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Platelet Endothelial Cell Adhesion Molecule-1 Mediates Endothelial-Cardiomyocyte Communication and Regulates Cardiac Function

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***Background*—**Dilated cardiomyopathy is characterized by impaired contractility of cardiomyocytes, ventricular chamber dilatation, and systolic dysfunction. Although mutations in genes expressed in the cardiomyocyte are the best described causes of reduced contractility, the importance of endothelial-cardiomyocyte communication for proper cardiac function is increasingly appreciated. In the present study, we investigate the role of the endothelial adhesion molecule platelet endothelial cell adhesion molecule (PECAM-1) in the regulation of cardiac function.

***Methods and Results*—**Using cell culture and animal models, we show that PECAM-1 expressed in endothelial cells (ECs) regulates cardiomyocyte contractility and cardiac function via the neuregulin-ErbB signaling pathway. Conscious echocardiography revealed left ventricular (LV) chamber dilation and systolic dysfunction in PECAM-$1^{-/-}$ mice in the absence of histological abnormalities or defects in cardiac capillary density. Despite deficits in global cardiac function, cardiomyocytes isolated from PECAM-$1^{-/-}$ hearts displayed normal baseline and isoproterenol-stimulated contractility. Mechanistically, absence of PECAM-1 resulted in elevated NO/ROS signaling and NRG-1 release from ECs, which resulted in augmented phosphorylation of its receptor ErbB2. Treatment of cardiomyocytes with conditioned media from PECAM-$1^{-/-}$ ECs resulted in enhanced ErbB2 activation, which was normalized by pre-treatment with an NRG-1 blocking antibody. To determine whether normalization of increased NRG-1 levels could correct cardiac function, PECAM-$1^{-/-}$ mice were treated with the NRG-1 blocking antibody. Echocardiography showed that treatment significantly improved cardiac function of PECAM-$1^{-/-}$ mice, as revealed by increased ejection fraction and fractional shortening.

***Conclusions*—**We identify a novel role for PECAM-1 in regulating cardiac function via a paracrine NRG1-ErbB pathway. These data highlight the importance of tightly regulated cellular communication for proper cardiac function. **(*J Am Heart Assoc*. 2015;4: e001210 doi: 10.1161/JAHA.114.001210)**

Key Words: cardiomyocyte • cardiomyopathy • endothelial cell • neuregulin-1 • PECAM-1

Endothelial cells (ECs) are in close physical proximity to cardiomyocytes in the heart, such that each cardiomyocyte is surrounded by a dense network of capillary vessels comprised of ECs. The endothelium releases factors such as neuregulin (NRG-1) and nitric oxide (NO), which can affect numerous parameters of cardiomyocyte function, including their contractility.[1] Cardiomyocytes, in turn, secrete factors that can also direct EC function.[2] Recent data provide a compelling link between EC dysfunction and the progression of dilated cardiomyopathy (DCM), a condition characterized by dilatation and impaired contraction of the heart that can lead to heart failure and sudden death.[3] Although critical for understanding the physiological and pathological processes of the heart, our knowledge of the pathways that regulate EC-cardiomyocyte crosstalk is limited. Importantly, we know that impaired release and/or activity of the molecules implicated in crosstalk can result in heart failure. For example, the knockout mouse of apelin, a protein released by ECs and important for a number of physiological functions, develops impaired contractility with age.[4–6] The NRG-1-ErbB signaling pathway represents another pertinent example. Interest in

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Received June 19, 2014; accepted November 13, 2014.

this pathway was generated following the observation that women treated with trastuzumab, a monoclonal antibody against the NRG-1 receptor ErbB2 (or HER2), have increased risk for development of heart failure.[7] Numerous studies have supported a role for NRG-1 and ErbB2 in heart failure; the ventricular-specific knockout of ErbB2 develops DCM with age[8] and treatment with NRG-1 improves cardiac function in models of cardiomyopathy.[9] Finally, studies have suggested a role for hemodynamic stress, hypoxia as well as mechanical stress in the development of DCM.[10]

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a cell adhesion and signaling molecule expressed in blood and vascular cells that has roles in regulation of endothelial junctional integrity; transendothelial migration; as well as angiogenesis.[11] Our own work has shown that PECAM-1 functions as a mechanosensor in ECs and confers the ability to sense and respond to the hemodynamic force of flowing blood,[12] as well as tensional force using a magnetic tweezers system.[13] Although PECAM-1 initiates mechanosignaling specifically in ECs, its presence (or absence) can have profound consequences on EC communication with other cell types, including vascular smooth muscle cells (VSMCs), and thus affect the physiology and pathology of the vessel as a whole. The PECAM-$1^{-/-}$ mouse is viable, but displays vascular defects in response to changes in hemodynamic forces. Specifically, PECAM-$1^{-/-}$ mice display blunted flow-mediated dilation due to misregulated NO production[14] and reduced VSMC relaxation.[15,16] In addition, our lab has shown that PECAM-1 deficiency results in impaired flow-mediated vascular remodeling that results in reduced VSMC activation and signaling.[17,18]

As EC-cardiomyocyte crosstalk is important in regulating cardiac function and cellular communication in the vessel wall is perturbed in the absence of PECAM-1, we hypothesized that cardiac function is impaired in PECAM-$1^{-/-}$ mice. Here, using a combination of knockout mouse models and cell culture systems, we show that increased NRG-1 release in PECAM-1 knockout ECs results in augmented ErbB2 phosphorylation in cardiomyocytes and impaired cardiac function, at least in part through effects on cardiomyocyte contractility. This novel PECAM-1/NRG-1/ErbB2 pathway may represent a fundamental mechanism that coordinates endothelial and cardiomyocyte signaling in the heart.

Materials and Methods

Animals

PECAM-$1^{-/-}$ C57BL/6 mice were kindly provided by Dr P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee), bred in house and used in accordance with the guidelines of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM-$1^{-/-}$ and age-matched wild-type PECAM-$1^{+/+}$ littermates were used for all studies.

Cell Culture

PECAM-1-knockout (PE-KO) cells and cells reconstituted with murine full-length PECAM-1 (PE-RC) were prepared as previously described.[14,19] The murine HL-1 cell line was a gift from Dr W.C. Claycomb (Louisiana State University Medical Centre, New Orleans, LA) and maintained as described.[20]

Cardiac Function and Hemodynamic Measurements

Conscious echocardiography was performed as previously described using a Vevo 2100 ultrasound biomicroscopy system.[21,22] LV dimension data represent the average of at least 3 independent waveforms. Blood pressure measurements were performed in conscious animals.[23] In vivo left ventricular function was assessed by PV catheter using a 1.2 French catheter-tip pressure transducer (Scisense, Inc, London, Ontario, Canada). The mouse was anesthetized with 2% isoflurane, intubated, and ventilated. Body temperature was maintained at 37°C. The chest was then opened and the apex of the left ventricle exposed. A 25-gauge needle was used to make a stab wound near the apex of the heart and the catheter was inserted. Baseline PV loops were recorded as well as vena cava inferior occlusions.[24]

RNA Isolation and Quantitative PCR

Total RNA was isolated from mouse left ventricles using the Qiagen AllPrep Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. First-strand cDNA was transcribed using random primers and SuperScript II Reverse Transcriptase (Invitrogen). Real-time quantitative PCR was performed using ABsolute SYBR Green ROX mix (Thermo Scientific). Primers used are found in Table 1.

Preparation of Lysates and Immunoblot Analysis

Hearts were homogenized and protein was extracted in a RIPA lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 2 mmol/L EDTA, 0.5% Triton X-100, 0.5% Na-Deoxycholate, 1% NP-40, 25 mmol/L β-glycerophosphate, 10% glycerol, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylsulphfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 mmol/L sodium fluoride, 1 mmol/L sodium pyrophosphate, pH 7.2). Protein extracts (30 μg) were subjected to Western blot

Table 1. Primers for Quantitative PCR

Primers	Sequence
ANP	
For	5′-TGGGACCCCTCCGATAGATC-3′
Rev	5′-TCGTGATAGATGAAGGCAGGAA-3′
βMHC	
For	5′-TTGAGAATCCAAGGCTCAGC-3′
Rev	5′-CTTCTCAGACTTCCGCAGGA-3′
αMHC	
For	5′-CTACGCGGCCTGGATGAT-3′
Rev	5′-GCCACTTGTAGGGGTTGAC-3′
αSKActin	
For	5′-CAGCTCTGGCTCCCAGCACC-3′
Rev	5′-AATGGCTGGCTTTAATGCTTCA-3′
18s	
For	5′-CATTCGAACGTCTGCCCTATC-3′
Rev	5′-CCTGCTGCCTTCCTTGGA-3′
PECAM-1	
For	5′-CACCTCGAAAAGCAGGTCTC-3′
Rev	5′-CGTTATACACCATCGCATCG-3′

βMHC indicates myosin heavy chain; ANP, atrial natriuretic peptide; PCR, polymerase chain reaction; PECAM, platelet endothelial cell adhesion molecule.

analysis with antibodies against pTyr877ErbB2, pTyr1284-ErbB4 (Cell Signaling); GAPDH (Millipore, Co, Bedford, MA). Vinculin was from Sigma. Immunocomplexes were detected using the Licor Odyssey secondary detection system. NRG-1β antibody was a gift from D. Sawyer. Media was concentrated as previously described[25] using Amicon Ultra-15 with Ultracel-3 membrane (Millipore, Co, Bedford, MA).

