Gene expression profiling of dilated cardiomyopathy in older male EP4 knockout mice

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Observations of dilated cardiomyopathy (DCM) have been created by either selectively knocking out (KO) or over expressing a gene (1, 3, 6, 10, 16, 22, 32, 36, 43). In most cases, the genes were chosen in a targeted approach because of studies showing functional significance of the gene product in vitro in myocytes. Often, the mice develop severe DCM very early and die 2–8 wk after birth (1, 4). A strength of these types of studies is that a single gene is examined in inbred strains of mice, minimizing the role of complex genetic variables. However, DCM is a multifactorial disease, and the environment and genetic background can be important contributors to disease progression and severity.

In this report, we characterize a new model of DCM, which develops in older mice with cardiac-specific deletion of EP4, a receptor for PGE2 (31). Histological analysis of these mice at 10–12 wk of age indicated that EF, myocyte cross-sectional area (MCSA), and interstitial collagen fraction (ICF) were not different from that in control littermates (WT). However, when subjected to myocardial infarction, the KO mice had reduced fibrosis and hypertrophy but an unexpected worsening of cardiac function, suggesting that EP4 was involved in either a survival pathway or compensatory hypertrophy. Another study also had suggested that EP4 was protective in a model of ischemia-reperfusion injury (39). Additional studies on myocardial infarction in older EP4 KO mice indicated an unusually high rate of mortality (unpublished observations). Thus it was of interest to analyze the development of cardiac dysfunction in these mice, examining the progression of the disease with age and whether it was sex dependent. In addition, whole genome gene expression arrays were used to examine differentially expressed genes in KO and WT mice to understand disease progression.

METHODS

Generation and Genotyping of KO Mice

Generation of cardiac myocyte-specific EP4 KO mice by a Cre-mediated process has been previously described (31). Genotyping and breeding details are provided in the Supplemental Data. (Supplemental data for this article are available online at the American Journal of Physiology-Heart and Circulatory Physiology website.)

Animal Protocols

All animal experiments were approved by the Henry Ford Health System Institutional Animal Care and Use Committee.

Protocol 1: Study of cardiac phenotype in old male and female KO mice. We used 23- to 33-wk-old male KO (n = 55) and WT littermates (n = 70) in these studies. Female KO (n = 7) and WT mice (n = 12) were studied at 30–32 wk of age. Mice were subjected to two-dimensional (2-D) M-mode echocardiography, and then hearts were harvested. Male KO and WT hearts were used for extraction of RNA and protein and for histological analysis.

THE PREVALENCE OF DILATED CARDIOMYOPATHY (DCM) is increasing every year and currently accounts for ~25% of cases of congestive heart failure. Its occurrence is greater in males and African Americans than in females or Caucasians. DCM is characterized by left ventricular dilatation and systolic dysfunction (i.e., impaired ejection fraction, EF). Ventricular wall thickness can be less than or equal to that of a normal heart, and fibrosis is present to a variable degree. The three major causes of DCM are inflammation, toxic stress (e.g., alcoholism), and genetics (autosomal dominant inheritance). For genetic forms of the disease, the penetrance is variable and age dependent (5).
Protocol 2: Development of DCM in male mice with age. To determine potential differences between male (n = 6) and female (n = 5) KO mice, we performed echocardiography every 4 wk beginning at 12 wk of age. Age-matched male (n = 5) and female (n = 8) WT mice were evaluated at the beginning and end of the study. At 28 wk of age, systolic blood pressure was measured to ensure that any changes in cardiac function could not be ascribed to alterations in blood pressure. Some KO and WT hearts were used for extraction of RNA and protein and for histological analysis.

Protocol 3: Isolation of adult mouse myocytes and measurement of length. Myocytes were isolated from 31- to 33-wk-old male WT and KO mice (n = 3 per group). Three hours after plating, images of myocytes were taken under bright-field microscopy, and their length and width were then measured using ImageJ software. The method is described in the Supplemental Data.

Protocol 4: Gene expression profiling of old male WT and KO mice. Twenty-four 30- to 32-wk-old mice were used for this study (11 WT and 13 KO). Mice were subjected to echocardiography, and then hearts were harvested for RNA and protein extraction for microarray and cytokine bead assays, respectively.

