Cy5) (405205; 1:500), PE-Sca-1 (108108; 1:200), allophycocyanin-cyanine-7 (APC-Cy7)-c-Kit (105826; 1:200), PE-Cy7-CD48 (103424; 1:200), and APC-CD150 (115910; 1:100) for the analysis of HPCs, LSK cells, and HSCs; streptavidin-APC-Cy7 (405208; 1:200), PE-anti-Sca-1 (108108; 1:200), APC-anti-c-Kit (105812; 1:200), and PE-Cy5-anti-fms-like tyrosine kinase 3 (Flt3) (135312; 1:200) for the analysis of lymphoid-primed multipotent progenitor (LMPPs); streptavidin-APC-Cy7 (1:200, 405208), PE-anti-Sca-1 (108108; 1:200), APC-anti-c-Kit (105812; 1:200), PE-Cy7-anti-CD16/32 (101318; 1:200), and BV421-anti-CD34 (152208; 1:200) for the analysis of common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), and megakaryocyte-erythroid progenitors (MEPs).[19,20]

5-Fluorouracil (5-FU) treatment

Mice were treated with 5-FU at a dose of 150 mg/kg body weight once every week for up to 3 weeks. Mice were monitored daily.

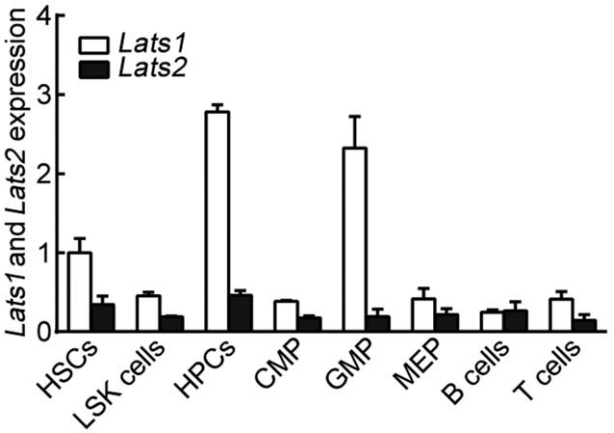

Figure 1: Quantitative RT-PCR analysis of *Lats1* and *Lats2* in primitive and mature murine hematopoietic cells from wild-type bone marrow. Data were normalized to those of the HSCs. *Actb* was used as an internal control. *Actb*: Actin, beta; B cells: B lymphocytes; CMP: Common myeloid progenitor; GMP: Granulocyte-monocyte progenitor; HPCs: Hematopoietic progenitor cells; HSCs: Hematopoietic stem cells; *Lats1*: Large tumor suppressor 1; *Lats2*: Large tumor suppressor 2; LSK cells: $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells; MEP: Megakaryocyte-erythroid progenitor; RT-PCR: Reverse transcription-polymerase chain reaction; T cells: T lymphocytes.

Statistical analysis

All quantitative data are presented as the mean ± standard deviation of $n = 5$–6 mice per genotype. Data are either representative of three experiments or from three experiments. GraphPad Prism 6 software (Graphpad Software, San Diego, CA, USA) was used for all statistical analyses. Statistical comparisons were conducted using Student's *t* test. *P* values of <0.05 were considered statistically significant.

Results

The core components Lats1 and Lats2 of the Hippo signaling pathway are expressed in HSPCs

To explore the role of *Lats1* and *Lats2* in hematopoietic cells, we first investigated the expression of *Lats1* and *Lats2* of the Hippo signaling pathway in the subsets of

