

**Azacitidine is a potential
therapeutic drug for
pyridoxine-refractory female
X-linked sideroblastic anemia**

(アザシチジンはピリドキシン不応性の
女性 X 連鎖性鉄芽球性貧血の
治療薬となり得る)

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Azacitidine is a potential therapeutic drug for pyridoxine-refractory female X-linked sideroblastic anemia

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Short title: XLSA amelioration by the demethylating agent AZA

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Key Points

- A patient-derived iPSC model recapitulates defective erythroid maturation in female XLSA.
- Azacitidine reactivates the silent wild-type *ALAS2* allele and ameliorates inefficient erythropoiesis in iPSC-derived HPCs from female XLSA.

Abstract

X-linked sideroblastic anemia (XLSA) is associated with mutations in the erythroid-specific δ -aminolevulinic acid synthase (*ALAS2*) gene. Treatment for XLSA is mainly supportive, except in pyridoxine-responsive patients. Female XLSA often represents a late onset of severe anemia, mostly due to the acquired skewing of X-chromosome inactivation. Here, we successfully generated active wild-type and mutant *ALAS2* induced pluripotent stem cell (iPSC) lines from the peripheral blood cells of an affected mother and two daughters in a family with pyridoxine-resistant XLSA due to a heterozygous *ALAS2* missense mutation (R227C). The erythroid differentiation potential was severely impaired in active mutant iPSC lines compared to that in active wild-type iPSC lines. Most of the active mutant iPSC-derived erythroblasts revealed an immature morphological phenotype, and some showed dysplasia and perinuclear iron deposits. Additionally, globin and *HO-1* expression and heme biosynthesis in active mutant erythroblasts were severely impaired compared to that in active wild-type erythroblasts. Furthermore, genes associated with erythroblast maturation and karyopyknosis showed significantly reduced expression in active mutant erythroblasts, recapitulating the maturation defects. Notably, the erythroid differentiation ability and hemoglobin expression of active mutant iPSC-derived hematopoietic progenitor cells (HPCs) were

improved by the administration of δ -aminolevulinic acid, verifying the suitability of the cells for drug testing. Administration of a DNA demethylating agent, azacitidine, reactivated the silent wild-type *ALAS2* allele in active mutant HPCs and ameliorated erythroid differentiation defects, suggesting that azacitidine is a potential novel therapeutic drug for female XLSA. Our patient-specific iPSC platform provides novel biological and therapeutic insights for XLSA.

Introduction

Congenital sideroblastic anemias are a group of heterogeneous inherited diseases characterized by impaired heme synthesis and mitochondrial iron overload with ring sideroblasts in the bone marrow. X-linked sideroblastic anemia (XLSA) is the most common hereditary sideroblastic anemia and is caused by mutations in the erythroid-specific δ -aminolevulinic acid synthase (*ALAS2*) gene, which is the first enzyme involved in heme biosynthesis in erythroid cells.^{1,2} Nearly 100 different *ALAS2* mutations have been reported to date.³ XLSA occurs preferentially in males and usually presents as asymptomatic or mild microcytic anemia. However, approximately one-quarter of the affected XLSA probands are females, and these patients show a distinct clinical presentation and mutation spectrum.⁴⁻⁶ Unlike males, females with XLSA often harbor heterozygous deleterious *ALAS2* mutations that are embryonic lethal in males and develop more severe clinical phenotypes with macrocytic anemia in mid-to-late adulthood.⁷ Their anemia is mostly associated with skewed X-chromosome inactivation, which increases with age.^{8,9} Most families with female XLSA have multiple affected members, indicating a familial predisposition to acquired X-chromosome skewing.³

One treatment option is pyridoxine, which is metabolized to pyridoxal 5'-phosphate, the cofactor for ALAS2. However, pyridoxine refractoriness is reported in nearly half of the cases, including those with a R227C mutation.^{1,10} The severe loss of function due to this mutation may explain why no male carriers have been identified in affected families.¹¹ Therapies for XLSA in patients who do not respond to pyridoxine are mostly aimed at supportive care. δ -aminolevulinic acid (ALA) administration could theoretically ameliorate the heme biosynthesis defects that occur in XLSA, because ALAS2 catalyzes

the condensation of glycine and succinyl-CoA into ALA. However, a recent study of a single case showed no improvement in the hemoglobin level even at the in vitro effective concentration.¹² Therefore, other treatment approaches should be explored.

Several cell and animal models of male XLSA have been developed previously.¹³⁻¹⁸ However, there are no reported models of female XLSA with heterozygous *ALAS2* mutations. In the present study, we established active wild-type induced pluripotent stem cell (WT iPSC) and active mutant induced pluripotent stem cell (MT iPSC) lines from the peripheral blood cells of an affected mother and her two daughters in a family with XLSA due to a heterozygous *ALAS2-R227C* mutation. We then differentiated the iPSCs into erythroblasts to model the pathophysiology of female XLSA. Using this iPSC-based platform, we found that the demethylating agent azacitidine (AZA) induces the expression of wild-type *ALAS2* in active mutant iPSC-derived hematopoietic progenitor cells (MT HPCs) to ameliorate abnormal erythroid differentiation caused by the heme biosynthesis defects.

Materials and methods

Generation of iPSCs from XLSA patients

This study was approved by the Ethics Committees of the Graduate School of Medicine, Kyoto University, and Kyoto University Hospital. Written informed consent was obtained from all patients, and the study was performed in accordance with the Declaration of Helsinki. XLSA-iPSCs were established from three patients harboring a heterozygous *R227C* mutation using episomal vectors, as previously described.¹⁹ Briefly, the patients' peripheral blood mononuclear cells (PBMCs) were transfected with episomal vector

mixtures including pCXLE-hOCT3/4-shp53, pCXLE-hSK, pCXLE-hUL, and pCXWB-EBNA1, and then seeded onto mouse embryonic fibroblasts feeder cells. The iPSC IDs of all XLSA-iPSC lines used in the differentiation experiment are described in supplemental Table 1. The WT and MT iPSC lines derived from SA1 are denoted as WT1-iPSC1, 2, and 3 and MT1-iPSC1, 2, 3, and 4, respectively. Similarly, those from SA3 are denoted as WT3-iPSC1 and 2 and MT3-iPSC1 and 2, respectively. We used one iPSC line derived from a healthy donor (692D2) as the control.¹⁹ Chromosomal G-band analysis of the XLSA-iPSCs was performed at the Nihon Gene Research Laboratories (Sendai, Japan). Animal experiments for the teratoma formation of XLSA-iPSCs were performed as previously described, in accordance with the code of ethics of the Animal Research Committee of Kyoto University.²⁰

Human androgen receptor (HUMARA) assay and Sanger sequencing

Genomic DNAs from iPSCs, CD43⁺CD34⁺ iPSC-derived HPCs, and magnetic-activated cell sorting (MACS)-sorted CD34⁺ and CD235a⁺ bone marrow cells were extracted for the HUMARA assay using the DNeasy Blood & Tissue kit (Qiagen). DNA samples (200 ng) were incubated overnight at 37 °C in 20 µL reactions containing 10 U HhaI and 10 U RsaI. The digested DNA samples were purified using QIAquick spin columns (Qiagen) and amplified using a Veriti Thermal Cycler (Thermo Fisher Scientific). PCRs and analysis of the PCR products were performed as previously described.^{21,22} Peripheral red blood cells were separated using Polymorphprep (Abbott Diagnostics Technologies AS) following the manufacturer's protocol. Sanger sequencing of exon 6 of the *ALAS2* gene was performed as previously reported.⁶

Hematopoietic differentiation and erythroid differentiation assays

The hematopoietic differentiation of iPSCs was performed using embryoid body (EB) formation, and feeder-free erythroid differentiation was performed as previously reported.^{23,24} For feeder-free erythroid differentiation with AZA, fluorescence-activated cell sorting (FACS)-sorted iPSC-derived HPCs on day 15 were differentiated in StemPro-34 medium (Invitrogen). The cytokines used for the differentiation were as follows: on days 15-23, human stem cell factor (hSCF; 100 ng/mL, R&D Systems), human erythropoietin (hEPO; 4 U/mL, Kyowa Kirin Co., Ltd.), and human IL-3 (hIL-3; 5 ng/mL, R&D Systems); on days 23-26, hSCF (100 ng/mL) and hEPO (4 U/mL); and on days 26-29, hEPO (4 U/mL). Erythroid differentiation on OP9 stromal feeder cells was performed as previously described with some modifications.^{16,18,25} Briefly, FACS-sorted HPCs on day 15 were plated onto OP9 cells and cultured and maintained in alpha-minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum, 100 mM glutamine (Invitrogen), 100 μ M monothioglycerol (Sigma Aldrich), 5 μ g/mL transferrin (Roche), and 50 μ g/mL ascorbic acid (Sigma Aldrich). The cytokines and chemical compounds used during the differentiation were as follows: on days 15-20, hSCF (100 ng/mL), human Fms-like tyrosine kinase 3 ligand (hFlt-3; 100 ng/mL, R&D Systems), hEPO (4 U/mL), and human thrombopoietin (hTPO; 50 ng/mL R&D Systems); on days 20-27, hSCF (100 ng/mL) and hEPO (4 U/mL); and on days 27-34, hEPO (4 U/mL) and sodium ferrous citrate (SFC; 250 μ M, Nihon Generic Co., Ltd).

Treatment with ALA and AZA

From the start of erythroid differentiation, cultures were administered 500 μ M ALA (SIGMA) or 100 or 500 nM AZA (Nacalai Tesque). For the ALA-treated cells, ALA was

administered daily to the erythroid differentiation medium. For the AZA-treated cells, AZA was administered to the medium at the time of the medium change (in the EB-feeder-free method, on days 19, 23, and 26; in the EB-OP9 method, on days 23 and 27). For the methylcellulose assay, AZA was added to the media at the beginning of the colony-forming unit (CFU) assay.²⁶

Statistical analyses

GraphPad Prism 9 (GraphPad Software) was used to perform all statistical analyses. $P < 0.05$ was considered significant.

Data sharing statement

RNA sequencing (RNA-seq) data are available at GEO under the accession number GSE176008. For the original data, please e-mail the corresponding authors.

Results

Late-onset female XLSA patients show preferential inactivation of the paternal X chromosome at the stage of hematopoietic progenitor cells

We previously identified a family with XLSA harboring a heterozygous *ALAS2-R227C* mutation (c.679C>T, NCBI NM_000032.4) (Figure 1A).²⁷ The clinical features of the proband (SA1) and her mother (SA3), who were diagnosed with XLSA due to the mutation, have been described previously.⁶ Her younger sister (SA2) also presented late-onset severe macrocytic anemia and evidence of iron overload (Table 1). Sanger sequencing of the genomic DNA from SA2 buccal cells demonstrated the same heterozygous *ALAS2* missense mutation as the proband. We also checked the *ALAS2*

cDNA sequence of her peripheral blood erythrocytes and found no *ALAS2* mutation (Figure 1B). The bone marrow aspirate showed erythroid hyperplasia with ring sideroblasts and dyserythropoiesis, such as megaloblastic changes, nuclear budding, and karyorrhexis, but no apparent abnormalities of megakaryocytes and granulocytes (Figure 1C). While the CD34⁺ and CD235a⁺ bone marrow cells of SA2 revealed apparent skewing toward the mutant *ALAS2* allele, the peripheral blood erythrocytes expressed only wild-type *ALAS2* (Figure 1D). Her condition was diagnosed as XLSA. We also performed a bone marrow aspirate of SA3, verifying similar erythroid-restricted dysplasia (Figure 1C-D). The clinical characteristics of the patients are shown in Table 1, including laboratory data at the time of diagnosis. SA1 was administered 60 mg of pyridoxal phosphate hydrate for 3 months with no improvement in her anemia (supplemental Figure 1), Therefore, a clinical trial of ALA administration was performed with the consent of SA1 and SA2. They were administered 150 mg of ALA-phosphate (kindly provided by SBI Pharmaceuticals, Tokyo, Japan) for 12 and 24 weeks. There were no side effects of ALA administration; however, the anemia did not improve (supplemental Figure 2).

