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EPO-R+ myelodysplastic cells with ring sideroblasts produce high erythroferrone levels to reduce hepcidin expression in hepatic cells

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ABSTRACT

Recently, a new erythroid regulator, erythroferrone (ERFE), which downregulates hepatic hepcidin production, has been identified. However, the relationship between ERFE and abnormal iron metabolism in MDS is unclear. In this study, we examined the level of ERFE mRNA during ex vivo erythroid differentiation using cord blood CD34+ cells and we further analyzed whether ERFE could be produced by MDS cells using a public database (GSE58831). ERFE mRNA was increased during normal erythroid differentiation. An analysis of GSE58831 indicated that ERFE expression in bone marrow (BM) MDS cells was higher than that in healthy volunteer (HV)–derived BM cells. ERFE expression significantly and positively correlated with the expression of erythropoietin (EPO) receptors (EPO-R), ALAS2 (5'-Aminolevulinate Synthase 2), STEAP3 (STEAP family member 3) and the presence of ring sideroblasts or the SF3B1 mutation. These results suggest that EPO-R+ MDS cells with ring sideroblasts or an SF3B1 mutation produce high levels of ERFE that may be associated with a reduction in hepcidin.

1. Introduction

Multiple genomic mutations in bone marrow (BM) hematopoietic stem/progenitor cells are involved in the development of myelodysplastic syndrome (MDS) [1–3]. Of these, founder gene mutations, including *TET2* and *DNMT3A*, were associated with preclinical clonal hematopoiesis, even that seen in the BM of healthy volunteers (HV) [4,5]. Subsequently, driver gene mutations were found to determine the disease-specific phenotype and facilitate the clonal evolution of MDS stem/progenitor cells [6]. In this process, the frequency of genomic mutations may be reduced by scavengers of reactive oxygen species (ROS) [7], the nuclear DNA repair system and quiescent cells. Conversely, ROS [8], single cell polymorphisms of the DNA repair system [9], and genotoxic therapy, including chemotherapy and stromal dysfunction [10–12], may accelerate the accumulation of genomic mutations in MDS stem progenitor cells.

Oxidative DNA stress [13,14] and mitochondrial dysfunction [15] were found to be elevated in patients with transfusion-dependent and transfusion-independent MDS. Moreover, we have previously demonstrated that highly reactive ROS production, such as hydroxyl radicals (OH·), was also enhanced in BM MDS cells, while the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG) induced by OH· was increased in the genome of MDS-derived mononuclear cells [8]. The production of OH· is known to occur by Fenton or Haber–Weiss reactions using ferrous iron as a cofactor. Iron chelation can reduce the level of labile iron [16,17] and 8-OHdG in hematopoietic cells [8], and contribute to hematopoietic recovery [18,19], resulting in the improvement of the prognosis of MDS patients [20]. Excess iron in the BM could be an accelerator of oxidative DNA damage in patients with MDS, although the precise mechanism on how iron accumulation may occur in such patients is unknown.

Recently, it was shown that the level of serum hepcidin, a direct down-regulator of gastrointestinal iron absorption, was reduced in a proportion of patients with lower-risk MDS, such as MDS with ring sideroblasts (MDS-RS), or with single lineage dysplasia (SLD; MDS-RS-SLD), and MDS-RS with multilineage dysplasia (MLD; MDS-RS-MLD)

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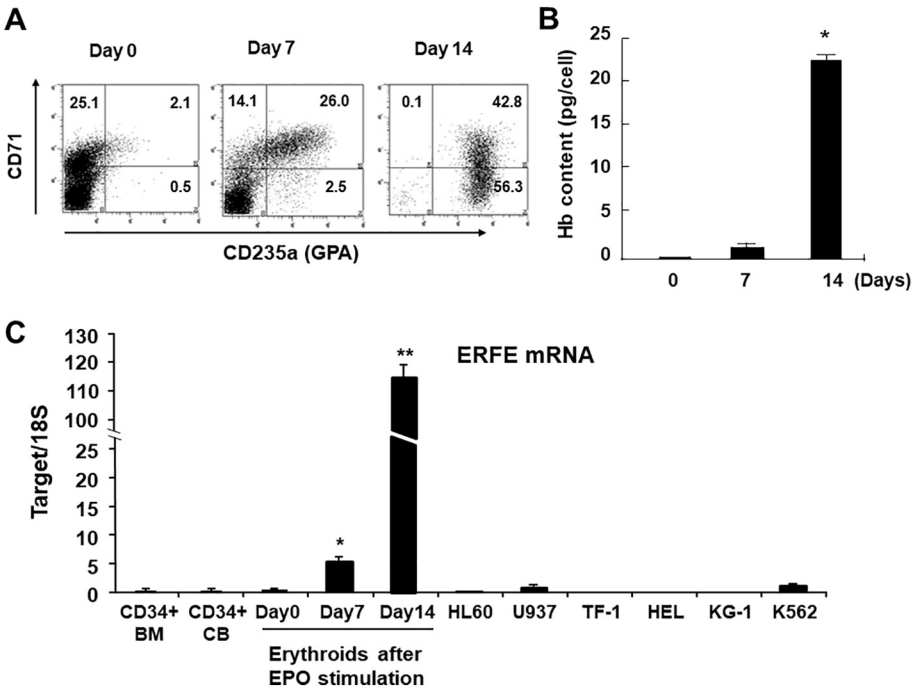