Histological Analysis and Immunohistochemistry

Hearts were perfused with 4% paraformaldehyde for 24 hours and then switched to 70% ethanol. The hearts were then paraffin embedded and sectioned into 5 μm sections and stained with hematoxylin and eosin to assess overall morphology.[21] For cross-sectional analysis of cardiomyocytes and determination of capillary density, heart sections were stained with TRITC (tetramethylrhodamine)-conjugated lectin (*Triticum vulgaris*) and examined by fluorescence microscopy as previously described.[21,22]

Cardiomyocyte Isolation/Functional Assays

Adult mouse cardiomyocyte isolation, experimentation, and analysis from PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ mice were performed as previously described.[26,27]

Intracellular Calcium Measurements

Myocytes were loaded with 5 to 10 μmol/L Fluo-4 AM (Molecular Probes) and placed in a heated chamber (35°C) on the stage of an inverted microscope and perfused with physiological Tyrode's solution containing 150 mmol/L NaCl, 5.4 mmol/L KCl, 1.2 mmol/L MgCl2, 10 mmol/L glucose, 2 mmol/L Na-pyruvate, 1 mmol/L CaCl2, and 5 mmol/l HEPES, pH 7.4. Myocytes were paced at 0.5 Hz and fractional shortening data was collected using edge detection. For $[Ca^{2+}]_i$ fluorescence measurements, the F0 (or F unstimulated) was measured as the average fluorescence of the cell 50 ms prior to stimulation. The maximal Fluo-4 fluorescence (F) was measured at peak amplitude. Isoproterenol (Sigma) was used at 1 μmol/L.

Blood Pressure Measurements

Blood pressure was measured non-invasively by a volume pressure-recording sensor and an occlusion tail-cuff (CODA System; Kent Scientific, Torrington, CT).

Tissue Hypoxia Staining

Tissue hypoxia was assessed using the Hypoxyprobe™-1 Kit (Hypoxyprobe, Inc, Burlington, MA) according to the manufacturer's instructions. Briefly, animals were injected i.p. with 60 mg/kg hypoxyprobe for 60 minutes. Hearts were then harvested and fixed in 4% PFA, embedded in paraffin and sectioned at 8 μm. Tissue sections were stained with the Hypoxyprobe-1 antibody and visualized using DAB.

Transmission Electron Microscopy

For electron microscopy, animals were euthanized and perfused with freshly made fixative containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.15 mol/L sodium phosphate buffer, pH 7.4. After perfusion, the hearts were removed and cut into 1 to 2 mm^3 cubes and stored from several hours to overnight in the fixative before processing for electron microscopy. Sections were observed using a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss SMT, Inc, Peabody, MA) and photographed using a Gatan Orius SC1000 Digital Camera and Digital Micrograph 3.11.0 (Gatan, Inc, Pleasanton, CA).

ROS Assays

Confluent endothelial cells were preincubated with 10 μmol/L of 2,7-dichlorodihydrofluoroscein diacetate (H_2-DCFDA) at 37°C for 30 minutes. Cells were rinsed with PBS and

lysed in PBS containing 0.2% Triton X-100 and 1 mmol/L N-acetylcystein. Fluorescence was measured using the 485 nm excitation/535 nm emission filter in a Wallac Victor 1420 MULTILABEL plate reader and normalized to total protein concentrations in the lysates which were determined using Bradford assay (Pierce).

Conditioned Media and Blocking Antibody Assays

A blocking antibody against human NRG-1 was purchased from R&D Systems (Minneapolis, MN). Endothelial media (0.1% FBS) was conditioned for 48 hours from a confluent monolayer. Thirty minutes prior to treatment of the HL-1 cells, 25 μg/mL of blocking antibody was added and incubated at 37°C. Cells were treated with the conditioned media for 15 minutes and then homogenized in RIPA lysis buffer. Protein extracts were subjected to Western blot analysis.[28]

Inhibition of NRG-1 In Vivo

Twelve-week old male PECAM$^{-/-}$ mice were treated with either saline or 4 μg of NRG-1 EGF-domain blocking antibody (R&D Systems) via intraperitoneal injections. Echos were performed at baseline and 24 hours post-injection.

Quantitation and Statistical Analysis

The band intensity was quantitated using ImageJ software. Each experimental group was analyzed using single factor analysis of variance (Excel; Microsoft). Probability values were obtained by performing a 2-tailed Student's *t* test using the same program. Mann-Whitney tests were also used (GraphPad Prism). Statistical significance was defined as $P<0.05$.

Results

PECAM-1$^{-/-}$ Mice Exhibit Both Systolic and Diastolic Dysfunction Indicative of Dilated Cardiomyopathy

To assess cardiac performance of PECAM-1$^{-/-}$ animals, we performed transthoracic echocardiography in conscious 14- to 16-week-old male mice (Figure 1, Table 2). We observed a significant enlargement of the left ventricular (LV) chamber at end diastole (LVID;d) in PECAM-1$^{-/-}$ mice compared to PECAM-1$^{+/+}$ mice (Figure 1A and 1B). Echocardiography also revealed impairment of LV function in PECAM-1$^{-/-}$ hearts, as evidenced by decreased ejection fraction and fractional shortening (Figure 1C). Interestingly, there was no significant difference in wall thickness at end diastole (Table 2). We did, however, observe an increase in left ventricular mass (Figure 1D). This phenotype was present at every post-natal time point evaluated (Figure 1E and 1F; Table 3). In summary, the echocardiographic data suggest that PECAM-1$^{-/-}$ mice have LV chamber dilation and systolic dysfunction, suggestive of dilated cardiomyopathy (DCM).

Cardiac function was further evaluated in anesthetized mice by in vivo cardiac catheterization. Representative images from PECAM-1$^{+/+}$ (black trace) and PECAM-1$^{-/-}$ (red trace) pressure-volume (PV) loops are shown (Figure 2A). PECAM-1$^{-/-}$ mice displayed increased end-systolic (ESV) and end-diastolic (EDV) volumes (Table 4). Conversely, there was a decrease in end-systolic pressure (ESP) (Table 4). These data are in agreement with observations made by echocardiography, further suggesting dilated cardiomyopathy in the PECAM-1$^{-/-}$ mice. Interestingly, the PV loop data also revealed a significant decrease in both the maximum and minimum derivatives of pressure development (+dP/dt and −dP/dt), indicating impaired rates of contraction and relaxation, respectively, in the PECAM-1$^{-/-}$ mice (Figure 2B). Importantly, we do not see an increase in diastolic pressures, suggesting that the PECAM-1$^{-/-}$ mice have compensated heart failure.

Reactivation of the fetal gene program is a common marker for various cardiac pathologies, including hypertrophy and DCM.[29] We therefore assessed fetal gene expression in adult PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ hearts by quantitative PCR. The expression of β-myosin heavy chain (βMHC), atrial natriuretic peptide (ANP) and α-skeletal actin (αSka) were significantly increased in PECAM-1$^{-/-}$ hearts compared with PECAM-1$^{+/+}$ littermates (Figure 3A). These data support our echocardiographic data and further indicate that genetic deletion of PECAM-1 results in DCM.

Studies indicate that increased blood pressure (hypertension) can lead to heart failure.[30,31] To exclude the possibility that differences in blood pressure account for the impaired cardiac function in the PECAM-1$^{-/-}$ mouse, we measured mean arterial pressures (MAP) in conscious 12- to 14-week-old mice. There was no significant difference in pressures between genotypes (Figure 3B), suggesting that cardiac dysfunction in the PECAM-1$^{-/-}$ animals is not associated with or a direct result of hypertension.

