Mmrn1 expression defines a novel subset of hematopoietic stem cells and leukemia stem cells with great self-renewal potential

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Abstract

Hematopoietic stem cells (HSC) are critical for lifelong blood cell generation. After mutation accumulation and functional disruption, HSC may transform into leukemic stem cells (LSC), leading to malignant hematological disorders. However, both HSC and LSC are highly heterogeneous, which hinders our comprehensive understanding of their biological characteristics and clinical application. Here, we identified multimerin 1 (Mmrn1) as a reliable marker for the most primitive HSC and LSC. We found that *Mmrn1* was abundantly present in human and mouse HSC. Interestingly, HSC with high levels of *Mmrn1* displayed increased quiescence and regenerative capacity, accompanied by megakaryocytic lineage commitment. Importantly, *Mmrn1* deficiency gradually impairs HSC self-renewal under stress of transplantation due to reduced quiescence. Additionally, we noticed that *Mmrn1* was specifically upregulated in acute myeloid leukemia (AML) cells, and its overexpression predicted poor patient prognosis. Further investigation revealed that Mmrn1 marked a subset of quiescent LSC responsible for AML initiation and development, and that deletion of *Mmrn1* delays AML progression. Collectively, these data broaden our knowledge of stem cell heterogeneity in the context of normal and malignant hematopoiesis and advance the precision diagnosis and therapy of AML in the clinic.

Introduction

Hematopoietic stem cells (HSC) are a rare cell population that possesses the capacity to self-renew and differentiate into all lineages of blood cells throughout life.[1] The classical tree-like hierarchical model of hematopoiesis has been instrumental in understanding the step-by-step differentiation process of HSC, but it raises confusing questions, such as the divergent states and functions of HSC with similar immunophenotypes.[2,3] This heterogeneity poses a major challenge in elucidating the intrinsic properties and regulatory mechanisms.[4] Although recent single-cell RNA sequencing (scRNA-seq) technologies have provided insight into the characteristics of different HSC subpopulations, experimental evidence is still lacking.[5]

At present, a new model of hematopoietic differentiation, in which lineage determination occurs at the HSC level, has been proposed.[6,7] Some markers have been applied to discriminate distinct HSC subsets, but they are still often used in combination to improve identification accuracy.[8-10] In addition, HSC can be transformed into leukemic stem cells (LSC), also known as leukemia-initiating cells (LIC), when genetic and epigenetic mutations accumulate, therefore resulting in the development and progression of acute myeloid leukemia (AML).[11] Therefore, LSC share many characteristics with HSC, including cell cycle quiescence and strong self-renewal ability.[12,13] For instance, the presence of a dormant LSC subset is the root cause of AML

chemoresistance and recurrence.[14] Therefore, identifying specific markers that can refine the analysis of HSC and LSC is urgent and of significant practical importance.

Multimerin 1 (Mmrn1), a member of the EMILIN protein family, is present in multiple tissues, including brain, muscle, skin and bone marrow (BM), as shown by the Human Protein Atlas database. Initially, Mmrn1 was reported to promote the adhesion of platelets via binding to von Willebrand factor.[15] Additionally, Mmrn1 participates in modulating cell viability and thus affects tissue regeneration and repair.[15] Notably, *Mmrn1* has been found to be overexpressed in a variety of cancers including cervical cancer and colorectal cancer, and its upregulation is positively correlated with poor patient prognosis.[16] In the hematopoietic system, *Mmrn1* overexpression is also associated with the hemorrhagic symptoms of Quebec platelet disorder (QPD) and adverse outcomes in pediatric AML.[17,18] Although research has made some progress, the exact role of Mmrn1 in HSC and LSC remains to be fully elucidated.

In this study, we demonstrated that *Mmrn1* was enriched predominantly in HSC. However, HSC did not express *Mmrn1* uniformly, and those with high levels of *Mmrn1* exhibited increased quiescence, regenerative capacity and megakaryocytic lineage commitment. Importantly, knockout of *Mmrn1* reduced HSC quiescence, therefore impairing HSC self-renewal under stress of transplantation. Furthermore, *Mmrn1* expression could identify LSC with high leukemic initiation potential, and its deficiency inhibits AML progression. Collectively, our findings reveal that Mmrn1 is a reliable marker in distinguishing HSC heterogeneity and provide an effective observational indicator for the development and treatment of AML.

Methods

Animals

Wild-type (WT) C57BL/6J mice were purchased from Laipite Biotechnology Company (Chongqing, China). $Mmrn1^{+/eGFP}$ and NOD-$Prkdc^{scid}$ $Il2rg^{em1/Smoc}$ (M-NSG) mice were obtained from the Model Organisms Center (Shanghai, China). $Mmrn1^{-/-}$ mice were purchased from Cyagen Biosciences Inc (Suzhou, China). Congenic CD45.1 mice were kindly gifted by Prof. Jinyong Wang. All mice were male and used at 6-8 weeks of age unless otherwise specified. All animal experiments were approved by the Animal Care Committee of the Third Military Medical University (Chongqing, China).

Human samples

Human umbilical cord blood (UCB) samples were obtained from Southwest Hospital (The First Affiliated Hospital of Third Military Medical University), and then $CD34^+$ cells were isolated using the Human CD34 Positive Selection kit (Stem Cell Technologies, Vancouver, Canada). BM specimens from AML patients and healthy volunteers were obtained from Daping Hospital (The Third Affiliated Hospital of Third Military Medical University). All participants provided informed consent, and the use of human UCB and BM samples were approved by the institutional review board of the Third Military Medical University (Chongqing, China).

Flow cytometry

Analysis of mouse or human hematopoietic cell phenotype, cell cycle, bromodeoxyuridine incorporation, apoptosis and ploidy distribution was carried out as we previously described.[19,20] Flow cytometry detection was conducted using a FACSVerse flow cytometer (BD Biosciences, San Jose, CA, USA), and data analysis was performed via FlowJo version 10.0 software (TreeStar, San Carlos, CA, USA). Cell sorting was performed on a FACSAria III sorter (BD Biosciences).

RNA sequencing

Total RNA was isolated from $Mmrn1^{-/low}$ and $Mmrn1^{high}$ long-term HSC (LT-HSC). After quality assessment, samples were subjected to RNA sequencing (RNA-seq) analysis at Sinotech Genomics Co., Ltd (Shanghai, China). Genes with a fold change >2 and a *P* value <0.05 were considered differentially expressed genes (DEG). The methods for generating scatter plots and performing gene set enrichment analysis (GSEA) were described in our previous works.[21,22] All the data have been deposited in the Gene Expression Omnibus (GEO) database (number GSE233828).

Statistical analysis

The experimental data were analyzed using the GraphPad Prism 10.1.2 software (La Jolla, CA, USA). The within-group variances were similar. Unpaired two-tailed Student's *t* tests were used for two-group comparisons, and one-way analysis of variance (ANOVA) followed by Tukey's test was used for multiple-group comparisons. Kaplan-Meier survival curves were compared via the log-rank test. Correlation analysis was conducted by Spearman's correlation test. Each experiment was independently performed at least three times. The data are presented as the mean ± standard deviation (SD). **P*< 0.05; ***P*<0.01; and ****P*<0.001 are considered statistically significant.

Additional methods

Additional methods are provided in the *Online Supplementary Appendix*. All antibodies and primers are listed in the *Online Supplementary Tables S1* and *S2*, respectively.

Results

Mmrn1 is highly expressed in primitive human and mouse hematopoitic stem cells

Studies have shown that Mmrn1 is expressed in megakaryocytes/platelets and participates in regulating the

