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SUPPLEMENTARY MATERIAL

HEART FAILURE-ASSOCIATED ANEMIA: BONE MARROW DYSFUNCTION AND RESPONSE TO ERYTHROPOIETIN

Willem-Peter T. Ruifrok*[1], Cheng Qian*[1], Herman H.W. Silljé[1], Harry van Goor[2], Dirk J. van Veldhuisen[1], Wiek H. van Gilst[1], Rudolf A. de Boer[1]

* These authors contributed equally to this work

[1] Department of Cardiology, University Medical Center Groningen, University of Groningen, The Netherlands

[2] Department of Pathology, University Medical Center Groningen, University of Groningen, The Netherlands

Supplemental methods

Animal model

We employed 6 week old, male, homozygous TGR(mREN2)27 rats. These rats over express the mouse renin gene (*ren-2d*), and have a phenotype of severe hypertension and left ventricular (LV) hypertrophy, which culminates into heart failure (HF) over the course of 12-16 weeks [1]. The rats were obtained from the Max Delbrück Center for Molecular Medicine, Berlin Buch, Germany (Prof. Dr. M. Bader). Age-matched Sprague Dawley (SD) rats were used as controls (Harlan, The Netherlands). Animals were housed under standard condition.

Experimental protocol

At baseline, three weeks and six weeks, blood was drawn from the tail vein for a full blood count: hemoglobin (Hb), hematocrit (Ht), white blood cell count (WBC), red blood cell count (RBC), red blood cell indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)], platelet count (PLT) and reticulocytes (RET) were measured. In a subset of rats, we analyzed serum iron status at sacrifice to rule out deficiencies. To assess hypertension, each week systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured in conscious rats using a noninvasive blood pressure monitoring system (CODA-6, Kent Scientific Corporation, Torrington, CT) as described before [2]. Mean arterial pressure (MAP) was calculated ((2xDSP+1xSBP)/3). Rats were trained for blood pressure measurement two weeks prior to the start of the experiment. Prior to sacrifice hemodynamic function was assessed invasively by introducing a 2-French microtip pressure-volume transducer (Millar Instr. Inc., Houston, TX, USA) in the right carotid artery and the left ventricle (LV) [3]. Heart rate (HR), LV end diastolic pressure (LVEDP) and maximal indices of contraction and relaxation of the LV (dPdtmax and dPdtmin) were measured. Thereafter, blood was drawn, and hearts were rapidly excised and weighed. Heart tissue was processed for analysis by snap-freezing in liquid nitrogen at -80°C.

Cardiac function was prospectively assessed by echocardiography at baseline, three weeks and six weeks (Vivid 7, GE Healthcare, Chalfont St Giles, UK; equipped with a

10-MHz phase array linear transducer). The echocardiographic measurements were performed under general anesthesia with 2% isoflurane. Both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing were obtained. Long-axis views were obtained, ensuring that the mitral and aortic valves and the apex were visualized. Short-axis views were recorded at the level of mid-papillary muscles. LV end-systolic diameter (LVESD) and LV end-diastolic diameter (LVEDD) were measured from the M-mode. LV fractional shortening was (automatically) calculated as (LVEDD − LVESD)/LVEDD × 100%. Cardiac output was obtained by echo Doppler measurements over the aortic valve.

EPO plasma levels prior to sacrifice were measured with ELISA according to protocol (Quantikine EPO Immunoassay MEP00, R&D Systems Inc, Minneapolis, MN, USA).

