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Disruption of Integrin Function in the Murine Myocardium Leads to Perinatal Lethality, Fibrosis, and Abnormal Cardiac Performance

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The molecular mechanisms that regulate the cardiac hypertrophic response and the progression from compensated hypertrophy to decompensated heart failure have not been thoroughly defined. Alteration in cardiac extracellular matrix is a distinguishing characteristic of these pathological processes. Integrins, cell surface receptors that mediate cellular adhesion to the extracellular matrix, are signaling molecules that possess mechanotransduction properties. Therefore, we hypothesized that integrins are likely candidates to play an important role in cardiac function. To test this hypothesis, transgenic mice were constructed in which normal integrin function was disrupted by expression of a chimeric molecule encoding the transmembrane and extracellular domains of the Tac subunit of the IL-2 receptor, fused to the cytoplasmic domain of β_{1A} integrin (Tacβ_{1A}). Using the α myosin heavy chain promoter to target expression of this chimera to the cardiac myocyte, transgenic mice were generated that had varied levels of transgene expression. Multiple transgenic founders that expressed the transgene at high levels, died perinatally and exhibited replacement fibrosis. Lines that survived showed 1) hypertrophic changes concordant with reduction in endogenous β_1 integrin levels, or 2) reduced basal contractility and relaxation as well as alterations in components of integrin signaling pathways. These data support an important role for β_1 integrin in normal cardiac function. ***(Am J Pathol 2001, 158:1079–1090)***

Pressure or volume overload of the myocardium results in initial compensatory responses that can progress to decompensated heart failure. The molecular mechanisms and specific cellular signaling pathways that coordinate these responses are poorly understood. In the course of hemodynamic loading, cardiac myocytes are stretched to a greater degree than that present in the normal heart. Further, alteration in the extracellular matrix that surrounds cardiac myocytes occurs in the overloaded heart. Therefore, transmembrane proteins that respond to mechanical stimuli and interface between cells and extracellular matrix are likely to be important components of this response pathway.

Integrins are heterodimeric cell-surface receptors composed of α and β subunits, that function as adhesive and signaling molecules, as well as mechanotransducers.[1,2] In non-cardiac cells, it has been demonstrated that integrins respond to abnormal strain in a manner similar to that which would be found during pressure or volume overload in the heart.[3] β_1 integrin is a dominant integrin β subunit expressed in heart. Two of the four splice variants of β_1 integrin, β_{1A} and β_{1D} are expressed on cardiac myocytes. They are identical with the exception of the last 24 amino acid residues of their respective cytoplasmic domains. The expression of β_{1A} and β_{1D} isoforms is developmentally regulated in cardiac cells. β_{1A} is expressed during embryogenesis while β_{1D} expression begins late in development and eventually becomes the dominant β_1 integrin isoform expressed on adult cardiac myocytes. In previous work, we demonstrated that both isoforms could participate in the hypertrophic response of cultured cardiac ventricular myocytes.[4,5] Increased expression of β_1 integrin augmented both morphological and biochemical characteristics of the hypertrophic response. In contrast, expression of the chimeric protein Tacβ_{1A}, a protein that disrupts integrin

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adhesion and signaling,[6] suppressed the expression of atrial natriuretic factor (ANF), a marker gene of hypertrophic induction. These data implicated integrin mediated adhesion and signaling in the *in vitro* cardiac hypertrophic response pathway.

Based on these results, we hypothesized that alteration of integrin function in the cardiac cell, through cardiac myocyte-specific expression of the Tacβ_{1A} chimeric molecule in the transgenic mouse could provide novel insights into the role of integrins in the heart. We produced lines of transgenic animals that expressed varied amounts of Tacβ_{1A} in the cardiac myocyte. Multiple transgenic founders which expressed the transgene at high levels, died perinatally with significant replacement fibrosis. Lines that survived showed 1) hypertrophic changes concordant with reduction in endogenous β_1 integrin levels, or 2) reduced basal contractility and relaxation, as well as alterations in components of integrin signaling pathways. These data suggest that β_1 integrin expression and signaling are required for normal murine cardiac form and function.

Materials and Methods

Animals

All animals were housed in compliance with the NIH Guide to Care of Laboratory Animals in an AALAC approved facility.

Transgene Construction

A construct encoding a chimeric protein consisting of the extracellular and transmembrane domain of the Tac subunit of the human interleukin-2 (IL-2) receptor fused to the cytoplasmic domain of β_{1A} integrin (Tac-β_{1A}) in plasmid pcDNA was a kind gift of S. LaFlamme and K. Yamada (NIDR, NIH, Bethesda, MD) and has been described previously.[4] The Tac-β_{1A} fragment was subcloned into the *Sal*I site of an α myosin heavy chain (αMHC) promoter construct (clone 26, obtained from J. Robbins, University of Cincinnati, Cincinnati, OH). To generate the Tac-α_5 construct, the β_{1A} cytoplasmic domain portion of the Tacβ_{1A} construct was removed by digestion with *Hin*dIII and *Xho*I and replaced with the α_5 cytoplasmic domain. The cytoplasmic domain of α_5 was generated by reverse transcriptase polymerase chain reaction (PCR) from RNA isolated from the human 293 cell line (ATCC). Oligonucleotides used for the amplification contained a *Hin*dIII site on the 5′ end of the forward primer and an *Xho*I site on the 5′ end of the reverse primer.

Southern and Northern Blots and Polymerase Chain Reaction

Southern blotting or PCR was used to identify potential founders that had integrated the transgene.[7] For these procedures, genomic DNA was isolated from weanling mice by tail or toe clips. For Northern blot studies, total RNA was extracted from freshly isolated cardiac tissue with the TRIzol Reagent (Life Technologies, Rockville, MD). For Southern and Northern blotting, a 760 bp *Sst*I-*Hin*dIII fragment composed of the human interleukin-2 receptor extracellular and transmembrane domain was excised from pBluescript-Tac$\beta_{1A,}$ and used as a probe. The ANF probe was as described previously.[8] β-myosin heavy chain (βMHC) transcript was detected using an oligonucleotide probe(5′-CAAAGGCTCCAGGTCTGAGGGCTTCAC-3′). For Southern blotting, hybridizations were performed in 6× standard saline citrate (SSC), 5× Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 100 μg/ml of herring sperm DNA at 60°C overnight, followed by washing in 0.1× SSC, 0.1% SDS at 62.5°C. Similar conditions were used for Northern blotting, but the post-hybridization washing temperature was increased to 65°C for the ANF and βMHC probes. For PCR, two primers specific for the coding region of Tac subunit portion of the transgene were synthesized: forward primer: 5′-CATACCTGCTGATGTGGGGAC-3′, and reverse primer: 5′-CCCTGCAGTGACCTGGAAGGC-3′. Tac-β_{1A} transgenic animals were identified by the presence of a 371-bp product.

