The transcription factor Zfp90 regulates the self-renewal and differentiation of hematopoietic stem cells

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Abstract
Hematopoietic stem cells (HSCs) can give rise to all blood cells that are essential to defend against pathogen invasion. The defective capability of HSC self-renewal is linked to many serious diseases, such as anemia. However, the potential mechanism regulating HSC self-renewal has not been thoroughly elucidated to date. In this study, we showed that Zfp90 was highly expressed in HSCs. Zfp90 deficiency in the hematopoietic system caused impaired HSPC pools and led to HSC dysfunction. We showed that Zfp90 deletion inhibited HSC proliferation, while HSC apoptosis was not affected. Regarding the mechanism of this effect on HSC proliferation, we found that Zfp90 interacted with Snf2l, a subunit of the NURF complex, to regulate Hoxa9 expression. Ectopic expression of Hoxa9 rescued the HSC repopulation capacity in Zfp90-deficient mice, which indicates that Hoxa9 is the downstream effector of Zfp90. In summary, our findings identify Zfp90 as a key transcription factor in determining the fate of HSCs.

Introduction
Hematopoietic stem cells (HSCs) generate all types of mature blood cells, which are essential for defense against pathogen infection. Although HSCs mostly exist in a quiescent state, they can quickly expand and differentiate in response to intrinsic or extrinsic cues, such as infection1. To maintain normal hematopoiesis, HSCs must maintain a balance between self-renewal and differentiation to preserve a constant hematopoietic stem progenitor cell (HSPC) pool and enough terminal hematopoietic cells. Hematopoiesis is elaborately regulated by signals and transcription factors2,3. Disorder of the regulation network often leads to the abnormal proliferation of HSCs and symmetric division. Dysregulation of particular transcription factors may lead to HSC exhaustion. Therefore, it is highly necessary to define the mechanism of transcriptional regulation in HSCs.

Zinc finger proteins (ZFP) are a diverse family of proteins, which conduct various biological functions. The ZFPs’ functional domains require at least one zinc ion to stabilize the integration of the protein itself4. Zinc finger-containing domains usually bind to DNA, RNA, proteins or small molecules to execute specific biological functions. The ZFP family can regulate gene expression in many tissues4. A previous study showed that Zfp90, a zinc finger protein, is involved in the regulation of cardiac development5. However, the role of Zfp90 in the hematopoietic system remains largely unknown. We found that Zfp90 is specifically highly expressed in HSCs compared with MPPs. Hence, we hypothesized that Zfp90 may play an essential role in HSC maintenance by regulating the expression of specific genes.

Chromatin modifiers have been shown to be important for gene expression during hematopoiesis. In most cases, chromatin is not accessible for transcription-factor binding and transcription initiation. Thus, chromatin remodeling is a prerequisite for gene expression7. Based on the...
common SWI/SNF-related catalytic ATPase subunit, chromatin remodeling complexes can be classified into four major subfamilies, including SWI/SNF, ISWI, CHD, and INO80\(^8\). Among these chromatin remodeling complexes, the nucleosome remodeling factor (NURF) complex, which contains three subunits of Bptf, Snf2l, and Rbbp4 in mammals, can use the energy from ATP hydrolysis to modify the chromatin structure to increase accessibility. Chromatin remodeling factors specifically associate with sequence-specific transcription factors to regulate gene expression\(^9,10\). A previous study has showed that the NURF complex participates in thymocyte maturation and HSC differentiation\(^11\). However, the function of the NURF complex in HSC maintenance has not been thoroughly elucidated to date. In this study, we showed that Zfp90 deletion causes rapid exhaustion of...
HSPC pools. Deletion of Zfp90 using the CRISPR/Cas9 technology impairs the abilities of HSC self-renewal and repopulation. Zfp90 promotes HSC self-renewal via a Hoxa9-dependent fashion. Zfp90 associates with the NURF complex on the promoter of Hoxa9 to initiate Hoxa9 expression.

