ORIGINAL RESEARCH

HE4 Predicts Progressive Fibrosis and Cardiovascular Events in Patients With Dilated Cardiomyopathy

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BACKGROUND: Cardiac fibrosis plays a crucial role in the pathogenesis of dilated cardiomyopathy (DCM). HE4 (human epididymis protein 4) is a secretory protein expressed in activated fibroblasts that exacerbates tissue fibrosis. In the present study, we investigated the clinical utility of HE4 measurement in patients with DCM and its pathophysiological role in preclinical experiments in vivo and in vitro.

METHODS AND RESULTS: We measured serum HE4 levels of 87 patients with DCM. Endomyocardial biopsy expressed severe fibrosis only in the high HE4 group (P<0.0001). Echocardiography showed that left ventricular end-diastolic diameter tends to decrease over time (58±7.3 to 51±6.6 mm; P<0.0001) in the low HE4 group (<59.65 pmol/L [median value]). HE4 was significantly associated with risk reduction of mortality and cardiovascular hospitalization in multivariate Cox model. In vivo, HE4 was highly expressed in kidney and lung tissue of mouse, and scarcely expressed in heart. In genetically induced DCM mouse model, HE4 expression increased in kidney but not in heart and lung. In vitro, supernatant from HE4-transfected human embryonic kidney 293T cells enhanced transdifferentiation of rat neonatal fibroblasts and increased expression of fibrosis-related genes, and this was accompanied by the activation of extracellular signal-regulated kinase signaling in cardiac fibroblasts. Treatment with an inhibitor of upstream signal of extracellular signal-regulated kinase or a neutralizing HE4 antibody canceled the profibrotic properties of HE4.

CONCLUSIONS: HE4 functions as a secretory factor, activating cardiac fibroblasts, thereby inducing cardiac interstitial fibrosis. HE4 could be a promising biomarker for assessing ongoing fibrosis and a novel therapeutic target in DCM.

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Dilated cardiomyopathy (DCM) is one of the most common causes of heart failure (HF) and is associated with significant morbidity and mortality.\(^1\) Pathophysiological remodeling of the left ventricle is an important hallmark of DCM, and one of the most important indicators of adverse events.\(^2\) Furthermore, left ventricular reverse remodeling (LVRR), characterized by a decrease in dimensions and normalization of shape, is associated with significant improvement in pump function and a favorable prognosis.\(^3\) However, the causes and predictive variables of left ventricular (LV) remodeling and LVRR remain undetermined.\(^4\)
A key cellular event in the pathophysiology of cardiac fibrosis and remodeling is activation of fibroblasts with subsequent deposition of extracellular matrix proteins.\textsuperscript{5} Fibroblasts are activated in response to various stress stimuli, which facilitates their differentiation into myofibroblasts that express \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA).\textsuperscript{6} Myofibroblasts excessively secrete extracellular matrix proteins, leading to development of DCM.\textsuperscript{7} Therefore, a noninvasive test to determine the expression of myofibroblasts has the potential to evaluate the degree or severity of “ongoing” fibrosis in patients with DCM. Late gadolinium enhancement via cardiac magnetic resonance (CMR) imaging is an effective tool for detection of myocardial fibrosis and one of the most useful modalities contributing to the diagnosis of DCM.\textsuperscript{2} However, late gadolinium enhancement is not ideal in the evaluation of ongoing tissue fibrosis, as it has been shown to reflect “accomplished” myocardial fibrosis.\textsuperscript{8}

Recent studies showed that HE4 (human epididymis protein 4), also known as WAP (whey acidic protein) 4-disulfide core domain 2, is specifically expressed by activated myofibroblasts and secreted into the circulation.\textsuperscript{9} Its concentration in the serum of patients with renal diseases correlates with renal fibrosis, and treatment of mice with anti-HE4 antibodies was shown to improve renal fibrosis by reducing the multiple protease inhibitor activity characteristic of HE4.\textsuperscript{9} In the current study, we measured circulating serum HE4 concentration in patients with DCM to determine its utility in the detection of ongoing cardiac fibrosis and future LV remodeling or adverse cardiac events. In addition, to determine whether HE4 has an impact on cardiac fibrosis, we conducted both in vivo and in vitro experiments.

**CLINICAL PERSPECTIVE**

**What Is New?**
- Low levels of serum HE4 (human epididymis protein 4) were significantly associated with future left ventricular reverse remodeling and favorable outcomes in patients with dilated cardiomyopathy.
- HE4 is upregulated at kidney tissue in heart failure with reduced ejection fraction model mice.
- The supernatant from HE4-transfected human embryonic kidney 293T cells introduced cardiac fibroblast activation and upregulation of fibrosis-related genes, which were accompanied by the activation of extracellular signal-regulated kinase signaling.

**What Are the Clinical Implications?**
- HE4 is a key regulator of novel cardiorenal interaction and ongoing fibrosis of cardiac tissue, and can be a new biomarker contributing to a novel treatment strategy aimed at left ventricular reverse remodeling in patients with dilated cardiomyopathy.
- The data of present study suggest that HE4 could be a novel therapeutic target in dilated cardiomyopathy.

**METHODS**

For each paragraph, additional details can be found in Data S1. The data that support the findings of this study are available from the corresponding author on reasonable request.

**Study Population of Clinical Study**

Eighty-seven consecutive patients with DCM, scheduled to undergo cardiac catheterization for an assessment of hemodynamic status or a diagnostic workup for HF at Kumamoto University Hospital between January 2009 and December 2018, were enrolled (Figure S1). DCM was diagnosed on the basis of clinical and physical examination, including enhanced CMR and myocardial biopsy. Of 87 patients, 60 (69%) underwent myocardial biopsy, and 2 cases showed lymphocyte infiltration suggestive of inflammatory dilated cardiomyopathy. HF was defined by the American College of Cardiology/American Heart Association, and only those with HF stage B or C were included.\textsuperscript{10} Patients with neoplasms and those on hemodialysis were excluded. Fifty-nine patients without HF, confirmed by normal coronary angiography on cardiac catheterization, comprised our control group.
Ethical Statement
All procedures were conducted in accordance with the Declaration of Helsinki and its amendments. The study protocol was approved by the institutional review board of Kumamoto University (approval No.: Senshin 2259). Opt-out materials are available at: https://kumadai-junnai.com/archives/clinical.

Procedures
Blood samples were collected in the stable phase during the first admission, and the serum HE4 levels were measured using the Clinical Laboratory Improvement Amendments method (Abbott).

Follow-Up and Study End Points
After baseline blood sampling, patients were followed up in the outpatient clinic for a median of 897 days (interquartile range, 421–3022 days). The study primary end point was a composite of all-cause death, LV assist device implantation, and hospitalization for HF events. Furthermore, a composite of all-cause death and LV assist device implantation was defined as secondary end point.

Echocardiography
Echocardiography was performed under stable conditions on admission and during the follow-up period (median follow-up period, 639 days) (Vivid 7; GE-Vingmed Ultrasound). Changes over time in LV dimension and systolic function were evaluated by serial echocardiography measurements. Echocardiographic parameters were assessed in alignment with the recommendations of the American Society of Echocardiography.11 LV end-diastolic diameter (LVEDD) index was calculated as LVEDD divided by body surface area. LV end-diastolic volume index (LVEDVi) and LV end-systolic volume index (LVESVi) were calculated as LV end-diastolic volume and LV end-systolic volume divided by body surface area. LV ejection fraction (LVEF) was calculated by the modified Simpson method (Vivid 7; GE-Vingmed Ultrasound).

Statistical Analysis of Echocardiographic Data
Sixty-five patients (74%) underwent follow-up echocardiography and were analyzed by the degree of changes in echocardiographic parameters. Univariable linear regression, logistic regression, and multivariable analysis for ΔLVEDVi (follow-up LVEDVi–baseline LVEDVi), ΔLVESVi (follow-up LVESVi–baseline LVESVi), and LVRR was performed using HE4 and other variables involved in LV remodeling.12 LVRR was defined as the combined presence of the following: (1) an increase in LVEF of at least 10 points or a follow-up LVEF ≥50%; and (2) a decrease in LVEDD index of at least 10% or an LVEDD index ≤33 mm/m.3

CMR Image Acquisition and Image Analysis
Sixty-six patients (76%) underwent CMR and were checked for the presence of late gadolinium enhancement.

Myocardial Biopsy and Calculation of Collagen Volume Fraction and Extracellular Space
The samples were taken from the mid interventricular septum, and at least 2 samples per patient were submitted for light microscopic examination. All biopsy specimens were stained with Masson trichrome. The collagen area and total myocardial area were measured, and the collagen area fraction was expressed as percentage of total area. (ImageJ version 1.52a).