Generation of MT and isogenic WT iPSC lines from XLSA female patients

We generated iPSCs from the peripheral blood cells of all three patients. Because the *ALAS2* gene is located on the X chromosome, one of the two *ALAS2* genes is randomly inactivated by lyonization in female patients. Therefore, the cells in which the X chromosome with mutated *ALAS2* is inactivated, namely, WT cells, and the cells in which the X chromosome with normal *ALAS2* is inactivated, namely, MT cells, were established from the same patient (Figure 2A and supplemental Table 1). We generated 58 iPSC lines from SA1, 94 iPSC lines from SA2, and 47 iPSC lines from SA3. HUMARA assays

demonstrated that the PBMCs of all three patients underwent skewed X-chromosome inactivation with preferential inactivation of the X chromosome with wild-type *ALAS2* (Figure 2B). The percentage of MT iPSC lines was similar to the percentage of X-chromosome inactivation in PBMCs for all XLSA patients (Figure 2B). The gene expression of pluripotent markers *OCT3/4*, *SOX2*, and *NANOG* was verified in three WT and four MT iPSC lines derived from SA1 using quantitative reverse transcription (RT)-PCR analysis with the primers in supplemental Table 2 (Figure 2C). These seven iPSC lines expressed the pluripotent markers SSEA-4 and TRA1-60 and formed teratomas in vivo (supplemental Figure 3A-B). Karyotype analysis revealed no abnormalities (supplemental Figure 3C). Using the HUMARA assay and immunostaining of H3K27me3, we demonstrated that the maternal X chromosome of the WT iPSC lines was inactivated, whereas the paternal X chromosome of the MT iPSC lines was inactivated (Figure 2D). The X-chromosome inactivation status was retained in the iPSC lines after passaging. The maternal X chromosome of the WT iPSC lines, which was inactivated immediately after iPSC establishment (passage 5), remained inactivated at passages 20 and 30. The paternal X chromosome of the MT iPSC lines also retained the inactivated state after repeated passages. Notably, X-chromosome inactivation was maintained after hematopoietic differentiation and further differentiation into erythroblasts (Figure 2E).

Erythroid differentiation is impaired in MT iPSCs

Next, we differentiated XLSA-iPSCs into HPCs by EB formation. The hematopoietic maturation capacity was assessed using CFU assays in iPSC-derived HPCs from SA1. No difference was observed in the total colony count between the two types of HPCs (Figure 3A). Although erythroid and mixed colonies were observed in the CFU assay of

WT iPSC lines, no erythroid colonies and only a few mixed colonies were observed in the MT iPSC lines (Figure 3B-C). Lentiviral transduction of wild-type *ALAS2* improved erythroid colony formation in MT HPCs (Figure 3D and supplemental Figure 4A). We further investigated the erythroid differentiation defects in MT iPSC lines using a feeder-free erythroid culture system. Hematopoietic differentiation was comparable between the two types of iPSC lines (Figure 3E). However, further differentiation into CD235+ erythroid cells was significantly impaired in the MT iPSC lines compared to that in the WT iPSC lines derived from both SA1 and SA3 (Figure 3F and supplemental Figure 4B-C). In our feeder-free erythroid differentiation method, most MT HPCs died at an early stage of differentiation (supplemental Figure 4D). As a result, we could neither identify morphological characteristics, such as ring sideroblasts or megaloblastic changes, nor perform a precise gene expression analysis of the erythroblasts expressing mutant *ALAS2*. Therefore, we designed a co-culture system with OP9 feeder cells to successfully recapitulate the morphological features of mutant *ALAS2*-expressing erythroblasts. In this system, most MT HPCs survived for extended periods (supplemental Figure 4E-F). The pellet of active wild-type iPSC-derived erythroblasts (WT erythroblasts) was red, whereas that of active mutant iPSC-derived erythroblasts (MT erythroblasts) was white (Figure 4A). Iron staining revealed ring sideroblasts in MT erythroblasts, but not in WT erythroblasts (Figure 4A). May Grunwald-Giemsa staining showed that most WT erythroblasts were mature, whereas most MT erythroblasts exhibited immature morphological phenotypes. In addition, dysplastic changes, such as nuclear budding and karyorrhexis, were observed in MT erythroblasts, similar to the primary erythroblasts of the patient (Figure 4B). Although the majority of the WT erythroblasts were orthochromatic erythroblasts, most of the MT erythroblasts were polychromatic

erythroblasts and megaloblasts (Figure 4C). In addition, although the *o*-dianisidine staining of WT erythroblasts was normal, MT erythroblasts were only slightly stained (Figure 4D), suggesting that heme synthesis was highly impaired in MT erythroblasts. Furthermore, quantitative RT-PCR analysis using the primers in supplemental Table 2 demonstrated that *HBB*, *HBG*, *HO-1*, and *ALAS2* expression in MT erythroblasts was significantly decreased (Figure 4E and supplemental Figure 4G). By contrast, the expression of these genes in WT erythroblasts was comparable to that in the control erythroblasts.

ALA improves the erythroid differentiation capacity

We next explored the potential application of our iPSC-based disease model for drug testing. Heme synthesis starts with the polymerization of succinyl-CoA and glycine followed by the synthesis of ALA in the mitochondria. *ALAS2* encodes an enzyme that catalyzes the first step in the heme synthetic pathway. Thus, the administration of ALA to MT HPCs is expected to improve the heme synthesis capacity. Indeed, we observed a noticeable increase in the erythroid differentiation of MT iPSCs after the administration of ALA from the start of erythroid differentiation (supplemental Figure 5A-C). Additionally, the pellet of these erythroid cells was red, supporting the conclusion that ALA restored heme biosynthesis (supplemental Figure 5D). Moreover, quantitative RT-PCR analysis demonstrated that ALA administration restored the expression of *HBB*, *HBG*, and *HO1* (Figure 4F). These results support the use of our iPSC model for drug testing.

Expression of erythroid maturation-related genes is impaired in MT erythroblasts

To compare the transcriptional expression patterns during erythroid differentiation in WT and MT cells, we performed RNA-seq of the iPSCs, CD34⁺ cells on day 8, CD43⁺CD34⁺CD38⁻Lin⁻ cells on day 15, and CD235a⁺ erythroblasts on day 34 in the three WT and four MT iPSC lines (Figure 5A). No differences were observed between the WT and MT cells at the stages of iPSCs, CD34⁺ cells, and CD43⁺CD34⁺38⁻Lin⁻ cells. However, erythroblasts were divided into two clusters according to hierarchical clustering (Figure 5B). A principal component analysis (PCA) of differentiated erythroblasts from the seven iPSC lines and bone marrow erythroblasts from a healthy donor and from SA3 revealed that iPSC-derived erythroblasts have cluster patterns similar to corresponding primary bone marrow erythroblasts. MT erythroblasts and SA3 bone marrow erythroblasts were located on the positive side of PC2, whereas WT erythroblasts and healthy donor bone marrow erythroblasts were located on the negative side (Figure 5C). To verify this observation, the cells were assessed using Gene Ontology (GO) analysis of the RNA-seq data. The upregulated genes in WT cells showed enrichment for mature erythroblast-related GO terms including Ras GTPase binding, Rho GTPase binding, and actin binding, and iron-transporter-regulated GO terms, such as transition metal ion transmembrane transporter activity (Figure 5D). This analysis indicated that WT erythroblasts differentiated into more mature stages than MT erythroblasts despite the same differentiation method. We also determined the enrichment of gene expression in WT erythroblasts and MT erythroblasts using gene set enrichment analysis (GSEA) for a set of genes differentially expressed during erythroid differentiation. The heme metabolism dataset was significantly enriched in WT erythroblasts (Figure 5E and supplemental Table 3). Hierarchical clustering analysis of iPSC-derived and bone marrow erythroblasts for genes specifically

expressed in early orthochromatic erythroblasts was performed (Figure 5F).²⁸ The expression of these genes was lower in MT erythroblasts and SA3 bone marrow erythroblasts than that in WT erythroblasts and healthy donor bone marrow erythroblasts. These gene expression data support the findings of the phenotype experiments, which indicated that erythroblasts derived from MT iPSCs stopped at the immature erythroid stage.

AZA treatment ameliorates heme synthesis failure and erythroid differentiation defects in MT iPSCs

We hypothesized that the pharmacological reactivation of the silent wild-type inactive X chromosome in the *ALAS2* gene could ameliorate heme synthesis failure in XLSA patients. In a previous study, it was suggested that the administration of a DNA demethylating agent, decitabine, reactivates the silent *LAMP2* gene in iPSCs in female patients with Danon disease.²⁹ Notably, in that study, other silent wild-type X-chromosome genes, including *ALAS2*, were also demethylated by decitabine. Therefore, we administered decitabine to the erythroid differentiation culture medium but found no improvement in the erythroid differentiation capacity of MT HPCs (data not shown). Next, we administered another DNA demethylating agent, AZA, at the erythroid differentiation stage (Figure 6A). The administration of AZA significantly improved the generation of CD235a⁺ erythroblasts in MT HPCs (Figure 6B and supplemental Figure 5E). Moreover, in *o*-dianisidine staining, erythroblasts differentiated from MT HPCs without AZA showed no positivity, whereas a considerable number of the same cells with AZA were positively stained (Figure 6C). We collected mRNA from AZA-treated and DMSO-control erythroblasts and performed cDNA sequencing of the *ALAS2* gene. AZA

reactivated the silent wild-type *ALAS2* allele in MT HPCs and the silent mutant *ALAS2* allele in active wild-type HPCs (WT HPCs), suggesting the demethylation of the *ALAS2* gene after AZA treatment (Figure 6D and supplemental Figure 5F). We also performed CFU assays with AZA treatment using iPSC-derived HPCs. There was no difference in the total colony counts between treated and control cells (Figure 6E). No erythroid colony and few mixed colonies were observed in MT HPCs with DMSO. On the other hand, erythroid and mixed colony formation was significantly improved by AZA treatment in MT HPCs, although it did not reach the levels seen in WT HPCs (Figure 6F-G). These results show that AZA treatment ameliorates the defective heme synthesis and ineffective erythropoiesis in MT HPCs.

Discussion

The majority of female XLSA patients exhibit a severe anemia phenotype, as observed in the patients presented in this study.^{5,30,31} This pathology is caused by an increase in erythroblasts expressing a markedly defective mutant *ALAS2* enzyme in the bone marrow because of the skewed inactivation of the X chromosome harboring the wild-type *ALAS2* gene in hematopoietic stem cells later in life. In such cases, few mutant erythroblasts in which mutant *ALAS2* is expressed differentiate into reticulocytes and reach circulation. Therefore, peripheral blood erythrocytes mainly represent descendant cells of the small number of clones with an active wild-type *ALAS2* allele.^{5,32} Consistent with these reports, both CD34⁺ and CD235a⁺ bone marrow cells from SA2 and SA3 showed preferential inactivation of the X chromosome carrying wild-type *ALAS2*. By contrast, peripheral blood erythrocytes from SA2 and SA3 were derived exclusively from a few erythroblasts expressing the wild-type *ALAS2* gene.

WT and MT iPSC lines have been established for several X-linked diseases in which X-chromosome inactivation is maintained after reprogramming.^{29,33,34} However, it remains unclear whether the same is true for XLSA. In the present study, we generated iPSC lines with an active mutant *ALAS2* allele and isogenic iPSC lines with an active wild-type *ALAS2* allele from the peripheral blood cells of three female patients with XLSA. All female XLSA-iPSC lines were found to have extremely skewed X-chromosome inactivation patterns and consistently expressed either the wild-type or mutant allele, but not both, of the *ALAS2* gene after erythroid differentiation.

Consistent with the severe ineffective erythropoiesis seen in our XLSA patients, MT HPCs were difficult to differentiate into erythroblasts in our feeder-free condition, because most of them died at an early stage of the erythroid differentiation. In a previous report, erythroblasts derived from XLSA-iPSCs exhibited ring sideroblasts by co-culturing with stromal cells.¹⁶ Therefore, we adopted the OP9 co-culture method to perform precise morphological and gene expression analyses of the MT erythroblasts. According to another report, the co-culture of adult CD34⁺ cells with OP9 feeder cells increases the proliferation ability of erythroid cells by delaying erythroid differentiation.³⁵ We therefore speculated that stromal cell co-culture maintains MT erythroblasts in a more immature state than the feeder-free condition, preventing their death as maturation proceeds. MT erythroblasts failed to synthesize heme and exhibited maturation defects with ring sideroblast formation in contrast to WT erythroblasts (Figure 7). RNA-seq revealed that MT erythroblasts showed decreased expression of genes associated with erythroid maturation, which is consistent with the immature morphology of MT

erythroblasts and female patient bone marrow erythroblasts. To the best of our knowledge, this is the first report to demonstrate the defective erythroid differentiation of *ALAS2*-affected HPCs from female XLSA patients with a heterozygous *ALAS2* missense mutation. A previous report of *ALAS2*-knockout murine embryonic stem cells showed that *ALAS2* deficiency does not interfere with the maturation of definitive erythroid cells.¹⁵ One explanation for this phenomenon could be the differences in mice and human patients with XLSA. In a previous study, male XLSA patient-derived iPSCs were able to differentiate into orthochromatic erythroblasts, and the erythroid maturation defect was not observed.¹⁶ Another study showed that human iPSC-derived erythroid progenitor (HiDEP) cells and XLSA patient bone marrow cells lacking the *ALAS2* enhancer region had increased expression levels of anti-apoptosis genes.¹⁸ However, our MT erythroblasts stopped at the early stage of erythroid differentiation and did not show the same expression profiles in RNA-seq (data not shown). Although the reason for the observed discrepancies is unclear, this may be because of the degree of *ALAS2* functional loss between our model and the previous models. Alternatively, sex differences could be a factor. Overall, our iPSC model may be a useful tool for understanding the roles of *ALAS2* and heme synthesis in maintaining the erythroid cell transcriptome and morphology associated with XLSA.