Adenoviral Production and Culture of Neonatal Ventricular Myocytes

Adenoviral construction and culture of neonatal rat ventricular myocytes was performed as described previously.[4,5]

Histology and Immunofluorescent Microscopy

Sections (5 μm) of paraffin-embedded hearts were stained with hematoxylin and eosin or with Masson's Trichrome. β_{1D} integrin protein was detected in 5-μm cryosectioned specimens of murine heart by immunostaining with a polyclonal isoform-specific antibody (no. 186) that has been previously characterized.[5] Microscopic analysis was performed using a Nikon Diaphot microscope equipped with epifluorescent optics.

Lysates and Western Blot Analysis

Lysate preparation and Western blotting was performed as previously described.[9] Hearts were dissected, rinsed in PBS, and immediately homogenized on ice in 1 ml ice-cold RIPA buffer (158 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.2, 1 mmol/L EGTA, 1 mmol/L orthovanadate, 1% Triton-X100, 1% Na deoxycholate, 0.1% SDS, 100 μmol/L leupeptin, 5 IU/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride) and incubated for 10 minutes on ice. Lysates were clarified by centrifugation (16,000 × *g*, 15 minutes, at 4°C). Protein content of the lysate was determined using the BCA protein assay (Biorad Laboratories, Hercules, CA). For immunoblotting, equal amounts of protein (10–20 μg) were electrophoresed on an 8 to 16% gradient SDS-polyacrylamide gel electrophoresis gel (Novex, Carlsbad, CA) and transferred onto

nitrocellulose. Immunoblotting was performed using the following antibodies: monoclonal 7G7/B6 (ATCC) to detect the Tac extracellular domain, polyclonal MC555, specific for the β_{1A} cytoplasmic domain, polyclonal no. 186 specific for the β_{1D} cytoplasmic domain,[5] monoclonal anti-phosphotyrosine 4G10, monoclonal anti-Src GD11, polyclonal anti-FAK (all Upstate Biotech, Lake Placid, NY), polyclonal p44/42 MAP kinase, and monoclonal phospho-p44/42 MAP kinase E10 (New England Biolabs, Beverly, MA). Signals were quantitated by densitometry using Alphaease software (Alpha Innotech, San Leandro, CA).

Hemodynamics and Transverse Aortic Constriction

Eight- to 20-week-old male and female transgenic and negative littermate control mice were used for all studies, with ages matched for a particular analysis. Animals were anesthetized via intraperitoneal injection of 100 mg/kg ketamine and 5 mg/kg xylazine and placed on a warming pad. Heart rate and temperature were continuously monitored. Surgery was performed and hemodynamic measurements were obtained as previously described.[10]

Briefly, after anesthetization and intubation, the right carotid artery was exposed. A 1.4 French Millar catheter (Millar Instruments, Houston, TX) was inserted and advanced until a left ventricular pressure tracing was visualized. The catheter was adjusted so that no catheter trapping was evident. The animals were recovered from the initial procedure and baseline pressure measurements were obtained. Sequential injections of isoproterenol (0.01, 0.02, and 0.05 μg) were administered with at least a 5-minute recovery period between injections. Measurements were acquired with Hem Software (Notocord Systems, Croissy, France). Heart rate and left ventricular pressure were recorded 150 seconds after the injections and averaged over a 10-second period. Maximum and minimum dP/dT were calculated during this 10-second period. The animals were given a lethal dose of KCl to terminate the experiment.

Pressure overload (POL) hypertrophy was induced via transverse aortic constriction, using previously published techniques.[8] Animals were anesthetized and monitored as above. A midline cervical incision was made and the trachea was exposed. Animals were intubated with a 20 gauge blunt-tipped needle and then connected to a mechanical ventilator. The chest was entered at the left second intercostal space. The thymus was deflected to expose the aorta. A constriction was made in the transverse aorta by tying a 7-0 silk suture over a 27-gauge needle. The pneumothorax was evacuated and the chest was closed. The animals were extubated approximately 20 minutes after the surgery and allowed to recover before being returned to their cages. Sham animals underwent an identical procedure without aortic constriction. On postoperative day 7, the animals were again anesthetized and intubated. The carotid arteries were exposed and cannulated with flame-stretched PE50 tubing. Carotid pressures were recorded to assess for adequate gradients between the left and right carotid pressures. The animals were then sacrificed, and the heart chambers were weighed at the conclusion of the procedure.

Determination of Calcium Sensitivity Using Skinned Fiber Bundles

Calcium sensitivity was determined as previously described.[11] Briefly, hearts were isolated from transgenic or age-matched wild-type mice (10–13 days or 21–24 days old) and rinsed in cold high relaxing (HR) solution (10 mmol/L EGTA, 2 mmol/L $MgCl_2$, 79.2 mmol/L KCl, 5.4 mmol/L Na_2ATP, 12 mmol/L Creatine phosphate, 20 mmol/L MOPS, pH 7.0 [ionic strength 150 mmol/L]) plus protease inhibitors (2.5 μg/ml pepstatin A, 1 μg/ml leupeptin, and 50 μmol/L phenylmethylsulfonyl fluoride). Left ventricular papillary muscles were removed and dissected further into fiber bundles approximately 150 μm in diameter and 4 to 5 mm in length. Bundles were incubated in 1% Triton-X100 for 30 minutes to skin the fiber bundle and subsequently sarcomere length was set to 2.0 μm as determined by the laser diffraction pattern. All fibers used had a final maximal contraction that was at least 90% of the initial maximal contraction. The force-pCa (log of molar $[Ca^{+2}]$) relation was fit to the Hill equation with nonlinear regression analysis (Prizm, GraphPad, San Diego, CA) to derive pCa_{50} and the Hill coefficient. One fiber bundle per animal was analyzed.

Statistics

Data are presented as mean ± SEM. Statistical differences were determined by *t*-test with $P < 0.05$ indicating significant differences.

Results

Expression of Free β_{1A} Integrin Cytoplasmic Domains Results in Alteration of Cultured Cardiac Myocyte Morphology and Detachment from Extracellular Matrix

Previous studies have shown that expression of free cytoplasmic domains of β_1 integrin in various cultured cell lines disrupts normal integrin function.[6,12–15] To further investigate the role of β_1 integrin in cardiac myocytes, we assessed the effect of expression of Tac-β_{1A} in primary cultured cardiac myocytes. High-level expression of Tac-β_{1A} or Tac-α_5 in cardiac myocytes was obtained by infection of cultured myocytes with recombinant adenoviral expression vectors (Figure 1A). Expression of the control Tac-α_5 chimeric protein does not inhibit integrin function in noncardiac cells.[6] In cultured neonatal ventricular myocytes, it similarly did not produce any significant morphological alterations or affect myocyte adhesion to the substrate as compared to uninfected cells (Figure 1, B and C). This indicates that expression of the Tac extracellular transmembrane domain does not affect car-

