Dilated cardiomyopathy in Erb-b4-deficient ventricular muscle


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Dilated cardiomyopathy, a leading cause of heart failure, is believed to result from an abnormal remodeling of cardiac tissue in response to a variety of stressors (for reviews see Refs. 8, 31). Although the majority of dilated cardiomyopathies are of unrecognized etiology, an increasing number of genes have been implicated in this process (31). We present in vivo evidence for the critical role of Erb-b4 signaling in cardiomyocyte differentiation and maintenance of normal postnatal cardiac structure and function.

METHODS

Generation of erb-b4+/− mice. The targeting construct was made from a 15-kb genomic DNA including the entire coding sequence of exon 2 and flanking intronic sequence (obtained from M. Gassmann, MRC Labs, London) cloned into the targeting vector pFLRT. In the pFLRT-erb-b4 targeting construct, two loxP sequences flanked exon 2, while downstream was the positive neomycin (neo) selection marker flanked by two fRT sites. After positive/negative selection of pFLRT-erb-b4-transfected R1 embryonic stem cells (27) with G418 and 1-[2-deoxy-2-fluoro-D-arabinofuranosyl]-5-iodouracil and screening for homologous recombination by Southern blot analysis, 20–30 R1 cells were microinjected into each of 12–16 C57Bl/6 blastocysts, which were subsequently transferred into the uterus of timed-pseudopregnant CD1 females (18). Chimeric mice were bred with C57Bl/6 females to establish germ-line transmission of the targeted allele.

Breeding and analysis of erb-b4 gene-targeted mice. All experiments were performed in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” (US DHHS Publication No. 85-23, Revised 1996, Protocol No. 1-159). The erb-b4+/− mice were crossed into erb-b4−/−;MLC2vCreKI+/− mice and maintained in colonies. The genotype of individual animals was determined by PCR on tail DNA. Southern and Northern analyses were performed as previously described (15). RT-PCR was performed in 0.5 μg of total RNA isolated from ventricles using reverse transcription (Life Technologies, Rockville, MD) and followed by PCR amplification using primers corresponding to exons 1 and 4 of erb-b4 cdNA (a gift from M. Gassmann). Ventricles from a mouse of the same genotype were pooled and homogenized or treated for cardiomyocyte isolation following established protocols (13). Western blot analysis for Erb-b4 and Erb-b2 detection was performed in immunoprecipitates using antibodies against Erb-b4 (rabbit polyclonal 0615 and 0618, obtained from C. Lai) or Erb-b2 (Santa Cruz Biotechnol-
ogy, Santa Cruz, CA), as described elsewhere (14). Briefly, equivalent amounts of proteins from ventricles or isolated cardiomyocytes were lysed in 0.4 ml of buffer containing 1% Triton X-100 and protease inhibitors. The membranes were blotted with primary antibodies, washed, incubated with peroxidase-coupled goat anti-rabbit antibody (Amersham), and developed using enhanced chemiluminescence (ECL, Amersham). Relative amounts of Erb-b4 protein per sample represent the ratio of Erb-b4 to Erb-b2 band intensity. Animals of the following genotypes were used as controls because of their similar Erb-b4 protein level: erb-b4+/+;MLC2v-CreKI+/−, erb-b4F/F;MLC2v−/−, erb-b4F/F;MLC2v−/−, and erb-b4F/F;MLC2vCreKI−/−.

