Erythropoietin stimulates murine and human fibroblast growth factor-23, revealing novel roles for bone and bone marrow

Erica L. Clinkenbeard,[1] Mark R. Hanudel,[2] Keith R. Stayrook,[3] Hitesh Nidumanda Appaiah,[1] Emily G. Farrow,[4] Taryn A. Cass,[1] Lelia J. Summers,[1] Colin S. Ip,[1] Julia M. Hum,[1] Joseph C. Thomas,[1] Mircea Ivan,[5] Briana M. Richine,[6] Rebecca J. Chan,[6] Thomas L. Clemens,[7] Ernestina Schipani,[8] Yves Sabbagh,[9] Linlin Xu,[10] Edward F. Srour,[6] Marta B. Alvarez,[11] Melissa A. Kacena,[11] Isidro B. Salusky,[2] Tomas Ganz,[12] Elizabeta Nemeth[12] and Kenneth E. White[1]

**ELC, MRH and KRS contributed equally to this work [1]Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN; [2]Department of Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles, CA; [3]Department of Pharmacology and Toxicology, Indiana University School of Medicine, Indianapolis, IN; [4]Center for Pediatric Genomic Medicine at Children's Mercy Hospital, Kansas City, and Department of Pediatrics, University of Missouri-Kansas City School of Medicine, Kansas City, MO; [5]Department of Medicine/Hematology-Oncology, Indiana University School of Medicine, Indianapolis, IN; [6]Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN; [7]Department of Orthopaedic Surgery, The Johns Hopkins University and the Baltimore Veterans Administration Medical Center, Baltimore, Maryland, MD; [8]Department of Medicine/Endocrinology, University of Michigan School of Medicine, Ann Arbor, MI; [9]Rare Diseases, Sanofi Genzyme, Framingham, MA; [10]Department of Microbiology and Immunology, IUSM, Indianapolis, IN; [11]Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN and [12]Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA*

Correspondence: kenewhit@iupui.edu
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Supplementary Information

Animal studies: Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) for Indiana University, and comply with the NIH guidelines for the use of animals. *Jck* mice were previously derived and characterized as a spontaneously-arising model with a *Nek*-8 mutation[1,2]. C57BL/6 ('C57') wild-type controls were purchased (Jackson Labs) and acclimated prior to all studies. Genotyping included PCR amplification and subsequent BseYI digestion overnight as previously described (http://jaxmice.jax.org/protocolsdb/f?p=116:2:0::NO:2:P2_MASTER_PROTOCOL_ID,P2_JRS_CODE:2120,002561). All mice received a normal rodent diet from the IUSM animal facility (2018SX, Harlan Teklad) containing 0.7% phosphorus and 200mg/kg iron. Recombinant human erythropoietin (EPO; BioLegend, Inc.) or saline was injected into 6-week-old C57BL/6, or 6-week-old homozygous *Jck* female mice at 25 U/g body weight to 250 U/g body weight i.p. daily for three consecutive days and analyzed 4 hours after the final injection. For iron supplementation studies, *Jck* and C57BL/6 mice were divided into four treatment groups: Group one received saline (100 µl) daily for three consecutive days; Group two was injected with rhEPO for three consecutive days; Group three received ferric carboxymaltose (FCM) on day 2 (80 mg/kg body weight; Monofer) with saline on days 1 and 3; and Group four received EPO and FCM in combination: EPO on all three days and FCM on day two. Mice were euthanized by CO_2 inhalation/cervical dislocation, and blood was collected by cardiac puncture for serum and plasma (collected in EDTA tubes). Where indicated, mice were also tail bled for complete blood counts (CBCs) and facial vein bled for serum intact FGF23 concentrations.

Human Studies: Four elderly patients (70-88 years old) with normal kidney function who were classified as anemic (Hb 9.4-10.3 g/dL) were administered EPO (20,000-40,000 U)

subcutaneously between 08:30-10:30am for all patients. Serum samples were collected every 6 hours over a 48 hour time period after injection.

Serum biochemistries: Commercial ELISAs were used to measure serum intact FGF23 (iFGF23) and C-terminal FGF23 (cFGF23) concentrations in humans and mice (Quidel, Corp.). Human serum hepcidin levels were measured by competitive ELISA (Intrinsic LifeSciences, La Jolla). Human iron and serum phosphate were analyzed by routine automation. Standard mouse serum biochemistries were measured using an automated COBAS MIRA Plus Chemistry Analyzer (Roche Diagnostics; Indianapolis, IN). Mouse CBCs were performed on whole blood using a Mascot HemaVet950FS automated processor. Quantitative determination of erythropoietin (EPO) in serum samples was performed using the Quantikine ELISA mouse EPO Immunoassay Kit (R&D Systems); mouse erythroferrone (ERFE) was measured as described[3].