Mouse Models and Procedures
Wild-type (WT) male mice and genetically induced DCM model mice on a BALB/cA background were used in this study. All procedures were performed in accordance with the Kumamoto University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (publication No. 85-23, revised 1996). The study was approved by the Animal Research Ethics Committee of Kumamoto University (No. A2019-122). WT male mice with BALB/cA background were purchased from Kyudo Company. The mice were anesthetized with isoflurane in all models. Two models of mouse HF with reduced ejection fraction (HFrEF) were produced. The mouse myocardial infarction model was generated as previously described.13,14 The mouse DCM model was generated using knock-in mice on the genetic background of BALB/cJ, in which 3 base pairs coding for K210 in cardiac troponin T were deleted from the endogenous Tnnt2 gene, as previously described.15 At 6-week-old WT mouse, at 4 weeks after myocardial infarction surgery mouse, and at 6-week-old DCM model mouse were anesthetized with overdose isoflurane, and hearts, kidney, lung, and liver were rapidly excised, and freeze clamped for subsequent analyses.

Echocardiography, In Vivo
At 1 day before harvest, echocardiography was performed using the Xario system (Toshiba) with a 12-MHz linear array transducer.
Cell Culture and Harvest and Incubation of Neonatal Rat Cardiomyocytes and Fibroblasts

Primary neonatal rat cardiomyocytes and fibroblasts were isolated from 2-day-old Wistar rats (Japan SLC, Inc), as described previously.14 Cardiomyocyte and fibroblast isolation and primary culture were approved by the Committee on the Use of Live Animals in Research in Kumamoto University.

Cell Culture, Transfection of Plasmid in Human Embryonic Kidney 293T Cells, and Culture Supernatant Transfer

Human embryonic kidney 293T (HEK293T) cells were cultured in high-glucose (4-g/L) DMEM containing 10% fetal bovine serum. The dish was replaced with fresh low-glucose DMEM with 0.1% fetal bovine serum, and then the control or HE4 plasmid (pcDNA3-HE4, No. 18101, Addgene) was introduced into HEK293T cells using Lipofectamine 3000 (Life Technologies), according to the manufacturer’s instructions. The pcDNA3-HE4 was a gift from Ronny Drapkin (Addgene plasmid No. 18101; http://n2t.net/addgene:18101; Research Resource Identifier: Addgene_18101). At 12 hours after transfection, the medium was replaced with fresh DMEM with 0.1% fetal bovine serum. After 24 hours, the culture supernatant from the HEK293T cells was transferred to previously harvested rat neonatal cardiomyocytes and cardiac fibroblasts. In addition, U0126 (No. 9903S, CST), an mitogen-activated protein kinase kinase (MEK)1/2 inhibitor, was used to inhibit the phosphorylation of extracellular signal-regulated kinase (ERK) in rat neonatal cardiac fibroblasts. Anti-HE4 antibody (ab200828, Abcam) was used to inhibit the HE4 contained in the culture supernatant from HE4-overexpressing HEK293T cells. After 10 minutes, the plates were rinsed twice in PBS and lysis buffer was added, containing 1% SDS and protease inhibitor cocktail or protease and phosphatase inhibitors from Thermo Scientific for Western blotting to evaluate intracellular signaling. After 24 hours, RLT buffer (RNaseasy Plus Mini Kit, Qiagen) was added for quantitative reverse transcription–polymerase chain reaction (PCR) or lysis buffer was added for Western blotting or immunofluorescence staining, as described above, to evaluate fibrosis-related genes and proteins.

Quantitative Real-Time PCR Analysis

Total RNA was prepared by Qiagen RNaseasy kit using manufacturer protocols. cDNA was produced using the PrimeScript RT Master mix (Takara), according to the manufacturer’s directions. A quantitative reverse transcription–PCR was performed. Primer sequences are listed in Table S1.

Western Blot Analysis

Western blot analysis was performed as described previously.16 Antibodies used were as follows: anti-HE, anti–type I collagen, anti-cSMA, anti-GAPDH, ERK, phosphorylated ERK, Protein Kinase B (Akt), phosphorylated Akt, Smad2/3, phosphorylated Smad2, phosphorylated Smad3, c-Jun-NH2-terminal kinase (JNK), phosphorylated JNK, p38, and phosphorylated p38.

Immunofluorescence Staining for Fibroblast Phenotyping

Immunofluorescence staining was performed as described Data S1. To assess the degree of differentiation, cells were double stained for F-actin using rhodamine-phalloidin (1:1000 dilution, P1951-1MG, Sigma) and for cSMA using an antibody against cSMA (1:500 dilution, No. 102M4804V, Sigma), to characterize stress fibers. The coverslips were mounted using Prolong Gold antifade with 4′,6-diamidino-2-phenylindole (1:1000, NX034, Dojindo). Degree of differentiation was evaluated by counting the number of cells positive for either F-actin or cSMA stress fibers in 3 randomly chosen images with a minimum of 80 cells counted per sample. Results from these 3 samples were averaged.

Statistical Analysis

All data are presented as individual samples with mean values or as mean±SD. Unpaired t tests or Mann-Whitney U tests were used to compare groups. HE4, B-type natriuretic peptide, highsensitivity cardiac troponin T, and CRP (C-reactive protein) concentrations showed skewed distributions and are therefore expressed as median (interquartile range) and log transformed before Pearson correlation analysis, linear and logistic regression, and Cox regression analysis, when appropriate. Categorical and noncontinuous variables were presented as frequencies or percentages and compared via χ2 test. Echocardiographic parameters at baseline and those at follow-up period were compared using a paired t test. The Kaplan-Meier method, log-rank test, and the simple and multiple Cox regression analyses were used to assess prognostic association. Unpaired t tests were used to compare the collagen area fractions (collagen area/total area) used to estimate cardiac fibrosis between the high and low HE4 groups. Pearson correlation was used to analyze the correlation between the collagen area fraction and serum HE4 levels. The significance level of a statistical hypothesis test was set at P<0.05. All data were statistically analyzed using the Statistical Package for the Social Sciences v24 for Windows (SPSS Japan Inc) or GraphPad Prism 8.0. In regard to the basic
Table 1. Baseline Characteristics of the Study Patients

| Characteristics                                      | All (n=87)     | High HE4 (n=43) | Low HE4 (n=44) | P value |
|------------------------------------------------------|----------------|-----------------|----------------|---------|
| Age, y                                                | 60±15          | 64±15           | 56±13          | 0.006   |
| Male sex, n (%)                                       | 62 (71)        | 31 (71)         | 31 (71)        | 0.866   |
| NYHA functional class, n (%)                         |                |                 |                |         |
| I                                                    | 21 (24)        | 7 (16)          | 14 (32)        |         |
| II                                                   | 44 (51)        | 19 (44)         | 25 (57)        |         |
| III                                                  | 16 (18)        | 12 (28)         | 4 (9)          |         |
| IV                                                   | 6 (7)          | 5 (12)          | 1 (2)          |         |
| ≥III                                                  | 22 (25)        | 17 (40)         | 5 (11)         | 0.003   |
| Body mass index, kg/m²                                | 23.8±4.2       | 23.1±4.0        | 24.5±4.4       | 0.123   |
| Systolic blood pressure on admission, mm Hg           | 114±17.2       | 109±14          | 118±19         | 0.012   |
| Hypertension, n (%)                                   | 30 (35)        | 15 (35)         | 15 (34)        | 0.938   |
| Diabetes mellitus, n (%)                              | 15 (17)        | 8 (19)          | 7 (16)         | 0.701   |
| Dyslipidemia, n (%)                                   | 34 (40)        | 14 (33)         | 20 (47)        | 0.186   |
| Current smoking, n (%)                                | 14 (16)        | 6 (14)          | 8 (18)         | 0.592   |
| Atrial fibrillation, n (%)                            | 22 (25)        | 14 (33)         | 8 (18)         | 0.123   |
| Ventricular tachycardia, n (%)                        | 20 (23)        | 11 (26)         | 9 (21)         | 0.570   |
| Ventricular fibrillation, n (%)                       | 3 (3)          | 3 (7)           | 0 (0)          | 0.075   |
| Prior HF hospitalizations, n (%)                      | 31 (36)        | 21 (49)         | 10 (23)        | 0.011   |
| Laboratory examination parameters                     |                |                 |                |         |
| White blood cell, /μL                                 | 6357±1938      | 6319±1885.7     | 6455±2007.2    | 0.746   |
| Hemoglobin, g/dL                                      | 14.2±2.18      | 13.6±2.31       | 14.8±1.83      | 0.008   |
| hs-cTnT, ng/mL                                        | 0.015 (0.009–0.030) | 0.023 (0.015–0.043) | 0.011 (0.007–0.016) | 0.004 |
| BNP, pg/mL                                            | 249.0 (72.7–654.3) | 446.0 (194.0–987.1) | 134.9 (31.0–480.1) | <0.0001 |
| Albumin, g/dL                                         | 3.9±0.5        | 3.8±0.5         | 4.1±0.3        | <0.0001 |
| Sodium, mEq/L                                         | 139±2.6        | 139±3.1         | 140±1.7        | 0.050   |
| Creatinine, mg/dL                                     | 0.93±0.27      | 1.03±0.31       | 0.84±0.17      | 0.001   |
| eGFR, mL/min×m²                                       | 65±15.4        | 58±15.9         | 72±11.4        | <0.0001 |
| T-bil, mg/dL                                          | 1.0±0.59       | 1.1±0.75        | 0.93±0.37      | 0.149   |
| CRP, mg/mL                                            | 0.13 (0.05–0.36) | 0.18 (0.10–0.63) | 0.06 (0.03–0.22) | 0.003 |
| HbA1c (%NGSP)                                         | 5.8±0.7        | 5.9±0.8         | 5.7±0.7        | 0.331   |
| Medications at baseline, n (%)                        |                |                 |                |         |
| β-Blockers on admission                               | 51 (59)        | 27 (63)         | 24 (55)        | 0.435   |
| β-Blockers on discharge                               | 83 (95)        | 42 (98)         | 41 (93)        | 0.317   |
| ACE-I or ARB on admission                             | 59 (68)        | 33 (77)         | 26 (59)        | 0.078   |
| ACE-I or ARB on discharge                             | 84 (97)        | 43 (100)        | 41 (93)        | 0.081   |
| Aldosterone antagonist on admission                   | 34 (39)        | 23 (54)         | 11 (25)        | 0.006   |
| Aldosterone antagonist on discharge                   | 54 (62)        | 28 (65)         | 26 (59)        | 0.563   |
| Diuretics on admission                                | 44 (51)        | 25 (58)         | 19 (43)        | 0.163   |
| Tolvaptan on admission                                | 3 (4)          | 2 (6)           | 1 (3)          | 0.474   |
| Anticoagulant on admission                            | 23 (26)        | 15 (35)         | 8 (18)         | 0.077   |
| Statin on admission                                   | 11 (13)        | 5 (12)          | 6 (14)         | 0.778   |
| Amiodarone on admission                               | 11 (13)        | 8 (19)          | 3 (7)          | 0.098   |
| Pimobendane on admission                              | 4 (5)          | 3 (7)           | 1 (2)          | 0.306   |
| ECG parameters                                        |                |                 |                |         |
| Heart rate, bpm                                       | 78±18.5        | 79±20           | 76±17          | 0.514   |
| CLBBB, n (%)                                          | 13 (15)        | 9 (21)          | 4 (9)          | 0.121   |