**Results**

**Zfp90 is essential for the maintenance of HSPC pools**

HSCs are the source of all lineages of hematopoietic cells. Upon sensing differentiation signals, HSCs can differentiate toward multipotent progenitor cells (MPP) and MKE followed by common lymphoid progenitor cells (CLP) or common myeloid progenitor cells (CMP) [12,13]. To maintain the hematopoietic cell pool, HSCs need to maintain a balance between differentiation and self-renewal. Aberrant HSC self-renewal leads to impaired hematopoietic cell pools followed by serious nosohemia. To understand the regulatory mechanism of HSC self-renewal, we analyzed microarray data that was available online regarding HSCs and MPPs in Seita’s cohort [GSE34723] using R language and Bioconductor approaches [14,15]. Surprisingly, we found that many transcription factors were especially highly expressed between differentiation and self-renewal. Aberrant HSC self-renewal leads to impaired hematopoietic cell pools (Fig. 1b).

To ensure that the number of Zfp90 was successfully_dt inserted in the CRISPR/Cas9 vector, we sequenced the genome of the vector control. As expected, Zfp90 was successfully inserted into the genome of the vector control. In addition, we confirmed that the number of Zfp90 was successfully expressed in the CRISPR/Cas9 vector by western blotting.

Next, we analyzed the hematopoietic cells in peripheral blood and found that the lymphocytes and granulocytes were decreased in the Zfp90-deleted mice compared with that in the Zfp90+/+ mice (Fig. 1i). Furthermore, we found that the HSCs in the spleen and splenocytes were also decreased in the Zfp90+/− mice (Fig. 1j). However, we observed that the Zfp90 deletion did not affect the differentiation of HSCs toward either myeloid, erythroid or lymphoid lineages because the GMP/MEP and CMP/CLP ratios in the Zfp90+/− and Zfp90+/+ mice were similar (Fig. 1k). In summary, Zfp90 deletion in mice leads to decreased HSCs and impaired hematopoiesis.

**Zfp90 is indispensable for HSC self-renewal and repopulation capacity**

We showed above that the number of HSCs was decreased in Zfp90−/− mice. The decrease in cell number may be caused by cell death or proliferation. To explore the mechanism, we analyzed HSC apoptosis in Zfp90−/− mice via Annexin V/PI and active caspase 3 staining. We found no obvious change in cell apoptosis after Zfp90 deletion (Fig. 2a,b). However, the proliferation rate of HSCs was reduced when Zfp90 was deleted. We found that the number of Ki67+ HSCs decreased in the Zfp90−/− mice (Fig. 2c). The Zfp90−/− HSCs incorporated much less BrdU than those from the Zfp90+/+ mice (Fig. 2d).

When the ability of HSC proliferation was impaired by Zfp90 deletion, we explored whether the differentiation
and reconstitution capacities of HSCs were affected by Zfp90. First, we performed colony-forming cell (CFC) assays using MethoCult™ GF M3434 to define the potential of myeloid lineage colony formation. We found that Zfp90-deleted HSCs produced much fewer colonies in vitro, such as CFU-GM (Colony-forming unit-granulocyte-macrophage), CFU-M (Colony-forming unit-macrophage), BFU-E (Burst-forming unit-erythroid) and CFU-G (Colony-forming unit-granulocyte) colonies (Fig. 2e). Next, we conducted competitive bone marrow transplantation (BMT) assays to evaluate the capacity of HSC reconstitution. We transplanted a 1:1 mixture of CD45.2+ Zfp90++/− and CD45.1+ WT BM cells into lethally irradiated CD45.1+ recipients. At indicative time points after the transplantation, we analyzed the percentages of CD45.2+ BM cells in the peripheral blood. Our data revealed that the Zfp90−/− BM cells produced fewer blood cells (Fig. 2f). In addition, we performed serial BMT assays to analyze the role of Zfp90 on HSC maintenance. We transplanted 1 × 10⁷ Zfp90++/− or Zfp90−/− HSC (CD45.2+) from chimeras along with 5 × 10⁶ CD45.1+ WT helper BM cells into a lethally irradiated CD45.1+ recipient. After 16 weeks of the transplantation, we calculated the number of donor-derived HSCs in the chimeras. We found that the Zfp90−/− HSCs decreased after serial transplantation (Fig. 2g). Taken together, these findings indicate that Zfp90-deleted HSCs showed reduced proliferation potential and impaired repopulation capacity. The cells rested on an abnormally quiescent status.

