Supporting Information

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SI Methods

Cell Culture and Human Skin Equivalents. Primary human fibroblasts (Fb) and keratinocytes (Kc) were isolated from human neonatal foreskin as previously described (1). The acquisition of all human skin specimens was approved by the appropriate institutional review boards. Primary human Fb were grown in DMEM/10%FBS (Invitrogen Life Technologies), and primary human Kc were grown in serum-free medium plus supplements (Invitrogen Life Technologies). Human skin equivalents (HSE) were generated as previously reported (1, 2). Briefly, 75,000 Fb (50% MDR-positive in some experiments) were mixed with 3 mL of type I bovine collagen (Organogenesis Inc.) mixture and poured into a Transwell-COL permeable support (cat. # 3492; Costar) to generate the dermis equivalent. After culturing for 5–7 days with DMEM/10%FBS at 37 °C and 5% CO_2, 1×10^6 genetically modified Kc (50% MDR-positive) were seeded onto the dermis equivalent and cultured for 2–4 days submerged in Epithelialization medium. HSE were raised to the air–liquid interface with Cornification medium beneath the Transwell support for 2 days before grafting onto mice or for up to 8 days for in vitro experiments.

Retroviral Expression Vectors and Gene Transfer. The bicistronic retroviral vector pQCXIX (Clontech) was used to generate control (Ctl-MDR) and therapeutic (Ther-ANP-MDR; Fig. 1*A*) vectors. To generate Ctl-MDR vector, the multidrug resistance (MDR1) cDNA was first retrieved from pHaMDR1/A vector [kindly provided by Michael Gottesman, National Institutes of Health (NIH)] using restriction enzymes and a MLU1 linker (5′-pGGACGC-GTCCGC-3′). The MDR1 fragment then was cloned in the second position of the pQCXIX vector after digestion with MLU1 and XhoI (New England Biolabs, Inc). The Ther-ANP-MDR vector was generated by cloning an atrial natriuretic peptide (ANP) cDNA, previously amplified from a human heart cDNA library (Ambion), using primer sets 5′-AGC GGC CGC TTG GAT TGC TCC TTG ACG ACG-3′ (forward) and 5′-CGG GAT CCC GTC CTC CCT GGC TGT TAT CTT CAG-3′ (reverse), into the first position of the Ctl-MDR vector, after digestion with NotI and BamHI (New England Biolabs, Inc). Retroviral vectors were transfected into the Phoenix amphotropic packaging cell line (Clontech) using FuGENE 6 (Roche). Twenty-four hours after transfection, colchicine (50 ng/mL) (Sigma-Aldrich) was added into the culture medium for 14 days. The stable clone with highest viral titer was identified for each vector (Ctl-MDR and Ther-ANP-MDR) and was expanded for virus production. Retroviral transduction of early-passage Fb and Kc with Ctl-MDR or Ther-ANP-MDR viruses was achieved by centrifuging the target cells and the virus together at $200 \times g$ for 1 h (32 °C) followed by incubation at 37 °C in 5% CO_2 for 3 h. The culture medium was changed, and cells were incubated overnight. The cells were reexposed to Ctl-MDR or Ther-ANP-MDR virus on day 2 using the same protocol.

Flow Cytometry Analysis. Five days posttransduction, Fb and Kc were collected after trypsinization for rhodamine assay or anti-ANP antibody labeling using mAb ANP (ab20893; Abcam) as the primary antibody and rat anti-mouse IgG_1-PE (BD Biosciences) as the secondary antibody. For the rhodamine assay, Fb or Kc were replated into 6-well plates and incubated overnight. The following day, monolayer cultures of Fb or Kc were incubated for 20 min with rhodamine (250 ng/mL) (Sigma-Aldrich) and then in fresh cell medium for 2 h. Before intracellular ANP detection, the cells were incubated with BD Golgistop (BD Biosciences) at 37 °C in 5% CO_2 for 4 h. We used 7-AAD labeling to exclude dead cells, and cells were analyzed using the FACScalibur flow cytometry system (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc.).