Histochemical and morphological analyses. Dissected hearts from female and male control and KO mice were washed in saline phosphate buffer and then treated as follows. For electron microscopy, pieces of ventricular tissue were fixed in 5% glutaraldehyde and then in 1% osmium tetroxide and embedded in epoxy resins. For optical microscopy, hearts were fixed in 4% paraformaldehyde and then in 1% osmium tetroxide and embedded in paraffin. Paraffin-embedded 5-μm-thick serial tissue sections were stained with hematoxylin and eosin. The number of myocyte nuclei and the morphometric analyses of free ventricular walls and interventricular septum diameter were determined with an image analyzer on 40 consecutive fields in heart sections stained with hematoxylin and eosin. The morphometric analysis of individual cardiomyocytes was performed in adult myocytes isolated using an alkaline dissociation method (33). The intercalated disks (IDs) were counted in 20 consecutive fields of 28,542 μm² per sample in tissue sections immunostained with antibodies against ID-related proteins (catenin and connexin isoforms). The cardiomyocyte ploidy index was determined in Feulgen-stained tissue sections on 300 myocytes per sample, which contained longitudinally oriented nuclei and were compared with diploid cells, and was analyzed in digital images (VIDAS Kontron, Zeiss) (35). Cardiomyocyte synthesis of DNA and apoptosis were analyzed by bromodeoxyuridine incorporation (14) (Zymed, San Francisco, CA) and TdT-mediated dUTP nick end-labeling reaction (15) (Intergen), respectively. Immunohistochemical analyses were performed in paraffin-embedded tissue sections by indirect immunostaining using antibodies against Erb-b4 (Labvision, Fremont, CA), Erb-b2 and ZO-1 (Santa Cruz Biotechnology), and sarcomeric α-actinin and vinculin (Sigma, St. Louis, MO). The primary antibodies were detected by fluorescent labeling with biotin-coupled antibodies and streptavidin-FTIC (Amersham), as previously described (13, 14). Immunostained sections were analyzed by confocal microscopy.

Statistical significance between groups was analyzed by ANOVA and Student’s t-test.

Contractility determination. Isolated hearts from 3-mo-old female and male wild-type (WT, n = 5) and erb-b4-KO (n = 5) mice were perfused according to the Langendorff technique at constant temperature (37°C), flow (4 ml/min), and heart rate (360 beats/min), as previously described (30). The basal mechanical data obtained in erb-b4-KO mice were age and gender matched to control, WT or heterozygous, mice. The mechanical activity was assessed through an intracardiac water-filled latex balloon connected to a pressure transducer (Perceptible disposable transducer, Namic), delivering 0.5 mA pulses for 0.2 ms. Additional override pacing was achieved with an SSi pulse for 200 ms and two extrastimuli for 90 and 70 ms. Bipolar electrograms were acquired with an EXXER multichannel recorder.

RESULTS

Generation of ventricular muscle-specific erb-b4-KO mice. We generated mice containing a “floxed” erb-b4 allele, in which a 3-kb region, including exon 2 of the erb-b4 gene, was flanked by loxP sites. The Cre-recombinase-mediated excision of exon 2 introduced a shift in the reading frame, resulting in the early termination of translation (Fig. 1A). To induce the specific ablation of the erb-b4 floxed alleles, we employed a strain of mice carrying Cre-recombinase coding sequences in the locus of the ventricular myosin light chain MLC2v-Cre knock-in (KI) (6). Coincident with the early expression of MLC2v in ventricular muscle, the MLC2v-Cre-driven recombination events occurred no later than embryonic day 8.5 (7). Mice heterozygous for the MLC2v-CreKI allele display normal cardiac development and function (26). The erb-b4 floxed allele recombination (F°) was observed in the ventricles of heterozygous and homozygous erb-b4-KO mice, but not in other tissues analyzed (Fig. 1, B and C). Genotypic analyses of the offspring showed that erb-b4-KO mice were present at the expected Mendelian ratios, indicating that the loss of this receptor in the ventricles did not result in embryonic lethality.

