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% H_2O_2, 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 Ribo-Zero 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]

References

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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	CGCTTCTGGAACGTCTGAGGTTAT
	Reverse	CCAGGAGCTTGAAGTTCTCAGGAT
HO-1	Forward	CTCAAACCTCCAAAAGC
	Reverse	TCAAAAACCACCCCAACCC
ALAS2	Forward	CTGCCAGGGTGCGAGATT
	Reverse	TTGGCTGCTCCACTGTTACG
GAPDH	Forward	ACGAATTTGGCTACAGCAAC
	Reverse	CAGTGAGGGTCTCTCTCTTC
OCT3/4	Forward	CCCCAGGGCCCCATTTTGGTACC
	Reverse	ACCTCAGTTTGAATGCATGGGAGAGC
SOX2	Forward	TTCACATGTCCCAGCACTACCAGA
	Reverse	TCACATGTGTGAGAGGGGCAGTGTGC
NANOG	Forward	AGTGCAGTGGCGCGGTCTTGGCTCAC
	Reverse	AGGCGGGCGGATCACAAGGTCAGGAG

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 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: ×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: ×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

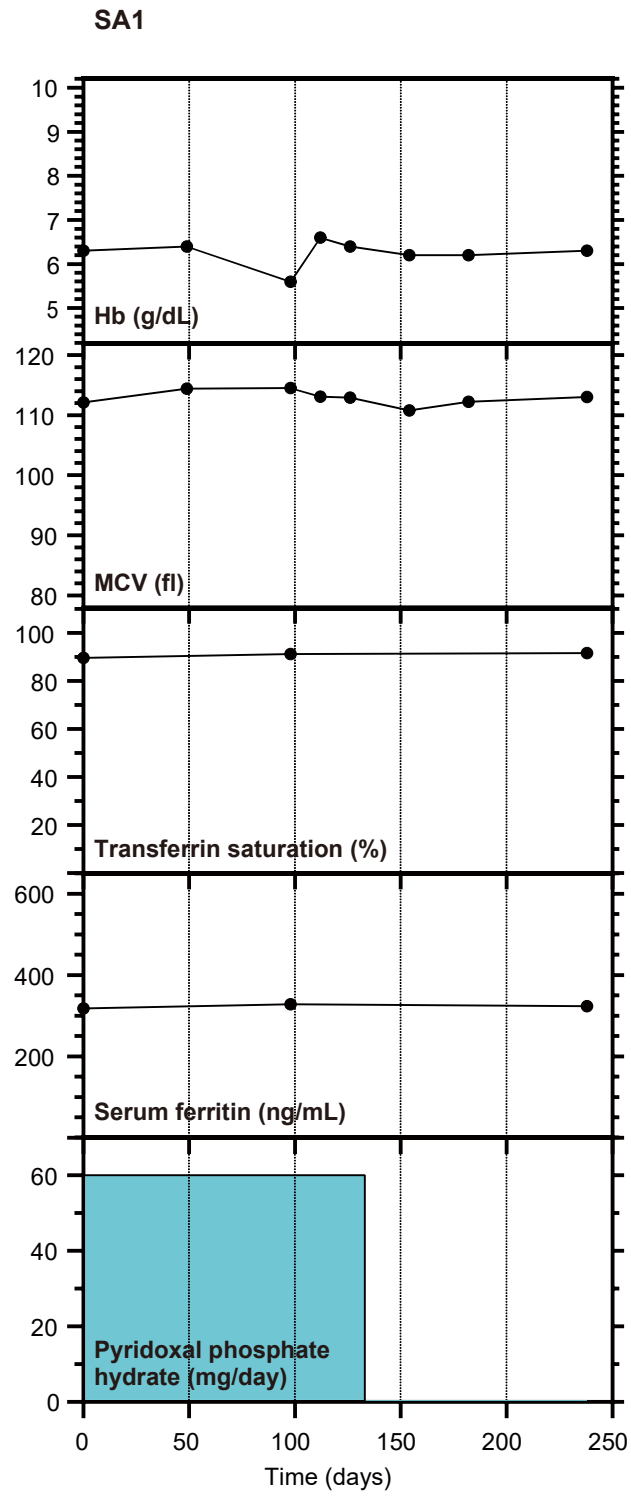
and WT3-iPSC2) or two MT iPSC lines (MT3-iPSC1 and MT3-iPSC2). **(C)** Representative flow cytometry data showing the co-expression of CD235a and CD71 in erythroblasts differentiated using the feeder-free method. **(D)** Cell viability was assessed using the trypan blue exclusion test. Time course of 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 ± 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.