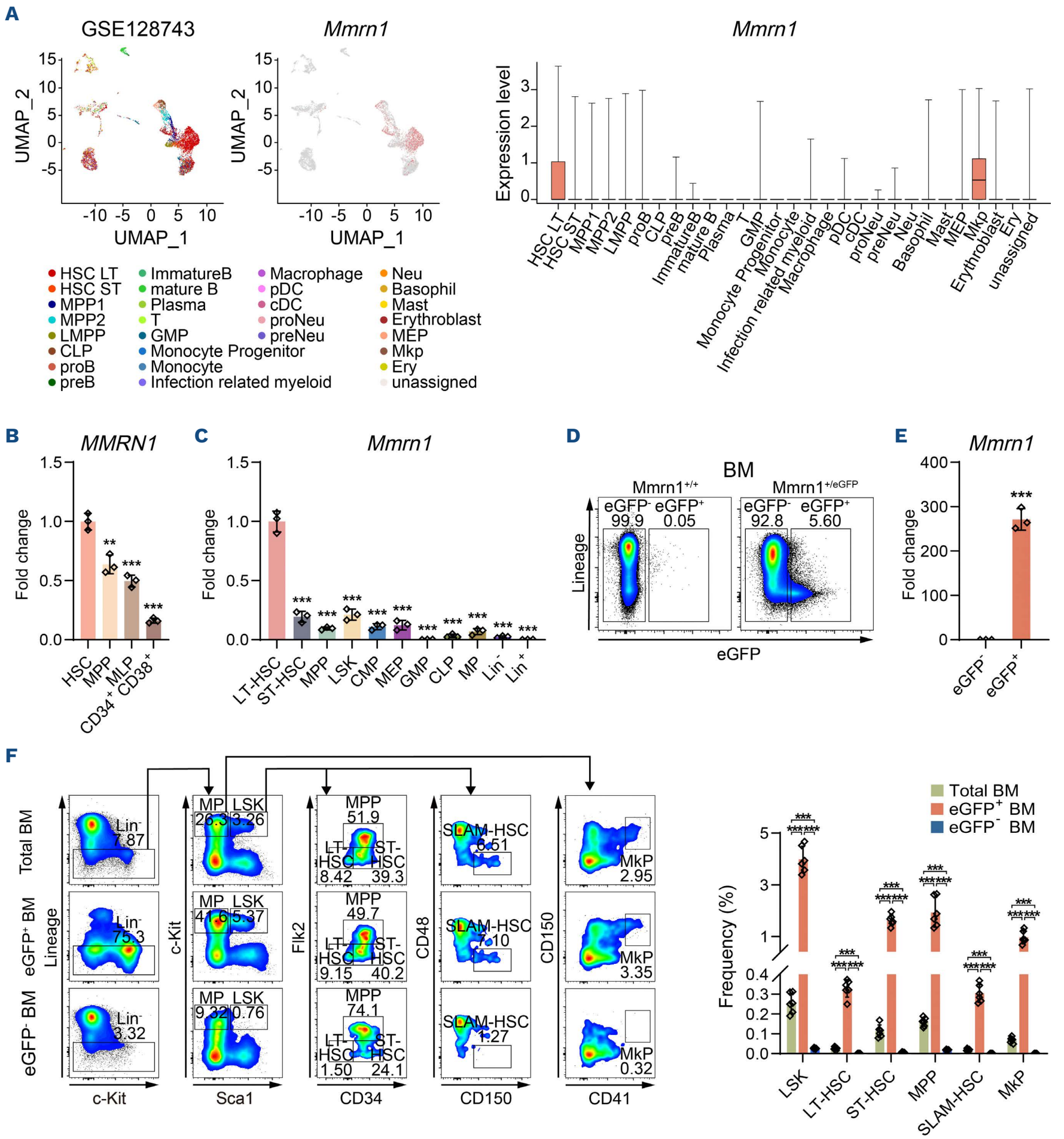

Figure 1. *Mmrn1* is highly expressed in primitive human and mouse hematopoietic stem cells. (A) Single cell RNA sequencing (scRNA-seq) analysis of the expression of *Mmrn1* in mouse bone marrow (BM) cells (GSE128743) from the Atlas of Blood Cells database. (B) Quantitative polymerase chain reaction (qPCR) analysis of *MMRN1* mRNA expression in the indicated hematopoietic cell populations purified from umbilical cord blood (UCB) cells (N=3). The flow cytometric gating strategies are provided in *Online Supplementary Figure S1C*. Statistical differences were analyzed by comparing each group with hematopoietic stem cells (HSC). (C) qPCR analysis of *Mmrn1* mRNA expression in the indicated hematopoietic cell populations purified from mouse bone marrow (BM) (N=3). The flow cytometric gating strategies are provided in *Online Supplementary Figure S1D*. Statistical differences were analyzed by comparing each group with long-term HSC (LT-HSC). (D) Representative flow cytometric plots showing the frequency of eGFP- and eGFP$^+$ BM cells from Mmrn1$^{+/+}$ and Mmrn1$^{+/eGFP}$ mice. (E) qPCR analysis of *Mmrn1* mRNA expression in the sorted eGFP$^-$ or eGFP+ BM cells from Mmrn1$^{+/eGFP}$ mice (N=3). (F) The percentages of the indicated HSPC populations in total, eGFP$^-$ and eGFP$^+$ BM cells from Mmrn1$^{+/eGFP}$ mice (N=6). HSPC were distinguished by CD34 and Flk2 or SLAM family markers (CD150 and CD48), respectively. Representative flow cytometric plots are shown in the left. Data are shown as the mean ± standard deviation. ***P*<0.01; ****P*<0.001.

process of blood clotting.[15,23] Interestingly, by analyzing the Atlas of Blood Cells database, we found that Mmrn1 is also enriched in human and mouse hematopoietic stem and progenitor cell (HSPC) populations (Figure 1A; *Online Supplementary Figure S1A*). Similar results were observed in the BloodSpot database (*Online Supplementary Figure S1B*). To confirm these findings, we purified various hematopoietic cells from human UCB and mouse BM by flow cytometry (*Online Supplementary Figure S1C, D*). Subsequently, quantitative polymerase chain reaction (qPCR) analysis verified that *Mmrn1* expression was higher in primitive HSC than in other non-megakaryocytic hematopoietic cells (Figure 1B, C).

To further elucidate the characteristics of Mmrn1, we engineered a transgenic mouse model (Mmrn1$^{+/eGFP}$) by knocking eGFP into the *Mmrn1* locus (*Online Supplementary Figure S2A*). The transgenic manipulation did not interfere with Mmrn1 expression or the hematopoietic homeostasis in the mice (*Online Supplementary Figure S2B-E*). Notably, we detected a small eGFP^{+} population in the BM of Mmrn1$^{+/eGFP}$ mice, where *Mmrn1* expression was observed in eGFP^{+} but not eGFP^{-} cells (Figure 1D, E). These data suggest that Mmrn1$^{+/eGFP}$ mice may be an ideal model for tracking *Mmrn1* expression *in vivo*. Consistent with the above results, HSC from these mice displayed relatively high mean fluorescence intensity (MFI) of eGFP (*Online Supplementary Figure S2F*). Alternatively, the majority of HSPC, as well as megakaryocyte progenitors (MkP), were significantly present within the eGFP^{+} BM cells obtained from Mmrn1$^{+/eGFP}$ mice (Figure 1F). Therefore, Mmrn1 may be a reliable marker for distinguishing primitive HSC.

Mmrn1 expression synchronizes with the hematopoietic stem cell state after various stresses

HSC behavior is closely related to the cell cycle state.[21,24] However, how to effectively identify the HSC state is also an issue that urgently needs to be addressed. Given that the expression of c-Kit is markedly disrupted following irradiation (IR) or 5-fluorouracil (5-FU) treatment, we utilized EPCR in conjunction with SLAM instead of conventional markers to define HSC and therefore referred these cells as ESLAM-HSC (*Online Supplementary Figure*