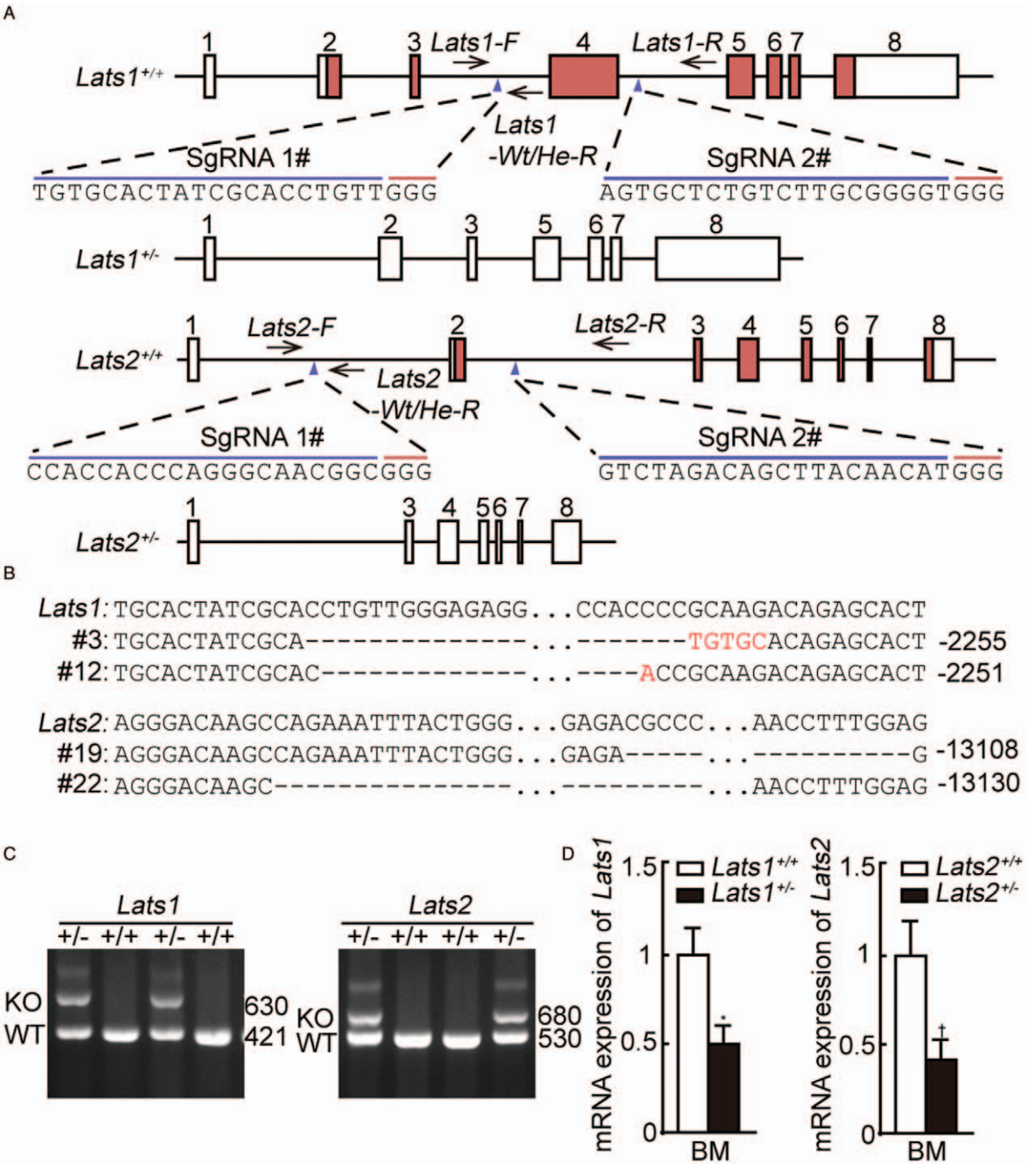

Figure 2: Targeted depletion of *Lats1* or *Lats2* in mice by CRISPR/Cas9 system and genotyping. (A) Schematic representation of the targeting strategy of *Lats1* or *Lats2*. *Lats1* and *Lats2* loci, the sgRNA sequences, the genotyping primers, and the targeted loci are indicated. Exon 4 of *Lats1* or exon 2 of *Lats2* were deleted using two sgRNAs, which generated exon 4 or exon 2 deletion and a frameshift mutant, respectively. (B) Multiple sequence alignments of *Lats1* alleles from #3 and #12 strains of F1 with wild-type sequence (top panel) and multiple sequence alignments of *Lats2* alleles from #19 and #22 strains of F1 with wild-type sequence (bottom panel). Dashed lines indicate deletion mutations. Red letters indicate substitution mutations. The numbers in the right panel indicate the length of the deletion nucleotides in the indicated strains. (C) Genotyping of offspring obtained from heterozygote inter-crosses. DNA was prepared from mice tails biopsies The numbers in the right panel indicate the size of nucleotide band. (D) Quantitative RT-PCR analysis of *Lats1* and *Lats2* expression in bone marrow. Data were normalized to those of the wild-type. *Actb* was used as an internal control. $^{*}P < 0.01$, $^{+}P < 0.001$ compared with their wild-type controls. *Actb*: Actin, beta; BM: Bone marrow; Cas9: CRISPR associated protein 9; CRISPR: Clustered regularly interspaced short palindromic repeats; F1: First filial generation; KO: Knockout allele; *Lats1*: Large tumor suppressor 1; *Lats2*: Large tumor suppressor 2; *Lats1-F*: Forward primer of *Lats1* mice genotyping; *Lats1-R*: Reverse primer for *Lats1* mouse genotyping; *Lats1-Wt/He-R*: Reverse primer for distinguishing homozygotes from heterozygotes of *Lats1* mice; *Lats2-F*: Forward primer for *Lats2* mouse genotyping; *Lats2-R*: Reverse primer for *Lats2* mouse genotyping; *Lats2-Wt/He-R*: Reverse primer for distinguishing homozygotes from heterozygotes of *Lats2* mice; RT-PCR: Reverse transcription-polymerase chain reaction; sgRNA: Small guide RNA; WT: Wild-type allele.