Fig. 1. Differentiation of erythroids from bone marrow CD34 + cells that had expanded above bone marrow mesenchymal stromal cells and expression levels of ERFE mRNA. (A) Flow cytometric analysis of hematopoietic cells after expansion (day 0), and after days 7 and 14 of erythroid induction in the presence of erythropoietin (EPO). Hematopoietic cells were immunolabeled with fluorescein isothiocyanate (FITC)–conjugated CD71 and phycoerythrin (PE)-conjugated CD235a antibodies on the indicated days. (B) Comparison of the hemoglobin (Hb) content per cell (mean corpuscular hemoglobin; MCH) on days 0, 7 and 14 after the start of erythroid differentiation. Each bar represents the mean ± standard deviation (SD) in 10^6 hematopoietic cells. *$p < 0.05$ vs. day 0 in the respective group. (C) Analysis of the expression of erythroid regulators in erythroid cells and leukemic cell lines. The expression levels of erythroferrone (ERFE) mRNA were examined by Taqman quantitative real-time (qRT)–PCR). Results are expressed as mean ± SD. *$p < 0.05$, **$p < 0.01$ vs. day 0 in the respective group. BM, bone marrow; CB, cord blood; EPO, erythropoietin.

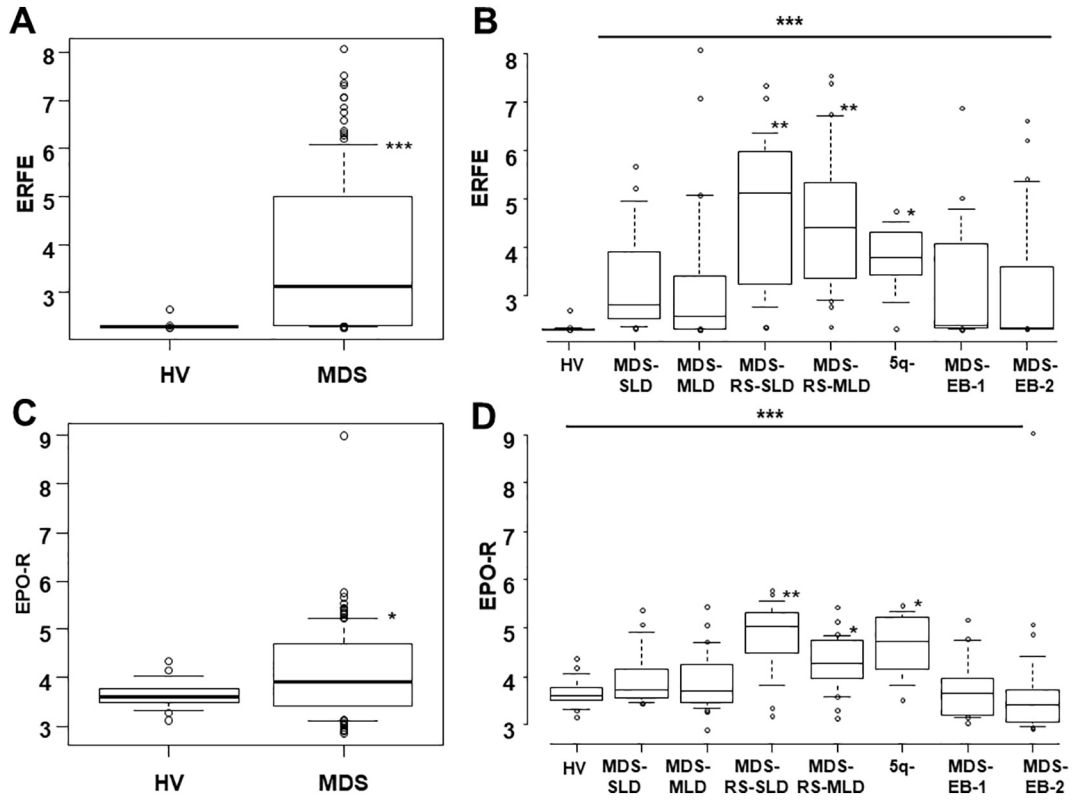

Fig. 2. Analysis of ERFE and EPO-R mRNA, and hematological parameters using a public database. The MDS1 data set was used for these analyses. (A) The level of ERFE mRNA in patients with myelodysplastic syndrome (MDS; $n = 130$) was compared with that in healthy volunteers (HV; n = 17). ***$p < 0.001$, HV vs. MDS. (B) Analysis of ERFE mRNA levels in World Health Organization (WHO) categories of MDS. Results are expressed as dot plots. Horizontal bars indicate the mean value for each group. *$p < 0.05$, HV vs. MDS with isolated del(5q). **$p < 0.01$, HV vs. MDS-RS-MLD, HV vs. MDS-RS-SLD. ***$p < 0.001$, HV vs. all phenotypes of MDS. Data was analyzed by a one-way ANOVA test (the expression of ERFE mRNA in MDS vs. HV). (C) The level of erythropoietin (EPO) receptor (EPO-R) mRNA in patients with MDS (n = 130) as compared with that in HV (n = 17). *$p < 0.05$, HV vs. MDS. (D) Analysis of EPO-R mRNA in WHO categories for MDS. *$p < 0.05$, HV vs. MDS with isolated del(5q). *$p < 0.01$, HV vs. MDS-RS-MLD. **$p < 0.01$, HV vs. MDS-RS-SLD. ***$p < 0.001$, HV vs. all phenotypes of MDS. Data was analyzed by a one-way ANOVA test. MDS-SLD, MDS with single lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts and single lineage dysplasia; MDS-RS-MLD, MDS-RS with multilineage dysplasia; 5q-, MDS with isolated del(5q); MDS-EB, MDS with excess blasts.