The responsiveness of patients with XLSA to pyridoxine supplementation varies considerably according to the nature and position of the amino acid substitution in *ALAS2*.¹¹ In our model, the supplementation of pharmacologic levels of pyridoxal hydrochloride or pyridoxine hydrochloride did not improve the erythroid differentiation capacity of MT HPCs, coinciding with the unresponsiveness to pyridoxine in this patient

(data not shown). This finding suggests that our iPSC-based platform is useful for precisely assessing pyridoxine sensitivity in XLSA patients with various *ALAS2* mutations.

Previous reports have shown that ALA restores heme synthesis by HiDEP cells and K562 cells.³⁶ Using our iPSC platform, we demonstrated that ALA not only restores heme synthesis but also ameliorates the erythroid differentiation efficiency of MT iPSC lines in vitro. However, the oral administration of ALA did not improve anemia in our patients, consistent with a reported case.¹² This may be due to the fact that the concentration of ALA in the bone marrow was not sufficient to improve the pathophysiology of MT cells. Drugs that increase the cellular uptake of ALA, especially in early erythroid cells, may improve the drug efficacy. In addition, a dose-escalation clinical study may be useful to assess the safety and efficacy of ALA in pyridoxine-resistant XLSA patients.

Lastly, we focused on the unusual disease mechanism of XLSA in females, which is caused by the acquired skewing of X-chromosome inactivation in hematopoietic stem cells. We speculated that the restoration of the expression of wild-type *ALAS2* in a considerable number of affected erythroblasts could potentially ameliorate disease phenotypes in female XLSA patients. This study provides the first proof-of-concept that the silent wild-type *ALAS2* allele in female subjects could be partially reactivated by the DNA demethylating agent AZA, which serves as a potential novel therapeutic approach for the treatment of refractory anemia in female XLSA patients (Figure 7). Although the demethylating effect of AZA is global and off-target side effects may occur in both hematopoietic and non-hematopoietic tissues, AZA is currently being used in clinical

practice to treat hematological malignancies with acceptable adverse effects. However, the efficacy of AZA in our XLSA model is limited, and further studies using DNA demethylating agents are needed for future therapeutic use.

In summary, this study provides novel biological and therapeutic insights into XLSA. Using a patient-specific iPSC platform, we showed that a severe loss-of-function *ALAS2* mutation could cause maturation defects in erythroid cells that resemble the phenotype of female XLSA. Furthermore, we revealed that the demethylating agent AZA ameliorates the disease phenotype of female XLSA by reactivating the silenced wild-type *ALAS2* allele. Considering the acceptable toxicity in some cancer patients, DNA demethylating agents may be promising new therapeutic candidates for female XLSA patients.

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Author Contributions

Y.M., K.C., and Y.Y. conceived and designed the research; Y.M. performed most of the experiments, analyzed the data, and wrote the manuscript; K.C. performed some experiments, analyzed the data, and wrote the manuscript; C.O. analyzed the RNA-seq data; M.Nishikawa, M.Narita, and A.I. provided essential experimental support; M.Y-M., K.N., and M.Nagao assisted with the hematopoietic cell staining; H.K. collected patient blood samples and advised on the experimental design; A.T-K. advised on the experimental design and wrote the manuscript; Y.Y. supervised the overall research and wrote the manuscript. All authors interpreted the data and reviewed and approved the final manuscript.

Disclosure of Conflicts of Interest

Y.M., K.C., H.K., A.T-K., and Y.Y. are the inventors of JP patent applications (JP2019/525414 and JP2020/218557). H.K. and A.T-K. received honoraria from Nippon Shinyaku outside the submitted work. A.T-K. and Y.Y. received grants from Nippon Shinyaku outside the submitted work.

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Table

Table 1. Clinical and hematological characteristics of the family members

Figure Legends

Figure 1. Identification of XLSA in a family harboring the heterozygous *ALAS2*-*R227C* mutation.

(A) Pedigree of the family. Squares indicate males, and circles indicate females. Closed circles denote patients confirmed using *ALAS2* sequencing. The arrow indicates the proband. (B) Sanger sequencing data of *ALAS2* genomic DNA from SA2 buccal cells (top) and *ALAS2* cDNA from SA2 peripheral blood erythrocytes (bottom) are shown. (C) Morphology of SA2 and SA3 bone marrow cells. An increased number of ring sideroblasts was observed based on Prussian blue staining (left). The arrows show ring sideroblasts. Megaloblastic change was detected by May Grunwald-Giemsa staining (middle and right). Magnification of the objective lens: $\times 100$ (left and middle) and $\times 40$ (right). Scale bars, 25 μm . (D) Summary of HUMARA assays of CD34⁺ and CD235a⁺ bone marrow cells and *ALAS2* cDNA Sanger sequencing of peripheral blood erythrocytes. The schematic diagram of erythropoiesis was created using BioRender.com.

Figure 2. Generation of XLSA patient-derived iPSCs and confirmation of X-chromosome inactivation.

(A) Schematic representation of the iPSC generation from PBMCs of XLSA female patients harboring a heterozygous *ALAS2* mutation. The figure was created using BioRender.com. (B) Summary of HUMARA assays of PBMCs from XLSA patients and the percentage of MT iPSC lines among established iPSCs. (C) Expression levels of *OCT3/4*, *SOX2*, and *NANOG* genes in PBMCs, one control iPSC line derived from a healthy donor, three WT iPSC lines, and four MT iPSC lines. Expression levels of PBMCs were set to 1. Each line was tested in three independent experiments. (D) HUMARA

assays of WT and MT iPSCs (left). The undigested control is shown at the bottom left. Representative immunofluorescence staining images for Hoechst expression and H3K27me3 expression in XLSA-patient derived iPSCs (right). Magnification of the objective lens: $\times 20$. Scale bars, 50 μm . **(E)** HUMARA assays of representative iPSC lines (WT1-iPSC3 and MT1-iPSC2) after 5, 20, and 30 passages and HPCs derived from the iPSC lines. cDNA Sanger sequencing data of erythroblasts derived from the iPSC lines are shown on the far right.

Figure 3. Impaired erythropoiesis in MT iPSCs.

(A) Colony formation assay on day 14 of hematopoietic differentiation. Each line was tested in three independent experiments. **(B)** Erythroid and mixed colony counts in **(A)**. **(C)** Representative images of erythroid (left) and mixed (right) colonies derived from WT1-iPSC2. Magnification of the objective lens: $\times 4$. Scale bars, 200 μm . **(D)** Erythroid and mixed colony counts in MT HPCs transduced with wild-type *ALAS2*. Each line was tested in three independent experiments. **(E)** Percentages of CD43⁺ hematopoietic cells. Each line was tested in three independent experiments. **(F)** Percentages of CD235a⁺ erythroblasts. Each line was tested in three independent experiments. All data are presented as the mean $\pm$ standard error of the mean (SEM). *P*-values were calculated using the unpaired, two-tailed Student's *t*-test. *****P* < 0.0001; N.S., not significant.

Figure 4. Maturation defects of erythroblasts derived from MT iPSCs.

(A) The pellets (left) and iron staining (right) of erythroblasts derived from WT1-iPSC2 and MT1-iPSC3. The arrow shows a ring sideroblast. CD235a⁺ cells were sorted on day 34 using FACS. Magnification of objective the lens: $\times 100$. Scale bars, 10 μm . **(B)** May

Grunwald-Giemsa staining of erythroblasts derived from WT1-iPSC2 (top) and MT1-iPSC4 (bottom). CD235a⁺ cells were sorted on day 34 using FACS. Magnification of the objective lens: $\times 100$. Scale bars, 25 μm . **(C)** Percentages of polychromatic megaloblasts (Poly-M), polychromatic erythroblasts (Poly-E), and orthochromatic erythroblasts (Ortho-E) from three WT and four MT iPSC lines. **(D)** The pellets (top) and photomicrographs (bottom) of *o*-dianisidine-stained erythroblasts derived from WT1-iPSC3 and MT1-iPSC3 (bottom). CD235a⁺ cells were sorted on day 34 using FACS. Magnification of the objective lens: $\times 10$. Scale bars, 200 μm . **(E)** The expression levels of *HBB*, *HBG*, and *HOI* genes in erythroblasts derived from one control iPSC line, three WT iPSC lines, and four MT iPSC lines. Each line was tested in three independent experiments. Expression levels were normalized to the level of *GAPDH*. **(F)** The expression levels of *HBB*, *HBG*, and *HOI* genes in WT1-iPSC1- and MT1-iPSC2-derived erythroblasts treated with DMSO or ALA relative to the expression levels of non-treated erythroblasts derived from a control iPSC line. Each line was tested in three independent experiments. Expression levels were normalized to the level of *GAPDH*. All data are presented as the mean $\pm$ SEM. *P*-values were calculated using the unpaired, two-tailed Student's *t*-test. ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; N.S., not significant.

Figure 5. Gene expression in erythroblasts derived from WT and MT iPSCs.

(A) Schema of the protocol for the erythroid differentiation from iPSCs and sample collection time points. Images of HPCs and OP9 cells were created using BioRender.com. **(B)** A heatmap of the sample-to-sample distance and hierarchical clustering of iPSCs, CD34⁺ cells, CD43⁺CD34⁺CD38⁻Lin⁻ cells, and erythroblasts derived from three WT and four MT iPSC lines with RNA-seq. **(C)** PCA of erythroblasts derived from the three

WT and four MT iPSC lines, bone marrow erythroblasts from a healthy donor and from SA3. **(D)** Two GO analyses showing molecular function terms enriched for WT erythroblasts (top) and MT erythroblasts (bottom). Differentially expressed genes between WT erythroblasts and MT erythroblasts were used. ($|\text{fold change}| > 2$, adjusted $P < 0.05$). **(E)** GSEA of the heme metabolism dataset showed an enrichment of WT erythroblasts. **(F)** A heatmap of 13 genes characteristically expressed at the orthochromatic stage in the erythroblasts described in (C). For WT1-erythroblasts and MT1-erythroblasts, the heatmap shows the average expression levels of the three and four lines, respectively.

Figure 6. Amelioration of heme synthesis failure and maturation defects of MT erythroblasts with AZA treatment.

(A) Schema of a protocol for erythroid differentiation with AZA administration. The image of sorted HPCs was created using BioRender.com. **(B)** Percentages of CD235a⁺ erythroblasts treated with DMSO or AZA. The generation of CD235a⁺ erythroblasts in MT HPCs was significantly improved after the administration of AZA, whereas the generation of CD235a⁺ erythroblasts in WT HPCs was unchanged. Each line was tested in three independent experiments. **(C)** The pellets of unstained (top left) and *o*-dianisidine-stained (top right) erythroblasts derived from MT1-iPSC2 treated with DMSO or AZA. *o*-dianisidine staining images of erythroblasts derived from WT1-iPSC1 (left) and MT1-iPSC2 (right) treated with DMSO or AZA. CD235a⁺ cells were sorted on day 34 using FACS. Magnification of the objective lens: $\times 20$. Scale bars, 100 μm . **(D)** Representative Sanger sequencing data of *ALAS2* cDNA from erythroblasts derived from WT iPSC (WT1-iPSC1) and MT iPSC (MT1-iPSC2) lines treated with

DMSO or AZA. **(E)** Colony formation assay on day 15 of hematopoietic differentiation in WT1-iPSC1- and MT1-iPSC2-derived HPCs treated with DMSO or AZA. Each line was tested in three independent experiments. AZA 100, 100 nM AZA; AZA 500, 500 nM AZA. **(F)** Erythroid and mixed colony counts in (E). **(G)** A representative image of mixed colonies derived from MT1-iPSC2 with AZA. Magnification of the objective lens: $\times 4$. Scale bars, 200 μm . All data are presented as the mean $\pm$ SEM. *P*-values were calculated using the one-way ANOVA with Tukey's correction and unpaired, two-tailed Student's *t*-test. **P* < 0.05; ***P* < 0.01; N.S., not significant.

Figure 7. Schematic representation of our results.