Bone marrow isolation and MACs sorting [4]

BM was isolated from tibia and femurs of REN2 and SD rats by flushing bone marrow cavity with Dulbecco's phosphate buffered saline (PBS). Mononuclear cell fraction was obtained by density gradient centrifugation on lympholyte-rat (CEDARLANE, Sanbio BV, Canada). C-kit positive (c-kit$^+$) cells were isolated according to protocol. Briefly, the cell pellet was washed twice with PBS containing 2 mM EDTA and 1% fetal bovine serum (FBS) to remove platelets. Cells were labeled with polyclonal rabbit anti-c kit antibody (Santa Cruz Biotechnology, CA, USA; SC-5535; 1:25 dilution) followed by goat anti-rabbit IgG Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany, 1:5 dilution) for 30 min. at room temperature. The anti-c-kit-labeled cells were sorted by using Mini-magnetic activated cell sorting columns (MS-MACs, Miltenyi Biotech, Bergisch Gladbach, Germany) in a magnetic field. The effluent was collected in chilled IMEM medium and combined with effluents from three washes. c-kit$^+$ cells were collected into a 5 mL polystyrene round-bottom tube with 1 mL chilled IMEM medium and cell number was counted.

Quantitative real-time PCR [5]

Total RNA from c-kit$^+$ cells and LV tissue was extracted using the Nucleospin RNA II kit, according to manufacturer's protocol (Machery-Nagel, Düren, Germany). cDNA synthesis was performed with 0.5 µg total RNA using a specific cDNA synthesis kit

according to manufacturers protocol (Quantitect Rev. Transcriptase kit, Qiagen, Venlo, The Netherlands). Quantitative real-time PCR (RT-qPCR) was performed using SYBR Green mix according to protocol (Absolute SYBR Green ROX mix, Thermo Scientific, Breda, The Netherlands, on C1000 Thermal Cycler CFX384 Real-Time PCR Detection System, Bio-Rad Laboratories, Veenendaal, The Netherlands). All targets were evaluated under the same reaction conditions: 95°C for 15 minutes, then 36 cycles of 95° for 15 seconds and 60°C for 30 seconds. Samples were analyzed by quantification software (Bio-Rad CFX Manager 1.6). mRNA levels were expressed in relative units based on a standard curve obtained with serial dilutions of a calibrator cDNA mixture. To normalize expression data, multiple reference genes were used as described by Vandesompele et al [6]. Reference genes were chosen with little sample-to-sample variability (B2M and GAPDH). Please table S2 for a list of primers used for RT-qPCR.

Supplemental results

S1. Iron status.

	SD-PL (N=8)	**REN2-PL (N=5)**
Iron (µmol/l)	30.9±1.5	27.2±1.7
Folic acid (nmol/l)	>45	>45
Vitamin B12 (pmol/l)	>1476	>1476

Table shows serum iron levels at sacrifice. Data are expressed as mean±SEM. PL = placebo. Folic acid and vitamin B12 serum levels were both above upper detection limit. There was no insufficiency in iron, folic acid or vitamin B12 status.

S2. List of primers used for RT-qPCR.

	RT-qPCR primer, 5' to 3'	
Gene name	**Forward**	**Reverse**
B2M	CTC GGT GAC CGT GAT CTT TC	AGT TGG GCT TCC CAT TCT CC

GAPDH	CAT CAA GAA GGT GGT GAA GC	ACC ACC CTG TTG CTG TAG
ANP	ATG GGC TCC TTC TCC ATC AC	TCT ACC GGC ATC TTC TCC TC
LMO2	TGC AGG CGA GAC TAT CTC AGG	CGC GCA TCG TCA TCT CAT AGG
GATA-1	ATG CCT GCG GCC TCT ACT AC	CAG ATG CCT TGC GGT TCC TC
MMP9	CGG GAA CGT ATC TGG AAA TTC G	CATG GCA GAA ATA GGC CTT GTC
SDF-1	CCG ATT CTT TGA GAG CCA TGT C	TTC GGG TCA ATG CAC ACT TGT C
Ferroportin	GCT GTT TGC AGG AGT CAT TG	TGG AGT TCT GCA CAC CAT TG
TFR	CTT CCG TGC TAC TTC TAG AC	ACA TAG GGT GAC AGG AAG TG