primitive and mature murine hematopoietic cells. In general, *Lats1* and *Lats2* were widely expressed in hematopoietic cells, with higher expression levels found in primitive hematopoietic cells than in mature cells, such as $CD4^+$ or $CD8^+$ T cells and $B220^+$ B cells [Figure 1]. Notably, the expression of *Lats1* and *Lats2* was higher in hematopoietic progenitor cells (HPCs: $Lin^-Sca\text{-}1^-c\text{-}Kit^+$) than in long-term HSCs (LT-HSCs) ($Lin^-Sca\text{-}1^+c\text{-}Kit$

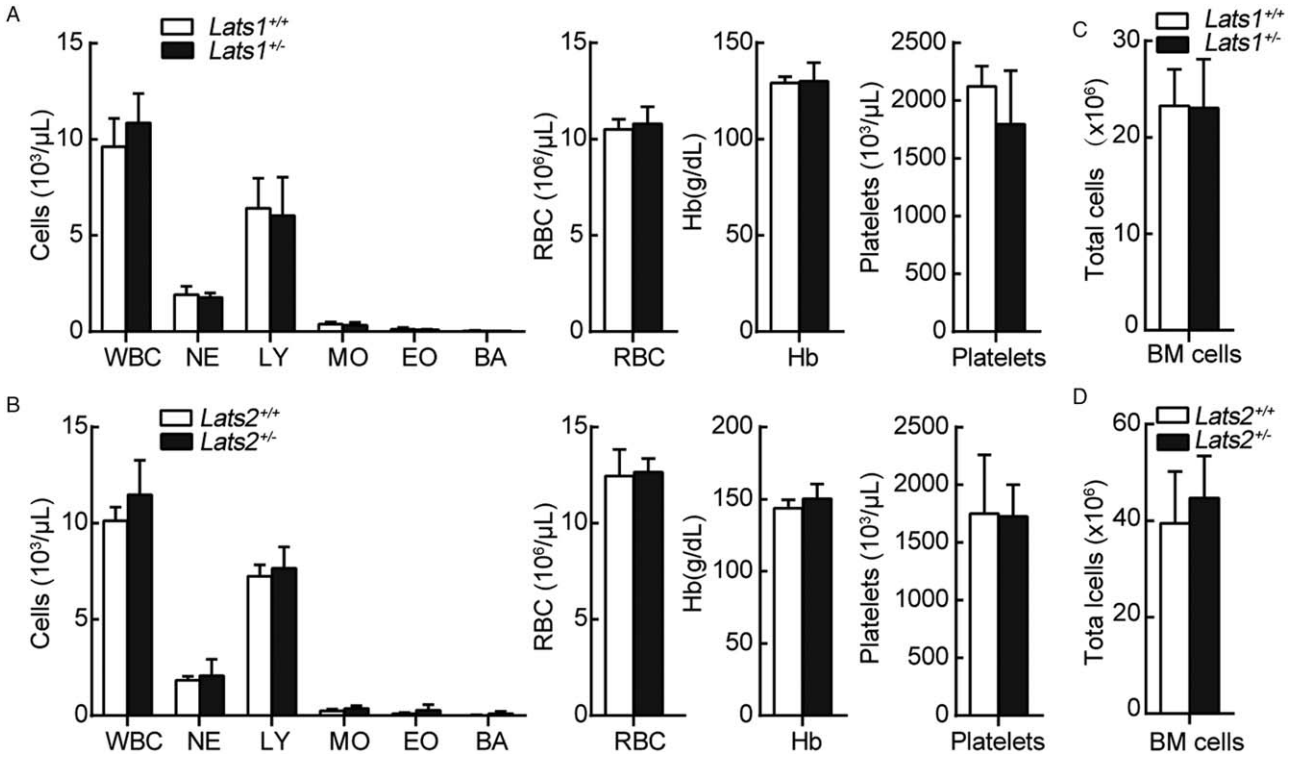

Figure 3: Peripheral blood cell count. (A, B) Absolute total number of WBC, LY, NE, BA, MO, and EO (left), as well as RBC and concentration of Hb (middle) and PLT, in peripheral blood from 8-week-old *Lats1* or *Lats2* heterozygotes and their littermates. (C, D) Total BM cells in *Lats1* or *Lats2* heterozygotes and their littermates. BA: Basophils; BM: Bone marrow; EO: Eosinophils; Hb: Hemoglobin; *Lats1*: Large tumor suppressor 1; *Lats2*: Large tumor suppressor 2; LY: Lymphocytes; MO: Monocytes; NE: Neutrophils; PLT: Platelets; RBC: Red blood cells; WBC: White blood cells.

