Effects of erythropoietin on fibroblast growth factor 23 in mice and humans

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL RESULTS:

Associations between serum EPO and circulating FGF23 levels in kidney transplant recipients

As presented in the main text, we assessed associations between serum EPO and circulating FGF23 levels in a large cohort of adult renal transplant recipients (RTR, n=680). We observed that Log EPO was independently and positively associated with Log cFGF23. We repeated the analyses in an independent replication cohort consisting of 592 RTRs, whose baseline characteristics are described in **Supplemental Table 3**. In this cohort, Log EPO was univariately associated with Log cFGF23 levels ($\beta = 0.32$, $p < 0.001$). In multivariable analysis, Log EPO remained a major determinant of Log cFGF23 levels, independent of adjustment for age, sex, eGFR, time since transplantation, phosphate, Log PTH, hemoglobin, and Log CRP levels ($\beta = 0.21$, $p < 0.001$). Mediation analysis showed only 14.6% mediation by hemoglobin on the association between total FGF23 and EPO, independent of potential confounders (**Supplemental Table 4**). In a sensitivity analysis, we excluded kidney transplant patients on ESA (n=13), and repeated the multivariable model. Again, Log EPO remained independently and positively associated with Log cFGF23 ($\beta = 0.22$, $p < 0.001$).

Supplemental Table 1. Baseline characteristics of UCLA non-dialysis CKD and dialysis cohort

	UCLA Non-dialysis CKD Cohort	**UCLA Dialysis Cohort**
Number	42	79
Sex	40% male, 60% female	70% male, 30% female
Age Group	57% pediatric, 43% adult	48% pediatric, 52% adult
Dialysis Modality	n/a	85% HD, 15% PD
Age (years)	37 ± 28	40 ± 27
eGFR (ml/min/1.73m^2)	34 ± 18	n/a
C-terminal (total) FGF23 (RU/ml)	119 (92, 205)	1648 (467, 4812)
Intact FGF23 (pg/ml)	105 (70, 165)	1054 (302, 3934)
Erythropoietin (U/l)	9.7 (7.1, 15.7)	n/a
Weekly rhEPO dose/kg	n/a	214 (116, 367)
Calcium (mg/dl)	9.1 ± 0.6	8.9 ± 0.8
Phosphate (mg/dl)	4.0 ± 1.0	5.5 ± 1.6
Parathyroid hormone (pg/ml)	97 (54, 152)	304 (157, 611)
Transferrin saturation (%)	23 ± 10	35 ± 17
Ferritin (ng/ml)	60 (31, 128)	562 (252, 895)
Hemoglobin (g/dl)	12.1 ± 1.7	12.0 ± 1.5
C-reactive protein (mg/l)	1.4 (0.6, 5.3)	3.3 (0.9, 7.4)

Data are presented as mean ± standard deviation, or median (interquartile range). CKD: chronic kidney disease, HD: hemodialysis, PD: peritoneal dialysis, eGFR: estimated glomerular filtration rate, FGF23: fibroblast growth factor 23; rhEPO: recombinant human erythropoietin.

Supplemental Table 2. Mediation analysis of hemoglobin in the association between erythropoietin and C-terminal FGF23 in 680 RTRs

Potential mediator	Outcome	Effect (path)*	Multivariable model**	
			Coefficient (95% CI)†	**Proportion mediated§**
Hemoglobin	**cFGF23**	Indirect effect (*ab* path)	0.02 (0.006; 0.04)	**8.3%**
		Total effect (*ab* + *c*' path)	0.23 (0.14; 0.32)	
		Unstandardized total effect	0.19 (0.14; 0.26)	

* The coefficients of the indirect *ab* path and the total *ab* + *c'* path are standardized for the standard deviations of the potential mediator, erythropoietin, and outcome.

**All coefficients are adjusted for age, sex, eGFR, time since transplantation, serum phosphate, and hs-CRP levels

§The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

†95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2000 bootstrap samples.

