Materials and Methods

Generation of hBAC-S100 mice. The bacterial artificial chromosome clone CTD - 2120014 (pBeloBAC11-2120014, Invitrogen) containing 60kbp of human DNA of the S100 gene cluster on human chromosome 1q21 including only the genes for S100A12 (=1.89k bp), S100A8 (=1.15kbp), S100A9 (=3.17kbp) with 40kbp of regulatory DNA upstream and 20kbp downstream of the S100A12 gene, as well as 1.4k bp of plasmid DNA was amplified using NucleoBond PC20 (Macherey-Nagel, Germany). Purified DNA was injected into pronuclei of fertilized C57BL/6J mouse oocytes at the University of Chicago transgenic core facility. Seven founder mice were determined by PCR analysis of genomic DNA extracted from tail biopsies using primers specific to human S100A12 cDNA: 5' AGCATCTGGAGGGAATTGTCA and 5'GCAATGGCTACCAGGGATATGAA. Human S100A12 protein was measured in the serum by Sandwich ELISA with specific antibodies against human S100A12 (abcam 37657, and R&D BAF1052) in all 7 founder mice. Two founder hBAC-S100 mice were mated with wild type (WT) C57BL/6J (Jackson Laboratory) and lines were propagated. Hemizygote hBAC-S100 and littermate WT not expressing the transgene were used for all experiments.
hBAC-S100/RAGE$^{-/-}$ mice were generated by breeding with homozygote RAGE-KO mice (also in the C57BL/6J strain) received as gift from Dr. Ann Marie Schmidt, NYU, NY (1). F4 mice (hBAC-S100/RAGE$^{-/-}$ and WT/RAGE$^{-/-}$ littermate) were used for all experiments. All mice were housed at all times in a pathogen-free barrier facility and maintained on normal rodent chow with free access to food and water. Murine IL-6 and IL-22 were measured in the plasma by ELISA (R&D, E Bioscience, respectively)

CKD. Chronic kidney disease was induced in ten-week-old mice (25-30 mice per group) using a model of reversible unilateral ureteral obstruction as previously reported (2, 3). Briefly, the right ureter was obstructed for 6 days and the clip was re-located every other day to prevent irreversible obstruction. After 7 days, the right ureter clip was removed to allow recovery of right kidney, followed by irreversible ligation of the left ureter. Sham-operated mice underwent repeated anesthesia and laparotomy with inspection of the ureter, but without instrumentation to the ureter. Blood urea nitrogen (BUN), calcium, phosphorus and creatinine were measured bi-weekly in the core facility using the Synchron CX system (Beckman Coulter). Only mice with at least a 2-fold increase in BUN at 7 days after the last surgery were included in the CKD group, which was achieved in approximately 90%. Mice were subjected to chronic kidney disease for approximately 10 weeks until being euthanized at the age of 22 weeks. Serum FGF23 was measured at sacrifice by ELISA (Millipore). All procedures were carried out with the approval of the Institutional Animal Care and Use Committee of The University of Chicago and confirm to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Human serum samples. Human serum was obtained with informed consent and approved by the University of Chicago Institutional Review Board from 10 patients with CKD stage III undergoing coronary angiography for stable coronary artery disease. Patients with acute coronary syndrome or acute infections were excluded. Arterial blood was collected in an EDTA-containing vacuum tube and the serum was separated by centrifugation and stored at -80°C prior use for S100A12-ELISA.

Non-invasive blood pressure measurement. Arterial blood pressure was measured by tail-cuff plethysmography with volume pressure recording (CODA Standard, Kent Scientific Corporation) at the end of the study. Blood pressure was recorded for 10 min in awake mice on five consecutive days and analyzed by CODA software. The average systolic and diastolic pressure readings obtained in the last 5 min of each recording period were reported.

In vivo ultrasound. At the end of the experiment, mice were anesthetized with inhaled 1-2% isoflurane titrated to a heart rate of 470-500 beats per minute and prepared for ultrasound using a 40 MHz high frequency Visual Sonics Vevo 660 ultrasound machine. The heart was visualized in one plane from the aortic valve to the transverse aorta in a parasternal long axis. Pulse wave Doppler mode was used to measure mean and peak aortic valve velocity, and mitral valve early (E) and atrial (A) velocity. Continuous Doppler M-mode imaging was used to measure left ventricular end systolic (LVESD) and end-diastolic (LVEDD) diameter and posterior wall thickness during diastole. Systolic left ventricular function is expressed as fractional shortening using the equation: FS (%) = [(LVEDD-LVESD)/LVEDD] x100.