$^{+}$CD48^{-}CD150^{+}), LSK (Lin^{-}Sca-1^{+}c-Kit^{+}) cells, CMPs (Lin^{-}Sca-1^{-}c-Kit^{+}CD16/32^{-}CD34^{+}), MEPs (Lin^{-}Sca-1^{-}c-Kit^{+} CD16/32^{-}CD34^{-}), and GMPs (Lin^{-}Sca-1^{-}c-Kit^{+} CD16/32^{+}CD34^{+}). In addition, *Lats1* had higher expression levels than *Lats2* in hematopoietic cells, suggesting that *Lats1* plays a more important role in hematopoietic cells.

Generation of mouse models for the Lats1 or Lats2 knockout

To investigate the function of *Lats1* and *Lats2* in normal hematopoiesis, *Lats1* or *Lats2* knockout mice were obtained using the CRISPR/Cas9 system. Two sgRNAs for each gene were designed to target exon 4 of *Lats1* or exon 2 of *Lats2*. Two knockout lines for each gene (#3 and #12 for *Lats1* and #19 and #22 for *Lats2*) were obtained [Figure 2A]. For *Lats1* knockout mice, deletions of 2255 bp plus five point mutations (#3) and deletions of 2251 bp (#12) plus one point mutation led to frameshift mutations. Analogously, deletions of 13,108 and 13,130 bp resulted in frameshift mutations in the *Lats2* knockout mice [Figure 2B]. The following studies were conducted with mice derived from two independent lines. *Lats1* or *Lats2* homozygous mutants were generated by inter-crossing heterozygous mice. However, no homozygotes were born from the *Lats1* or *Lats2* knockout lines [Figure 2C]. Heterozygous mice were viable and fertile and did not show any visible differences compared with their wild-type littermates, although the expression level of *Lats1* and *Lats2* was found to be halved in the BM [Figure 2D]. These results were identical to those obtained by previous studies, which reported that the *Lats1* or *Lats2* homozygous mutation resulted in embryonic lethality.[9,10]

Normal hematopoiesis in Lats1 or Lats2 knockout heterozygotes

As mentioned above, the expression level of *Lats1* and *Lats2* decreased by half in the BM [Figure 2D]. As such, we investigated whether the single allele of *Lats1* or *Lats2* was sufficient for normal hematopoiesis. To determine the consequences of the *Lats1* or *Lats2* heterozygous mutation within the hematopoietic system, several key hematological arguments were analyzed in 8 to 12 week-old mice. No difference was found in numbers of circulating white blood cells, neutrophils, lymphocytes, monocytes, platelets, and red blood cells, as well as hemoglobin concentration [Figure 3A and 3B]. The total number of BM cells was similar between heterozygous mice and their littermate controls [Figure 3C and 3D].

Similarly, flow cytometry analyses revealed no obvious differences in the frequency or total number of early HPCs (HPC, CMP, GMP, MEP, LMPP, and LSK) or HSCs (LT-HSCs and CD34 negative LSK [CD34^{-}LSK]) [Figures 4 and 5]. Overall, based on the analysis of the expression of the cell markers and hematopoietic stem and progenitor cell assays, the hematopoietic profiles were normal in *Lats1* or *Lats2* heterozygotes. These results suggest that a single allele of *Lats1* or *Lats2* is sufficient for normal hematopoiesis.