Supplemental Table 3. Baseline characteristics of 592 renal transplant recipients (RTRs) across tertiles of erythropoietin levels

	All patients (n=592)	Tertiles of erythropoietin (IU/L)			*P* value
		T1 (n=197) [4.0-13.7]	T2 (n=199) [13.8-21.5]	T3 (n=196) [21.6-195.0]	
Age (years)	51±12	48±12	52±12	55±11	<0.001
Male sex (n, %)	325 (55)	113 (57)	111 (56)	101 (52)	0.49
Body mass index, kg/m^2	26±4	26±4	26±4	27±4	0.009
Alcohol use (n, %)	295 (50)	102 (52)	94 (47)	99 (51)	0.47
Smoking status					0.24
Never smoker (n, %)	211 (36)	65 (33)	80 (40)	66 (34)	
Former smoker (n, %)	249 (42)	83 (42)	85 (43)	81 (41)	
Current smoker (n, %)	130 (22)	48 (24)	34 (17)	48 (25)	
Time since transplantation (yrs)	6.0 (2.6-11.5)	4.7 (2.2-9.3)	6.5 (3.5-11.6)	6.6 (2.7-13.8)	0.002
Diabetes mellitus (n, %)	104 (18)	37 (19)	27 (14)	40 (20)	0.18
Systolic blood pressure (mmHg)	153±23	151±21	151±21	157±25	0.02
Diastolic blood pressure (mmHg)	90±10	90±10	90±9	90±11	0.79
Laboratory measurements					
cFGF23 (RU/mL)	139 (94-218)	119 (79-169)	138 (89-204)	194 (115-356)	<0.001
Hemoglobin (mmol/L)	8.6±1.0	8.8±1.0	8.6±1.0	8.4±1.0	<0.001
MCV (fL)	91±6	90±5	91±6	92±8	<0.001
Ferritin (µg/L)	155 (76-283)	151 (82-321)	166 (98-283)	135 (64-256)	0.07
Total cholesterol (mmol/L)	5.6±1.1	5.7±0.9	5.6±1.2	5.5±1.1	0.39
Phosphate (mmol/L)	1.1±0.2	1.1±0.2	1.1±0.2	1.1±0.2	0.34
eGFR (ml/min/1.73m^2)	46±15	48±15	47±14	44±15	0.02
Creatinine (µmol/L)	147±58	145±55	143±53	154±65	0.13
Proteinuria (>0.5g) (n, %)	161 (27)	46 (23)	55 (28)	60 (31)	0.24
hs-CRP (mg/L)	2.0 (0.8-4.8)	1.5 (0.6-4.0)	1.9 (0.8-3.9)	3.0 (1.2-7.5)	<0.001
Treatment					
ACE-inhibitors (n, %)	199 (34)	83 (42)	57 (29)	59 (30)	0.008
Bèta-blocker (n, %)	365 (62)	120 (61)	127 (64)	118 (60)	0.74
Calcium channel blockers (n, %)	225 (38)	78 (40)	68 (34)	79 (40)	0.54
Diuretic use (n, %)	261 (44)	77 (39)	76 (38)	108 (55)	0.001

Values are means ± standard deviation, medians (interquartile range) or proportions (%). Abbreviations: ACE, angiotensin converting enzyme; cFGF23, C-Terminal fibroblast growth factor 23; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; MCV, mean corpuscular volume.

Supplemental Table 4. Mediation analysis of hemoglobin in the association between erythropoietin and C-terminal FGF23 in replication cohort of 592 renal transplant recipients (RTRs)

