ONLINE SUPPLEMENT

CORIN IMPROVES CARDIAC FUNCTION, HEART FAILURE AND SURVIVAL IN MICE WITH DILATED CARDIOMYOPATHY

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Methods

Assessment of corin function in vivo. Anesthesia was induced with 3% followed by 1.5% isoflurane. Mice were mechanically ventilated as described. Body temperature was maintained at 37°C by a warming pad. Mice (age-matched females) were randomly assigned to receive intravenous pro-ANP in PBS or PBS alone. Hemodynamics were measured as we have described. Retro-orbital blood was collected ≥ 1 week before and immediately after hemodynamic measurement into EDTA (2ul, 500mM) siliconized (AquaSil, Pierce, Rockford, IL) tubes (Natelson, Fisher Scientific, Pittsburgh, PA) and spun at 1460 x g at 4°C for 20 mins. Plasma was stored at -20 ºC for assay.

Pro-ANP production. Pro-ANP was expressed recombinantly with a C-terminal V5 tag in human embryonic kidney HEK293 cells in serum-free conditioned medium as described, lyophilized and dissolved in sterile PBS. Pro-ANP was characterized by immunoprecipitation followed by Western blot and ELISA as described.

Enzyme immunoassay for mouse corin and cGMP in plasma. Plasma cGMP and corin levels were measured in duplicate or triplicate by immunoassays (Assay Designs, Inc., Ann Arbor, MI and USCN Life Science Inc., China).

Northern analysis and DNA probes. Total mouse heart RNA was isolated with TRIZol reagent (Invitrogen) according to the manufacturer’s protocol. RNA (10 µg) electrophoresed on 1.0% formaldehyde-agarose gels and transferred to nitrocellulose. After pre-hybridization (30 min.) Northern blots were hybridized (ExpressHyb, BD Biosciences, San Diego, CA) for 60 minutes with a corin-(32P)dCTP probe (2 x 10⁶ cpm/ml) labeled with Ready-To-Go DNA labeling beads, Amersham Biosciences, Piscataway, NJ). After washing blots were visualized by a Storm 840 (Amersham Biosciences). Results were normalized to GAPDH control.

Real-time polymerase chain reaction (RT-PCR). Total RNA was extracted from whole hearts using the RNeasy® Mini Kit (Qiagen). First strand cDNA synthesis was performed with 1 µg of total RNA (Transcriptor First Strand cDNA Synthesis Kit, Roche). Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler® 480 System following the manufacturer’s protocol. Specific primers were: ctggaaggattgttgagag and aegctctgctgtctctca for corin; tcatcagagggtcactg and gcctgtgaaggggtgatta for BNP; cacagctgtgattcaaga and ctcttcacattgccg for ANP; gggacgtttaaccgcgcga and gcatctccatctgtggtt for CREB; catgttaaccttccagcact and gcacgtaaccttgattc for collagen 1; gcacgtaaccttggagtg for corin; gacgttaccttcaccttggtga for MMP-9; cagcagcagcacacgttgcttggttggg for TGFbeta1; tggagacatctctctcctctc and cgtgcctccaatgatctctc for CMA1; acgacatagacggcatcag and gctggttcagttggtt for furin. PCR was performed at: 95°C for 5 min, followed by 40 cycles of 95°C (10 s), 60°C (30 s), and 72°C (10 s). PCR products were confirmed by melting curve analysis using the Lightcycler Software 4.0 and samples normalized to a β-actin control. Experiments were performed in triplicate and the qRT-PCR was subjected to log transformation as recommended to achieve a normal distribution.

Immunohistological Analysis. Frozen mouse hearts were embedded in OCT (Sakura Finetek U.S.A. Inc., Torrance, CA) and cut into 5 µm cryosections. Slides were fixed (10% formalin, 20 min), rinsed in PBS and incubated with 0.3% H₂O₂ for 30 min. Non-specific antibody binding was blocked with 5% goat serum for 30 min. Corin was detected with rabbit polyclonal anti-mouse corin protease domain antibodies (1:700 dilution in PBS containing 5% goat serum) for 60 min at 21°C followed by biotinylated goat anti-rabbit secondary antibody (Vector Lab., Burlingame, CA) and avidin horseradish peroxidase (Vector Lab.). Immuno-
reactivity was demonstrated by 3,3'-diaminobenzidine (DAB, Vector Lab.). Nuclei were identified by hematoxylin counterstaining. Slides were scanned (Aperio's ScanScope) and images were taken using ImageScope software (MAN-0001, revision G). In control sections primary antibodies were replaced with pre-immune rabbit serum. Images (10 random fields from each mouse) were analyzed using ImagePro Plus 6.2 software (Media Cybernetics, Bethesda, MD)

Masson’s trichrome stain was used to detect fibrosis. Both perivascular and interstitial fibrosis were measured in 5 hearts from each group. The digital images of ten random ventricular fields (40 x) were analyzed using ImagePro Plus 6.2 software and the mean fibrosis for each mouse was determined (Media Cybernetics, Bethesda, MD).

Lung Edema and Lung Water Retention Analysis. Formalin-fixed lung sections were stained by hematoxylin and eosin. Images (10 random fields from each mouse) were analyzed using ImagePro Plus 6.2 software (Media Cybernetics, Bethesda, MD) to determine the percent of total alveolar area free of congestion in each field (20 x) by comparison to normal wild-type controls as a reference. Lung edema was also assessed by wet/dry lung weight ratios as described 7. Right and left lungs were excised and rapidly weighed (wet weight) and then were oven dried (65°C; Fisher Isotemp, Fisher Scientific) for 72 h to a stable dry lung weight. Data are presented as the ratio of right+left lung wet weight/ right+left lung dry weight.

