GFI1 regulates chromatin state essential in human endothelial-to-haematopoietic transition

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Abstract

Objectives: During embryonic haematopoiesis, haematopoietic stem/progenitor cells (HSPCs) develop from hemogenic endothelial cells (HECs) though endothelial to haematopoietic transition (EHT). However, little is known about how EHT is regulated in human. Here, we report that GFI1 plays an essential role in enabling normal EHT during hematopoiesis.

Results: GFI1 deletion in hESCs leads to a complete EHT defect due to a closed chromatin state of hematopoietic genes in HECs. Mechanically, GFI1 directly regulates important signaling pathways essential for the EHT such as PI3K signaling.

Conclusions: Together, our findings reveal an essential role of GFI1 mediated epigenetic mechanism underlying human EHT during hematopoiesis.
1 | INTRODUCTION

During development, haematopoietic stem cells (HSCs) with multi-lineage and long-term repopulating potential are generated in the aorta-gonad-mesonephros (AGM) region. Studies in mice and zebrafish demonstrated that haematopoietic stem or progenitor cells (HSPCs) are produced from hemogenic endothelial cells (HECs) through the endothelial-to-haematopoietic transition (EHT) process during which the flattened endothelial cells gradually acquire the morphology and characteristics of haematopoietic cells.

Understanding the underlying mechanisms to control EHT could benefit the in vitro generation of HSPC from human pluripotent stem cells (hPSCs), including human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs). To date, a panel of signalling pathways and factors was found to be involved and regulate EHT. For instance, the signalling pathways such as Notch, retinoic acid and TGF-beta were shown to be crucial in HSC development from HECs. In addition, transcription factors such as Gata2 also play a central role to ensure EHT as Gata2 deletion results in EHT blockage in mice. Other critical factors like Ncor2 and Mel17 also play important roles to regulate EHT. Deletion of growth factor independent-1(Gfi1) and Gfi1b led to the impairment of EHT in mice. However, deletion of Gfi1 or Gfi1b alone did not block the HSPC generation, indicating that these two proteins functionally compensate with each other during haematopoiesis. At the mean time, how human EHT is regulated remains poorly understood due to limited research material. We previously reported a chemically defined, mono-layer approach to differentiate hPSCs into HSPCs, which recapitulates major developmental stages during embryogenesis, such as mesoderm, HEC and EHT. This defined haematopoietic differentiation protocol provides an in vitro model to understand human EHT. Indeed, we revealed an essential role of GFI1-mediated epigenetic mechanism for EHT, such as PI3K signalling in HECs to enable EHT. Our findings reveal an essential role of GFI1-mediated epigenetic mechanism underlying human EHT during haematopoiesis.

2 | MATERIALS AND METHODS

2.1 | Cell lines

The hESC lines used in this study include H1 hESCs, H1-GFI1<sup>−/−</sup>, H1-GATA2<sup>−/−</sup>, H1-GATA2<sup>−/−</sup>-GFI1<sup>−/−</sup>, H1-GFI1<sup>−/−</sup>-GFI1(FUW). H1 hESCs were obtained from WiCell Research Institute, and other cell lines above were derived in our lab through gene editing from the H1 hESCs.

2.2 | Cell culture

The hESCs, including H1 and their derivatives, were maintained in mTeSR1 medium (Stemcell Technologies) on matrigel-coated plates (Corning), at 37°C and with 5% CO2. The hESCs were passaged every 3-4 days with 0.5 mM ethylene Diamine Tetra acetic Acid.

2.3 | Gene targeting

Gene knockout of GFI1 was performed by the CRISPR/Cas9 system in H1 and H1-GATA2<sup>−/−</sup>-GFP cell lines. The px330-U6-PGK-hSpCas9 vector, containing the sgRNA targeting the exon 2 or exon 6 of GFI1 gene designed by sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), was electroporated into hESCs, using Nucleofector™ 2b Device (Lonza). After that, the cells were seeded onto the matrigel-coated 6-well plate in the presence of ROCK inhibitor—Y27632 (5 μM, Sigma). After clonal expansion for about 10 days, each individual hESC clone was picked for genotype analysis. Then, we used PCR and Sanger sequencing to detect the gene mutation of each clone. In detail, the PCR products of the gene targeting region were ligated to the pCE2-TA-Blunt-Zero cloning vector (Vazyme). Then, the ligation products were transformed into DH5α Competent Cells. After that, individual Es.coli clones were picked for Sanger sequencing to analyse the genotype of each hESCs clone. Finally, we harvested 3 clones with GFI1 mutation in double alleles. These GFI1-mutated clones were further confirmed by the RT-qPCR. Then, we performed fluorescence-activated cell sorting (FACS) analysis, immunostaining, teratoma formation, and karyotype analysis to analyse the pluripotency of these GFI1-mutated clones.