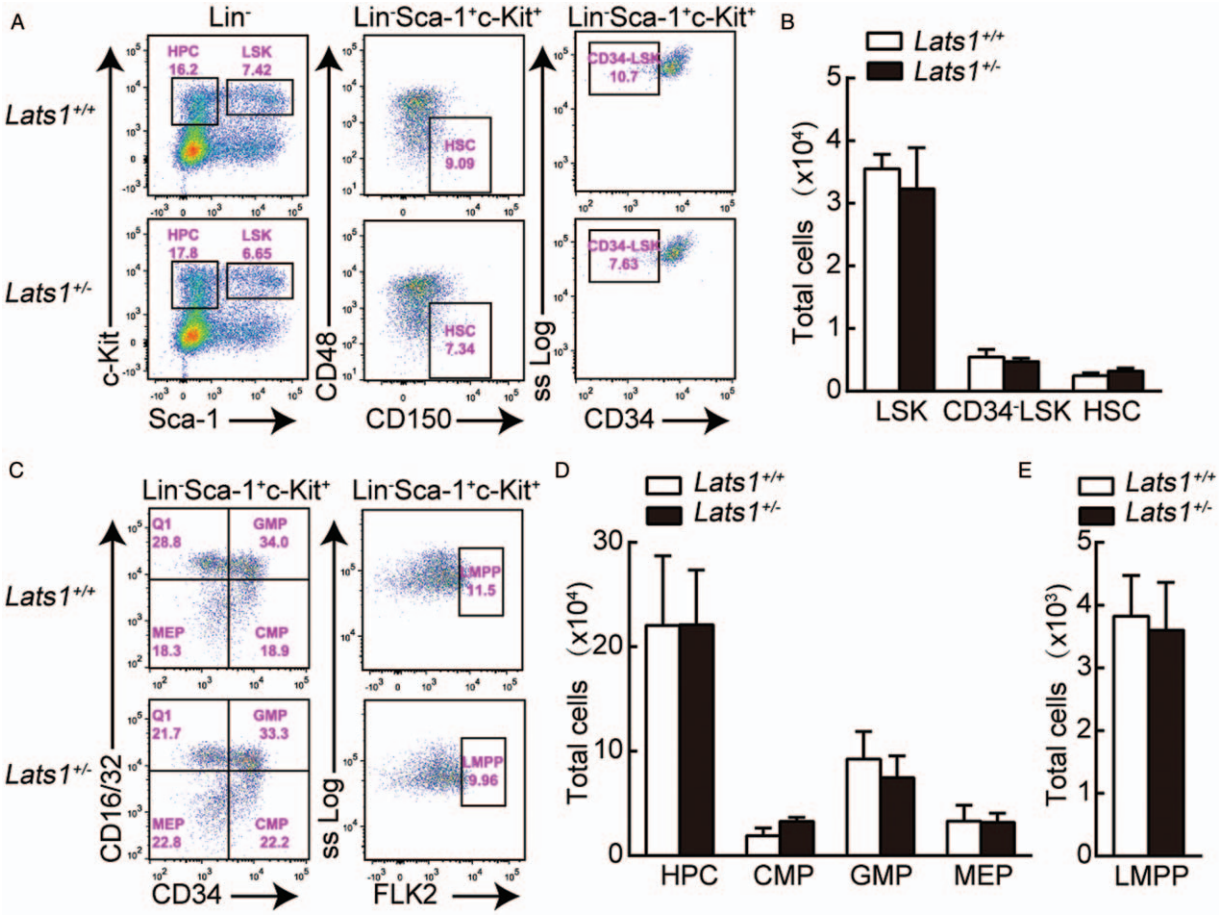

Figure 4: The size of the HPCs and HSCs pools were not impaired in the *Lats1* heterozygotes. (A) Flow cytometry analysis of bone marrow cells from *Lats1*$^{+/+}$ and *Lats1*$^{+/-}$ mice. The numbers indicate the frequency of LSK (Lin$^-$c-kit$^+$Sca-1$^+$) cells or HPCs (Lin$^-$c-kit$^+$Sca-1$^-$) (left), or LT-HSCs (Lin$^-$c-kit$^+$Sca-1$^+$CD48$^-$CD150$^+$) (middle) or CD34$^-$LSK cells (right). (B) The total number of LSK, LT-HSCs, and CD34$^-$LSK cells in the bone marrow of 8-week-old *Lats1*$^{+/+}$ and *Lats1*$^{+/-}$ mice. (C) Flow cytometry analysis of myeloid progenitor cells and HPCs from *Lats1*$^{+/+}$ and *Lats1*$^{+/-}$ mice. The numbers indicate the frequency of MEPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^-$CD34$^-$) (bottom left), CMPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^-$CD34$^+$) (bottom right), GMPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^+$CD34$^+$) (top right), or LMPPs (Lin$^-$c-kit$^+$Sca-1$^+$Flk2$^+$) (right). (D, E) Total HPCs, GMPs, CMPs, and MEPs (D), and LMPPs (E) in the bone marrow of 8-week-old *Lats1*$^{+/+}$ and *Lats1*$^{+/-}$ mice. CD34$^-$LSK: CD34 negative LSK; CMP: Common myeloid progenitor; GMP: Granulocyte-monocyte progenitor; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; *Lats1*: Large tumor suppressor 1; Lin$^-$: Lineage negative cell; LSK: Lin$^-$c-kit$^+$Sca-1$^+$; LT-HSC: Long term hematopoietic stem cell; MEP: Megakaryocyte-erythroid progenitor; SS Log: Side scatter log value; LMPP: Lymphoid-primed multipotent progenitor.