[21–23]. Importantly, erythroid regulators such as growth differentiation factor 15 (GDF15) and erythroferrone (ERFE), which downregulates hepatic hepcidin production, have recently been identified. Of these, only the expression of ERFE was upregulated after hemorrhage and hemolysis in response to erythropoietin (EPO) stimulation. Moreover, not only GDF15, but ERFE may contribute to suppress the level of hepcidin and enhance gastrointestinal iron absorption to deliver iron into the BM in a mouse model of beta-thalassemia [24,25]. Based on these findings, we hypothesized that ERFE could be involved in iron delivery to the BM in a subset of patients with MDS via a reduction of hepcidin in hepatocytes.

Here, we evaluated the expression levels of ERFE mRNA in erythroid cells generated from cord blood (CB) CD34+ cells or sodium butyrate (SB)–treated K562 cells using an ex vivo culture system. We found that the ERFE mRNA level was dramatically elevated during erythroid differentiation as compared with that in CD34+ cells. We further analyzed the ERFE mRNA level in BM CD34+ cells in HV or patients with MDS using public Gene Expression Omnibus (GEO) data sets (GSE58831) and assessed the effect of ERFE on hepcidin expression in hepatocytes.

2. Materials and methods

2.1. Reagents, cell lines and human CD34+ cells

SB was purchased from Sigma Chemical Corp. (St. Louis, MO). StemPro®-34 (Life Technologies, Carlsbad, CA) was used as a serum-free medium. Total RNA derived from human BM CD34+, CB CD34+ and human mesenchymal stromal (MSCs) cells was purchased from AllCells, LLC (Toronto, Canada). Human myeloid leukemia cell lines, HEL, HL60 and U937, were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum (FCS; Gibco BRL, Rockville, MD), 2 mM L-glutamine, 0.1% penicillin (100 U/mL) and streptomycin (100 mg/mL). The CD34+ leukemia cell lines, TF-1 and KG-1 (American Type Culture Collection, Manassa, VA), were cultured in RPMI1640 containing 20% heat-inactivated FCS, 2 mM L-glutamine, 1 mM pyruvate, 0.1% penicillin (100 U/mL) and streptomycin (100 mg/mL). For the long-term culture of TF-1, 10 U/mL interleukin-3 (IL-3; R&D systems, Minneapolis, MN) was added to the complete medium. The human erythroleukemia cell line K562 (RCB0027; sensitive to SB) was obtained from RIKEN BRC CELL BANK (Tsukuba, Japan). K562 cells were cultured in Nutrient Mixture F-12 Ham (HamF12) containing 10% heat-inactivated FCS, and 2 mM L-glutamine without antibiotics. The diagnostic criteria of MDS in the public database was according to the 2016 revision to the World Health Organization (WHO) 2016 Classification of myeloid neoplasms and acute leukemias [26].

2.2. Preparation of erythroblasts following stem/progenitor expansion ex vivo

Primary human MSCs (4×10^5 cells) were plated in a 25-cm^2 flask and cultured until they reached over 90% confluency. On the first day of coculture, the MSCs were washed five times with phosphate-buffered saline (PBS) before adding CB CD34+ cells. These CD34+ cells were seeded on a monolayer of human MSCs in 5 ml of serum-free StemPro®-34 medium (Life Technologies) supplemented with 50 ng/mL thrombopoietin (TPO), 10 ng/mL human stem cell factor (SCF) and 50 ng/mL human Fms-related tyrosine kinase 3 ligand (FLT3LG; all R&D Systems) and maintained at 37 °C under 5% CO_2 for 14 days (stem/progenitor cell expansion). All hematopoietic cells that had been expanded above MSCs were collected by gentle pipetting as previously described (day 0 cells) [27]. To avoid the presence of MSCs in the resulting cell suspensions, a 30-minute adhesion procedure was performed at 37 °C as described previously [28,29].

Subsequently, erythroid differentiation was carried out in a serum-free liquid culture system without a human stromal layer. In the first step, hematopoietic progenitor cells at 1×10^6 cells/mL were suspended in erythroid induction medium, which consisted of StemPro®-34 medium with 10 ng/mL SCF, 4 U/mL EPO, 100 U/mL IL-3, 20 ng/mL insulin-like growth factor (IGF)-1 (R&D Systems) and 500 μg/mL iron-saturated transferrin (Sigma Chemical Corp.). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 7 days (day 7 cells). In the second step, progenitor cells that had been induced toward the erythroid lineage were suspended in erythroid maturation medium, which consisted of erythroid induction medium that did not contain IL-3 and SCF. The cells were incubated at 3×10^6 cells/mL in a humidified atmosphere of 5% CO_2 for 7 days (day 14 cells) [30].

2.3. Analysis of mRNA

For reverse transcription, total RNA was prepared from cells using Trizol reagent according to the manufacturer's instructions (Invitrogen, Waltham, MA). Total RNA (1 μg) was reverse transcribed with a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) for Taqman PCR or SYBR® Green PCR. Taqman Assays IDs (Applied Biosystems, Foster City, CA) and Real-time SYBR® Green PCR, primer set IDs are shown in Supplementary Tables 1 and 2. Quantitative real-time (qRT)–PCR was performed in triplicate using the ABI PRISM®7300 Sequence Detection System (PE Applied Biosystems, Waltham, MA) in a 50 μL reaction volume. Relative gene expression was calculated as the signal ratio of the target gene (*FAM*) to *S18* cDNA.