Promoter construction: The -5 kb segment of the mouse *Fgf23* promoter was amplified using the KOD Xtreme Hotstart kit (Millipore) according to manufacturer's instructions from purified mouse genomic DNA with a -5032 forward primer containing a KpnI restriction site and a -1 reverse primer containing a XhoI restriction site (primer sequences available upon request). The resulting PCR product was purified using ExoSAP-It (Affymetrix). The promoter cDNA as well as the pGL4.1 firefly luciferase vector (Promega) were digested with *Kpn*I and *Xho*I (New England Biolabs) and purified with gel extraction (Qiagen), then subsequently ligated using T4 DNA ligase according to directions (Cell Signaling). Positive colonies were assessed with both PCR and restriction digest methods. Maxi-plasmid preps of the positive colonies (IBI Maxi prep extraction kit; Midwest Scientific) were used in subsequent luciferase assays.

Cell transfections and Luciferase assays: ROS17/2.8 cells were seeded in 12-well plates at $2x10^5$ cells/well in α-MEM media supplemented with 10% FBS (Atlanta Biologicals), 1% L-

glutamine (Hyclone) and 1% Penicillin/Streptomycin (Hyclone). After overnight incubation, cells were transfected with the -5kb *Fgf23*-pGL4 reporter construct together with pcDNA3.1 or human EPO receptor (EPOR; Origene). Twenty four hours post transfection, cells were treated with 10^{-8}M 1,25$(OH)_2$ vitamin D (Sigma) or 100-1000 U/mL rhEPO (BioLegend) for an additional 24 hours. Cells were collected in 1X Passive Lysis Buffer, analyzed with the Dual Luciferase Assay reagent (Promega) in a Luminoskan (Thermo-Fisher Scientific), and firefly-luciferase levels were normalized to Renilla-luciferase. UMR-106 cells were maintained in DMEM/F-12 (Hyclone) supplemented with 10% FBS, 1% L-glutamine, 1% Sodium Pyruvate (Hyclone) and 1% Penicillin/Streptomycin. UMR cells were seeded $2x10^5$ cells/well in a 12 well plate. For iron chelation treatment, cells were incubated with 50 uM DFO or vehicle for 24 hours. For transfection procedures, after overnight incubation, the cells were transfected with 0.5 ug EPO receptor expression plasmid using Fugene HD (Promega). Twenty-four hours later, the cells were treated with either vehicle or 100U/mL EPO for an additional 24 hours. All UMR cells underwent RNA extraction using the Qiagen RNeasy Mini kit (Qiagen) according to instructions.

Western blots: UMR-106 cells were seeded at $2x10^5$ cells/well in 12 well plates. After overnight incubation, the cells were transfected with the EPOR expression plasmid (Origene) using the Fugene HD protocol (Promega). The following day, the cells were treated with vehicle control, 100U/mL or 1000U/mL EPO and collected in 1X lysis buffer (Cell Signaling) containing AEBSF (Sigma) 5, 10 and 15 minutes after treatment. Membranes were blotted over night for phospho-Stat5 (Novus Biologicals); total Stat5 (Novus Biologicals); phospho-ERK1/2 (Cell Signaling); and total ERK (Promega), with subsequent incubation with goat-anti Rabbit-HRP antibody (Cell Signaling). Anti-β Actin-HRP antibody (Sigma) was used as a loading control. Chemilumiescent images were collected with the GE AB1600 digital imager.

Marrow ablation: C57BL/6 mice at 8 weeks of age were treated with carboplatin (2.5 mg/mouse) or water control via i.p. injection. 24 hours later, mice were injected with either EPO (125 U/g body weight) or saline control for the standard three day time course. At necropsy, remaining marrow cells were flushed from the femur prior to homogenization and RNA extraction.

Marrow cell ex vivo culture: For marrow cell isolation, tibia, femur and pelvis were dissected following euthanasia from WT mice at 8-10 weeks of age. Marrow was flushed with RPMI-1640+2% BSA. Marrow cells were incubated with 1X Red blood lysis buffer (BioLegend) for 10 minutes according to manufacturer's instructions. The cells were resuspended in IMDM (Hyclone) supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin/Streptomycin and seeded into 24 well plates at $1x10^6$ cells/well. 30 minutes after plating, the cells were treated with 100U/mL EPO (BioLegend) or vehicle control. Two and 4 hours after treatment, the cells were collected and RNA was extracted using the Qiagen RNeasy Mini kit.