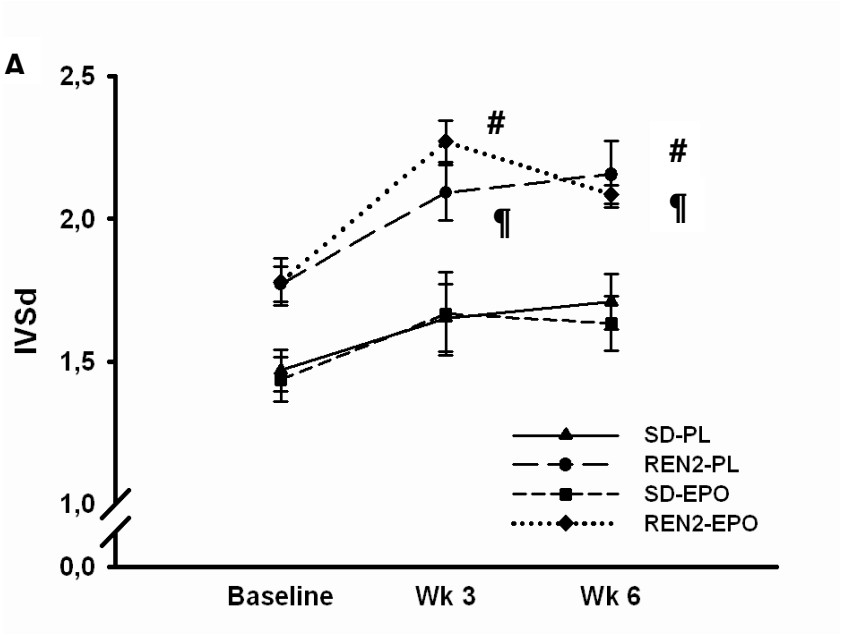
B2M = β_2-microglobulin, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, ANP = atrial natriuretic peptide, LMO = lim-domain partner of TAL, GATA = zinc finger factor that binds GATA sequences, MMP = matrix metalloproteinase, SDF = stromal cell-derived factor, TFR = transferrin receptor.

