

## Cardiac Myocyte-Specific Excision of the $\beta 1$ Integrin Gene Results in Myocardial Fibrosis and Cardiac Failure

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**Abstract**—Integrins link the extracellular matrix to the cellular cytoskeleton and serve important roles in cell growth, differentiation, migration, and survival. Ablation of  $\beta 1$  integrin in all murine tissues results in peri-implantation embryonic lethality. To investigate the role of  $\beta 1$  integrin in the myocardium, we used Cre-LoxP technology to inactivate the  $\beta 1$  integrin gene exclusively in ventricular cardiac myocytes. Animals with homozygous ventricular myocyte  $\beta 1$  integrin gene excision were born in appropriate numbers and grew into adulthood. These animals had 18% of control levels of  $\beta 1$  integrin protein in the heart and displayed myocardial fibrosis. High-fidelity micromanometer-tipped catheterization of the intact 5-week-old  $\beta 1$  integrin knockout mice showed depressed left ventricular basal and dobutamine-stimulated contractility and relaxation (LV  $dp/dt_{max}$  and LV  $dp/dt_{min}$ ) as compared with control groups ( $n=8$  to 10 of each,  $P<0.01$ ). Hemodynamic loading imposed by 7 days of transverse aortic constriction showed that the  $\beta 1$  integrin knockout mice were intolerant of this stress as they had 53% survival versus 88% in controls ( $n=15$  each). By 6 months of age, mice with depressed ventricular expression of  $\beta 1$  integrin developed a dilated cardiomyopathy that was not evident in any control animals and had patchy decrease in glucose metabolism as determined by positron emission tomography. Myocyte membrane integrity as determined via Evan's blue dye staining was disrupted in the  $\beta 1$  integrin knockout mice. This model provides strong evidence for the importance of  $\beta 1$  integrin in cardiac form and function and indicates that integrins can be linked to development of cardiomyopathies. (*Circ Res.* 2002;90:458-464.)

**Key Words:** extracellular matrix ■ homologous recombination ■ Cre recombinase  
■ heart ■ positron emission tomography

Integrins are a large family of heterodimeric cell surface receptors composed of  $\alpha$  and  $\beta$  subunits. They function in cell-extracellular matrix (ECM) adhesion and cell-cell adhesion, and signal bidirectionally across the cell membrane.<sup>1,2</sup> Further, they serve as mechanotransducers, converting mechanical signals to biochemical ones.<sup>3</sup> This combination of properties allows integrins to play important roles in cell growth, differentiation, migration, and survival<sup>4</sup> and also makes them attractive candidates for essential roles in the developing and postnatal heart.

Our previous work has shown that  $\beta 1$  integrins are linked to the hypertrophic response of cultured ventricular myocytes and also that dominant-negative disruption of integrin function in transgenic mice resulted in cardiac fibrosis and abnormal cardiac function.<sup>5-7</sup> Ablation of  $\beta 1$  integrin expression in all murine tissues resulted in gastrulation defects and death by E5.5 of the 21-day gestation period.<sup>8,9</sup> Chimeric

mice as well as embryoid bodies constructed from  $\beta 1$  integrin-null cells showed delayed development and differentiation of  $\beta 1$ -deficient cells along the cardiac lineage, as well as abnormal sarcomerogenesis of these cardiac-like cells.<sup>10</sup> Although a few  $\beta 1$  integrin-null cells were detected in the myocardium of chimeric mice, cellular debris was always detected along with the null cells. These null cells were completely lost from the myocardium of the chimeric mouse heart by 6 months of age.

To more specifically evaluate the role of  $\beta 1$  integrin in the myocardium, we used a Cre-loxP gene targeting approach. Cre recombinase expression driven by the myosin light chain-2 ventricular (*MLC-2v*) promoter caused  $\beta 1$  integrin gene excision exclusively in ventricular cardiac myocytes.<sup>11</sup> Our results in these  $\beta 1$  knockout ( $\beta 1$ KO) mice showed the following: (1) the  $\beta 1$  integrin gene was excised only in the heart; (2)  $\beta 1$ D integrin protein in the myocardium was

Original received September 14, 2001; revision received January 16, 2002; accepted January 16, 2002.

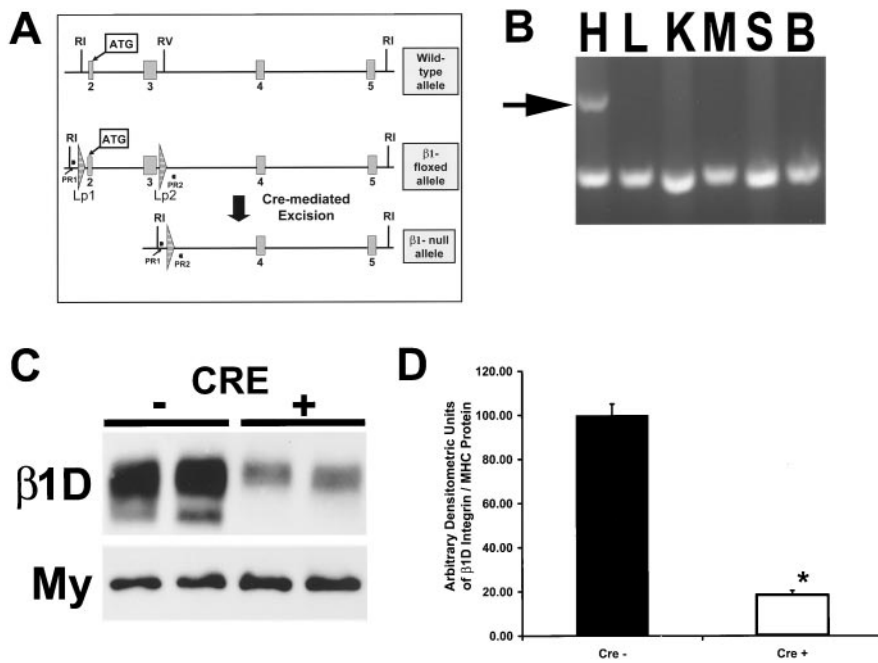
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DOI: 10.1161/hh0402.105790