2.4. Immunophenotyping of ex vivo cultured hematopoietic cells

Aliquots of cells were stained with fluorescein isothiocyanate (FITC)– and/or phycoerythrin (PE)-conjugated monoclonal antibodies, including isotype control antibodies (BD Biosciences, San Jose, CA). Cells were incubated with PE-conjugated anti-glycophorin A (GPA; Immunotech, Marseille, France) and FITC-conjugated anti-CD71 (BD Biosciences, Tokyo, Japan) antibodies at 4 °C for 60 min, and then washed twice with PBS containing 0.1% BSA. The cells were analyzed by flow cytometric analysis using FACSCanto (Becton Dickinson, Mountain View, CA) and dead cells were gated out by propidium iodide (PI) staining.

2.5. Analysis of public database

For the analysis of CD34+ cells, Gene Expression Omnibus (GEO) data sets (GSE58831) of gene expression were downloaded and quintiles normalized using an affy Bioconductor package (R commander version 3.5.2). Since this data set included acute myeloid leukemia with myelodysplasia-related changes (AML–MRC, n = 7), chronic myelomonocytic leukemia (CMML, n = 7), MDS with excess blasts (MDS-EB) with an unknown percentage of blasts (n = 14) and unknown diagnosis (n = 1), the data for these diseases was excluded by dplyr package (R commander) to form a new database (MDS1 data set) consisting of HV (n = 17), MDS-SLD (n = 13), MDS-MLD (n = 27), MDS-RS-SLD (n = 20), MDS-RS-MLD (n = 22), MDS with isolated del(5q) (n = 6), MDS-EB-1 (n = 14) and MDS-EB-2 (n = 28).

To detect differentially expressed genes (DEGs), we first identified DEGs showing a more than four-fold change between HV and MDS by analysis using a limma software package. To avoid selecting false negatives, we used a low *p*-value between HV and MDS in data set MDS1 according to the Bonferroni method (*p*-value = 0.003). The DEGs were shown by using a 3dheatmap software package in Supplementary Fig. 1. We focused on 57 DEGs and several iron metabolism–related genes (*p*-value = 0.05, HV vs. MDS) for further analysis.

2.6. Statistical analysis

Each data set was first evaluated for the normality of distribution by the Komolgorov–Smirnov test to decide whether a non-parametric

Table 1
Multiple regression analysis of relationship between ERFE mRNA expression and gene mutations.

	Estimate	Std. error	t value	*p* value
(Intercept)	3.31 (2.86–3.76)	0.2300	14.5900	0.0000
ASXL1	0.80 (−0.14–1.75)	0.4800	1.6800	0.0960
BCOR	1.10 (−0.71–2.92)	0.9200	1.2100	0.2300
CDKN2A	−3.37 (−7.57–0.83)	2.1100	−1.5900	0.1100
CUX1	0.47 (−2.65–3.58)	1.5700	0.3000	0.7700
DNMT3A	−0.43 (−1.43–0.57)	0.5000	−0.8500	0.4000
EP300	−0.76 (−3.76–2.24)	1.5100	−0.5000	0.6200
ETV6	−1.00 (−4.01–2.00)	1.5100	−0.6600	0.5100
EZH2	−0.36 (−1.64–0.93)	0.6400	−0.5500	0.5800
GATA2	−1.89 (−4.92–1.14)	1.5200	−1.2400	0.2200
IDH1	2.08 (−0.92–5.09)	1.5100	1.3800	0.1700
IDH2	−0.89 (−2.78–1.01)	0.9500	−0.9300	0.3600
JAK2	0.02 (−1.63–1.68)	0.8300	0.0300	0.9800
KDM6A	2.34 (−0.66–5.35)	1.5100	1.5500	0.1300
KRAS	1.66 (−1.34–4.67)	1.5100	1.1000	0.2700
SF3B1	0.95 (0.25–1.66)	0.3500	2.6900	0.0085
SH2B3	0.90 (−2.22–4.01)	1.5700	0.5700	0.5700
STAG2	−0.37 (−1.71–0.97)	0.6700	−0.5500	0.5900

The MDS1 data set (subclass of GSE58831) consisting of HV (n = 17), MDS-SLD (n = 13), MDS-MLD (n = 27), MDS-RS-MLD (n = 22), MDS-RS-SLD (n = 20), MDS with isolated del(5q) (n = 6), MDS-EB-1 (n = 14) and MDS-EB-2 (n = 28) was used for these analyses. The left column indicates gene symbols used in this analysis. MDS, myelodysplastic syndrome; HV, healthy volunteers; MDS-SLD, MDS with single lineage dysplasia; MDS-MLD, MDS with multilineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts and single lineage dysplasia; MDS-RS-MLD, MDS-RS with multilineage dysplasia; 5q-, MDS with isolated del (5q); MDS-EB, MDS with excess blasts.

Table 2
Multiple regression analysis relationship between ERFE mRNA and significant DEGs including other iron metabolism–related genes of MDS1 data set.

	Estimate	Std. error	t value	*p* value
(Intercept)	6.890046	3.167040	2.175547	0.031383
ALAS2	0.193845	0.071596	2.707453	0.007684
EPOR	0.388125	0.139967	2.772964	0.006365
FLJ38379	−0.259499	0.063317	−4.098365	0.000072
GDF15	0.468594	0.174680	2.682587	0.008247
METRNL	−0.157468	0.044303	−3.554320	0.000527
NFS1	−0.595363	0.246839	−2.411951	0.017253
PAX5	0.408648	0.129993	3.143615	0.002064
PCBP2	−0.588894	0.184708	−3.188240	0.001790
STEAP3	0.592674	0.136508	4.341685	0.000028
VPREB3	−0.170977	0.066717	−2.562717	0.011516
ABCB7	0.011898	0.085390	0.139342	0.889393
FXN	0.072213	0.209625	0.344489	0.731030
PCBP1	−0.091173	0.209557	−0.435074	0.664224
TFR2	0.015347	0.126235	0.121580	0.903417
TFRC	0.031388	0.166073	0.189005	0.850381