(Continued)
science experiments, all values were presented as the mean±SD. Differences between groups were analyzed by the Welch t test with multiple comparison correction (Bonferroni method).

**RESULTS**

**Patient Clinical Characteristics**

Baseline characteristics of control and DCM groups are listed in Table S2. Patients in the control group were older than those in the DCM group in our cohort. Fewer patients in the control group had underlying renal or cardiovascular disease, including atrial fibrillation. In the DCM group, the LVEF was significantly lower (33% versus 65%; \(P<0.0001\)), whereas the LVEDD was significantly higher (60 versus 44 mm; \(P<0.0001\)). Median serum HE4 levels of the DCM group were significantly higher compared with those of the control group (59.65 versus 44.1 pmol/L; \(P<0.0001\)) (Figure S2).

Patients with DCM were divided into high and low HE4 groups, using the median values (Table 1). Patients in the high HE4 group were older than those in the low HE4 group (64 versus 56 years; \(P=0.006\)) and had a higher incidence of prior HF-related hospitalizations (21 [49%] versus 10 [23%]; \(P=0.011\)). There were no significant differences in sex or the prevalence of coronary risk factors between the 2 groups. Patients in the high HE4 group had higher serum creatinine, CRP, high-sensitivity cardiac troponin T, and plasma B-type natriuretic peptide levels. Patients in the high HE4 group also had lower hemoglobin and serum albumin levels compared with the low HE4 group. Echocardiographic findings, including LVEDD (61 versus 59 mm; \(P=0.339\)), were not significantly different between the 2 groups at entry point, although LVEF was lower in the high HE4 group (30% versus 35%; \(P=0.020\)). The prevalence of late gadolinium enhancement in CMR did not differ between the 2 groups. However, Masson trichrome–stained sections from endomyocardial biopsy revealed that the degree of cardiac fibrosis, as estimated by the collagen area fraction, was positively correlated with serum HE4 levels (Figure 1). Most of the study participants were prescribed \(\beta\)-blockers and renin-angiotensin-aldosterone system inhibitors at the time of discharge.

**Association of HE4 With LV Remodeling**

Sixty-five patients (29 in the high HE4 group and 36 in the low HE4 group) underwent serial echocardiographic
assessments during a median follow-up period of 639 days after diagnosis of DCM (Table 2). Follow-up echocardiography showed slight or insignificant improvement in LV remodeling and function compared with baseline data in the high HE4 group. However, in the low HE4 group, LV dimensions were decreased and LVEF was drastically elevated, establishing a possible link between HE4 and ongoing cardiac fibrosis. Multivariate linear regression analysis showed that HE4 correlated significantly with ΔLVEDVi and ΔLVESVi, independent of factors that have an association with LV remodeling (Tables S3 and S4). During follow-up, LVRR was observed in 33 of 65 (50.8%) individuals who underwent serial echocardiography. Univariate and multivariate logistic regression analyses revealed that HE4 was an independent predictor of subsequent LVRR (Table S5).

Table 2. Echocardiographic Parameters at Baseline and Follow-Up

| Parameters         | High HE4 (n=29) | Low HE4 (n=36) |
|--------------------|-----------------|----------------|
|                    | Baseline | Follow-up | P value | Baseline | Follow-up | P value |
| LVEDD, mm          | 59±7.6   | 58±9.3   | 0.095   | 58±7.3   | 51±6.6   | <0.0001 |
| LVESD, mm          | 51±9.6   | 48±12.6  | 0.035   | 49±8.5   | 38±8.1   | <0.0001 |
| LVEF, %            | 33±11.1  | 39±14.0  | 0.006   | 35±9.5   | 49±9.4   | <0.0001 |
| LVEDVi, mL/m²      | 97±34.1  | 90±34.6  | 0.118   | 90±28.7  | 59±14.6  | <0.0001 |
| LVESVi, mL/m²      | 68±30.7  | 59±32.4  | 0.034   | 59±22.8  | 31±10.2  | <0.0001 |
| LAD, mm            | 42±7.8   | 43±7.9   | 0.426   | 40±7.4   | 38±7.0   | 0.011   |

Values are mean±SD. The parameters at baseline and follow-up were compared using a paired t test. HE4 indicates human epididymis protein 4; LAD, left atrium diameter; LVEDD, left ventricular end-diastolic diameter; LVEDVi, left ventricular end-diastolic volume index; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic diameter; LVESVi, left ventricular end-systolic volume index.
The Prognostic Value of HE4

During the follow-up period, 22 of 87 (25%) patients died or were hospitalized for HF events; 4 of 87 (4.6%) patients died, 4 of 87 (4.6%) patients required LV assist device implantation, and 14 of 87 (16.1%) patients were hospitalized for HF decompensation. The Kaplan-Meier curve demonstrated a significantly higher probability of cardiovascular hospitalization and all-cause mortality in the high HE4 group compared with the low HE4 group (Figure 2A and 2B). As shown in Table 3 and Table S6, Cox regression analysis demonstrated that HE4 was significantly associated with risk reduction of mortality and cardiovascular hospitalization.

Circulating HE4 Was Derived Mainly From Kidney in the Mouse HF Models

We examined the roles of HE4 in pathophysiology of cardiac fibrosis and first examined the expression profile of HE4 by quantitative reverse transcription–PCR in WT BALB/cA male mice and our 2 mouse models of HFrEF, the genetically induced DCM model and the myocardial infarction–induced model. By echocardiography, mice in both models showed significantly larger LV dimensions and lower LV contraction compared with control mice (Tables S7 and S8). HE4 was scarcely expressed in heart tissues and was highly expressed in kidney tissue of WT mice in physiological condition (Figure S3A). These data showed similar findings to that of a previous study. Furthermore, the expression of HE4 in kidney tissue was increased in these 2 HFrEF models (Figure S3B and S3C), although those in heart, lung, and liver tissue were not increased. These data suggested that the main source of circulating HE4 in situation of HFrEF is not so much the heart, but instead other organs, such as kidney.