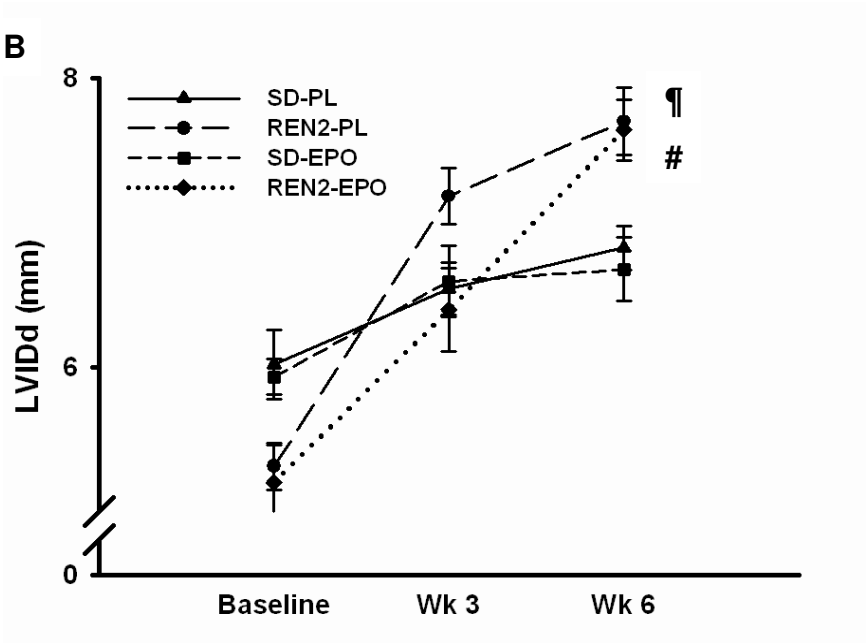
S3. Plasma EPO levels

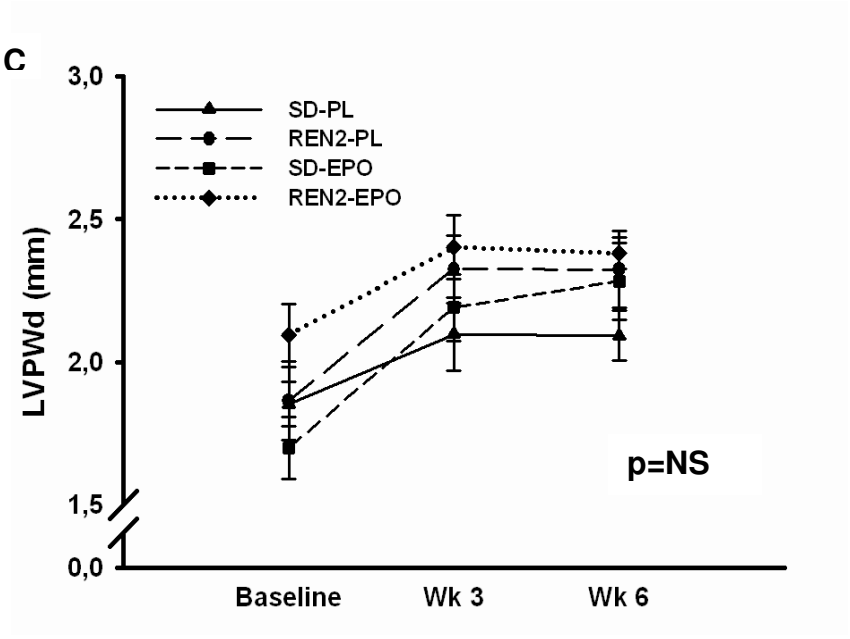
	SD-PL (N=12)	**REN2-PL (N=12)**	**SD-EPO (N=12)**	**REN2-EPO (N=15)**
EPO levels (pg/mL)	13±3	10±4	44±18 *	35±5 †

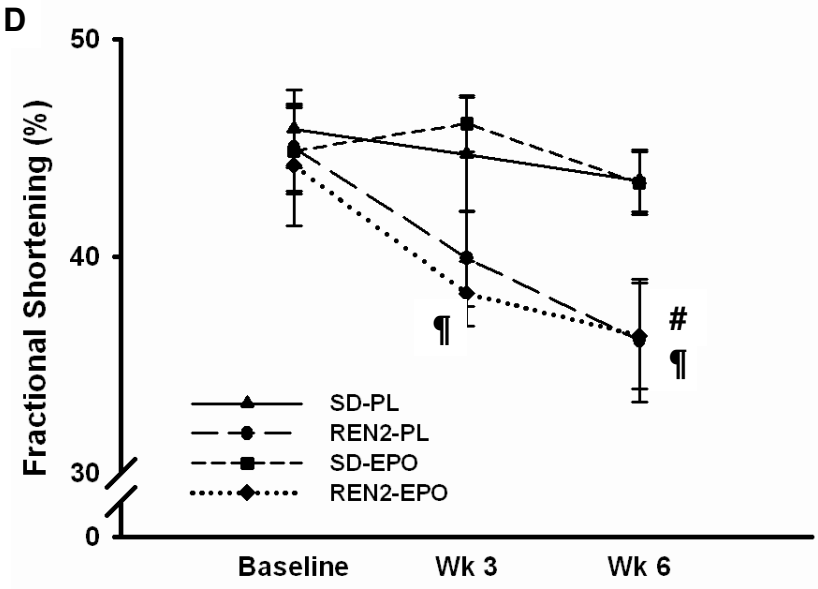
Table shows the plasma EPO levels. PL = placebo. * = $p<0.05$ vs. SD-PL, † = $p<0.05$ vs. REN2-PL. Data is expressed as mean±SEM.

S4. Echocardiographic data during experiment.









IVSd = thickness of the interventricular septum in diastole (A). LVIDd = left ventricular internal diameter in diastole (B), LVPWd = thickness of the left ventricular posterior wall in diastole (C), Fractional shortening (D). PL = placebo, Wk = week. # = $p<0.05$ REN2-PL vs. SD-PL and SD-EPO, ¶ = $p<0.05$ REN2-EPO vs. SD-PL and SD-EPO. Data is expressed as mean±SEM.

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