SUPPLEMENTARY APPENDIX

Iron overload impairs normal hematopoietic stem and progenitor cells through reactive oxygen species and shortens survival in myelodysplastic syndrome mice

Xin Jin,[1*] Xiaoyuan He,[1*] Xiaoli Cao,[2] Ping Xu,[3] Yi Xing,[2] Songnan Sui,[3] Luqiao Wang,[3] Juanxia Meng,[3] Wenyi Lu,[3] Rui Cui,[3] Hongyan Ni[4] and Mingfeng Zhao[3,1**]**

[1]Nankai University School of Medicine, Tianjin; [2]Tianjin Children's Hospital; [3]Department of Hematology, Tianjin First Central Hospital and [4]Department of Radiology, Tianjin First Central Hospital, Tianjin, PR China

*XJ and XYH, and **HYN and MFZ contributed equally to this work.

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Correspondence: mingfengzhao@sina.com

Supplementary data

Supplementary Methods

Reagents and antibodies

PLAT-E cell line and retrovirus packaging kit were purchased from Cyagen Biosciences (USA). αMEM medium was purchased from Thermo Fisher scientific (USA). 5-fluorouracil (5-Fu) was purchased from Sigma-Aldrich (USA). Iron dextran injection was purchased from Pharmacosmos (Denmark). Methocult M3434 was purchased from Stem Cell Technologies (Canada). The Mouse Ferritin and GDF11 ELISA Kit were purchased from CUSABIO (USA). Anti-RUNX1 and anti-α-Tublin antibodies, human IL-6, mouse IL-3, and mouse stem cell factor (SCF) were purchased from R&D Systems (USA). Mouse antibodies to CD45.1, CD45.2, Gr1, Mac1/CD11b, Ter119, CD71, B220, CD19, CD3, CD5, CD41, CD117/c-Kit, Sca1 and CD34 were purchased from eBioscience or BioLegend (USA). And lineage mixture solution contains CD3ε, Ter119, CD11b, CD45R/B220, and Gr1.

Identification of MDS mice that underwent transplantation

Engraftment of bone marrow (BM) cells infected with retroviruses was confirmed by measuring the percentage of GFP^+ cells in peripheral blood (PB) obtained every 4 weeks after transplantation. 24 weeks post transplantation, several mice were sacrificed, and their tissue samples, including PB, BM, femur, spleen, and liver were analyzed. Circulating blood cells were counted by an analyzer. Morphology of PB cells and cytospin preparations of BM cells were stained by air-dried smears with Wright's Staining Solution. The femur, spleen and liver were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E), to study the pathological changes.

Quantitative Real-Time PCR analysis

Quantitative expression of the *NOX4*, *GPX1*, *GDF8*, *GDF11*, *GDF15*, *Activin A*, *Activin B*, *Acvr2b*, *ALK4*, *ALK*5 mRNA levels was conducted by real-time RT-PCR with a LightCycler 96 system. The primers were presented in Supplementary Table 1.

ELISA analysis

The evaluation of ferritin and GDF11 was performed according to the manufacturer's instruction of the Mouse Ferritin and GDF11 ELISA Kit, respectively. Briefly, use a serum separator tube to collect PB and allow the sample to clot for two hours at room temperature or overnight at 4℃ before centrifugation for 15 minutes at 1000xg. Remove serum and assay immediately.

Perl's iron staining

Iron deposition in BM, liver and spleen was evaluated by Perl's iron staining. Briefly, the specimens were fixed in 10% buffered formalin, embedded in paraffin, sectioned, dyed with Perl's stain, and visualized by microscope.

ROS analysis

The levels of ROS were detected by Arnplex red Hydrogen Peroxide assay Kit referring to the manufacturer's instruction. Briefly, the supernatant of BM was collected and the red cells in BM were sorted. After preparation of the samples and reaction mixtures, measure the fluorescence using a microplate reader at 590 nm.

Apoptosis analysis

For detection of apoptosis, 1×10^6 BM cells were prepared and washed twice with precold PBS buffer. These cells were incubated with Annexin-V-APC antibody for 15min and PI for 5 min, and then analyzed by flow cytometry.

Colony-forming assay and replating assay

Colony-forming assay was performed in Methocult M3434 medium. The number of colony-forming unit erythroid (CFU-E), burst-forming unit erythroid (BFU-E), colony-forming unit granulocyte-macrophage (CFU-GM), and colony-forming unit granulocyte, erythroid, macrophage, megakarycyte (CFU-GEMM) was counted at day14 of culture. For a serial replating assay, cells were scored from the first plating at day14 and 1×10^4 cells were replated into the same medium. For these two assays, fluorescence microscope was used to differentiate normal (GFP^-) and mutant (GFP^+) colonies.

Competitive repopulation assay

For competitive transplantation, 1×10^6 $CD45.2^+$ donor cells from RX291/FE and RX291/NS were transplanted into lethally irradiated recipient mice together with 1×10^6 $CD45.1^+$ competitor BM cells. The proportion of $CD45.2^+$ cells and multilineage analysis in the PB were determined every 4 weeks post transplantation. After 16 weeks, BM cells were collected and analyzed.

Supplementary Table 1. Primers used in this study

Gene	Primer sequence
NOX4	F: 5’-GATTTCTGGACCTTTGTGCCTTT-3’ R: 5’-TGATGGTGACAGGTTTGTTGCT-3’
GPX1	F: 5’-TGCTCATTGAGAATGTCGCGTCTC-3’ R:5’-AGGCATTCCGCAGGAAGGTAAAGA-3’
GDF8	F: 5’-CTGTAACCTTCCCAGGACCA-3’ R: 5’-TCTTTTGGGTGCGATAATCC-3’
GDF11	F: 5’-CATCACACCTTGTTCCCTGA-3’ R: 5’-ACCCTGACTGCCCTTCTCTT-3’
GDF15	F: 5’-GCACAGGACAGACAGTGGT-3’ R: 5’-GGAGTGTAGGTGAGGAGCAG-3’
Activin A	F: 5’-TGGTGCCAGTCTAGTGCTTC-3’ R: 5’-CCGTCACTCCCATCTTTCTT-3’
Activin B	F: 5’-ACAGGCCCTTTGTAGTGGTG-3’ R: 5’-GCTGCCCTCACAGTAGTTCC-3’
Acvr2b	F: 5’-CAGTCGTGGCAGAGTGAAC-3’ R: 5’-GGAGCCCTTGTCGTGGAAG-3’
ALK4	F: 5’-CGCTCCAGGATCTCGTCTAC-3’ R: 5’-AACCAAGACCGTTCTTCACG-3’
ALK5	F: 5’-ACCTTCTGATCCATCGGTTG-3’ R: 5’-TTCCTGTTGGCTGAGTTGTG-3’
GAPDH	F: 5’-ACGGCAAATTCAACGGCACAGTCA-3’ R: 5’-TGGGGGCATCGGCAGAAGG-3’