Tissue preparation for histology. The heart with adjacent aorta was removed after in situ fixation with10% formalin at 20 cm hydrostatic pressure prior to processing for immunohistochemistry using at least 10 mice per group. Five μm thick serial sections of the heart and ascending aorta were each stained for H&E, Verhoeff van Giessen (VVG), Masson's trichrome (MT), Alizarin Red (AR) and Fibroblast Growth factor 23 (FGF23 IgG, Santa Cruz, sc-16849). Calcification foci were counted on approximately 50 AR-stained sections (25-200 μm apart) from the apex through the aortic valve per heart. The size of "calcification foci" varied and was counted only if it contained at least two red-stained cells. Single spots that could possibly represent a staining artifact were not counted. Aortic valve thickness was measured as maximal valve thickness of the three leaflets on serial short-axis section (25 μm apart with 6-9 sections of the AV per animal). All images were acquired with a digital camera connected to a microscope (Zeiss) using iVision 4.0 software (BioVision technologies). H&E stained slides of the right kidneys were scored in a blinded manner for glomerulonephritis, interstitial fibrosis/tubular atrophy and interstitial inflammation by a board-certified pathologist as previously reported (2). Briefly, scores of 0-3 were used for semi-quantitative assessment with 0 =<5%, 1= 6-25%, 2= 26-50%, and 3=>50% involvement.

Tissue preparation for immunoblotting and RT-PCR. The heart was cannulated in situ immediately after euthanasia and flushed with normal saline for 5 min prior to tissue removal using at least 10 mice per group. The left ventricles were cut from the apex to the base into two equal-sized portions and were stored at -80°C for extraction of whole tissue lysates (Pierce, supplemented with Triton X 0.1%) and for extraction of RNA.

Immunoblotting was performed on 20 μg protein using standard methods with anti FGF23 (Santa Cruz, sc-16849) and anti-beta-actin IgG (Cell signaling, 4970).

Quantitative real time RT PCR. Total RNA was isolated from the left ventricle (RNeasy mini kit, Qiagen) and from white blood cells (QIAampRNA blood mini kit, Qiagen) including a DNAse step. RNA quality was determined by the ratio of absorbance at

260nm/280nm. cDNA was synthesized from 1 μg RNA using Superscript first strand synthesis system (Invitrogen) followed by PCR amplification using SYBR GreenER (Invitrogen) with a IQ5 cycler (BioRAD). All PCR amplifications were carried out in triplicate. Primers were designed on the primer bank MGH library and are available upon request. The ΔC_T value was used to describe the difference between the hBAC-S100 and WT normalized to the house keeping gene β-actin. Relative mRNA expression was estimated as 2exp (ΔC_T target gene- ΔC_T housekeeping gene). There was no significant difference in absolute C_T values for the amplification of β-actin among the different experimental groups (C_T ranging from 19.59-20.52), indicating that RNA quality and abundance of this house keeping gene was not affected by the experimental design.

Isolation of neonatal and adult cardiac fibroblasts and myocytes. Ventricles from 1-day old mice or 6-week old mice were separated from 5 hearts and were minced. Tissue was pooled for digestion in balanced salt solution containing 0.1% collagenase type 2 (Worthington Biochemical) as previously described(4). Cardiac fibroblast (CF, passage 3-5) and myocytes (passage 0, day 3-5) were cultured in six well plates in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco). Confluent CF were treated as indicated with rS100A12 (1 μg/ml; R&D Systems) with or without osteogenic induction DMEM containing Dexamethasone (10nM; Sigma), Ascorbic acid (50μg/ml; Sigma), β-Glycerophosphate (10mM; Sigma), 10% FBS and 1% antibiotic-antimycotic (Gibco) for 1-20 days with medium changes every 48 hours.
Confluent CF were placed in serum free DMEM for 24h, followed by stimulation with cytokines IL-6 (25-100ng/ml), TNFα (25-100 ng/ml), LPS (100ng/ml), or 3% serum harvested from hBAC-S100 mice for 6h prior harvesting of RNA, or for 24h prior harvesting of cell protein and culture supernatant.

Statistics: At least 10 mice were analyzed per group, and all cell culture experiments were performed three times in triplicates. All continuous data were reported as mean ± SEM and discrete variables were summarized by percentage. Mann-Whitney U-test, independent sample t-test and one-way analysis of variance were used for mean comparison between two or multiple groups, respectively. The Bonferroni correction was used to adjust for multiple comparisons. Two tailed probability values of P less than 0.01 were considered statistically significant for each test to ensure an overall study significance level of P less than 0.05. Fisher's exact test was used to analyze the difference frequency of categorical data in mice.

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