**Zfp90 deletion-mediated HSC loss is cell intrinsic**

To determine whether Zfp90-deletion-mediated HSC abnormality is cell intrinsic or extrinsic, we performed BMT assays. We transplanted 1 × 10⁶ GFP+ Zfp90++/− or
Zfp90−/− BM cells (CD45.2+) into lethally irradiated CD45.1+ recipients (Fig. 3a). After 16 weeks later, we analyzed the percentages of LT-HSCs, ST-HSCs, MPPs, CLPs, and MPs. We found that chimeras reconstituted with the Zfp90−/+ or Zfp90−/− BM cells exhibit reduced HSPCs compared with those reconstituted with the Zfp90+/+ BM cells (Fig. 3b–d). Moreover, there was a decrease in the numbers of T cells, B cells, NK cells and granulocytes generated by Zfp90−/− BM cells in blood (Fig. 3e). Furthermore, in the competitive BM transplantation assay, we found that Zfp90 deletion led to reduced percentages of LT-HSCs, ST-HSCs and MPPs in chimera BM (Fig. 3f). Collectively, Zfp90 acted as an intrinsic factor for HSC maintenance.

Zfp90 associates with the NURF complex by interacting with Snf2l

To explore the molecular mechanism through which Zfp90 regulated HSC maintenance, we performed a screen with mouse cDNA library using Zfp90 as a bait via the yeast two-hybrid approach. We identified Snf2l as a new potential candidate to interact with Zfp90 (Fig. 4a). Snf2l, also termed Smarca1, is an important component of the NURF complex that catalyzes nucleosome sliding and interacts with transcription factors to regulate gene expression. In mice, the NURF complex has three subunits of Bptf, Snf2l and Rbbp4. We confirmed the interaction of Zfp90 with the NURF complex via a co-immunoprecipitation (co-IP) assay (Fig. 4b). Our data showed that Myc-tagged Zfp90 enriched HA-Snf2l, His-Rbbp4, and Flag-Bptf (Fig. 4b). To examine the interaction in vivo, we conducted co-IP assays using BM cell lysates. We found that endogenous Zfp90 also interacted with Snf2l and Bptf (Fig. 4c). In addition, Zfp90 was co-localized with Snf2l in the nucleus of HSCs (Fig. 4d). To confirm whether the interaction of Zfp90 with NURF was direct or not, we purified the GST-Zfp90, His-Snf2l, His-Rbbp4, and Flag-Bptf proteins. Next, we performed pull-down assays and found that Zfp90 directly bound to Snf2l, but not to Bptf or Rbbp4 (Fig. 4e). In summary, we showed that Zfp90 associated with the NURF complex by directly binding to Snf2l.
Zfp90 cooperates with the NURF complex to regulate Hoxa9 expression