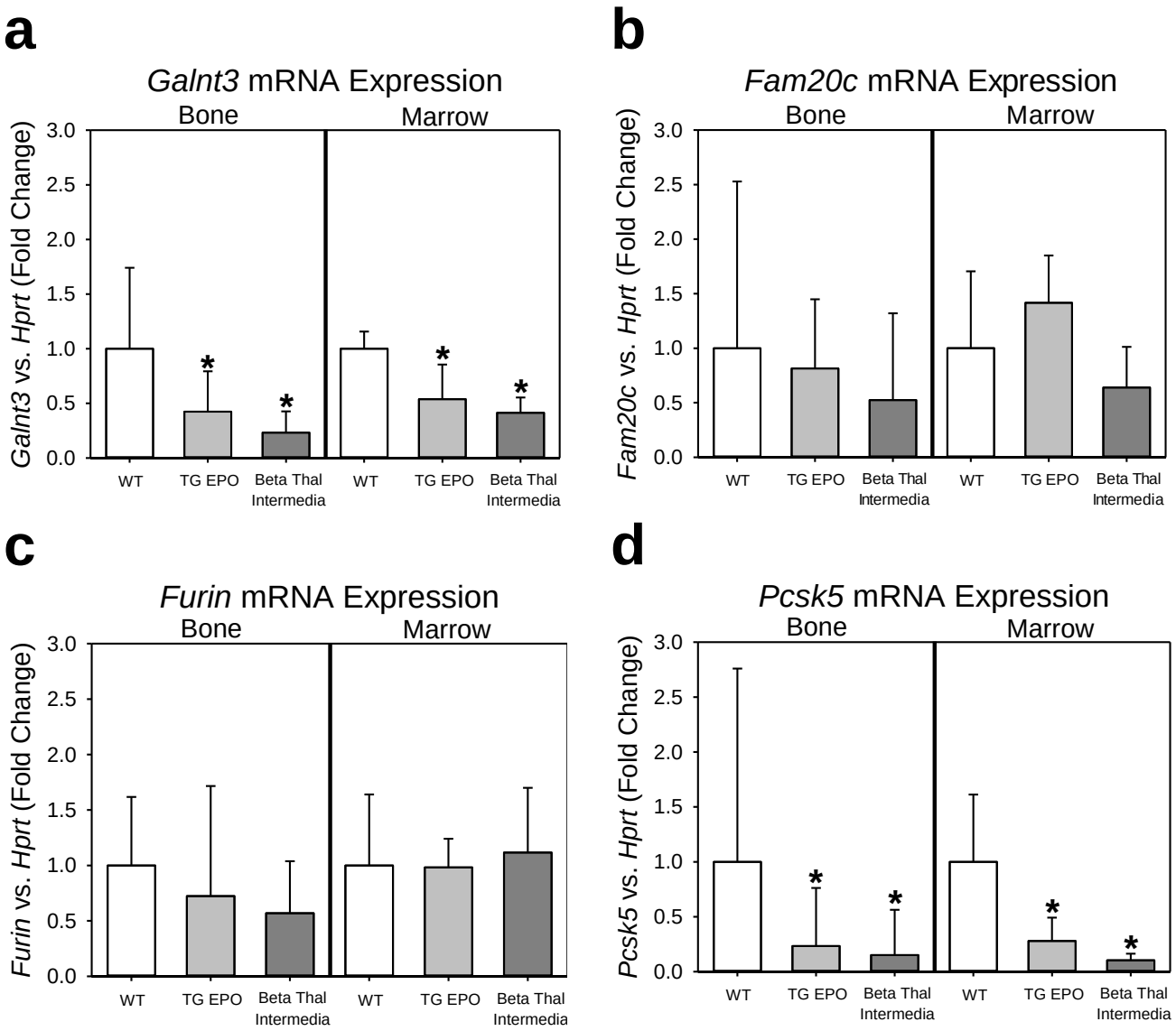
Potential mediator	**Outcome**	**Effect (path)***	**Multivariable model****	
			Coefficient (95% CI)†	**Proportion mediated§**
Hemoglobin	**cFGF23**	Indirect effect (*ab* path)	0.04 (0.01-0.07)	**14.6%**
		Total effect (*ab* + *c*' path)	0.25 (0.16-0.32)	
		Unstandardized total effect	0.29 (0.18-0.40)	

* The coefficients of the indirect *ab* path and the total *ab* + *c'* path are standardized for the standard deviations of the potential mediator, erythropoietin, and outcome.