We established two types of iPSC lines derived from individual female XLSA patients harboring the *ALAS2-R227C* mutation. WT iPSCs showed mature erythroid differentiation, and MT iPSCs stopped at the immature erythroblast stage, recapitulating the pathogenesis of XLSA. AZA administration reactivated the silent wild-type *ALAS2* allele in MT HPCs and ameliorated erythroid differentiation defects. The figure was created using BioRender.com.

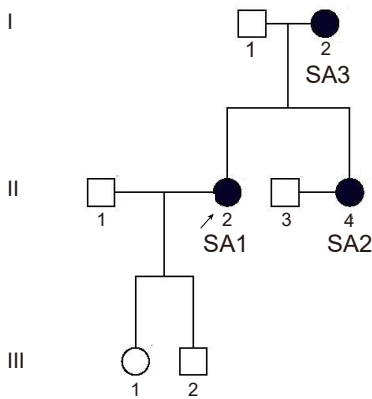
Table 1 Clinical and hematological characteristics of the family members

Case number	Age at diagnosis (years)	Hb (g/dl)	RBC ($\times 10^{12}/L$)	MCV (fL)	WBC ($\times 10^9/L$)	Platelets ($\times 10^9/L$)	Ferritin (ng/mL)	TS (%)	RS (%)	Karyotype	<i>ALAS2</i> mutation	Skewed XCI	RBC transfusion dependency
SA1 (Proband)	42	6.3	1.65	112.2	5.28	297	318	89.6	12	46,XX [20]	R227C	Yes	Yes
SA2 (Younger sister)	40	5.9	1.67	112	3.18	264	645.7	90.3	18	46,XX [20]	R227C	Yes	Yes
SA3 (Mother)	70	9.1	2.38	110.9	5.5	357	263.1	77.3	17	46,XX [20]	R227C	Yes	No

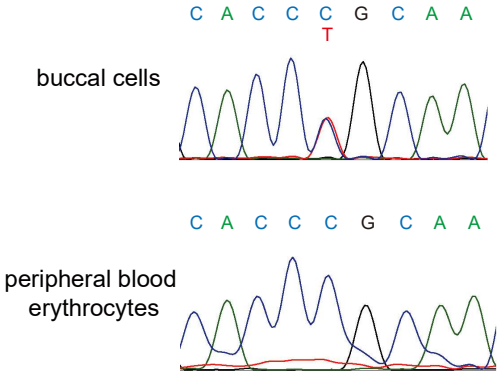
Abbreviations: TS = Transferrin saturation; RS = Ring sideroblasts; XCI = X chromosome inactivation.

Figure 1

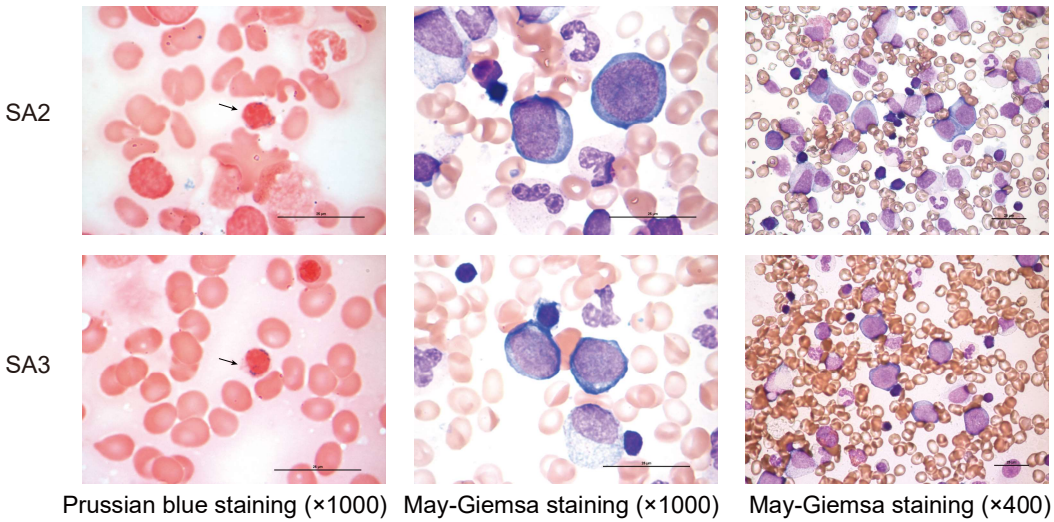
A



B



C



D

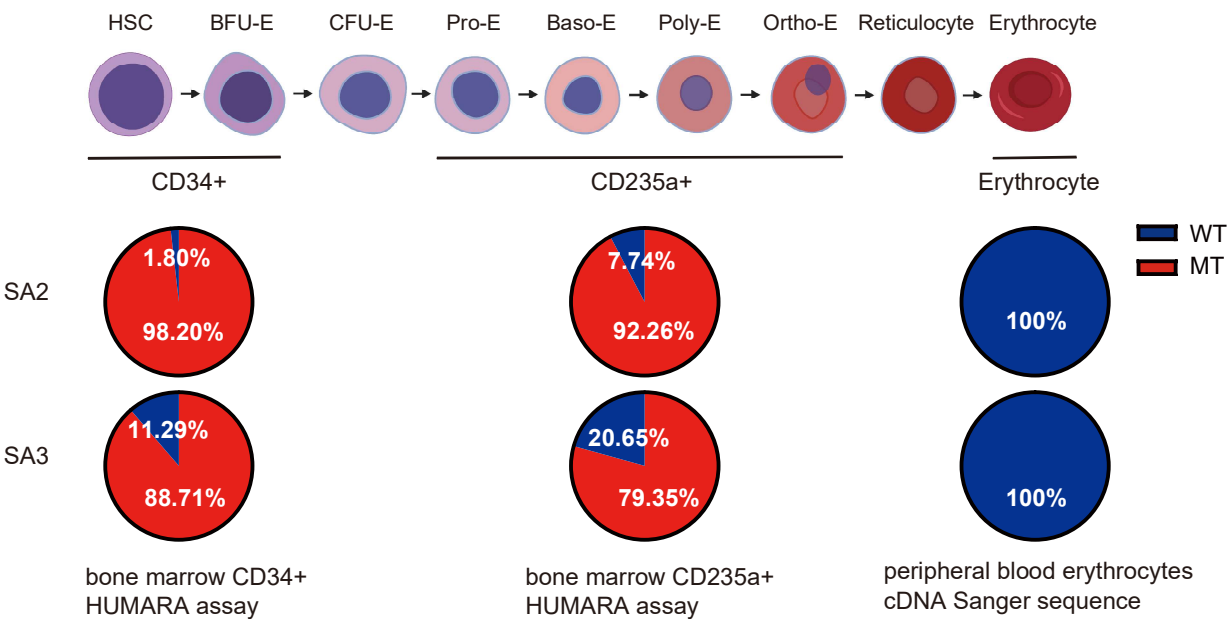
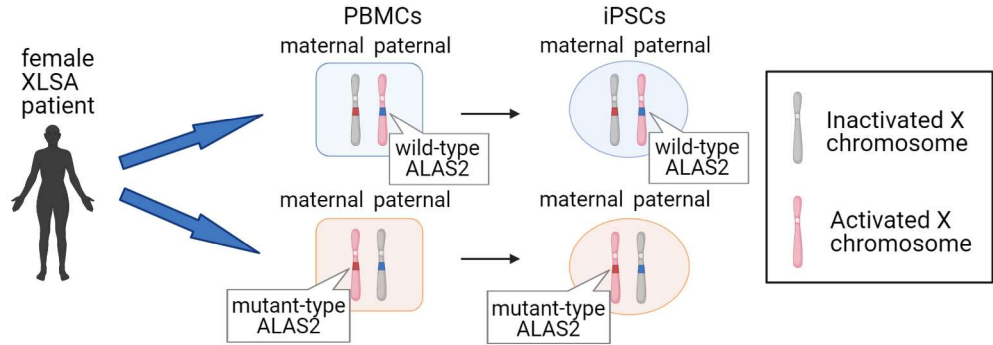
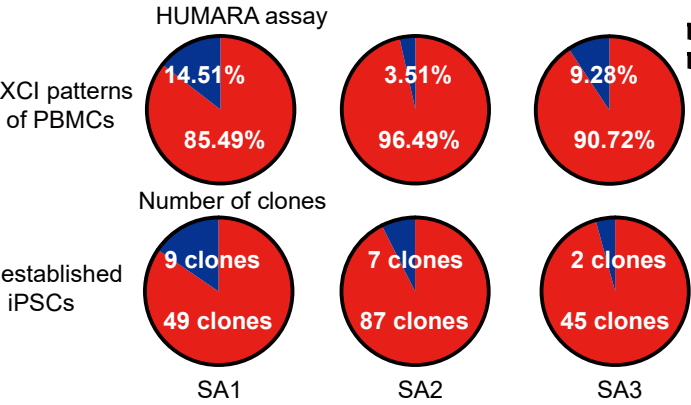


Figure 2

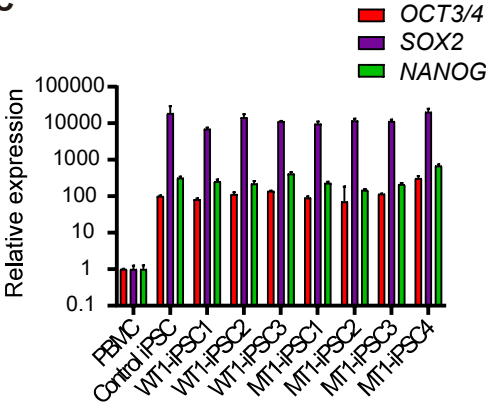
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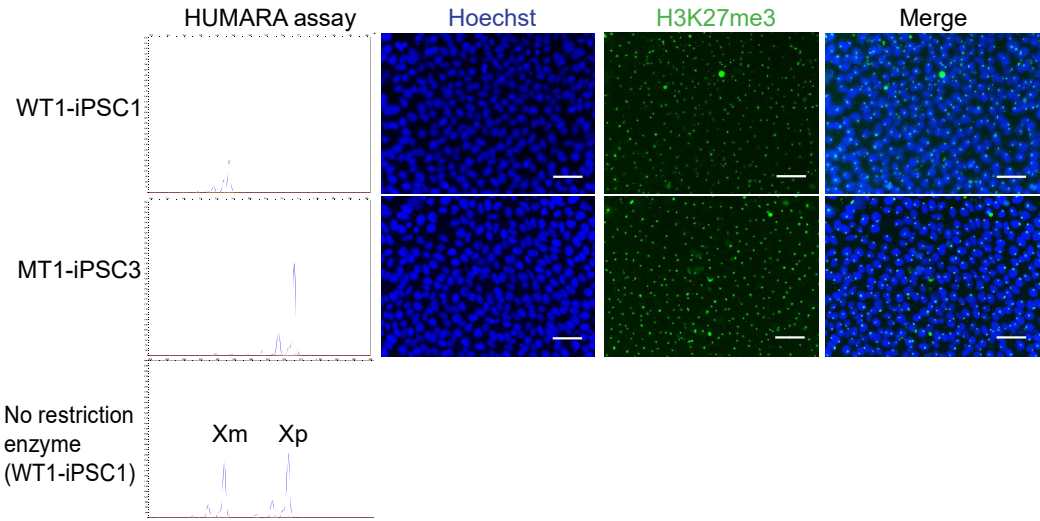
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E