Next, we sought to explore how Zfp90 cooperates with the NURF complex to regulate HSC maintenance. Previous studies have shown that many transcription factors (TFs) are involved in the regulation of HSC self-renewal, such as Myc, Hoxa9, Gata2, Runx1, Gata3, and Lmo2. We explored whether Zfp90 regulates their expression in HSCs. Thus, we purified Zfp90+/+ and Zfp90−/− HSCs and analyzed the expression levels of these TFs via RT-qPCR. Surprisingly, we found that the Zfp90 deletion impaired Hoxa9 expression (Fig. 5a). Considering that Zfp90 interacts with the NURF complex, we explored whether the NURF complex also regulates Hoxa9 expression. We deleted Bptf, Snf2l or Rbbp4 in HSCs via the CRISPR/Cas9 technology using two different sgRNAs and determined the Hoxa9 expression levels. We validated the knockout of Bptf, Snf2l and Rbbp4 in BM cells via western blot analysis (Supplementary Fig. 1f) and found that the deletion of Bptf, Snf2l or Rbbp4 by either sgRNA also led to decreased mRNA levels of Hoxa9 (Fig. 5b-d). For further confirmation, we isolated LSKs (Lin− Sca-1+ c-Kit+) that contain all HSCs to perform chromatin immunoprecipitation (ChIP) assays. We found that Zfp90 was enriched on the Hoxa9 promoter (−750 to −550 bp) (Fig. 5e). In addition, we confirmed their direct interaction through EMSA assays (Fig. 5f). Next, we conducted luciferase assays using the region (−2000 to 0 bp from transcription start site) of the Hoxa9 promoter and found that Zfp90 overexpression promoted the luciferase activity, whereas deletion of the region (−800 to −550) in the luciferase reporter plasmid abrogated this trend (Fig. 5g).
Next, we evaluated how Zfp90 cooperated with the NURF complex to regulate Hoxa9 expression. We performed ChIP assays with Bptf or Snf2l antibody and found that Zfp90 deletion impaired Bptf and Snf2l enrichment on the Hoxa9 promoter (Fig. 5h, i). Moreover, Zfp90 or Bptf deletion decreased the chromatin accessibility of the Hoxa9 promoter to DNase I digestion (Fig. 5j). Collectively, Zfp90 recruits the NURF complex to the Hoxa9 promoter, and they cooperate to regulate Hoxa9 expression.

**Hoxa9 rescues HSC self-renewal capacity in Zfp90 deficient mice**

To determine whether Hoxa9 is essential for HSC proliferation, we analyzed the effect of Hoxa9 ectopic expression via retrovirus in Zfp90−/− HSCs. We found that overexpressing Hoxa9 restored the differentiation ability of the Zfp90−/− HSCs in CFC assays (Fig. 6a). Moreover, we performed BMT assays. We transplanted 1 × 10³ Zfp90−/− HSCs infected with Hoxa9-overexpressing virus into lethally irradiated recipients.
along with $5 \times 10^5$ CD45.1$^+$ WT BM cells. Sixteen weeks after transplantation, we conducted BrdU incorporation assays and found that Hoxa9 overexpression enhanced the BrdU incorporation by HSCs (Fig. 6b). In addition, ectopic Hoxa9 expression increased the number of LT-HSCs, ST-HSCs, MPPs, CLPs, and CMPs (Fig. 6c). As expected, Hoxa9 expression also restored the reconstitution capacity of the $Zfp90^{-/-}$ HSCs (Fig. 6d). Notably, we showed that the overexpression of Hoxa9 in $Zfp90^{+/+}$ HSCs further promoted HSC expansion (Fig. 6c–d), which was consistent with a previous study. $Zfp90$ regulates HSC homeostasis via recruiting the NURF complex to initiate Hoxa9 expression. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ by two-tailed Student’s $t$ test. All data presented are shown as the means±SD collected from three independent experiments.

**Discussion**

In this study, we identified a novel transcription factor, $Zfp90$, involved in the maintenance of HSCs. We demonstrated that $Zfp90$ played a key role in maintaining HSC self-renewal and repopulation potential in vivo. $Zfp90$ intrinsically regulated HSC proliferation but not apoptosis. Regarding the mechanism, we identified that $Zfp90$ interacted with the NURF complex and synergistically regulated the chromatin accessibility of the Hoxa9 promoter. Hoxa9 transcription activation was directly initiated by $Zfp90$ and the NURF complex. Hoxa9 acts as a downstream effector of $Zfp90$.