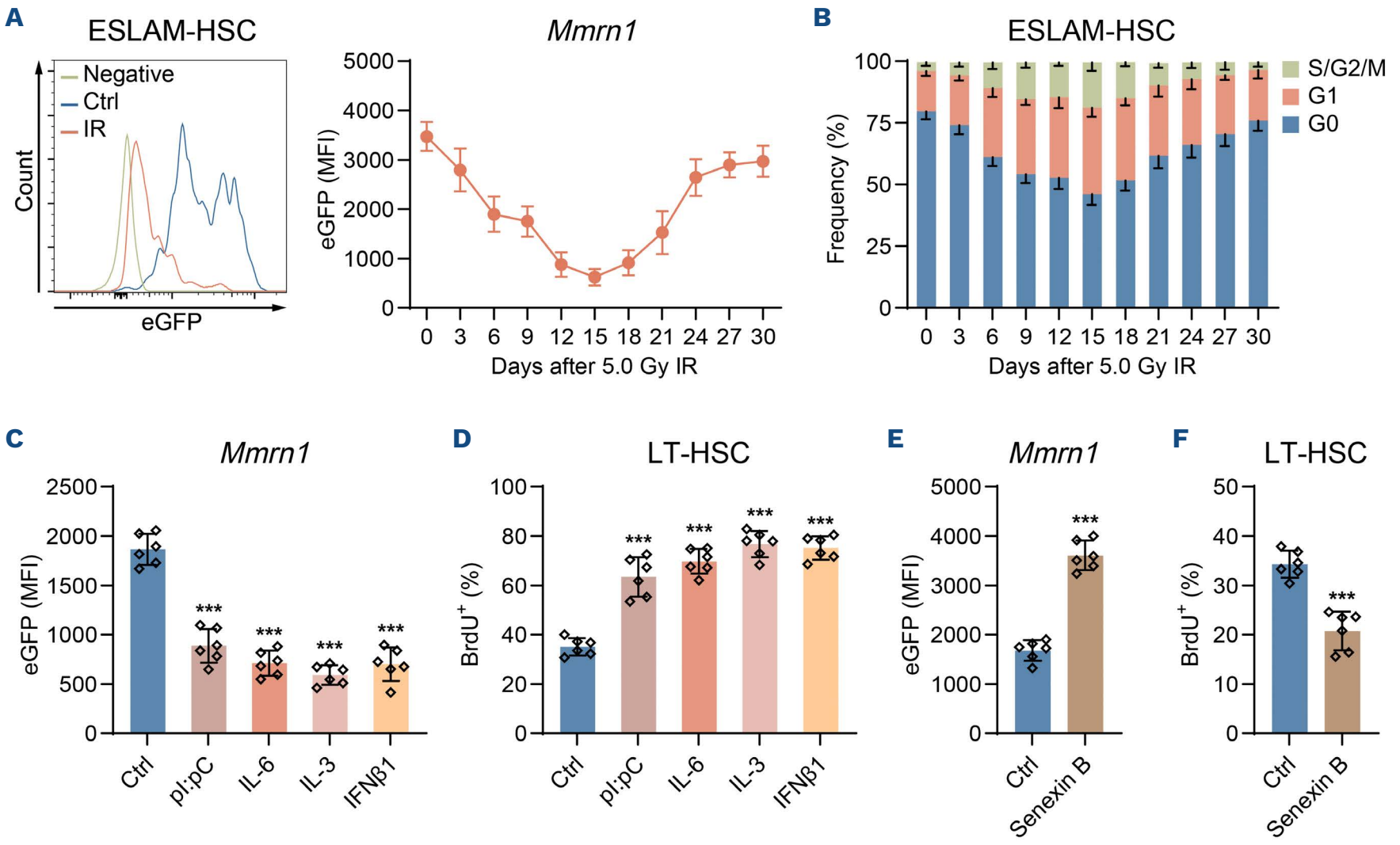

Figure 2. *Mmrn1* expression is synchronized with the hematopoietic stem cell state after various stresses. (A) Flow cytometric analysis of *Mmrn1*-eGFP expression in ESLAM-hematopoietic stem cells (HSC) from the bone marrow (BM) of Mmrn1$^{+/eGFP}$ mice at the indicated time points after 5.0 Gy irradiation (IR) (N=6 per time point). Wild-type (WT) mice served as negative controls (Ctrl). Representative flow cytometric plots at 15 days after 5.0 Gy IR are shown on the left. MFI: mean fluorescence intensity. (B) Cell cycle analysis of ESLAM-HSC from the BM of Mmrn1$^{+/eGFP}$ mice at the indicated time points after 5.0 Gy IR (N=6 per time point). The flow cytometric gating strategies are provided in *Online Supplementary Figure S3A*. (C-F) LT-HSC sorted from the BM of Mmrn1$^{+/eGFP}$ mice were cultured with polyinosinic:polycytidylic acid (pI:pC; 1 μg/mL), IL-6 (50 ng/mL), IL-3 (20 ng/mL), IFNβ1 (1000 units/mL) or Senexin B (5 μM), a CDK19 inhibitor, for 48 hours (N=6). (C, E) *Mmrn1*-eGFP expression and (D, F) the percentage of BrdU^{+} cells in cultured LT-HSC were analyzed by flow cytometry. Data are shown as the mean ± standard deviation. ***P<0.001.

S3A). Intriguingly, the expression of *Mmrn1* tended to first decrease but then increase in ESLAM-HSC, which was consistent with the proportion of cells in G0 phase at the indicated time points (Figure 2A, B; *Online Supplementary Figure S3B, C*). Additionally, CD86 was used to replace Sca-1 as a reliable HSC marker under lipopolysaccharide (LPS) treatment condition, which can significantly affect Sca1 expression and result in its inability to stably label HSC.[25] In the L86K-HSC of the Mmrn1$^{+/eGFP}$ mice treated with LPS, the changes in *Mmrn1* expression and G0 phase proportion were still strongly correlated (*Online Supplementary Figure S3D-F*). In particular, the expression of *Mmrn1* increased with age, as HSC tended to be more quiescent (*Online Supplementary Figure S3G, H*). Furthermore, *in vitro* culture experiments with the addition of known cell cycle regulatory factors also showed a clear negative correlation between *Mmrn1* expression and HSC proliferation (Figure 2C-F). Hence, these results suggest that *Mmrn1* expression may also be used to reflect the state of HSC.

Mmrn1 expression distinguishes a distinct population of hematopoietic stem cells

HSC exhibit high heterogeneity and can be grouped into different subpopulations using various marking strategies.[3,26] Flow cytometry analysis of Mmrn1$^{+/eGFP}$ mice revealed that eGFP expression was variable in HSC, which could be divided into Mmrn1$^{-/low}$ and Mmrn1^{high} groups (Figure 3A). A significant difference in *Mmrn1* mRNA expression was detected between the two subsets (Figure 3B). Of note, we found that compared with Mmrn1$^{-/low}$ LT-HSC, Mmrn1^{high} LT-HSC had a higher percentage of cells in the G0 phase and incorporated less BrdU, suggesting that Mmrn1^{high} LT-HSC are more quiescent (Figure 3C; *Online Supplementary Figure S4A*). However, apoptosis rates did not significantly differ between these two groups (*Online Supplementary Figure S4B*). Similar phenomena were observed in HSC marked by SLAM (*Online Supplementary Figure S4C-E*).

Next, Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC were isolated and subjected to RNA-seq analysis (*Online Supplementary Figure*

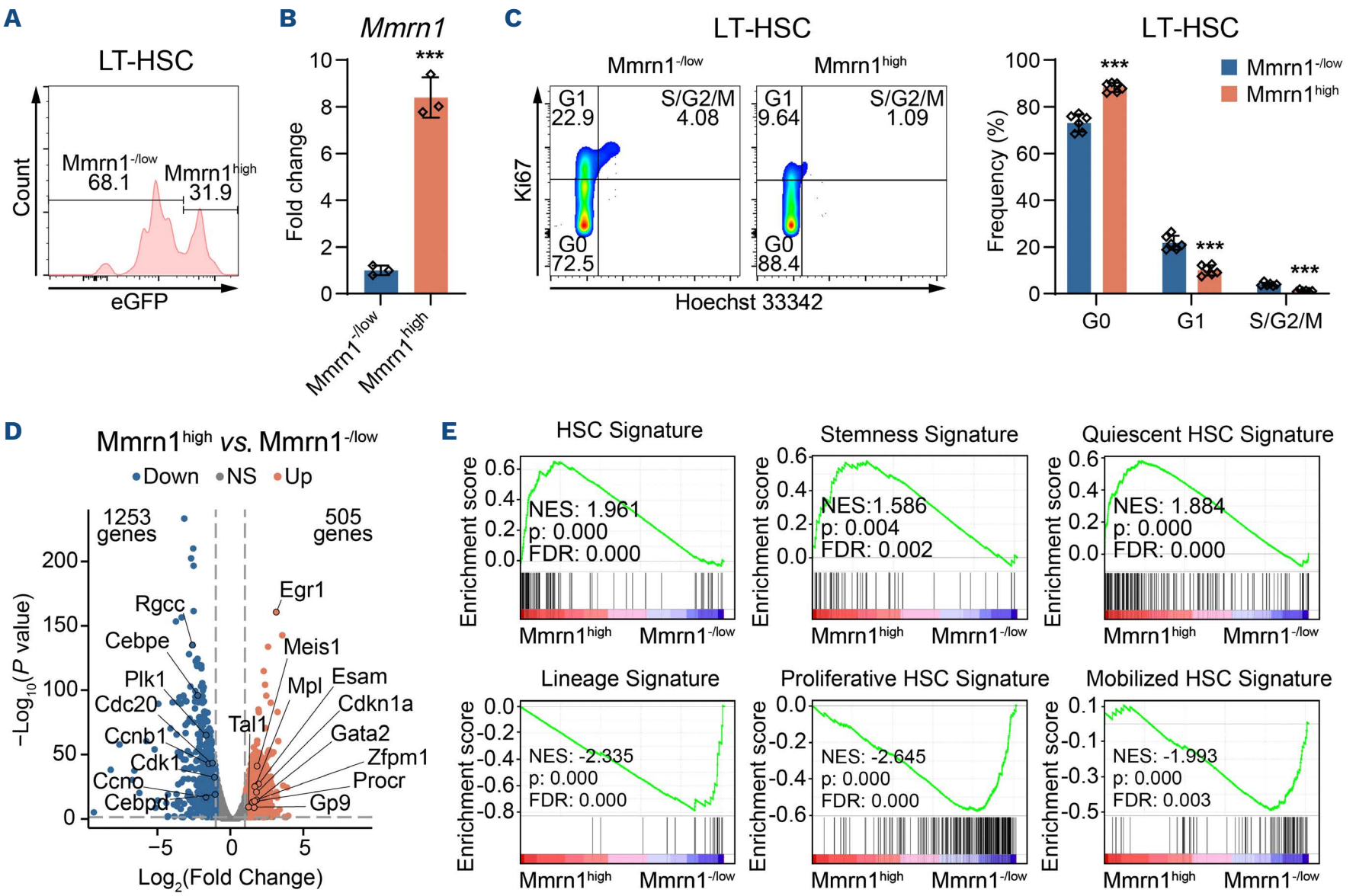

Figure 3. *Mmrn1* expression distinguishes a distinct population of hematopoietic stem cells. (A) The gating strategies for subsequent flow cytometric analysis and sorting of Mmrn1$^{-/low}$ and Mmrn1^{high} long-term hematopoietic stem cells (LT-HSC) from the bone marrow (BM) of Mmrn1$^{+/eGFP}$ mice. (B) Quantitative polymerase chain reaction (qPCR) analysis of *Mmrn1* mRNA expression in Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC sorted from the BM of Mmrn1$^{+/eGFP}$ mice (N=3). (C) Cell cycle analysis of Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC from the BM of Mmrn1$^{+/eGFP}$ mice (N=6). (D) The volcano plot showing differentially expressed genes (DEG) between Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC. Representative DEG are indicated in the plot. (E) Gene set enrichment analysis (GSEA) of the RNA-sequencing data using HSC-related gene sets. Data are shown as the mean ± standard deviation. ***P<0.001.