Protocol 5: Cytokine/chemokine gene expression in young vs. old male KO mice. To study alterations in gene expression before DCM development, we used real-time RT-PCR to measure expression of the chemokines Ccl8, Ccl12, Ccl21b, and Cx3c1l and the cytokines galectin-3 and growth differentiation factor (GDF)-15 in hearts of 30- to 32-wk-old male WT and KO mice (n = 3–10) and 10- to 12-wk-old male WT and KO mice (n = 6 KO and 3 WT).

The cardiac function of all mice was assessed by echocardiography using an Acuson 256 system (Mountain View, CA) with a 15-MHz linear transducer, as reported previously (31). Mice were conscious during the procedure. Diastolic measurements were made at the maximum left ventricle cavity dimension, whereas systolic parameters were measured during maximum anterior motion of the posterior wall. All echocardiography was performed by investigators who were blinded to the genotype.

After echocardiography, mice were euthanized with pentobarbital sodium (100 mg/kg ip) and hearts were injected with 15% KCl to stop them in diastole. Hearts were trimmed of atria and weighed. If hearts were used for RNA extraction, they were stored in RNAlater at −80°C. For histological analysis, hearts were frozen in isopentane and stored at −80°C. For protein extraction, pieces of heart were snap-frozen in liquid N2 and stored at −80°C.

For a subset of mice, systolic blood pressure was obtained by the tail-cuff method using a MC 4000 blood pressure analysis system for mice (Hatteras Instruments, Cary, NC), as described previously (41).

Histological Assessments of ICF, Collagen, MCSA, and Macrophage Infiltration

Mouse hearts were harvested and sectioned transversely into four slices from apex to base. Preparation, staining, and analysis of sections is described in detail in the Supplemental Data.

Western Blot Analysis

In selected mouse hearts, we assessed Cre protein by Western blotting. Briefly, 20-mg portions of whole heart ventricle were homogenized, and protein concentration was determined using the Coomassie blue assay. A 15-μg protein sample was electrophoresed under reducing conditions and transferred to a polyvinylidene difluoride membrane overnight at 4°C and 23 V. Cre protein was detected using a rabbit polyclonal antibody (Novagen) at a dilution of 1:10,000, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000 dilution). After washing, membranes were developed using an enhanced chemiluminescent detection method (Pierce).

RNA Isolation and Microarray Analysis

Gene expression differences were analyzed between KO (n = 13) and WT mice (n = 11). Six of the KO mice were classified as KO-low (EF range of 14–34%) and seven as KO-high (EF range 46–76%) based on their EF. The RNA isolation procedure and gene array methods are provided in the Supplemental Data. Both raw and normalized gene array data and a spreadsheet of data with the 1,343 statistically significant Illumina gene identifications associated with each of the three groups have been submitted to the Gene Expression Omnibus (GEO; accession no. GSE16909 [www.ncbi.nlm.nih.gov/geo]).

Real-Time RT-PCR

To confirm some of the gene array data, we analyzed 12 genes (see Table 2) by quantitative real-time RT-PCR using a SYBR green method. Predesigned mouse-specific primers from SA Biosciences (Frederick, MD) were used for all PCR reactions. One microgram of DNase-treated total RNA sample was reverse transcribed using random primers and Omniscript reverse transcriptase (Qiagen, Valencia, CA). Two microliters of the reverse transcription reaction were then amplified in a Roche version 2.0 LightCycler PCR instrument (Indianapolis, IN) using SYBR green dye (SA Biosciences) and specific primers according to the SA Biosciences protocol. At the end of PCR cycling, melting curve analyses were performed and representative PCR products were run on agarose gels and visualized by ethidium bromide staining. RT-PCR of GAPDH was used for normalization of all data. A relative quantitation method (ΔΔCt) (37) was used to evaluate expression of each gene in KO heart relative to WT. Primers for the detection of Cre, galectin-3, and GAPDH were designed by TIB MolBiol (Adelphia, NJ), as described in the Supplemental Data.