Normal Vascular and Cardiomyocyte Architecture in PECAM-1$^{-/-}$ Hearts

Recent studies have suggested a role for defective vascularization in the pathogenesis of DCM.[3] Because PECAM-1 is expressed in ECs, and ECs line blood vessel walls, we hypothesized that differences in cardiac vascularity might account for differences in cardiac function of PECAM-1$^{-/-}$

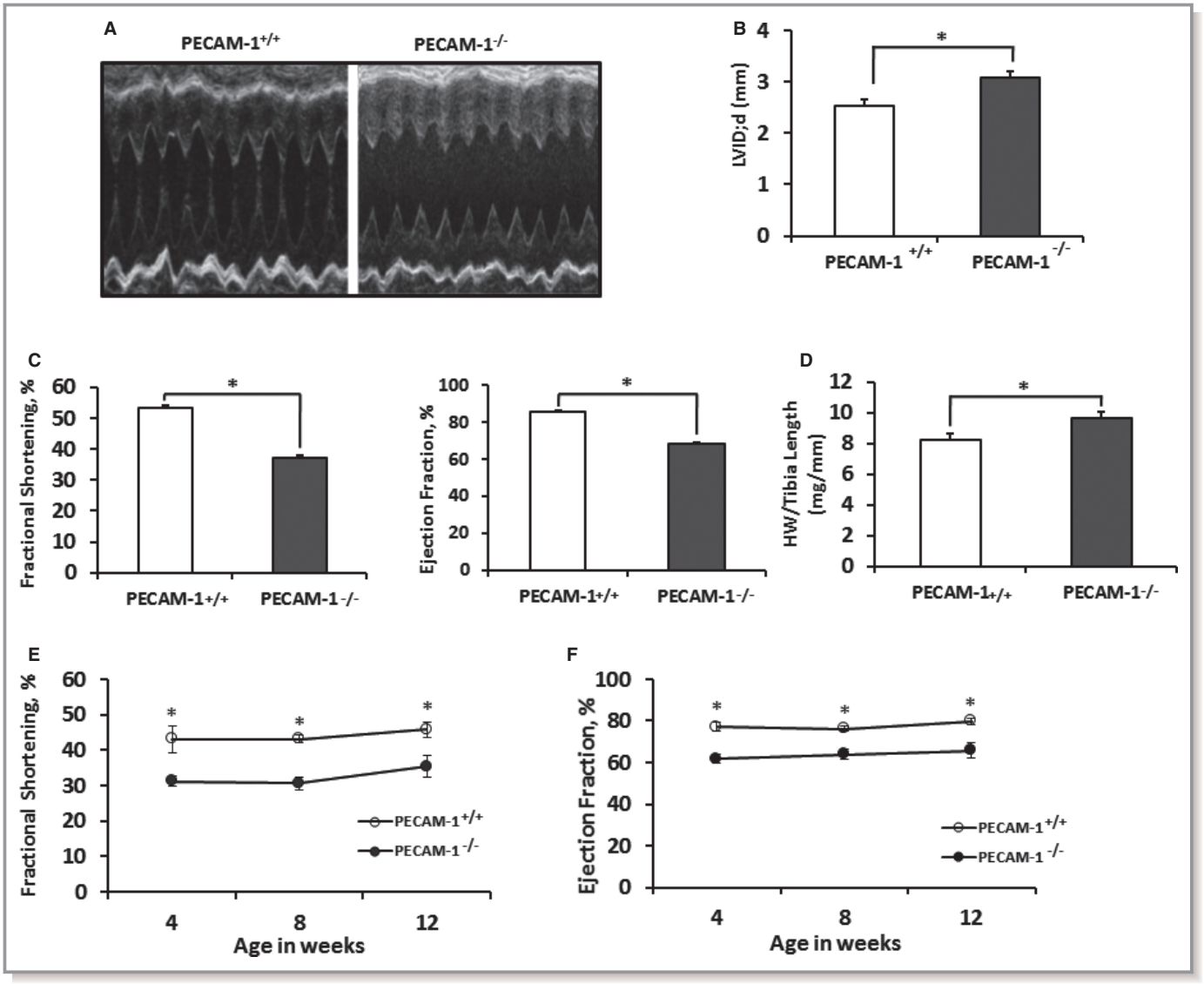

Figure 1. PECAM-1$^{-/-}$ mice have increased chamber size with systolic and diastolic dysfunction. A, Representative image of M-Mode echocardiography for baseline heart function in age-matched adult PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice. B and C, Echocardiographic measurement assessment of left ventricular dilation and cardiac function. Echocardiography data represent at least 10 mice per group (*P<0.01). Measurements were collected at the level of the papillary muscle. D, Analysis of left ventricle weight to tibia length to normalize left ventricular mass (n=10/genotype, *P<0.005). E and F, Echocardiographic measurement assessment of left ventricular function by fractional shortening (E) and ejection fraction (F). Echocardiography data represent 10 mice per group (*P<0.01). LVID indicates left ventricular internal dimension; PECAM-1, platelet endothelial cell adhesion molecule.

animals. There were no obvious defects in coronary artery structure or number in PECAM-1$^{-/-}$ hearts (data not shown) and no indication of hypoxia in PECAM-1$^{-/-}$ heart tissue (Figure 4). Measurements of capillary density by TRITC-lectin staining revealed a small increase in PECAM-1$^{-/-}$ hearts (Figures 5A and 5B), suggesting that cardiac defects are not attributable to differences in capillary density.

The fundamental contractile unit of cardiac muscle is the sarcomere. Previous studies have shown that defects in sarcomere protein expression and structure can lead to DCM.[32] In order to determine if alterations in sarcomere structure could explain the impaired contractility of PECAM-1$^{-/-}$ hearts, we performed transmission electron microscopy (TEM). We did not detect any differences in sarcomere organization or size between PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice (Figure 5C). When taken together, our studies suggest that the impaired cardiac function in PECAM-1$^{-/-}$ animals is not associated with structural or architectural defects in the heart and raise the possibility that impaired cardiac function might be a result of defective intercellular signaling or communication.

Table 2. Echocardiographic Data From PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ Mice

	PECAM-1$^{+/+}$ (n=9)	PECAM-1$^{-/-}$ (n=10)
Heart rate (BPM)	710±16	660±13*
IVS;d (mm)	1.0±0.02	1.1 ±0.02
IVS;s (mm)	1.7±0.02	1.6 ±0.02
LVPW;d (mm)	0.96±0.01	0.99±0.02
LVPW;s (mm)	1.52±0.02	1.4±0.03
LVID;d (mm)	2.9±0.2	3.3±0.1*
LVID;s (mm)	1.4±0.1	2.06±0.08
LV Vol;d (μL)	34.9±5.1	44.6±3.9
LV Vol;s (μL)	5.3±1.0	14.2±1.4
EF%	85.5±0.9	68.2±1.1†
FS%	48.3±2.4	37.2±0.9†

The values presented represent the mean±SE. EF indicates ejection fraction; FS, fractional shortening; HR, heart rate; IVS, interventricular septum; LV vol, left ventricular volume; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall; PECAM, platelet endothelial cell adhesion molecule.
*P<0.005 PECAM-1$^{-/-}$ vs PECAM-1$^{+/+}$.
†P<0.01 PECAM-1$^{-/-}$ vs PECAM-1$^{+/+}$.

Cardiomyocytes Isolated From PECAM-1$^{-/-}$ Hearts Display Normal Contractility

EC-cardiomyocyte communication is thought to play an important role in regulating cardiac function, as ECs not only provide a lifeline for cardiomyocyte survival but they also can regulate cardiomyocyte signaling and function via secretion of growth factors, cytokines and other signaling molecules.[2] PECAM-1 has previously been reported to be absent from cardiomyocytes,[33,34] and our own data verified that PECAM-1 is not expressed in cardiomyocytes (Figure 6). We therefore hypothesized that absence of PECAM-1 in ECs leads to impaired EC-cardiomyocyte communication. Accordingly, we hypothesized that cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts (and not under the influence of ECs) are indistinguishable from those isolated from PECAM-1$^{+/+}$ hearts. To test this, we isolated cardiomyocytes from PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ hearts and measured baseline contraction. As shown in Figures 7A and 7C, cardiomyocytes isolated from PECAM-1$^{+/+}$ and PECAM-

Table 3. Aging Echocardiographic Data From PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ Mice