Immunohistochemistry for ANP. Paraffin-embedded sections of normal and genetically modified HSE were H&E stained. Unstained sections also were incubated in an antigen-unmasking agent, Retrieve-All (Signet Laboratories), and ANP was detected by immunohistochemistry using anti-ANP mAb (Abcam), VECTASTAIN elite ABC kit (Vector Laboratories, Inc), diaminobenzidine (DAKO), and Mayer's solution (Sigma-Aldrich) to counterstain nuclei.

cGMP Assay. During in vitro culture of HSE, 1 mL of medium was taken 4 h after the medium was changed, and 500 μL was incubated at 37 °C in 5% CO_2 for 4 h with 1×10^6 NCI-H1688 cells (corin-expressing cell line) that can convert inactive precursor ANP (pro-ANP) to active α-ANP. BHK-21 cells that express the α-ANP receptor, NPR-A, and can be activated by α-ANP subsequently were incubated with a 100-μL aliquot of medium that was either exposed or not exposed to corin-expressing NCI-H1688 cells for 10 min at 37 °C in 5% CO_2. The BHK-21 cells then were lysed, and a cGMP assay was performed using a BioTrak kit (Amersham Biosciences). Results were obtained using a spectrophometric plate reader (Bio-Tek Instrument, Inc.) at 630 nm and Kcjunior software (Bio-Tek Instrument, Inc.). Vasopressin (10 nM) was used as a negative control, and sodium nitroprusside (1 mM) was used as a positive control to induce cGMP.

ANP Measurements in Culture Medium and Mouse Plasma. The amount of ANP secreted into the medium was measured by RIA (Amersham Biosciences) following the manufacturer's protocol. For in vivo studies, human ANP plasma levels were determined at 4 months, 7 months, and 12 months postgrafting, using an RIA (Shionoria ANP; Shionogi & Co) that is specific for human ANP. Serial measurements of human ANP at 4 months and 7 months were performed in the same individual mice when possible. Peripheral blood was collected into a 1.5-mL Eppendorf tube containing 1,000 KIU/mL aprotinin (MP Biomedicals, LLC) and 1 mg/mL EDTA (Sigma-Aldrich). Plasma was separated from the red blood cells by centrifugation at $2,000 \times g$ for 30 min at 4 °C. The human ANP level in 100 μL of plasma was determined using the immunoradiometric assay for human ANP as previously described (3).

Human Skin Equivalent Grafting. Four- to 5-week-old immunocompromised NIH male Swiss *nu/nu* mice (Taconic Farms), housed and used in accordance with NIH institutional guidelines, were grafted with genetically modified HSE. All animal studies were approved by the National Cancer Institute Animal Care and Use Committee. Grafts were placed on the muscle fascia in the correct anatomical orientation (epidermis side up), covered with sterile Vaseline gauze (Sherwood Medical Industries), and secured with a 0.75-inch × 3-inch tape dressing (Johnson & Johnson). The dressing was changed after 2 weeks and was removed after an additional 1 or 2 weeks. Successful graft take usually was evident ≈4–6 weeks after grafting.

In Vivo Topical Colchicine Treatment. A chamber was constructed as depicted in Fig. S5. A 0.1-g dose of colchicine cream (450 μg/g) was applied to human skin grafts for a total of 4 weeks (45 μg colchicine 3 times per week). After each application the chamber was covered with a Band-Aid (Johnson & Johnson) to enhance penetration into the grafted HSE. Colchicine cream was pre-

pared as previously described (4). Briefly, colchicine (Sigma-Aldrich) was dissolved in DMSO, diluted 1:100 with distilled deionized water, and mixed with Velvachol cream (Healthpoint) by stirring to obtain a final dosage of 450 μg/g.

Renin Assay. Plasma renin levels were determined in mice 6 months after grafting. Renin levels were determined in individual grafted mice at 2-week intervals for 4 weeks to control for variability in individual mice; then averaged values for each mouse were calculated. Blood was collected from unanesthetized mice into 75-μL hematocrit tubes that contained 1 μL 125 mM EDTA. Plasma was separated from red blood cells by centrifugation and was frozen until used for renin determination. After a 5-fold dilution of 2 μL of plasma, renin concentration was measured by an RIA (Gammacoat; DiaSorin) that detected the generation of angiotensin-I from an excess of rat substrate angiotensinogen, with final plasma dilutions varying between 1:500 and 1:1,000. Angiotensin-I generation was determined for a 1-h incubation period at 37 °C and was expressed as an hourly average.

