Efficacy estimation of erythropoiesis-stimulating agents using erythropoietin-deficient anemic mice

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Supplementary Appendix

"Efficacy estimation of erythropoiesis-stimulating agents using erythropoietin-deficiency anemic mice" by Norio Suzuki, et al.

Methods

Mice

Inherited super anemia mouse/mice (ISAM, $Epo^{GFP/GFP}$:$Tg^{3.3K\text{-}EpoE}$ genotype) and ISAM-REC mice ($Epo^{GFP/GFP}$:$Tg^{3.3K\text{-}EpoE3}$:$\underline{R}osa26^{LSL\text{-}tdTomato}$:$Tg^{\underline{Epo}\underline{C}re}$ genotype)[1,2] were backcrossed with the C57Black/6 strain more than 6 times, and male mice were used for experiments. In the ISAM-REC mice, a *GFP* cDNA is homozygously knocked into the *Epo* gene (*EpoGFP*), and EpoGFP expression in REP cells is strongly activated by severe anemia. Additionally, anemia-activated *EpoCre* transgene expression efficiently causes recombination of the $Rosa26^{LSL\text{-}tdTomato}$ locus and induces the expression of the tdTomato fluorescent protein from the recombined $Rosa26^{LSL\text{-}tdTomato}$ locus in REP cells permanently.[2] All mice were maintained under the Regulations for Animal Experiments and Related Activities of Tohoku University.

Blood analysis

Peripheral blood (0.2–0.3 mL) was collected from the mouse heart or submandibular vein into a 1.5-mL tube containing 5.0 μL of 0.5 M EDTA. To measure long-term changes of the hematocrit values in living mice, approximately 60 μL of peripheral blood was taken weekly from the tail using a heparinized microtube (Drummond) followed by centrifugation. The effect of this weekly small-volume phlebotomy was negligible.

Measurement of iron indices

Serum iron levels were measured using an automatic biochemistry analyzer (TBA-2000FR, Toshiba). Serum hepcidin levels were measured by a sensitive liquid chromatography/electrospray ionization tandem mass spectrometry method using a Triple Quad 5500 system (AB Sciex) equipped with a Prominence UFLCXR system (Shimadzu) as previously reported (the lower limit of quantitation is 10 ng/mL).[3,4] The hepatic and splenic iron contents were measured via inductively coupled plasma atomic emission spectroscopy using an Optima 8000 spectrometer (PerkinElmer).

Erythropoiesis stimulating agents (ESAs)

rHuEPO (epoetin beta, Epogen, Chugai Pharmaceutical), Darbepoetin alpha (DA, NESP, Kyowa Hakko-Kirin) and C.E.R.A. (epoetin beta pegol, Mircera, Chugai Pharmaceutical) were reconstituted with PBS containing 0.02% Tween 80. Each ESA was subcutaneously injected at a dose of 3.0 μg/kg body weight (BW). The dose corresponds to the peptide weight of each ESA, excluding their carbohydrate chains, to compare the efficacies of the ESAs at the same molar concentrations.

DA contains 2 additional glycans by genetic modification compared with rHuEPO, and C.E.R.A. is a methoxy polyethylene glycol (PEG)-conjugated rHuEPO. These carbohydrate modifications reduce the affinities between ESAs and EPOR, which determine the plasma half-lives of ESAs because EPO-EPOR complexes are degraded following their endocytosis into erythroid cells after signal transduction.[5] PEG may also affect the stability of glycans in ESAs, and asialoglycans of ESAs may be associated with ESA half-life through degradation in hepatocytes after the capture of asialo-ESAs by asialoglycoprotein receptors.[6]

Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted using ISOGEN (Nippon Gene). cDNAs was synthesized using a SuperScript III system (Invitrogen). Quantitative PCR (qPCR) was performed with the primers listed in Table 1 using the FastStart reagent (Roche). *Hprt* mRNA expression levels were used as an internal control for the qPCR experiments.

Dorsal chamber window and surface PO_2 measurement

A dorsal chamber window was prepared in the mouse back skin as previously described.[7,8] To monitor the oxygen concentration inside the back skin, oxygen sensor foil (PreSens, Germany) was placed between the hole and the cover glass, and the surface oxygen tension was measured using a VisiSens A1 detector camera and imaging software (PreSens).[9]

Histological analyses

Heart size was measured as the maximum width using a slide caliper. Sections (4 μm thickness) were prepared from paraffin-embedded formalin-fixed organs. Hemosiderin

deposition was assessed using Berlin blue staining.[3] The heart sections were stained with hematoxylin-eosin (Muto). To detect fluorescent protein expression, frozen kidney sections (10 μm thickness), which were fixed in 4% paraformaldehyde for 4 hours at 4 ºC, were observed after DAPI counterstaining using a BZ9000 microscope (Keyence).