	PECAM-1$^{+/+}$ (n=9)			PECAM-1$^{-/-}$ (n=10)		
	4 weeks	8 weeks	12 weeks	4 weeks	8 weeks	12 weeks
Heart Rate (BPM)	684±24	737±8	725±9	612±24	664±22	678±17
IVS;d (mm)	0.73±0.01	0.88±0.03	0.98±0.03	0.70±0.03	0.86±0.02	0.99±0.02
IVS;s (mm)	1.2±0.03	1.46±0.03	1.62±0.02	1.07±0.05*	1.35±0.04*	1.57±0.04
LVPW;d (mm)	0.7±0.02	0.8±0.03	0.91±0.03	0.62±0.03†	0.82±0.04	0.92±0.04
LVPW;s (mm)	1.15±0.04	1.33±0.04	1.49±0.05	0.93±0.02†	1.2±0.06	1.34±0.05
LVID;d (mm)	2.4±0.1	2.7±0.07	2.9±0.05	2.8±0.1*	3.3±0.2†	3.2±0.13*
LVID;s (mm)	1.36±0.08	1.5±0.05	1.53±0.06	1.89±0.09†	2.2±0.2†	2.1±0.1†
LV Vol;d (μL)	21.3±1.87	27.1±1.68	31.2±1.25	29.5±2.8*	46.8±6.0†	42.5±4.1*
LV Vol;s (μL)	4.9±0.6	6.17±0.5	6.52±0.63	11.4±1.4†	14.9±3.2†	14.7±2.5‡
EF%	76.9±2.2	77.3±1.0	79.8±1.5	61.6±2.1†	64.9±2.5†	65.2±2.4†
FS%	44.1±2.0	44.3±0.9	47.2±1.5	31.9±1.5†	34.7±1.7	34.9±1.8†

The values presented represent the mean±SE. EF indicates ejection fraction; FS, fractional shortening; HR, heart rate; IVS, interventricular septum; LV vol, left ventricular volume; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall.
*P<0.05, †P<0.005, ‡P<0.01. All statistics were calculated PECAM-1$^{-/-}$ vs PECAM-1$^{+/+}$ at corresponding age.

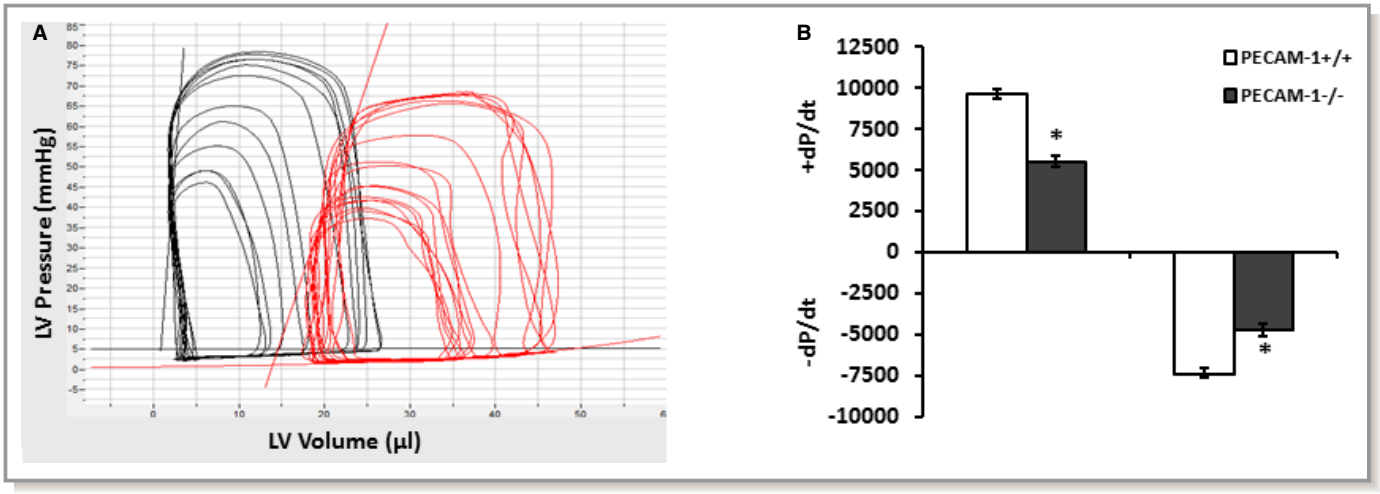

Figure 2. Decreased contractility and relaxation in PECAM-$1^{-/-}$ hearts. A, Representative left ventricular (LV) pressure-volume (PV) loops during preload reduction (vena cava occlusion) in PECAM-$1^{+/+}$ (black trace) and PECAM-$1^{-/-}$ mice (red trace). B, Maximum and minimum rates of pressure change in the left ventricle (n=8/genotype, *P<0.005). PECAM-1 indicates platelet endothelial cell adhesion molecule.

$1^{-/-}$ hearts had identical baseline contraction. Furthermore, no differences were observed in either peak systolic $[Ca^{2+}]_i$ transients (Figures 7B and 7D) or the rate of $[Ca^{2+}]_i$ transient decay (Figures 7B and 7E). Similarly, no differences were observed in the average resting length of cardiomyocytes from either genotype (data not shown). Finally, cardiomyocytes isolated from PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ hearts responded similarly to the β-adrenergic agonist isoproterenol. These findings show that cardiomyocytes isolated from PECAM-$1^{-/-}$ or PECAM-$1^{+/+}$ hearts are indistinguishable in their ability to contract (baseline and in response to isoproterenol) in the absence of EC input; these data provide support for the notion that an endothelial-derived signal may be responsible for the cardiac dysfunction in the PECAM-$1^{-/-}$ mouse.

Misregulated Neuregulin-ErbB Signaling in the PECAM-$1^{-/-}$ Mice

To further test the hypothesis that EC-cardiomyocyte communication is altered in PECAM-$1^{-/-}$ mice, we examined the involvement of known mediators of paracrine signaling. Of particular interest is the endothelial-derived signaling molecule NRG-1. This EGF-family protein is cleaved at the EC membrane, which releases the active, EGF-domain-containing peptide that is capable of binding to ErbB receptors expressed

Table 4. Hemodynamic Measurements From PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ Mice

	PECAM-$1^{+/+}$ (n=8)	PECAM-$1^{-/-}$ (n=8)
HR, BPM	552±7	504±6*
ESP, mm Hg	75.5±2.3	62.4±2.1†
EDP, mm Hg	4.3±0.3	4.1±0.4
ESV, µL	17.8±2.6	26.0±2.0*
EDV, µL	39.8±2.4	47.4±1.7*
EF, %	57.5±4.5	45.6±2.4†
ESPVR slope	7.2±0.8	6.7±1.0
ESPVR intercept	0.6±4.2	13.5±1.8*
PRSW intercept	16.6±2.4	28.4±2.4†
PVA vs EDV intercept	17.0±2.7	27.1±2.4*

The values presented represent the mean±SE. EDP indicates end-diastolic pressure; EDV, end-diastolic volume; EF, ejection fraction; ESP, end-systolic pressure; ESPVR, end-systolic pressure-volume relationship; ESV, end-systolic volume; HR, heart rate; PRSW, preload recruitable stroke work; PVA, pressure-volume area.
*P<0.005 PECAM-$1^{-/-}$ vs PECAM-$1^{+/+}$.
†P<0.01 PECAM-$1^{-/-}$ vs PECAM-$1^{+/+}$.

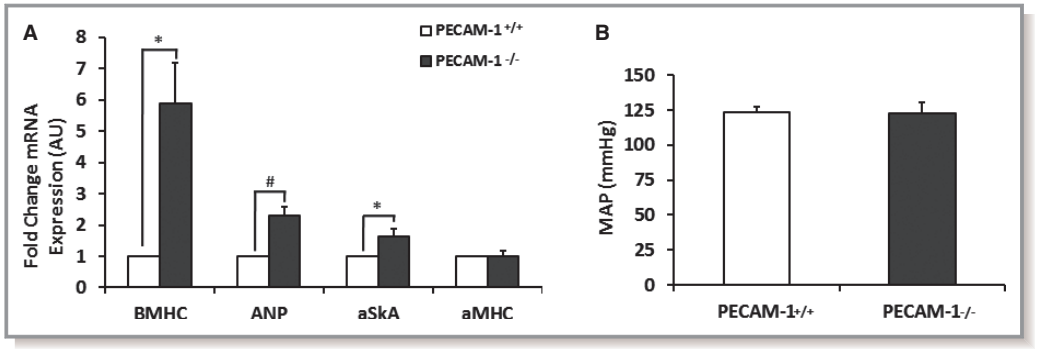

Figure 3. Characterization of PECAM-$1^{-/-}$ gene expression and blood pressure. A, Quantitative real-time RT-PCR of cardiac fetal gene expression in 12-week-old male mice. All mRNA species were quantified relative to 18s housekeeping gene expression and presented as fold change (arbitrary units [AU]) relative to PECAM-$1^{+/+}$ mice. (n=3/genotype, *P<0.01 #P<0.05). B, Blood pressures from PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ mice were performed by tail-cuff measurement (n=10/genotype). αMHC indicates α myosin heavy chain; αSka, α skeletal actin; βMHC, β-myosin heavy chain; ANP, atrial natriuretic peptide.