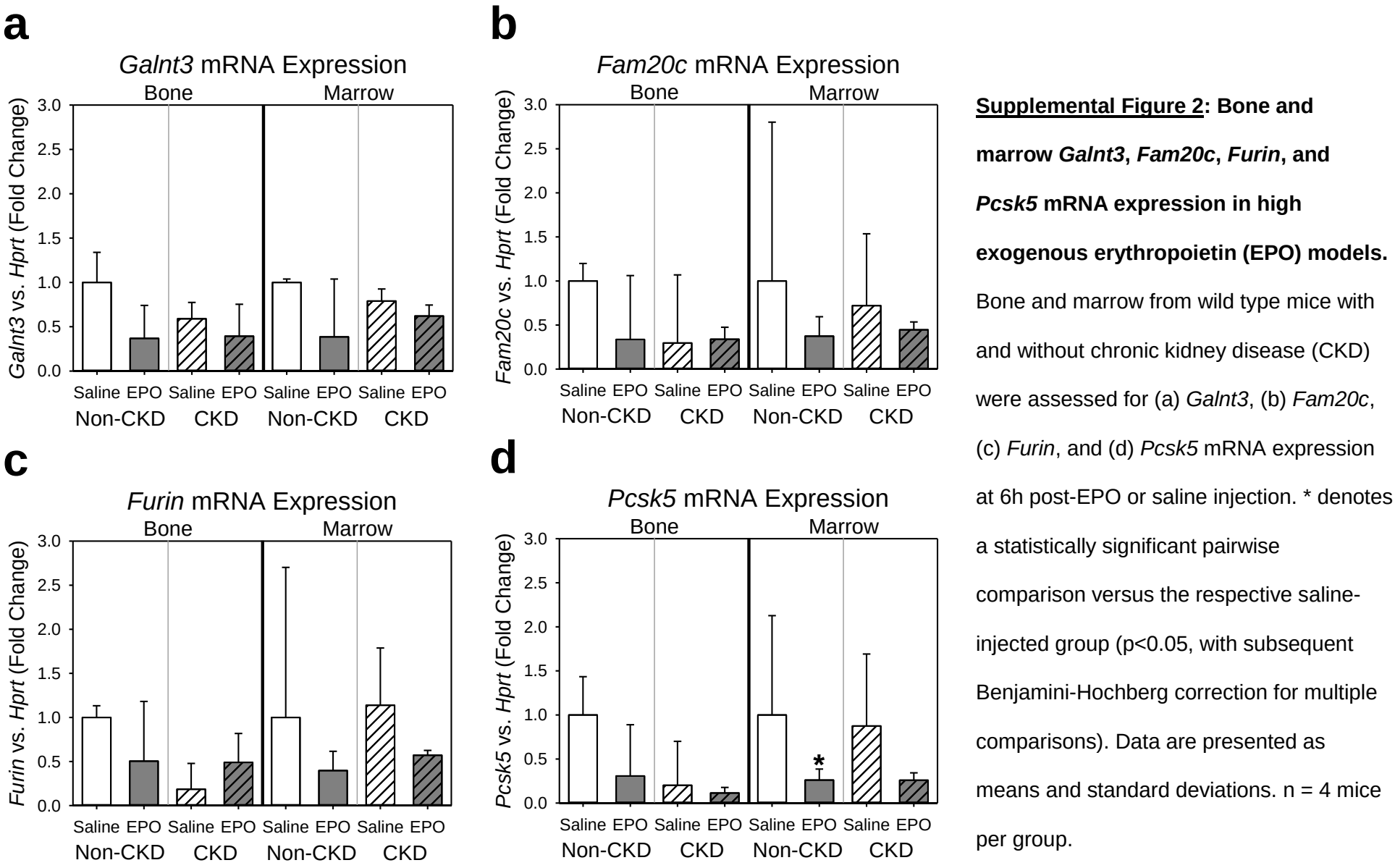
**All coefficients are adjusted for age, sex, eGFR, time since transplantation, serum phosphate, and hs-CRP levels

§The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

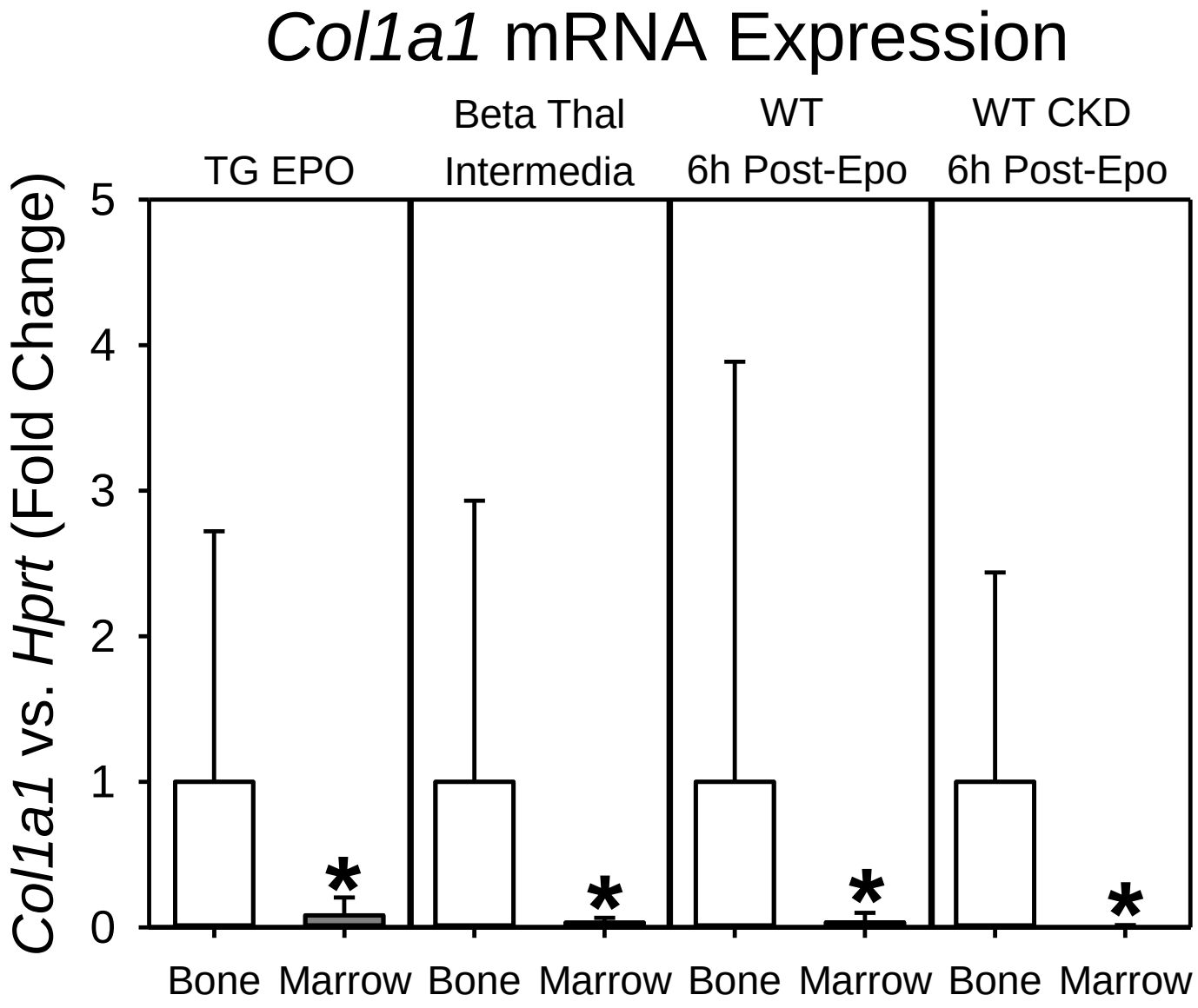
†95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2000 bootstrap samples.