In adult mammals, hematopoiesis relied on a rare group of HSCs that rest in the bone marrow and possess the potential to self-renew and differentiate toward all lineage blood cells. The self-renewal and differentiation of HSCs were elaborately regulated by internal and external signals. The cell cycle is essential for HSC proliferation and self-renewal. However, HSCs need to maintain a balance between proliferation and quiescence to maintain normal hematopoiesis. For example, abnormal constitutive activation of Wnt signal leads to early HSC exhaustion. Excessive activation of the Wnt/$\beta$-catenin signal promotes HSCs to enter into the cell cycle and lose the abilities of lineage differentiation and reconstitution. In addition, previous reports showed...
that HSCs were activated during chronic infection through IFN-γ signaling. Constitutive IFN-γ signaling also causes HSC exhaustion. In contrast, loss of proliferation also leads to HSC dysfunction. Many transcription factors have been demonstrated to be essential for HSC proliferation, such as Hoxa9. Hoxa9 deficiency results in impaired proliferation of HSCs. Here, we found that Zfp90 deletion led to the obvious inhibition of HSC proliferation and turnover rates. Zfp90 deletion reduced the expression levels of many transcription factors involved in the regulation of HSC proliferation including Hoxa9. However, the deletion of Zfp90 did not change HSC apoptosis. Thus, our study suggests that the decrease in HSC number in Zfp90-deleted mice is due to impaired proliferation.

Hoxa9 is a member of the Hox gene family that contains a well conserved homeodomain and functions as transcription factors involved in embryonic development. Previous research has showed that Hoxa9 plays an essential role in hematopoiesis. Hoxa9 is preferentially expressed in HSCs and other progenitors and is down-regulated when HSCs differentiate. Hoxa9 deletion in hematopoietic cells leads to a decrease in the number of CMPs. In addition, another study showed that Hoxa9-deficient HSCs displayed impaired proliferation in vitro and did not differentiate into downstream progenitors, especially myeloid lineages. Overexpressing Hoxa9 can rescue the proliferation and differentiation ability of HSCs. In vivo assays also showed that Hoxa9 deletion weakened the HSC repopulating ability. Hoxa9−/− HSCs produced ~60% less myeloid cells compared to WT after bone marrow transplantation. However, Hoxa9-transgenic mice showed more number of HSCs and other progenitors in the BM.

Thus, Hoxa9 plays an indispensable role in HSC maintenance and differentiation, especially toward myeloid cells. Nevertheless, the regulation mechanism of Hoxa9 expression remains unknown. In this study, our data show that the phenotype of Zfp90-deleted mice is similar to that of Hoxa9-mutant mice. Zfp90 acts as an upstream transcription factor to modulate Hoxa9 expression. However, Zfp90 is highly expressed only at the HSC stage, unlike Hoxa9.

Epigenetic modifications including posttranslational modulation of histones, histone variant incorporation, DNA methylation and nucleosome remodeling activity regulate many biological processes, such as gene expression. Exchange of histone variants and nucleosome remodeling rely on the existence of ATP-dependent chromatin remodeling complexes. Many chromatin remodeling complexes have been reported to regulate hematopoiesis. The SWI/SNF-like BAF complex is indispensable to regulate HSC survival. Nucleosome remodeling deacetylase (NURD) is involved in HSC maintenance. In this study, we found that Zfp90 binds to Snf2L, another subunit of the NURF complex, and recruits the NURF complex to the Hoxa9 promoter. By promoting Hoxa9 expression, Zfp90 and the NURF complex cooperate to control HSC proliferation and self-renewal. Our data reveal an additional function of the NURF complex in HSC maintenance.

**Materials and methods**

**Cell culture**

Human 293T cells (ATCC) were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 4 mM L-glutamine. Mouse multipotent HSC/MPP-like cell line EML cells (ATCC) were cultured in IMDM supplemented with 4 mM L-glutamine, 100 ng/ml mSCF, 20% FBS, 100 µg/ml streptomycin and 10U/ml penicillin.