LSK enrichment/isolation: 6-week-old C57BL/6 mice were injected with a three-day protocol of 125 U/g rhEPO or saline control. At necropsy, pelvis, tibia, femur and humerus were collected and flushed of marrow using IMDM media supplemented with 5% FBS (Hyclone) and 2% Pen/Strep (Sigma, Inc.). For saline replicates, bones from two mice were pooled, whereas bones from three mice were pooled per replicate for EPO treatments. Low density mononuclear cells (LDMNCs) were collected by Ficoll gradient. After washing, the LDMNCs were either isolated through the hematopoietic progenitor enrichment kit (StemCell Technologies) or stained (as previously performed[4]), then isolated using a Becton-Dickinson FACS Aria multicolor cell sorter. In addition to Lineage markers: a cocktail of lineage specific markers that identifies differentiated hematopoietic cells within specified cell lineages such as T cells (CD3, CD4, and CD8), B cells (B220), myeloid cells (GR-1, Mac1, CD11b, CD14), and erythroid cells (Ter119); Sca1 and CD117 (c-kit), cells were also stained with CD34 and FCgammaR. Common myeloid

progenitors (CMP) were defined as $Lin^{-}ckit^{-}Sca1^{low}CD34^{+}FCgammaR^{low}$ [5]. Remaining bone fragments were digested with collagenase A (Roche) for 15 minutes at 37°C and the media subsequently discarded. Another round of collagenase digestion occurred for 90 min at 37°C. The media with osteoblastic cells was removed and centrifuged to isolate the cells; cells were washed twice with PBS. RNA from the flow cytometry sorted cells and isolated bone cells were extracted using the RNeasy Micro kit (Qiagen, Inc.).

RNA preparation and quantitative RT-PCR (qPCR): RNA was isolated from mouse spleen and femur, as well as from cell lines as previously published[6]. For cortical bone and flushed marrow preparations, one femur was placed in a tube containing sterile PBS and spun for 3 minutes at 10,000 rpm. Both the bone fraction and marrow cells were processed individually with Trizol according to manufacturer's standard protocol and resuspended in sterile water. RNA samples were tested with TaqMan One-Step RT-PCR kit (ThermoScientific) using primers specific for mouse or rat Fgf23, transferrin receptor (TfRc), EpoR, hepcidin or erythroferrone (Erfe). β-actin, Gapdh or Hprt was used as an internal control as indicated in figure legends. qPCR primers and probes were either purchased as pre-optimized reagents (Life Technologies) or designed and validated by our laboratory.

Statistical analysis: Significance between groups for mouse studies was assessed by *t*-test or ANOVA where appropriate. Human data was assessed with rank sum tests comparing medians. Significance for all tests was set at $p<0.05$. Data are presented as means ± standard error of the mean (SEM) unless otherwise noted.

Supplemental references:

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Supplementary Tables

Supplementary Table 1. Patient serum iron and CBC measurements prior to EPO treatment

	Patient #1 (70 M)	**Patient #2 (88 F)**	**Patient #3 (86 M)**	**Patient #4 (77 F)**
Iron (mcg/dl)	52 (23-202)	110 (23-182)	125 (23-202)	46 (23-182)
Total Iron Binding Capacity (mcg/dl)	248 (240-520)	310 (240-520)	286 (240-520)	442 (240-520)
Transferrin Saturation (%)	21 (20-50)	35 (20-50)	44 (20-50)	***10*** (20-50)
Ferritin (ng/ml)	***966*** (10-210)	***171*** (8-150)	***335*** (10-210)	12 (8-150)
Hepcidin (ng/ml)	71 (29-254)	179 (17-286)	88 (29-254)	NA
Hemoglobin (g/dl)	***10.0*** (12.3-16.3)	***10.3*** (11.5-14.6)	***9.9*** (12.3-16.3)	***9.4*** (11.5-14.6)
Hematocrit (%)	***31.4*** (37.4-47.0)	***32.1*** (34.0-42.1)	***29.5*** (37.4-47.0)	***30.5*** (34.0-42.1)
Red Blood Cell Count (million cells/ul)	***3.20*** (4.21-5.61)	***3.44*** (3.76-4.93)	***2.95*** (4.21-5.61)	***3.56*** (3.76-4.93)
Mean Corpuscular Volume (fl)	***98.1*** (79.0-95.0)	93.3 (79.0-95.0)	***100.0*** (79.0-95.0)	85.7 (79.0-95.0)
Mean Corpuscular Hemoglobin (pg)	31.3 (26.0-36.2)	29.9 (26.0-36.2)	33.6 (26.0-36.2)	26.4 (26.0-36.2)
MCH Concentration (g/dl)	31.8 (31.7-35.5)	32.1 (31.7-35.5)	33.6 (31.7-35.5)	***30.8*** (31.7-35.5)
Red Blood Cell Distribution Width-CV (%)	13.9 (10.7-15.5)	12.5 (10.7-15.5)	15.1 (10.7-15.5)	15.3 (10.7-15.5)
Reticulocyte Count (%)	1.91 (0.60-2.06)	1.05 (0.55-2.08)	0.87 (0.60-2.06)	1.57 (0.55-2.08)