Distant Organ–Derived HE4 Also Activated Cardiac Fibroblasts and Induced Type I Collagen Deposition

Accordingly, to examine the role of HE4 as a mediator of multiorgan linkage, the culture supernatant of HE4 that overexpressed HEK293T cells (Figure S4A) was transferred to rat neonatal cardiomyocytes and cardiac fibroblasts. Changes in gene and protein expression and intracellular signaling related to pathological hypertrophy, fibrosis, and fibroblast differentiation were examined. Increased HE4 was observed by both quantitative reverse transcription–PCR and Western blotting analyses of culture medium after overexpression of HE4 in HEK293T cells (Figure S4B and S4C). In contrast, mRNAs of major proinflammatory and fibrotic cytokines, such as tumor necrosis factor-α, transforming growth factor-β1, and interleukin-6, were not increased (Figure S4D).

Figure 2. Elevated levels of serum HE4 (human epididymis protein 4) were associated with a high risk of future cardiovascular events.

Kaplan-Meier analysis for the probability of primary end point (A) and secondary end point (B) in patients with high and low HE4 levels. Primary end point: composite of all-cause death, left ventricular assist device (LVAD) implantation, and hospitalization for heart failure events. Secondary end point: composite of all-cause death and LVAD implantation.
Meanwhile, addition of the supernatant that contained myofibroblast transdifferentiation (Figure 3E and 3F). Factor- and fibrotic factors, such as transforming growth factor-β1 and interleukin-6, drive canonical and non-canonical intracellular signaling and induce ongoing fibrosis. We then used U0126, an MEK1/2 inhibitor, to inhibit the activation of ERK. ERK phosphorylation in cardiac fibroblasts was markedly attenuated by treatment with 10 μmol/L U0126 (Figure S6B). As shown in Figure 4C and 4D, the increase in protein expression of type I collagen by addition of supernatant that contained HE4 was completely inhibited by U0126 treatment. Furthermore, an anti-HE4 antibody was used to exclude the possibility that factors other than HE4, contained within the supernatant, induced these phenotypes, as previously reported.19 ERK activation and enhanced fibroblast differentiation by addition of the supernatant were obviously inhibited by anti-HE4 antibody treatment (Figure 4E through 4H). These data suggest that distant organ–derived HE4 facilitated cardiac fibroblast activation through ERK signaling and can lead to ongoing fibrosis in the heart. In other words, HE4 is an independent key driver of fibrosis involving linkage of multiple organs and ongoing fibrosis (Figure 5).

### DISCUSSION

The main findings of the present study were as follows: (1) the high HE4 group showed a lower rate of LVRR; (2) high serum HE4 was associated with future adverse cardiovascular events; (3) upregulated HE4 gene expression in kidney accompanied HFrEF; and (4) HEK293T cell–derived HE4 enhanced the expression of fibrosis-related genes and protein in cardiac fibroblasts, which was accompanied by activation of ERK. Our present findings are thus all compatible with the hypothesis that HE4 is involved in an endocrine manner in cardiac fibrosis and remodeling, and that circulating serum HE4 could be a useful biomarker for the detection of ongoing cardiac fibrosis in patients with DCM.

LVRR has a favorable prognostic value in cardiac diseases.20 However, methods to predict future LVRR have not been established. In the present study, we found that HE4 was specifically expressed in activated fibroblasts, or “myofibroblasts,” and that serum levels of HE4 had a direct correlation with disease severity and with the degree of cardiac interstitial fibrosis in patients with DCM. More interestingly, the present study also showed that HE4 can serve as a significant predictor of the degree of future LVRR, making it unique compared with conventional markers that reflect past rather than ongoing fibrosis. Ultimately, measurement of circulating HE4 levels could potentially contribute to a novel treatment strategy aimed at LVRR in patients with HF.

To date, there have been 2 studies investigating the prognostic impact of HE4 in patients with HF. These studies showed that HE4 concentration correlated with disease severity and poor prognosis in patients with

### Table 3. Univariate Cox Regression Analysis for the Primary End Point

| Variable                              | HR   | 95% CI       | P value |
|---------------------------------------|------|--------------|---------|
| Log HE4                               | 5.85 | 2.77–12.35   | <0.0001 |
| Age                                   | 1.00 | 0.97–1.03    | 0.818   |
| NYHA functional class ≥ II (yes)      | 2.90 | 1.24–6.74    | 0.014   |
| Systolic blood pressure, mm Hg        | 0.96 | 0.93–0.99    | 0.013   |
| Heart rate, bpm                       | 1.01 | 0.99–1.04    | 0.309   |
| Prior HF hospitalizations (yes)       | 4.39 | 1.79–10.80   | 0.001   |
| Atrial fibrillation (yes)             | 1.34 | 0.54–3.34    | 0.526   |
| Log BNP                               | 1.51 | 1.05–2.15    | 0.225   |
| Albumin, g/dL                         | 0.44 | 0.17–1.12    | 0.084   |
| Hemoglobin, g/dL                      | 0.97 | 0.81–1.17    | 0.764   |
| Sodium, mEq/L                         | 0.78 | 0.69–0.88    | <0.0001 |
| Log creatinine                        | 9.05 | 2.02–40.6    | 0.004   |
| T-bil, mg/dL                          | 2.43 | 1.22–4.85    | 0.012   |
| Log CRP                               | 1.36 | 1.02–1.83    | 0.037   |
| QRS duration, ms                      | 1.01 | 0.99–1.02    | 0.165   |
| CLBBB (yes)                           | 1.44 | 0.53–3.93    | 0.476   |
| LVEF, %                               | 0.94 | 0.90–0.99    | 0.015   |
| LVEDD, mm                             | 1.09 | 1.04–1.15    | <0.0001 |
| LVESD, mm                             | 1.09 | 1.04–1.14    | <0.0001 |
| LGE (yes)                             | 2.74 | 0.97–7.72    | 0.057   |

BNP indicates B-type natriuretic peptide; bpm, beats per minute; CLBBB, complete left bundle-branch block; CRP, C-reactive protein; HE4, human epididymis protein 4; HF, heart failure; HR, hazard ratio; LGE, late gadolinium enhancement; LVEDD, left ventricular end-diastolic diameter; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-systolic diameter; NYHA, New York Heart Association; and T-bil, total bilirubin.
acute and chronic HF, and could improve risk assessment in those patient populations.\textsuperscript{21,22} However, they did not evaluate the underlying mechanism of those results. In our present study, the serum HE4 concentration quantified the magnitude of cardiac fibrosis and future LV structural change. In addition, we investigated the role of HE4 in the pathophysiology of HF by in vivo and in vitro experiments. Our in vitro data demonstrated that HE4 activates cardiac fibroblasts and promotes extracellular matrix deposition as an endocrine factor. In addition, our in vivo data suggested HFrEF itself induced HE4 upregulation in kidney regardless of the means of induction of HFrEF. The data from the in vitro and in vivo study may shed light on the novel mechanism of cardiorenal association via circulating HE4.

Matrix metalloproteinases and tissue inhibitor of matrix metalloproteinases are key factors in cardiac extracellular matrix turnover.\textsuperscript{6,23} HE4 has 2 WAP domains consisting of disulfide linkages and can function as an inhibitor of multiple proteinases. Indeed, a previous study showed that HE4 suppressed matrix metalloproteinase expression in fibrotic kidney.\textsuperscript{9} Unlike these previous reports, however, we found that HE4 directly induced differentiation from fibroblast to myofibroblast and increased collagen deposition. Furthermore, we examined the underlying mechanism of HE4-induced fibroblast activation and revealed the involvement of ERK signaling, which has been reported to trigger cardiac fibroblast proliferation, leading to cardiac fibrosis.\textsuperscript{18} Furthermore, the profibrotic phenotype induced by the supernatants from HE4 overexpressing HEK293T cells was inhibited by anti-HE4 antibody, suggesting that HE4 per se acts as a profibrotic factor in these multiorgan linkages. Taken together, our in
vitro data suggest that HE4 exerts a profibrotic effect through ERK-dependent signaling. Although previous studies have shown the profibrotic effect of HE4 in kidney tissue, to the best of our knowledge, this is the first report revealing pathophysiological role of HE4 in cardiac fibrosis.

In the present cohort, although the HF medications were optimized in both the high and low HE4 groups, only the low HE4 group showed significant future LVRR and low adverse events. These clinical findings, in conjunction with our experimental findings described above, suggest that inhibition of HE4 can lead to favorable outcomes. In fact, previous studies have reported that inhibition of HE4 led to the amelioration of the kidney fibrosis.\(^5\) The utility of HE4 in the clinical setting as a potential cardiac fibrosis–reducing therapy may lead to novel treatment strategies for patients with DCM.

We acknowledge several limitations of the current study. First, this was a retrospective single-center study with a small cohort size and small number of cardiovascular events. Although we performed multivariate analysis to the extent possible, as shown in Table S6, confounding factors have not been completely excluded. Further multicenter studies of larger populations are needed to confirm the present results. Second, given the retrospective nature of the study, gene analysis, myocardial biopsies, follow-up echocardiography, and CMR were not performed uniformly in all patients. So, we should take care in interpreting the results of

Figure 4. HE4 (human epididymis protein 4) facilitated cardiac fibroblast activation through extracellular signal-regulated kinase (ERK) signaling.