The MDS1 data set (subclass of GSE58831) was used. The left column indicates the gene symbol used in this analysis. Deferentially expressed genes (DEGs) and iron metabolism–related genes, including *ALAS2*, *EPOR*, *PCBP1*, *PCBP2*, *STEAP3*, ATP Binding Cassette Subfamily B Member 7 (*ABCB7*), Transferrin Receptor (*TFRC*), Transferrin Receptor 2 (*TFR2*) and Frataxin (*FXN*), were analyzed. *FLJ38379* is an uncharacterized RNA gene and is affiliated with the non-coding RNA class. METRNL: Meteorin-like, Glial Cell Differentiation Regulator.

rank–based or a parametric analysis should be used. The significance of differences between groups was assessed by one-way ANOVA test with a post-hoc Tukey Honestly Significant Difference Test. Results are expressed as the mean ± standard deviation (SD). The significance of differences was assessed by either the Student's *t*-test or the Mann-Whitney *U*–test, and a *p*-value < 0.05 was considered as statistically significant. All statistical analyses were performed with R commander or EZR (Easy R) software, which is a graphical user interface for R [31].

3. Results

3.1. The mRNA expression of erythroid regulators in erythroblasts and leukemic cell lines

We first investigated the expression level of ERFE and GDF15 in ex vivo cultured erythroblasts and leukemic cell lines to obtain insights into the specificity of erythroid expression. In this culture system, we used MSCs to expand stem/progenitor cells before erythroid differentiation. Importantly, MSCs was almost completely removed by a 30-minute adhesion procedure [27,28]. Moreover, we have recently analyzed transcriptome of MSC (GEO108186) [32] and confirmed that ERFE and GDF15 mRNA was quite low and ignorable in this experiment. Erythroid cells were differentiated ex vivo from CB CD34+ cells in the presence of SCF, IL-3 and EPO (Fig. 1A) [30,33,34]. The hemoglobin (Hb) content per cell (mean corpuscular hemoglobin, MCH) was significantly elevated during culture for 14 days (Fig. 1B; $p < 0.05$). Remarkably, *ERFE* mRNA was approximately a thousand-fold higher 14 days after the EPO stimulation of erythroblasts (Fig. 1C; $p < 0.01$). In addition, *ERFE* mRNA in leukemic cells, including K562 erythroblastic leukemia cells, remained at a low level. The expression of *GDF15* mRNA was thirty-fold higher in erythroid cells 14 days after EPO stimulation (Supplementary Fig. 2; $p < 0.01$). Based on these findings, we focused on ERFE and further examined its expression in MDS.

3.2. Analysis of ERFE mRNA levels in CD34+ MDS cells using a GEO database

To check the gene expression of ERFE in CD34+ MDS cells, we employed an MDS1 data set (a subset of GSE58831) in which the transcriptomes in CD34+ MDS and CD34+ normal BM cells were analyzed. *ERFE* gene expression in CD34+ MDS cells was significantly higher than that in CD34+ cells derived from HV (Fig. 2A; $p < 0.001$ and Supplementary Fig. 3) although the level of ERFE expression was variable in CD34+ MDS cells. Probably, MDS has been recently recognized as syndromes which includes several specific types of hematological disorders such as MDS-RS and MDS with isolated del(5q). Therefore, we evaluate the level of ERFE in each WHO category. ERFE expression in CD34+ cells derived from MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q) was higher than that in other subgroups of MDS (Fig. 2B and Supplementary Fig. 3). Interestingly, when we analyzed whole data set of GSE58831, we found that AML-MRC exhibited a low level of ERFE expression, although some CMML and MDS-EB cells exhibited a high level of ERFE expression (Supplementary Fig. 4). Considering the involvement of ring sideroblasts that correlated with the *SF3B1* mutation, the MDS1 data set was further analyzed by adding supplementary information of clinical and sequencing data (https://www.nature.com/articles/ncomms6901). As expected, multiple regression analysis revealed that only the *SF3B1* mutation positively correlated with a higher expression of ERFE mRNA (Table 1). Moreover, we selected highly expressed genes in MDS from the MDS1 data set as compared with those expressed in HV, and subsequently analyzed the statistical correlation between ERFE and these genes using multiple regression analysis. As a result, several iron metabolism–related genes, including *ALAS2* (*5′-Aminolevulinate Synthase 2)*, *EPO-R* and *STEAP3* (STEAP family member 3), which encodes the protein involved in transferrin-dependent iron uptake in erythroid cells by reducing Fe^{3+} to Fe^{2+}) significantly and positively correlated with ERFE. Conversely, *PCBP2,* which encodes a cytosolic ferrous iron carrier protein, weakly and negatively correlated with ERFE (Table 2). In addition, the expression of EPO-R and ALAS2 in CD34+ MDS cells was slightly higher than that in CD34+ cells derived from HV (Fig. 2C; $p < 0.05$ and Supplementary Fig. 5) although the level of TFRC (transferrin receptor 1) and PCBP2 in CD34+ MDS cells was largely same with that in CD34+ cells derived from HV (Supplementary