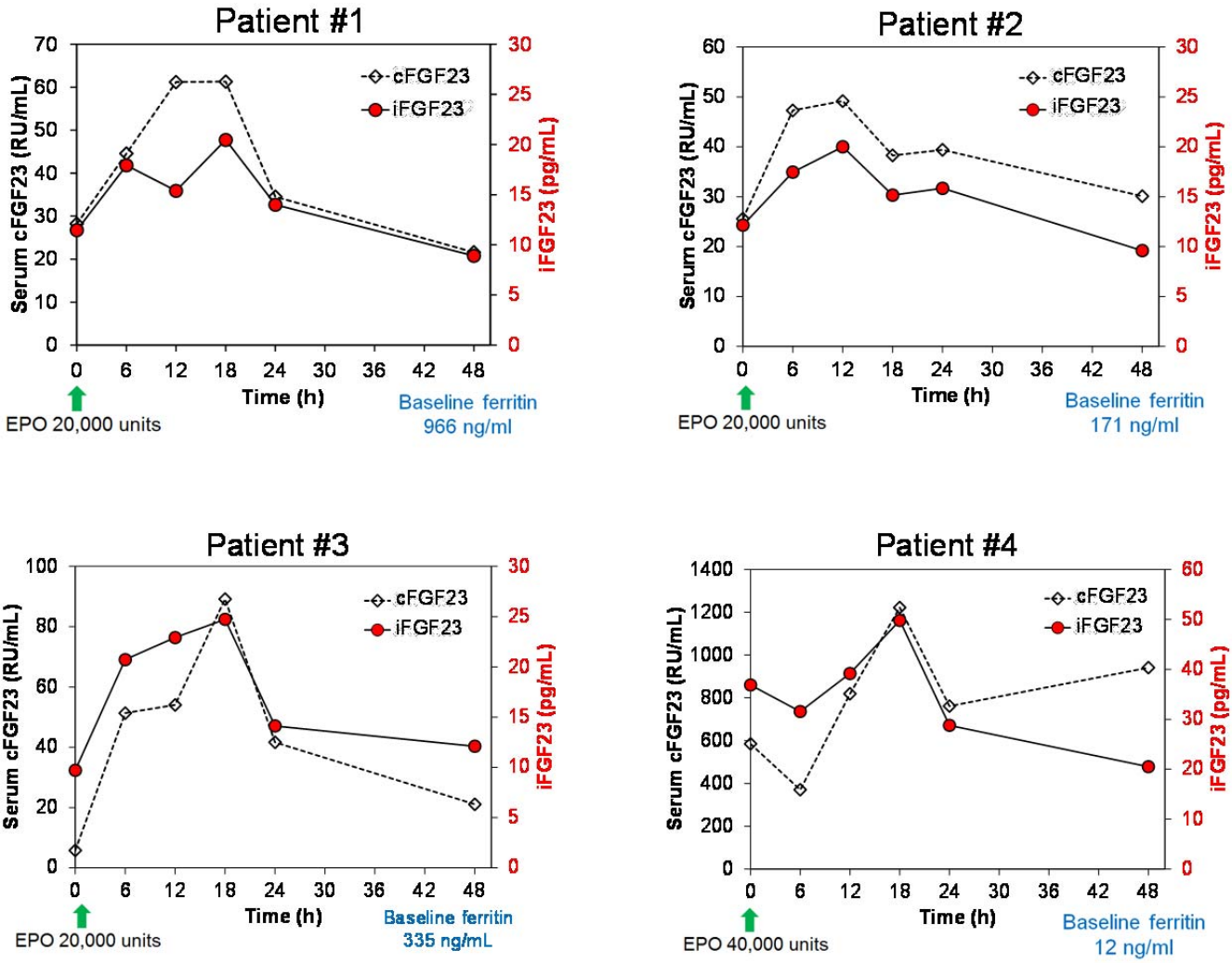
Parentheses include the normal male or female reference ranges.