There were no detectable differences in the Erb-b4 expression level or the distribution pattern from hearts of WT or erb-b4 floxed allele mice (data not shown). RT-PCR analysis in total RNA prepared from whole ventricles (mice at postnatal day 10) revealed the expression of long transcripts spanning exons 1–4 of the WT or floxed erb-b4 gene and short transcripts corresponding to the excised erb-b4 allele in exon 2 of homozygous erb-b4-KO mice (Fig. 1D). To examine the loss of Erb-b4 in KO compared with WT myocytes, we determined the relative protein level in extracts from ventricles at embryonic day 14.5 (22 ± 5%, n = 4) or isolated adult cardiomyocytes (18 ± 4%, n = 4; Fig. 1E). The reduction of Erb-b4 protein was comparable to the MLC2v-Cre-recombination events when used in conjunction with RXXα (7), gp130 (15), and VEGF-A floxed allele mice (12).
Subcellular localization of Erb-b4. Inasmuch as the precise sites of receptor expression may provide functionally relevant clues, we analyzed the subcellular distribution of Erb-b4 and Erb-b2 in cardiac ventricular tissue prepared from 3- to 4-wk-old mice. Confocal analysis of immunofluorescent staining revealed detectable levels of Erb-b4 and Erb-b2 expression in myocytes of trabecular and interventricular septum. Both transmembrane protein receptors localized to Z lines and IDs of cardiomyocytes (Fig. 2, A and B). This clustering represents the continuous invaginations of the sarcolemma into the transverse t tubules, labeled by the staining of transmembrane ZO-1 proteins (Fig. 2C). The t tubules are located in close apposition to the sarcomeric Z lines marked by α-actinin (Fig. 2D), representing sites of functional protein interactions involved in the electrical-contraction coupling of cardiomyocytes (for review see Refs. 3, 19). In KO mice, there was virtually no staining for Erb-b4 protein in the myocardium (Fig. 2E). Erb-b2 was targeted to Z lines and abnormally accumulated along the cardiomyocyte membrane compared with control mice at 1 mo of age (Fig. 2F). Immunostaining for ZO-1 and α-actinin showed a regular alignment in Z lines (Fig. 2, G and H). The localization of proteins associated with the IDs in 3- to 4-wk-old erb-b4-KO mice showed a broader pattern in trabeculae and septum as visualized by the immunostaining of vinculin, localized to costameres and to N-cadherin-mediated junctions (Fig. 3, top). This result was verified by electron microscopy, showing less compact interactions among cardiomyocytes in the interventricular septum (Fig. 3, bottom).

Erb-b4-deficient mice displayed dilated cardiomyopathy and hypertrophy. The erb-b4-KO neonatal mice exhibited normal thickness of right and left ventricular walls and septum (Table 1). However, ventricular chamber dilation was clearly evident by the third mo, as manifested by the significant reduction in wall thickness and the enlargement of the hearts (Fig. 4A, Table 1). Despite relatively normal behavior and activity, erb-b4-KO mice die within the first year of life (Fig. 4B). We investigated a possible reduction in the number of myocytes that could account for the postnatal thinning of the ventricular walls. There were no differences in cell number when nuclei of KO and WT mice were counted at birth (Table 1). There were no changes in cardiomyocyte proliferation or apparent apoptosis monitored by bromodeoxyuridine incorporation into DNA or the TdT-mediated dUTP nick end-labeling reaction, respectively.

The erb-b4-KO mice at 3 mo of age exhibited higher heart-to-body weight ratios than WT, heterozygous (control)
mice (8.78 ± 2.55 vs. 5.58 ± 0.80 mg/g) without significant differences in total body weight (30.65 ± 3.2 vs. 30.46 ± 2.9 g), indicative of a hypertrophic growth. The increase in cardiac tissue mass was correlated with the expression of hypertrophy-related genes (29), including skeletal actin and atrial natriuretic factor and brain natriuretic peptide (Fig. 4). The number of IDs and nuclei per unit area of ventricular muscle (Table 1) was reduced as a result of the myocyte elongation (136.5 ± 34.4 vs. 181.6 ± 50.3 μm, P < 0.05; Fig. 4, D and E). The erb-b4-KO cardiomyocytes showed enlarged nuclei with a significantly higher rate of polyploidy at 3 mo of age (Fig. 4E, Table 1), a parameter also altered in human dilated cardiomyopathy (24). Ultrastructural analysis revealed dilation of the myocyte membrane structures with apparently normal myofibrils and mitochondrial morphology (Fig. 4F).