Depletion of Lats1 or Lats2 did not affect the overall survival of heterozygotes

To further evaluate the effects of the depletion of *Lats1* or *Lats2* on the hematopoietic reconstitution of mice, we treated *Lats1* or *Lats2* heterozygous and control mice weekly with injections of 5-FU,[21] which killed rapidly proliferating cells and replenished the hematopoietic system. These mice were monitored on a daily basis for 3 weeks. Both heterozygotes and wild-type control mice died gradually after the second injection of 5-FU, with the remaining mice dying after a third 5-FU injection [Figure 6]. There were no significant differences in overall survival between heterozygotes and wild-type control mice. These results indicated that the depletion of *Lats1* or *Lats2* did not affect the long-term self-renewal of HSCs.

Discussion

Hippo is a key regulator of organ size and stem cell function in the majority of tissues.[22] *Lats1* and *Lats2* are key mediators of Hippo signaling, which function to negatively regulate *YAP* and *TAZ*. To identify whether Hippo signaling is essential for hematopoiesis and the regulation of HSCs, we investigated two knockout mouse models of *Lats1* and *Lats2* mice. The knockout of *Lats1* or *Lats2* resulted in early embryonic lethality, which is in line with the results of previous studies.[9,10] We demonstrate that hematopoiesis or HSC function is normal in the heterozygotes of *Lats1* and *Lats2*. *Lats1* and *Lats2* are considered key stemness genes; however, *Lats1* and *Lats2* are not haploinsufficiency genes. The functions of *Lats1* and *Lats2* are not dosage dependent and the remaining *Lats1* or *Lats2* are sufficient for normal hematopoiesis and HSC function.

Haploinsufficiency describes the situation in which a single functional copy of a gene is insufficient to preserve normal function, which is the main cause of dominant diseases. It has been reported that many genes are haploinsufficient in normal hematopoiesis, such as *Apc*, *Npm1*, and *macroH2A1.1*.[23-25] The haploinfufficiency of *Apc* expands the HSC populations and reduces the capacity to regenerate hematopoiesis.[23] Our findings