S4F). The results showed that 505 genes were upregulated and 1,253 genes were downregulated in Mmrn1^{high} LT-HSC (Figure 3D). The GSEA results indicated that stemness and quiescence-associated signatures were enriched in Mmrn-1^{high} LT-HSC, whereas lineage differentiation, proliferation and mobilization-associated signatures were enriched in Mmrn1$^{-/low}$ LT-HSC (Figure 3E). In line with these data, qPCR analysis also substantiated changes in genes involved in the cell cycle modulation (*Online Supplementary Figure S4G*). Overall, the data suggest that these two HSC populations may play different roles in the hematopoietic system.

Mmrn1^{high} hemtopoietic stem cells exhibit robust hematopoietic regenerative capacity

To further delineate the differences between Mmrn$^{1-/low}$ and Mmrn1^{high} LT-HSC, we conducted additional assessments of these subsets. The *in vitro* assays show that Mmrn1^{high} LT-HSC were capable of generating more daughter cells, accompanied by increased colony-forming ability (Figure 4A, B). Moreover, HSC transplantation (HSCT) experiments

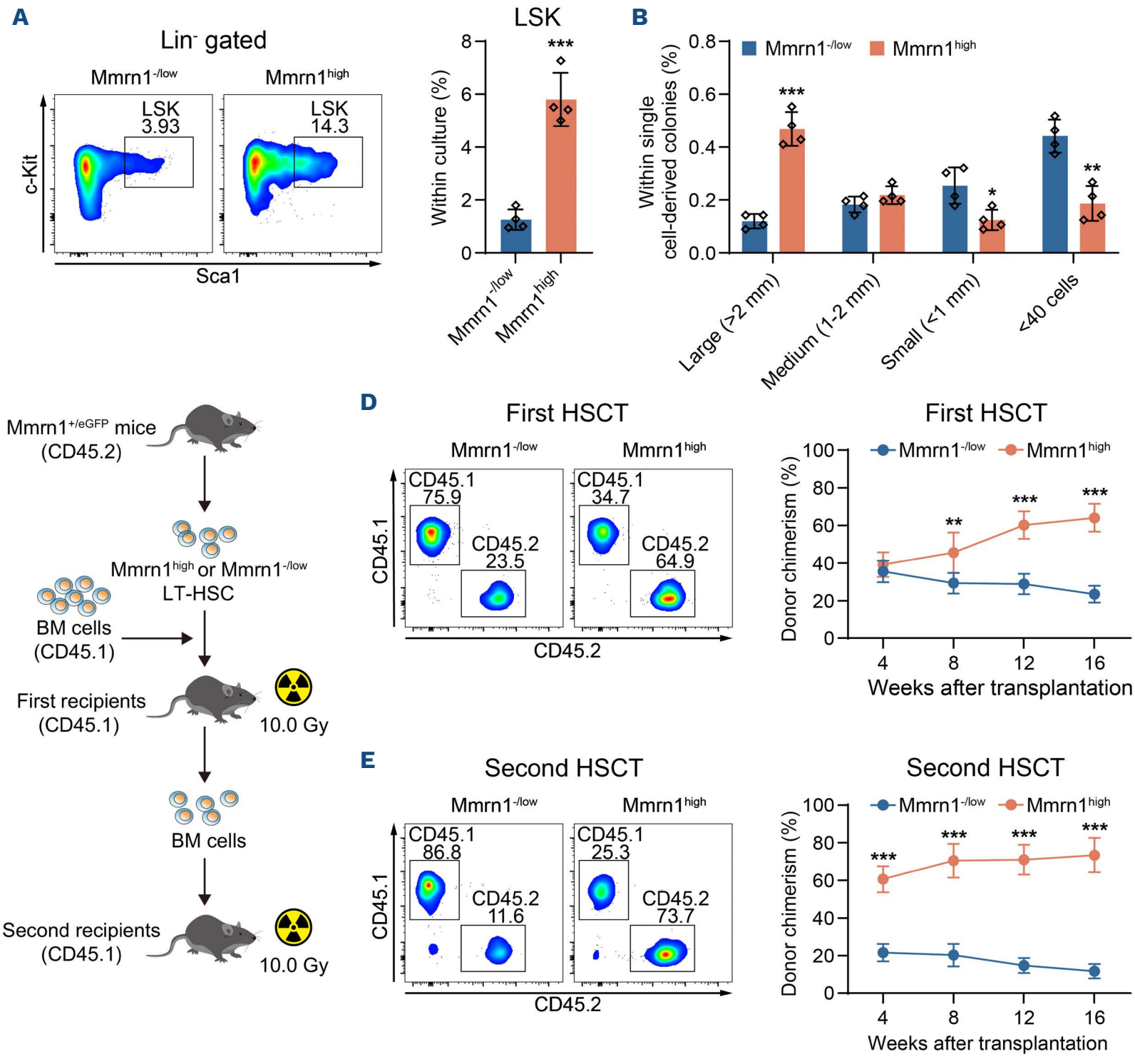

Figure 4. Mmrn1^{high} hematopoietic stem cell exhibit robust hematopoietic regenerative capacity. (A) Mmrn1$^{-/low}$ and Mmrn1^{high} long-term hematopoietic stem cells (LT-HSC) were sorted from the bone marrow (BM) of Mmrn1$^{+/eGFP}$ mice, and then cultured in SFEM medium (1×10^3 cells per well) for 10 days. The percentage of LSK in culture was analyzed by flow cytometry (N=4). (B) The single-cell clonal formation analysis of Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC in methylcellulose medium (N=4). (C-H) 5×10^2 Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC were mixed with 5×10^5 CD45.1 mouse BM cells and then transplanted into lethally irradiated CD45.1 mice. Sixteen weeks later, 1×10^6 BM cells from the first recipients were transplanted into lethally irradiated second CD45.1 recipients. (C) Schematic diagram of the first and second hematopoietic stem cell transplantation (HSCT). (D, E) The percentage of donor-derived cells in the peripheral blood (PB) of recipients at the indicated time points after the (D) first and (E) second HSC (N=6). Representative flow cytometric plots at 16 weeks after the first and second HSCT are shown in the left, respectively. Data are shown as the mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001.

were performed to clarify the functional heterogeneity of LT-HSC subpopulations divided on the basis of *Mmrn1* expression (Figure 4C). It was found that Mmrn1^{high} subpopulation-derived cells exhibited a gradually increasing percentage in the peripheral blood (PB) of recipient mice after the first and second HSCT (Figure 4D, E). Substantially different chimerism levels were also observed in the BM cell populations of the recipients, although lymphoid and myeloid lineage commitment were comparable between the two groups (*Online Supplementary Figure S5A-E*). It is known that the homing of HSC to the BM microenvironment is a critical step for hematopoietic reconstitution after transplantation. However, the results indicated that the difference in transplantation outcomes did not originate from distinct homing abilities (*Online Supplementary Figure S5F, G*). Collectively, HSC with high *Mmrn1* expression may contribute more strongly to the maintenance of long-term hematopoiesis.