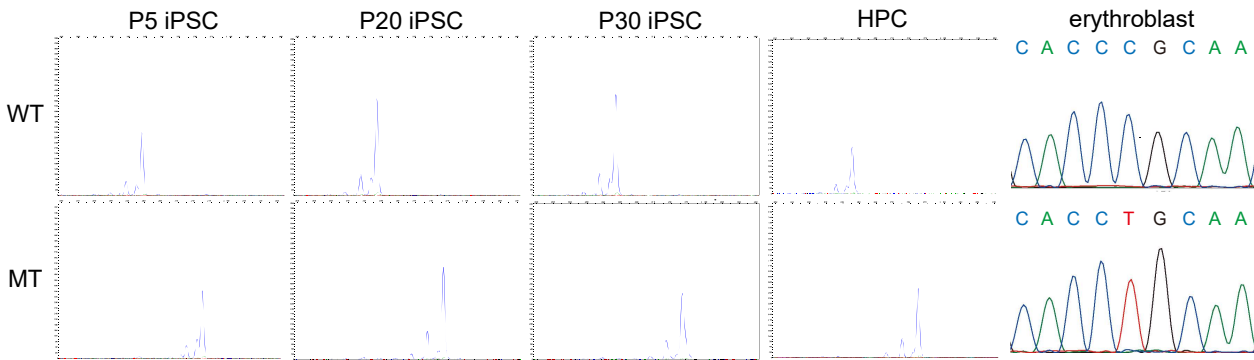
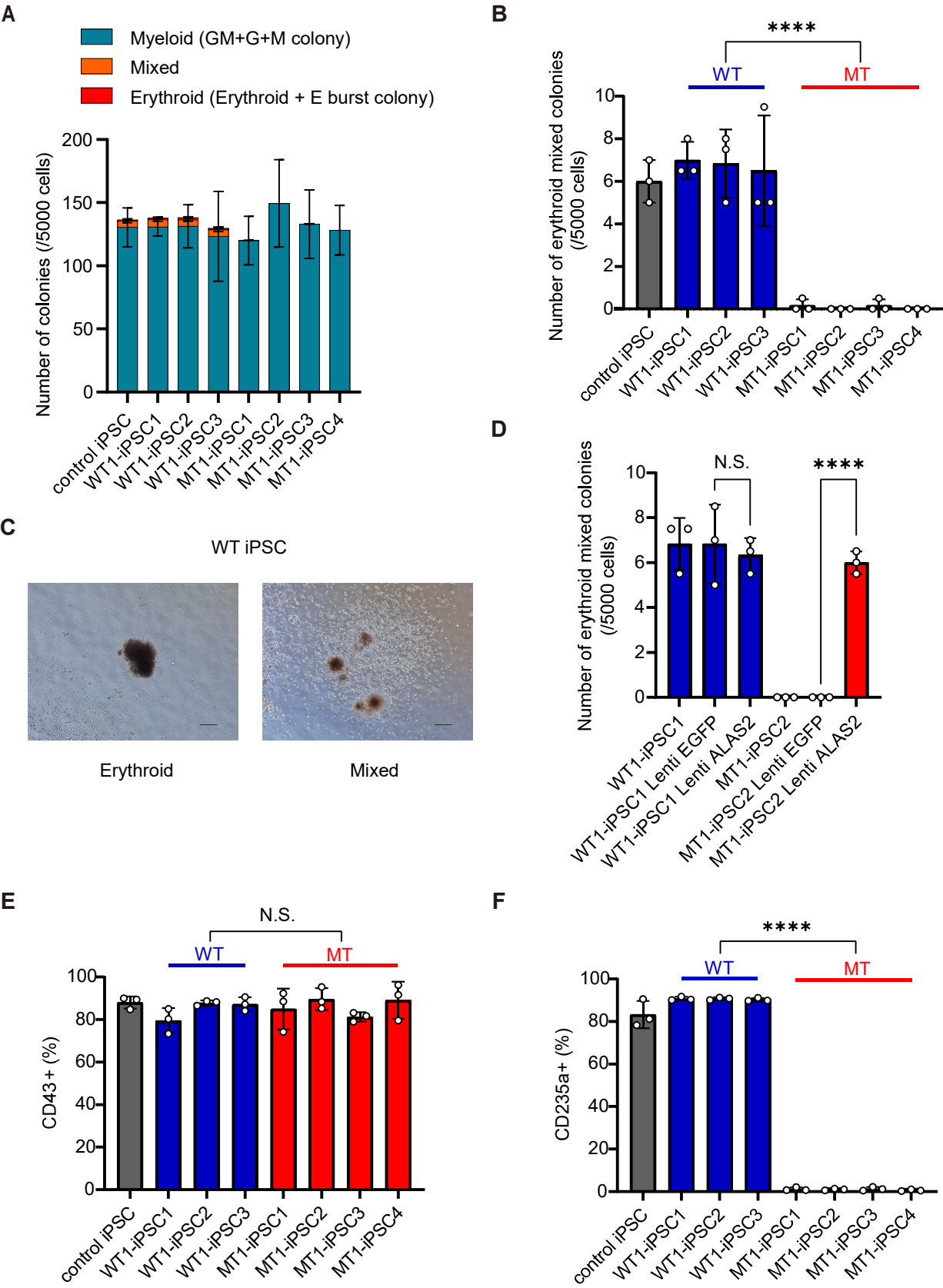


Figure 3



A

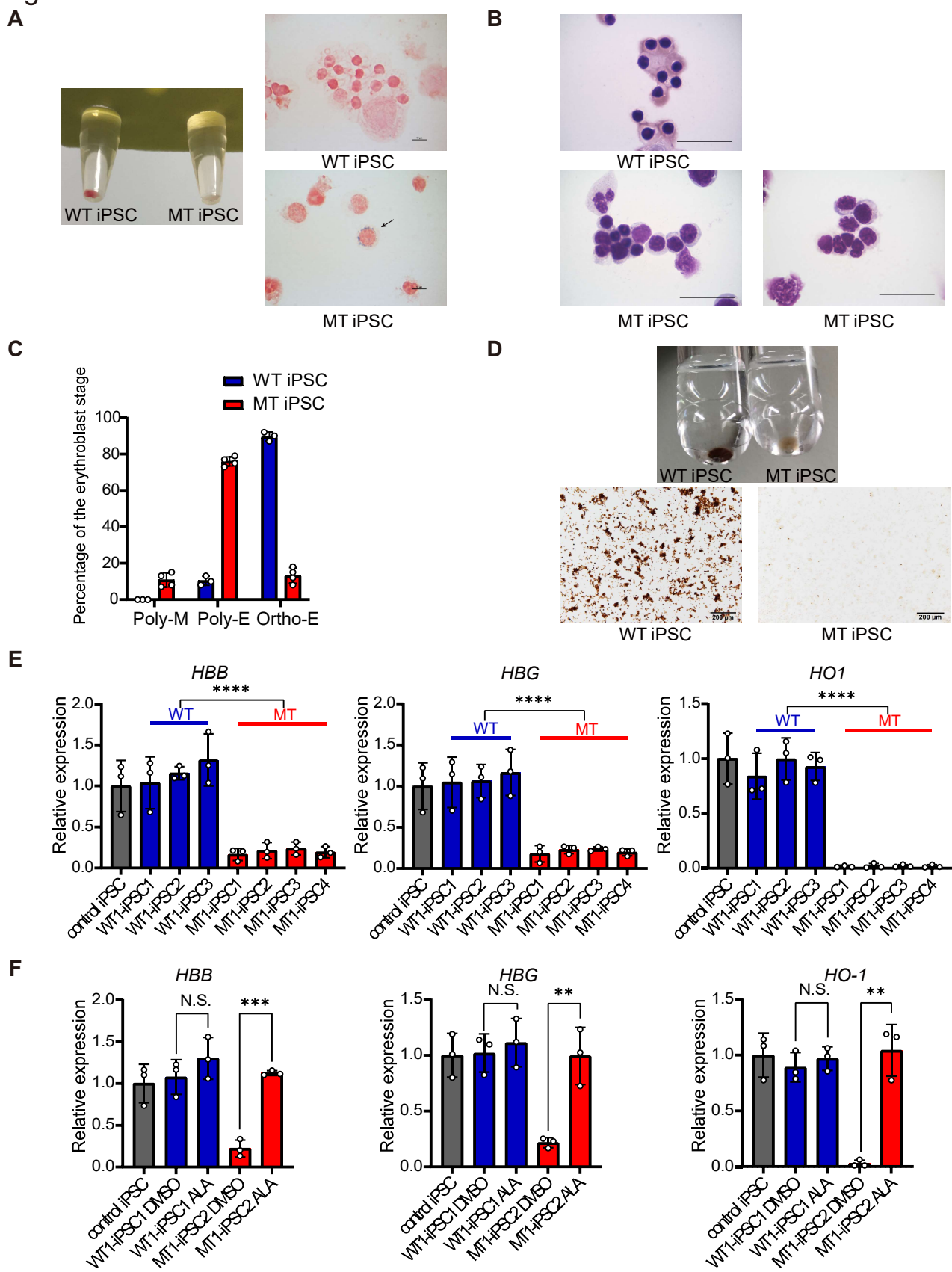
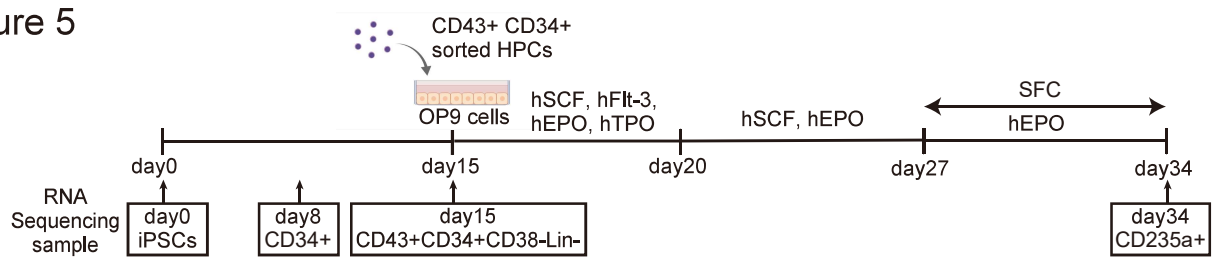
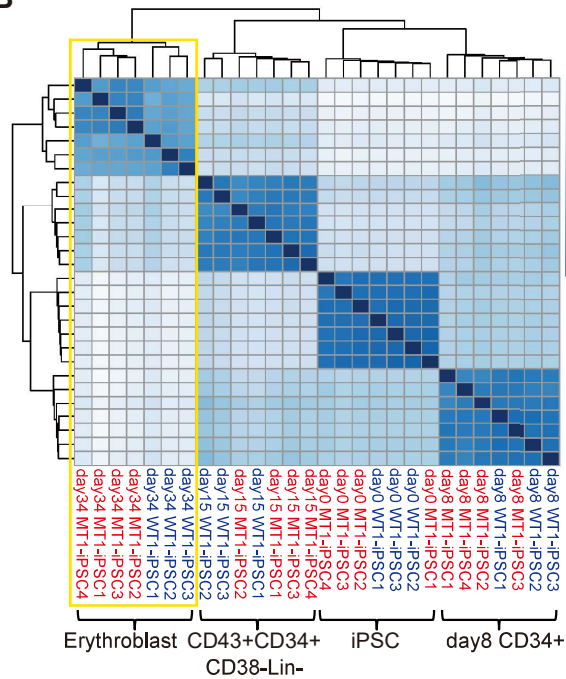


Figure 5

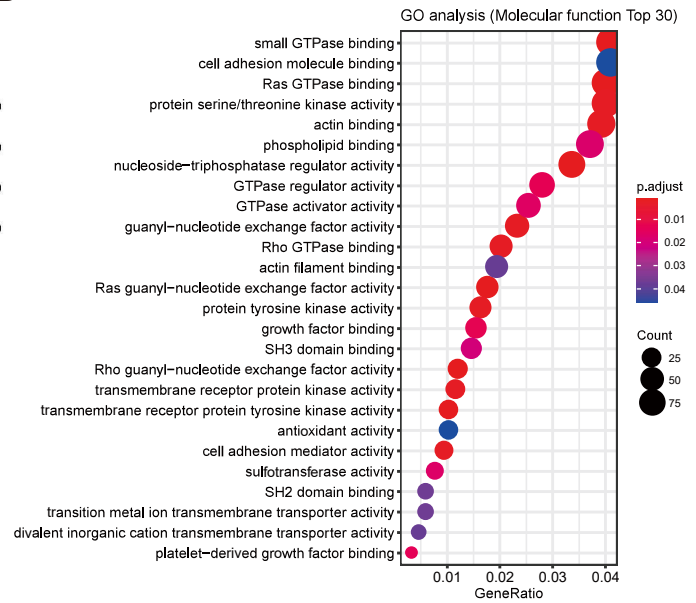
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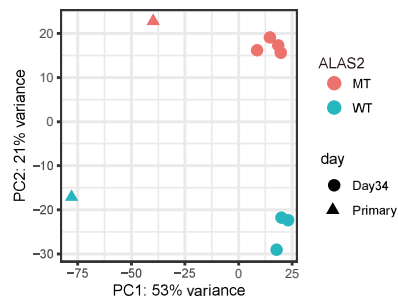
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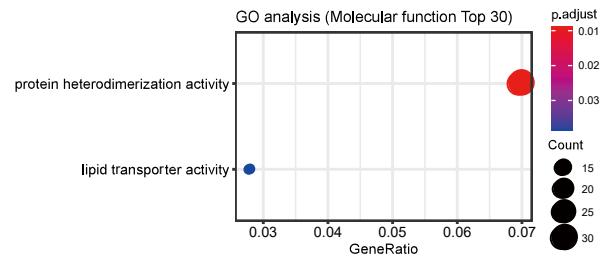
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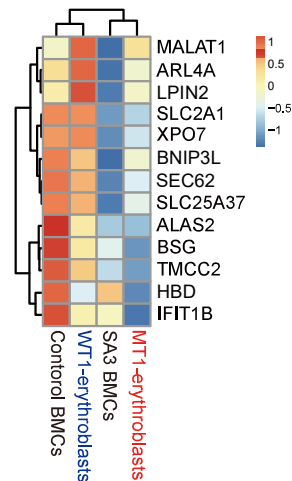
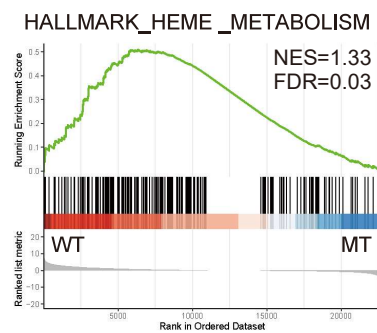