Cardiac function was assessed in erb-b4-KO compared with WT hearts. Retrograde perfusion of the left ventricle in isolated erb-b4-KO hearts showed a reduction of the maximal left ventricular developed pressure (49.16 ± 3.05 and 24.58 ± 5.29 mmHg in WT and KO mice, respectively) and +dP/dt (1,241.34 ± 107.94 and 587.02 ± 117.57 mmHg/s in WT and KO mice, respectively), demonstrating depressed myocardial contractility (Fig. 5). Although basal +dP/dt data in WT mice appeared relatively low, these values are comparable to and within the range reported for mice of different genetic backgrounds (17, 30). There were no differences in ventricular relaxation, determined by t½, between KO and WT hearts (Fig. 5). The response to β-adrenergic agonists was assayed at a maximal dose of isoproterenol, showing an inotropic response in KO and WT hearts (146.94 ± 20.07 and 150.15 ± 18.98% of basal +dP/dt in WT and KO hearts, respectively). This result suggests that Erb-b4 signaling is not required for this physiological response.

The contractile performance in isolated cardiomyocytes appeared normal, inasmuch as there were no detectable differences in Ca²⁺ transients or fractional shortening in response to increasing Ca²⁺ concentrations or pacing frequency. (Supplemental data can be found at http://ajpheart.physiology.org/cgi/
VENTRICULAR DELETION OF **ERB-B4** LEADS TO CARDIOMYOPATHY

**Fig. 4.** Erb-b4 deficiency results in severe dilation of ventricular chambers. **A:** transverse ventricular sections of 3-mo-old control WT and KO mice stained with hematoxylin-eosin. Note severe dilation of right and left ventricles (RV and LV) in KO heart. **B:** survival rate in 139 WT and heterozygous (Het) and 48 KO mice from 23 litters. **C:** Northern blots of total RNA from adult ventricles of WT, heterozygous, and KO hearts hybridized with skeletal (sk) actin, atrial natriuretic factor (ANF), and brain natriuretic peptide (BNP) cDNA probes. The same blots were stripped and hybridized with GAPDH cDNA probe for sample loading control. **D:** isolated cardiomyocytes from WT and KO hearts. Myocyte length is increased in KO compared with WT hearts. **E:** high magnification of ventricular wall from WT (Ct) and KO mice in A. Scale bar, 20 μm. Note enlarged nuclei in KO myocytes compared with Ct. **F:** electron-microscopic images of ventricles from WT (left) and KO (right) mice. WT myocytes contain myofibrils and mitochondria of normal morphology. Note dilation of tubular membrane system (arrows) in KO myocytes and normally appearing myofibrils and mitochondria. Scale bar, 2.5 μm.