Supplementary Table 2. *Jck* and C57 mouse CBCs

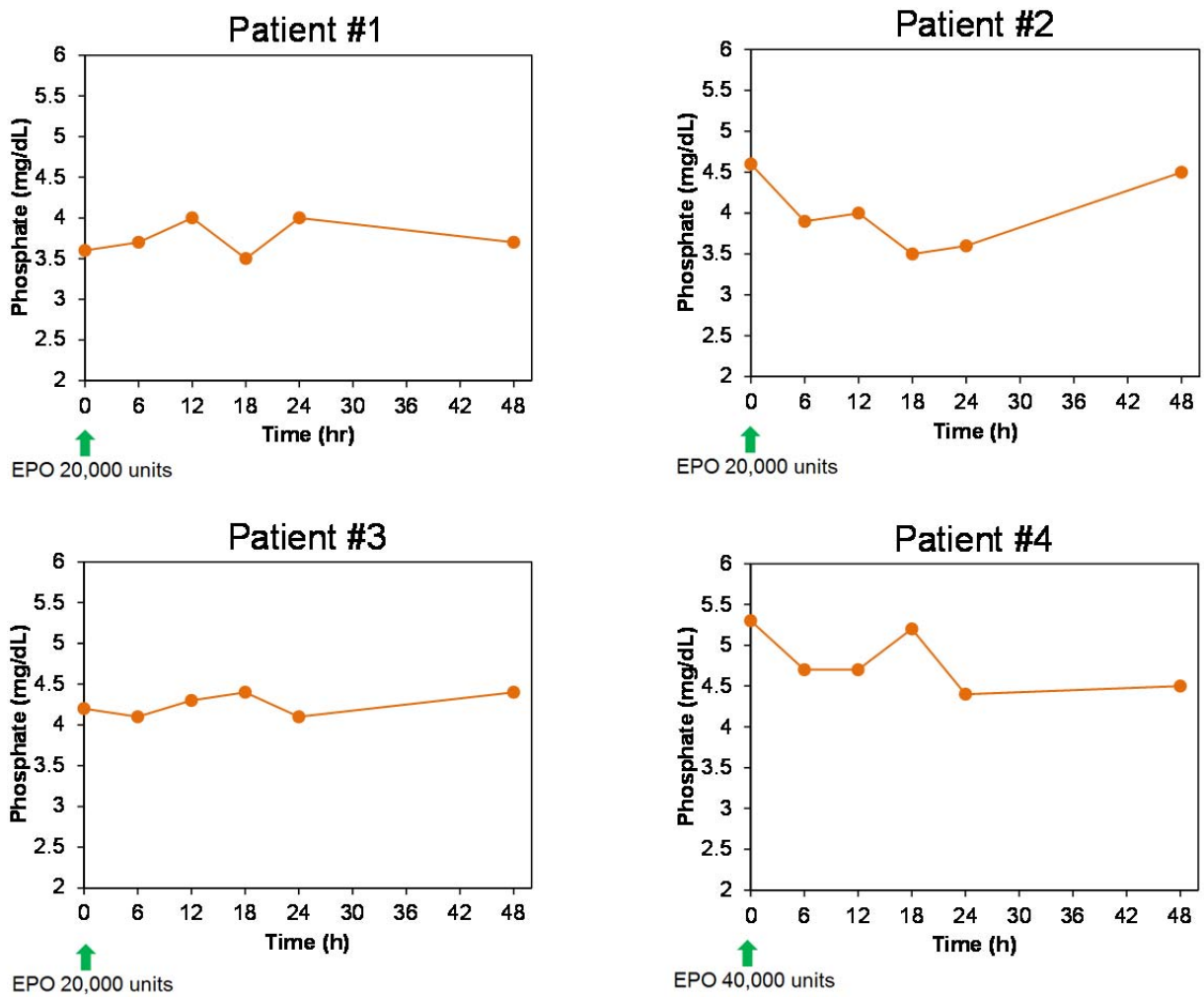
CBC	4 week		8 week		12 week		16 week		>20 week	
	C57(n=5)	*Jck* (n=5)	C57(n=6)	*Jck* (n=5)	C57(n=5)	*Jck* (n=9)	C57(n=4)	*Jck* (n=6)	C57(n=9)	*Jck* (n=7)
RBC	12.4±0.2	9.0±0.18***	11.6±0.36	8.7±0.2***	10.8±0.29	8.2±0.32***	11.8±0.4	8.2±0.67**	8.2±0.45	6.6±0.38*
Hb	18.2±0.2	13.8±0.27***	16.9±0.68	12.2±0.42***	15.8±0.41	10.7±0.57***	15.5±0.63	10.7±1.04*	11.2±0.69	7.8±0.56**
HCT	57.2±0.97	45.4±0.73***	50.1±1.41	37.3±1.15***	54.4±1.3	34.9±1.81***	51.4±2.12	34.8±3.24**	36.4±2.23	24.5±1.59**
MCV	46.3±0.29	50.3±0.57	43.2±0.22	42.9±0.63	50.3±0.59	42.6±1.04***	43.4±0.38	41.9±0.69	43.8±0.48	36.9±0.46***
MCH	14.7±0.16	15.4±0.34	14.5±0.17	14.0±0.16	14.6±0.04	13.0±0.3**	13.1±0.21	12.8±0.23	13.6±0.19	11.8±0.19***
MCHC	31.7±0.23	30.6±0.81	33.8±0.51	32.6±0.42	29.0±0.29	30.6±0.49	30.3±0.32	30.5±0.27	31.06±0.33	31.9±0.29
RDW	19.5±0.23	23.6±0.58	17.8±0.21	17.7±0.42	18.1±0.13	16.6±0.19***	17.6±0.15	16.5±0.29*	16.5±0.07	16.3±0.24