on cardiomyocytes. Within cardiomyocytes, ErbB receptors initiate signal transduction pathways that ultimately regulate cardiomyocyte contractility.[28,35]

To determine if the NRG-ErbB pathway is altered in the absence of PECAM-1, NRG-1 release from ECs was assessed. We measured the cleaved fragment of NRG-1 present in conditioned cell media from PECAM-1 knockout (PE-KO) and PECAM-1 expressing (PE-RC) ECs by immunoblot analysis. We observed a small but significant increase in NRG-1 in the conditioned media of PE-KO cells compared to PE-RC cells (Figure 8A). To verify that increased NRG-1 also occurs in vivo, heart lysates from PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ animals were assessed for NRG-1 levels. Similar to our observations in vitro, we found higher levels of NRG-1 in PECAM-$1^{-/-}$ hearts (Figure 8B). In further support for the elevated NRG-1 in PECAM-$1^{-/-}$ hearts in vivo, we observed a tendency for increased cardiomyocyte area in the PECAM-$1^{-/-}$ hearts (Figure 5B), consistent with previous studies showing that NRG-1 promotes hypertrophy.[36] Additionally, we observed increased reactive oxygen species (ROS) production in PECAM-1 KO ECs, consistent with the observation that NRG-1 release is mediated by ROS[37] and the finding that coronary arteries from PECAM-$1^{-/-}$ mice have increased ROS production[16] (Figure 8C). To provide additional mechanistic insight into the regulation of NRG-1 release from the PE-KO cells, we treated both PE-RC and PE-KO cell either with diphenyleneiodonium (DPI), a ROS inhibitor, or L-NG-nitroarginine methyl ester (L-NAME), an NO inhibitor. We found that blockage of either ROS or NO significantly reduced NRG-1 levels in media from the PE-KO

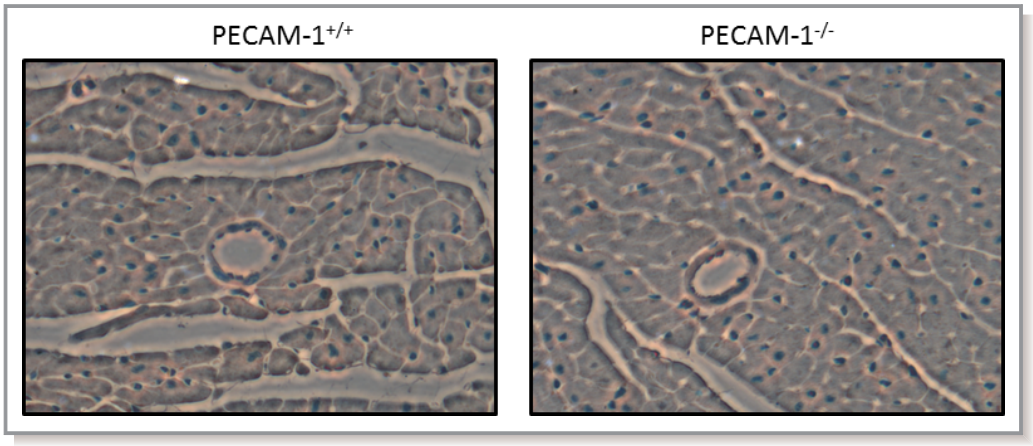

Figure 4. No evidence for hypoxia in PECAM-$1^{-/-}$ hearts. PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ were injected with Hypoxyprobe-1™ prior to sacrifice. The tissue was fixed, paraffin-embedded and sectioned. There was no apparent positive staining in either genotype. PECAM-1 indicates platelet endothelial cell adhesion molecule.

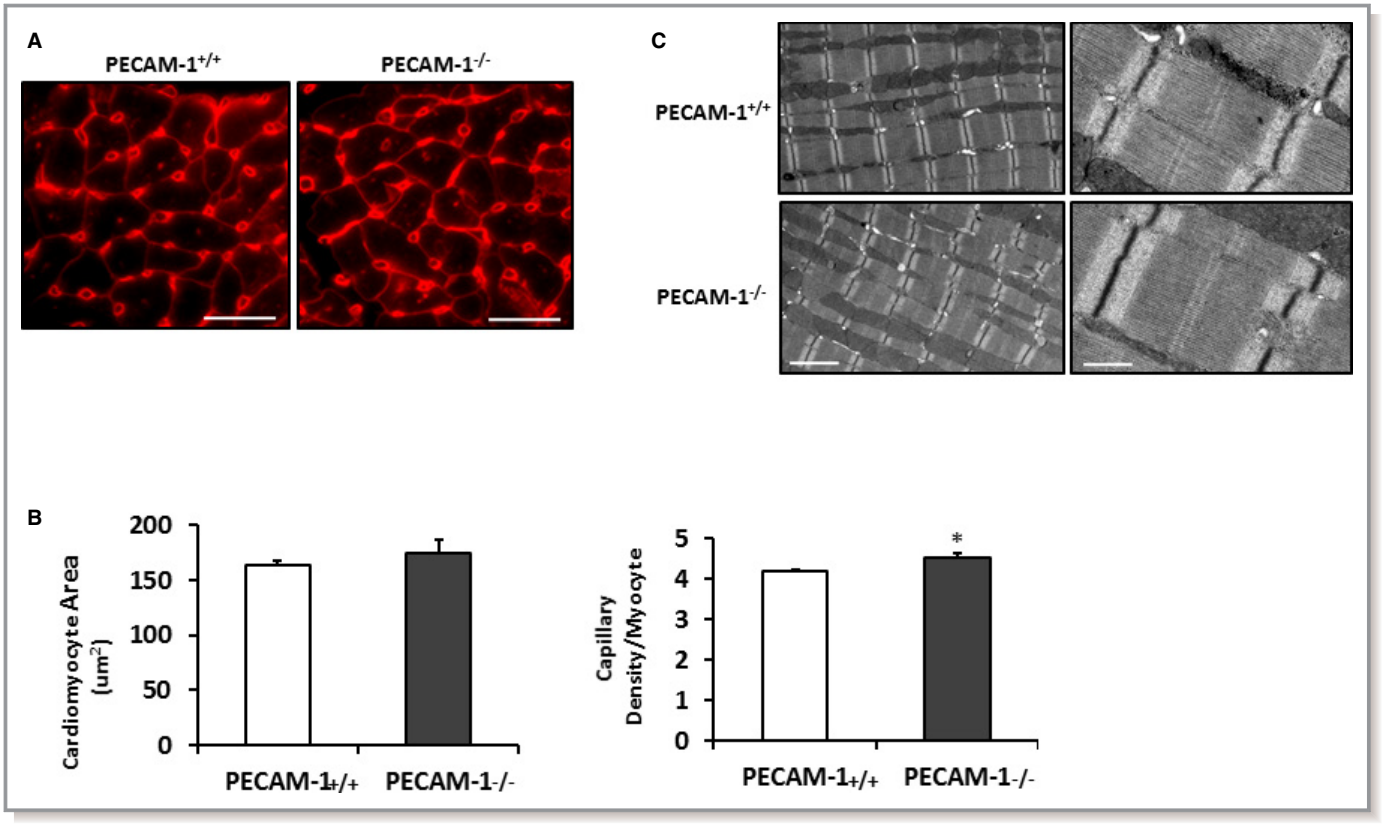

Figure 5. PECAM-1$^{-/-}$ hearts have normal tissue and ultrastructural architecture. A, TRITC (tetramethylrhodamine)-lectin stained sections for baseline cardiomyocyte cross-sectional area and capillary density in PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice. Scale bar 20 μm. B, Quantitation of images shown in (A), (n=4 PECAM-1$^{+/+}$ and n=3 PECAM-1$^{-/-}$, *P<0.01). C, The ultrastructure of cardiac muscle from PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice by transmission electron micrograph. ×10000 and ×25000 representative images shown, scale bars 2 and 1 μm, respectively. PECAM-1 indicates platelet endothelial cell adhesion molecule.

cells (Figure 8D). These data suggest that elevated NO/ROS signaling from PE-KO cells contribute to the elevated NRG-1 signaling in PECAM-1$^{-/-}$ animals.