**Figure 1.** Generation of mice with cardiac-specific excision of  $\beta 1$  integrin gene. A, Partial maps of wild-type  $\beta 1$  integrin allele,  $\beta 1$  integrin-floxed allele, and  $\beta 1$  integrin-null allele. Exons and loxP sequences are indicated as rectangles and triangles, respectively. Translational start site (ATG) is located in the second exon as shown. Primers used for PCR confirmation of gene excision are designated as PR1 and PR2. Expression of Cre recombinase leads to deletion of the  $\beta 1$  integrin gene between LoxP1 and LoxP2, causing deletion of exons 2 and 3. RI indicates *EcoRI*; RV, *EcoRV*; and Lp, *loxP*. B, Ethidium bromide visualization of PCR products generated from genomic DNA derived from various tissues of the  $\beta 1$ KO mouse. Duplex PCR was performed using 2 primer sets. One primer set as shown in panel A (PR1 and PR2) detected a 480-bp band only after excision of the portion of the  $\beta 1$  integrin gene between loxP site 1 (Lp1) and loxP site 2 (Lp2). Without excision, this PCR product would be 3300 bp. The 480-bp band (arrow) was only

detected in heart. The lower 330-bp band was detected with a second primer set that amplified a portion of the Cre transgene, indicating that it was present in all tissues. Thus, Cre expression and subsequent Cre-mediated excision only occurred in heart. H indicates heart; L, lung; K, kidney; M, skeletal muscle; S, spleen; and B, brain. C, Western blot analysis of  $\beta 1D$  integrin protein (top panel) as compared with sarcomeric myosin (My) heavy chain (bottom panel) in murine ventricle shows reduction of integrin protein only in mice expressing Cre recombinase (+). Data are representative of 3 independent experiments analyzing 9 samples in each group performed in 6-week-old animals. D, Densitometric analysis of Western blots showed that the normalized  $\beta 1D$  integrin protein in hearts of Cre recombinase-expressing mice (Cre+) was reduced to <20% of the value of comparable samples obtained in mice that did not express Cre recombinase (Cre-) (n=9 each). Data are arbitrary densitometric units of  $\beta 1$  integrin protein normalized to sarcomeric myosin heavy chain protein. \* $P < 0.001$ .

reduced to 18% of control levels; (3)  $\beta 1$ KO mice developed postnatal cardiac fibrosis, had depressed cardiac function as determined by in vivo catheterization, and were intolerant of hemodynamic loading; (4) the hearts had abnormal myocardial glucose metabolism as determined by positron emission tomography (PET); (5) animals developed spontaneous heart failure by 6 months of age; and (6) animals showed abnormal myocyte membrane integrity as determined by Evan's blue dye (EBD) staining. These results demonstrate a critical function of  $\beta 1$  integrin in the postnatal myocardium and link this transmembrane protein to development of cardiomyopathy.

## Materials and Methods

### Antibodies and Reagents

The anti- $\beta 1D$  integrin antibody was created in our laboratory.<sup>6</sup> Anti-talin and anti-myosin (MF20) monoclonal antibodies were from Sigma and the Developmental Studies Hybridoma Bank, respectively. FITC- and rhodamine-labeled secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. Fluorescein-conjugated wheat germ agglutinin (F-WGA) was from Molecular Probes.

### Construction of $\beta 1$ Integrin-Floxed Mice and Ventricular-Specific Excision of the $\beta 1$ Integrin Gene

Two clones 14 and 17 kb in length were isolated from a 129/SVJ mouse genomic library (Stratagene) and used for construction of the floxed  $\beta 1$  integrin gene-targeting vector, as shown in Figure 1A. RI embryonic stem cells<sup>12</sup> were electroporated with the targeting vector, and successful homologous recombination was confirmed by Southern blot analysis using probes derived separately from intron 1 and exon 5 of the  $\beta 1$

integrin gene. (Data are shown in the expanded Materials and Methods section available online at <http://www.circresaha.org>.) The targeted embryonic stem cells were subjected to a second electroporation with pMC-Cre<sup>13</sup> and selected for resistance to 1-2'-deoxy-2'-fluoro- $\beta$ -D-arabinofuranosyl-5'-iodouracil (FIAU [Moravek]). Colonies with Cre-mediated type II deletion were confirmed by polymerase chain reaction (PCR) analyses and injected into blastocysts to generate the targeted mice with a floxed  $\beta 1$  integrin gene. Mice with successful germline transmission of the targeted allele were identified and termed  $\beta 1$  integrin-floxed mice.

$\beta 1$  integrin-floxed mice were mated to MLC2v-Cre mice<sup>11,14</sup> to generate animals with ventricular-specific  $\beta 1$  integrin gene inactivation ( $\beta 1^{\text{floxed/floxed}}/MLC2v^{\text{Cre/+}}$  or  $\beta 1$ KO). Mice were analyzed in a 129SVJ/Black-Swiss background throughout the study, but results were confirmed in a 129/SVJ background. All animals were housed in an AALAC-approved facility.