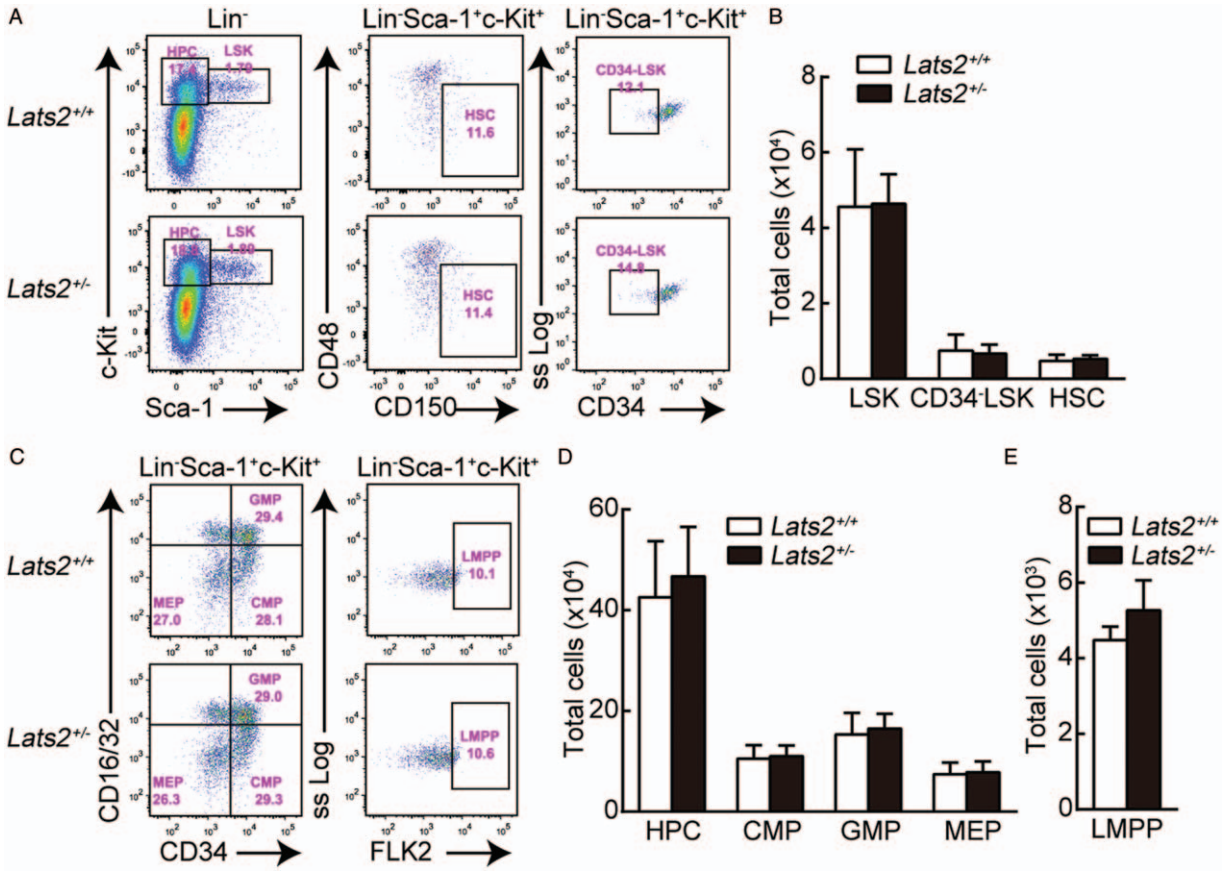

Figure 5: The size of the HPCs and HSCs pools were not impaired in the *Lats2* heterozygotes. (A) Flow cytometry analysis of bone marrow cells from *Lats2*$^{+/+}$ and *Lats2*$^{+/-}$ mice. The numbers indicate the frequency of LSK (Lin$^-$c-kit$^+$Sca-1$^+$) cells or HPCs (Lin$^-$c-kit$^+$Sca-1$^-$) (left), LT-HSCs (Lin$^-$c-kit$^+$Sca-1$^+$CD48$^-$CD150$^+$) (middle) or CD34$^-$LSK cells (right). (B) The total number of LSK, LT-HSCs, and CD34$^-$LSK cells in the bone marrow of 8-week-old *Lats2*$^{+/+}$ and *Lats2*$^{+/-}$ mice. (C) Flow cytometry analysis of myeloid progenitor cells and HPCs from *Lats2*$^{+/+}$ and *Lats2*$^{+/-}$ mice. The numbers indicate the frequency of MEPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^-$CD34$^-$) (bottom left), CMPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^-$CD34$^+$) (bottom right), GMPs (Lin$^-$c-kit$^+$Sca-1$^-$CD16/32$^+$CD34$^+$) (top right), or LMPPs (Lin$^-$c-kit$^+$Sca-1$^+$Flk2$^+$) (right). (D, E) Total HPCs, GMPs, CMPs, and MEPs (D), and LMPPs (E) in the bone marrow of 8-week-old *Lats2*$^{+/+}$ and *Lats2*$^{+/-}$ mice. CD34$^-$LSK: CD34 negative LSK; CMP: Common myeloid progenitor; GMP: Granulocyte-monocyte progenitor; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; *Lats2*: Large tumor suppressor 2; Lin$^-$: Lineage negative cell; LMPP: Lymphoid-primed multipotent progenitor; LSK: Lin$^-$c-kit$^+$Sca-1$^+$; LT-HSC: Long term hematopoietic stem cell; MEP: Megakaryocyte-erythroid progenitor; SS Log: Side scatter log value.

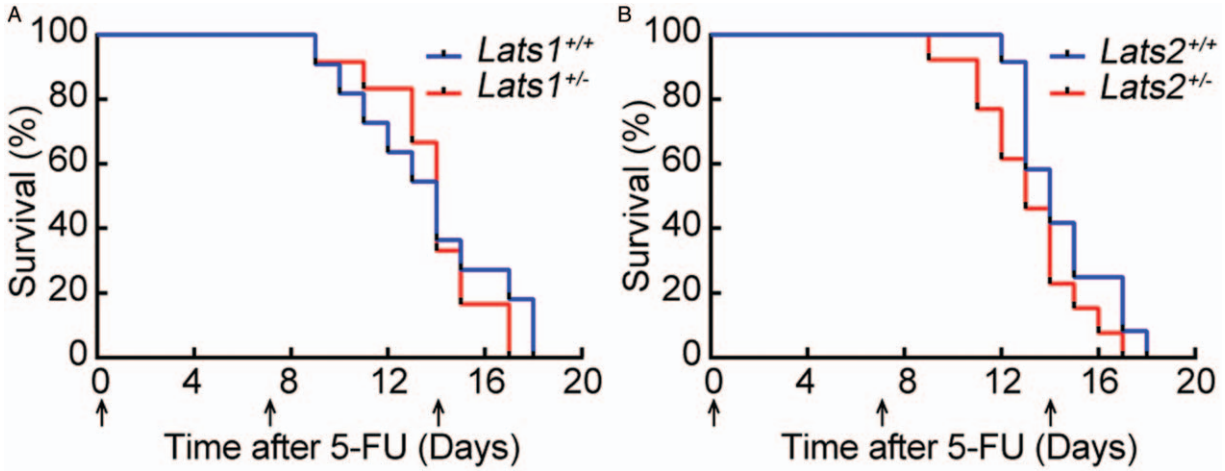

Figure 6: (A and B) Survival curves of *Lats1* or *Lats2* heterozygotes and their wild-type littermates ($n = 11–13$ for each genotype) after three injections (arrowheads indicate 5-FU injection time: 0, 7, and 14 days) of 5-FU, presented as Kaplan-Meier curves. 5-FU: 5-Fluorouracil; *Lats1*: Large tumor suppressor 1; *Lats2*: Large tumor suppressor 2.