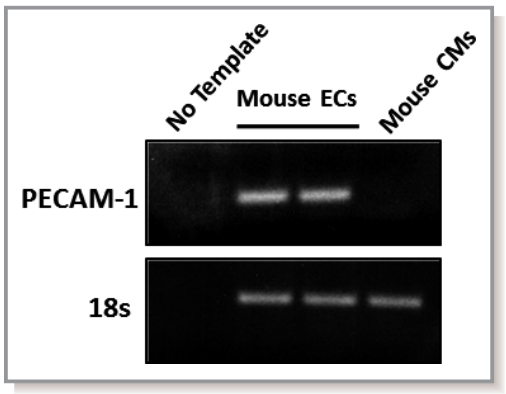

Figure 6. Adult mouse cardiomyocytes do not express PECAM-1. qPCR was performed on RNA isolated from 2 lines of mouse endothelial cells as well as mouse cardiomyocytes (HL1) cells. No expression was detected in the cardiomyocytes (CMs) compared to the robust signal from the endothelial cells. PECAM-1 indicates platelet endothelial cell adhesion molecule.

Binding of NRG-1 induces rapid tyrosine phosphorylation of the ErbB receptor expressed in cardiomyocytes. We therefore evaluated if the increased NRG-1 observed in PECAM-1$^{-/-}$ hearts results in elevated phosphorylation of its receptor in vivo. Importantly, we observed enhanced ErbB2 tyrosine phosphorylation in PECAM-1$^{-/-}$ hearts (Figure 8E), suggesting that the misregulated NRG-1 release leads to hyperactivation of ErbB receptor signaling. We extended these studies further by developing an in vitro system to test the hypothesis that increased NRG-1 release from PECAM-1$^{-/-}$ ECs is responsible for hyperactivation of the ErbB receptor in cardiomyocytes. We incubated mouse cardiomyocytes with conditioned media from PE-KO or PE-RC cells and compared ErbB2 phosphorylation levels. As shown in Figure 9A, incubation of cardiomyocytes with conditioned media from PE-KO ECs resulted in a significant increase in pTyr877ErbB2 levels compared to when cardiomyocytes were incubated with conditioned media from PE-RC ECs (3.7-fold versus 10.9-fold). Importantly, pre-incubation of PE-KO conditioned media with an NRG-1 blocking antibody significantly reduced ErbB2 phosphorylation levels by ≈50% (Figures 9A and 9B), closer to the levels observed

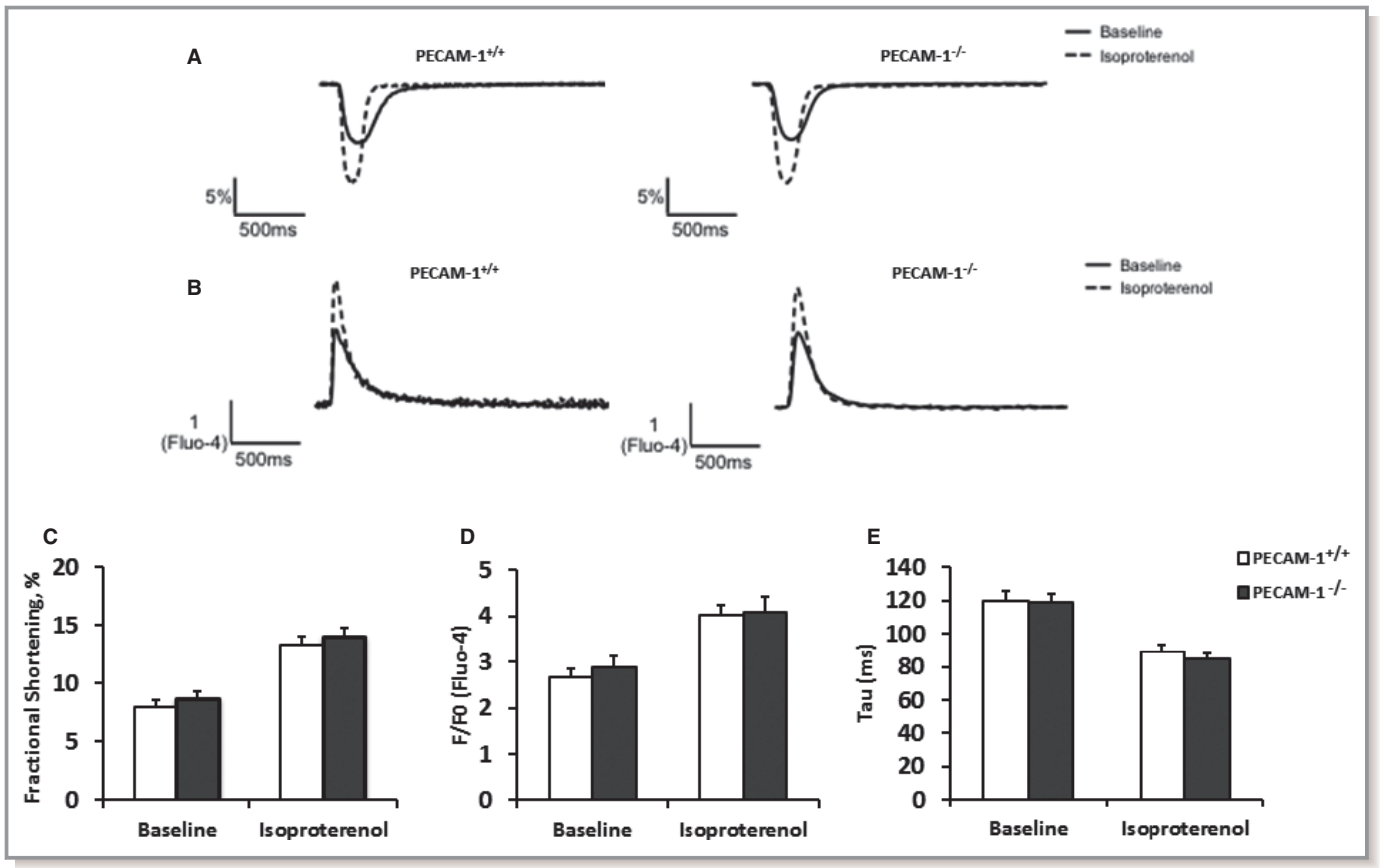

Figure 7. Cardiomyocytes isolated from PECAM-1$^{-/-}$ hearts have preserved contractility and calcium transients. A and B, Representative examples of contractions (A) and calcium transients (B) from cardiomyocytes isolated from PECAM-1$^{+/+}$ or PECAM-1$^{-/-}$ mice at baseline and after isoproterenol treatment. C through E, Average data measuring fractional shortening (C), peak calcium transients (D), and calcium decay rate (E) in isolated cardiomyocytes from PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice±isoproterenol. PECAM-1 indicates platelet endothelial cell adhesion molecule.

in cardiomyocytes treated with conditioned media from PECAM-1-expressing ECs. These data provide further credence to the misregulated NRG-1/ErbB2 pathway in PECAM-1$^{-/-}$ hearts.

NRG-1 Blockade Restores Cardiac Function in PECAM-1$^{-/-}$ Mice

Our results so far have revealed that absence of PECAM-1 is associated with increased NRG-1 release and increased ErbB2 phosphorylation; we have also shown that the increased NRG-1 released from PECAM-1 KO ECs is responsible for the increased ErbB2 phosphorylation, as treatment of isolated cardiomyocytes with conditioned media from PECAM-1 KO ECs results in enhanced ErbB2 phosphorylation that can be normalized by an NRG-1 blocking antibody. We hypothesized that aberrant NRG-1 signaling in ECs contributes to the cardiac dysfunction of PECAM-1$^{-/-}$ mice, and if this notion was correct, we posited that NRG-1 blocking antibody administration might be cardioprotective. Age-matched, adult PECAM-1$^{-/-}$ mice were treated with the NRG-1 blocking antibody or saline (Figures 9C and 9D; Table 5). Conscious echocardiography revealed that PECAM-1$^{-/-}$ mice demonstrated a significant improvement in left ventricular ejection fraction and fractional shortening after NRG-1 blocking antibody treatment, compared to saline control-treated PECAM-1$^{-/-}$ mice (fractional shortening: 40.9% versus 33.0%; ejection fraction: 72.8% versus 62.4%). These data show that the excess NRG-1 levels observed in PECAM-1$^{-/-}$ ECs and hearts and the ensuing elevated ErbB activation, play a key causative role in the impaired cardiac function observed in the PECAM-1$^{-/-}$ mice.