### Western Blot Analyses

Protein lysate preparation and Western blot were performed via standard methods.<sup>7</sup> Densitometry of samples (n=9 each group) was performed via use of either AlphaEase (Alpha Innotech) or ImageQuant (Molecular Dynamics) software.

### Hemodynamic Analysis, Transverse Aortic Constriction (TAC), and Echocardiography

Age-matched 5-week-old  $\beta 1$ KO and control mice were used for both of these types of studies. Hemodynamic analyses at baseline and after dobutamine infusion were performed using in vivo catheterization with a micromanometer-tipped catheter.<sup>7</sup> For studies assessing survival after aortic constriction or sham operation, animals recovered from operative anesthesia, were returned to their cages, and were checked twice daily. Echocardiography was performed utilizing an Apogee CX echocardiographic machine (ATL Interspec).<sup>15</sup>

## Micro-Positron Emission Tomography (MicroPET)

A microPET scanner developed at the UCLA Crump Institute for Biological Imaging, also available through Concorde Microsystems, was used to image the mice. Animals were anesthetized with 225 mg/kg avertin (2,2,2-tribromoethanol) (IP) and injected 20 minutes later with 200 to 233 mCi of [<sup>18</sup>F]fluorodeoxyglucose (FDG). After an uptake period of 20 minutes, animals received additional anesthesia (ketamine, 100 mg/kg) and were imaged prone, in a long-axis orientation, with the microPET scanner. Scanner bed position was directly over the heart. Images were reconstructed using the maximum *a posteriori* reconstruction algorithm, which provides a resolution of  $\approx 1.2$  mm.<sup>16</sup>

## Assay of Membrane Integrity

Six-month-old mice were injected intraperitoneally (50  $\mu$ L/10 g body weight) with a 2% solution of EBD (Sigma) and euthanized 2 days after injection. Cryosections of myocardial tissue (5 to 7  $\mu$ m) were examined under epifluorescent optics (as above) and viewed as a red image by using green activation (546 nm) and barrier (590 nm) filters, respectively.<sup>17</sup>

## DNA Laddering and Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling (TUNEL) Assays

DNA was extracted from 6-month-old  $\beta$ 1KO and control mouse hearts and analyzed by agarose gel electrophoresis for nucleosomal DNA fragmentation. TUNEL assay was performed on heart cryosections by using Cardiotacs kit reagents (Trevigen), and visualization of TUNEL-positive cells was performed with FITC-ExtrAvidin (Sigma). Cardiomyocytes were detected with an anti-sarcomeric myosin antibody (MF20), and nuclei were localized by staining with DAPI (Sigma). Staining was visualized by immunofluorescent microscopy. TUNEL-positive cells were counted in multiple sections from each heart and recorded as TUNEL-positive myocytes/10 000 myocytes.

## Statistics

The Student *t* test or ANOVA was used for analyses.  $P < 0.05$  was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at <http://www.circresaha.org>.

## Results

### Generation of Ventricular Muscle-Specific $\beta$ 1KO Mice

To generate mice that carry ventricular myocyte-restricted inactivation of the  $\beta$ 1 integrin gene, we used a Cre-loxP strategy.<sup>18</sup> On the basis of previous successful ablation of the  $\beta$ 1 integrin gene using a conventional knockout approach,<sup>9</sup> we constructed a floxed  $\beta$ 1 integrin gene-targeting vector so that Cre recombinase-mediated excision would delete exon 2 (containing the translational start site) and exon 3, of the  $\beta$ 1 gene (Figure 1A). This excision event would prevent expression of all  $\beta$ 1 integrin isoforms since splice variation occurs downstream from these exons. We detected appropriate targeting and subsequent excision of the selectable markers in embryonic stem cells and produced gene-targeted mice via standard techniques.<sup>19</sup> Mice homozygous for a floxed  $\beta$ 1 integrin gene were phenotypically normal and gave birth to litters of normal sizes.

To excise the  $\beta$ 1 integrin gene specifically in cardiac ventricular myocytes, we bred the homozygous floxed  $\beta$ 1 integrin mice to *MLC2v* Cre recombinase “knockin” mice

that had been previously characterized.<sup>11,14</sup> Analysis of adult animals (ages 6 to 8 weeks) confirmed  $\beta$ 1 integrin gene excision only in ventricular tissue derived from homozygous  *$\beta$ 1 integrin flox*  $\times$  *MLC2v-Cre* mice ( $\beta$ 1<sup>Flox/Flox/MLC2v<sup>+/Cre</sup></sup>) (Figure 1B).  $\beta$ 1D integrin protein levels in the 6- to 8-week-old  $\beta$ 1<sup>Flox/Flox/MLC2v<sup>+/Cre</sup></sup> mice were reduced to  $\approx 20\%$  of the control levels ( $18.6\% \pm 1.9\%$  versus controls,  $n=9$  each,  $P < 0.001$ ), which were wild-type mice, homozygous floxed animals ( $\beta$ 1<sup>Flox/Flox</sup>) that had not been crossed with the *MLC2v-Cre* mice, or mice doubly heterozygous for the floxed  $\beta$ 1 integrin allele and *MLC2v-Cre* ( $\beta$ 1<sup>Flox/+/MLC2v<sup>+/Cre</sup></sup>) (not shown) (Figures 1C and 1D). This amount of residual  $\beta$ 1D integrin protein is in agreement with other studies using *MLC2v-Cre*-mediated excision.<sup>11,20</sup>