Cytokine Bead Array

The cytometric bead array mouse inflammation kit (BD Biosciences, San Jose, CA) was used to detect cytokines in the heart (34). This kit detects IL-6, IL-10, monocyte chemotactic protein (MCP)-1, IFN-γ, TNF-α, and IL-12p70. Hearts were homogenized on ice in 300 μl of assay diluent plus protease inhibitors and were then centrifuged at 10,000 g for 10 min at 4°C. The supernatant was removed and used for subsequent analysis as described by the manufacturer. Detection was performed using the FL-2 channel of an upgraded LSR flow cytometer (Becton Dickinson) with six-color capability set to capture 2,000 events using a low flow rate. The concentration of each cytokine in the heart samples was calculated from standard curves of mean fluorescence intensity and corrected for protein concentration.

Statistical Analysis

To determine differences between WT and KO mice, we performed a t-test, with a P value <0.05 considered significant. For comparisons of more than two groups of normally distributed data, we performed ANOVA followed by Tukey’s method for multiple pairwise comparisons. Where conditions of normality were not satisfied, we performed ANOVA on ranks using the Kruskal-Wallis test followed by Dunn’s method for multiple comparisons.

RESULTS

Effect of EP4 KO on Cardiac Structure and Function in Male and Female Mice

Echocardiography was performed once on 23- to 33-wk-old male mice. EF was significantly lower in KO vs. WT mice (WT: 80 ± 0.6%, n = 70; KO: 60 ± 2.7%, n = 55; P < 0.001). The frequency distribution of EF values suggested that there were two groups of KO mice (Fig. 1), one with EF >
70% (KO >70) and the other with EF < 70% (KO <70), with 70% as the approximate cutoff between the two groups as determined by evaluation of the data using a spline interpolation to draw a smooth curve through the data. Table 1 shows that the KO <70 group had decreased EF, increased left ventricular mass, and altered structure [left ventricular dimension at systole (LVDs), left ventricular dimension at diastole (LVDd), and posterior wall thickness at diastole (PWTd)] vs. the WT controls. There was also a tendency for increased heart-to-body weight ratio (HW/BW) in KO mice. Echocardiography analysis of female mice indicated a modest but significant decline in EF, accompanied by a slight increase in LVDs (Table 1).

To determine whether individual male and female KO mice exhibited age-dependent alterations in cardiac function, we performed echocardiography every 4 wk on mice from 12 to 28 wk of age. During this time, the mean EF of male but not female KO mice declined with age (Fig. 2B). EF data for individual male KO mice were graphed, showing that some mice had a more rapid decline in EF (Fig. 2B). These data coupled with the data in Table 1 indicate that the decline in EF begins at ~16 wk of age in males but after 24–28 wk of age in females.

At 28 wk of age, systolic blood pressure was not different between male and female WT mice (122.5 ± 2.8 vs. 119.6 ± 3.0 mmHg) and KO mice (120.7 ± 2.9 vs. 116.6 ± 3.0 mmHg). Also, Cre mRNA and protein levels in heart extracts were similar in male and female WT and KO mice (Supplemental Fig. 1).

**Effect of EP4 KO on Myocyte Size, Collagen Fraction, and Macrophage Infiltration in Male Mice**

Histology was performed on additional sets of KO and WT control mice. The ICF values of the KO groups were higher than those of WT mice, with a 39% increase in ICF (7.5 ± 0.8% for KO <70, 6.9 ± 0.6% for KO >70, and 5.4 ± 0.2% for WT; *P < 0.05*) (Fig. 3A). Picrosirius red staining of collagen demonstrated greater collagen deposition in KO hearts, verifying the ICF data (Fig. 3, B and C). Regarding the size of the myocytes, MCSA was not different among the groups, but HW/BW was increased in KO hearts (Fig. 3D). To understand this discrepancy, we measured the length and width of isolated myocytes from 31- to 33-wk-old male WT and KO hearts (n = 3 each). Length of myocytes from KO hearts was significantly increased compared with WT hearts, whereas myocyte width was not different (Fig. 4).

Gross histological analysis of heart sections indicated that there was myofiber disarray and patches of infiltrating cells in the KO mice (Supplemental Fig. 2). This was consistently observed in all six slides of heart sections from KO mice. In contrast, there was no myofiber disarray observed in heart sections from seven WT mice, and only one of seven WT mice showed a very minimal infiltrate. In addition, heart sections were stained for macrophages, and we found that the percentage of positive staining was not different between KO and WT mice (1.47 ± 0.19% for WT vs. 1.59 ± 0.16% for KO mice, n = 6).