Mmrn1^{high} hemapoietic stem cells display megakaryocyte-biased differentiation

Considering that *Mmrn1* is also present in megakaryocytes/platelets, we speculated that HSC with high *Mmrn1* expression may be directed towards differentiating into megakaryocytic lineage. As anticipated, GSEA of the RNA-seq data indicated that genes associated with megakaryocyte development and differentiation programming were enriched in Mmrn1^{high} LT-HSC (Figure 5A). Indeed, Mmrn1^{high} LT-HSC expressed high levels of recognized megakaryocyte-related genes, accompanied by increased CD41^{+} frequency (Figure 5B, C). Consistently, treatment with thrombopoietin, a master cytokine that promotes megakaryopoiesis, induced *Mmrn1* expression in the LT-HSC of Mmrn1$^{+/eGFP}$ mice (*Online Supplementary Figure S6A*). Studies have shown that there is a shortcut of megakaryopoiesis during stress-induced hematopoiesis, in which megakaryocytes can be directly generated from HSC without experiencing intermediate differentiation processes.[27-29] Therefore, the differentiation rate of the megakaryocytic lineage is significantly faster than that of other hematopoietic lineages in the early stages of hematopoietic reconstitution. In view of this finding, we then examined HSC-derived platelet production *in vivo* through short-term transplantation experiments. It was observed that Mmrn1^{high} LT-HSC generated more premegakaryocyte/erythroid progenitors (PreMegE) and MkP in recipient mice (Figure 5D). Moreover, Mmrn1^{high} LT-HSC had the potential to generate highly polyploid (≥8 N) megakaryocytes, which is in line with the *in vitro* culture results (Figure 5E, F). Of particular interest, analysis of the scRNA-seq data revealed that the expression of *Mmrn1* was significantly upregulated in high-ploidy megakaryocytes, which are mainly responsible for producing platelets (*Online Supplementary Figure S6B, C*). Expectedly, the platelet chimerism level was significantly increased in the PB of the recipients receiving Mmrn1^{high} LT-HSC (*Online Supplementary Figure S6D*). These findings prove that Mmrn1 may be helpful for mapping the development and maturation of megakaryocytes.

Mmrn1 deficiency leads to the loss of hemtopoietic stem cell self-renewal potential

To provide functional evidence, we generated *Mmrn1* knockout (KO) mice (*Online Supplementary Figure S7A, B*). It was found that blood cell parameters were largely normal in Mmrn1-deficient mice at steady-state (*Online Supplementary Figure S7C-G*), which is consistent with a previous study.[23] Further analysis revealed that *Mmrn1* deficiency only slightly but did not statistically significantly affect the phenotypes of HSPC in the BM of mice (Figure 6A; *Online Supplementary Figure S7H*). Of note, we observed that the proportion of G0 phase cells was decreased in HSC with Mmrn1 deficiency (Figure 6B). To assess whether Mmrn1 regulates HSC function, we performed a competitive transplantation assay (Figure 6C). Recipient mice transplanted with Mmrn1$^{-/-}$ BM cells displayed a gradual decrease in donor-derived chimerism levels in the PB (Figure 6D). Consistent with these data, limiting dilution assays revealed that the frequency of functional, although not phenotypic, HSC was decreased in Mmrn1$^{-/-}$ mice (Figure 6E). The results were also obtained from human CD34^{+} cells with *MMRN1* knockdown (*Online Supplementary Figure S7I, J*). These phenomena were similar to those from other HSC stemness regulators, such as Hlf, Ndn and Foxo3a.[30-32] Taken together, these data confirmed that *Mmrn1* is involved in maintaining the self-renewal of HSC under the stress of transplantation.

Mmrn1 is upregulated in acute myeloid leukemia and its high expression predicts poor patient prognosis

Previous studies reported that MMRN1 expression is correlated with the diagnosis, staging and prognosis of multiple cancers.[18,33] Interestingly, the analysis of public databases revealed that *MMRN1* was remarkably upregulated in AML, whereas it was decreased in most cancers (Figure 7A). The results from other databases and our clinical samples validated the above findings (*Online Supplementary Figure S8A, B*). Meanwhile, high expression of *MMRN1* was detected in different leukemia cell lines (*Online Supplementary Figure S8C*). On the basis of the French-American-British (FAB) classification, *MMRN1* was found to be upregulated mainly in non-M3 AML (*Online Supplementary Figure S8D*). Further analysis of the AML dataset demonstrated that LSC enriched for CD34 and/or CD38 expressed higher levels of *MMRN1* (*Online Supplementary Figure S8E*). Similarly, the scRNA-seq data showed that *MMRN1* was highly expressed in the malignant cells of AML specimens (*Online Supplementary Figure S8F*). A previous study reported that there are two types of LSC, lymphoid-primed multipotent progenitor (LMPP)-like LSC and granulocyte-monocyte progenitor (GMP) LSC, which exhibit distinct functional behaviors.[34] However, compared with GMP-like LSC, MMRN1 expression

was only slightly but not statistically significantly increased in LMPP-like LSC (*Online Supplementary Figure S8G*).

We next analyzed the correlation between the expression of MMRN1 and the prognosis of AML patients. The receiver operating characteristic (ROC) curve clearly revealed that *MMRN1* expression had good diagnostic performance in predicting AML prognosis (Figure 7B; *Online Supplementary Figure S8H*). In addition, we attempted to divide AML

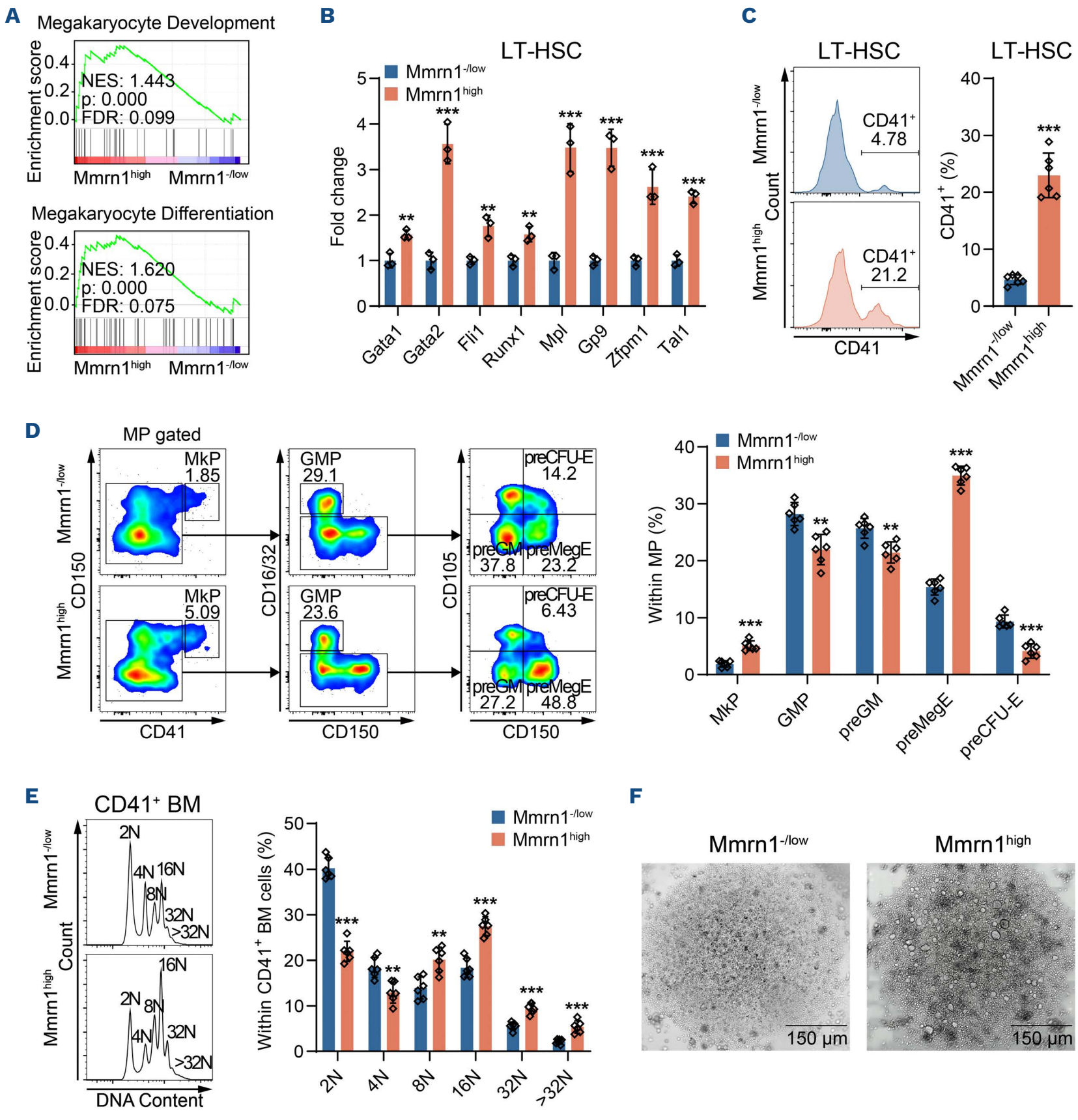

Figure 5. Mmrn1^{high} hematopoietic stem cell display megakaryocyte-biased differentiation. (A) Gene set enrichment analysis (GSEA) of the RNA-sequencing data using megakaryocyte-related gene sets. (B) Quantitative polymerase chain reaction (qPCR) analysis of mRNA expression of the indicated genes in Mmrn1$^{-/low}$ and Mmrn1^{high} long-term hematopoietic stem cells (LT-HSC) sorted from the bone marrow (BM) of Mmrn1$^{+/eGFP}$ mice (N=3). (C) The percentage of CD41^{+} cells in Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC from the BM of Mmrn1$^{+/eGFP}$ mice (N=6). Representative flow cytometric plots are shown in the left. (D, E) 5×10^{2} freshly sorted Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC were transplanted into lethally irradiated CD45.1 mice along with 5×10^{5} CD45.1 mouse BM cells, and follow-up analysis was performed at 2 weeks after transplantation. (D) The percentages of the indicated hematopoietic progenitor cell populations from CD45.2^{+} BM of the recipients (N=6). Representative flow cytometric plots are shown in the left. (E) The ploidy distribution in CD41^{+} cells from the CD45.2^{+} BM of recipients (N=6). Representative flow cytometric plots are shown in the left. (F) Representative images of Mmrn1$^{-/low}$ and Mmrn1^{high} LT-HSC at 7 days after culture *in vitro*. Data are shown as the mean ± standard deviation. ***P*<0.01; ****P*<0.001.