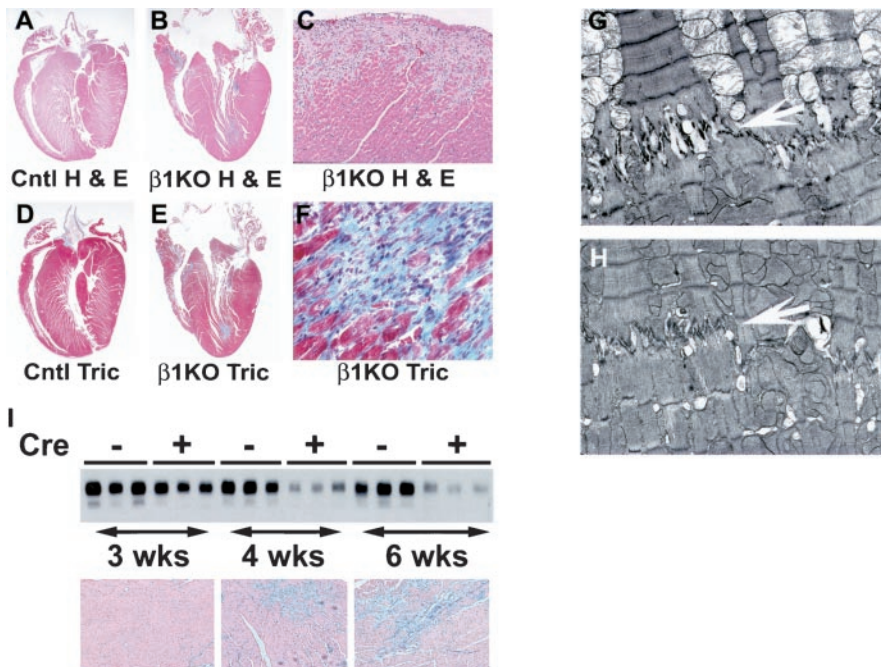
### Conditional Gene Targeting of $\beta$ 1 Integrin Results in Cardiac Fibrosis and Ultrastructural Abnormalities in the Myocardium

We next evaluated the phenotype in the adult  $\beta$ 1KO mice. Histological studies showed that patchy fibrosis developed in the ventricular wall of the  $\beta$ 1<sup>Flox/Flox/MLC2v<sup>+/Cre</sup></sup> mice (Figures 2A and 2F). No comparable results were detected in age-, sex-, and strain-matched wild-type or  $\beta$ 1<sup>Flox/Flox</sup> mice. In concert with identical reduction in  $\beta$ 1 integrin protein expression, ventricular fibrosis was also detected when the  $\beta$ 1<sup>Flox/Flox</sup> mice were crossed with a second cardiac-specific Cre mouse, in which the  $\alpha$ -myosin heavy chain ( *$\alpha$ MHC*) (5.5 kb) promoter drove Cre recombinase expression<sup>21</sup> (data not shown). Transmission electron microscopic analysis of affected areas of the  $\beta$ 1KO myocardium showed focal dissolution of myofibrils and intercalated disks as well as mitochondrial swelling, with disruption and loss of cristae. No similar areas were detected in control animals (Figures 2G through 2H).

Temporal analysis showed that  $\beta$ 1 integrin protein expression was reduced to  $62.7 \pm 5.6\%$  of control values ( $n=6$ ) in the 2- to 3-week-old  $\beta$ 1KO ventricle, to  $23.4 \pm 5.7\%$  of control values ( $n=3$ ) in the 4-week-old ventricle, and to  $18.6 \pm 1.9\%$  of control values ( $n=9$ ) by 6 to 8 weeks of age (Figure 2I). No histological abnormalities were detected in 3-week-old mouse hearts, whereas  $22.1 \pm 6.8\%$  fibrosis developed by age 6 to 8 weeks. These results suggested that  $\beta$ 1 integrin is required for maintenance of cardiac myocyte and myocardial integrity.

### $\beta$ 1 Integrin Protein Deficiency Leads to Abnormal Cardiac Function, Intolerance to Hemodynamic Loading, and Development of Heart Failure by 6 Months of Age

Cardiac function of the  $\beta$ 1KO mice was evaluated with *in vivo* cardiac catheterization. Mice were analyzed at 5 weeks of age, when histological abnormalities were not extensive. Contractility and relaxation of the left ventricle in the  $\beta$ 1<sup>Flox/Flox/MLC2v<sup>+/Cre</sup></sup> mice were significantly impaired as compared with any of the control groups (Figures 3A and 3D). Left ventricular end-diastolic pressure and heart rate did not vary significantly between groups. Because integrins can link transmission of force from the ECM to the cellular cytoskeleton, we challenged the  $\beta$ 1KO mice by hemodynamic loading through TAC (Figure 3E). After 7 days of aortic