### Table 1. Baseline characteristics of old male and female mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Controls</th>
<th>KO (EF &gt;70)</th>
<th>KO (EF &lt;70)</th>
<th>WT Controls</th>
<th>KO (All)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>40.5±0.81</td>
<td>37.9±1.24</td>
<td>43.0±1.17†</td>
<td>27.6±0.76</td>
<td>28.6±1.31</td>
</tr>
<tr>
<td>Age, wk</td>
<td>29±0.4</td>
<td>28±0.4</td>
<td>28±0.5</td>
<td>31±0.3</td>
<td>31±0.2</td>
</tr>
<tr>
<td>EF, %</td>
<td>79.8±0.58</td>
<td>79.8±0.59</td>
<td>46.7±2.52†</td>
<td>80.3±1.30</td>
<td>68.7±3.47*</td>
</tr>
<tr>
<td>LVDs</td>
<td>1.35±0.04</td>
<td>1.41±0.07</td>
<td>2.72±0.18†</td>
<td>1.19±0.06</td>
<td>1.56±0.12*</td>
</tr>
<tr>
<td>LVDd</td>
<td>3.20±0.04</td>
<td>3.01±0.06</td>
<td>4.01±0.14†</td>
<td>2.51±0.08</td>
<td>2.81±0.12</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>652±6.0</td>
<td>663±8.8</td>
<td>629±8.8†</td>
<td>678±13</td>
<td>683±10</td>
</tr>
<tr>
<td>PWTd</td>
<td>1.00±0.01</td>
<td>0.97±0.03</td>
<td>0.91±0.01†</td>
<td>0.89±0.03</td>
<td>0.92±0.02</td>
</tr>
<tr>
<td>Total n</td>
<td>70</td>
<td>22</td>
<td>33</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>3.32±0.09</td>
<td>3.45±0.22</td>
<td>3.83±0.25</td>
<td>3.84±0.06</td>
<td>3.86±0.18</td>
</tr>
<tr>
<td>Heart mass, mg/g</td>
<td>2.48±0.08</td>
<td>2.50±0.13</td>
<td>3.50±0.31†</td>
<td>2.38±0.12</td>
<td>2.76±0.16</td>
</tr>
<tr>
<td>HW n</td>
<td>56</td>
<td>19</td>
<td>27</td>
<td>7</td>
<td>12</td>
</tr>
</tbody>
</table>

Baseline echocardiography data in old male and female mice. Ejection fraction (EF) values for female EP4 receptor knockout (KO) mice are not divided into 2 groups because of their limited number. All female mice were 32 wk old. Heart weight (HW) refers to the weight of the ventricles (left ventricle, right ventricle, and septum). The last row of n values (HW n) is for heart-to-body weight ratio (HW/BW) and heart mass data only. LVDs, left ventricular dimension at systole; LVDd, left ventricular dimension at diastole; HR, heart rate; PWTd, posterior wall thickness at diastole. Values are means ± SE. *P < 0.05 vs. WT, †P < 0.05 vs. KO >70% EF. Statistical analysis was performed using ANOVA followed by Tukey’s test of pairwise multiple comparisons for normally distributed data and by Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn’s method of pairwise comparisons for data that did not fit a normal distribution.
Fig. 2. Comparison of the development of cardiac dysfunction in male and female KO and WT mice. KO mice were evaluated every 4 wk by echocardiography from 16 to 28 wk of age. WT mice were evaluated at the beginning and end of the study. A: EF is plotted on the y-axis vs. age in wk on the x-axis for the 4 groups of mice. *P < 0.05, WT vs. KO. B: EF of each of the 6 male KO mice was plotted individually. One mouse died shortly after 24 wk of age.

**Gene Expression Profiling of Hearts of Old Male WT and KO Mice**

For this study, KO mice were divided into two groups, both with mean EF values <70% [EF for KO-high = 56.8 ± 3.9% (n = 7) and for KO-low = 24.8 ± 2.8% (n = 6)]. The echocardiography data of these mice are shown in Supplemental Table 1. Supplemental Fig. 3A is a density plot of raw Illumina intensity data for all 45,000 probes for all 24 mice, and Supplemental Fig. 3B is the density plot of the normalized data. With the use of a modified one-way analysis of variance from the limma package on the normalized microarray data, 1,343 probe sequences across the three preassigned groups (WT, KO-high, KO-low) were significantly differentially expressed (Bonferroni-adjusted P value <0.05). After genes with multiple probe sequences were identified, there were 965 genes and 141 unidentified expressed sequences (ESTs) representing the 1,343 differentially regulated probes. These data along with their moderated t-statistics and P values are available on the GEO website.