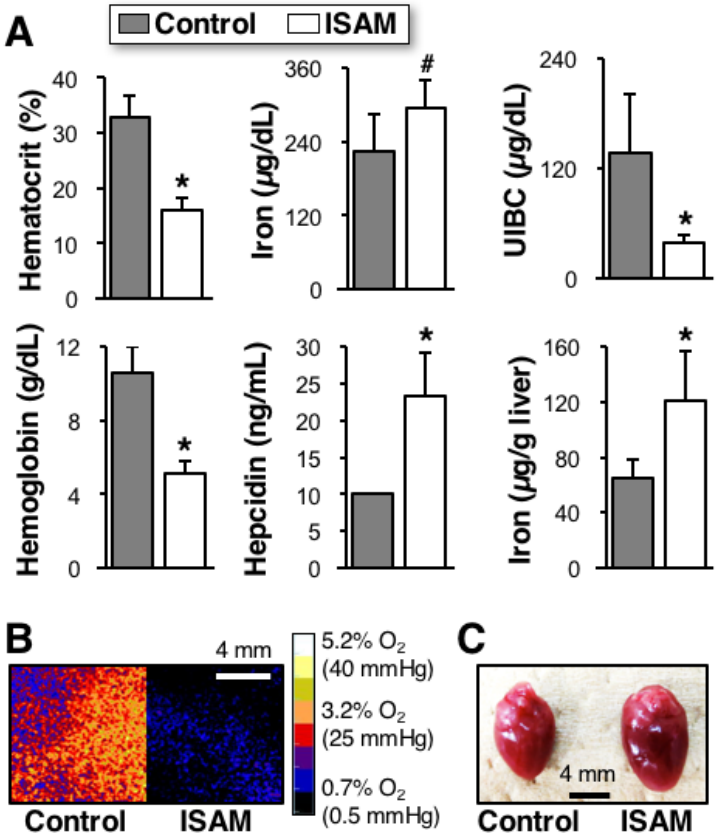
Statistics

The data are presented as the means ± standard deviation (SD). The *P* values were calculated using two-tailed, unpaired Student's *t* tests. Dunnett's test or the nonparametric Steel test were also used for multiple comparison.

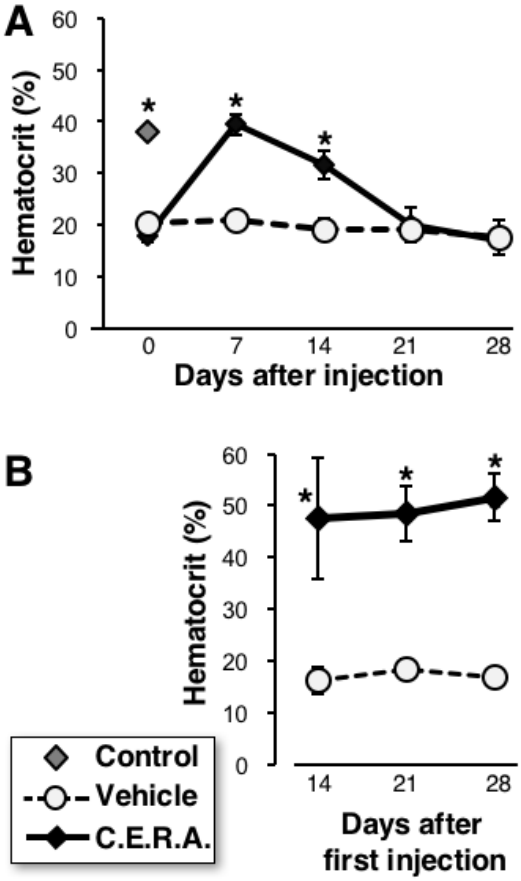
Online Supplementary Table S1.

Sequences of the oligonucleotide primers used in the RT-qPCR analyses.