**Figure 2.** Abnormalities in mouse hearts from  $\beta 1$ KO mice. A through F, Heart sections from 8-week-old control (Cntl;  $\beta 1^{Lox/Lox}$ , no Cre) (A and D) and from  $\beta 1$ KO mice (B, C, E, and F). A through C, Hematoxylin and eosin (H&E); D through F, Masson's trichrome (Tric). Patchy fibrosis was evident in  $\beta 1$ KO ventricle (B and C), and collagen deposition was detected in trichrome-stained specimens (E and F). G and H, Electron micrographs of specimens from 4-month-old  $\beta 1$ KO mouse heart (G) and control heart (H). Focal dissolution of myofibrils and intercalated disks (arrows), as well as mitochondrial swelling, was detected only in  $\beta 1$ KO specimens but not in control animals. I, Temporal changes in  $\beta 1$  protein expression and development of fibrosis in  $\beta 1$ KO mice. Top panel, Western blot analysis of  $\beta 1$ D integrin expression in  $\beta 1$ KO mice (Cre+) compared with control mice ( $\beta 1^{Lox/Lox}$  and  $MLC2v^{+/+}$  (Cre-)). Bottom panels show histology of heart sections from corresponding-age  $\beta 1$ KO mice. Whereas no abnormalities were histologically visible in myocardium of 3-week-old  $\beta 1$ KO mice, increasing fibrosis was detected with age to  $22.1 \pm 6.8\%$  at 6 to 8 weeks of age (n=5).

constriction, only 53% of the  $\beta 1$ KO mice survived, whereas 88% survival was found in hemodynamically loaded  $\beta 1^{Fllox/Fllox}/MLC2v^{+/+}$  mice. No mortality occurred in sham-operated animals. These data suggested that  $\beta 1$  integrin is required for the murine ventricle to maintain normal function and that excessive hemodynamic demands are not tolerated by the  $\beta 1$  integrin-deficient ventricle.

When the  $\beta 1$  integrin-deficient mice reached  $\approx 6$  months of age, many of the animals showed clinical signs of congestive heart failure, including pleural effusions and liver congestion, and died spontaneously. Similar findings were evident in many  $\beta 1$ KO postpartum female animals. Representative 6-month-old animals were examined pathologically, histologically, and echocardiographically. Histologically hearts showed dilation, replacement fibrosis, and even calcification within the ventricular wall and thrombi in enlarged atria (Figures 4A and 4B). M-mode echocardiography showed left ventricular chamber dilation, increased septal and free wall thicknesses, and depressed ventricular function (reduction in both percentage fractional shortening and velocity of circumferential fiber shortening) in  $\beta 1$ KO animals as compared with controls (Figures 4C and 4D and the Table).

We evaluated cardiac metabolism in these  $\beta 1$ -deficient animals by microPET (Figures 4E and 4F).  $\beta 1$ KO mice displayed abnormal glucose metabolism in the ventricular tissue with patchy uptake of FDG throughout the ventricular myocardium and obvious dilation of the ventricular chamber in the  $\beta 1$ KO mouse as compared with control littermates.

#### Distribution of Talin and Membrane Permeability Are Disrupted, but No Increase in Apoptosis Is Detected in $\beta 1$ KO Mouse Hearts

Because integrins link the ECM and cellular cytoskeleton and could stabilize cell membranes, we evaluated whether  $\beta 1$

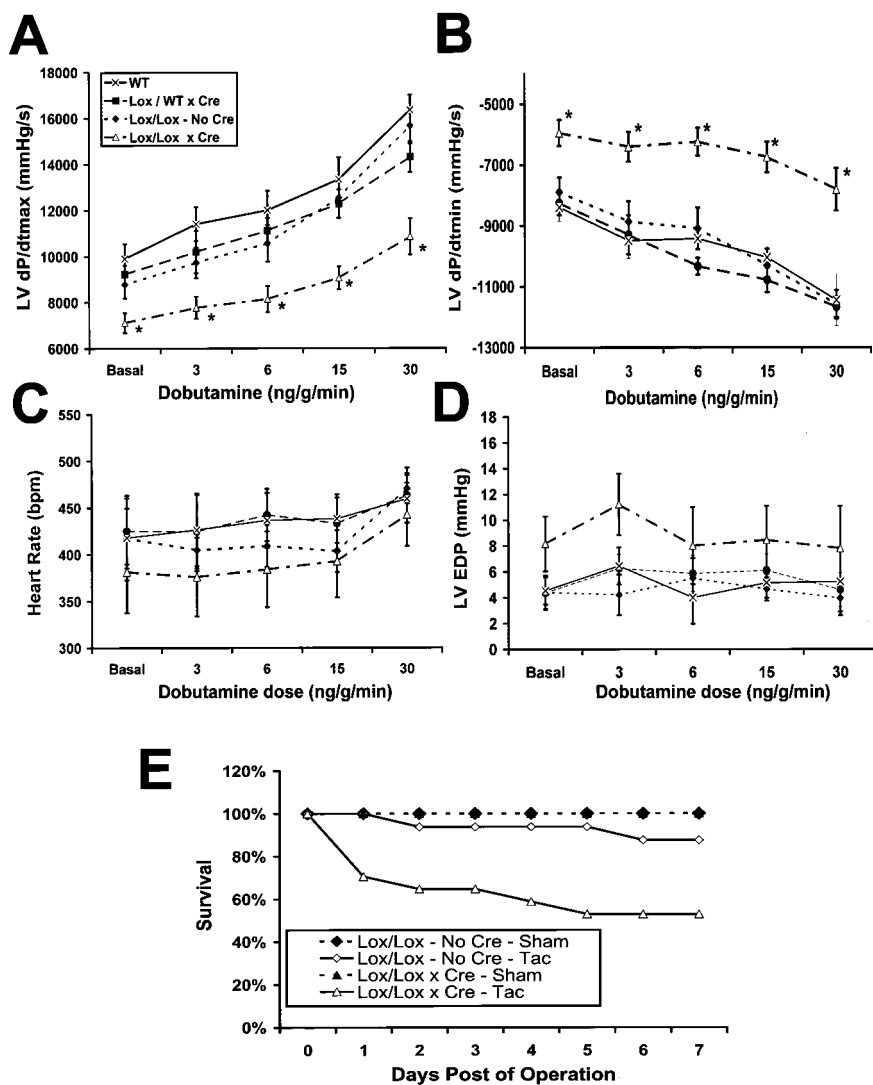
integrin-deficient mice had altered membrane or actin binding protein characteristics. Talin binds directly to the  $\beta 1$  integrin cytoplasmic domain.<sup>22</sup> Immunostaining of heart tissue was performed with anti- $\beta 1$ D integrin and anti-talin antibodies (Figure 5A). Both  $\beta 1$  integrin and talin staining were uniformly visualized in the membranes of myocytes from control mice. In myocardial sections from the  $\beta 1^{Fllox/Fllox}/MLC2v^{+/+}/Cre$  mice, although residual  $\beta 1$ D integrin protein was detected in some myocytes, its pattern even in those cells was non-uniform. This result was in agreement with our Western blot analyses (above). Normally even distribution of talin expression was also disrupted in the  $\beta 1$ D integrin-deficient myocytes.