Blood Pressure Measurement by Telemetry. Telemetry studies to monitor real-time blood pressure in individual mice were performed 8–10 months after the ANP-expressing HSE were grafted. Telemetric transmitters were activated magnetically >24 h before implantation. HSE-grafted mice were anesthetized with ketamine and xylazine (90 and 10 mg/kg, respectively), and the left carotid artery was isolated. The telemetric catheter was inserted into the left carotid artery and advanced to the aortic arch. The telemeter body (model TA11PA-C20; Data Sciences International) was placed in an s.c. pocket on the right flank (5, 6). One day after surgery, each animal was returned to its home cage with food and water ad libitum for the duration of the study. The transmitter signal was captured and processed using a model RPC-1 receiver, a 20-channel data-exchange matrix, APR-1 ambient pressure monitor, and a Dataquest ART 2.3 acquisition system (Data Sciences International). The recording room was maintained at 21–22 °C with a 12:12-h light-dark cycle. Blood pressure recording began 1 week after surgery and continued for 1 week, during which time the mice ate a normal diet. Some mice then were fed a high-salt diet for 3 weeks, during which time blood pressure was again recorded.

Statistical Analysis. Initial telemetry data analyses were performed using the Dataquest A.R.T. 2.3 analysis program (Data Sciences International). Data are expressed as means ± SE. Statistical comparisons were done by paired and unpaired Student's *t*-test for comparisons of ANP level, renin level, mean arterial pressure, systolic arterial pressure, diastolic arterial pressure, heart rate, pulse pressure, and mean activity between control (Kc-MDR/Fb-MDR) and ANP-expressing (Kc-ANP/Fb-MDR, Kc-MDR/Fb-ANP, and Kc-ANP/Fb-ANP) mice. *P* values ≤0.05 were considered to be significant. Error bars represent the SD (Figs. 4*A*, 5, and 6), except for bar graphs (Figs. 3 *A* and *B* and 4 *B–D*) where average deviation is shown.

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2. Garlick JA, Taichman LB (1994) Fate of human keratinocytes during reepithelialization in an organotypic culture model. *Lab Invest* 70:916–924.
3. Clerico A, et al. (1996) Analytical performance and clinical usefulness of a commercially available IRMA kit for measuring atrial natriuretic peptide in patients with heart failure. *Clin Chem* 42:1627–1633.
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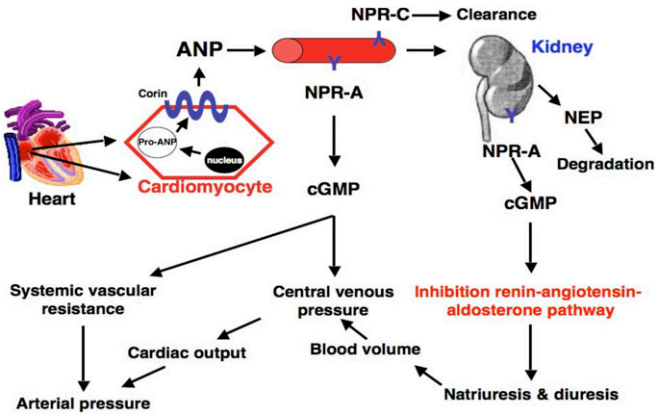

Fig. S1. ANP regulation of arterial pressure in humans. Briefly, ANP is produced and packaged into granules as pro-ANP in cardiomyocytes located in the right atrium. Following elevation of central venous blood pressure, pro-ANP is secreted and converted by the corin receptor, also present on the cardiomyocytes, into active α-ANP. α-ANP binds its specific receptor, NPR-A, expressed on endothelial and kidney cells and induces cGMP production. Decreased arterial pressure results from inhibition of the renin-angiotensin-aldosterone pathway with decreased volume and/or decreased systemic vascular resistance and central venous pressure.