**Antibodies and reagents**

Anti-CD127 (A7R34), anti-CD34 (RAM34), anti-c-Kit (2B8), anti-Sc-1 (D7), anti-CD16/32 (93), lineage cocktail (consisting of anti-CD3, anti-B220, anti-CD11b, anti-Ter119, and anti-Gr1, 88-7772), anti-CD150 (mShad150), anti-CD48 (HM48-1), anti-NK1.1 (PK136), anti-CD11b (M1/70), anti-CD3 (17A2), anti-CD19 (eBio1D3 (ID3)), anti-BrdU (BU20A) and anti-Ki67 (SolA15) were purchased from eBioscience. Anti-active caspase 3 (550821) was purchased from BD Bioscience. Antibodies against Myc (9E10) and GST (1-109) were purchased from Santa Cruz Biotechnology. Antibodies against Flag-tag (M1), β-actin (SP124), anti-Zfp90 (SAB2103688) and His-tag (6AT18) were obtained from Sigma-Aldrich. Anti-Snf2l (PA5-41440) and anti-Bptf (730026) were purchased from Invitrogen Antibodies. Antibodies conjugated with Alexa-488 (A11008) or Alexa-594 (A11012) were purchased from Molecular probes Inc. Hoechst 33342 (14533) were purchased from Sigma-Aldrich. Annexin V-FITC/PI apoptosis detection kit (BMS500FI) was purchased from eBioscience.

**Mouse strains, lentiviral production and bone marrow transplantation**

Eight-week-old male C57BL/6 (CD45.2+) or SJL (CD45.1+) mice (~20 g) were purchased from Charles River and maintained under pathogen-free conditions. We replaced GFP with puromycin in lentiCRISPRv2 (Addgene, 52961) plasmid and cloned an sgRNA guide sequence targeting Zfp90 into this vector. To produce lentivirus, lentiCRISPRv2-vector, lentiCRISPRv2-scramble or lentiCRISPRv2-sgRNA was co-transfected into 293T cells with packaging plasmids, pMD2.G (Addgene, 12259) and psPAX2 (Addgene, 12260), as described previously. For Zfp90 deletion in vivo, CD45.2+ WT BM cells were infected with 2 × 10⁹
infective units of lentiviruses in the presence of 2 µg/ml polybrene and were cultured in StemSpan SFEM (Stem-Cell Technologies) supplemented with 50 ng/ml murine Tpho and 50 ng of murine Scf (both Peprotech) for 2 days. Next, 2 × 10^6 GFP+ BM cells were isolated through FACS and intravenously injected into lethally irradiated CD45.1+ recipients.

For bone marrow transplantation, 1 × 10^6 GFP+ Zfp90+/+ or Zfp90−/− cells isolated from the Zfp90+/+ or Zfp90−/− chimeras were collected via FACS and intravenously injected into lethally irradiated CD45.1+ SJL recipients, as previously reported. In brief, for the first transplantation, 1 × 10^6 GFP+ CD45.2+ Zfp90+/+ or Zfp90−/− cells isolated from the Zfp90+/+ or Zfp90−/− chimeras were collected via FACS and intravenously injected into lethally irradiated (10 Gy) CD45.1+ SJL recipients, as previously reported. For competitive transplantation, 5 × 10^5 CD45.2+ GFP+ Zfp90+/+ or Zfp90−/− BM cells obtained as described above were intravenously injected into lethally irradiated CD45.1+ recipients together with 5 × 10^5 CD45.1+ helper cells, as previously described. At indicated time points post-transplantation, the percentages of donor-derived peripheral blood cells and other indicative cells were examined via FACS. Blood cells were obtained from the tail vein and were stained and analyzed via FACS.

Serial competitive transplantation was performed, as described previously. In brief, for the first transplantation, 1 × 10^5 GFP+ HSCs (CD45.2+) were generated as described above and injected into a lethally irradiated CD45.1+ recipient supplemented with 5 × 10^5 fresh isolated CD45.1+ helper BM cells. For the second or third transplantation, 1 × 10^5 HSCs (CD45.2+Lin−c-Kit−Sca1−CD150+) isolated from chimeras derived from the last transplantation were injected into a lethally irradiated CD45.1+ recipient supplemented with 5 × 10^5 fresh isolated CD45.1+ helper BM cells. Sixteen weeks after transplantation, the number of HSCs was counted via FACS. All animal experiments were approved by the Institutional Animal Care and Use Committees at Academy of Military Medical Sciences. All animal experiments were conducted in accordance with the relevant guidelines and regulations of the Institutional Animal Care and Use Committees at Academy of Military Medical Sciences. Animals and protocols were approved by the Institutional Animal Care and Use Committees at Academy of Military Medical Sciences.