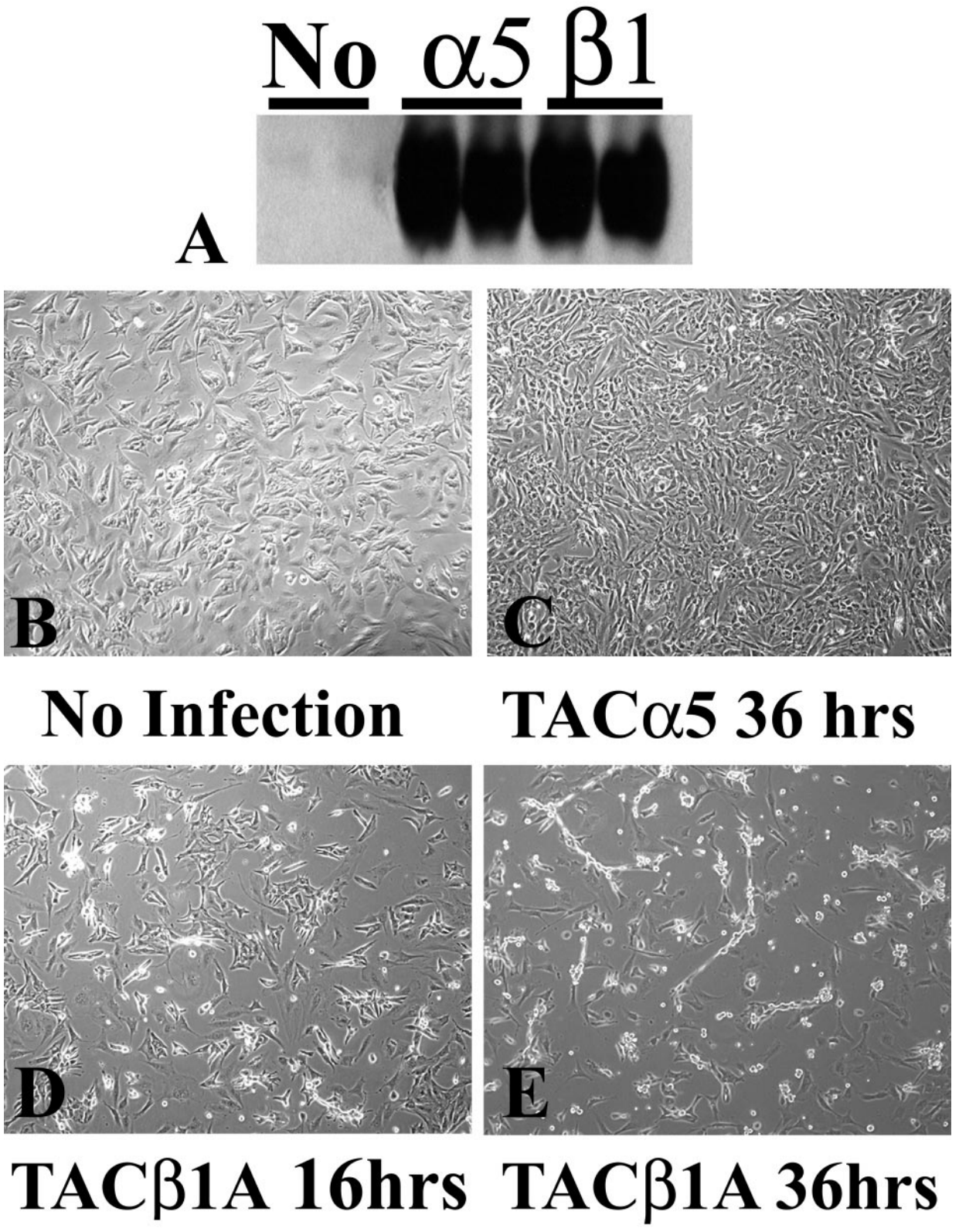

Figure 1. High-level expression of free β_1 integrin but not α_5 integrin cytoplasmic domains results in altered adhesion of cultured neonatal ventricular myocytes. **A:** Western blot analysis of protein extracts from myocytes either not infected (NO) or infected with recombinant adenoviruses which express either Tacα_5 (α_5) or Tacβ_{1A} (β_1) show high level expression of the recombinant protein only in the infected cells. **B:** Uninfected neonatal ventricular myocytes plated on laminin and photographed under phase contrast optics. **C:** Myocytes infected with Tacα_5 recombinant adenovirus (MOI = 50) for 36 hours show no morphological alterations as compared to uninfected cells. **D** and **E:** Myocytes infected with Tacβ_{1A} recombinant adenovirus (MOI = 50) for 16 hours (**D**) or 36 hours (**E**) show altered cell morphology and eventual loss of adhesion from the laminin-coated culture matrix.

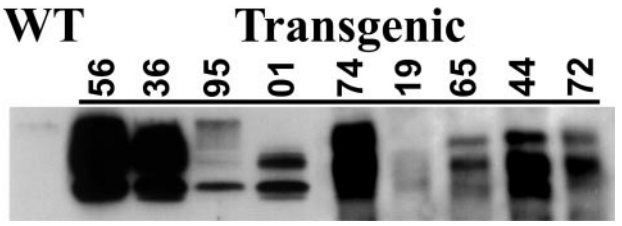

Figure 2. Transgene expression in cardiac tissue from multiple independent transgenic lines. Western blot analysis of cardiac protein extract from 9 independent transgenic lines and a wild-type animal was performed using an anti-IL2 receptor antibody and reveals no detectable signal in the control (WT) animal and high-level expression of Tacβ_{1A} transgene in myocardial protein from numerous transgenic αMHC Tacβ_{1A} mice.

diac myocytes. In contrast, high-level expression of Tac-β_{1A} produced significant morphological alterations in the infected cells by 16 hours post infection. At later time points, Tac-β_{1A} expression resulted in myocyte detachment (Figure 1, D and E). This is consistent with our previous finding that expression of the Tac-β_{1A} chimera in primary cultured cardiac myocytes disrupted adrenergically induced hypertrophic signaling events.[4]

Cardiac-Specific Expression of β_{1A} Integrin Cytoplasmic Domains Results in Perinatal Death

Based on these and our previous results,[4] we hypothesized that transgenic expression of free β_{1A} integrin cytoplasmic domains in the cardiac myocyte would disrupt integrin function and signaling in the targeted cells and provide important information about the role of integrins in the intact heart. The αMHC promoter construct used for generation of these mice has been previously well characterized and is known to become up-regulated in the murine ventricle perinatally and continue expression in the adult.[16,17] The αMHC -Tacβ_{1A} construct was used to generate 15 independent transgenic lines with varied level of transgene expression as assessed via Western blot analysis. (Figure 2). Six independent founder animals, which had the highest relative level of transgene expression, all died perinatally and had diffuse fibrotic replacement of the myocardium (Figure 3, C and D). Additional lines of animals with high-level transgene expression (nos. 36 and 56 shown in Figure 2) survived and bred appropriately, but showed evidence of a dilated and hypertrophic phenotype (Figure 3, E and F) and expressed molecular markers of hypertrophic induction (Figure 4), whereas other lines (eg, nos. 44 and 74 in Figure 2) showed no basal histological or molecular abnormalities (data not shown).