Discussion

The findings presented here support a model in which PECAM-1 modulates cardiac contractility via a paracrine mechanism that involves the NRG-1/ErbB2 pathway. Although PECAM-1$^{-/-}$ animals display impaired cardiac function, surprisingly, cardiomyocytes isolated from these mice show normal contractility and calcium handling. These data suggest that

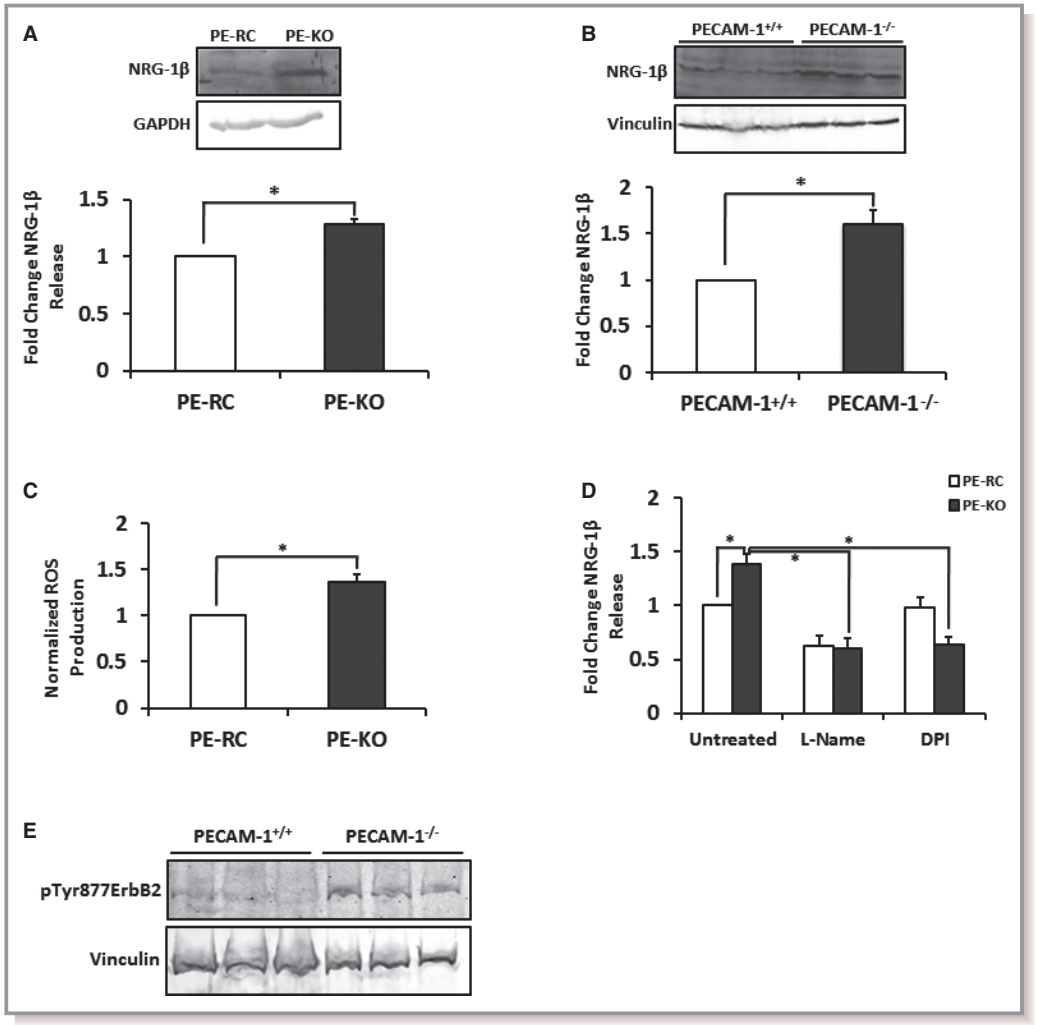

Figure 8. Misregulated NRG-1/ErbB signaling in PECAM-$1^{-/-}$ hearts. A, Quantitation of Western blot for NRG-1β release from PE-RC and PE-KO cells. Media was collected after 24 hours and concentrated. Concentrated media was then run on a polyacrylamide gel (n=5, *P<0.05). B, Quantitation of Western blot for NRG-1β in heart tissue from PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ hearts. (P<0.05). C, Measurement of reactive oxygen species (ROS) production from PE-RC and PE-KO cells using 2,7-H_2DCFDA. Fluorescence was measured using a plate reader and normalized to total protein levels in the lysate (n=7, *P<0.01). D, Quantification of Western blots for NRG-1β from PE-RC and PE-KO cells treated with either DPI or L-NAME for 24 hours (n=3, *P<0.05). E, Representative Western blots for pTyr877ErbB2 (n=6/genotype). DCFDA indicates 2,7-dichlorodihydrofluoroscein diacetate; DPI, diphenyleneiodonium; NRG-1, neuregulin.

the reduced contractility observed in vivo is due to an endothelial-derived signal, as cardiomyocytes from PECAM-$1^{-/-}$ mice exhibit impaired contractility only when under the influence of ECs. Our data indicated that the absence of PECAM-1 results in elevated NO/ROS signaling and enhanced NRG-1 release from ECs that, in turn, leads to aberrant activation of the ErbB2 receptor in cardiomyocytes. In support of this model, treatment of PECAM-$1^{-/-}$ mice with NRG-1 EGF-domain blocking antibody resulted in improved cardiac function, as evidenced by increased left ventricular fractional shortening and ejection fraction. Overall, these results provide support for the model that precise spatiotemporal activation of the NRG-1/ErbB signaling cascade is crucial for normal cardiac function and that absence of PECAM-1 from ECs results in augmented NRG-1 release and impaired cardiomyocyte contractility which can be acutely reversed by an NRG-1-blocking antibody.

The rather intuitive consensus in the field is that EC signaling can affect global cardiac function. However, studies supporting this dogma are limited. There are only a handful of reports of EC-specific knockout animals demonstrating impaired cardiac contractility. Importantly, not all EC knockout models display a basal cardiac phenotype. For example, the EC-specific HIF2α knockout has normal contractility, but