Genes in the data set with the highest level of expression included cardiac-specific gene transcripts, such as cardiac troponin C (Tnnc1), α2-actinin (Actn2), cardiac α-actin (Actc1), α-myosin heavy chain (Myh6), muscle lim protein (Mlp or Csrp3), myosin light chain 2 (Myl2), myosin light chain 3 (Myl3), and cardiac Ca2+-ATPase (Atp2a2). When the data set was sorted to identify the most highly expressed genes in the KO-low group, then the natriuretic peptide gene Nppb (BNP) was found among the most highly expressed. In addition, β-myosin heavy chain (Myh7) expression was upregulated and α-myosin heavy chain (Myh6) downregulated in KO mice. The changes in Nppb, Myh6, and Myh7 are consistent with their changes in many animal models of cardiac disease (12, 23, 29).

Real-time RT-PCR was used to confirm in part the microarray gene expression data. The expression patterns were similar...
between the array data and the RT-PCR data for 12 genes tested (Table 2).

Using the normalized expression data, we found that 156 genes had at least a twofold higher expression in the KO-low group compared with the WT group. Supplemental Table 2 is a subset of upregulated genes in KO hearts, including genes involved in remodeling, inflammation, and oxidative stress. The natriuretic peptide BNP and the cytokine GDF-15, markers of cardiac disease, were also upregulated. Four chemokine genes, Cx3cl1 (fractalkine), Ccl8 (monocyte chemoattractant-2 or MCP-2), Ccl12 (monocyte chemoattractant-5 or MCP-5), and Ccl21 (chemokine ligand 21), were overexpressed, as was the profibrotic and proinflammatory factor galectin-3. In addition to upregulated genes, the normalized microarray data also indicated that 79 genes were downregulated in the KO hearts (i.e., the expression level in WT hearts was at least 2-fold higher than in the KO-low group; Supplemental Table 3). These genes will not be discussed further.

Finally, two-way cluster analysis was used to generate a heat map to visualize patterns of gene expression in the 24 mice (Fig. 5). The clusters separated mice according to their genotype (WT vs. KO) and the degree of cardiac dysfunction (KO-high = KO-07 and KO-09–KO-13 mice; KO-low = KO-01–KO-06 mice). The KO-08 mouse clustered more with the KO-low group than the KO-high group despite the fact that

Table 2. Real-time RT-PCR validation of gene array data

<table>
<thead>
<tr>
<th>Gene</th>
<th>Relative Induction (KO-L vs. WT)</th>
<th>RT-PCR</th>
<th>Gene array data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace</td>
<td>4.7±0.8</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Col 3α1</td>
<td>0.8±0.8</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Col 3β1</td>
<td>14.9±3.6</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Ctgf</td>
<td>13.9±1.8</td>
<td>6.3</td>
<td></td>
</tr>
<tr>
<td>Gadd15</td>
<td>14.4±1.9</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Lox</td>
<td>20.2±4.2</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>NcoA4</td>
<td>11.8±2.2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Nppb</td>
<td>7.4±0.5</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Ptgis</td>
<td>3±0.1</td>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>Sparc</td>
<td>3.8±0.7</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>Taz</td>
<td>0.5±0.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Timp 1</td>
<td>5±0.5</td>
<td>12.3</td>
<td></td>
</tr>
</tbody>
</table>

Relative (fold) induction of genes from the microarray data compared with the real-time RT-PCR data. Values are means ± SE; n = 5–10 per group. Gene identifications are defined in Supplemental Tables 2 and 3.
its EF was 47% and the EF of the KO-low group averaged 25%. Of interest, three KO mice had EF values that were 60% or greater (KO-11, 60%; KO-12, 61%, and KO-13, 76%), yet they all had gene expression profiles more similar to the KO groups than to WT mice.