Also, the 2 × flag-tag knockin into the GFI1 stop code upstream region was performed using the same strategy as the knockout above. Briefly, in addition to the px330, we used a targeting vector, which contains two homology arms about 1 kb upstream and downstream of the end of the GFI1 last exon, respectively. In addition, there was a 2 × flag tag and a loxp-flanked PGK puromycin cassette between the homology arms. After electroporating px330 and targeting vector, the cells were selected with puromycin (0.5 μg/ml), and the positive individual clones were picked for further screening. Then, the positive clones were verified and further electroporated with 400 ng Cre-mRNA to remove the loxp-flanked PGK-puromycin cassette. Thereafter, the H1-GFI1<sup>−/−</sup>-<sup> Flag</sup> clone was expanded for the pluripotency analysis as discussed above.

2.4 | Teratoma formation

The teratoma-formation experiments have been reviewed and approved by IACUC at Guangzhou Institutes of Biomedicine and
Health, Chinese Academy of Sciences. In detail, approximately 1 million hESCs suspended in 50% cold matrigel (BD, diluted by DMEM/ F12 medium [Hyclone]) were subcutaneously injected into NOD-SCID mice (Vital River). After 6-to-8 weeks, the teratoma was harvested and fixed in 4% paraformaldehyde (PFA). Furthermore, the haematoxylin/eosin (H&E) staining was performed to detect the three germ-layer formation potential of the hESCs.

2.5 | Haematopoietic differentiation

Before haematopoietic differentiation, hPSCs were dissociated by Accutase (Sigma) and plated on growth factor-reduced Matrigel (Corning)-coated plates with thiazovivin (0.1 μM, Selleck). Firstly, at day 0, 40 ng/ml of BMP4 (Peprotech), 30 ng/ml of ACTIVINA (Sino Biological Inc.), 20 ng/ml of bFGF (Sino Biological Inc.), 6 μM CHIR99021 (Selleck) and 10 μM LY294002 (Selleck) were added to the basic medium (BM, mimics of the CustomTeSR1) of Dulbecco’s-modified Eagle’s medium/F-12 (GIBCO) supplemented with 1% insulin–transferrin–selenium (GIBCO), 70 μg/ml of vitamin C (Sigma). Second, 30 ng/ml of BMP, 1 μM A8301 (Selleck) and 2 μM IWR-1-endo (Selleck) were added to the BM on day 1. Then, on days 2–4 of differentiation, 40 ng/ml of vascular endothelial growth factor (Sino Biological Inc.) and 50 ng/ml of bFGF were added to the BM. Finally, 40 ng/ml of vascular endothelial growth factor, 50 ng/ml of bFGF, 10 μM SB431542 (Selleck), 10 ng/ml of stem cell factor (Peprotech), 50 ng/ml of thrombopoietin (Sino Biological Inc.), 10 ng/ml of interleukin 3 (Sino Biological Inc.) and 50 ng/ml of interleukin 6 (Sino Biological Inc.) were added in the BM at days 4–6 of differentiation and further haematopoietic commitment and maturation.

2.6 | CFU assay

Single cells of the certain cell number were suspended in the 100 μl of Iscove’s modified Dulbecco’s medium (Gibico) supplemented with 2% FBS (Biological Industries) and then mixed with 1 ml of Methocult H4435 (Stem Cell Technologies) in 35-mm ultra-low attachment plates (Stem Cell Technologies). After the cell culture at 37°C and with 5% CO₂ of 14 days, the CFUs were classified and counted according to their morphology.