samples into MMRN1^{low} and MMRN1^{high} groups and found that *MMRN1* expression was significantly associated with BM blasts, FAB classification, cytogenetics, cytogenetic risk and NPM1 mutation (*Online Supplementary Table S3*). Besides, AML patients with high *MMRN1* expression presented a relatively poor risk of relapse and a low overall survival rate (Figure 7C, D; *Online Supplementary Figure S8I, J*). In agreement with these findings, MMRN1^{high} AML cells were enriched with several pathways linked to leukemia development and progression, such as the PI3K-Akt signaling pathway and calcium signaling pathway (Figure 7E). To assess how *MMRN1* is upregulated in AML, we performed transcription factor analysis using published chromatin immunoprecipitation-sequencing (ChIP-seq) data. It was observed that multiple hematopoietic transcription factors could bind to the promoter region of the *MMRN1* gene, among which ERG had the highest correlation with MMRN1 in AML samples (Figure 7F; *Online Supplementary Figure S9A, B*).

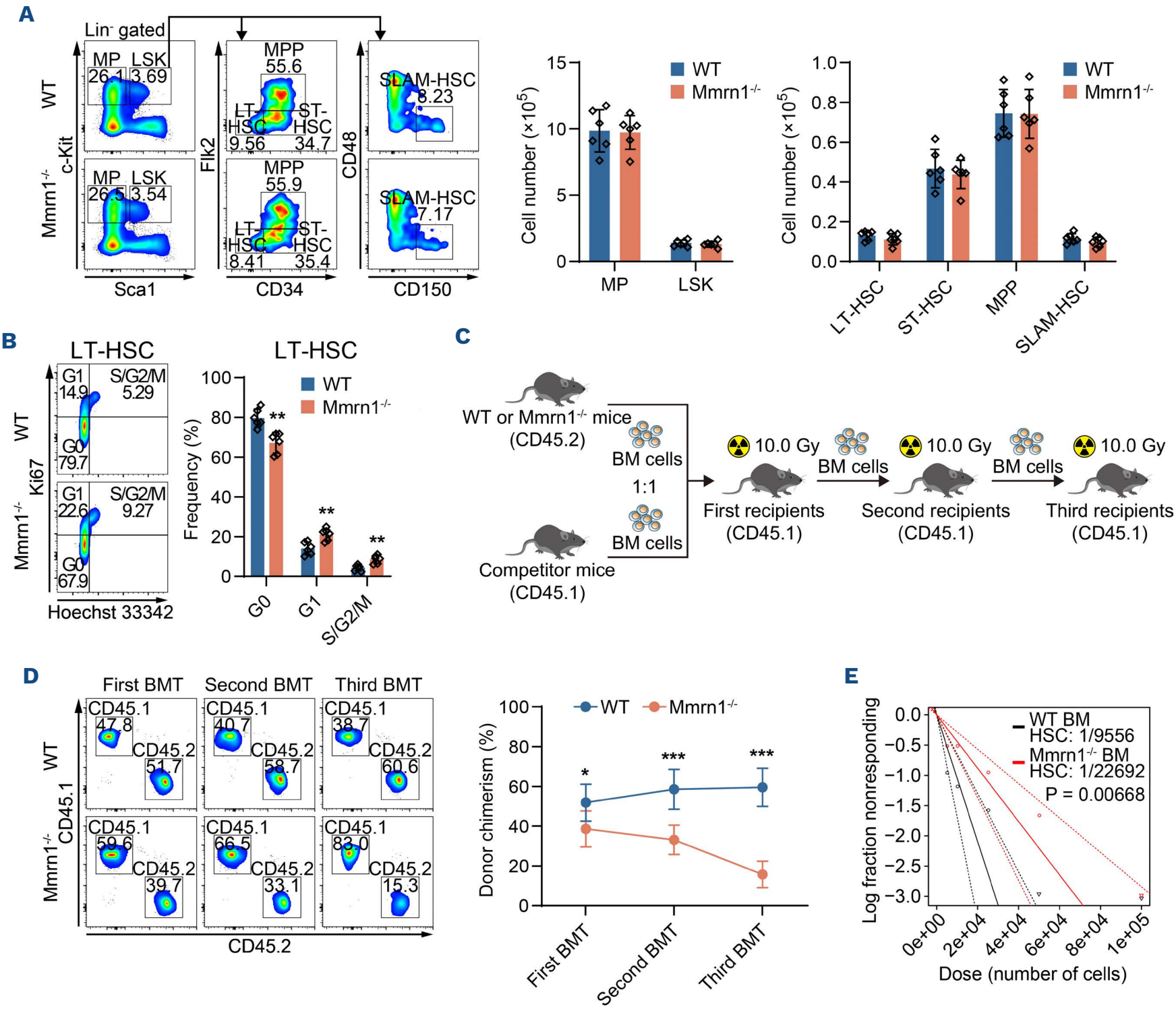

Figure 6. *Mmrn1* deficiency leads to the loss of hematopoietic stem cell self-renewal potential. (A) The numbers of myeloid progenitors (MP), LSK, long-term hematopoietic stem cells (LT-HSC), short-term HSC (ST-HSC), murine multipotent progenitors (MPP) and SLAM-HSC in 2 tibias and femurs from wild-type (WT) and Mmrn1$^{-/-}$ mice (N=6). Representative flow cytometric plots are shown in the left. (B) Cell cycle analysis of LT-HSC from the bone marrow (BM) of WT and Mmrn1$^{-/-}$ mice (N=6). (C, D) 5×10^5 BM cells from the BM of WT and Mmrn1$^{-/-}$ mice were mixed with 5×10^5 CD45.1 mouse BM cells and then transplanted into lethally irradiated CD45.1 mice. Sixteen weeks later, 1×10^6 BM cells from the first recipients were transplanted into lethally irradiated second CD45.1 recipients. Then, the third round of transplantation was carried out in the same manner. (C) Schematic diagram of competitive BM transplantation. (D) The percentages of donor-derived cells in the peripheral blood (PB) of recipients at 16 weeks after the first, second and third HSC transplnatation (HSCT) were analyzed by flow cytometry (N=6). Representative flow cytometric plots are shown in the left. (E) Limiting dilution analysis of the functional HSC frequency in the BM of WT and Mmrn1$^{-/-}$ mice (N=10). Data are shown as the mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001.