Cytokine Production in Old Male WT and KO Mouse Hearts

Since the microarray data indicated no change in expression of two cytokines involved in remodeling (Ccl2 or MCP-1 and IL-6), an analysis of their protein levels was done. Using a cytokine bead array, we detected both IL-6 and MCP-1 in the KO and WT hearts, but there were no differences between the strains (IL-6: WT = 0.25 ± 0.06 and KO = 0.26 ± 0.03 pg/µg protein; MCP-1: WT = 0.49 ± 0.02 and KO = 0.60 ± 0.06 pg/µg protein; n = 10–11).

Cytokine/Chemokine Gene Expression in Young vs. Old Male KO Mice

Because of the presence of infiltrates in KO mouse hearts and evidence of cytokine and chemokine overexpression from the microarray data, the expression of several chemokines/cytokines was assessed by real-time RT-PCR. The chemokines Cx3C11, Ccl8, Ccl12 and Ccl21b were upregulated in the KO hearts from old males, and galectin-3 and GDF-15 were each upregulated more than 15-fold (Fig. 6).

To determine whether these chemokines/cytokines play a role in the development of the DCM phenotype or function as a biomarker of the disease, we analyzed their expression in the hearts of 10- to 12-wk-old male KO and WT mice. Echocardiography indicated that there were no differences in EF, LVDs, LVDd, and PWTd between young male WT and KO mice (data not shown). Both Ccl8 and Ccl12 expression were not reproducibly detectable in 40 cycles of PCR. Moreover, there were no differences in cardiac expression of Ccl21b, Cx3C11, and galectin-3 between young KO and WT male mice (Fig. 7). However, GDF-15 expression was significantly up-regulated threefold in KO hearts, although this relative increase was less than that in old DCM mice (15-fold vs. WT).

DISCUSSION

This report describes a novel mouse model of age- and sex-dependent DCM that results from knockdown of the EP4 receptor in cardiac myocytes. These KO mice had no obvious cardiac phenotype at 12 to 14 wk of age, but their EF was further decreased after myocardial infarction (31). Other studies in mice also suggested that EP4 was protective (14, 39). Thus it was expected that knockdown of EP4 in the heart might prove deleterious in response to other stressors, such as aging.

![Fig. 6. Real-time RT-PCR analysis of chemokines and cytokines in old male mice. The y-axis is normalized expression plotted as fold induction, and the x-axis indicates WT, KO mice with an EF range of 14–34% (KO-L), and KO mice with an EF range of 46–76% (KO-H). Cardiac expression of the chemokines Ccl8, Ccl12, Ccl21b, and Cx3c11 (fractalkine), GDF-15, and galectin-3 is shown in 30- to 32-wk-old male WT vs. KO mice (n = 3–10 per group). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.005 compared with WT mice. #P < 0.05 compared with KO-H.](http://ajpheart.physiology.org/)

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Cardiac Remodeling

Old male KO mice had significant changes in the size and shape of the heart. Male KO mice demonstrated an increase in cardiac mass and evidence of increased HW/BW. However, wall thickness was reduced, and there was no change in MCSA. Given that isolated myocytes from old KO hearts had increased length with no change in width (Fig. 4), altogether these observations suggest that the EP4 KO mice are developing eccentric hypertrophy.

The hearts of the old male KO mice were characterized by fibrosis and dilatation. Dilatation of the ventricle occurs as a result of breakdown of the collagen matrix by metalloproteinases (MMPs), resulting in a thinning of the ventricle wall. MMPs with collagenolytic activity include MMP-1, MMP-8, MMP-13, MMP-2 (gelatinase), and MMP-14 (also known as MT-MMP-1) (26). In this study, MMP-2 and MMP-14 were upregulated in the hearts of old KO mice. MMP-2 activity seems to be important in wound healing and remodeling in mouse hearts, since MMP-2 deletion reduces both cardiac rupture and remodeling (24). In addition, MMP-14 has been identified in the heart of patients with nonspecific and ischemic DCM (35), in pig hearts subjected to ischemia-reperfusion injury (7), and in mouse hearts overexpressing EMMPRIN (extracellular MMP inducer) (42). Both cardiac myocytes and fibroblasts have been shown to synthesize MMP-14 (7, 11, 35). MMP-14 also has been shown to process and/or activate a number of proinflammatory and profibrotic chemokines and cytokines, including MCP-3, connective tissue growth factor (CTGF), and galectin-3 (25, 26). Thus it will be of particular importance to investigate the roles of these MMPs, especially MMP-14, in the progression of DCM.