Hemodynamic Abnormalities Are Present in Tac-β_{1A} Transgenic Animals that Appear Phenotypically Normal

Integrins are known to be important mechanotransducers in non-cardiac cells.[18] To further explore the role of integrins in the cardiac myocyte, we analyzed transgenic animals from lines that had high transgene expression (lines 44 and 74 of Figure 2), but showed no basal morphological or molecular abnormalities. First we evaluated the hemodynamic properties of these mice, as compared to littermate control animals using invasive techniques. Basal left ventricular contractility and relaxation were significantly depressed in the transgenic animals as compared to controls, yet following infusion of the inotropic agent isoproterenol, this abnormality could be overcome (Figure 5). No significant difference was seen in left ventricular pressure or heart rate between the transgenic or control groups. To assess whether this functional abnormality of the myocardium was a direct consequence of intrinsic myocyte dysfunction, we evaluated the Ca^{2+} sensitivity of isometric tension in skinned fiber bundles from transgenic and control animals. No difference in the pCa-tension relationship or Hill coefficient was detected in the transgenic fiber bundles (data not shown).

Next, we performed POL via transverse aortic constriction[8] on transgenic animals to test whether transgene expression would alter the ability of hemodynamic loading to effect hypertrophic induction. The αMHC-Tacβ_{1A} animals developed similar degrees of left ventricular hypertrophy after 7 days of POL (22.4 $\pm$ 2.9% increase in left ventricular weight/body weight over sham control) as compared to wild-type littermate control animals (21.4 $\pm$ 2.1% increase in left ventricular weight/body weight over sham-operated control).

Altered Phosphorylation of Focal Adhesion Kinase (FAK) and Extracellular Regulated Kinase (ERK), as Well as Reduction of Endogenous β_1 Protein, Were Found in Tac-β_{1A} Transgenic Animals

Integrins are devoid of intrinsic kinase activity and rely on the assembly of cytoskeletal and signaling proteins following integrin ligation for efficient signal transduction. To assess the effect of transgene expression on signaling events in the cardiac myocyte, we evaluated the phosphorylation profile of proteins that are known components of integrin signaling cascades. Basal (sham-operated) protein values were assessed and compared to the protein values after hemodynamic loading caused by aortic constriction. Densitometry of Western blot analyses was performed for each signaling molecule, where the activated form of each protein was normalized to the total amount of that particular protein.

We first examined the effect of transgene expression on the phosphorylation of FAK, a cytoplasmic tyrosine kinase that is thought to be a key intermediary of signaling through integrins.[19] The level of phosphorylated FAK in sham-operated transgenic animals was significantly reduced compared to sham-operated controls. Further, in the wild-type control animals, FAK phosphorylation was reduced after 7 days of hemodynamic loading, whereas no change was detected in the POL transgenic mice (Figure 6, A and B).

As integrin signaling pathways have been demonstrated to activate ERK, perhaps independently of FAK,[20] and ERKs have been implicated in the hypertrophic re-

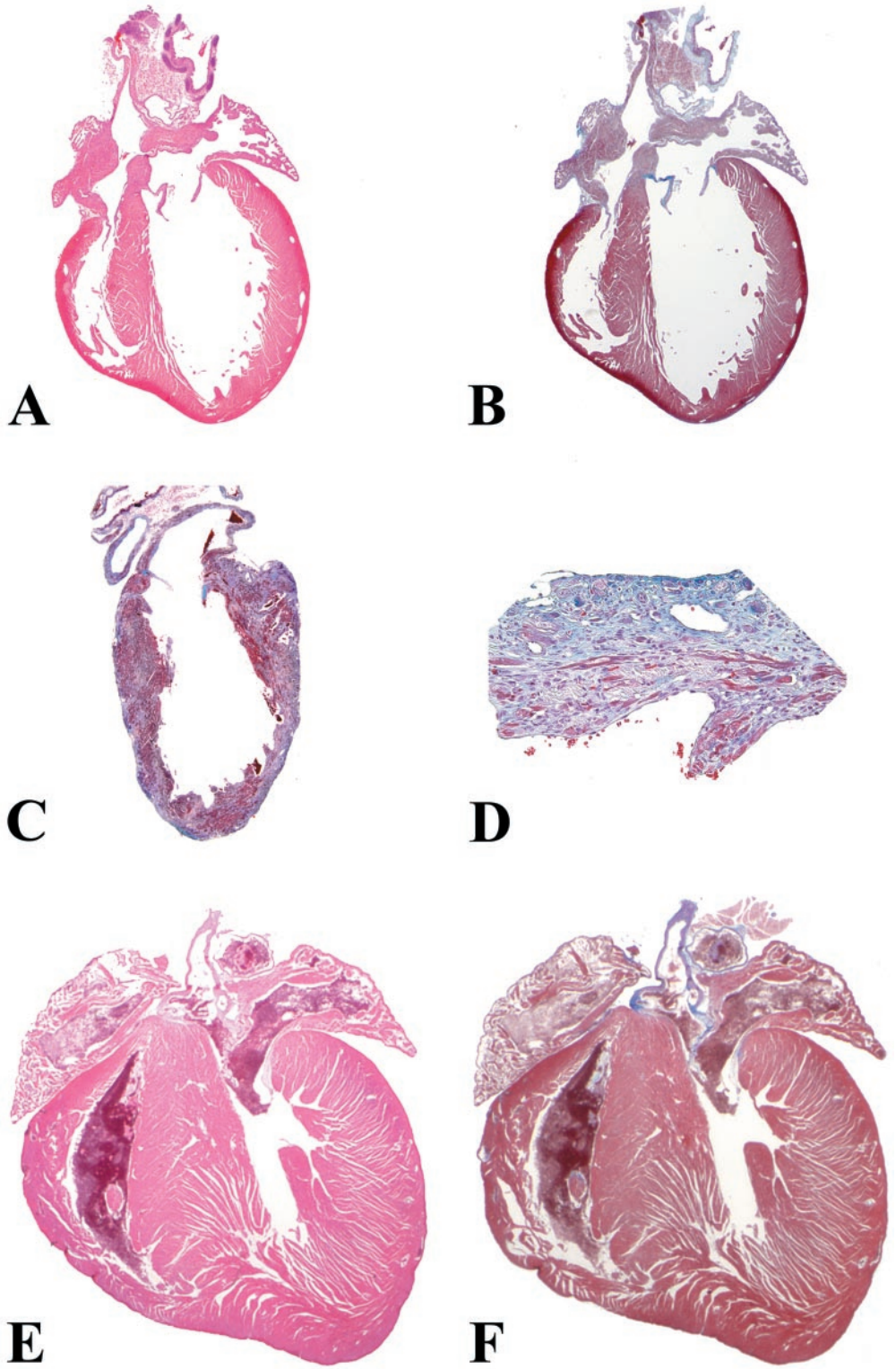

Figure 3. αMHC Tacβ_{1A} transgene expression results in both perinatal fibrosis and dilated hypertrophy of transgenic myocardium. **A** and **B:** Hematoxylin and eosin (**A**) and Masson's Trichrome (**B**) staining of sections from wild-type hearts. **C** and **D:** Masson's Trichrome staining of sections from a representative transgenic animal that exhibited high-level transgenic protein expression and died perinatally. Low-power (**C**) and high-power (**D**) views reveal dramatic replacement fibrosis in this specimen. **E** and **F:** Hematoxylin and eosin (**E**) and Masson's Trichrome (**F**) staining of sections from a representative transgenic line with high level transgenic protein expression (no. 56 in Figure 2) displays a dilated and hypertrophic phenotype.