Measurements include Red blood cell count (RBC; M/μL); Hemoglobin (Hb; g/dL); Hematocrit (HCT; %); Mean corpuscular volume (MCV; fL); Mean corpuscular hemoglobin (MCH; pg); Mean corpuscular hemoglobin concentration (MCHC; g/dl); Red cell distribution width (RDW; %). Mean and standard error (SE) are shown for each group and time point; significant differences are indicated compared to control C57: *p<0.05, **p<0.005, ***p<0.0005.

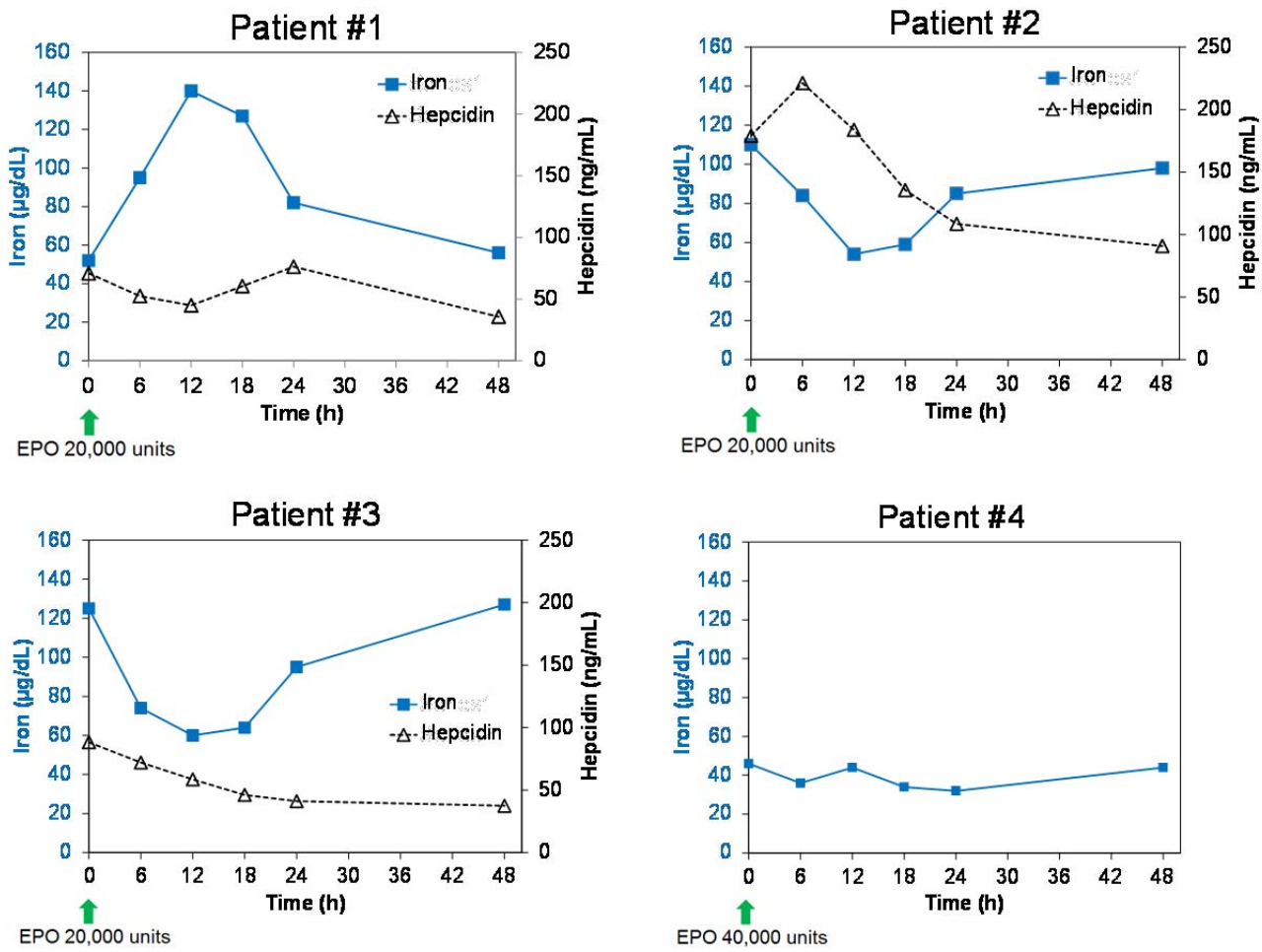