2.7 | Virus production and transduction

GFI1 open-reading frame was cloned into TetO-FUW-EBHX-P2a-EGFP-T2a-Puro lentiviral vectors. Lentiviral particles were produced by transfecting 293T cells with the packaging plasmids pMDL, VSVG and pRSV-Rev. Viruses were harvested at 36 and 60 h after transfection and concentrated by ultracentrifugation at 50,000 g for 2.5 h at 4°C. Viruses were resuspended with DMEM/F12 medium (Gibico). Transduction was carried out in 6-well plates with FUW-rtTA when the confluent of the cell line H1-GFI1−/− was about 40%. Doxycycline (dox) (Sigma-Aldrich) was added at a concentration of 20 μg/ml for transgene induction. After the 48 h of Dox adding, puromycin (0.5 μg/ml) was applied to select positive individual clones.

2.8 | RT-qPCR

Total RNA was isolated by the TRIzol (Sigma) following the manufacturer’s instructions. Then, the RNA was reverse transcribed into cDNA by the HiScript® III RT SuperMix for RT-qPCR (Vazyme). The RT-qPCR was performed on a CFX96 machine (Bio-Rad) with the SYBR Green Mix (Vazyme). PCR cycle conditions were at 95°C for 10 min for denaturation, 40 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20s. All experiments were done in triplicate. Primers used in this study were listed in Supporting Information Table S1.

2.9 | Fluorescence-activated cell sorting

The cells were digested into single cells by accutase (Sigma) and suspended in PBS with 2% FBS. Then, cell suspensions were incubated with the antibody for 30 min at 4°C. After the incubation, the marker expressions in this study were analysed by the CytoFLEX Flow Cytometer (Beckman). For cell sorting, cells were sorted by the Moflo (Beckman). Antibodies used in this study were listed in Supporting Information Table S2.

2.10 | Immunostaining

The cells were incubated with 4% paraformaldehyde at room temperature for 2 min. Then, cells were washed with PBS and incubated with the primary antibody in PBS with 10% bovine serum albumin (BSA) and 0.3% TritonX-100 overnight at 4°C. Then, the cells were washed twice and further staining with secondary antibody for 1 h in PBS with 1% BSA at room temperature. After the incubation, nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma) for an additional 5 min. Finally, the cells were imaged with a Confocal laser scanning microscope (Zeiss LSM 710).

2.11 | RNA-Seq

H1-GATA2w/eGFP and H1-GATA2w/eGFP-GFI1−/− HECs at day 4 of differentiation were lysed with TRIzol (Sigma) to isolated total RNA, and sequencing libraries were prepared by the TruSeq RNA Sample Prep Kit (Illumina) under the manufacturer’s recommendations. The samples were run on a NextSeq system with NextSeq 500 Mid Output kit (Illumina). RNA-Seq data were processed as follows. Reads were aligned to an index generated from the Ensembl transcriptome version 74 (hg38), using HISAT2, and gene expression was analysed with SAMtools and htseq-count, normalised with EDAsSeq. A threshold of at least 20 average row read counts was used to filter lowly
expressed transcripts. Differential expression was performed using DESeq2 and genes were considered significant if they had a Benjamini-Hochberg corrected p value < .05. Gene ontology was performed using clusterProfiler; heatmap was prepared using heatmap and Gene set enrichment analysis was analysed using the GSEA software.

**Figure 1** Legend on next page.
2.12 | ATAC-Seq

ATAC-seq and data analysis were performed as follows. 50,000 cells of each sample were collected to generate DNA libraries with a Nextera DNA library preparation kit (Illumina) according to the manufacturer’s recommendation. Then, the DNA libraries were run on a NextSeq system with NextSeq 500. Then, the sequencing data were mapped onto human genome (UCSC hg38) using bowtie2. Signals were compiled using MACS2 call peak, and MACS2 was used to call open peaks. Signal density heatmap and profile were plotted using deep Tools, motifs were found using homer and gene ontology analysis was performed using DAVID.

2.13 | CUT&Tag

CUT&Tag DNA library was prepared using Hyperactive pG-Tn5 Transposase kit (Vazyme), following the manufacturer’s instructions, and the DNA libraries for sequencing by NextSeq 500. All sequencing data were mapped onto hg38 using bowtie2; peaks were called using MACS2; and gene ontology analysis was performed using DAVID.