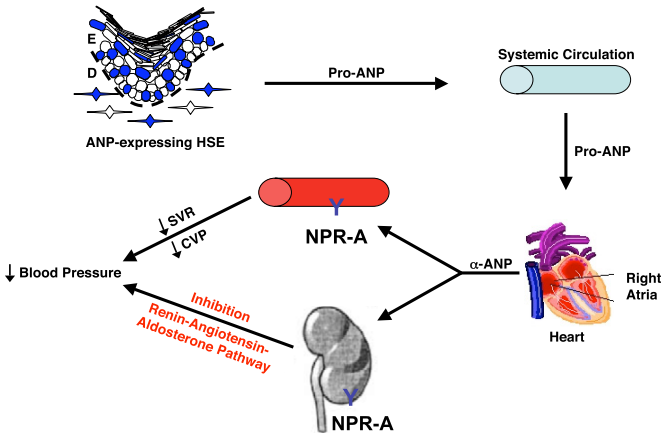

Fig. S2. Mechanisms of blood pressure regulation in mice grafted with HSE that secrete human pro-ANP.

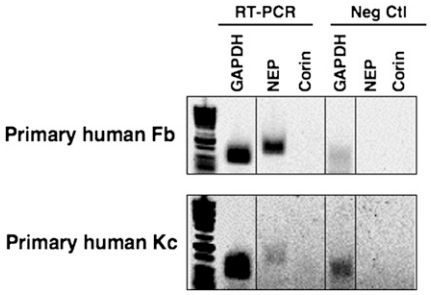

Fig. S3. RT-PCR analysis of neutral endopeptidase (NEP) and corin receptor expression by human Fb and human Kc. Briefly, total RNA was isolated from primary Kc and Fb using TRIzol reagent and a RNAeasy mini kit (Qiagen), and RT-PCR was performed using primer sets for GAPDH (5′-GTC AAT GTC CCA AAC GTC ACC AGA-3′ and 5′-ATT TCG GGA ATG CTG AGA AAA CAG ACA GA-3′), NEP (5′-TGT GGC CAG ATT GAT TCG TC-3′ and 5′-TTG TAG GTT CGG CTG AGG CT-3′), and corin (5′-AAC AAA AGG ATC CTT GGA GGT CGG ACG AGT-3′ and 5′-CGG AGC CCC ATG AAG TTA ATC CA-3′).

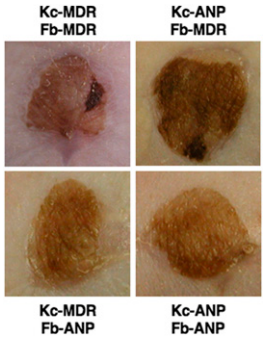

Fig. S4. A representative photograph of HSE grafted to immunocompromised mice (Kc-MDR/Fb-MDR, Kc-MDR/Fb-ANP, Kc-ANP/Fb-MDR, and Kc-ANP/Fb-ANP). The skin pigmentation pattern is heterogeneous because the epidermis in the HSE contains melanocytes derived from individuals with variable degrees of pigmentation.