A. Western blotting (WB) for ERK in cardiac fibroblasts treated with human embryonic kidney 293T (HEK293T) culture medium. B. Quantification of phosphorylated ERK (pERK)/total ERK WB analysis. C. WB for type 1 collagen in whole cell lysate of cardiac fibroblast stimulated by culture medium of HEK293T cells, and U0126 or dimethyl sulfoxide (DMSO). GAPDH was used as an internal control. D. Quantification of WB analysis. E. WB for ERK in whole cell lysate of cardiac fibroblast stimulated by culture medium of HEK293T cells, and anti-HE4 antibody or IgG. GAPDH was used as an internal control. F. Quantification of WB analysis. G. Overlay of images of fibroblast cultured with supernatant of HEK293T cells, and anti-HE4 antibody or IgG stained for 4′,6-diamidino-2-phenylindole (DAPI) (blue), F-actin (green), and \(\alpha\)-smooth muscle actin (\(\alpha\SMA\)) (red).

Relative expression

\[ \begin{align*}
\text{Control} & : \text{GAPDH} \\
\text{HE4} & : \text{GAPDH}
\end{align*} \]

\[ \begin{align*}
\text{Control} & : \text{Type 1 collagen}/\text{GAPDH} \\
\text{HE4} & : \text{Type 1 collagen}/\text{GAPDH}
\end{align*} \]

\[ \begin{align*}
\text{Control} & : \text{pERK} \div \text{Total ERK} \\
\text{HE4} & : \text{pERK} \div \text{Total ERK}
\end{align*} \]

\[ \begin{align*}
\text{Control} & : \text{\(\alpha\SMA\)} \text{-positive cells} \\
\text{HE4} & : \text{\(\alpha\SMA\)} \text{-positive cells}
\end{align*} \]

Unpaired \(t\) tests with Welch correction and multiple comparison correction (Bonferroni method) were used to compare each group.
the details of clinical data. Third, we failed to assess the pathophysiological role of HE4 in cardiac fibrosis in vivo examinations. Further studies are needed to confirm the involvement of HE4 to cardiac fibrosis using HE4-null mouse. Finally, our in vitro analysis was limited as we did not examine the signal cascades related to the profibrotic property of HE4. To address these limitations and to validate our above findings, a large-scale DCM patient analysis and more detailed basic science evaluations are warranted to further validate the present findings.

CONCLUSIONS

The present study demonstrated that in patients with DCM, circulating levels of HE4 were associated with cardiac remodeling and future adverse cardiovascular events and that HE4 contributed to the cardiac fibrosis or remodeling. These data suggest that HE4 has great potential as both a biomarker of ongoing cardiac fibrosis and a novel therapeutic target for patients with DCM.

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Disclosures

None.

Supplementary Material

Data S1
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SUPPLEMENTAL MATERIAL
**Supplemental Materials and Methods**

| Procedure and Follow up of clinical study | Blood samples were collected in the stable phase during the first admission, and the serum human epididymis protein 4 (HE4) levels were measured using the CLIA method (Abbott). The coefficient of variation of HE4 among samples was less than 10%. The study primary endpoint was a composite of all-cause death, left ventricular assist device (LVAD) implantation, and hospitalization for heart failure (HF) events. Furthermore, composite of all-cause death and LVAD implantation were defined as secondary endpoint. Death, LVAD implantation and heart failure events were identified by searching the medical records and confirmed by direct contact with the patients, relatives, and caring physicians. |
|-------------------------------------------|---|
| Echocardiography of clinical study        | Left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), thickness of the interventricular septum, posterior ventricular wall and left atrial diameter were obtained from M-mode or two-dimensional images of parasternal long axis views. Left ventricular end-diastolic volume (LVEDV) and left ventricular end-systolic were (LVESV) were measured in apical 4-and 2-chamber windows by the Simpson method. Left ventricular (LV) ejection fraction (LVEF) was calculated by the modified Simpson method (Vivid 7®; GE-Vingmed Ultrasound). |
| Statistical analysis of Echo data         | Univariable linear regression and logistic regression analysis for ΔLVEDVi (follow-up LVEDVi – baseline LVEDVi), ΔLVESVi (follow-up LVESVi – baseline LVESVi), and left ventricular reverse remodeling (LVRR) was performed using HE4 and other variables involved in LV remodeling. Multivariable analysis was performed using the variables achieving significance at p< 0.05 on univariable analysis or clinically important variables to determine the factors associated with ΔLVEDVi, ΔLVESVi, and LVRR. LVRR was defined as the combined presence of: (1) an increase in LVEF of at least 10 points or a follow-up LVEF ≥ 50%; and (2) a decrease in LVEDDi of at least 10% or an LVEDDi ≤ 33 mm/m. |
| CMR image acquisition and Image analysis  | 66 patients (76%) underwent cardiac magnetic resonance (CMR) and were checked for the presence of late gadolinium enhancement (LGE). All images were acquired using a 3.0 T scanner (Achieva 3.0 T X-series TX; Philips Medical Systems). We used electrocardiogram-gated cine imaging techniques with a segmented steady-state free precession sequence in the short and three
long cardiac axes with LGE imaging as described previously. Approximately 10 min after injection of 0.1 mmol/kg of a gadolinium-based contrast agent (Magnevist; Bayer Healthcare), we acquired two-dimensional inversion-recovery sequences, including the LV from base to apex. CMR images were independently analyzed by a cardiologist and a radiologist. Patients were then classified into LGE-positive or -negative groups.

| Mouse models and Procedures | Wild-type (WT) male mice on a BALB/cA background were used in this study. All procedures were performed in accordance with the Kumamoto University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (publication No. 85-23, revised 1996). The study was approved by the Animal Research Ethics Committee of Kumamoto University (#A2019-122). WT male mice with BALB/cA background were purchased from Kyudo company (Saga, Japan). The mice were housed in a temperature- and humidity-controlled (24°C) room on a 12 h light/dark cycle. The 8-week-old mice were anesthetized with isoflurane. The mouse myocardial infarction (MI) model was generated as previously described. Briefly, the trachea was cannulated with a polyethylene tube connected to a respirator (tidal volume, 0.6 mL; frequency, 110 breaths per minute). A left thoracotomy was performed between the fourth and fifth ribs. The pericardial tissue was removed, and the left anterior descending artery was visualized under a microscope and permanently ligated with 7-0 silk suture. Sham-operated mice underwent surgery but not left anterior descending artery ligation. At 4 weeks after MI surgery, mouse body weight, echocardiographic data, and urine output were analyzed prior to sacrifice. The mouse DCM model was generated using knock-in mice on the genetic background of BALB/cJ, in which three base-pairs coding for K210 in cTnT were deleted from the endogenous Tnnt2 gene as previously described. Homozygous mutant mice and WT mice were obtained by crossing heterozygous mutant mice, and were used as DCM and control models, respectively. MI surgery model and six-week old DCM model mouse were anesthetized with overdose isoflurane, and hearts, kidney, lung and liver were rapidly excised, and freeze clamped for subsequent analyses. In vivo analysis and post-euthanasia myocardial histological and molecular analyses were performed by investigators who were blinded to the experimental groups. |
| Echocardiography, in vivo | At 1 day before harvest, echocardiography was performed using the Xario system (Toshiba, Tokyo, Japan) with a 12-MHz linear array transducer. Heart rates and respiratory rates were continuously monitored. LV wall thickness and LV systolic and diastolic dimensions were measured in M-mode. LV percent fractional shortening were calculated. These analyses were performed by investigators who were blinded to the mice models. |
| Cell culture, harvest and incubation of neonatal rat cardiomyocytes and fibroblasts | Primary neonatal rat cardiomyocytes and fibroblasts were isolated from 2-day-old Wistar rats (Japan SLC, Inc). The hearts were harvested and minced and allowed to digest in 1 mg/ml Type II collagenase (Sigma Chemical Co.). After digestion, cardiomyocytes and fibroblasts were separated by Percoll density gradient centrifugation and incubated under 5% CO2 and 37°C in 1 g/L glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), ampicillin (10 U/µl), streptomycin (10 µg/µl), and amphotericin B (25 µg/ml). |
| Quantitative real time PCR analysis | RNA was extracted using a RNeasy Mini Kit (QIAGEN). cDNA synthesis was performed using PrimeScript RT Master mix (TAKARA) according to the manufacturer’s directions. A quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was carried out for CoI1a1, CoI3a1, alpha smooth muscle actin (αSMA), plasminogen activator inhibitor-1 (PAI-1), fibroblast growth factor 2 (FGF2), smooth muscle protein 22 (SM22), periostin, fibronectin, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6). The reactions were carried out in technical duplicates. Primers were utilized with SYBR Green PCR Master Mix (BIO-RAD) in CFX384 Real-Time System (BIO-RAD). The data processing is based on a standard curve-based method for relative qRT-PCR. Measurements were standardized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S. For in vivo studies, qRT-PCR was carried out for HE4 and GAPDH. Table S1 lists the primer sequences used in this study. |
| Western blot analysis | Cells were scraped and lysed with 1% SDS lysis buffer containing protease inhibitor cocktail (Thermo). The samples were centrifuged at 20400 g for 15 min. The supernatant was collected, and protein concentrations were determined using a Pierce BCA Protein assay Kit (Code: 23225, Thermo). After proteins were transferred to a PVDF Blotting membrane (GE Healthcare Life Sciences), the membrane was blocked with 100 mM Tris-HCl, pH 7.5, 0.9% NaCl, and 0.1% Tween 20 (TBST) containing 5% nonfat dry milk for 1 hour and then |
incubated with primary antibodies at 4°C overnight. The primary antibodies were as follows: anti-HE4 (ab200828, Abcam), anti-type I collagen (#84336S, 1, CST), anti-αSMA (ab5694, Abcam), anti-GAPDH (#2118, CST), ERK (#9102, CST), p-ERK (#4377, CST), Akt (#9272, CST), p-Akt (#9271, CST), Smad2/3 (#8685, CST), p-Smad2 (#18338, CST), p-Smad3 (#9520, CST), JNK (#9252, CST), p-JNK (#9251, CST), p38 (#9211, CST), p-p38 (#4511, CST). Membranes were then incubated with HRP-secondary antibodies for 1 hour at room temperature. Immunoreactive proteins were detected using ECL Prime (GE Healthcare UK Ltd.) with LAS-4000 Imaging system (FUJIFILM).