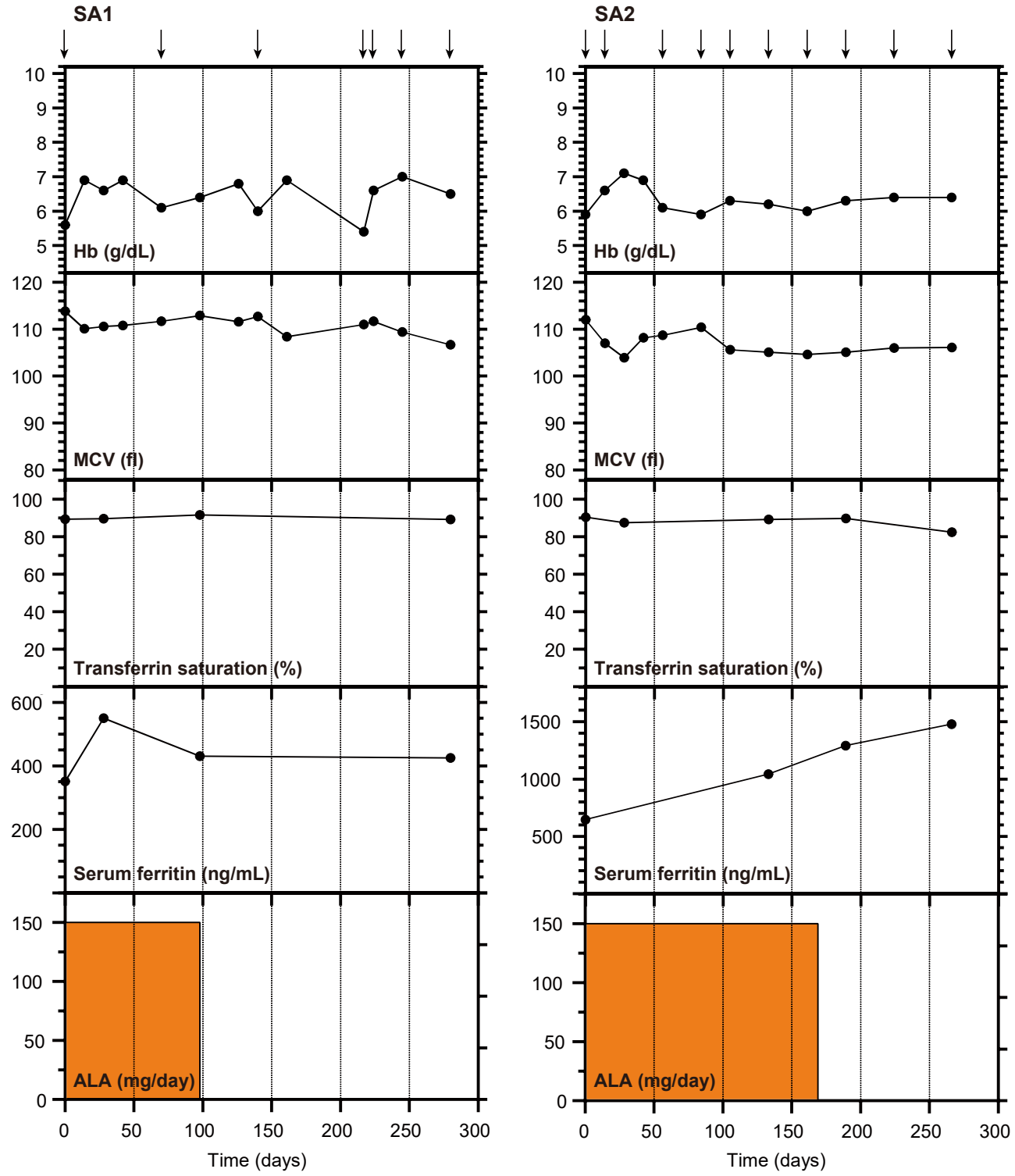
(A) Schema of the protocol for erythroid differentiation with ALA administration. (B) Percentages of CD235a+ cells treated with DMSO or ALA. Each line was tested in three independent experiments. **(C)** Representative flow cytometry data of erythroblasts differentiated from MT1-iPSC2 using the feeder-free 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 ± SEM. *P*-values were calculated using the unpaired, two-tailed Student's *t*-test. **P* < 0.05; *****P* < 0.0001.