Figure 6

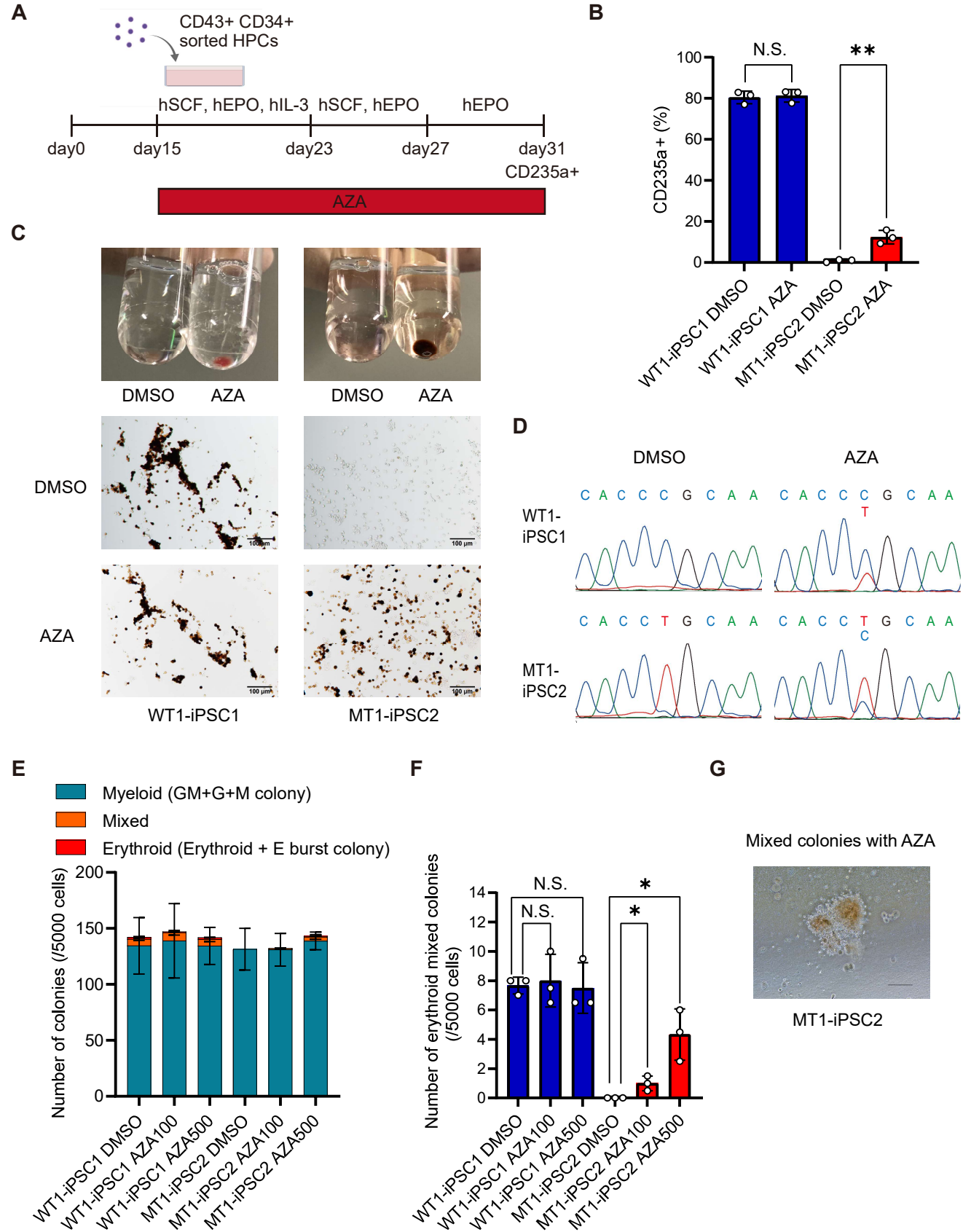
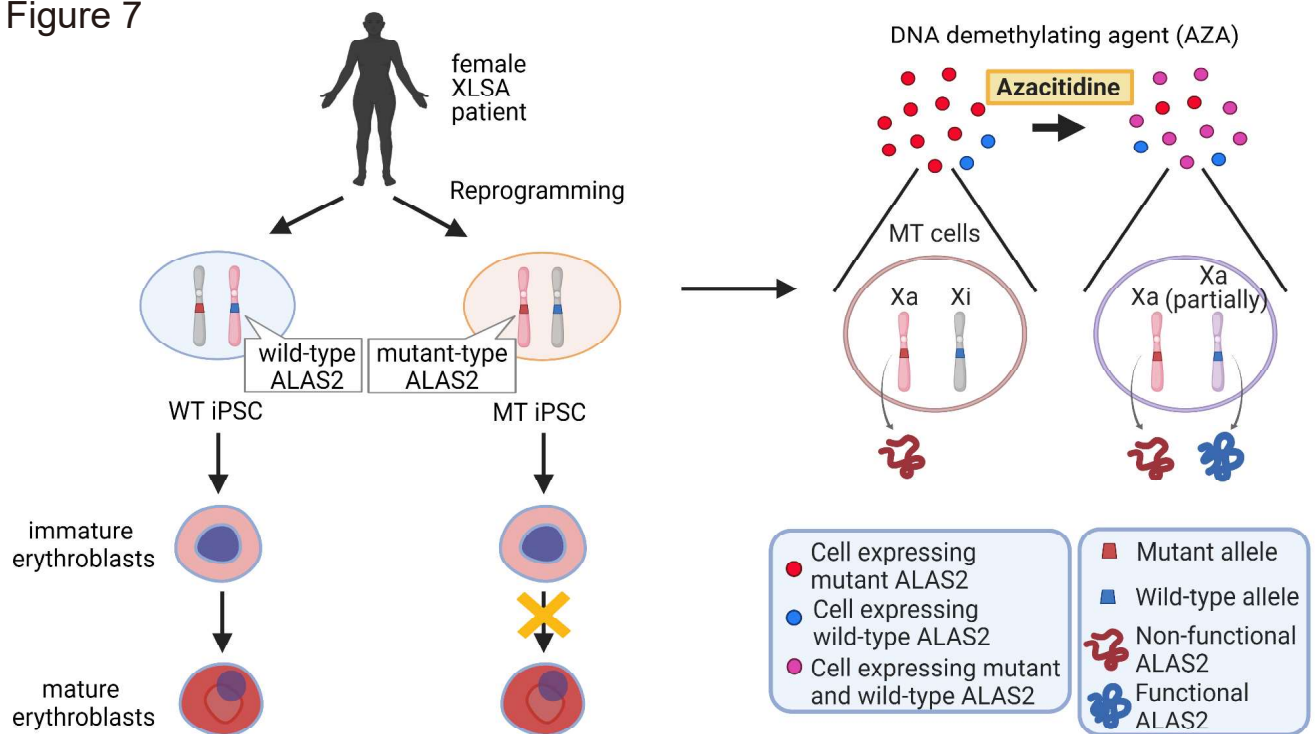


Figure 7



Supplemental Figure Legends

Supplemental Figure 1. Changes of hematological and iron parameters in SA1 during pyridoxal phosphate hydrate administration.

Supplemental Figure 2. Changes of hematological and iron parameters in SA1 and SA2 during ALA administration.

Arrows indicate RBC transfusion days.

Supplemental Figure 3. Characteristics of XLSA patients and patient-derived iPSCs.

(A) Representative immunofluorescence staining of SSEA-4 expression (top left) and TRA1-60 expression (top right) in MT1-iPSC3. The nuclei of all cells were stained with DAPI (bottom). Magnification of the objective lens: $\times 10$. Scale bars, 200 μm . (B) Histological analysis of teratomas derived from MT1-iPSC1. Teratomas had tissues of all three germ layers, including pigment epithelium (ectoderm, top), cartilage (mesoderm, middle), and gut-like epithelium (endoderm, bottom). Magnification of the objective lens: $\times 20$. Scale bars, 100 μm . (C) Karyotype analysis of all XLSA patient-derived iPSC lines showed no abnormalities (46, XX). Representative data for MT1-iPSC1 are shown.

Supplemental Figure 4. Hematopoietic and erythroid differentiation capacity of WT and MT iPSC lines.

(A) Colony formation assay on day 14 of WT1-iPSC1, MT1-iPSC2, and MT1-iPSC2 transduced with EGFP or wild-type *ALAS2*. Each line was tested in three independent experiments. (B) Percentages of CD235a⁺ erythroblasts derived from the SA3 iPSC lines. The data represent three independent experiments from two WT iPSC lines (WT3-iPSC1

and WT3-iPSC2) or two MT iPSC lines (MT3-iPSC1 and MT3-iPSC2). **(C)** Representative flow cytometry data showing the co-expression of CD235a and CD71 in erythroblasts differentiated using the feeder-free method. **(D)** Cell viability was assessed using the trypan blue exclusion test. Time course of erythroid differentiation of iPSC-derived HPCs using the feeder-free method. Each line was tested in three independent experiments. **(E)** Percentages of CD235a⁺ cells. Erythroblasts were differentiated from iPSC-derived HPCs using the OP9 co-culture method. Each line was tested in three independent experiments. **(F)** Cell viability was assessed using the trypan blue exclusion test. Time course of erythroid differentiation of iPSC-derived HPCs using the OP9 co-culture method is shown. Each line was tested in three independent experiments. **(G)** The expression level of *ALAS2* in iPSC-derived erythroblasts derived from one control iPSC line, three WT iPSC lines, and four MT iPSC lines is shown. Each line was tested in three independent experiments. The expression levels were normalized to that of *GAPDH*. All data are presented as the mean $\pm$ SEM. *P*-values were calculated using the unpaired, two-tailed Student's *t*-test. *****P* < 0.0001; N.S., not significant.

Supplemental Figure 5. Improvement of erythroid differentiation capacity with ALA and AZA treatment.

(A) Schema of the protocol for erythroid differentiation with ALA administration. **(B)** Percentages of CD235a⁺ cells treated with DMSO or ALA. Each line was tested in three independent experiments. **(C)** Representative flow cytometry data of erythroblasts differentiated from MT1-iPSC2 using the feeder-free erythroid differentiation method with DMSO or ALA. **(D)** Pellets of erythroblasts derived from MT1-iPSC2. CD235a⁺ cells were sorted using FACS on day 34. **(E)** Representative flow cytometry data of

erythroblasts differentiated from MT1-iPSC2 using the feeder-free erythroid differentiation method with DMSO or AZA. **(F)** Representative Sanger sequencing data of *ALAS2* cDNA from erythroblasts derived from MT1-iPSC1 treated with DMSO or AZA using the OP9 co-culture method. All data are presented as the mean $\pm$ SEM. *P*-values were calculated using the unpaired, two-tailed Student's *t*-test. **P* < 0.05; *****P* < 0.0001.

Supplemental Methods

Maintenance of iPSC lines

The maintenance of all iPSC lines was performed on STO feeder cells in primate ES cell medium (ReproCell) supplemented with 4 ng/mL recombinant bFGF (Wako), as previously reported.¹ The iPSC lines were differentiated after culturing on Matrigel-coated plates for one passage to remove feeder cells.

Lentiviral transduction

Wild-type *ALAS2* cDNA was produced from bone marrow cells from a healthy donor using the following primers: forward, 5'-CACCATGGTGACTGCAGCCATG-3', and reverse, 5'-TCAGGCATAGGTGGTGAC-3', and subcloned into the pENTR-D-TOPO vector (Thermo Fisher Scientific). *ALAS2* was transferred from the entry clone into the pLenti6.3/TO/V5-DEST destination vectors (Thermo Fisher Scientific) using a gateway LR reaction, according to the manufacturer's instructions. FACS-sorted HPCs on day 15 were transfected lentivirally using wild-type *ALAS2* and a RetroNectin Plate (Takara) according to the manufacturer's instructions.

CFU assay

The CFU assay was performed based on a previous report.² Lineage assignment was determined by morphologic analysis, and some typical colonies (especially erythroid and mixed colonies) were picked up and assessed using May Grunwald-Giemsa staining.