### Table 1. Phenotypic modifications and cellular hypertrophy

<table>
<thead>
<tr>
<th>Days</th>
<th>n</th>
<th>Genotype</th>
<th>LV, μm</th>
<th>IVS, μm</th>
<th>RV, μm</th>
<th>ID</th>
<th>Nuclei</th>
<th>Ploidy Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>WT</td>
<td>483 (25)</td>
<td>478 (15)</td>
<td>301 (15)</td>
<td>ND</td>
<td>78 (5)</td>
<td>301 (2)</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>KO</td>
<td>490 (13)</td>
<td>470 (12)</td>
<td>278 (18)</td>
<td>ND</td>
<td>73 (6)</td>
<td>301 (2)</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>WT</td>
<td>556 (55)</td>
<td>512 (37)</td>
<td>325 (25)</td>
<td>ND</td>
<td>31 (5)</td>
<td>302 (3)</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>KO</td>
<td>617 (63)</td>
<td>528 (45)</td>
<td>338 (34)</td>
<td>ND</td>
<td>27 (5)</td>
<td>300 (2)</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>WT</td>
<td>822 (45)</td>
<td>725 (31)</td>
<td>521 (25)</td>
<td>92 (5)</td>
<td>21 (6)</td>
<td>300 (4)</td>
</tr>
<tr>
<td>21</td>
<td>3</td>
<td>KO</td>
<td>745 (57)</td>
<td>700 (30)</td>
<td>460 (34)</td>
<td>87 (5)</td>
<td>22 (5)</td>
<td>345 (7)</td>
</tr>
<tr>
<td>90</td>
<td>4</td>
<td>WT</td>
<td>1,260 (29)</td>
<td>1,105 (79)</td>
<td>622 (7)</td>
<td>117 (5)</td>
<td>11 (4)</td>
<td>280 (13)</td>
</tr>
<tr>
<td>90</td>
<td>6</td>
<td>KO</td>
<td>618 (19)*</td>
<td>529 (15)*</td>
<td>222 (15)*</td>
<td>66 (4)*</td>
<td>6 (3)*</td>
<td>452 (17)*</td>
</tr>
</tbody>
</table>

Values are means (SD); n, number of mice. LV, left ventricle; RV, right ventricle; IVS, interventricular septum; ID, intercalated disk; WT, wild type; KO, erb-b4 cardiac knockout; ND, not determined. Ploidy index was used as a measure of DNA content. *P < 0.001.
The primary finding of this study is that, in adult mice, specific lack of erb-b4 expression in ventricular muscle leads to a severe dilated cardiomyopathy, resulting in a shortened lifespan. The cardiac dysfunction was characterized by ventricular dilation, reduced contractility, and delayed conduction. These observations are consistent with the cardiac phenotype mediated by the conditional inactivation of the erb-b2 gene (9, 28). Together, these mutant mice illustrate that the loss of neuregulin signaling through Erb-b2 and Erb-b4 heterodimeric receptors prevents normal postnatal cardiac remodeling and function. These murine models may prove useful in the understanding of progressive cardiac failure. For example, acquired cardiomyopathy has been observed in a subset of breast cancer patients undergoing combined treatments with anthracycline derivatives and the monoclonal anti-Erb-b2 antibody Herceptin (32). The observed dysfunction in erb-b4-KO mice indicates that Herceptin antibody may affect myocardial function not only as a modifier of anthracycline cardiotoxicity but because of impaired neuregulin signaling through Erb-b2 and Erb-b4.

**Discussion**

The primary finding of this study is that, in adult mice, specific lack of erb-b4 expression in ventricular muscle leads to a severe dilated cardiomyopathy, resulting in a shortened lifespan. The cardiac dysfunction was characterized by ventricular dilation, reduced contractility, and delayed conduction. These observations are consistent with the cardiac phenotype mediated by the conditional inactivation of the erb-b2 gene (9, 28). Together, these mutant mice illustrate that the loss of neuregulin signaling through Erb-b2 and Erb-b4 heterodimeric receptors prevents normal postnatal cardiac remodeling and function. These murine models may prove useful in the understanding of progressive cardiac failure. For example, acquired cardiomyopathy has been observed in a subset of breast cancer patients undergoing combined treatments with anthracycline derivatives and the monoclonal anti-Erb-b2 antibody Herceptin (32). The observed dysfunction in erb-b4-KO mice indicates that Herceptin antibody may affect myocardial function not only as a modifier of anthracycline cardiotoxicity but because of impaired neuregulin signaling through Erb-b2 and Erb-b4.