<u>Supplemental Figure 1</u>: Bone and marrow *Galnt3*, *Fam20c*, *Furin*, and *Pcsk5* mRNA expression in high endogenous erythropoietin (EPO) models. Bone and marrow from wild type mice (WT), transgenic erythropoietin overexpressing mice (TG EPO), and beta thalassemia intermedia mice were assessed for (a) *Galnt3*, (b) *Fam20c*, (c) *Furin*, and (d) *Pcsk5* mRNA expression. * denotes a statistically significant pairwise comparison versus the WT group ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). Data are presented as means and standard deviations. n = 7-9 mice per group.



<u>Supplemental Figure 2</u>: Bone and marrow *Galnt3*, *Fam20c*, *Furin*, and *Pcsk5* mRNA expression in high exogenous erythropoietin (EPO) models. Bone and marrow from wild type mice with and without chronic kidney disease (CKD) were assessed for (a) *Galnt3*, (b) *Fam20c*, (c) *Furin*, and (d) *Pcsk5* mRNA expression at 6h post-EPO or saline injection. * denotes a statistically significant pairwise comparison versus the respective saline-injected group ($p<0.05$, with subsequent Benjamini-Hochberg correction for multiple comparisons). Data are presented as means and standard deviations. n = 4 mice per group.



<u>Supplemental Figure 3</u>: Bone and marrow *Col1a1* mRNA expression. Bone and marrow from high endogenous/exogenous EPO groups (transgenic EPO, beta thalassemia intermedia, wild type mice without CKD 6h post-EPO injection, and wild type mice with CKD 6h post-EPO injection) were assessed for *Col1a1* mRNA expression, a bone marker. *p<0.05 for pairwise comparison of marrow versus bone. Data are presented as means and standard deviations. n = 4-7 mice per group.

COMPLETE METHODS:

Animal studies:

Mouse experiments

Experiments were conducted in accordance with UCLA Division of Laboratory Animal Medicine guidelines, and the study protocol was approved by the UCLA Office of Animal Research Oversight. Mice were housed at UCLA, in standard cages with wood chip bedding that was changed twice weekly. Animal housing rooms were temperature and humidity controlled, with a 12-hour light cycle.

Transgenic EPO-overexpressing mice

Transgenic C57BL/6 mice overexpressing human EPO (Tg6 mice)[1] were maintained on diets containing standard iron concentrations. We compared transgenic EPO mice to their wild type littermates, at the age of 7-11 weeks. A subset of transgenic EPO mice received a single intraperitoneal dose of 10 mg iron dextran (Sigma-Aldrich, St. Louis, MO) 24 hours prior to euthanasia. At the time of euthanasia, we collected whole blood, plasma, serum, livers, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Beta thalassemia intermedia mice

