Supplemental methods

Ethics statement

All experimental procedures were performed according to the animal welfare regulations of Yamagata University School of Medicine. The study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the Guide for the Care and Use of Laboratory animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

Human Studies

This study included 27 patients with heart failure and 5 control patients who were assessed to rule out cardiomyopathy and had normal cardiac function. Written informed consent was obtained from all patients before entry into the study. The protocol was performed in accordance to the Helsinki Declaration and was approved by the human investigations committee of our institution. Biopsy samples were immediately washed in phosphate buffered saline (PBS) before being snap-frozen in liquid nitrogen for immunofluorescent co-staining and biochemical measurements, as previously described (1).

Materials and Reagents

Angiotensin II (Ang II) was purchased from Sigma-Aldrich. A specific ataxia telangiectasia mutated (ATM) inhibitor, KU55933, was purchased from Tocris Bioscience (Bristol, UK) and was used for both *in vitro* and *in vivo* experiments. The compound used for *in vitro* and *in vivo* experiments was dissolved in dimethyl sulfoxide (DMSO) and stocked at -20°C as a 10 mM solution. Luciferase reporter constructs (atrial natriuretic peptide (ANP) luc; hANP/luc and brain natriuretic peptide (BNP) luc; BNP/luc) were generated and used to examine the effect of nuclear High-mobility group box 1 (HMGB1) on fetal gene expression (1).

Animal Models

Mice with cardiac-specific overexpression of HMGB1 (HMGB1-Tg) and their wild-type (WT) littermates were bred in our laboratory using standard techniques, as previously reported (1,2). Cardiac hypertrophy was induced in 10 – 12 week-old HMGB1-Tg and WT mice by chronic infusion of Ang II (1.5 mg/kg/day) or saline with osmotic minipumps (Alzet, model 2002, DURECT Corporation, Cupertino, CA, USA; 0.5 μL/h for 14 days) as previously described (3). For ATM inhibitor experiments, KU55933 (5 mg/kg) or vehicle (DMSO) was injected intraperitoneally to HMGB1-Tg mice at 2, 5, 8, and 11 days after Ang II infusion. After 2 weeks, blood pressure, cardiac function, and dimension were measured. The mice were sacrificed by intraperitoneal

injection of a combination of ketamine (1 g/kg) and xylazine (100 mg/kg), and the hearts were removed for examination of histological changes and biochemical analysis of various protein expression levels.

Echocardiography determination

Transthoracic echocardiography was performed before and after Ang II infusion under anesthesia following intraperitoneal injection of pentobarbital sodium (30 mg/kg) using a Vevo2100 (VisualSonics, Toronto, Canada). Left ventricular diastolic dimension (LVDd), systolic dimension (LVDs), interventricular septum diameter (IVSd), and posterior wall diameter (PWd) were measured digitally on the M-mode tracings and averaged from at least three cardiac cycles. Left ventricular fractional shortening (FS) was calculated as [(LVDd-LVDs) / LVDd] × 100 (%) (4). The transmitral Doppler velocity ratio of early to atrial wave (E/A ratio) was measured by pulse-wave Doppler images of mitral inflow from the apical-4 chamber view.

Blood pressure measurement

Blood pressure was measured before and after Ang II infusion using a non-invasive tail-cuff blood pressure system (Muromachi Kikai Co, Ltd, MK-2000ST NP-NIBP Monitor, Tokyo, Japan). Blood pressure was measured when mice were conscious. Systolic, diastolic, mean blood pressure, and heart rate were recorded.

Assessment of the histology of the heart

Tissues were excised from mice and immediately fixed with 4% paraformaldehyde in PBS for 24 hours, embedded in paraffin, and cut serially to 5 μm. The heart sections were stained with hematoxylin and eosin (H&E) to assess myocyte cross-sectional area. The degree of cardiac fibrosis was assessed by Masson's trichrome staining. The images were analyzed using NIH ImageJ software (NIH, Bethesda, MD, USA).

Cell culture and treatment

Primary culture of neonatal rat cardiomyocytes (NRCMs) was performed as described previously (5,6). Hearts were excised from 1 to 2-day-old Sprague-Dawley rat pups, promptly after euthanasia by decapitation. To isolate cardiomyocytes, the tissues were incubated in a balanced salt solution containing collagenase type 2 (Worthington Biochemical, Lakewood, NJ, USA) for 10 minutes at 37°C. The digestion buffer was replaced 10 times, after which the tissues were completely digested. The dispersed cells were incubated in 10-cm culture dishes to remove non-myocytes. The unattached viable cells, which were enriched for NRCMs, were cultured on collagen-coated dishes at 37°C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 μg/mL), and 10 μM cytosine 1-β-D-arabinofuranoside (Ara C), and cultured for 2 days. Rat H9C2 cells were

also cultured on collagen-coated dishes at 37°C in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). After serum starvation for 24 hours, cardiomyocytes were stimulated with 1 μM Ang II for 24 hours to induce cardiac hypertrophy, and samples were collected to perform each experiment. For ATM inhibitor experiments, cardiomyocytes were pre-treated with KU55933 (10 μM) for 1 hour before Ang II stimulation.