sponse of myocytes, we also evaluated ERK signaling in sham-operated transgenic and wild-type control mice and following aortic constriction. As shown in Figure 6, C and D, when wild-type animals underwent aortic constriction, they significantly increased phosphorylated p42 ERK relative to total p42 ERK protein and trended toward

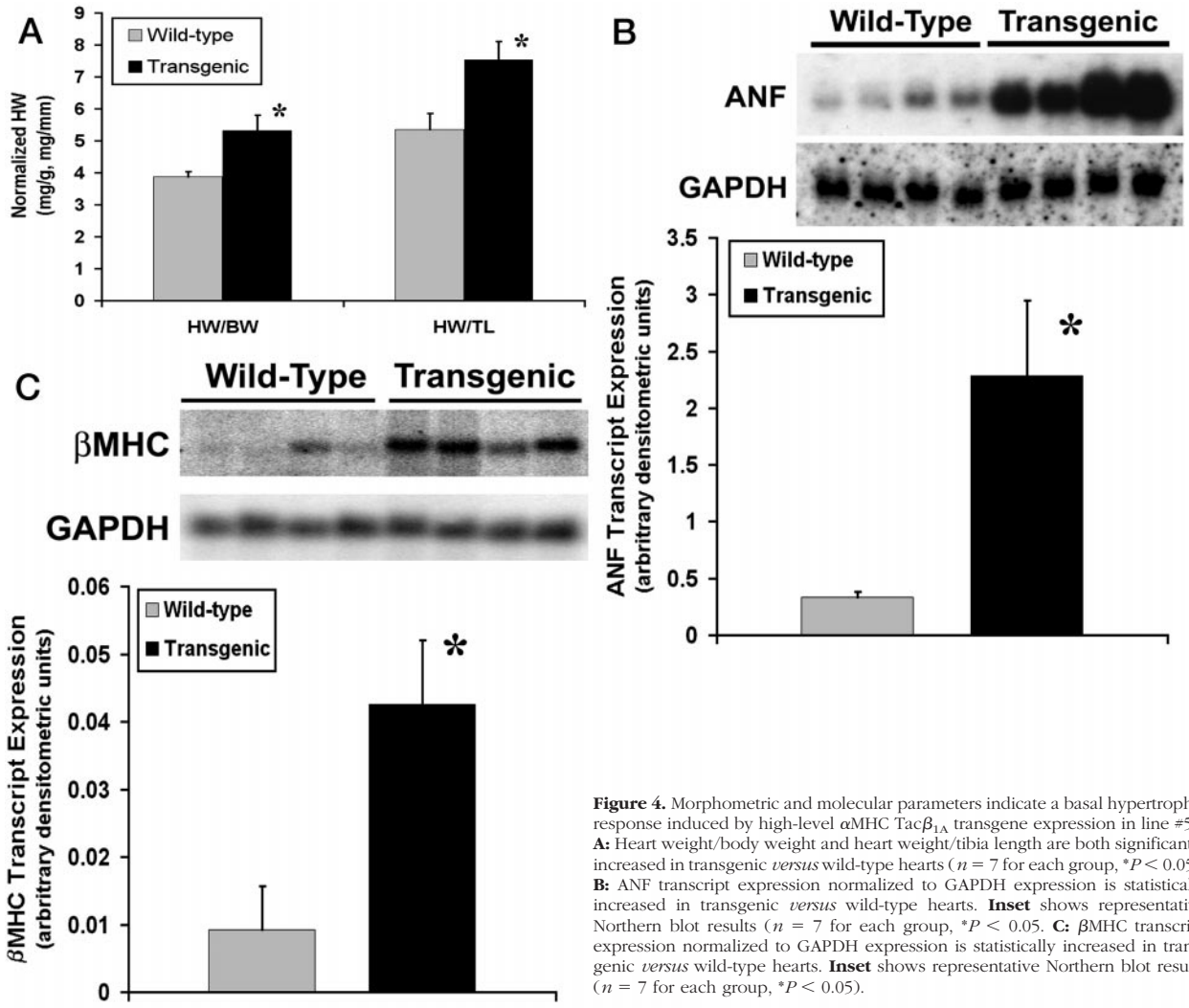

Figure 4. Morphometric and molecular parameters indicate a basal hypertrophic response induced by high-level αMHC Tacβ_{1A} transgene expression in line #56. **A:** Heart weight/body weight and heart weight/tibia length are both significantly increased in transgenic *versus* wild-type hearts ($n = 7$ for each group, $^*P < 0.05$). **B:** ANF transcript expression normalized to GAPDH expression is statistically increased in transgenic *versus* wild-type hearts. **Inset** shows representative Northern blot results ($n = 7$ for each group, $^*P < 0.05$. **C:** βMHC transcript expression normalized to GAPDH expression is statistically increased in transgenic *versus* wild-type hearts. **Inset** shows representative Northern blot results ($n = 7$ for each group, $^*P < 0.05$).

similar increases in the p44 isoform phosphorylation. In contrast, no significant change was noted in the phosphorylation of either ERK isoform in the hemodynamically loaded transgenic animals. Similarly, we evaluated phosphorylation of c-Src in sham-operated or aortic constricted wild-type and transgenic animals (Figure 6, E and F). No significant differences were noted in c-Src normalized phosphorylation in the wild-type animals after aortic constriction, and no differences were noted between the wild-type and transgenic animals.

Finally, we evaluated the expression level of endogenous β_{1D} integrin protein in the context of the transgenic animals. As shown in Figure 7, the level of expression of the endogenous β_1 integrin was reduced in those animals with high-level expression of the Tacβ1A transgene. The reduction of the endogenous β_1 integrin expression was apparent by western blotting (Figure 7A) and by immunostaining of cardiac tissue (Figure 7, B and C).

Discussion

In this study, the role of β_1 integrin in normal cardiac function was investigated by targeting the expression of autonomous β_{1A} cytoplasmic domains to cardiac myocytes in transgenic mice. The major findings were as follows. First, multiple transgenic founders that expressed the highest relative levels of the autonomous β_{1A} cytoplasmic domain protein died perinatally with significant replacement fibrosis. Second, surviving lines with the highest levels of transgene expression developed a compensatory hypertrophy without any physiological stimulus. Third, lines with lower levels of transgene expression possessed abnormalities in basal contractility and relaxation as well as alterations in the phosphorylation of several effectors of integrin signaling. These data support an important role for β_1 integrin-mediated adhesion and signaling in cardiac function.