**Immunofluorescence staining for fibroblast phenotyping in vitro**

After 24 hours in culture, cells were fixed with 4% paraformaldehyde diluted in PBS for 20 minutes at room temperature. Further, cells were permeabilized with 0.1% Triton X-100. To assess the degree of differentiation, cells were double stained for F-actin using rhodamine-phalloidin (1:1000 dilution, P1951-1MG, Sigma) and for α-smooth muscle actin, using an antibody against αSMA (1:500 dilution, #102M4804V, Sigma), to characterize stress fibers. The coverslips were mounted using Prolong Gold anti-fade with DAPI (1:1000, NX034, Dojindo). Fluorescence imaging was done using a confocal microscope TCS SP8 LS with 20X/0.4 objective. Degree of differentiation was evaluated by counting the number of cells positive for either F-actin or αSMA stress fibers in three randomly chosen images with a minimum of 80 cells counted per sample. Results from these 3 samples were averaged.
Table S1. Primer sequences used for quantitative real-time PCR.

|                | Forward Primer                        | Reverse Primer                        |
|----------------|---------------------------------------|---------------------------------------|
| HE4 (human)    | CCCCAATGATAAGGAGGGT                   | ATTTCATCTGGCCAGGAC                    |
| HE4 (mouse)    | AACCAATTACGGACTGTGTT                  | TCGCTCGGTCCATTAGGCT                   |
| αSMA (rat)     | GGGATCCTGACCCTGAAG                    | AGTGGTGCCAGATCTTTT                   |
| PAI-1 (rat)    | ACATCCTGGAACTGCCCT                    | TGGTCATGTTGCTTCTTC                   |
| FGF2 (rat)     | CGCCTGGAGTCCAATAAC                    | ACAGTATGGCCTTCTGT                   |
| SM22 (rat)     | GGAACAGGTGGCTCAATTCT                  | CCAAAGGCATTAGCTCT                    |
| collagen1α1 (rat) | GATGGACTCAACGGTCTCA                | GGCAGGAAGCTGAAGTCA                   |
| collagen3α1 (rat) | ATGCATGTTTCTCCGGTTT                  | CTCGGAATTGCAGAGACC                   |
| TGF-β1 (rat)   | CGGACTACTACGCCAAAG                    | TTCCCGAATGTCTGACGT                   |
| TGF-β1 (human) | GCGTGCTAATGGTGGAACC                   | GCTTCTCGGAGCTTGATGT                  |
| Periostin (rat) | CAAACCACTTTCACGGGACCT                 | TTGTTCAACAGGCCTACAGA                 |
| Fibronectin (rat) | CAGCCCCTGATTTGGAGTC                 | TGGTGACACCTGAGTGAC                    |
| TNF-α (human)  | GACCTCTCTCTCTAATCAGCC                | TGAAGAGGACCTGAGTGAGA                 |
| IL-6 (human)   | TACATCTGCACGGCATCTC                   | TGGCTTTCTCCTACATCCT                  |
| GAPDH (rat)    | TCAAGAAGGTGGCTGAAGCAG                 | AGGTGGAAGTATGGGGATTG                 |
| 18S (human)    | CGGCTACCCATCACCAGAAGA                 | GCTGGAATTACCACGGGAG                   |
| ANP (rat)      | AGGCCATTATGGAGCAATACT                 | CATTTTCTCCCTCCAGTGTT                 |
| β-MHC (rat)    | CTGGCACCTGGACTGACTACAAAT              | GCCCTTGCTCAGAGTGCA                    |

PCR, polymerase chain reaction; HE4, human epididymis protein 4; αSMA, alpha smooth muscle actin; PAI-1, plasminogen activator inhibitor-1; FGF2, Fibroblast growth factor 2; SM22, smooth muscle protein 22; TGF-β1, Transforming Growth Factor-β1; TNF-α, tumor necrosis factor-α; IL-6, Interleukin-6; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; ANP, atrial natriuretic peptides; β-MHC, β-myosin heavy chain
Table S2. Baseline characteristics of the control and DCM groups.

|                          | Control (n = 59) | DCM (n = 87) | p value  |
|--------------------------|-----------------|--------------|----------|
| HE4, pmol/L              | 44.1 [35.6-52.9] | 59.65 [49.0-86.2] | <0.0001  |
| Age, y                   | 69 ± 3          | 60 ± 15      | <0.0001  |
| Male sex, n (%)          | 30 (51)         | 62 (71)      | 0.012    |
| Body Mass Index, kg/m2   | 23.8 ± 3.9      | 23.8 ± 4.2   | 0.902    |
| Systolic blood pressure on admission, mmHg | 123 ± 17 | 114 ± 17.4 | 0.002 |
| Hypertension, n (%)      | 36 (61)         | 30 (35)      | 0.002    |
| Diabetes mellitus, n (%) | 17 (29)         | 15 (17)      | 0.105    |
| Dyslipidemia, n (%)      | 43 (73)         | 34 (40)      | <0.0001  |
| Current smoker, n (%)    | 14 (24)         | 14 (16)      | 0.250    |
| Atrial fibrillation, n (%) | 3 (5)       | 22 (25)      | 0.001    |
| Non-Sustained ventricular tachycardia, n (%) | 1 (2)   | 20 (23)      | <0.0001  |
| Ventricular fibrillation, n (%) | 0 (0)   | 3 (3)        | 0.150    |
| Prior HF hospitalizations, n (%) | 0 (0)  | 31 (36)      | <0.0001  |
| Laboratory examination parameters | | | |
| White blood cell, /μL    | 6066 ± 1798.6   | 6387 ± 1938  | 0.313    |
| Hemoglobin, g/dL         | 13.9 ± 1.64     | 14.2 ± 2.16  | 0.493    |
| hs-cTnT, ng/mL           | 0.007 [0.003-0.010] | 0.015 [0.009-0.029] | 0.013 |
| BNP, pg/mL               | 16.6 [9.9-29.8] | 249.0 [72.7-654.3] | <0.0001 |
| Albumin, g/dL            | 4.2 ± 0.33      | 3.9 ± 0.5    | <0.0001  |
| Serum sodium, mEq/L      | 140 ± 1.8       | 139 ± 2.6    | 0.011    |
| Creatinine, mg/dL        | 0.71 ± 0.16     | 0.93 ± 0.27  | <0.0001  |
| eGFR, mL/min*m²          | 76 ± 11.8       | 65 ± 15.4    | <0.0001  |
| T-bil, mg/dL             | 0.8 ± 0.29      | 1.0 ± 0.59   | 0.009    |
| Parameter                        | Value                  |
|---------------------------------|------------------------|
| CRP, mg/ml                      | 0.04 [0.02-0.08]       |
| HbA1c (NGSP)                    | 6.0 ± 1.00             |
| Electrocardiogram parameters    |                        |
| Heart rate, bpm                 | 68 ± 12.1              |
| CLBBB, n (%)                    | 0 (0)                  |
| QRS duration, msec              | 99 ± 11.6              |
| Electrophysiological parameters |                        |
| Heart rate, bpm                 | 68 ± 12.1              |
| CLBBB, n (%)                    | 0 (0)                  |
| QRS duration, msec              | 99 ± 11.6              |

| Parameter                        | Value                  |
|---------------------------------|------------------------|
| HbA1c (NGSP)                    | 0.13 [0.05-0.36]       |
| Electrocardiogram parameters    |                        |
| Heart rate, bpm                 | 78 ± 18.5              |
| CLBBB, n (%)                    | 13 (15)                |
| QRS duration, msec              | 114.5 ± 29.3           |
| Echocardiogram parameters       |                        |
| LVEF, %                         | 65 ± 4.6               |
| LVEDD, mm                       | 44 ± 5.0               |
| LVESD, mm                       | 27 ± 4.2               |
| Intraventricular septal thickness, mm | 9.7 ± 1.5         |
| LV posterior wall thickness, mm | 9.7 ± 1.6              |
| LVEDVi, ml/L/min/m²             | 38 ± 15.0              |
| LVESVi, ml/L/min/m²             | 13 ± 5.9               |
| LAD, mm                         | 34 ± 5.1               |

Data are number of patients (%), mean ± standard deviation (SD), and median (interquartile range).