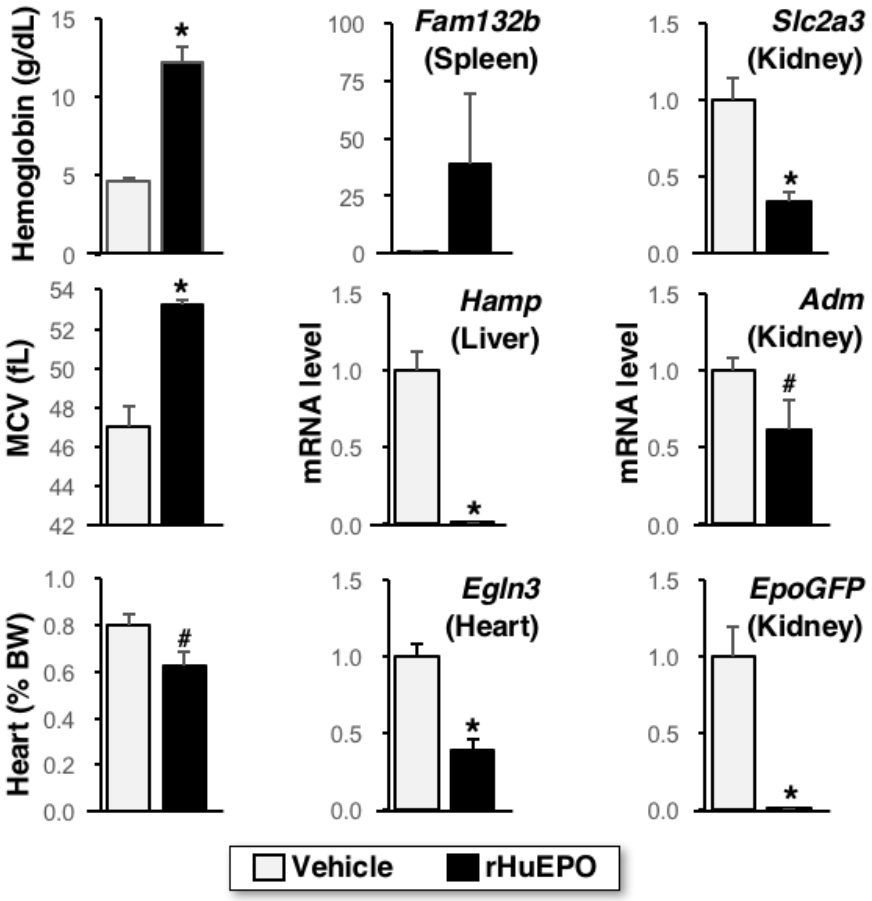
Target gene	Primer 1 (5'-3')	Primer 2 (5'-3')
Hprt	GCTGGTGAAAAGGACCTCTC	CACAGGACTAGAACACCTGC
	(FAM-labeled probe: ATCCAACAAAGTCTGGCCTGTATCCAAC)	
Hamp	TCTTCTGCATTGGTATCGCA	GAGCAGCACCACCTATCTCC
Fam132b	ACTCACCGAGCAGCCAAGAAAG	TGTTCTCCAGCCCCATCACAG
Egln3	CTATGTCAAGGAGCGGTCCAA	GTCCACATGGCGAACATAACC
Slc2a3	CTGGACCTCCAACTTTCTGG	GCAGCGAAGATGATAAAAACG
EpoGFP	GAAGACTTGCAGCGTGGAC	GGTGGATCCTAAAGCAGCAG
tdTomato	CTCTTTGATGACCTCCTCGC	TCTGACTGACCGCGTTACTCC
Pdgfrb	GTGGTGAACTTCCAATGGACG	GTCTGTCACTGGCTCCACCAG
Adm	GAGCGAAGCCCACATTCGT	GAAGCGGCATCCATTGCT



Online Supplementary Figure S1. The anemic phenotype appears in ISAM approximately 4 weeks after birth. (**A**) The levels of hematocrit, hemoglobin (Hb), iron, unsaturated iron binding capacity (UIBC), and hepcidin were measured in the peripheral blood of 4-week-old ISAM and control mice. The iron concentrations in the livers were also determined. n=9 and n=5 for the control and ISAM, respectively. **P*<0.01, #*P*<0.05 using Student's *t* test. (**B**) The surface oxygen concentrations on the inside of the back skin of 12-week-old control and ISAM were measured using a VisiSens A1 oxygen imaging system. (**C**) A gross view of the hearts demonstrates that the ISAM hearts are larger than the control hearts at 12 weeks of age.



Online Supplementary Figure S2. Long-term observation of hematocrit values after C.E.R.A. administration into ISAM. (**A**) C.E.R.A. (3.0 μg/kg BW) or vehicle was subcutaneously injected into ISAM on day-0, and the change in hematocrit values was measured on the indicated days. On day-28, the increased hematocrit values returned to the levels of untreated ISAM. Data from untreated control mice are also shown. n=3 for each time point. (**B**) Weekly C.E.R.A. administration sustains a normal level of erythropoiesis in ISAM. C.E.R.A. (3.0 μg/kg BW) or vehicle was injected into ISAM-REC mice every 7 days beginning at day-0, and the hematocrit value was measured on day-14, 21 and 28. The weekly injection of C.E.R.A. is optimal to maintain the normal hematocrit value in ISAM, not inducing polycythemia. n=4 for each time point. Male mice at 12 to 16 weeks of age were analyzed. *P<0.01 compared with vehicle-treated mice at each time point using Student's t test.



Online Supplementary Figure S3. Cardiomegaly and hypoxic milieu of ISAM are corrected by 1-week administration of rHuEPO. rHuEPO (3.0 μg/kg BW) or vehicle was injected into ISAM every 2 days (day-0, 2, 4 and 6), and the mice were analyzed for the indicated parameters at 7 days after the first injection (day-7). n=3 for each group. *P<0.01, $^{\#}P$<0.05 compared with vehicle-treated ISAM using Student's t test.

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