Beta thalassemia intermedia mice (*Hbb*$^{th3/+}$;[2] The Jackson Laboratory, Bar Harbor, ME; JAX stock #003253) were maintained on diets containing standard iron concentrations. Mice were euthanized at a median age of 17 weeks (range 9-28 weeks), and we collected whole blood, plasma, serum, livers, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Wild type mouse models, diets, and treatments

Wild type C57BL/6 mice (Jackson Laboratories) were used for experiments assessing the effects of a single rhEPO dose on FGF23. Mouse diets were obtained from Harlan Teklad (Indianapolis, IN), and contained

sufficient iron (50 ppm) and standard phosphate concentrations. For groups of mice in which CKD was induced, the diets also contained 0.2% w/w adenine, as previously described.[3-7] Diets were started at 4-6 weeks of age, and provided ad libitum. Mice remained on the diets for ~5 weeks, then the experiments were conducted. Groups of mice, with and without CKD, received a single intraperitoneal dose of ~67 units/gram rhEPO (BioLegend, San Diego, CA) or saline (vehicle) and were euthanized 6 or 24 hours post-injection. Separate groups that received no injections provided baseline data. At the time of euthanasia, we collected whole blood, plasma, serum, and tibias, from which we flushed the bone marrow with saline solution and 28G syringes.

Mouse biochemical parameters

Complete blood counts were measured by the Hemavet® 950 automated processor (Drew Scientific, Oxford, CT). Colorimetric methods were used to assay serum urea nitrogen (BioAssay Systems, Hayward, CA), phosphate (for Tg6 and *Th3*/+ mice, Stanbio Laboratory, Boerne, TX; for wild type mice treated with EPO, Alfa-Wassermann ACE® Alera and Axcel Systems, West Caldwell, NJ), and iron (Genzyme, Cambridge, MA). Plasma human EPO (in the Tg6 mice) and serum mouse EPO (in the *Th3*/+ mice) were assayed using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN). Plasma C-terminal (total) FGF23 (cFGF23) and intact FGF23 (iFGF23) concentrations were assayed using rodent-specific ELISA kits (Immutopics, San Clemente, CA and Quidel, San Diego, CA). Whereas the cFGF23 assay detects both the full-length, intact hormone and its inactive C-terminal proteolytic fragments, thus functioning as a surrogate measure of overall FGF23 production, the iFGF23 assay detects only the full-length, biologically active form. Percentage iFGF23 was calculated by dividing the iFGF23 values (measured in pg/ml) by the cFGF23 values (also measured in pg/ml) and multiplying by 100, as previously described.[7]

Quantitative hepatic iron concentration

Harvested livers were snap-frozen in liquid nitrogen and stored at -80°C. Small pieces of the livers (~100 mg) were weighed and homogenized. Protein precipitation solution (0.53N HCl and 5.3% trichloroacetic acid in ddH2O) was added, and the samples were boiled and centrifuged. Iron concentrations in the supernatants

were measured by a colorimetric assay (Genzyme), then normalized to the weights of the original samples to yield liver iron concentration.

Quantitative real-time PCR

Flushed tibias and isolated bone marrow were homogenized in Trizol (Invitrogen, Life Technologies, CA) immediately after tissue collection. RNA was then isolated according to the manufacturer's protocol. We performed quantitative RT-PCR using the iScript RT-PCR kit (Bio-Rad, Hercules, CA) and primers specific for mouse *Fgf23*, *Galnt3*, *Fam20c*, *Furin*, *Pcsk5*, and *Col1a1*. Collagen 1a1 (*Col1a1*) mRNA expression was evaluated to assess for residual bone-derived cells in the marrow samples. We used the following PCR conditions: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 10 minutes. Gene expression was normalized to that of hypoxanthine-guanine phosphoribosyltransferase (*Hprt*),[8] and each RNA sample was analyzed in duplicate.

Mouse primer sequences used were:

Fgf23 forward: 5'-ACAGGAGCCATGACTCGAAG-3';

Fgf23 reverse: 5'-GCAATTCTCTGGGCTGAAGT-3';

Galnt3 forward: 5'-ACCAGGGAGGCAAACCATTG-3';

Galnt3 reverse: 5'-TCCTTCTGGATGTTGTGCCG-3';

Fam20c forward: 5'-AACCCATGAAGCAGACGAGAG-3';

Fam20c reverse: 5'-GGAGGGACTCTGCGGAAATC-3';

Furin forward: 5'-GCGCTCGTCCGGAAAAGTT-3';

Furin reverse: 5'-GGACAGGGTAAGGGCCAGAT-3';

Pcsk5 forward: 5'- ACTGCTTACACTACTACTAC-3';

Pcsk5 reverse: 5'- GCCATATTTACAGGAGAGG-3';

Col1a1 forward: 5-CCTCAGGGTATTGCTGGACAAC-3';

Col1a1 reverse: 5-CAGAAGACCTTGTTTGCCAGG-3';

Hprt forward: 5'-CTGGTTAAGCAGTACAGCCCCAA-3';

Hprt reverse: 5'-CAGGAGGTCCTTTTCACCAGC-3'.

Statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (San Jose, CA). Mouse data are presented as means ± standard deviations.

For the mouse studies involving high endogenous EPO models presented in Figure 1, the following four t-tests were performed: (1) wild type (WT) group vs. transgenic EPO group, (2) WT group vs. transgenic EPO group treated with iron dextran, (3) WT group vs. beta thalassemia intermedia group, and (4) transgenic EPO group vs. transgenic EPO group treated with iron dextran. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

For the mouse studies involving high endogenous EPO models presented in Supplemental Figure 1, the following four t-tests were performed: (1) bone samples, WT group vs. transgenic EPO group, (2) bone samples, WT group vs. beta thalassemia intermedia group, (3) marrow samples, WT group vs. transgenic EPO group, (4) marrow samples, WT group vs. beta thalassemia intermedia group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

For the mouse studies involving rhEPO injection presented in Figure 2, within the CKD and non-CKD cohorts, the following four t-tests were performed: (1) baseline group vs. EPO-treated 6-hour time point group, (2) baseline group vs. EPO-treated 24-hour time point group, (3) EPO-treated 6-hour time point group vs. saline-treated 6-hour time point group, and (4) EPO-treated 24-hour time point group vs. saline-treated 24-hour time point group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used. For the mouse studies involving rhEPO

injection, given non-normal data distributions, plasma FGF23 levels were log-transformed prior to statistical analysis.

For the mouse studies involving rhEPO injection presented in Supplemental Figure 2, the following four t-tests were performed: (1) bone samples from non-CKD mice, EPO-treated group vs. saline-treated group, (2) bone samples from CKD mice, EPO-treated group vs. saline-treated group, (3) marrow samples from non-CKD mice, EPO-treated group vs. saline-treated group, (4) marrow samples from CKD mice, EPO-treated group vs. saline-treated group. A p-value of <0.05 was considered to be statistically significant; however, given multiple comparison testing, Benjamini-Hochberg correction was used.

Human studies:

UCLA human studies

The study cohort was comprised of adults and children receiving outpatient care for CKD stages 2–5 (estimated glomerular filtration rate (eGFR) <90 ml/min/1.73m^2) and 5D (dialysis-dependent). We recruited adult and pediatric non-dialysis CKD patients from UCLA general nephrology clinics. We recruited adult and pediatric dialysis patients from UCLA-affiliated Davita dialysis centers. The study was approved by the UCLA Institutional Review Board; informed consent was obtained from adult patients; and informed consent and assent were obtained from parents and pediatric patients, respectively.

Exclusion criteria were: (1) previously diagnosed non-renal cause of anemia; (2) evidence of active bleeding; (3) blood transfusion within four months of enrollment; (4) history of malignancy, end-stage liver disease, or chronic hypoxia; and (5) hospitalization or infection requiring antibiotics within four weeks of enrollment.

Following inclusion, we obtained demographic data and medication history from the medical records. Patients receiving rhEPO were on stable doses for at least four weeks prior to study enrollment, and all rhEPO administered was epoetin alfa (Epogen®, Amgen, Thousand Oaks, CA). We collected whole blood, plasma,

and serum from all enrolled patients. In the dialysis patients, blood was obtained at the initiation of the hemodialysis session. This cohort was first assembled and characterized as part of a cross-sectional assessment of circulating hepcidin levels across the CKD spectrum.[9]

University Medical Center Groningen human studies

Further, we analyzed data from a cohort of post-transplant CKD patients. All kidney transplantations took place at the University Medical Center Groningen (Groningen, the Netherlands). Renal transplant recipients (RTRs) that were more than 1 year post-transplantation were approached for participation during outpatient clinic visits between 2008 and 2011, as described previously.[10] Written informed consent was obtained from 707 (87%) of the 817 initially invited RTRs. For current analyses, we excluded RTRs with missing data on EPO levels (n=27), resulting in 680 RTRs eligible for analysis.

As an independent replication cohort, we repeated the analyses in another RTR cohort with data available on EPO and cFGF23 levels. The replication cohort consisted of 606 RTRs, also all with a functional graft for more than 1 year post-transplant, which had been recruited between 2001 and 2003 at the University Medical Center Groningen. The study has been described in detail previously.[11] For current analyses, we excluded RTRs with missing data on EPO levels (n=14), resulting in 592 RTRs eligible for analysis.