Transfection with HMGB1 siRNA and HMGB1 expression vectors

Small interfering RNAs (siRNAs) specific for HMGB1 (5′-GUCCUUUCUCUUUUACAUUtt-3′, sense; 5′-AAUGUAAAAGAGAAAGGACtt-3′, antisense) were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Transfection into cardiomyocytes was performed using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Non-specific control siRNA (Invitrogen) was used as a negative control. Transfection of the HMGB1 expression vector or a control vector into cardiomyocytes was also performed using Lipofectamine 3000 Reagent according to the manufacturer's instructions. After 4 hours of transfection the media was replaced by DMEM supplemented with 10% FBS, penicillin, and streptomycin.

Luciferase reporter gene assay

Ang II induced ANP and BNP promoter activities were evaluated by luciferase reporter gene assay with hANP/luc and BNP/luc, qRL-TK vector, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) (1). Expression vector transfection was performed using Lipofectamine 3000 Reagent (Invitrogen) according to the manufacturer's instructions. Rat H9C2 cells were co-transfected with hANP/luc of BNP/luc and HMGB1 siRNA or non-specific control siRNA. ANP and BNP promoter activities were evaluated after Ang II stimulation for 24 hours.

Protein extraction and western blotting

Total protein was extracted from the left ventricle of mice or NRCMs by homogenization in ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 0.1% NP-40, 1 mM DTT, 0.1% SDS, 100 mM PMSF, 100 mM NEM, 100 mM iodoacetamide, 2% protease inhibitor, and 1% phosphatase inhibitor. Nuclear proteins on NRCMs were extracted with nuclear extraction reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. The protein concentration of each sample was determined using the BCA protein assay (BioRad Laboratories, Inc., Hercules, CA). Equal amounts of protein were electrophoresed on 4% – 14% sodium dodecyl sulfate-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes (GE Healthcare UK Ltd,

Little Chalfont, UK). Membranes were blocked with 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.1% Tween (TBS-T) and 5% milk or 5% bovine serum albumin (BSA) and then probed with primary antibodies diluted in TBS-T. After incubation with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T containing 5% milk or 5% BSA, immunoreactive bands were detected using an ECL kit (Amersham Biosciences, Piscataway, NJ, USA). The following antibodies were used in this study; anti-HMGB1 (Shino-Test Corporation, Sagamihara, Japan), anti-ERK 1/2, phospho-ERK 1/2 (p-ERK 1/2), NF-κB, phospho-NF-κB (p-NF-κB), γ-H2AX, Histone H3, and β-tubulin (Cell Signaling Technology, Danvers, MA, USA), anti-ATM (Santa Cruz, Dallas, TX, USA), anti-phospho-ATM (p-ATM) (Rockland, Limerick, PA, USA).

Immunofluorescence

Cultured NRCMs were fixed with 4% paraformaldehyde in PBS for 10 min. For in human and *in vivo* experiments, heart samples were cut serially to 5 μm. Sample sections were treated with 1 mM EDTA in boiling water to retrieval antigen. Samples were blocked with 5% FBS and 1% Triton-X100 for 1 hour, and subsequently, incubated with primary antibodies overnight at 4°C. After washing with PBS, secondary antibodies conjugated to Alexa Fluor 488 or 555 (Thermo Fisher Scientific) were added

and incubated for 1 hour. Foci numbers of p-ATM and γ-H2AX were assessed using NIH ImageJ software (NIH, Bethesda, MD, USA). The following antibodies were used in this study; anti-HMGB1 (Shino-Test Corporation, Sagamihara, Japan, or BioLegend, San Diego, CA, USA), anti-p-ATM (Millipore, Temecula, CA, USA), anti-γ-H2AX (Cell Signaling Technology, Danvers, MA, USA, or Abcam, Cambridge, UK), anti-sarcomeric α-actinin (Abcam, Cambridge, UK, or Sigma-Aldrich, St. Louis, MO, USA).

Co-immunoprecipitation

After samples were collected, protein extracts were prepared in modified RIPA buffer. Normal rabbit or mouse IgG was used as a negative control. Lysates were incubated with anti-HMGB1 (Shino-Test Corporation, Sagamihara, Japan), or anti-ATM (Santa Cruz, Dallas, TX, USA) antibodies overnight and then incubated for 1 hour with protein A/G-agarose beads. Samples were washed 4 times with buffer and subjected to western blot analysis (5).

Reference

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