Supplementary Figures



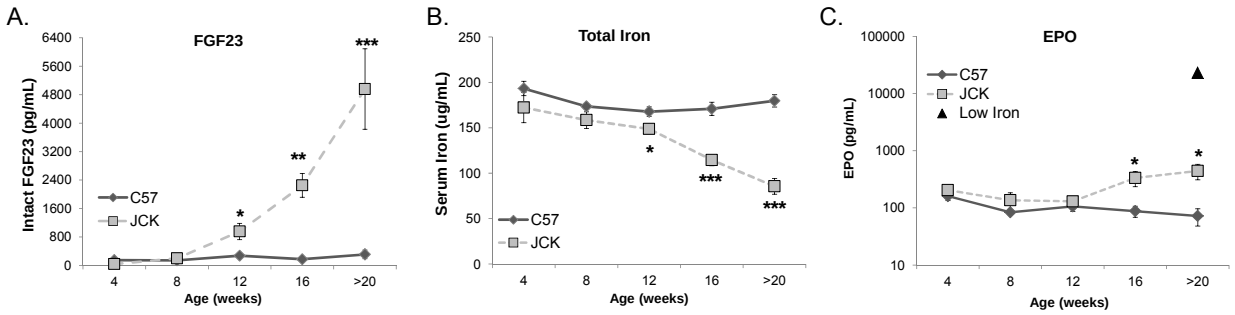
Supplementary Figure 1. Four patients were provided EPO injections at time zero (doses indicated below Time 0) and monitored over a 48 hour time course for cFGF23 (open diamonds) and iFGF23 (red circles).



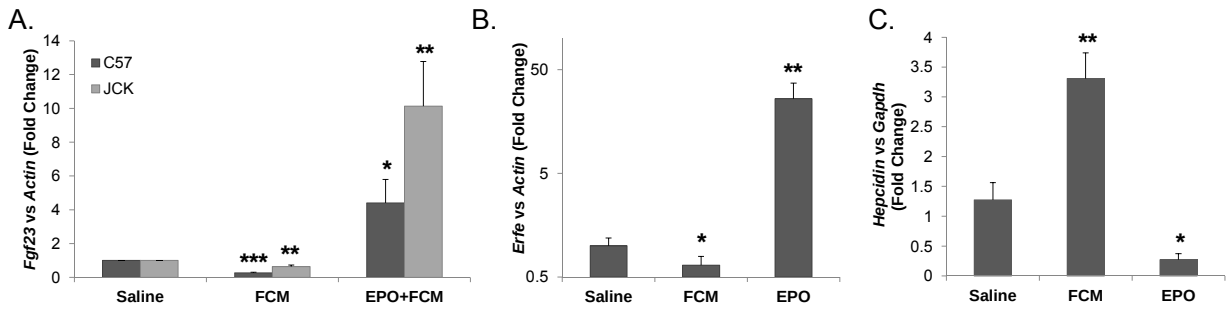
Supplementary Figure 2. Serum phosphate was measured in each patient at baseline and throughout the 48 hour time course after EPO treatment.