PGE2 has been associated with alterations in MMP activity. This effect appears to be cell-type specific, but little is known about how this prostanoid influences MMP activity and/or expression in the remodeled heart. In one of the few studies of cardiac remodeling, an EP4 agonist was recently shown to reduce MMP-2 and -9 activities and to improve cardiac function in a rat model of ischemia-reperfusion injury (14). If myocytes are an important source of secreted MMPs or of factors that regulate MMPs, then there could be a connection between EP4 deletion and the increased expression of MMPs in our DCM model. Important future studies must focus on the role of PGE2 and EP4 in the regulation of extracellular matrix proteins, MMPs, and growth factors and cytokines in cardiac myocytes and fibroblasts.

Chemokines, Cytokines, and Inflammation

Our microarray data indicated that the chemokines fractalkine, Ccl8, Ccl12, and Ccl21 were upregulated in hearts of old male KO mice. Chemokines are produced by several different cell types, but most typically by resident and infiltrating cells at sites of inflammation. Chemokines amplify chronic inflammatory reactions and sustain responses in autoimmune diseases. Their nonchemotactic functions include remodeling, angiogenesis, and immune regulation.

Ccl8 (MCP-2) has been shown to be a specific serum marker for the early and accurate diagnosis of graft-vs.-host disease in humans (15), and Ccl12 and Ccl21 also have been implicated in transplant graft disease (33). Whether these chemokines are expressed by infiltrating immune cells, fibroblasts, or myocytes in the EP4 KO mouse hearts is unknown. The unidentified infiltrate in the KO hearts might be the source of the chemokines, although our data have excluded macrophages in that infiltrate as the source. Preliminary studies indicate that Ccl8, Ccl12, and Ccl21b are expressed in both rat neonatal ventricular myocytes and adult mouse myocytes (Harding P and LaPointe MC, unpublished data), so it is possible that myocytes contribute to the production of chemokines during the development of DCM. However, it is doubtful that these chemokines are causative of the disease process, since their expression levels were not elevated in young male KO mice. Nonetheless, they could play important roles in remodeling during DCM.

Only recently has the relationship between PGE2 and chemokine expression been studied in the heart. A selective EP4 receptor agonist (EP4RAG) was shown to reduce chemokine expression, T-cell proliferation, and macrophage activation in a mouse model of cardiac transplantation (28), suggesting that EP4 has a role in preventing cardiac rejection. Additional studies are needed to understand whether PGE2 and EP4 regulate chemokine gene expression in myocytes or whether an EP4-dependent process regulates a factor that has an impact on chemokine expression in another cardiac cell type.

GDF-15, a stress-responsive member of the TGF-β family, was strongly induced in the KO heart. More importantly, its expression was increased in hearts of young male mice, suggesting that it may be involved in development of the disease or serve as a biomarker for disease development. In a population of apparently healthy blood donors, only 2% had elevated GDF-15 levels (21). However, this factor was induced in the hearts of patients with ischemia-reperfusion injury and associated with a higher risk of death in patients with congestive heart failure (20), as well as serving as a biomarker of adverse outcome in patients with acute chest pain and other cardiovascular diseases (8, 38). In general, elevated GDF-15 also is associated with inflammation, age, diabetes, and smoking in humans (38). Increased GDF-15 expression has been described in mouse models of DCM (68) and in myocytes subjected to stress (19). Thus one might infer that GDF-15 is deleterious.

Despite the fact that GDF-15 is a biomarker of cardiovascular disease, several studies in animal models suggest that it is cardioprotective and that its upregulation is compensatory.
Neither GDF-15-deficient nor GDF-15 cardiac-overexpressing mice had any obvious cardiac phenotype up to 4 mo of age (19, 40). However, GDF-15 KO mice exhibited a greater infarct size after coronary artery ligation (31) and enhanced cardiac hypertrophy following pressure overload (40). In contrast, GDF-15-overexpressing transgenic mice had a reduced hypertrophic response to pressure overload, and recombinant GDF-15 infusion into a mouse model of DCM improved cardiac function (40). The antihypertrophic effect of GDF-15 has been verified in an in vitro model of neonatal ventricular myocyte hypertrophy (40). Since there does not appear to be a hemodynamic stressor in our DCM model (given that blood pressure is normal and there is no apparent target organ damage in young male KO mice), an antihypertrophic role for GDF-15 does not seem likely.