Supplemental Figure 1



Supplemental Figure 2



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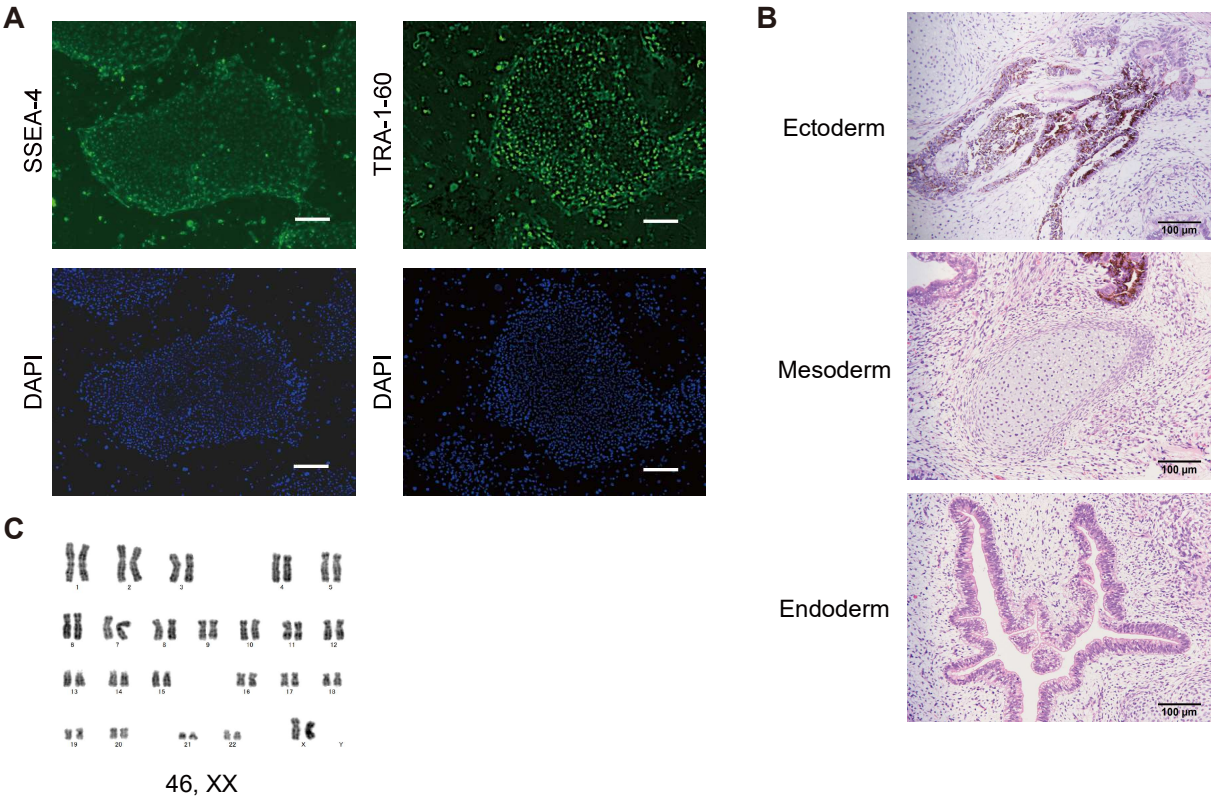
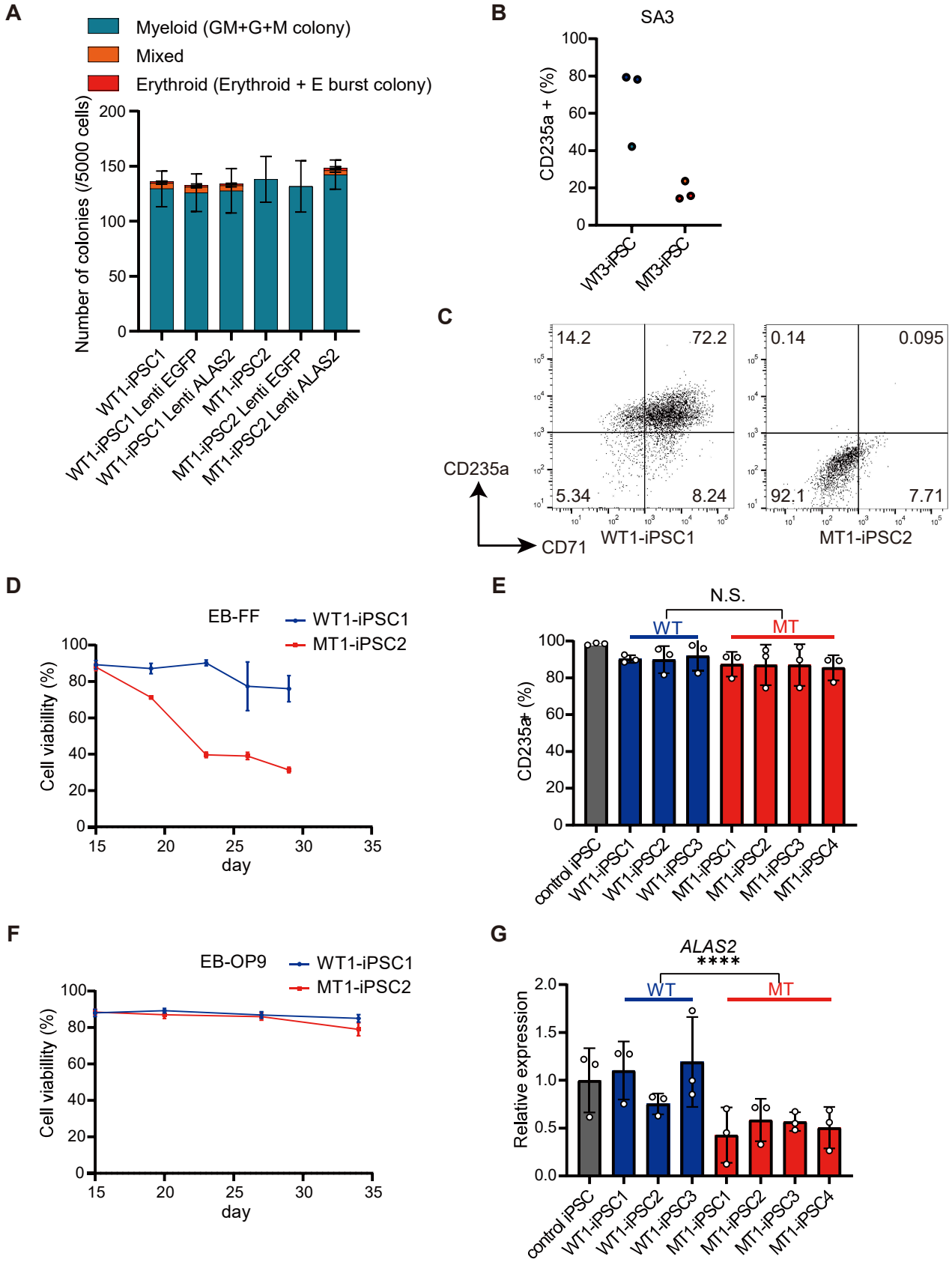


Supplemental Figure 4



Supplemental Figure 5