3 | RESULT

3.1 | GFI1 is essential in HPC generation during hESC differentiation

To investigate the role of GFI1 in human haematopoiesis, we generated GFI1 deletion in H1 hESCs using the CRISPR/Cas9 gene editing. Small-guide RNAs (sgRNAs) were designed to target Exon 2 and Exon 6, respectively (Figure 1A). After clone selection, we generated three mutated hESC clones in both alleles of GFI1 (H1-GFI1−/−) (Figure 1A). These GFI1−/−-mutated clones displayed normal hPSC phenotype in terms of pluripotent marker expression and teratoma-formation potential (Figure S1) compared to the wild-type (WT) H1 hESCs. However, three GFI1−/−-mutated clones showed almost complete defect in generation of CD34+ HPCs during haematopoietic differentiation of hESCs based on our previously reported monolayer differentiation protocol16 (Figure 1B). While the CD34+ CD31− CD43− endothelial cells were relatively normal in GFI1 mutant clones (Figure 1B), indicating GFI1 deletion leads an EHT defect during human hematopoietic differentiation. Indeed, both morphology and immunostaining confirmed the absence of EHT in GFI1-deleted cells (Figure 1C–E). Further RT-qPCR analysis confirmed the loss of GFI1, but not GFI1B expression during the haematopoietic differentiation of GFI1-deleted cells, and showed that expression of the early mesoderm gene T and hematovascular mesoderm gene KDR were not affected in GFI1-deleted cells (Figure 1F). FACS analysis further confirmed GFI1-deleted cells underwent normal mesoderm, endothelial commitment but HPC defect based on markers for mesoderm (T), endothelial (CD31) or HPC (CD43) (Figure 1G). Indeed, loss of CD43+ HPCs led to the absence of colony-forming unit (CFU) of the H1-GFI1−/− cells (Figure 1H). In particular, the CFU-E could express both the fetal and adult globins (Figure 1I), hinting that hESCs with GFI1 mutation might lose the primitive and definitive haematopoiesis potential. Together, these data indicate GFI1 deletion alone leads to haematopoietic defect in human differentiation.

3.2 | GFI1 is required for EHT process

To analyse the mechanisms underlying haematopoietic defect in GFI1-mutated hESCs, we deleted GFI1 in another hESC line with GATA2/eGFP knockin (H1-GATA2w/eGFP-GFI1−/−) (Figure 2A), as we have previously shown that GATA2/eGFP specifically labels HECs in hESCs differentiation. H1-GATA2w/eGFP-GFI1−/− showed normal hPSC characteristics in terms of pluripotent gene expression (Figure S2A–C), karyotype (Figure S2D) as well as teratoma formation (Figure S2E). Upon haematopoietic differentiation, H1-GATA2w/eGFP-GFI1−/− generated normal CD34+ CD31− CD43− endothelial cells, but impaired CD43+ HPCs as well as no CFUs (Figure 2B–D). The percentage of GATA2/eGFP+ cells in CD34+ CD31− CD43− endothelial population (G2EC) that were enriched with hESCs showed no substantial difference between H1-GATA2w/eGFP-GFI1−/− and wild-type cells (Figure 2E). However, the G2ECs derived from H1-GATA2w/eGFP-GFI1−/− failed to undergo EHT upon replating into the EHT condition (Figure 2F).
suggesting GFI1 is essential to ensure EHT rather than HECs committing. In addition, we proved that re-expression of GFI1 in GFI1\(-/-\) cells substantially rescue the HPC generations, confirming that the impairment of EHT of GFI1\(-/-\) cells was indeed GFI1 dependent. (Figure S3B).