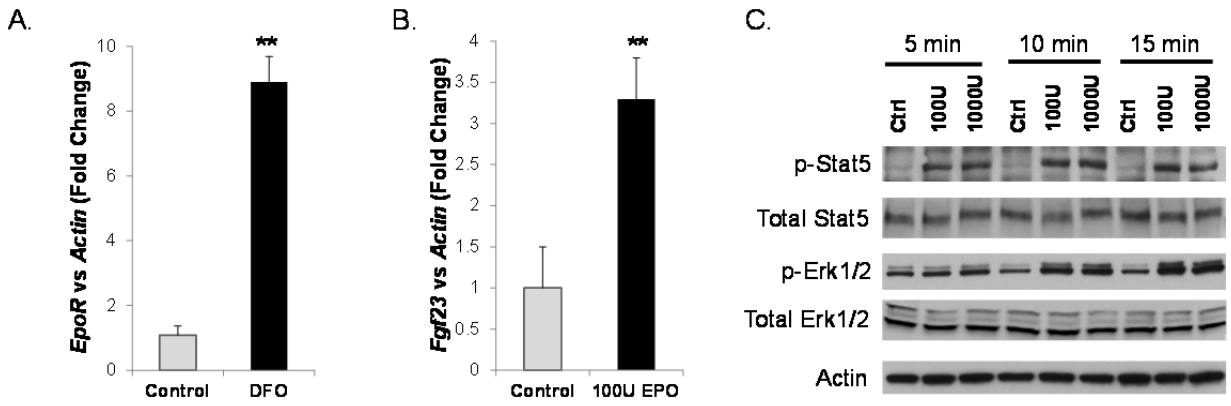
Supplementary Figure 3. Serum iron (blue squares) and hepcidin (open triangles) were measured in each patient over a 48 hour time course after EPO treatment. Hepcidin was not available for patient 4.



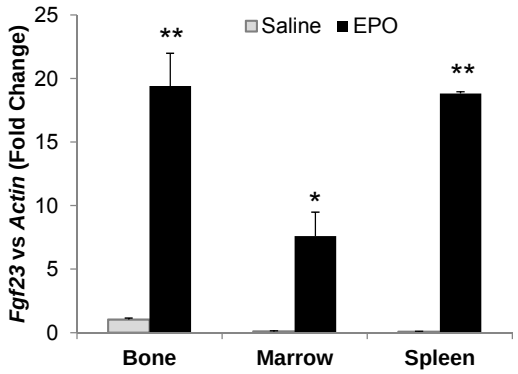
Supplementary Figure 4. *Jck* mice were studied from 4 to >20 weeks of age. (A) While maintained on a normal chow diet, *Jck* mice exhibited chronic renal dysfunction which resulted in a significant rise in serum intact FGF23 compared to C57 controls after 12 weeks of age. (B) *Jck* mice were also found to be hypoferremic, as serum iron was reduced versus C57 at 12 weeks and further declined to 50% of control by >20 weeks of age. (C) Serum EPO was found to increase in *Jck* mice at 16 and >20 weeks compared to C57 controls; however, this was only 2-fold increased over baseline and much lower compared to low-iron diet fed mice (triangle). (n=5-7 per group; *p<0.05, **p<0.005, and ***p<0.0005 vs. C57 at the same age).



Supplementary Figure 5. (A) C57 and *Jck* mice were treated with an i.v. injection of ferric carboxymaltose (FCM) on day 2 of the three-day regimen, plus either saline or EPO (EPO+FCM). Whole bone *Fgf23* mRNA expression was found to be significantly reduced in both C57 and *Jck* mice treated with FCM alone. Both genotypes had a significant increase in *Fgf23* mRNA with EPO+FCM compared to the same genotypes treated with saline (*p<0.02, **p<0.005 and ***p<0.0001 vs. saline treated mice of the same genotype). (B) As control, bone marrow *Erfe* mRNA from the C57 treated mice was found to be significantly decreased with FCM treatment alone compared to the induction observed with EPO treatment (Log scale). (C) Liver *hepcidin* levels (normalized to *Gapdh*) were observed to increase in C57 mice treated with FCM alone whereas EPO significantly suppressed this factor (n=5-6/group; *p<0.05 and **p<0.01 vs. Saline).



Supplementary Figure 6. (A) UMR-106 cells treated with DFO for 24 hours showed a significant up-regulation of *EpoR* mRNA expression compared to control treated cells (**p<0.01 vs. control treated cells). (B) To mimic this response, UMR-106 cells were transfected with a human EPOR expression plasmid and then treated with EPO for 24 hours which increased *Fgf23* mRNA expression (**p<0.01 vs. control treated cells). (C) UMR-106 cells overexpressing EPOR were also collected for protein analysis 5-15 minutes after treatment with EPO which showed phosphorylation of Stat5 and Erk1/2 signaling proteins.



Supplementary Figure 7. Cortical bone, flushed marrow and spleen were analyzed for *Fgf23* mRNA expression from wild-type mice treated with EPO (125U/g/d) or saline control. EPO significantly induced *Fgf23* mRNA levels in all tissues compared to control (n=4/group; *p<0.05 and **p<0.01 vs. saline treatment in the same tissue).