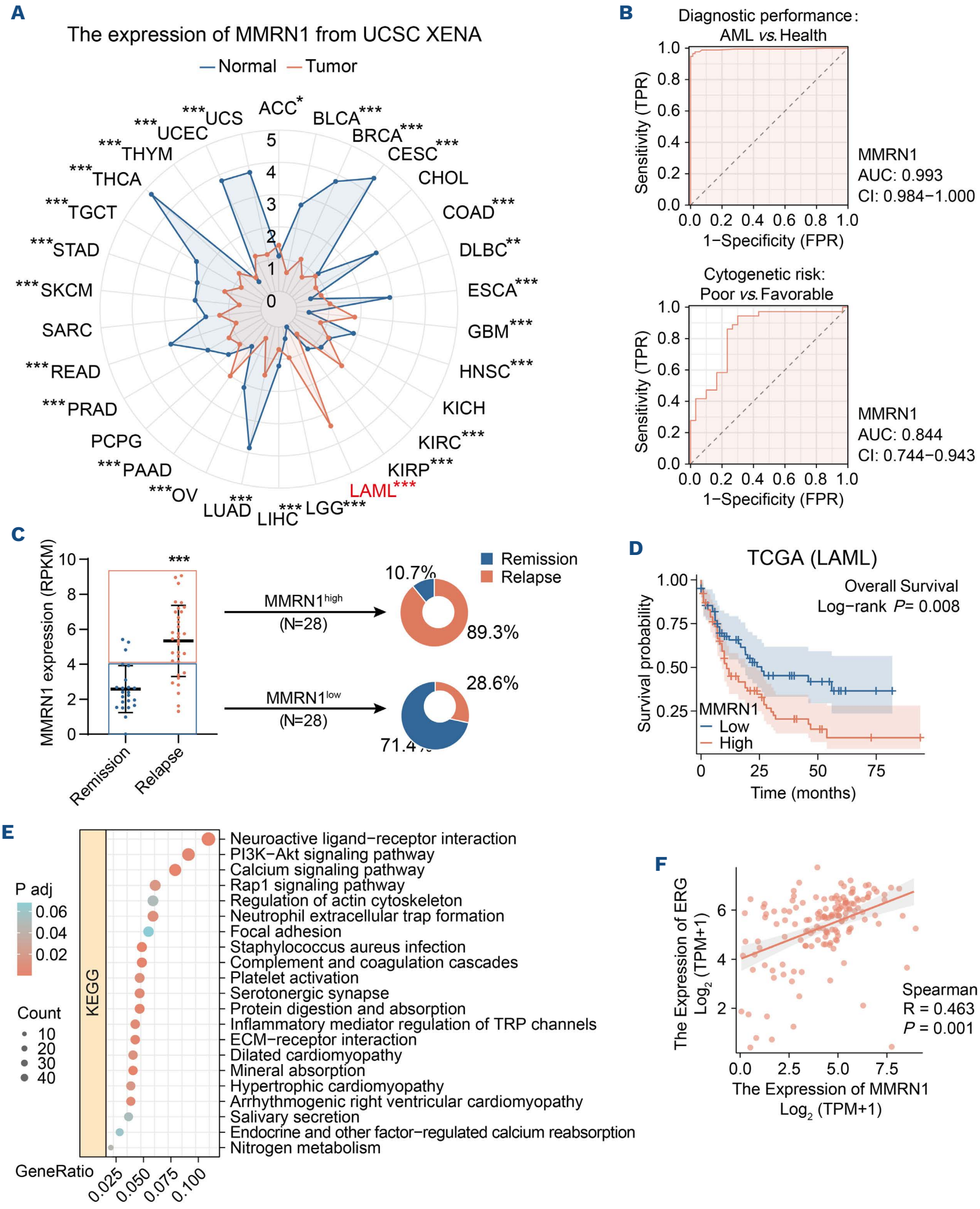

Figure 7. *Mmrn1* is upregulated in acute myeloid leukemia, and its high expression predicts poor patient prognosis. (A) The radar chart showing differential expression of MMRN1 between normal and tumor samples from the UCSC XENA database that integrates the data from TCGA (patients) and GTEx (healthy controls) database. (B) Reciever operating characteristics (ROC) analysis of the diagnostic performance of *MMRN1* for the indicated binary classification from the UCSC XENA database. (C) The distribution of *MMRN1* expression in the remission and relapse acute myeloid leukemia (AML) patients from the Vizome database, which is a visualization website of the BEAT-AML dataset. (D) Kaplan-Meier curves showing the survival rates of AML patients with low or high expression of *MMRN1*. The patient data were obtained from the TCGA database. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis showing upregulated pathways in MMRN1^{high} group (top 50%) compared with MMRN1^{low} group (bottom 50%) within AML samples from the TCGA database. (F) The correlation analysis of *MMRN1* and *ERG* expression in AML samples from the TCGA database. Data are shown as the mean ± standard deviation. ***P<0.001.

These results strongly suggest that MMRN1 can function as a biomarker in the diagnosis and prognosis of AML.

Mmrn1 high expression marks leukemic stem cells with increased quiescence and self-renewal

These findings prompted us to speculate that the high levels of *Mmrn1* may be used to identify functional LSC subpopulations. We then constructed an MLL-AF9 leukemia model using Mmrn1$^{+/eGFP}$ mice and noted that *Mmrn1* was differentially expressed in phenotypic LSC (Figure 8A; *Online Supplementary Figure S10A*). Consistent with the above data, the LSC subset with high *Mmrn1* expression was more quiescent than that with -/low *Mmrn1* expression (Figure 8B). Serial replating assays revealed that Mmrn1^{high} cells formed more colonies in each replating (Figure 8C). Importantly, we found that the survival rate of recipients receiving Mmrn1^{high} LSC was significantly declined (Figure 8D). In addition, the increases in mCherry^{+} cell percentage, white blood cell (WBC) count and Wright-Giemsa-stained leukemia cells indicated that Mmrn1^{high} LSC caused more serious leukemia symptoms in the recipients (Figure 8E; *Online Supplementary Figure S10B, C*). Meantime, compared with those in recipients of Mmrn1$^{-/low}$ LSC, more AML cell infiltration was found in the spleen, lung and liver of recipients transplanted with Mmrn1^{high} LSC (*Online Supplementary Figure S10D, E*). We also detected more progeny cells with leukemia initiation activity in the BM of recipients transplanted with Mmrn1^{high} LSC, which was further verified by limiting dilution assays (Figure 8F; *Online Supplementary Figure S10F*). The above effects were more pronounced after re-transplantation (*Online Supplementary Figure S10G-I*). On the other hand, we isolated CD34^{+} cells from human AML cases with high or low expression of MMRN1 and performed a xenotransplantation assay. Likewise, MMRN1^{high} cells exhibited higher activity to initiate leukemia development in M-NSG mice (*Online Supplementary Figure S11A, B*). Particularly, the functional assays using *Mmrn1*-deficient mice provided substantial evidence that Mmrn1 contributed to AML progression (*Online Supplementary Figure S11C-H*). Finally, to determine whether there is a direct link between *Mmrn1* expression and megakaryocytic lineage involvement in AML, Mmrn1^{high} and Mmrn1$^{-/low}$ LSC were isolated from mice and then cultured in vitro in the presence of thrombopoetin stimuli. It was observed that CD41 expression was comparable between the two groups after being cultured for 7 days (*Online Supplementary Figure S11I*), indicating that *Mmrn1*'s megakaryocyte bias is only relevant to normal hematopoiesis not leukemogenesis. In summary, these findings indicate that Mmrn1^{high} LSC have a greater advantage in promoting the development of leukemia.

Discussion

The rapid development of high-throughput sequencing and other innovative technologies has facilitated a deeper understanding of the stem cells within the hematopoietic system.[35] However, the heterogeneity of the immunophenotypically defined HSC and LSC hinders the progress of this research.[36,37] Thus, the precise identification of distinct stem cell subsets and an understanding of their unique biological properties are crucial for advancing medical research and clinical diagnosis and therapy.[37] In this study, we demonstrated that *Mmrn1* serves as a specific marker which not only enables the tracking of an HSC population with great self-renewal and megakaryocyte-biased differentiation potential but also reflects the severity and prognosis of AML.

Previous studies have indicated that *Mmrn1* is a megakaryocyte/platelet lineage-specific gene.[38,39] In the present study, by analyzing different transcriptomic data, we found that *Mmrn1* was also significantly enriched in human and mouse HSC. The subsequent generation of the *Mmrn1*-eGFP reporter mouse model allowed us to track *Mmrn1* expression easily *in vivo*. Under normal circumstances, most HSC located in the BM microenvironment are in a dormant state.[40] When the BM is subjected to various stress injuries, resting HSC can survive and then rapidly proliferate and differentiate to meet the body's need for blood cells.[41,42] Once hematopoietic reconstruction is complete, HSC can return to the quiescent state via an ERK-dependent negative feedback mechanism.[43] Interestingly, our experimental results demonstrated that the dynamic alteration in *Mmrn1* expression after irradiation, chemotherapy or LPS treatment was closely associated with the cell cycle state of HSC. Therefore, these findings support the notion that *Mmrn1* can function to determine the status of HSC.

Over the years, the classical model of hematopoiesis has been gradually revised based on the identification of various biomarkers, such as CD41, Hdc, CD63 and von Willebrand factor, which distinguish different functional HSC subpopulations.[8,19,44,45] However, potential hematopoietic markers remain inadequately understood due to the rarity of some HSC subsets or limitations in detection technology.[35,46] In this work, we noticed that *Mmrn1* was differentially expressed in phenotypic HSC populations and Mmrn1^{high} HSC presented increased quiescence and regenerative potential. These data imply that Mmrn1^{high} HSC may play a more critical role in hematopoietic recovery under stress conditions. After that, using the *Mmrn1* KO mice, we found that *Mmrn1* is nearly dispensable for normal hematopoiesis at steady-state but is required to maintain HSC self-renewal under stress of transplantation. Mmrn1 has been identified as a secretory protein containing an EGF-like domain, which is unique among EMILIN family members.[16] This domain is involved in the regulation of various physiological processes, including cell growth, adhesion, and signal transduction.[47] By integrating these findings, we reasonably assume that HSC may regulate themselves via the secretion of Mmrn1. Nevertheless, further experiments are needed to verify the