DCM, dilated cardiomyopathy; HE4, human epididymis protein 4; HF, heart failure; hs-cTnT, high-sensitivity cardiac troponin T; BNP, B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; T-bil, total bilirubin; CRP, c-reactive protein; CLBBB, complete left bundle branch block; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LV, left ventricular; LVEDVi, left ventricular end-diastolic volume index; LVESVi, left ventricular end-systolic volume index; LAD, left atrium diameter
Table S3. Univariate and multivariate linear regression analyses of ΔLVEDVi.

|                        | Univariate Analysis |            | Multivariate Analysis |            |
|------------------------|---------------------|------------|-----------------------|------------|
|                        | β-coefficient       | p Value    | β-coefficient         | p Value    |
| Log (HE4), per 1 pmol/L increment | 0.344               | 0.006      | 0.518                 | 0.001      |
| Age, per 1-year increment | 0.086               | 0.499      | -0.094                | 0.484      |
| NYHA class ≥ III       | -0.089              | 0.484      |                       |            |
| Systolic blood pressure on admission, 1 mmHg increment | 0.007               | 0.954      |                       |            |
| Hypertension           | -0.091              | 0.473      |                       |            |
| Diabetes mellites      | -0.029              | 0.818      |                       |            |
| β-blocker on discharge | -0.209              | 0.098      | -0.166                | 0.188      |
| ACE-I or ARB on discharge | -0.092             | 0.469      |                       |            |
| Log (BNP), per 1 pg/mL increment | -0.133              | 0.294      | -0.194                | 0.168      |
| Log (Creatinine), per 1 mg/dL increment | -0.033              | 0.794      | -0.192                | 0.146      |
| eGFR, per 1 mL/(min·m²) increment | -0.053              | 0.677      |                       |            |
| Log (CRP), per 1 mg/m increment | 0.107               | 0.407      |                       |            |
| QRS duration, per 1 mm increment | -0.057              | 0.657      |                       |            |
| CLBBB                  | 0.104               | 0.412      |                       |            |
| LVEF, per 1 % increment | 0.299               | 0.017      |                       |            |
| LVEDD, mm              | -0.381              | 0.002      | -0.359                | 0.009      |
| LVESD, mm              | -0.366              | 0.003      |                       |            |
| LGE                    | 0.039               | 0.778      | 0.091                 | 0.777      |

LVEDVi, left ventricular end-diastolic volume index; HE4, human epididymis protein 4; NYHA, New York Heart Association; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BNP, B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; CRP, c-reactive protein; CLBBB, complete left bundle branch block; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LGE, late gadolinium enhancement
Table S4. Univariate and multivariate linear regression analyses of LVESVi.

|                               | Univariate Analysis | Multivariate Analysis |
|-------------------------------|---------------------|-----------------------|
|                               | β-coefficient       | p value               | β-coefficient       | p value               |
| Log (HE4), per 1 pmol/L increment | 0.344               | 0.006                 | 0.508               | 0.001                 |
| Age, per 1-year increment      | 0.086               | 0.499                 | -0.072              | 0.592                 |
| NYHA class ≥ III               | -0.089              | 0.484                 |                      |                      |
| Systolic blood pressure on admission, 1 mmHg increment | 0.007               | 0.954                 |                      |                      |
| Hypertension                   | -0.091              | 0.473                 |                      |                      |
| Diabetes mellites              | -0.029              | 0.818                 |                      |                      |
| β-blocker on discharge         | -0.209              | 0.098                 | -0.153              | 0.227                 |
| ACE-I or ARB on discharge      | -0.092              | 0.469                 |                      |                      |
| Log (BNP), per 1 pg/mL increment | -0.133             | 0.294                 | -0.195              | 0.168                 |
| Log (Creatinine), per 1 mg/dL increment | -0.033             | 0.794                 | -0.175              | 0.192                 |
| eGFR, per 1 mL/(min*m2) increment | -0.053             | 0.677                 |                      |                      |
| Log (CRP), per 1 mg/m increment | 0.107              | 0.407                 |                      |                      |
| QRS duration, per 1 mm increment | -0.057             | 0.657                 |                      |                      |
| CLBBB                          | 0.104               | 0.412                 |                      |                      |
| LVEF, per 1 % increment        | 0.299               | 0.017                 |                      |                      |
| LVEDD, mm                      | -0.381              | 0.002                 |                      |                      |
| LVESD, mm                      | -0.366              | 0.003                 | -0.364              | 0.009                 |
| LGE                            | 0.039               | 0.778                 | 0.109               | 0.356                 |

LVESVi, left ventricular end-systolic volume index; HE4, human epididymis protein 4; NYHA, New York Heart Association; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BNP, B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; CRP, c-reactive protein; CLBBB, complete left bundle branch block; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LGE, late gadolinium enhancement
Table S5. Univariate and multivariate logistic regression analyses of LVRR positive.

|                                      | Univariate Analysis |         |          | Multivariate Analysis |         |
|--------------------------------------|---------------------|---------|----------|-----------------------|---------|
|                                      | B                   | p value |          | B                     | p value |
| Log (HE4), per 1 pmol/L increment    | -0.398              | 0.001   |          | -0.615                | <0.0001 |
| Age, per 1 year increment            | -0.209              | 0.094   |          | -0.032                | 0.830   |
| NYHA class ≥ III                     | -0.123              | 0.329   |          |                       |         |
| Systolic blood pressure on admission, 1 mmHg increment | 0.163       | 0.195   |          |                       |         |
| Hypertension                         | 0.150               | 0.235   |          |                       |         |
| Diabetes mellites                    | 0.072               | 0.569   |          |                       |         |
| β-blocker on discharge               | 0.003               | 0.983   |          | 0.074                 | 0.584   |
| ACE-I or ARB on discharge            | -0.070              | 0.585   |          |                       |         |
| Log (BNP), per 1 pg/mL increment     | 0.058               | 0.646   |          | 0.419                 | 0.008   |
| Log (Creatinine), per 1 mg/dL increment | -0.109          | 0.387   |          | 0.241                 | 0.096   |
| eGFR, per 1 mL/(min*m2) increment    | 0.148               | 0.238   |          |                       |         |
| Log (CRP), per 1 mg/m increment      | -0.151              | 0.239   |          |                       |         |
| QRS duration, per 1 mm increment     | -0.060              | 0.636   |          |                       |         |
| CLBBB                                | -0.177              | 0.158   |          |                       |         |
| LVEF, per 1 % increment              | -0.050              | 0.694   |          |                       |         |
| LVEDD, mm                            | -0.045              | 0.721   |          | -0.260                | 0.068   |
| LVESD, mm                            | -0.087              | 0.492   |          |                       |         |
| LGE                                  | 0.133               | 0.329   | 0.026    | 0.844                 |         |

LVRR, left ventricular reverse remodeling; HE4, human epididymis protein 4; NYHA, New York Heart Association; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BNP, B-type natriuretic peptide; eGFR, estimated glomerular filtration rate; CRP, c-reactive protein; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LGE, late gadolinium enhancement.
Table S6. Results of multivariate Cox regression analysis for the primary endpoint.