have indicated that the size of the HPC and HSC pools were not affected in the *Lats1* and *Lats2* heterozygotes. One possibility is that the downstream effectors of Hippo signaling, such as *Yap1* and *Taz*, are not essential in normal hematopoiesis. The ectopic expression of *Yap1* does not affect the number and function of HSCs neither in the steady state nor under stress conditions.[26] Moreover, the double knockout of *Yap1* and *Taz* does not affect physiological and malignant hematopoiesis.[27] As such, it seems that the Hippo signaling pathway is not essential in the hematopoietic system. *Lats1* and *Lats2* are considered to be stemness genes in many tissues, while the functions of *Lats1* and *Lats2* are clearly dependent on the context.[28] The Hippo signaling pathway is associated with various upstream regulatory molecules, the majority of which are still poorly characterized in mammalian cells. Hippo signaling plays a critical role in the cell-cell interactions that regulate cell proliferation through contact-mediated inhibition.[29] However, the cells in the hematopoietic system reside in a liquid environment, blood, and BM, which are distinct from solid organs. In solid organs, the contact-mediated inhibition of proliferation is critical for regulating tissue homeostasis, while this mechanism may not be effective in liquid tissue. This may explain why the depletion of *Lats1* or *Lats2* leads to overgrowth and aberrant cell proliferation in solid tissue, but not in hematopoietic cells.

Neurofibromatiosis2 (*NF2*) is a well-studied upstream regulator of Hippo signaling in mammalian cells. *Nf2* is essential for contact-mediated inhibition in mammalian cells and inhibits *Yap1* activity in the liver.[29] Loss of *Nf2* in the liver leads to an overgrowth phenotype in line with that observed in the livers of macrophage stimulating 1 (*Mst1*) and macrophage stimulating 2 (*Mst2*) depletion mice and mice with enforced expression of *Yap1*.[11] However, the loss of *Nf2* in hematopoietic cells does not affect the functions of progenitor cells and HSCs, which is consistent with our observations of *Lats1* or *Lats2* knockout.[30] In contrast, the depletion of *Nf2* has obvious effects on many non-hematopoietic cells of the BM microenvironment, resulting in secondary effects on the function and localization of HSCs.[30] One possibility is that *Nf2* functions through the Hippo signaling pathway in this specific context; therefore, it would be interesting to study the role of *Lats1* and *Lats2* within BM microenvironments.

In conclusion, our results show that the inhibition of the Hippo signaling pathway by the depletion of *Lats1* and *Lats2* does not affect hematopoiesis or HSC function. Our study has some limitations. This is a preliminary result on the role of *Lats1* and *Lats2* in normal hematopoiesis. The mRNA levels of *Lats1* and *Lats2* were found to decrease by half; however, the protein level and *Lats1 and Lats2* kinase activity level should be studied further. Although it is clear that the HSC function in the *Lats1* and *Lats2* heterozygotes is not influenced during steady and regeneration states, we cannot exclude the possibility that the HSC function can change under conditions of drastic hematopoietic stress, such as serial transplantations.[19] In addition, whether the depletion of *Lats1* or *Lats2*, in the context of serial transplantations, would affect the size of the HSC pool remains to be investigated further. More extensive and detailed work will be required to elucidate the function of *Lats1* and *Lats2* in a hematopoietic system-specific knockout mouse model.

Funding

This work was supported by grants from the National Key R&D Program of China (No. 2017YFA0106700), National Natural Science Foundation of China (No. 81670096), Natural Science Foundation of Chongqing (No. CSTC2016JCYJA0354), Army Major Scientific Research Projects (No. AWS17J007), Military Medical Innovation Program (No. SWH2018QNKJ-09, No. SWH2018LJ-07, and No. SWH2016ZDCX1010), and the Hubei Natural Science Foundation (No. 2018CFB434).

Conflicts of interest

None.

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How to cite this article: Li ZG, Fu XM, Chai CY, Sun FF, Xiao FF, Huang YX, Yao K, Chen JP, Hou Y. A single copy of large tumor suppressor 1 or large tumor suppressor 2 is sufficient for normal hematopoiesis. Chin Med J 2020;133:1943–1951. doi: 10.1097/CM9.0000000000000934