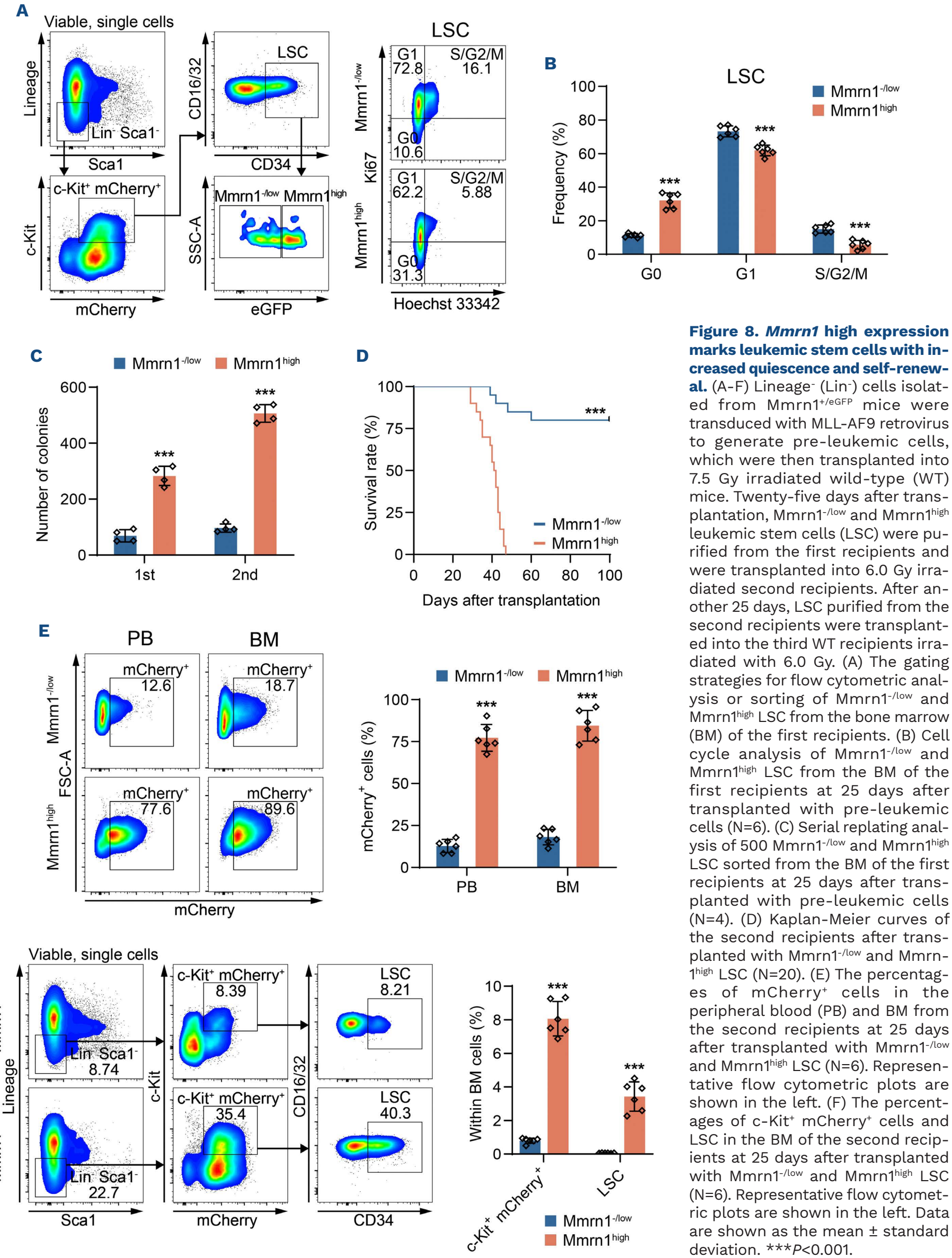

Figure 8. *Mmrn1* high expression marks leukemic stem cells with increased quiescence and self-renewal. (A-F) Lineage$^-$ (Lin$^-$) cells isolated from Mmrn1$^{+/eGFP}$ mice were transduced with MLL-AF9 retrovirus to generate pre-leukemic cells, which were then transplanted into 7.5 Gy irradiated wild-type (WT) mice. Twenty-five days after transplantation, Mmrn1$^{-/low}$ and Mmrn1^{high} leukemic stem cells (LSC) were purified from the first recipients and were transplanted into 6.0 Gy irradiated second recipients. After another 25 days, LSC purified from the second recipients were transplanted into the third WT recipients irradiated with 6.0 Gy. (A) The gating strategies for flow cytometric analysis or sorting of Mmrn1$^{-/low}$ and Mmrn1^{high} LSC from the bone marrow (BM) of the first recipients. (B) Cell cycle analysis of Mmrn1$^{-/low}$ and Mmrn1^{high} LSC from the BM of the first recipients at 25 days after transplanted with pre-leukemic cells (N=6). (C) Serial replating analysis of 500 Mmrn1$^{-/low}$ and Mmrn1^{high} LSC sorted from the BM of the first recipients at 25 days after transplanted with pre-leukemic cells (N=4). (D) Kaplan-Meier curves of the second recipients after transplanted with Mmrn1$^{-/low}$ and Mmrn1^{high} LSC (N=20). (E) The percentages of mCherry$^+$ cells in the peripheral blood (PB) and BM from the second recipients at 25 days after transplanted with Mmrn1$^{-/low}$ and Mmrn1^{high} LSC (N=6). Representative flow cytometric plots are shown in the left. (F) The percentages of c-Kit$^+$ mCherry$^+$ cells and LSC in the BM of the second recipients at 25 days after transplanted with Mmrn1$^{-/low}$ and Mmrn1^{high} LSC (N=6). Representative flow cytometric plots are shown in the left. Data are shown as the mean ± standard deviation. ***P<0.001.

mechanism that mediates the role of Mmrn1 in HSC.

Furthermore, our research revealed a significant association between *Mmrn1* expression and the megakaryocytic lineage commitment of HSC. This relationship is supported by the enrichment of megakaryocytic development and differentiation programs in Mmrn1^{high} HSC, as well as their increased capacity to generate platelets. These findings are consistent with those of a previous study showing that platelet-biased HSC reside at the apex of the hematopoietic hierarchy.[45] It is well established that there are two different pathways for megakaryopoiesis, namely, the direct and stepwise differentiation pathways.[48] Intriguingly, a recent study elucidated that direct and stepwise differentiation routes produce niche-supporting and immune megakaryocytes, respectively, and that both types of megakaryocytes usually have lower ploidy.[27] In contrast, platelet-producing megakaryocytes have high ploidy, and are collectively produced via these two routes. Here, our experiments revealed that Mmrn1^{high} HSC were more inclined to generate high-ploidy megakaryocytes, facilitating rapid platelet replenishment post-stress. However, *Mmrn1* deficiency had no effect on thrombopoiesis, hinting that Mmrn1 may be a non-functional marker for identifying HSC biased toward platelet-producing megakaryocytes. Overall, these findings link the expression of *Mmrn1* to the developmental program of HSC.

In addition to normal hematopoiesis, leukemia, a malignant clonal disease in living organisms, has attracted our attention.[13] The sequencing data from multiple databases as well as our experimental results revealed that *MMRN1* was specifically upregulated in AML cells and its expression is strongly correlated with the diagnosis and prognosis of AML, suggesting that MMRN1 can act as a new AML biomarker. There is a hypothesis that leukemia originates from LSC, which share many similarities with HSC but lack effective control of cell proliferation and differentiation.[37,49] Although there are still controversies, recent studies have provided evidence supporting LSC existence. Here, data from an MLL-AF9-derived AML mouse model emphasized that LSC with high *Mmrn1* expression presented enhanced activity to initiate leukemic development and its ablation suppressed leukemia progression, indicating that *Mmrn1* may be a functional marker for a more aggressive subset of LSC. Indeed, a previous study has shown that *MMRN1* is one of the LSC17 signature,[50] which further consolidates our findings. Based on our current data, transient inhibition of *Mmrn1* may pose a relatively low toxic risk to the normal hematopoiesis at steady-state. Therefore, targeted eradication of Mmrn1^{high} LSC may be a promising strategy for treating AML. However, our study did not employ other AML mouse models, which may limit the widespread use of *Mmrn1*. Moreover, future research should focus on exploring the downstream effector molecules and pathways of Mmrn1 in AML.

In conclusion, we delineated the multifaceted role of Mmrn1 in hematopoiesis and its potential significance in AML. By revealing *Mmrn1* as a marker for HSC status and function, these findings lay a foundation for future research into the molecular basis of HSC heterogeneity. Furthermore, the prognostic relevance of Mmrn1 in AML also provides an opportunity for the development of new diagnostic tools and targeted therapeutic strategies.

Disclosures

No conflicts of interest to disclose.

Contributions

NC and LY designed the study, performed experiments, analyzed data and wrote the paper. FC, HZ and XZ performed experiments and analyzed data. YL, ZC, MY and YL participated in some animal experiments. MS and MC participated in data analysis. YX, SW and XZ participated in the initial experimental design and discussed the manuscript. MH and JW conceived and supervised the study, and revised the manuscript.

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Data-sharing statement

The data and analyses conducted for this study are present in this article and supplementary material. Specifics of the bioinformatics analysis are detailed in the "Methods" section. The RNA-seq data of this article are available in the GEO database (number GSE233828).

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