We performed EBD and WGA staining to detect membrane abnormalities in the myocardium. None of the cardiac myocytes in control mice were permeated by EBD (Figure 5B). Yet in multiple regions of the  $\beta 1^{Fllox/Fllox}/MLC2v^{+/+}/Cre$  mouse heart, strong red staining indicated that EBD-albumin complexes had entered these myocytes through permeant cell membranes. Similarly, imaging of control mouse myocardium with F-WGA demonstrated intact staining of myocyte cell membranes, whereas  $\beta 1$ KO mouse heart sections showed disruption of cell membrane integrity.

Because disruption of cell adhesion can lead to apoptosis and decreased integrin expression can reduce cell adhesion, we evaluated for apoptosis in the  $\beta 1$ KO mice. Ventricular DNA from our  $\beta 1$ KO mice did not reveal DNA laddering (data not shown), nor were an increased number of TUNEL-positive myocytes detected in hearts of these mice ( $7 \pm 1.7$  [ $\beta 1$ KO] versus  $9.2 \pm 1.9$  [control] TUNEL-positive myocytes/10 000 myocytes; n=3 each group).

#### Discussion

Unlike the early embryonic lethality of the traditional  $\beta 1$ KO mice,<sup>9</sup> our cardiac myocyte-specific  $\beta 1$ KO mice survived to

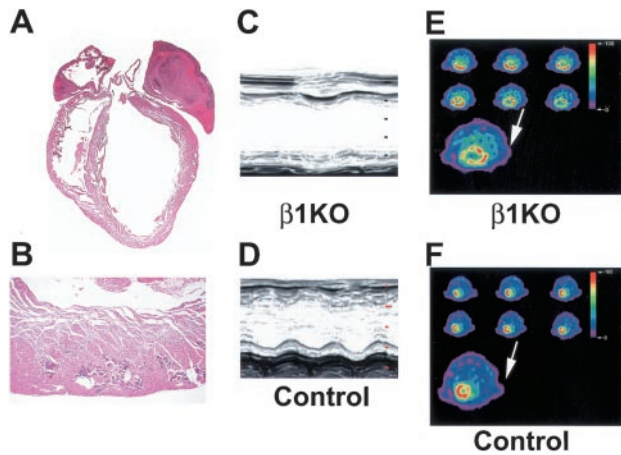


**Figure 3.**  $\beta 1$ KO mice have abnormal cardiac function and intolerance to mechanical loading. A through D, In vivo hemodynamic analysis of  $\beta 1$ KO and control mouse hearts. Cardiac catheterization was performed in intact, anesthetized mice of wild-type (WT,  $n=8$ ), double-heterozygous ( $\beta 1^{Lox/+}$ ,  $MLC2v^{Cre/+}$  [Lox/WT $\times$ Cre];  $n=8$ ), homozygous floxed ( $\beta 1^{Lox/Lox}$ ,  $MLC2v^{+/+}$  [Lox/Lox-No Cre];  $n=8$ ), and  $\beta 1$ KO ( $\beta 1^{Lox/Lox}$ ,  $MLC2v^{Cre/+}$  [Lox/Lox $\times$ Cre];  $n=10$ ) using a micromanometer-tipped catheter. No significant difference was detected in either heart rate or left ventricular (LV) end-diastolic pressure (LVEDP), but LV dP/dt<sub>max</sub> and LV dP/dt<sub>min</sub> were both depressed basally and after dobutamine stimulation of  $\beta 1$ KO mice compared with any other group. A, LV dP/dt<sub>max</sub>, an index of cardiac contractility. B, LV dP/dt<sub>min</sub>, an index of cardiac relaxation. C, Heart rate. D, LV end-diastolic pressure. Data are mean $\pm$ SEM. A and B,  $*P<0.01$ . E, Survival analysis of  $\beta 1$ KO mice after TAC. Shown are  $\beta 1^{Lox/Lox}$ ,  $MLC2v^{+/+}$  (Lox/Lox-No Cre, sham operation);  $\beta 1^{Lox/Lox}$ ,  $MLC2v^{+/+}$  (Lox/Lox-No Cre, TAC);  $\beta 1^{Lox/Lox}$ ,  $MLC2v^{Cre/+}$  (Lox/Lox $\times$ Cre, sham operation); and  $\beta 1^{Lox/Lox}$ ,  $MLC2v^{Cre/+}$ , Lox/Lox $\times$ Cre, TAC). All groups,  $n=15$ .  $\beta 1$ KO mice had 53% survival after hemodynamic loading compared with 88% of control animals.