**Erbb4 is critical for maintenance of ventricular function.** By 3 mo of age, erb-b4-KO mice exhibit the hypertrophic growth of myocytes with enlarged polyploid nuclei, as observed after volume overload. Expression of hypertrophy-related genes was detected in young mice, preceding the overt enlargement of the hearts. The absence of significant changes in the relative cell number suggests that the postnatal hypertrophic response may be related to early modifications in the cellular architecture, ultrastructurally visualized by dilation of the tubular membrane system and widening of the IDs. The restricted localization of Erb-b4 and Erb-b2 to the Z line and IDs, critical sites for the electrical-contraction coupling of the ventricular muscle (3, 19), provides clues as to how these receptors may be involved in regulating cardiomyocyte contractility. Functional studies demonstrated that deletion of erb-b4 in ventricular muscle affected the systolic contractile properties of the myocardium without apparent changes in relaxation or in the &beta;-adrenergic response. This dysfunction was also associated with the abnormally widened QRS complex and Q-Tc intervals. A delayed conduction and repolarization may result in the functional substrate for the observed increase in susceptibility to arrhythmias and death in adult erb-b4-KO mice. A shortened lifespan was also manifested in conditional erb-b2 mutants harboring a “null” erb-b2 allele (28) or under increased stress evoked by aortic banding (9). Together with the reported delay in the onset of dilation (9),

**Table 2. ECG measurements**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>R-R, ms</th>
<th>P-R, ms</th>
<th>QRS, ms</th>
<th>Q-T, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>123 (16)</td>
<td>45 (4)</td>
<td>13 (2)</td>
<td>80 (8)</td>
</tr>
<tr>
<td>KO</td>
<td>122 (16)</td>
<td>47 (5)</td>
<td>23 (2)</td>
<td>90 (12)*</td>
</tr>
</tbody>
</table>

Values are means (SD). *P < 0.05; †P < 0.001.
our findings suggest that conditional erb-b2-KO mice may display a less severe phenotype, probably because of functional Erb-b4 homodimeric receptor complexes in cardiac muscle.

How does Erb-b4 affect cardiomyocyte architecture? In erb-b4-KO myocytes, neuregulin signaling through Erb-b4 and Erb-b2 should be impaired. In this setting, Erb-b2 could potentially permit additional neuregulin-independent signaling events through interactions with other molecules, including the EGF receptor, gp130, and G protein-coupled receptors (1). Therefore, it is reasonable to assume that the defects likely stem from the loss of specific phosphorylation events performed by one or both of these receptors and from the loss of a single target or multiple targets recruited by activated Erb-b receptors. Erb-b4-mediated activation of the phosphatidylinositol 3-kinase/Akt pathway appears to be critical for growth of the developing heart and protection of myocytes from the cardiotoxic effects mediated by anthracycline derivatives or by oxidative stress (14, 10, 20). In this regard, the lack of cardiotoxic signs (e.g., cell apoptosis, relative metabolic changes, or early modifications in the number or structure of mitochondria and vacuoles) suggests that a highly cooperative signaling network might enhance myocyte survival in vivo under the stress conditions displayed in erb-b4-KO mice.

In addition, the loss of Erb-b4 could also disrupt protein-protein and phosphorylation-independent interactions critical for localized signaling of these receptors. Among these, the most notable are the interactions with PDZ domain-containing proteins (4, 5, 16), which may result in the localized targeting of most notable are the interactions with PDZ domain-containing protein and phosphorylation-independent interactions critical for growth of the developing heart and protection of myocytes from the cardiotoxic effects mediated by anthracycline derivatives or by oxidative stress (14, 10, 20). In this regard, the lack of cardiotoxic signs (e.g., cell apoptosis, relative metabolic changes, or early modifications in the number or structure of mitochondria and vacuoles) suggests that a highly cooperative signaling network might enhance myocyte survival in vivo under the stress conditions displayed in erb-b4-KO mice.

Overall, our findings suggest that an alteration in the intercellular coupling results in the impaired ability of the myocardium to develop tension without significant changes in Ca2+ handling or myofibrillar Ca2+ responsiveness in individual myocytes. An improved understanding of the molecular mechanisms underlying cardiomyocyte remodeling may lead to new therapeutic strategies. In this regard, these findings suggest that the development of agents that mediate Erb-b4 signaling may promote cardiac function and prevent progressive cardiac failure.

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