In our previous work, we found that increased expression of β_1 integrin augmented the hypertrophic response of cultured neonatal ventricular myocytes, whereas expression of the chimeric protein used in the current study, Tac-β_{1A}, inhibited this response.[4] The Tac-β_{1A} chimeric protein does not heterodimerize with integrin α subunits nor does it bind to extracellular matrix ligands. Previous studies with various cultured cell lines have demonstrated that expression of this or similar chimeric proteins acts to dominantly inhibit endogenous β_1 integrin func-

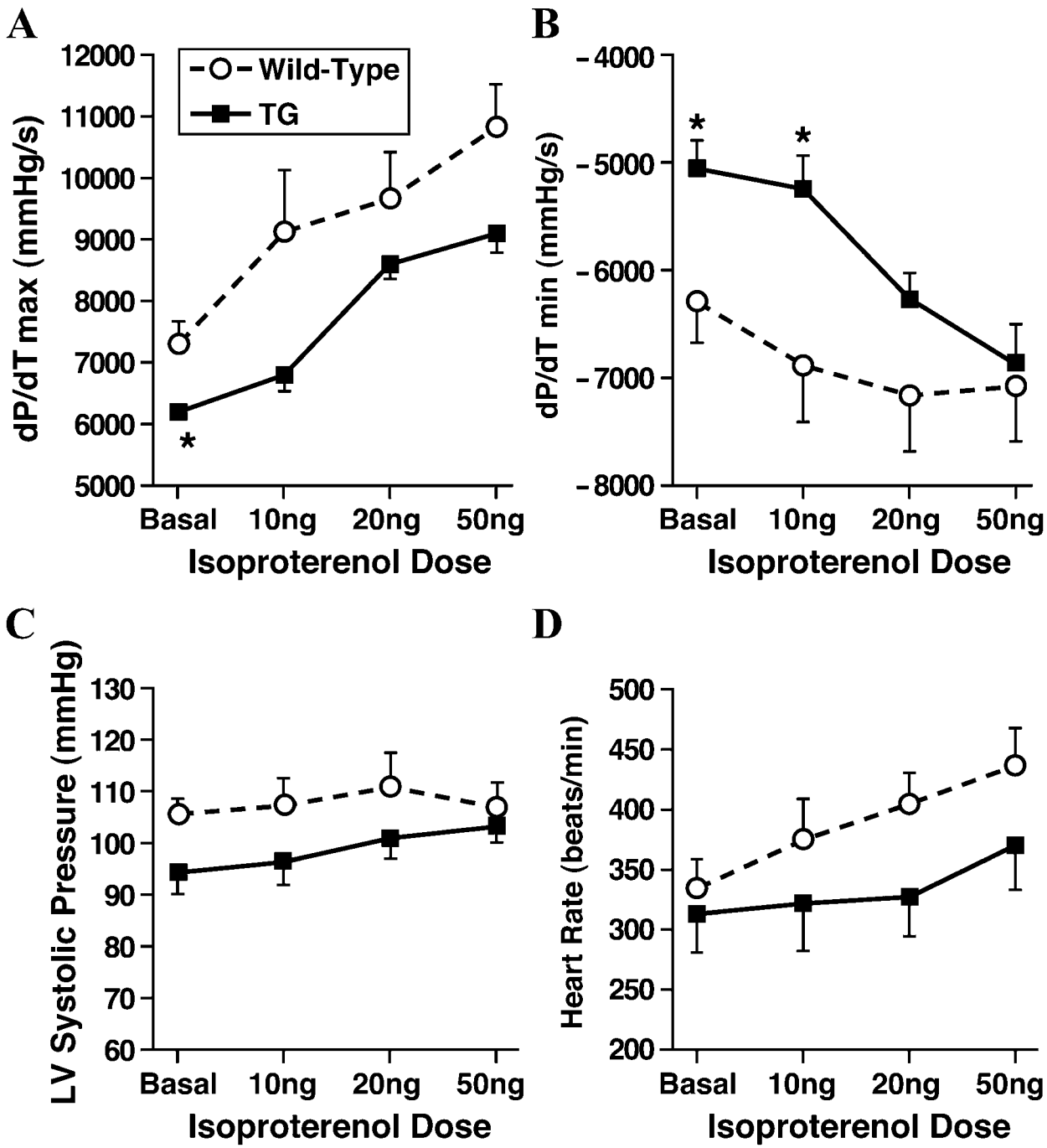

Figure 5. Basal myocardial contractility and relaxation are impaired in αMHC Tacβ_{1A} transgenic animals that show no morphological abnormalities. Cardiac catheterization was performed in anesthetized mice. Parameters were determined at baseline and following administration of the noted doses of isoproterenol. ($n = 6$ for each group) $^*P < 0.05$ of wild-type *versus* transgenic (TG). No significant differences were found in systolic pressure or heart rate between groups. **A:** Maximal first derivative of LV pressure, LV dP/dT max. **B:** Minimal first derivative of LV pressure, LV dP/dT min. **C:** Left ventricular systolic pressure. **D:** Heart rate.

tion, perhaps by titrating limiting amounts of cytoplasmic factors which bind to the β_1 integrin cytoplasmic tail and are required for normal β_1 integrin function[6,12,13,21–23]. Here we further demonstrated that high-level expression of Tac-β_{1A} in cardiac myocytes *in vitro* altered cell morphology and down-regulated adhesion to extracellular matrix. In contrast, expression of the control construct Tac-α_5 had no effect on myocyte morphology or adhesion, indicating that these alterations are due to the presence of free β_{1A} cytoplasmic domains rather than expression of the Tac component of the chimeric molecule. When the Tac-β_{1A} chimeric molecule is expressed, it must compete with the endogenous β_1 integrins that are present in the cells. Therefore, increased cellular expression of the Tac-β_{1A} chimera effectively titrates progressive alteration of endogenous integrin function. Relative