On the basis of our data in young and old male EP4 KO mice, both of which have enhanced GDF-15 expression, we would suggest that PGE2 acting through EP4 reduces the expression of GDF-15 and that cardiac EP4 knockdown releases GDF-15 from this inhibition. In fact, PGE2 inhibition of GDF-15 expression has been reported (26). Thus GDF-15 is either a marker of the stressed heart or contributes to the disease phenotype in the setting of EP4 knockdown. It also is possible that EP4 knockdown results in the upregulation of an inflammatory mediator, which stimulates expression of GDF-15. It would be of particular interest to understand whether cardiac cells respond differently to GDF-15 during disease progression, thus demonstrating both protective and deleterious effects. Thus understanding the regulation of GDF-15 might provide insight into the disease process.

**Sex Differences and DCM**

DCM developed in female KO mice, but it occurred at an older age and to a lesser extent than in male KO mice. Our result is consistent with published studies showing that female sex protects against both the severity and frequency of DCM (17, 32). Nieminen et al. (27) evaluated sex differences in patients hospitalized for acute heart failure in the EuroHeart Failure Survey II and found that men had DCM more frequently than women. Likewise, Haddad et al. (13) reported that men with idiopathic DCM undergoing transplantation showed a different gene expression profile than women. Thus the EP4 KO model, with its sexual dimorphism in DCM, may represent a good model to understand the molecular basis of sex differences in disease progression.

**Relationship to Human Disease**

Barth et al. (2) published a study in which they identified a common gene expression signature for DCM using two microarray studies of their own patient samples combined with two other data sets that had been deposited in the GEO database. Using prediction analysis for microarrays, the authors identified 27 genes that were sufficient to classify DCM and nonfailing hearts across the 4 studies. Eight of the marker genes were differentially expressed in our DCM model (the natriuretic peptide BNP (Nppb), α-myosin heavy chain (Myh6), nonmuscle myosin heavy chain (Myh10), CTGF, procollagen C-endopeptidase enhancer 2 (Pcolce2), Sparc (secreted protein, acidic, cysteine-rich), adipocyte enhancer binding protein (Aebp1), and pleckstrin homology-like A3 (Phlad3)], suggesting that our mouse model may reflect some of the changes seen in end-stage human disease.

**Effect of Genetic Background**

There could be several reasons for the age anomalies in disease progression in the male EP4 KO mice. First, it is possible that Cre is variably expressed in myocytes in the heart, resulting in incomplete EP4 excision. This could lead to a variable phenotype and be a possible explanation of the differences in disease severity in male KO mice. Alternatively, we may have two populations of KO mice that are developing DCM at different ages due to the effect of a modifier gene. Modifier genes have been implicated in cardiomyopathy, including angiotensin-converting enzyme (ACE) (18, 30) and the β-adrenergic receptor (9), but generally only alter the course and severity of the disease. However, our study design did not allow for identification of such modifiers.

**Summary**

In conclusion, we describe a mouse model of DCM that develops with age in cardiac-specific EP4 KO mice. These studies indicate that DCM development involves fibrosis, myocyte elongation, dilatation, and contractile dysfunction. DCM is also sex dependent, with females developing either a milder form of the disease or developing the disease at a later age. Gene expression profiling of our DCM mice during disease progression elucidated upregulated genes that may contribute to the disease process as well as reflect the remodeling and end stages of the disease. The increase in GDF-15 expression in young KO male mice suggests that further studies should be done to examine it as either an EP4-dependent causative factor or simply a biomarker of either the disease or the inflammation accompanying the disease. Limitations of our study are that we examined only gene expression, and we have yet to fully understand gene expression changes in young vs. old male KO mice and between females and males. It also will be important to understand how stressors, such as hypertension, impact DCM progression. Such studies are in progress.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**REFERENCES**

3. Bryant D, Becker L, Richardson J, Shelton J, Franco F, Peshock R, Thompson M, Giroir B. Cardiac failure in transgenic mice with myo-