3.3 | GFI1 sustained the haematopoietic program in HECs

To investigate the molecular mechanism underlying the functional deficiency in GFI1-deleted HECs, we compared the transcriptional...
FIGURE 3  GFI1 sustained the haematopoietic program in HECs. (A) MA plot for genes expression in WT versus GFI1−/− G2ECs. The x-axis shows log2 mean; y-axis shows log2 transformation of fold change and red points indicate significant genes. (B) Top Gene Ontology (GO) terms enriched in genes upregulated in GFI1−/− G2ECs (up), and upregulated in WT G2ECs (bottom). (C) Heatmap of the gene expression of the indicated GO terms normalised by Z score. (D) GSEA analysis for WT and GFI1−/− G2ECs
FIGURE 4  GFI1 promoted the chromatin accessibility on haematopoietic genes in HECs. (A) Distribution of the ATAC-seq peaks in the genomic regions of the WT and GFI1+/− G2ECs. (B) Volcano plot of the accessible peaks of the WT and GFI1+/− G2ECs; x-axis shows log2(fold change of the peak area); y-axis shows log10(p value of peak); the blue dots represent differentially accessible peaks in WT G2ECs; the red points indicate differentially accessible peaks in GFI1+/− G2ECs. (C) ATAC density heat map of the differential opened and closed peaks in WT and GFI1+/− G2ECs. (D) Selected genomic views of the ATAC-seq data at the indicated gene locus. (E) Selected GO terms enriched in genes downregulated (left) and upregulated (right) in GFI1+/− G2ECs compared to the WT G2ECs. (F) Known motif enrichment analysis of the accessible regions in WT and GFI1+/− G2ECs cells by homer. Fold-enrichment was calculated by target %/background %.
profile between the WT and GFI1−/− G2ECs through RNA-seq (Figure 3A). 264 genes were up-regulated GFI1−/− G2ECs and they were enriched the vascular biology and DNA replication-related functions (Figure 3B). Meanwhile, 142 genes were down-regulated in the GFI1−/− G2ECs and these genes are related to cell development (Figure 3B). For example, genes related to vascular and heart functions.

**FIGURE 5** GFI1 regulated EHT process through activating PI3K signalling. (A) Work flow of the GFI1 gene binding analysis: Generating a GFI1-flag-knock in hES cell line using CRISPR-Cas9 technology, and then sorting endothelial cells during hESCs blood differentiation by FACS, finally analysing its direct chromatin-binding through CUT&tag (B) Distribution of the GFI1 binding peaks in the genomic regions detected by Cut&tag. (C) Top KEGG terms enriched of GFI1 binding genes. (D) Cut&tag and ATAC-seq tracks of PI3K-Akt signalling pathway associated genes. (E) Experimental design to analyse the influence of PI3K inhibition on HPC generation. The canonical PI3K inhibitor—LY294002 was added from day4 to day8 during haematopoietic differentiation. (F) FACS analysis of the CD43+ HPC generation from the indicated group at day 8 of the haematopoietic differentiation. Statistics was determined using unpaired two-tailed Student’s t-tests, **p < .01. The data represent mean ± SD from three independent replicates (n = 3)
development, such as endothelium development (TJP1, NOTCH4, HEG1), cardiac ventricle development (FOXC1, DLL4, EGLN1) and artery morphogenesis (ADGRF5, ACVR1L, EFNB2) were up-regulated in GF1−/− G2ECs. The well-known master regulators in haematopoiesis like GATA2, KLF1, RUNX1, etc. were down-regulated in GF1−/− G2ECs (Figure 3C). Further Gene Set Enrichment Analysis (GSEA) analysis also demonstrated the gain of accelerated cell cycle, endothelial/vascular and accelerated cell cycle program and loss of haematopoietic profile in GF1−/− G2ECs (Figure 3D). In addition, we also identify the enrichment of NF-kappaB and IL6/JAK/STAT3 signalling in GF1−/− G2ECs (Figure 3D), suggesting that they might have a negative regulation in EHT. These data indicate GF1 deletion in HECs resulting elevated endothelial gene program and down-regulated haematopoiesis program.

### 3.4 | GF1 promotes the chromatin accessibility on haematopoietic genes in HECs

To investigate how GF1 deletion leads expression change in HECs, we examined chromatin assessbility in WT and GF1−/− G2ECs by transposase-accessible chromatin with sequencing (ATAC-seq) as chromatin accessibility change might precede the transcription.16 As expected, the detected peaks by ATAC-seq were close to the gene bodies, especially the transcriptional start site (TSS, overlapStart) (Figure 4A). Substantial number of differential accessible chromatin regions were identified between the WT and GF1−/− G2ECs (Figure 4B). These differential accessible peaks associated with 1141 genes down-regulated and 593 genes up-regulated in GF1−/− G2ECs (Figure 4C). The down-regulated haematopoietic genes like RUNX1 and FOXC1, etc. showed obvious decreased chromatin accessibility in GF1−/− G2ECs (Figure 4D). In general, blood cell development-regulated gene showed decreased chromatin accessibility while endothelial genes showed increased chromatin accessibility upon GF1 deletion (Figure 4E). Lastly, the accessible chromatin regions promoted by GF1 were enriched in many haematopoietic regulatory genes like FLJ1, ERG, ETV2, ETS1, etc. (Figure 4F). Together, these data suggest that GF1 promotes chromatin accessibilities on haematopoietic genes in human HECs.