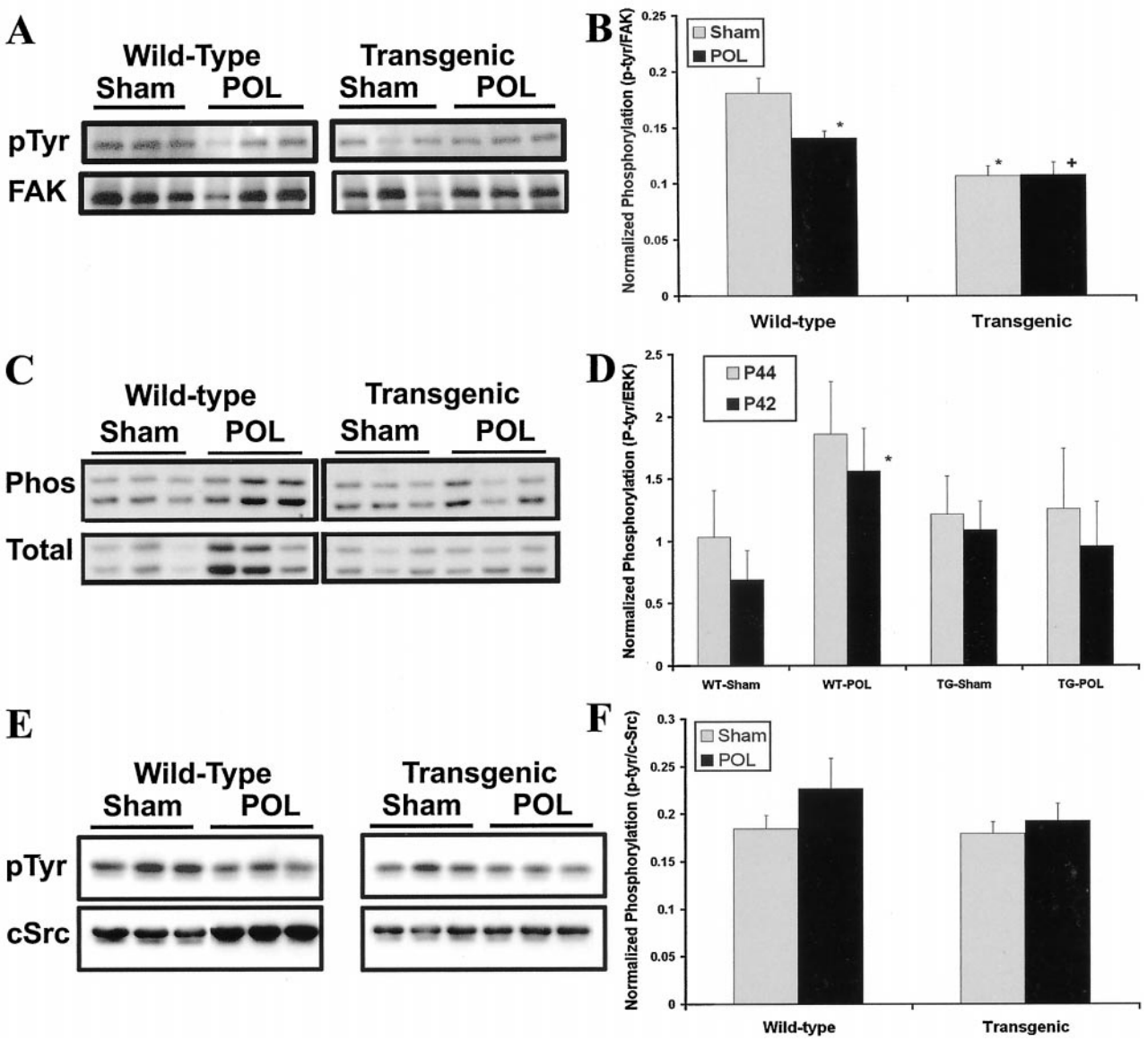

Figure 6. Phosphorylation of molecules implicated in integrin signaling are altered in morphologically normal αMHC Tacβ_{1A} transgenic animals as compared to controls. **A** and **B:** Phosphorylation of focal adhesion kinase (FAK) is reduced at baseline and is not altered by 7 days of POL induction via transverse aortic constriction in transgenic animals (TG) as compared to wild-type control animals. *, $P < 0.05$ *vs.* sham-operated wild-type animals; +, $P < 0.05$ *vs.* wild-type animals subjected to POL; pTyr, tyrosine phosphorylated FAK; FAK, total FAK protein. **C** and **D:** Phosphorylation of P42 ERK is significantly increased by 7 days after POL via transverse aortic constriction in wild-type animals but is not increased in the transgenic animals. *, $P < 0.05$ *vs.* sham-operated control; Phos, phosphorylated ERKs; Total, total ERK protein. **E** and **F:** Phosphorylation of c-Src is not significantly altered by transgene expression or pressure-overload. In all cases, immunoprecipitated phosphorylated species for each sample was normalized to the total protein level. **Insets** are representative Western blots. pTyr, tyrosine phosphorylated cSrc; cSrc, total cSrc protein.

to this, the phenotypes observed in Tac-β_{1A} transgenic mice correlated with the level of expression.

The highest relative expression of Tac-β_{1A} resulted in reduction of endogenous β_1 integrin expression with fibrotic replacement of the myocardium and perinatal mortality. Normal cell-matrix adhesion is required for appropriate cardiac development, as has been illustrated by the embryonic lethal phenotypes of several integrin and extracellular matrix knockout animals.[24–27] As overexpression of Tac-β_{1A} has been demonstrated to disrupt integrin receptor affinity for extracellular matrix, it is likely that the perinatal lethality in animals with the highest level of transgene expression was caused by abnormalities in myocyte adhesion to extracellular matrix. This work is consistent with the phenotype of transgenic mice that overexpress constitutively active ras-related c3 botulinum toxin substrate-1 (rac1) in the cardiac myocyte. These rac1 transgenic mice have been proposed to have alterations in cellular adhesion and in mechanotransductive properties of the heart.[28]

A second distinct phenotype was present in transgenic animals with substantial, but relatively less, transgene expression than those that died perinatally. Surviving lines with the highest level of transgene expression developed compensatory hypertrophy in the absence of any provoked hemodynamic stimulus. Other surviving