Both studies have been approved by the institutional review board (METC 2008/186 and 2001/039, respectively), and adhered to the principles of the WMA declaration of Helsinki.

UCLA human biochemical parameters

Complete blood counts, including hemoglobin measurements (Sysmex automated hematology analyzer), were performed. In the serum samples, we measured the following parameters: EPO (ELISA, R&D Systems, Minneapolis, MN), creatinine (Roche cobas® 8000 analyzer enzymatic assay, Switzerland), phosphate (Roche cobas® 8000 analyzer ammonium phosphomolybdate photometric assay, Switzerland), calcium (Roche cobas® 8000 analyzer 5-nitro-5'-methyl-BAPTA (NM-BAPTA) photometric assay, Switzerland), intact parathyroid

hormone (PTH; first generation Immutopics assay, San Clemente, CA), iron and total iron binding capacity (Roche cobas® 8000 analyzer Ferrozine colorimetric assay, Switzerland), ferritin (Roche cobas® 8000 analyzer electrochemiluminescence assay, Switzerland), and high sensitivity C-reactive protein (CRP; CardioPhase® hsCRP assay, Dade Behring, Deerfield, IL). Transferrin saturation (TSAT, %) was calculated by iron divided by total iron binding capacity. In the plasma samples, we measured total FGF23 (cFGF23; Immutopics/Quidel) and intact FGF23 (iFGF23; Immutopics/Quidel) concentrations. In the non-dialysis CKD patients, we also measured iFGF23 levels using both the Kainos Laboratories (Tokyo, Japan) assay. In adult patients, eGFR was calculated with the Modification of Diet in Renal Disease (MDRD) Study equation.[12] In pediatric patients, eGFR was calculated with the revised Schwartz equation.[13]

University Medical Center Groningen human biochemical parameters

Blood was drawn in the morning after an 8-12h overnight fast. Total (cFGF23) levels were measured in stored plasma samples using the human FGF23 (C-terminal) ELISA (Immutopics/Quidel). Intact FGF23 levels were measured in stored plasma samples by ELISA (Kainos Laboratories). cFGF23 data were available in both cohorts, but iFGF23 data were available only in the larger cohort of 680 RTRs. In both studies, EPO levels were measured using an immunoassay based on chemiluminescence (Immulite EPO assay, Los Angeles, CA). Further, we measured transferrin (Cobas c analyzer, Modular P system, Roche diagnostics, Mannheim, Germany), ferritin (Modular analytics E170, Roche diagnostics), and serum iron (Modular P800 system; Roche diagnostics). Transferrin saturation (%) was calculated as 100 x serum iron (µmol/L) / 25 x transferrin (g/L). Renal function was determined by estimating GFR by applying the Modification of Diet in Renal Disease (MDRD) Study equation.[12]

UCLA statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (San Jose, CA). Normally distributed data are presented as means ± standard deviation, skewed distributed data are presented as medians with interquartile range, and categorical data are presented as numbers (percentage). Multiple linear regression models, adjusted for demographic, anemia-related, and mineral metabolism covariables (age, eGFR, calcium,

phosphate, PTH, TSAT, ferritin, hemoglobin, and CRP), were developed to investigate the association between serum EPO levels and FGF23 in the non-dialysis CKD cohort, and between rhEPO dose and FGF23 in the dialysis cohort.

University Medical Center Groningen statistical analysis

Statistical analysis was performed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL) and STATA 14.1 (STATA Corp., College Station, TX). Normally distributed data are presented as means ± standard deviation, skewed distributed data are presented as medians with interquartile range, and categorical data are presented as numbers (percentage). Differences in baseline characteristics across EPO tertiles were evaluated with one-way ANOVA, Kruskal-Wallis test, or Chi-square test, as appropriate. Linear regression analysis was performed to assess whether EPO is a major determinant of cFGF23 or iFGF23 levels after adjustment for age, sex, eGFR, time since transplantation, calcium, phosphate, PTH, hemoglobin, ferritin, and CRP levels. In addition, we performed multivariable stepwise backward linear regression analysis to determine whether EPO remained a determinant of cFGF23 levels alongside known determinants. Finally, we determined in mediation analysis according to Preacher and Hayes,[14,15] based on logistic regression, whether hemoglobin mediated the association between EPO and cFGF23 independent of adjustment for potential confounders. In all analyses, a two-sided p-value <0.05 was considered significant.

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