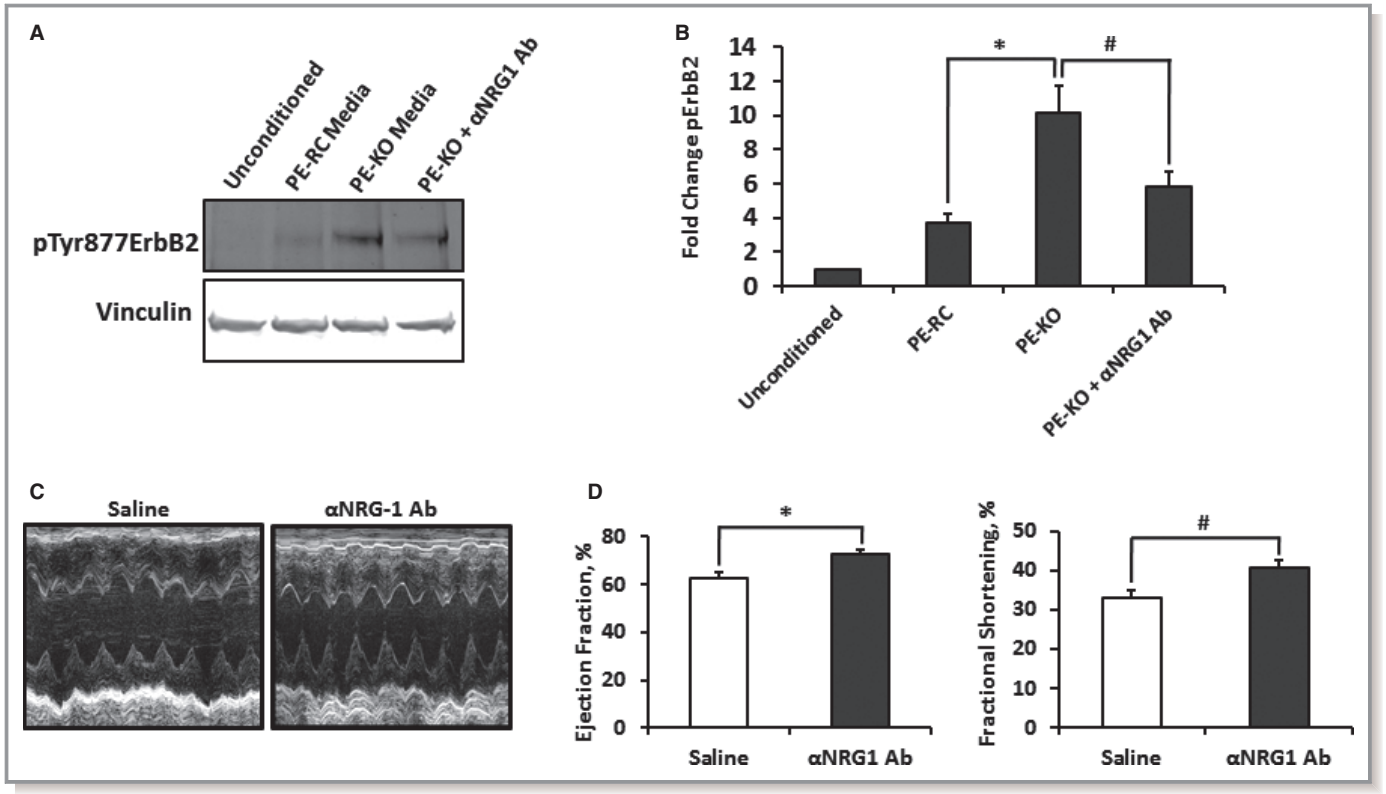

Figure 9. NRG-1 blockade normalizes ErbB2 phosphorylation and restores cardiac function in PECAM-1$^{-/-}$ mice. A, Western blot and quantitation of pTyr877ErbB2 in HL-1 cardiomyocytes treated with either unconditioned media or EC-conditioned media from PE-RC, PE-KO or PE-KO plus 25 μg/mL NRG-1 blocking antibody. B, Representative image of M-Mode echocardiography for heart function in adult PECAM-1$^{-/-}$ mice treated with vehicle or NRG-1 blocking antibody. C to D, Echocardiographic measurement assessment of left ventricular ejection fraction and fractional shortening. Echocardiography results (D) represent 10 mice per group (*P<0.05, $^{\#}P$<0.001). Measurements were collected at the level of the papillary muscle. EC indicates endothelial cells; NRG-1, neuregulin; PECAM-1 indicates platelet endothelial cell adhesion molecule.

Table 5. Echocardiographic Data From NRG-1 Blocking Antibody

	Saline (n=10)		αNRG-1 (n=10)	
	Baseline	Saline	Baseline	Blocking Ab
Heart rate, BPM	603±31	635±31	649±34	689±20
IVS;d (mm)	0.94±0.02	0.97±0.02	0.93±0.02	0.96±0.03
IVS;s (mm)	1.4±0.02	1.5±0.05	1.4±0.03	1.6±0.4*
LVPW;d (mm)	0.85±0.03	0.80±0.03	0.78±0.04	0.78±0.03
LVPW;s (mm)	1.25±0.03	1.20±0.06	1.22±0.07	1.28±0.04
LVID;d (mm)	2.95±0.13	3.28±0.15	3.33±0.16	3.09±0.09
LVID;s (mm)	1.98±0.11	2.20±0.13	2.20±0.14	1.83±0.08†,‡
LV Vol;d (μL)	34.6±3.9	44.9±5.5	46.5±5.2	38.4±2.8
LV Vol;s (μL)	13.1±2.0	17.4±3.0	17.2±2.5	10.5±1.1†,‡
EF%	62.7±2.6	62.4±2.7	63.6±2.9	72.8±2.1†,‡
FS%	32.9±1.9	33.0±1.9	34.1±2.3	40.9±1.7†,§

The values presented represent the mean±SE. EF indicates ejection fraction; FS, fractional shortening; HR, heart rate; IVS, interventricular septum; LV vol, left ventricular volume; LVID, left ventricular internal dimension; LVPW, left ventricular posterior wall; NRG-1, neuregulin.

*P<0.01 baseline vs blocking Ab.

$^{\dagger}P$<0.05 baseline vs blocking Ab.

$^{\ddagger}P$<0.05 blocking Ab vs saline.

$^{\S}P$<0.001 blocking Ab vs saline.

once challenged, there is rapid decompensation to heart failure.[38] Previously, work in the caveolin-1 global knockout mouse showed development of DCM at 5 months of age[39]; this was attributed to misregulation of eNOS/NO signaling in non-myocytes. Although it is accepted that ECs release molecules that can affect cardiomyocyte function, the mechanisms that regulate this release remain unclear. Our study places PECAM-1 as a central player in NRG-1-dependent intercellular communication between the endocardium and myocardium, which is crucial in controlling cardiac contractility and function.

Although we cannot exclude the possibility that other PECAM-1-expressing cells, such as leukocytes, may contribute to the impaired cardiac function of the PECAM-1$^{-/-}$ mice, the deletion of PECAM-1 in ECs is the likely culprit based on the observation of increased NRG-1 release from PECAM-1$^{-/-}$ ECs. In addition, several other pieces of data argue against a role for hematopoietic PECAM-1. First, previous studies have shown no difference in either leukocyte or platelet counts in PECAM-1$^{-/-}$ mice.[40] Second, on a C57Bl/6 background, no differences in platelet activation[41] or leukocyte emigration have been observed in PECAM-1$^{-/-}$ animals.[40] Furthermore, the contribution of leukocytes to the total cell population of a normal heart is minimal compared with that from either fibroblasts or endothelial cells.[42] Finally, each cardiomyocyte is in close physical contact with the surrounding EC-derived vasculature facilitating a unique interface for cellular crosstalk. Thus, we propose that endothelial PECAM-1 is essential for regulating cardiac function.

The effects of NRG-1 signaling in the cardiovascular system are strongly time- and tissue-dependent,[43] and NRG-1 has been reported to have both positive and negative effects on cardiac contractile function. Knockout models have indicated a requirement for NRG-1 in directing morphogenesis of the developing ventricles (trabeculation), as well as differentiation of cardiac stem cells. Within the adult heart, NRG-1 is important for paracrine signaling between ECs and cardiomyocytes to maintain adaptation of the heart to physiological and pathological stimuli. In support of this idea, administration of recombinant NRG-1 during acute cardiac injury and during chronic heart failure has beneficial effects on both survival and restoration of cardiac function.[9,44,45] These observations have been expanded into human models showing similar benefits to administration of NRG-1.[45,46] It is important to point out that the beneficial effects of NRG-1 have been observed in settings of induced heart failure; this is discordant with our model where no heart failure has been induced.

Despite ongoing studies on development of recombinant NRG-1 as a possible therapy for heart failure, our understanding of the NRG/ErbB signaling axis is still underdeveloped. NRG-1 is activated in the heart after injury[37] and by changes in hemodynamic load.[47] Interestingly, NRG-1 levels are higher in patients with stress-induced ischemia and higher serum NRG-1 levels are associated with poor prognosis in patients with chronic heart failure.[48] The elevated serum NRG-1 levels in heart failure could either be viewed as a favorable compensatory response or as pathogenic,[48] although more studies are needed to distinguish between these 2 possibilities. In addition, and perhaps antithetically to its cardioprotective effects, NRG-1 induces a negative inotropic response in adult cardiac muscle, at least in part, via nitric oxide (NO).[43,49] Interestingly, we,[14,17] and others,[50] have previously reported enhanced eNOS activation and NO production in PECAM-1$^{-/-}$ animals, although the role of NO in regulating cardiac contractility is somewhat conflicting.[51] The negative inotropic effects of NRG-1 suggest that the elevated NRG-1 signaling in the absence of PECAM-1 may be responsible for the observed depressed cardiac contractility in PECAM-1 $^{-/-}$ animals. Consistent with this hypothesis, administration of a blocking antibody against NRG-1 protected PECAM-1$^{-/-}$ animals and restored cardiac function to levels comparable to those observed in WT animals. Our observations allow us to surmise that precise fine-tuning of the NRG-1/ErbB pathway is crucial for maintenance of proper cardiac function.

This research furthers our understanding of the effects of PECAM-1 in various aspects of cardiovascular biology. It is unclear exactly how PECAM-1 directs NRG-1 cleavage and signaling. Future studies are aimed at understanding this relationship. Our data implicate a role for ROS production in this regulation but other aspects of PECAM-1 biology should be considered. For example, changes in hemodynamic forces in the heart have been implicated in cardiac dysfunction. Work from our lab and others have highlighted PECAM-1 as a critical endothelial mechanosensor. Thus, PECAM-1-dependent mechanosignaling may also influence cardiac function. Nevertheless, while it is possible that other aspects of PECAM-1 function may contribute to the observed cardiac phenotype in PECAM-1$^{-/-}$ mice, the work presented here clearly identifies PECAM-1 as a novel regulator of EC-cardiomyocyte crosstalk.

Acknowledgments

We would like to thank Victoria Madden (Microscopy Services Laboratory, University of North Carolina) for help with TEM preparation and imaging, Kirk McNaughton (Histology Research Core Facility, University of North Carolina) for help with immunofluorescence, and Dr Zhaokang Cheng and Dr Joan Taylor for help with tissue isolation and lysis. We gratefully acknowledge the help of Dr Luigi Pironti and Dr Howard Rockman with the initial cardiomyocyte contractility studies. We thank the Tzima laboratory colleagues for technical assistance, helpful discussion, and critical reading of the manuscript.

Sources of Funding

This work was supported in part by an NIH grant (HL088632) to Tzima, a NIH grant (T32 HL069768) to McCormick and an American Heart Association Predoctoral fellowship (4290007) to McCormick.

Disclosures

None.

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