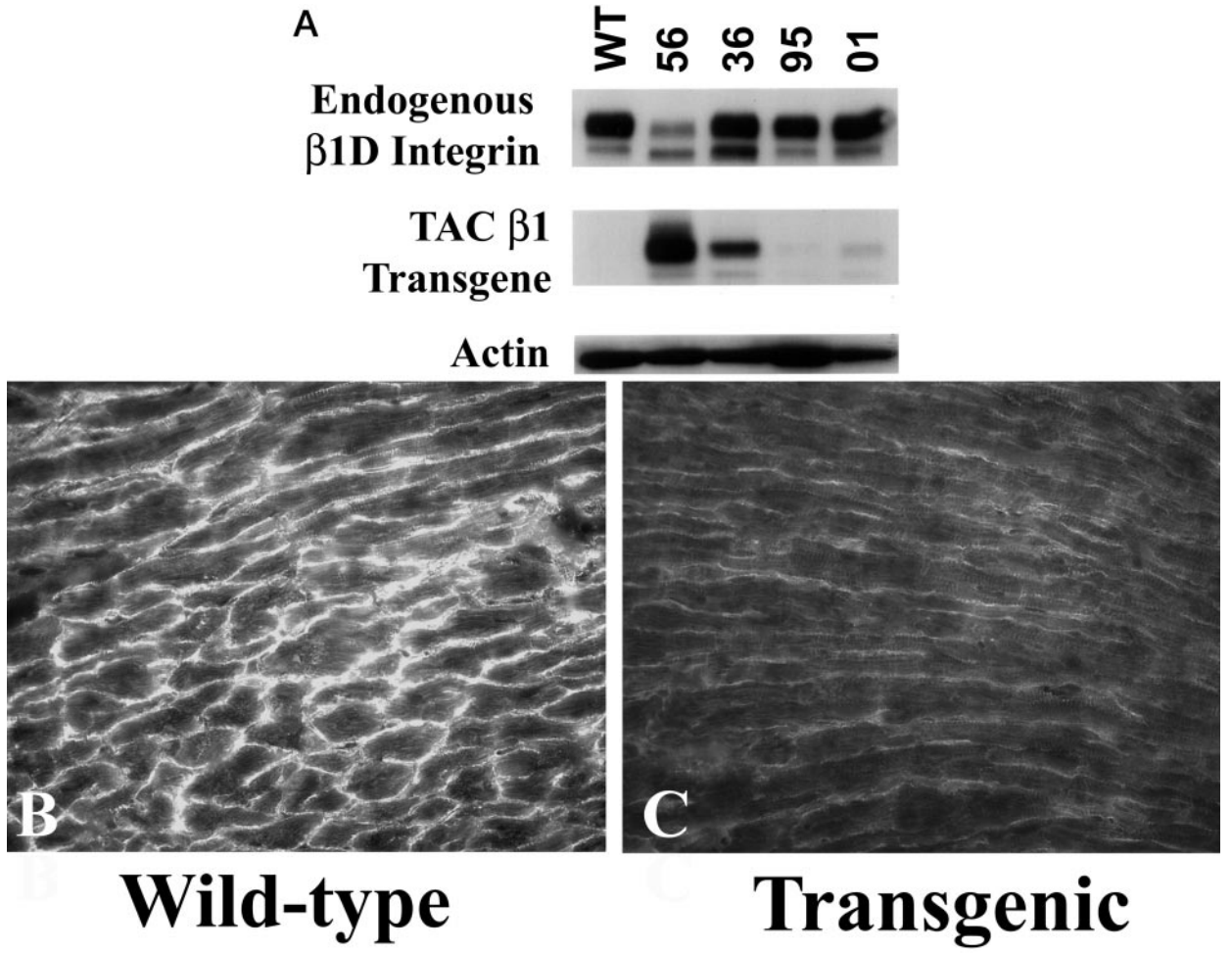

Figure 7. High-level transgenic protein expression inversely correlates with expression of endogenous β_{1D} integrin. **A:** Western blot analysis of myocardial protein performed to detect expression of endogenous β_{1D} protein, transgenic protein and actin (to control for gel loading conditions) reveals that the level of expression of the endogenous β1 was reduced in those animals with high level expression of the Tacβ1A transgene. **B** and **C:** Correlative results are detected by immunostaining of cardiac tissue from wild-type and transgenic hearts with an anti-β_{1D} integrin antibody in wild-type (**B**) and transgenic tissue (**C**).

lines with lesser relative chimera expression showed (i) no basal morphological abnormalities, (ii) depressed baseline positive and negative dP/dT, (iii) normal pCa-tension and Hill coefficient of fiber bundles isolated from hearts of transgenic animals, and (iv) disturbance in phosphorylation of several molecules implicated in integrin signaling.

Hypertrophy or remodeling of the myocardium to optimize the cardiac performance can result from mechanical stress on the heart from either pressure or volume loading, as well as cardiac myocyte death. Ventricular hypertrophy is an important adaptive mechanism that allows the heart to maintain its output. Recent data have shown modulation of integrin expression and extracellular matrix in the hypertrophied myocardium.[29–32] This is particularly noteworthy given the demonstration that integrins can function as mechanotransducers, translating mechanical signals to biochemical signals.[1,33] It is possible, therefore, that integrins may play a role in the translation of increased mechanical stress into the cardiac cellular response of hypertrophy.

We examined whether transgene expression would alter normal cardiac contractile function and prevent adequate compensatory hypertrophy from occurring in the transgenic animals. This concept was tested in the mice that had lower relative transgene expression. Abnormal contractility and relaxation were detected at rest, but no alterations in intrinsic function of the myofilaments were found. These functional abnormalities could be overcome by adrenergic stimulation of the transgenic myocardium. Additionally, after hemodynamic loading via aortic constriction, we found equal induction of cardiac hypertrophy in wild-type and transgenic hearts as measured by heart weight normalized to body weight or tibial length. This is in contrast to the transgenic animals with higher level transgene expression that developed a hypertrophic response without any provocation, indicative of a compensatory response for intrinsically impaired cardiac function. Therefore, it is likely that the animals tested for their hypertrophic response to aortic banding retained sufficient residual integrin function to effect relatively normal mechanotransduction.

In recent years, the role of the cardiac myocyte cytoskeleton in cardiac function has gained significant attention.[34,35] β_1 integrin is a critical component of the linkage between the extracellular matrix and cytoskele-

ton. In striated muscle, it is also localized at the junctional structures and may serve a unique role in orchestrating appropriate organization of the support structure of the contracting myocyte.[36] In this role, the cytoplasmic domain of β_1 has been shown to interact with several important structural components of the cytoskeleton such as vinculin and talin. Disturbance of vinculin function has been found to alter normal myofibrillar organization and, when fully ablated in the mouse, results in fetal death at embryonic day 9.5 to 10 with noncontracting hearts.[37,38] With the onset of heart failure, vinculin has been found to alter its localization away from intercalated disks into the cell body of myocytes.[39] Thus, it is possible that with disturbance of integrin function in our animals, this critical linkage was disrupted, leading to the unique phenotypes we saw both perinatally and in the adult animals. These results are in agreement with the data from chimeric animals composed of β_1 integrin null cells, where severe alterations in myofibril formation was noted in the few β_1 null cells found in the chimeric heart.[24] It is interesting to note that preliminary work by our own group using a cardiac-specific knockout approach to β_1 integrin ablation has shown similar results with replacement fibrosis and progression to heart failure in adult mice.

Determining the signaling pathways involved in cardiac hypertrophy is essential to the understanding of normal cardiac development and response of the heart to increased hemodynamic load. Ligand binding of integrins leads to their clustering. To propagate integrin signaling, focal adhesion complexes enriched in adapter and signaling molecules are subsequently assembled. Integrin signaling activates pathways and effector molecules known to be components of signaling from other cardiac receptors implicated in the hypertrophic response.[9,32,40] In addition, increased matrix deposition and altered integrin expression is a characteristic of *in vivo* models of hypertrophy.[29,30,41–43] Thus, we assayed for alterations in various molecules that are components of the integrin signaling cascades. Previous experiments that tested the effects of autonomously expressed β_1 integrin cytoplasmic domains showed that at modest expression levels, the chimera could interfere with phosphorylation of FAK.[13] At higher expression levels, the chimera could induce constitutive phosphorylation of FAK.[6,13] Consistent with these data are the results from our transgenic animals with moderate levels of transgene expression; FAK phosphorylation was depressed basally and was not changed after aortic constriction, as was detected in the wild-type animals. Normal FAK function has been suggested to be a cell survival factor.[44] Impaired induction of p42 ERK phosphorylation after hemodynamic loading was also observed in transgenic animals. These results suggest that expression of free β_{1A} integrin cytoplasmic domains disrupted several of the downstream events that are required for basal integrin signaling as well as events that may be crucial to modification of the myocyte stressed by a hemodynamic load, consistent with other investigators' work in the pressure-overloaded cat ventricle.[45]

In conclusion, this study has shown that cardiac myocyte-specific expression of free β_{1A} integrin cytoplasmic domains that alters normal integrin function leads to disturbed cardiac function, histological abnormalities, modification of molecules instrumental in integrin signaling, and perinatal death in a manner that correlates with expression level of the transgene.

This work is in agreement with the concept proposed by others that cytoskeletal disturbances of the cardiac myocyte may ultimately lead to a cardiomyopathic phenotype.[35] Further work is underway to alter β_1 integrin function conditionally in the intact myocardium to obtain further insights into the role of integrin-mediated adhesion and signaling in cardiac function.

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