adulthood likely because some  $\beta 1$  integrin protein was still present. Residual protein was present because of the incomplete nature of Cre-mediated excision effected by  $MLC2v^{+/+Cre}$  or  $\alpha MHC-Cre$ , and is in agreement with previous published work that used these two Cre mice to effect excision of the connexin 43 and gp130 genes.<sup>14,20</sup> It is likely that further reduction of  $\beta 1$  integrin protein in the cardiac myocyte below a yet-unidentified "critical level" would result in abnormal cardiac development and embryonic lethality. This is currently being tested by mating our  $\beta 1$  integrin-floxed mice with animals that may express Cre in a more efficient manner in the cardiac myocyte.

In younger  $\beta 1$ KO mice, there were few histological abnormalities, but in vivo catheterization detected abnormal cardiac function, and the mice were unable to tolerate hemodynamic loading. No evidence of morphometric hypertrophy or abnormal myocyte branching was detected in these animals. These findings demonstrate that  $\beta 1$  integrin is an important mechanotransducer in the cardiac myocyte as in other cell types.<sup>23</sup> Appropriate linkage of ECM and cytoskeleton through  $\beta 1$  integrins is essential for preservation of myocyte function.<sup>3</sup> When  $\beta 1$  integrin is reduced at the cell surface, this linkage is disrupted.

In older adult mice, we noted increased amounts of myocardial fibrosis and also found that the mice developed a dilated cardiomyopathy by 6 months of age. There was no consistent pattern of fibrosis within the ventricular wall, although some mice were found that displayed it in a subepicardial manner. Myocytes in the  $\beta 1$ KO mice displayed membrane abnormalities as detected by positive EBD staining. We hypothesize that the ECM-cytoskeletal linkage, which in part occurs through integrins, is essential for maintenance of the cardiac myocyte, which undergoes continual hemodynamic demands in the beating myocardium. When the integrin is lost from the membrane, this important equilibrium is altered, myocyte integrity is compromised in a progressive manner, and myocyte necrosis occurs, leading ultimately to fibrotic replacement in the areas of the ventricle with myocyte loss. Cell adhesion has been suggested to be required for cell survival. More specifically, loss of adhesion to the ECM has been linked to cell apoptosis, a process termed anoikis.<sup>24</sup> It is for this reason that we tested for accelerated apoptosis in our KO mice, but no increase over control values was detected at the time assessed. It is likely that the cardiomyopathy resulted not only from replacement



**Figure 4.**  $\beta 1$ KO mice develop cardiac failure and have abnormal glucose metabolism by microPET. A and B, Histological analysis of representative 6-month-old  $\beta 1$ KO mouse shows dilated chamber sizes, with clots in atria and fibrotic changes as well as calcium deposition in ventricular wall (as shown in higher-power view in panel B). C and D, Representative M-mode echocardiograms of  $\beta 1$ KO (C) and age-matched control littermate (D) hearts show depressed left ventricular function, dilated right and left ventricular chambers, and thickened ventricular walls in  $\beta 1$ KO heart. E and F, Results of microPET using FDG as a tracer of myocardial glucose utilization from a  $\beta 1$ KO (E) and littermate control mouse (F). Highest levels of tracer uptake are depicted by red-orange, and lowest levels by purple, as indicated by color scale at top right of each panel. FDG uptake is decreased in a patchy manner in ventricle of  $\beta 1$ KO hearts (E), which are also dilated compared with control animals (F). Arrows indicate enlarged view of denoted smaller images in panels E and F.

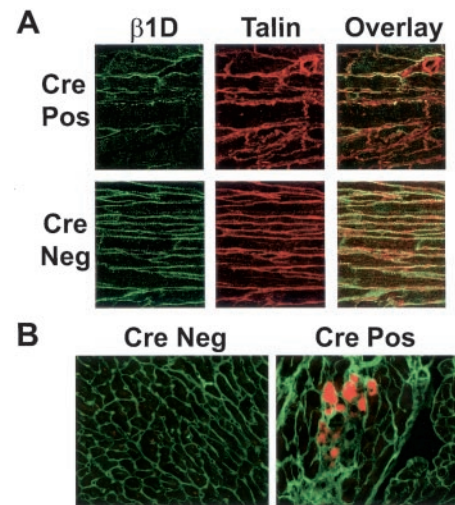
fibrosis of myocytes, but also from altered mechanotransduction of the  $\beta 1$  integrin-deficient myocytes. Deficiency of  $\beta 1$  integrin would lead to alterations in numerous  $\alpha\beta 1$  integrin heterodimer pairs, including  $\alpha 1\beta 1$ . It is interesting to note that  $\alpha 1$  integrin-null mice have increased expression of several matrix metalloproteinases (MMPs).<sup>25</sup> MMPs are linked to the remodeling that occurs during the development of cardiomyopathies, and thus it is possible that alterations in these molecules may also be present in our knockout mice. This is currently being investigated.