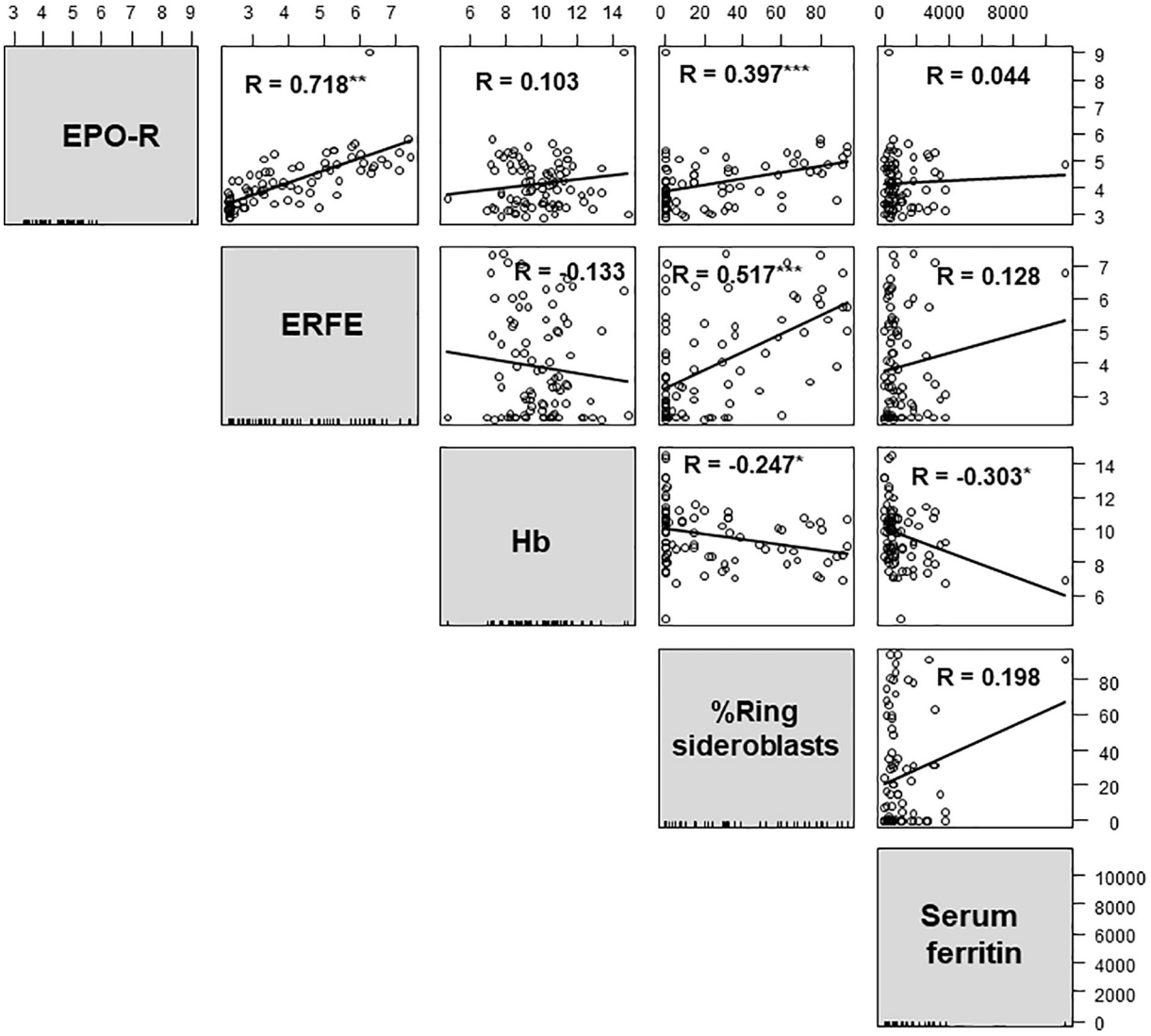

Fig. 3. Correlation matrix of different hematological parameters and percentage of ring sideroblasts. Scatter plots of all pairwise combinations of the variables: serum ferritin, hemoglobin (Hb), percentage of ring sideroblasts, erythroferrone (ERFE) and erythropoietin (EPO) receptor (EPO-R) in bone marrow (BM) CD34+ cells are given below the diagonal. The plots are arranged so that all plots in a row share a common Y-axis, whereas all plots in a column share a common X-axis. The names of the X- and Y-axes are shown in the gray boxes in bold letters at the top of the column and right of the row, respectively. The significant respective Spearman rank correlation coefficients are given as an overlay in the scatter plot. The correlations were statistically significant: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Fig. 5). Considering that ALAS2 is the first and rate-limiting enzyme in the mammalian heme biosynthetic pathway [35] and that EPO-R have been known to be expressed in the differentiation of erythroid progenitors [36], CD34+ fraction of MDS cells may have tendency to be biased into erythroid lineage. For the MDS1 data set, EPO-R mRNA expression in CD34+ cells derived from MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q) was significantly higher than that in HV (Fig. 2D and Supplementary Fig. 3). Moreover, EPO-R mRNA levels strongly and positively correlated with ERFE mRNA levels and the percentage of ring sideroblasts moderately correlated with ERFE mRNA levels (Fig. 3). These results suggested that ERFE mRNA expression may be correlated with the *SF3B1* mutation, the presence of ring sideroblasts and EPO-R mRNA expression in MDS cells.

