Original Research

Y-box binding protein-1 implicated in translational control of fetal myocardial gene expression after cardiac transplant

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Abstract

Peri-transplant surgical trauma and ischemia/reperfusion injury in accepted murine heterotopic heart grafts has been associated with myofibroblast differentiation, cardiac fibrosis and biomechanical-stress activation of the fetal myocardial smooth muscle α-actin (SMαA) gene. The wound-healing agonists, transforming growth factor β1 and thrombin, are known to coordinate SMαA mRNA transcription and translation in activated myofibroblasts by altering the subcellular localization and mRNA-binding affinity of the Y-box binding protein-1 (YB-1) cold-shock domain (CSD) protein that governs a variety of cellular responses to metabolic stress. YB-1 accumulated in polyribosome-enriched regions of the sarcoplasm proximal to cardiac intercalated discs in accepted heart grafts. YB-1 binding to a purine-rich motif in exon 3 of SMαA mRNA that regulates translational efficiency increased substantially in perfusion-isolated, rod-shaped adult rat cardiomyocytes during phenotypic de-differentiation in the presence of serum-derived growth factors. Cardiomyocyte de-differentiation was accompanied by the loss of a 60 kDa YB-1 variant that was highly expressed in both adult myocardium and freshly isolated myocytes and replacement with the 50 kDa form of YB-1 (p50) typically expressed in myofibroblasts that demonstrated sequence-specific interaction with SMαA mRNA. Accumulation of p50 YB-1 in reprogrammed, de-differentiated myocytes was associated with a 10-fold increase in SMαA protein expression. Endomyocardial biopsies collected from patients up to 14 years after heart transplant showed variable yet coordinately elevated expression of SMαA and p50 YB-1 protein and demonstrable p50 YB-1:SMαA mRNA interaction. The p60 YB-1 variant in human heart graft samples, but neither mouse p60 nor mouse or human p50, reacted with an antibody specific for the phosphoserine 102 modification in the YB-1 CSD. Modulation of YB-1 subcellular compartmentalization and mRNA-binding activity may be linked with reprogramming of contractile protein gene expression in ventricular cardiomyocytes that could contribute to maladaptive remodeling in accepted, long-term heart grafts.

Keywords: chronic rejection, smooth muscle α-actin, cardiac remodeling, fibrosis, mRNA translation, ischemia/reperfusion injury, cardiac intercalated disc

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Introduction

Cardiac allograft dysfunction is a poorly understood maladaptive response to transplant that ultimately impairs perfusion, performance and long-term survival of accepted cardiac allografts.[1,2] Retransplantation surgery to replace a dysfunctional heart graft often is the only recourse but impractical in terms of donor organ availability. Often described as 'chronic rejection', allograft dysfunction typically arises within 3–5 years after transplant in allografts that otherwise seem well tolerated by the immunosuppressed host.[3] Allograft dysfunction is a graft-remodeling syndrome that includes interstitial and perivascular fibrosis, accelerated coronary vasculopathy, and altered expression of muscle protein genes by coronary arterial smooth muscle cells and ventricular cardiomyocytes.[4,5] Importantly, remodeling is observed in accepted grafts despite the lack of sustainable leukocyte infiltration. Evaluation of accepted grafts in published accounts of small-animal allotransplant models have indicated a similar temporal disconnect between

immune cell infiltration and remodeling in several types of solid organ grafts.[6,7] A recent genome-wide survey of mRNA transcripts associated with future failure in accepted human renal allografts revealed that marker genes statistically linked to graft failure were related to wound healing-associated events including epithelial-to-mesenchymal transition, extracellular matrix remodeling and transforming growth factor β1 (TGF-β1) signaling.[8]

In a murine heterotopic cardiac transplant model, interstitial and perivascular cardiac fibrosis was a significant component of the allograft dysfunction that occurred early after transplant with subsequent detrimental effects on both coronary artery and myocardial structure and function.[9–11] Cardiac fibroblasts were highly susceptible to conversion to myofibroblasts in accepted grafts as indicated by robust *de novo* expression of the gene encoding the contractile protein isoform, smooth muscle α-actin (SMαA).[2,12] This actin isoform is a well-known indicator of myofibroblast differentiation and myocardial wound healing and one of the several fetal contractile protein isoforms that are re-expressed in adult cardiomyocytes in response to mechanical-stress injury.[13–16] SMαA-positive cardiac myofibroblasts (cMFB) could be detected in accepted murine allografts as soon as 3 days after transplant, with peak accumulation typically observed within 2–4 weeks.[1,2] Activation of SMαA gene expression during myofibroblast differentiation precedes up-regulation of type I collagen gene transcription in these cells by about 1–2 days, demonstrating the potential utility of SMαA as an early prognostic marker for cardiac fibrosis after transplant.[17] Ultimately, deposition of non-elastic scar tissue by activated myofibroblasts diminishes myocyte contractile function and alters the structural organization of the myocardium that could lead to premature graft failure.[18]