#### Echocardiographic Parameters in Anesthetized Mice

	Wild Type (n=5)	$\beta 1$ Lox/Lox NO Cre (n=9)	$\beta 1$ Lox/Lox Cre Positive (n=5)
LVEDD, mm	4.03±0.07	3.95±0.07	5.34±0.22*
LVESD, mm	2.87±0.09	2.69±0.05	4.89±0.20*
Sep th, mm	0.64±0.02	0.60±0.02	0.91±0.08*
PW th, mm	0.65±0.02	0.60±0.02	0.85±0.08*
FS, %	28.60±2.52	31.66±1.20	8.44±1.53*
Vcfc, circ/s	6.51±0.63	6.75±0.29	2.25±0.62*

Values are mean±SEM. n indicates number of mice; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; Sep th, ventricular septal thickness; PW th, left ventricular posterior wall thickness; FS, fractional shortening; Vcfc, velocity of circumferential fiber shortening; and circ/s, circumferences per second.

\* $P < 0.001$  by ANOVA with Bonferroni posttest.



**Figure 5.** Abnormal integrin and talin distribution and membrane integrity are seen in  $\beta 1$ KO hearts. A, Confocal microscopy with antibodies against  $\beta 1$ D and talin shows abnormal cellular structure and organization in  $\beta 1$ KO animals (Cre Pos) compared with age-matched littermate control samples (Cre Neg).  $\beta 1$ KO animals showed varying cell sizes and irregular myocyte membrane staining compared with control mice. There was patchy loss of  $\beta 1$  integrin and disordered talin expression in  $\beta 1$ KO animals. B, Representative images of EBD staining in  $\beta 1$ KO animals (Cre Pos) compared with age-matched littermate control samples (Cre Neg). Sections were made from EBD-injected 6-month-old mouse hearts stained with F-WGA to identify cell membranes. Membrane staining is uniform in Cre-negative mouse with irregularities in the Cre-positive ( $\beta 1$ KO) specimen. Control hearts did not show any red fluorescence (EBD/albumin complex penetration). On the contrary, Cre-positive ( $\beta 1$ KO) mouse hearts displayed areas with red fluorescence, indicating that the membrane had become permeant to the EBD-albumin complex in the  $\beta 1$ KO heart.

Parallels can be drawn between these integrin KO mice and changes that occur in muscular dystrophy. Like integrins, the dystrophin-glycoprotein complex also links the ECM and intracellular cytoskeleton. With loss of components of this complex, alteration in membrane permeability is seen and cardiomyopathy can result.<sup>17,26</sup> Although integrin mutations have been linked to skeletal muscle dystrophies in animal models and in humans, they have not been previously linked to cardiomyopathy.<sup>27-31</sup> Further, it is interesting to note that in muscles from *mdx* mice and patients with Duchenne and Becker dystrophies, increased expression of  $\alpha 7$  integrin transcript and protein were detected. Thus, integrins may compensate for absence of components of the dystrophin-dystroglycan complex. This has been recently shown in a mouse model of muscular dystrophy in which overexpression of  $\alpha 7$  integrin improved many of the clinical features of the disease.<sup>32</sup> Further,  $\alpha 7$  integrin-null mice develop progressive skeletal muscular dystrophy, although cardiac function has not been studied in this model. In contrast to integrin upregulation in the absence of dystrophin-dystroglycan complex proteins, no compensatory change in dystrophin, dystroglycan, or  $\alpha$ -sarcoglycan was detected in the  $\alpha 7$  integrin-null background.<sup>31</sup> Thus, this model gives further credence to the importance of integrins as an indispensable linkage between muscle fiber (and perhaps cardiac myocytes) and the ECM.

This linkage appears independent of the dystrophin-dystroglycan complex-mediated interaction of the cytoskeleton with the muscle basement membrane. Thus, there are clearly distinct functions of these two types of transmembrane proteins. This is emphasized by recent data that found that dystroglycan was necessary for initial binding of laminin to the cell surface, whereas  $\beta 1$  integrin was subsequently required for laminin matrix assembly after the binding.<sup>33</sup> We would suggest that integrin mutations could ultimately be detected in a subset of cardiomyopathy patients.

The strategy we used to target exons 2 and 3 of the  $\beta 1$  integrin gene reduces all isoforms of this protein. In the heart, the dominant postnatal isoform of  $\beta 1$  integrin is  $\beta 1D$ .<sup>6</sup> Previous work has shown that  $\beta 1D$  cannot substitute for  $\beta 1A$  integrin in all organs; doing so leads to fetal lethality with multiple developmental defects.<sup>34</sup> Conversely, full ablation of  $\beta 1D$  was suggested to lead to a "mild" cardiac phenotype represented by increased expression of atrial natriuretic factor in the  $\beta 1D$ -null adult mouse ventricle.<sup>34</sup> Our results provide more comprehensive analysis of the cardiac function in  $\beta 1$  integrin-deficient mice but cannot discriminate between absence of  $\beta 1A$  or  $\beta 1D$  as the prime cause of the resultant phenotype. Studies are currently underway to test the ability of cardiac-specific expression of  $\beta 1A$  or  $\beta 1D$ , to alter the  $\beta 1KO$  phenotype.

In summary, we have produced a murine model with cardiac-specific  $\beta 1$  integrin deficiency resulting in abnormal cardiac function and cardiomyopathy. This animal model will be useful for determination of integrin function in the myocardium and other tissues.

### Acknowledgments

This work was supported by NIH Grants HL57872 (to R.S.R.) and HL67938 (to J.C.L.) and by the UCLA Laubisch Cardiovascular Research Fund. We thank Dr Lutz Birnbaumer for assistance in embryonic stem cell work, Dr Joy Frank and Tony Mottino for assistance with electron microscopy, Dr Matthew Schibler for confocal microscopy support, and Dr Robb MacLellan for reagents and critical advice.

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