3.3. Analysis of relationship between serum ferritin, transfusion dependency, ring sideroblasts, SF3B1 mutation and ERFE expression in MDS cells

It has been shown that ERFE reduced hepatic hepcidin production, resulting in the enhancement of duodenal iron absorption via the iron transporter, ferroportin 1, and the elevation of hepatic iron storage. However, the ERFE level did not correlate with the serum ferritin level using the MDS1 data set (Fig. 3) probably because this data set contains transfusion-dependent MDS. Therefore, we conducted more detail analyses regarding the relationship between serum ferritin, transfusion dependency, ring sideroblasts, the SF3B1 mutation and ERFE expression. Regarding inflammation which enhances serum ferritin level, we did not analyze because MDS1 data set with supplementary clinical information did not included the data such as C reactive protein. As expected, the serum ferritin level in transfusion-dependent MDS was significantly higher than that in transfusion-independent patients (Fig. 4A). Further, the Hb level in transfusion-independent MDS was significantly higher than that in transfusion-dependent patients (Fig. 4B). Thus, iron overload by transfusion could lead to an increase in the serum ferritin level in transfusion-dependent patients. Conversely, the utilization of hepatic iron in non-anemic MDS patients decreased the serum ferritin level. We next confirmed the effect of the presence of ring sideroblasts and the SF3B1 mutation because MDS consisted of specific phenotypes such as MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q), these phenotypes have been known, to some extent, to

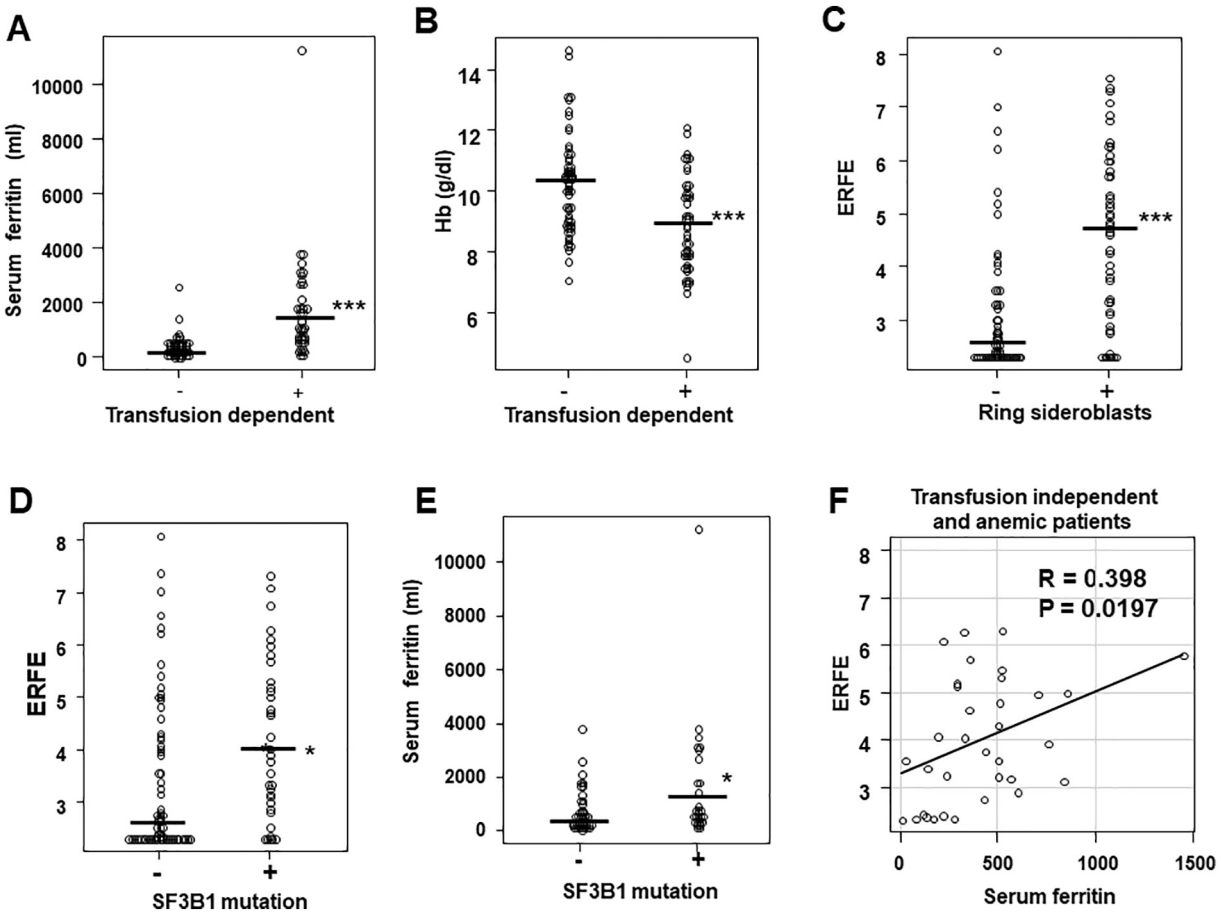

Fig. 4. Relationship between transfusion dependency, serum ferritin, Hb, ERFE and SF3B1 mutation. (A) The level of serum ferritin in myelodysplastic syndrome (MDS) between transfusion-independent and -dependent patients. ***$p < 0.001$, transfusion-independent vs. -dependent patients. (B) The level of hemoglobin (Hb) in patients with between transfusion-independent and -dependent MDS. ***$p < 0.001$, transfusion-independent vs. -dependent patients. (C) The level of erythroferrone (ERFE) in MDS with or without ring sideroblasts. The presence of ring sideroblasts was determined according to a World Health Organization 2016 classification. (D) The level of ERFE in MDS, with or without an SF3B1 mutation. *$p < 0.05$, SF3B1 mutation negative vs. SF3B1 mutation positive. (E) The level of serum ferritin in MDS, with or without an SF3B1 mutation. *$p < 0.01$, SF3B1 mutation negative vs. SF3B1 mutation positive. Data was analyzed by Student's two-tailed *t*-test. (F) Correlation between ERFE and the serum ferritin level in transfusion-independent and anemic MDS patients. The cut off Hb level (11.2 g/dL) was determined by a Receiver Operating Characteristic (ROC) curve, with or without an SF3B1 mutation to remove non-anemic patients without an SF3B1 mutation.