| Factor                        | Multivariate Analysis |
|-------------------------------|-----------------------|
|                               | HR   | 95% CI       | p value |
| Model 1                       |      |              |         |
| Log HE4                       | 7.91 | 3.49-17.94   | <0.0001 |
| Age (years)                   | 0.97 | 0.95-1.00    | 0.074   |
| Model 2                       |      |              |         |
| Log HE4                       | 5.07 | 2.25-11.43   | <0.0001 |
| NYHA class ≥ III              | 2.12 | 0.58-3.82    | 0.405   |
| Model 3                       |      |              |         |
| Log HE4                       | 4.92 | 2.34-10.35   | <0.0001 |
| Systolic blood pressure (mmHg)| 0.97 | 0.93-1.00    | 0.058   |
| Model 4                       |      |              |         |
| Log HE4                       | 5.09 | 2.31-11.19   | <0.0001 |
| Prior HF hospitalizations (yes)| 3.23 | 1.27-8.21   | 0.014   |
| Model 5                       |      |              |         |
| Log HE4                       | 4.29 | 1.85-9.94    | 0.001   |
| Sodium (mEq/L)                | 0.90 | 0.78-1.03    | 0.130   |
| Model 6                       |      |              |         |
| Log HE4                       | 5.09 | 2.05-12.64   | <0.0001 |
| Log Creatinine                | 1.57 | 0.28-8.76    | 0.606   |
| Model   | Log HE4     | HR      | 95% CI       | p-value |
|---------|-------------|---------|--------------|---------|
| Model 7 | 6.49        |         | 2.98-14.14   | <0.0001 |
|         | 2.32        |         | 1.26-4.28    | 0.007   |
| Model 8 | 6.68        |         | 2.43-18.37   | <0.0001 |
|         | 0.92        |         | 0.64-1.31    | 0.640   |
| Model 9 | 5.16        |         | 2.26-11.76   | <0.0001 |
|         | 1.16        |         | 0.77-1.75    | 0.474   |
| Model 10| 8.81        |         | 3.78-20.51   | <0.0001 |
|         | 1.12        |         | 1.06-1.18    | <0.0001 |
| Model 11| 9.13        |         | 3.78-22.08   | <0.0001 |
|         | 2.55        |         | 0.89-7.31    | 0.082   |

HR, hazard ratio; HE4, human epididymis protein 4; NYHA, New York Heart Association; HF, heart failure; T-bil, total bilirubin; CRP, c-reactive protein; BNP, B-type natriuretic peptide; LVEDD, left ventricular end-diastolic diameter; LGE, late gadolinium enhancement
Table S7. Parameters at harvest in BALB/cA WT and genetically induced HFrEF model mice (Homo).

| Parameter                                      | WT (n = 7)       | Homo (n = 7)     | p value |
|------------------------------------------------|------------------|------------------|---------|
| Age, week                                      | 6                | 6                | 1.000   |
| Body weight, g                                 | 18.3 ± 1.15      | 17.1 ± 1.76      | 0.134   |
| Heart rate, bpm                                | 715 ± 43.6       | 681 ± 57.8       | 0.243   |
| Echocardiogram parameters at 1 day before harvest |                  |                  |         |
| LVEDD, mm                                      | 2.63 ± 0.39      | 4.74 ± 1.03      | 0.001   |
| LVESD, mm                                      | 1.47 ± 0.42      | 3.91 ± 1.13      | 0.001   |
| Intraventricular septal thickness, mm          | 0.59 ± 0.09      | 0.44 ± 0.05      | 0.005   |
| LV posterior wall thickness, mm                | 0.63 ± 0.14      | 0.36 ± 0.05      | 0.001   |
| %FS, %                                         | 44.6 ± 11.1      | 18.5 ± 8.93      | <0.0001 |
| Organ weight at harvest                        |                  |                  |         |
| Heart/tibial length, mg/mm                    | 7.07 ± 0.62      | 13.5 ± 3.63      | 0.003   |
| Lung/tibial length, mg/mm                     | 8.92 ± 0.80      | 13.9 ± 5.93      | 0.070   |
| Kidney/tibial length, mg/mm                   | 8.93 ± 0.62      | 8.36 ± 0.52      | 0.091   |

Values are mean ± SD. WT, Wild-type; HFrEF, heart failure with reduced ejection fraction; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LV, left ventricular; %FS, % fractional shortening.
Table S8. Parameters at harvest 4 weeks after MI-induced HFrEF model mice in BALB/cA WT mice.

|                          | Sham-operated (n = 7) | MI (n = 7) | p value |
|--------------------------|------------------------|------------|---------|
| Age, week                | 12                     | 12         | 1.000   |
| Body weight, g           | 23.8 ± 0.93            | 23.8 ± 1.41| 0.976   |
| Heart rate, bpm          | 643 ± 59.0             | 677 ± 41.8 | 0.241   |
| Echocardiogram parameters at 1 day before harvest |                         |            |         |
| LVEDD, mm                | 3.00 ± 0.59            | 3.71 ± 0.42| 0.025   |
| LVESD, mm                | 1.30 ± 0.51            | 2.69 ± 0.53| <0.0001 |
| Intraventricular septal thickness, mm | 0.63 ± 0.10            | 0.30 ± 0.10| <0.0001 |
| LV posterior wall thickness, mm | 0.64 ± 0.08            | 0.50 ± 0.12| 0.021   |
| %FS, %                   | 58.0 ± 10.2            | 28.1 ± 7.32| <0.0001 |
| Organ weight at harvest  |                         |            |         |
| Heart/tibial length, mg/mm | 8.28 ± 0.76            | 9.35 ± 1.38| 0.103   |
| Lung/tibial length, mg/mm | 8.31 ± 0.38            | 8.58 ± 0.69| 0.387   |
| Liver/tibial length, mg/mm | 63.5 ± 5.07             | 66.0 ± 6.72| 0.443   |
| Kidney/tibial length, mg/mm | 12.3 ± 0.82             | 12.2 ± 2.03| 0.927   |

Values are mean ± SD. MI, myocardial infarction; HFrEF, heart failure with reduced ejection fraction; WT, Wild-type; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LV, left ventricular; %FS, % fractional shortening.
We excluded 9 patients because they were undergoing hemodialysis or had a tumor. According to the median value of HE4 (59.65 pmol/L), we divided all DCM patients into the high HE4 group (n = 43) and the low HE4 group (n = 44).

HE4: human epididymis protein 4, DCM: dilated cardiomyopathy
Figure S2. Serum HE4 levels in the control and DCM group.

Unpaired t-tests were used to compare groups.

HE4: human epididymis protein 4, DCM: dilated cardiomyopathy
Figure S3. HE4 is upregulated at kidney tissue in situation of HFrEF.

(A) The expression profile of HE4 in the heart, kidneys, lungs, and liver of BALB/cJ WT mice (n = 6). GAPDH was used as an internal control. (B) Quantitative evaluation of HE4 mRNA expression normalized to GAPDH mRNA expression in each tissue from DCM model mice (n = 7) and their WT littermates (n = 7) using the standard curve-method. The ratio of DCM mice to WT mice is shown. (C) Quantitative evaluation of HE4 mRNA expression normalized to GAPDH in heart, kidneys, lungs, and liver from MI model mice (n = 7) and sham operated mice (n = 7) using standard curve-method. The ratio of MI to sham operated mice is shown.

HE4: human epididymis protein 4, WT: wild type, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, MI: myocardial infarction, IA: infarcted area, NIA: non-infarcted area
Figure S4. Overexpression of HE4 have no impact on the expression of inflammatory-related and fibrosis-related genes in HEK293T cells.

(A) Experimental scheme for HE4 overexpression. (B) qRT-PCR analysis in HEK293T cells transfected with control or HE4 plasmid. 18S was used as an internal control. (C) WB for HE4 in supernatant of control or HE4 plasmid transfected HEK293T. (D) qRT-PCR analysis in HEK293T cells transfected with control or HE4 plasmid. 18S was used as an internal control.

Unpaired t-tests with Welch’s correction were used to compare groups.

WB: western blotting, HE4: human epididymis protein 4, HEK293T: human embryonic kidney 293T, qRT-PCR: quantitative reverse-transcription polymerase chain reaction, TNF-α: tumor necrosis factor-α, TGF-β1: transforming growth factor-β1, IL-6: interleukin-6
Figure S5. The addition of the supernatant that contained HE4 show no elevations of hypertrophy-related genes expression in cardiomyocytes.

(A) Experimental scheme for HE4 overexpression and transfer to cardiomyocyte. (B) Cardiac hypertrophy-related genes were evaluated by qRT-PCR. The measurements were standardized to expression of the GAPDH. Unpaired t-tests with Welch’s correction were used to compare groups.

HE4: human epididymis protein 4, HEK293T: human embryonic kidney 293T, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, ANP: atrial natriuretic peptides, β-MHC: β-myosin heavy chain, qRT-PCR: quantitative reverse-transcription polymerase chain reaction
Figure S6. HE4 does not affect the activity of Smad2, p38 MAP kinase, Akt, and JNK

(A) WB for intracellular signaling other than ERK in cardiac fibroblasts treated with HEK293T culture medium. (B) WB for ERK in cardiac fibroblasts treated with HEK293T culture medium and U0126, MEK 1/2 inhibitor, or DMSO.

WB: western blotting, ERK: extracellular signal-regulated kinase, HE4: human epididymis protein 4, HEK293T: human embryonic kidney 293T, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, DMSO: dimethyl sulfoxide