Plasmids

Mouse Bptf coding sequence was cloned into a p3× flag-CMV-9 expression vector. Mouse Rbbp4 full length was cloned into a pCDNA4-His expression vector. Mouse Snf2l was cloned into a pCDNA3-HA expression vector. Mouse Zfp90 was cloned into a pCDNA4-Myc expression vector. For recombinant protein purification, mouse Zfp90 was cloned into a pGEX-6P-1 vector, expressed in E. coli and purified with Glutathione Sepharose 4B beads, according to the manufacturer’s instruction. Mouse Snf2l was cloned into a Pet28a vector for E.coli recombinant expression and purification with Ni-NTA His-resins, according to the manufacturer’s instruction. Mouse Hoxa9 was cloned into a pMY-IREs-GFP vector for retrovirus production. Sg Snf2l (#1: 5′-ATTTTCTTTCCTCTGATATCCA-3′; #2: 5′-CTGCCCCAGGGAGCTTATAC-3′), sgBptf (#1: 5′-CGCGAGCGGAGGCCCCCTAT-3′; #2: 5′-TATGAGGTGTTGCCGAACTT-3′), sgSnf2l (#1: 5′-CTTCTTTAAGGTGGAGCCTT3′-3′; #2: 5′-CTTCTTCTGTACCCGGT3′-3′) were cloned into a lentCRISPRv2 vector. Mouse Hoxa9 promoter region was cloned into a pGL3 basic vector (Promega) for luciferase assays.

Analysis of peripheral blood cells

The peripheral blood was collected from anaesthetized mice and was analyzed using an XFA6030 automated hemacytometer. Cell numbers and percentages of each population were counted.

Flow cytometry

For BM cell analysis, the mice were sacrificed, and the BM cells were collected from the femurs in PBS containing 2% FBS. The cells were sifted through 70 µm cell strainers after removing red blood cells by suspending the cells in red cell lysis buffer. The cells were stained and analyzed via FACS. The staining strategy was performed as followed: LT-HSC (Lin−Sca-1−c-Kit−CD150+CD48−), ST-HSC (Lin−Sca-1−c-Kit−CD150−CD48−), MPP (Lin−Sca-1−c-Kit−CD48−CD150−), CLP (Lin−CD127−Sca-1−c-Kit−), CMP (Lin−c-Kit−Sca-1−CD34−CD16−32+), GMP (Lin−c-Kit−Sca-1−CD34+CD16−32+), MEP (Lin−c-Kit−Sca-1−CD34+CD16−32+) and granulocyte (Gr1+CD11b+). Macrophages were isolated, as previously described. For peripheral cell analysis, CD3+ T cells, CD19+ B cells, NK1.1+ NK cells and CD11b+ granulocytes in PBMCs were stained and evaluated via FACS. For cell cycle analysis, the HSCs were stained with indicated surface marker antibodies, followed by staining with Ki-67 and Hoechst lysis, the HSCs were stained with indicated surface marker antibodies, followed by staining with Ki-67 and Hoechst 33342. For apoptosis analysis, the HSCs were stained with the indicated surface marker antibodies, followed by staining with PI/Annexin V or active caspase 3 antibodies. All data were analyzed using the FlowJo 7.6.1 software.

Immunofluorescence assay

HSCs were isolated and placed on cationic slides and were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. For nuclear protein staining, the HSCs were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. DAPI was used for nuclei staining.
Images were obtained using an Olympus FV1000 laser scanning confocal microscope (Olympus, Japan). The ImageJ software was used for the quantitation of co-localization. For each experiment, at least 100 typical cells were observed.

Immunoprecipitation assay

In total 293 T cells were co-transfected with the corresponding plasmids, maintained for 36 h and harvested. Next, the 293 T cells were lysed with ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 2 mM PMSF, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 10 mg/ml pepstatin A, 150 mM benzamidine, and 1% Nonidet P-40) 4 °C for 2 h. The supernatant lysates were collected and incubated with indicated antibodies at 4 °C overnight. Finally, protein A/G agarose beads were added to the lysates, and immunoblotting was conducted.