Flow cytometry analysis and cell sorting

For the flow cytometry analysis or sorting of iPSC-derived hematopoietic cells, EBs were

dissociated using 0.25% trypsin followed by pipetting 10 to 20 times. The cells were subjected to flow cytometry analysis or cell sorting using FACS ARIA II (Becton-Dickinson). Dead cells were removed using 7-AAD (BD Pharmingen).

Antibodies

The antibodies CD34-PE (BD Biosciences, 348057), CD43-FITC (BD Biosciences, 555475), CD38-APC (BD Biosciences, 555462), Human Hematopoietic Lineage APC cocktail (eBioscience, 22-7776-72), CD235a-APC (BD Bioscience, 551336), CD71-FITC (BD Biosciences, 555536), and TRA1-60-PE (Stemgent, 09-0009) were used for FACS analysis and cell sorting. Glycophorin A MicroBeads, human (Miltenyi Biotec, 130-050-501) and CD34 MicroBead Kit, human (Miltenyi Biotec, 130-046-702) were used for the MACS. Histone H3K27me3 antibody (mAb) (Active Motif, 61017), Alexa Fluor 488 Mouse anti-Human TRA-1-60 Antigen (BD Biosciences, 560173), and Alexa Fluor 488 Mouse anti-SSEA-4 (BD Biosciences, 560308) were used for immunostaining.

RNA extraction and quantitative RT-PCR analysis

The cells were eluted and homogenized using QIAzol (Qiagen). RNA was extracted using the miRNeasy Micro Kit (Qiagen) following the manufacturer's protocol, and cDNA synthesis was performed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). The expression level of each gene was determined using TB Green Premix Ex Taq II (Takara) and the StepOne Real-Time PCR System (Applied Biosystems). Data were normalized to the expression level of GAPDH. The primers for the analysis are listed in supplemental Table 2.

Immunostaining

Immunostaining of H3K27me3, TRA-1-60, and SSEA-4 was performed using standard immunostaining protocols, as previously reported.³

May Grunwald-Giemsa staining

May Grunwald-Giemsa staining was performed using May-Grunwald stain solution (Wako) and Giemsa solution (Wako) following the manufacturer's instructions.

Prussian blue staining

Prussian blue staining was performed using the Fe stain kit (Muto Pure Chemicals Co., Ltd.) following the manufacturer's instructions.

***o*-Dianisidine staining**

o-Dianisidine staining was performed in 0.01 M sodium acetate, 0.65% H₂O₂, 40% ethanol, and 0.6 mg/mL *o*-dianisidine (Sigma) for 15 minutes.⁴

RNA-seq

RNA was extracted from TRA1-60+ iPSCs, CD34+ cells on day 8, CD43+CD34+CD38-Lin- cells on day 15, and CD235a+ erythroblasts on day 34 from three WT and four MT iPSC lines previously sorted using FACS. RNA was also extracted from MACS-sorted CD235a+ primary erythroblasts in patient bone marrow mononuclear cells isolated using Ficoll-Paque Plus (GE Healthcare) and healthy donor bone marrow mononuclear cells (Lonza). The library was constructed using the TruSeq Stranded Total RNA with the RiboZero Gold LT Sample Prep Kit, Sets A and B (Illumina), according to the manufacturer's

instructions. Sequencing was performed using the NextSeq 500/550 High Output Kit v2 (75 cycles) (Illumina). Reads of the adapter sequences were trimmed using cutadapt-1.15.⁵ Removal of the reads mapped to ribosomal RNA-seq was performed using Bowtie2 and Samtools.^{6,7} The reads were mapped to the human genome (GRCh38 from the UCSC Genome Browser) using STAR (version 2.5.4a), and RSeQC (version 2.6.4) was used for quality check.^{8,9} HTSeq (version 0.9.1) was used to count the reads with the GENCODE annotation file (version 27), and the counts were normalized using DESeq2 (version 1.24.0) in R (version 3.6.1).¹⁰⁻¹² We performed PCA and the Wald test using the DESeq2 package and GO analysis and GSEA using the clusterProfiler (version 3.12.0) and enrichplot (version 1.6.1) packages.^{13,14}

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Supplemental Tables

Supplemental Table 1. Human iPSC lines used in the differentiation experiments

Supplemental Table 2. List of primers used for RT-PCR

Supplemental Table 3. Results of GSEA (p.adjust < 0.05)

Supplemental Table 1 **Human iPSC lines used in the differentiation experiments**

ID	Name of iPSC lines	Source			HUMARA assay of iPSCs	sequencing of erythroblasts' cDNA	Teratoma formation	Karyotype	Pluripotent markers	Erythroid differentiation
		Patient	Origin	T / Non T cell						
CiRA01059A	WT1-iPSC1	SA1	PBMC	T	WT	WT	Yes	46, XX	Yes	Yes
CiRA01059B	WT1-iPSC2	SA1	PBMC	T	WT	WT	Yes	46, XX	Yes	Yes
CiRA01059C	WT1-iPSC3	SA1	PBMC	T	WT	WT	Yes	46, XX	Yes	Yes
CiRA01059D	MT1-iPSC1	SA1	PBMC	T	MT	MT	Yes	46, XX	Yes	Yes
CiRA01059E	MT1-iPSC2	SA1	PBMC	T	MT	MT	Yes	46, XX	Yes	Yes
CiRA01059E	MT1-iPSC3	SA1	PBMC	T	MT	MT	Yes	46, XX	Yes	Yes
CiRA01059F	MT1-iPSC4	SA1	PBMC	Non T	MT	MT	Yes	46, XX	Yes	Yes
CiRA01061A	WT3-iPSC1	SA3	PBMC	Non T	WT	N/A	Yes	46, XX	N/A	Yes
CiRA01061B	WT3-iPSC2	SA3	PBMC	Non T	WT	N/A	Yes	46, XX	N/A	Yes
CiRA01061C	MT3-iPSC1	SA3	PBMC	Non T	MT	N/A	Yes	46, XX	N/A	Yes
CiRA01061D	MT3-iPSC2	SA3	PBMC	T	MT	N/A	Yes	46, XX	N/A	Yes

Abbreviations and explanations: WT, expressing active wild-type allele; MT, expressing active mutant allele; N/A, not applicable

Supplemental Table 2

List of primers used for RT-PCR

Genes		Sequences (5'-3')
HBB	Forward	TGTCCACTCCTGATGCTGTTATGG
	Reverse	AGCTTAGTGATACTTGTGGGCCAG
HBG	Forward	CGCTTCTGGAAACGTCTGAGGTTAT
	Reverse	CCAGGAGCTTGAAAGTTCTCAGGAT
HO-1	Forward	CTCAAAACCTCCAAAAGC
	Reverse	TCAAAAACCAACCCCAACCC
ALAS2	Forward	CTGCCAGGGTGGGAGATT
	Reverse	TTGGCTGCTCCACTGTTACG
GAPDH	Forward	ACGAAATTTGGCTACAGCAAC
	Reverse	CAGTGAGGGTCTCTCTCTTC
OCT3/4	Forward	CCCCAGGGCCCCCATTTTGGTACC
	Reverse	ACCTCAGTTTGAATGCATGGGAGAGC
SOX2	Forward	TTCACATGTCCCCAGCACTACCAGA
	Reverse	TCACATGTGTGAGAGGGGCAGTGTGC
NANOG	Forward	AGTGCAGTGGCGGGTCTTGGCTCAC
	Reverse	AGGCGGCGGATCACAAAGTCAGGAG

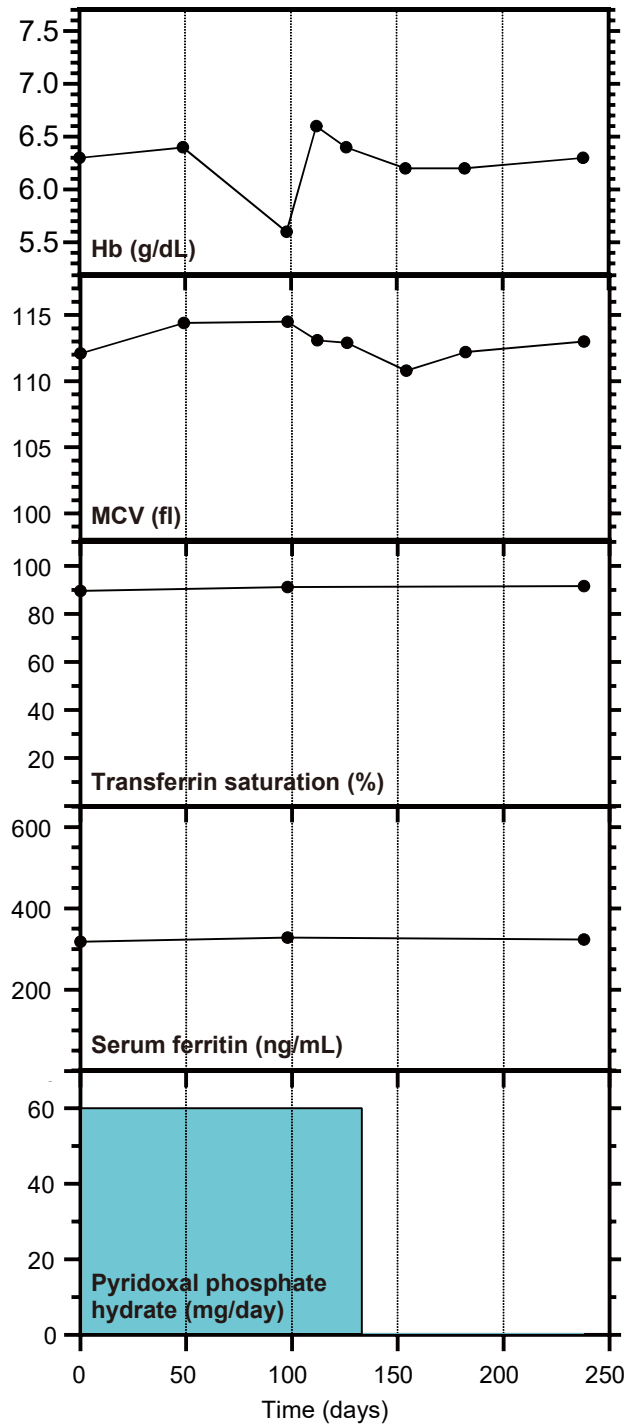
Supplemental Table 3

result of GSEA (p.adjust < 0.05)

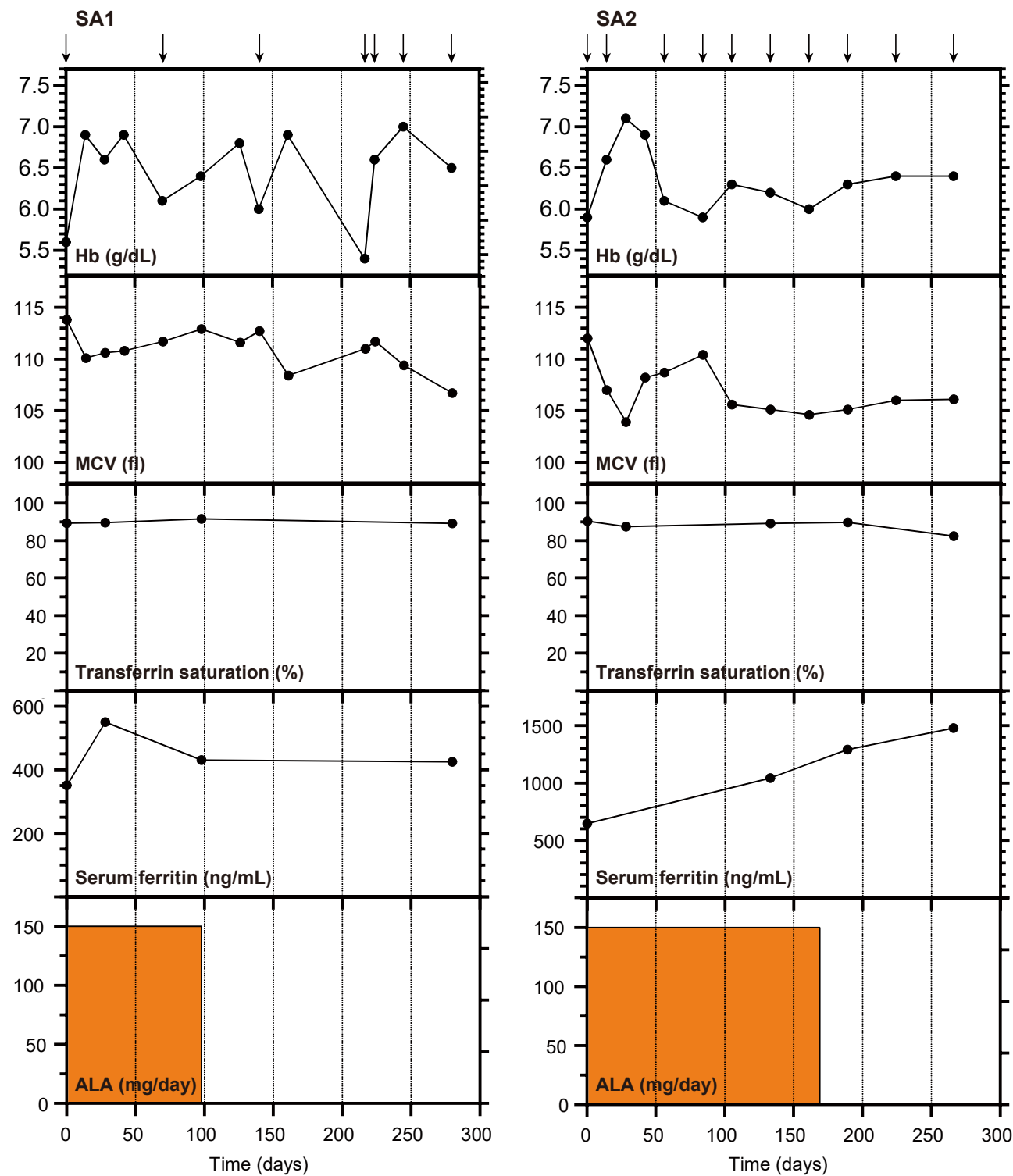
ID	Set size	Enrichment score	NES	p value	p.adjust	q values
HALLMARK_COAGULATION	137	0.590111815	1.525603	0.001015	0.033738	0.028411
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	200	0.534257692	1.398473	0.002006	0.033738	0.028411
HALLMARK_UV_RESPONSE_DN	144	0.554095302	1.438537	0.002024	0.033738	0.028411
HALLMARK_HYPOXIA	200	0.519666952	1.36028	0.003009	0.037613	0.031674
HALLMARK_HEME_METABOLISM	199	0.508982159	1.332112	0.004012	0.04012	0.033786
HALLMARK_IL2_STAT5_SIGNALING	198	0.502152014	1.314011	0.005015	0.041792	0.035193