possess SF3B1 mutations and ring sideroblasts. In fact, in the MDS1 data set, SF3B1 mutations in MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q) were 93.8%, 68.4%, and 33.3%, respectively. In addition, the presence of ring sideroblasts (> 15%) in MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q) was 100%, 90.5% (9.5%: > 5% with SF3B1 mutation positive), and 50.0%, respectively. As expected, the ERFE expression was markedly higher in MDS with ring sideroblasts (Fig. 4C) and an SF3B1 mutation (Fig. 4D). In addition, the serum ferritin level was significantly higher in MDS with an SF3B1 mutation (Fig. 4E). Based on these findings, we focused on transfusion-independent MDS patients with anemia and found that ERFE expression moderately and positively correlated with the serum ferritin level (Fig. 4F), suggesting that higher serum ferritin in MDS patients may be the result of higher ERFE and consequent decrease hepcidin expression.

4. Discussion

In the present study, we found that ERFE production is increased during ex vivo erythroid differentiation from CB CD34+ cells in the presence of EPO. Moreover, ERFE was highly produced by CD34+ MDS cells. In particular, ERFE mRNA expression significantly correlated with the SF3B1 mutation, the presence of ring sideroblasts and EPO-R mRNA expression in MDS cells.

Previously, it was found that excess tissue iron increased in the presence of ineffective erythropoiesis by analysis using iron radioisotopes, tissue iron staining and Magnetic Resonance Imaging (MRI) [37]. However, it was unclear whether the elevation of body storage iron could be attributed to increased gastrointestinal iron absorption [37,38]. However, a substitute marker of iron overload such as serum ferritin and MRI R2 clearly showed hepatic iron accumulation even in transfusion-independent MDS patients [39,40], suggesting that iron accumulation in MDS patients is not always dependent upon transfusion-associated iron loading. Consistent with these reports, the analysis of GEO data sets (GSE58831) indicated that a proportion of MDS patients, irrespective of WHO category, exhibited hyperferritinemia (Supplementary Fig. 6). These results suggested that certain molecular mechanisms may be implicated in excess tissue iron in patients with MDS [25]. In these analyses, we focused on the newly-identified erythroid regulator, ERFE, and found that ERFE mRNA was elevated in the CD34+ cells of MDS patients. These results suggest that ERFE is involved in abnormal iron metabolism in patients with MDS.

Next, we investigated the relationship between the ERFE level and the WHO category of MDS. In this study, as shown in Fig. 2, the level of ERFE was higher in MDS-RS-SLD, MDS-RS-MLD and MDS with isolated del(5q) as compared with others. When we analyzed the relationship between gene mutation and the ERFE level, the presence of an *SF3B1* mutation significantly correlated with ERFE expression (Table 1). Regarding the analysis of GEO data sets (GSE58831), ERFE expression in

CD34+ cells derived from transfusion-independent MDS and the average level of serum ferritin were highest in MDS-RS-SLD patients (Supplementary Fig. 6). These results were consistent with a previous report showing that patients with MDS-RS-SLD exhibited a high serum ferritin level [41]. Importantly, the level of hepcidin, which down-regulates the ferroportin transporter in the duodenum, was reduced in patients with MDS-RS-SLD [23], and GDF15 did not correlate with the hepcidin level [23]. These results seem to support our findings and results from GEO data sets (GSE58831) showing that the iron regulator, ERFE, was significantly elevated in the BM of MDS patients, especially those with MDS-RS-SLD and MDS-RS-MLD [23]. Thus, ERFE may contribute to reducing hepatic hepcidin production in MDS patients.

In the present study, the level of ERFE in CD34+ MDS cells strongly correlated with that of EPO-R and ALAS2 (Fig. 3 and Supplementary Fig. 5). Since ALAS2 and EPO-R have been known to be expressed in the differentiation of erythroid progenitors, CD34+ fraction of MDS cells may have tendency to be biased into erythroid lineage. Thereby, CD34+ MDS cells could highly express ERFE as compared with normal CD34+ cells. However, It is quite important to evaluate ERFE expression in the later stages of erythroid cells, since, ERFE mRNA in erythroid cells derived from human BM may be highly upregulated in response to EPO [42]. Further, blockade of JAK2/STAT5 signaling resulted in a reduction of the ERFE level [42]. Based on these findings, we hypothesized ERFE in erythroid cells derived from MDS may be increased in response to EPO via EPO-R. Comprehensive clinical studies are required to elucidate the relationship between ERFE in the later stages of erythroid cells, iron-related parameters, biomarkers of inflammation, the serum ERFE level, and serum hepcidin and iron absorption in any future studies.

5. Conclusion

ERFE expression by BM CD34+ cells from patients with MDS is higher than in normal BM. In particular, ERFE expression is greatest when MDS cells highly express EPO-R, and in the presence of RS or the SF3B1 mutation. The ERFE produced by erythroid cells may reduce the hepcidin level in hepatocytes. Thus, ERFE expression in EPO-R+ MDS cells with RS may contribute to aberrant iron metabolism.

Author contributions

Contribution: SM contributed to most experiments. MK designed experiments and drafted the paper. HH, SK, SI, KM conducted in vitro experiments. AG, HI and KT helped with the statistical analysis of the data. And KM helped with the analysis of GEO data base. JK edited the final version of this paper.

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Disclosure of conflicts of interest

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Appendix A. Supplementary data

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