Real-time qPCR

Total RNAs from different populations of mouse hematopoietic cells were extracted using the RNA mini-prep Kit (Tiangen, China) according to the manufacturer’s protocol. Next, 1 µg of total RNA per aliquot was used as a template for synthesizing cDNA with M-MLV reverse transcriptase (Promega, USA). For expression analysis of indicative genes, quantitative PCR analysis and data collection were performed using the ABI 7300 qPCR system. The qPCR primer sequences are available if requested.

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed according to a standard protocol (Upstate, USA). LSKs were purified, fixed in 1% formaldehyde for 10 min at 37 °C and lysed with ChIP SDS lysis buffer. DNA in the lysates were sheared into 200–500 bp by sonication. The lysates were incubated with 5 µg of the indicated antibody overnight at 4 °C, followed by immunoprecipitation with salmon sperm DNA/protein agarose beads. After washing, elution, cross-link reversal and purification, DNA from the ChIP sample was analyzed via qPCR.

DNasel accessibility assay

The BM cells were collected, and the LSKs were purified. The nuclei were isolated from the LSKs using the Nuclei isolating Kit (Sigma-Aldrich) according to the manufacturer’s protocol. The nuclei were resuspended in 150 µl of DNase 1 digestion buffer (1 mM EDTA, 0.1 mM EGTA, 5% sucrose, 1 mM MgCl₂, and 0.5 mM CaCl₂). Two equal aliquots of 75 µl of nuclei were treated with 0 or 2 units of DNase 1 (Sigma, USA) at 37 °C for 5 min. The reactions were stopped using 2 × DNase 1 stop buffer (20 mM Tris, pH 8.0, 4 mM EDTA, 2 mM EGTA). The DNA was extracted and analyzed using qPCR.

Yeast two hybrid screening

Yeast two-hybrid screening was performed using the Matchmaker Gold Yeast Tow-Hybrid system (Clontech laboratories, Mountain View, USA) following the manufacturer’s instruction. In brief, mouse Zfp90 was cloned into a pGBK7 plasmid as BD-Zfp90 bait. Yeast AH109 cells were transfected with BD-Zfp90 and plasmids containing mouse spleen cDNA library (Clontech). The candidates were further identified via DNA sequencing.

EMSA assay

EMSA experiments were conducted according to the manufacturer’s protocol using a Light Shift Chemiluminescent RNA EMSA Kit (Thermo Scientific). In brief, His-Zfp90 protein was incubated with a Biotin-labeled probe in the reaction system for 20 min at RT. The samples were analyzed in 4% polyacrylamide gel in 0.5 × TBE buffer. After being transferred on a nylon membrane (Amersham Biosciences), the labeled DNA was cross-linked by UV, checked with streptavidin-HRP conjugate and resolved using the detection substrate. The Hoxa9 promoter sequence for EMSA was 5'-TCTTCTTCTGGCCTCAAGCGAGGGGGTGTGGATCCCGGGAGCTTCGAGCCCCCTCTCT-3'.

BrdU incorporation

The mice were i.p. injected with one dose (200 µg) of BrdU and fed continuously with water containing 800 µg/ml BrdU and 5% glucose for 4 days. BrdU was detected via FACS using the BrdU labeling kit, as previously described.

Colony-forming assays

LSKs were isolated from wild-type C57BL/6 mice and infected with lentivirus containing Zfp90-sgRNA or a scramble sequence. One day later, GFP⁺ Zfp90⁺/+ or Zfp90⁻/⁻ HSCs (Lin⁻GFP⁺c-Kit⁻Sca1⁺CD150⁻) were sorted via FACS into a 96-well plate containing Methylcellulose Media (M3434; Stem cell technology). After incubation for 9–12 days, CFU-GM, CFU-M, BFU-E and CFU-G colonies were counted, as described previously.

Statistical analysis

An unpaired Student’s t-test was used for statistical analysis in this study. Statistical calculation was performed using Microsoft Excel or SPSS 13.

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