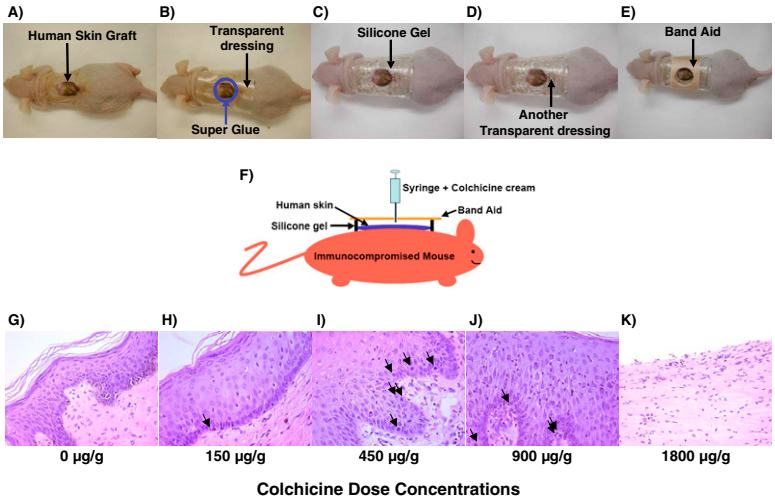

Fig. S5. Construction of a chamber that allows repetitive colchicine treatments of human HSE grafts on unanesthetized mice. (*A*) Immunocompromised mouse grafted with human skin. (*B*) A transparent dressing containing an opening is wrapped around the mouse to expose the HSE graft. Loctite Super Glue Control Gel (Loctite) is applied around the edge of the HSE graft. (*C*) Silicone gel containing an opening is placed on the top of the transparent dressing. (*D*) Another transparent dressing containing an opening then is wrapped around the mouse. **(***E*) A Sheer Comfort-flex adhesive bandage (BAND-AID, Johnson & Johnson) with a hole is added to facilitate the removal of a second Sheer Comfort-flex bandage that is used to seal the chamber when colchicine cream is applied. (*F*) Schematic showing the chamber with the second adhesive bandage as well as the syringe injection of colchicine cream. Colchicine doses were optimized by first determining the number of Kc blocked in mitosis following treatment with different doses, while tissue architecture was preserved. (*G*–*K*) H&E staining of human skin grafts treated with vehicle control (0 μg/g) (*G*) or with increasing concentrations of colchicine: 150 μg/g (*H*); 450 μg/g (*I*); 900 μg/g (*J*); and 1,800 μg/g (*K*). A dose of 45 μg (0.1 g of a 450-μg/g colchicine cream applied 3 times per week) was found to be optimal at blocking Kc mitosis while preserving epidermal architecture. Arrows indicate basal cells blocked in mitosis with condensed chromosomes and mitotic spindles. Magnification, 400×.

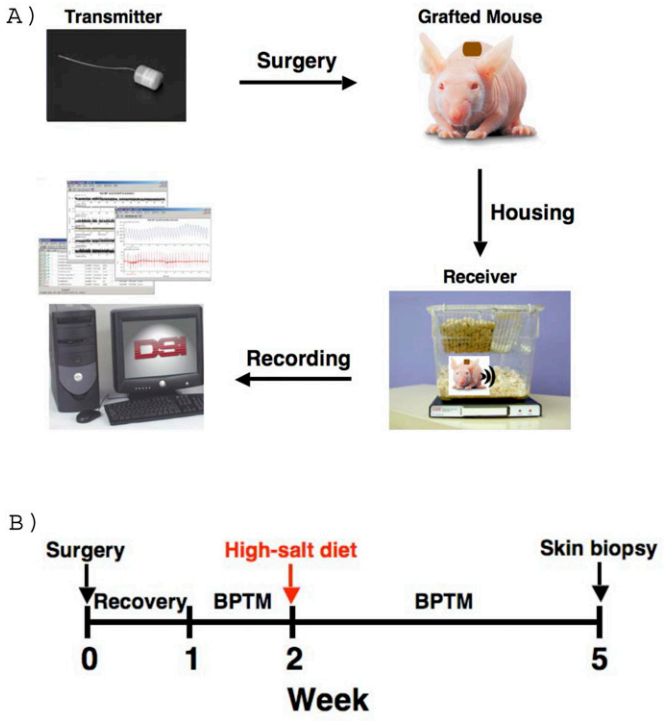

Fig. S6. Schematic of blood pressure telemetry system and experimental timeline. (*A*) Telemetric blood pressure monitoring following insertion of telemetric transducers and transmitters (model TA11PA-C20; Data Sciences International) into the left carotid arteries of the mice. The telemetric signal was processed using a model RPC-1 receiver, a 20-channel data-exchange matrix, APR-1 ambient pressure monitor, and a Dataquest ART 2.3 acquisition system (Data Sciences International). (*B*) Experimental protocol for blood pressure telemetric measurement (BPTM) to determine the basal blood pressure of each individual mouse between postoperative week 1 and 2. The mice then were fed a high-salt diet for 3 weeks, and BPTM was recorded. After 5 weeks, a biopsy of the human HSE graft was taken.