Western blot analysis. Equivalent amounts of protein extracted from whole frozen heart samples (n=2-4 per group) were subjected to reducing SDS-PAGE, electroblotted to Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) and probed with rabbit polyclonal anti-corin stem or protease domain antibodies as described 2, 6. Protein loading was normalized by α-actin (polyclonal anti-actin antibody, Santa Cruz) or Glut4 (Glug 4 mouse monoclonal antibody sc-53566, Santa Cruz).

Pro-ANP processing by mouse heart tissue. Frozen hearts were homogenized in 10 mM Tris-HCl, pH 7.4 buffer. The homogenate was centrifuged at 10,000 g for 30 min at 4 °C. Pellets were washed with ice-cold 10 mM Tris-HCl, pH 7.4 buffer and centrifuged. Washed pellets were combined with pro-ANP medium containing 1 mM EDTA, 20 mM CaCl2, 50 µM soybean trypsin inhibitor, 0.1% Triton X100, proceeded by ultra-sound for 10 inputs, and incubated for 5.5 h at 37 °C. The pH of reaction mixture was adjusted by 3 M Tris-HCL, pH 8.0. ANP was immunoprecipitated from the conditioned medium by a mouse monoclonal anti-V5 tag antibody (Invitrogen, Carlsbad, CA) coupled to protein A –Sepharose (Pierce, Rockford, IL) 6. Immunoprecipitated proteins were solubilized in SDS-PAGE sample buffer and analyzed by Western blotting under reducing conditions with an anti-V5 tag rabbit polyclonal antibody (Immunology Consultants Laboratory, Inc., Newberg, OR) followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit/or goat anti-mouse antibody, and detection by ECF substrate (Amersham Biosciences, Piscataway, NJ) 6.

Echocardiography. Transthoracic echoes were performed by an echocardiographer blinded to genotype with a VisualSonic Vevo 2100 Imaging Sytem (VisualSonic Inc. Toronto, Canada) as we previously described with some modifications 8. Briefly, female 3.5-month-old mice were sedated with 1.5% inhaled isoflurane; the hemithorax of each mouse was carefully shaved, and two-dimensional and M-mode images of LV at the long axis were recorded. M-mode images were analyzed using Vevo software; left ventricular end-diastolic dimension (LVEDD) and left ventricular end-systolic dimension (LVESD) were measured at least 6 times and averaged for each mouse. All measurements were performed using edge-to-edge convention adopted by the American Society of Echocardiography. The %FS and ejection fraction (%EF) were calculated.
Supplemental References


4. Ibebuogu UN, Gladysheva IP, Houng AK, Reed GL. Decompensated heart failure is associated with reduced corin levels and decreased cleavage of pro-atrial natriuretic peptide. *Circ Heart Fail*. 2011;4:114-120.


6. Gladysheva IP, Robinson BR, Houng AK, Kovats T, King SM. Corin is co-expressed with pro-anp and localized on the cardiomyocyte surface in both zymogen and catalytically active forms. *J Mol Cell Cardiol*. 2008;44:131-142.


Figure S1. Relative cardiac ANP expression in DCM and wild-type (WT) assessed by qRT-PCR analysis. Transcripts are means of averages of triplicate measures in 7 mice. *p<0.05.

Figure S2. Relative cardiac BNP expression in DCM and wild-type (WT) assessed by qRT-PCR analysis. Transcripts are means of averages of triplicate measures in 7 mice. **p<0.01.

Figure S3. Analyses of corin activity in the wild-type and corin Tg hearts by cleavage of recombinant pro-ANP with a carboxy-terminal V5-tag. The serum free conditioned medium containing pro-ANP was incubated with the pelleted heart tissue (membrane fraction) of wild-type and corin Tg mice (n=3 per group). Cleaved ANP was immunoprecipitated and analyzed by Western blot analysis with the use of anti-V5 tag antibody (top). The blots were subjected to image analysis (NIH Image Quant program). The percent cleavage of pro-ANP to ANP was calculated.
Figure S4. Comparison of heart weight (HW), body weight (BW) and BW:HW values between DCMc and DCMc, corin-Tg female, 14-15 weeks mice littermates (n=10-12 in each group).

Figure S5. Cardiac expression of ANP transcripts in DCMc and DCMc, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. *p<0.05.

Figure S6. Cardiac expression of BNP transcripts in DCMc and DCMc, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. ***p<0.001.
**Figure S7.** cGMP level in plasmas of DCMc and DCMc, corin-Tg mice assessed by ELISA. Plasma levels are means of averages of duplicate measures in 7 mice of each group. *p<0.05, unpaired t-test.

**Figure S8.** Cardiac expression of collagen 1 transcripts in DCMc and DCMc, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. **p<0.01.

**Figure S9.** Cardiac expression of collagen III transcripts in DCMc and DCMc, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. *p<0.05.
Figure S10. Cardiac expression of TGF beta transcripts in DCM<sup>c</sup> and DCM<sup>c</sup>, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. *p*=0.056.

Figure S11. Cardiac expression of CMA1 transcripts in DCM<sup>c</sup> and DCM<sup>c</sup>, corin-Tg. assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. *p*=1.00

Figure S12. Cardiac expression of MMP-9 transcripts in DCM<sup>c</sup> and DCM<sup>c</sup>, corin-Tg. assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. *p*=0.84
Figure S13. Cardiac expression of furin transcripts in DCMc and DCMc, corin-Tg assessed by qRT-PCR analysis, relative to wild-type. Transcripts are means of averages of triplicate measures in 7 mice. \( p=0.42 \)

Figure S14. Heart LV internal dimensions, LVID for DCMc (n=6) and DCMc, corin-Tg (n=7). \( p=0.84 \).