### 3.5 | GF1 regulates essential signalling pathways for EHT

To examine the direct binding targets of GF1 in regulating EHT, we analysed its direct chromatin-binding targets in CD34+/CD31−CD43− endothelial cells sorted in hESCs blood differentiation through cleavage under targets and tagmentation (CUT&tag) analysis (Figure 5A). We identified total 2926 GF1 binding peaks that associated to 2236 annotated genes in the hESCs derived CD34+/CD31−CD43− endothelial cells (Figure 5B). These targets contain many signalling pathways involved in HSC maintenance. such as MAPK21 and FoxO22 signalling pathways (Figure 5C). Among them, the PI3K-Akt signalling is the top 1 enriched pathway in GF1 targets (Figure 5C). Moreover, the GF1 bound PI3K-Akt signalling-related genes showed decreased chromatin accessibilities upon GF1 deletion (Figure 5D). Notably, previous studies have shown that PI3K signalling was involved in HSC maintenance.23 Thus, we examined PI3K signalling in EHT process and showed that its inhibition blocked the HPC generation during haematopoietic differentiation of hESCs (Figure 5E). However, activating PI3K signalling by SC79 could not rescue EHT defect in GF1−/− cells, indicating GF1 deletion might lead to defect in many other pathways essential for EHT (Figure 5A). Nonetheless, these results indicated that GF1 regulates essential signalling pathways for EHT process.

### 4 | DISCUSSION

During early development, definitive HSPCs are generated from HECs through the well-known EHT process that is demonstrated in various model systems like mice, zebrafish etc. However, how EHT is regulated in human remains largely unknown due to the limited research material. Differentiation of hPSCs to haematopoietic lineage cells in vitro might serve as a valuable model to understand human haematopoiesis. Based on hPSC differentiation model, couple pathways or regulators had been revealed to regulate human haematopoiesis, such as SOX17,24 HOX925 and vitamin C.16 In our previous study, we established an approach to differentiate hPSCs into HSPCs in a chemically defined, mono-layer condition that recapitulate major haematopoiesis developmental stages, such as mesoderm, HEC and EHT.18 In this report, based on this approach, we revealed that GF1 plays an essential role in human haematopoiesis to enable normal EHT through maintaining chromatin accessibility in critical haematopoietic regulatory genes such as PI3K signalling. Our findings highlight the important role of epigenetic machinism to ensure normal EHT, which has not been highly documented.

GF1 has been reported to involve in epigenetic regulation through recruiting histone methyltransferases (G9),26 histone demethylase (LSD1)15 and histone deacetylases27 to repress target gene transcription. In mouse haematopoiesis, Gfi1 deletion alone did not significantly impair EHT while deletion of both Gfi1 and Gfib lead severe EHT defect, as the Gfi1 proteins could recruit the chromatin-modifying protein LSD1 to epigenetically silence the endothelial program in HECs.15 Consistently, a recent study reported that blocking GF1 and/or GFib activity with a small molecule inhibitor of LSD1 could also abrogated all blood cell development from hiPSCs.28 While in this report, we further showed that GF1 deletion alone led complete EHT blockage, indicating the differential requirement of GF1 in different species. In fact, how exactly EHT accrues at the molecular level remains unclear. We failed to detect more genes with expression change in GF1−/− HECs compared with WT HECs (Figure 3). Interestingly, GF1−/− HECs exhibit reduced chromatin accessibilities in those haematopoiesis regulatory genes such as FLJ1, ERG, ETV2 and ETS1 indicating the critical haematopoietic genes have been predisposed at the chromatin level in endothelial cells without significant transcriptional change before EHT. The critical factors such as GF1 establish the predisposed haematopoietic gene program to ensure the subsequent EHT process for HSPCs generation.
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SUPPORTING INFORMATION
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