Supplemental Figure 1

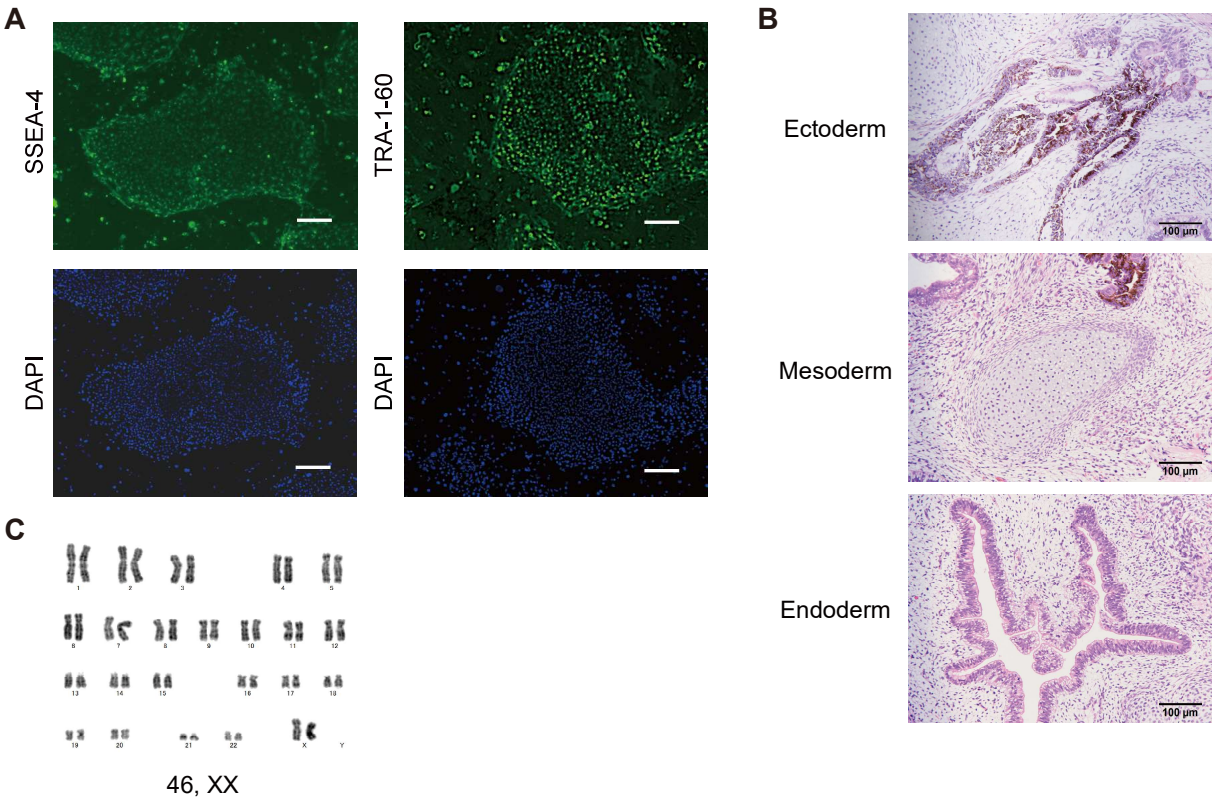
SA1



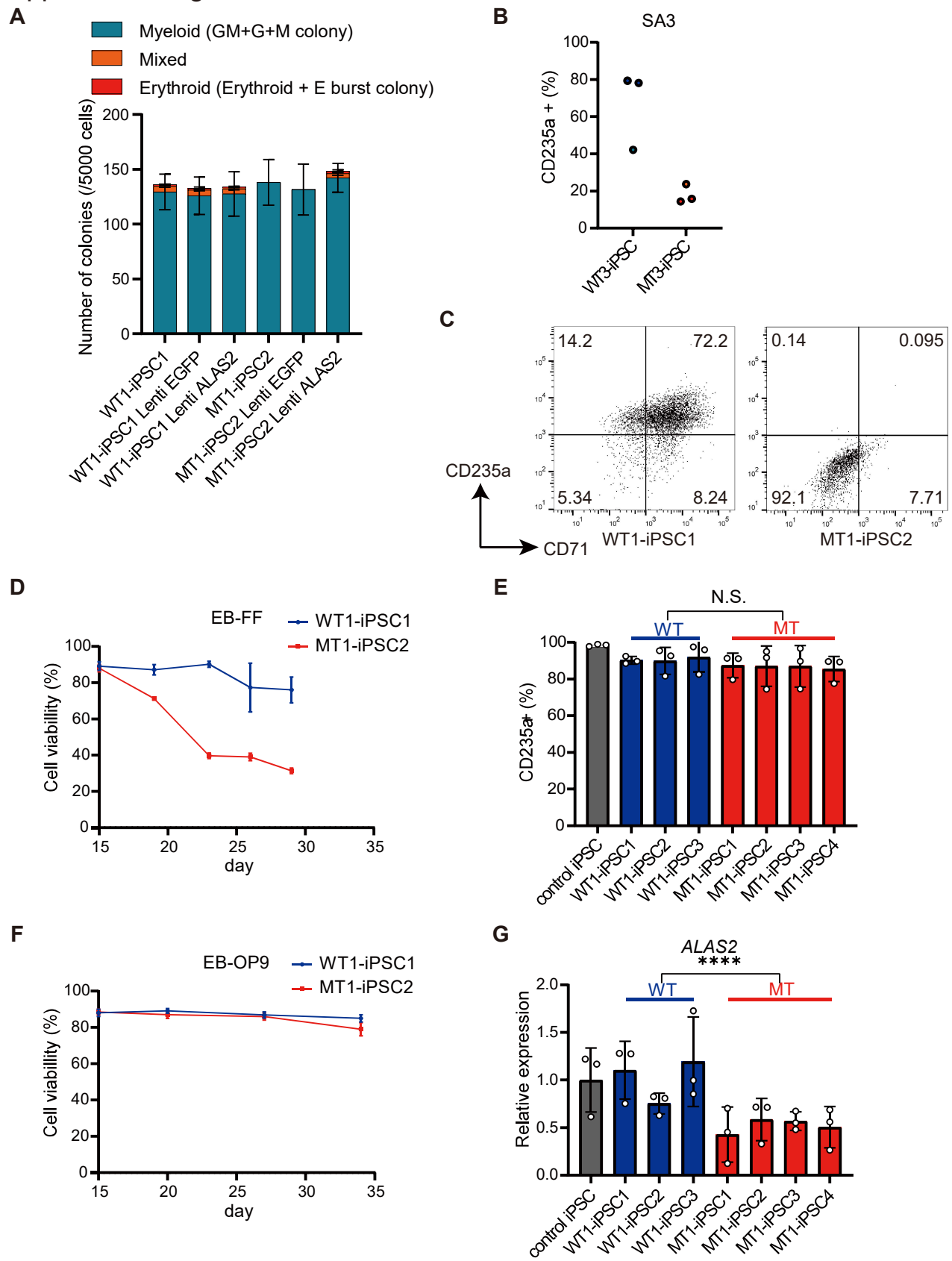
Supplemental Figure 2



Supplemental Figure 3

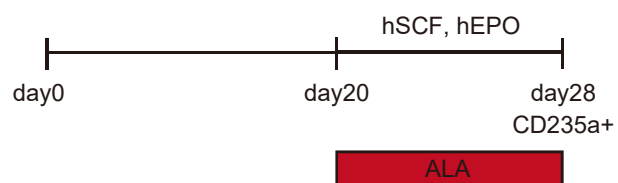


Supplemental Figure 4

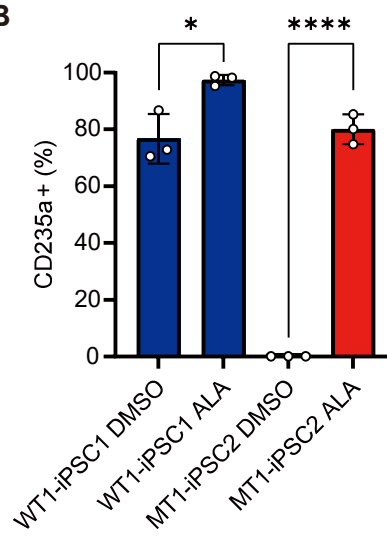


Supplemental Figure 5

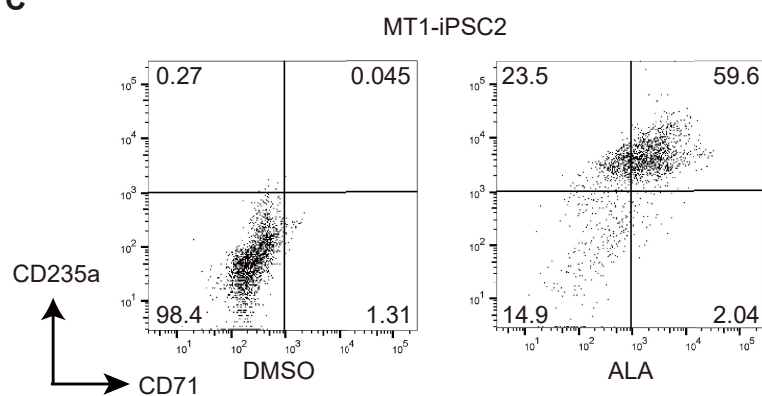
A



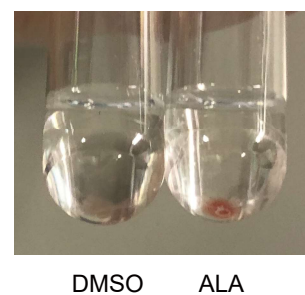
B



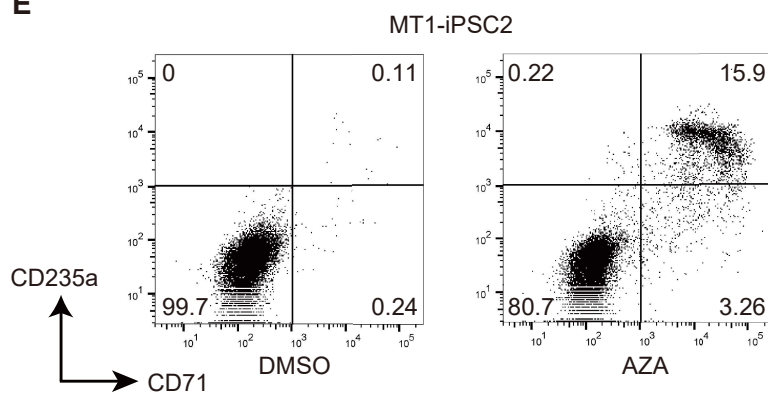
C



D



E



F