Recent findings in our laboratory implicate the YB-1 cold-shock domain (CSD) protein as a potentially important component in the molecular pathobiology of cardiac allograft dysfunction.[1,2,19] YB-1/DNA-binding protein B (dbpb)/mouse Y-box binding protein-1 (MSY-1) are alternate names for a highly conserved, single-strand-specific, nucleic acid-binding protein that regulates expression of multiple genes associated with metabolic stress and tissue repair, including those encoding SMαA, the α1 and α2 type I collagen subunits, the B-chain of platelet-derived growth factor, the epidermal growth factor receptor, matrix metalloproteinase-2, vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor, p21 and p53, cyclins A and B1 and the Fas death receptor.[20–25] In addition to its role as a nuclear transcription factor, YB-1 also accumulates in the cytosol to perform post-transcriptional regulatory tasks related to its ability to bind stem–loop structures in mRNA.[26,27] YB-1 has been implicated in controlling protein synthesis after tissue injury by forming complexes with other cytosolic proteins such as polypyrimidine tract binding protein-associated splicing factor (PSF), nuclear RNA-binding protein p54 (P54nrb), G-rich RNA sequence binding factor 1 (GRSF1) and polypyrimidine tract binding protein 1 (PTB-1)[27,28] that initiate translation from internal ribosome entry segments (IRES) as opposed to 5′-end cap structures in mRNA molecules that mediate ribosome scanning for AUG codons. One current idea is that the shift from cap-dependent to cap-independent programs of protein synthesis might be governed by hypoxic stress that specifically enhances translation of mRNAs containing IRES stem–loop structures that encode hypoxia-inducing factor alpha (HIFα), VEGF and Bcl2 required for cell growth and survival under low-oxygen conditions.[6,27,29,30] Whether cardiomyocyte response to peri-transplant ischemia/reperfusion (I/R) injury is similarly accompanied by a compensatory shift from cap-dependent to IRES-dependent mRNA translation is not known. Of additional relevance to cardiac allograft structure and function is the reported ability of YB-1 to sequester and protect mRNA molecules needed for homeostasis and cell survival during temporary periods of metabolic stress. YB-1 was recently identified as a constituent of stress granules where it may aid in the transport of mRNA along microtubules and microfilaments, as well as stabilize and store mRNA until metabolic conditions become more favorable for resumption of normal cell function.[31,32]

In this report, we show that heterotopic cardiac transplant in the mouse resulted in the accumulation of a 50-kDa YB-1 polypeptide (p50) within the polyribosome-enriched cytosol proximal to cardiac intercalated discs. We previously showed that retransplant of cardiac isografts into a second cohort of syngeneic hosts amplified I/R graft injury, leading to cardiac myofibroblast differentiation and extensive interstitial and peri-vascular fibrosis.[1] Using this 'two-hit' I/R injury model, we observed that accumulation of fetal SMαA protein in cardiac sarcomeres was associated with YB-1 deposition at cardiac intercalated discs. Elevated binding of YB-1 to SMαA mRNA in cardiomyocytes that were reprogrammed to reactive fetal gene expression by cultivation in serum-containing medium was associated with a nearly 10-fold increase in SMαA protein levels. The expression of YB-1 and fetal SMαA protein was coordinately elevated in endomyocardial biopsies collected from patients at various times after heart transplant with demonstrable binding of p50 YB-1 in these samples to SMαA mRNA. The results provide new insight regarding the molecular pathobiology of cardiac allograft dysfunction and suggest that changes in YB-1 subcellular compartmentalization and mRNA-binding activity may represent useful indicators for cardiomyocyte stress and dysfunctional remodeling after cardiac transplant.

Materials and methods

Murine heterotopic heart transplantation

Five-week-old, specific pathogen-free female DBA/2 (H-2d), FVB/N (H-2k) and C57BL/6 (H-2b) mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN, USA) or the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a barrier facility for use as heart donors and graft recipients. An Institutional Animal Care and Use Committee-approved animal protocol was used in this study in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Research Council (revised 1996).

Donor hearts (either DBA/2 or FVB/N strains) were heterotopically transplanted into the abdomen of gallium nitrate-immunosuppressed C57BL/6 recipient mice using an adaptation of the method of Corry *et al.*[2,9–11,33] The protocol for performing syngeneic cardiac retransplant (two-hit I/R model) has also been previously described.[1] Briefly, three groups of three FVB/N-to-FVB/N heart grafts were explanted 15 days after first transplantation, retransplanted into another cohort of FVB/N hosts, and then collected after 3, 7 or 11 days for immunohistochemical and biochemical analyses. The thoracic cavity of donor mice was exposed and 1.5 mL of heparin was injected into the inferior vena cava (IVC) proximal to the heart to arrest beating. The IVC and superior vena cava were ligated, the aorta and pulmonary artery transected, and the pulmonary veins ligated *en bloc*. Explanted donor hearts were placed in cold Ringer's lactate solution while the recipient was prepared by exposing and clamping the abdominal aorta and IVC. The infrarenal abdominal aorta and IVC were incised and end-to-end anastomoses were performed with the donor aorta and pulmonary artery respectively. Blood flow was re-established and contraction resumed in the grafted heart. The total ischemic time required from harvest to anastomosis was 20–25 min and the heart was covered with gauze soaked in cold Ringer's solution while the sutures were placed. Oxygenated blood entered the graft aorta as back flow to reach the coronary arteries and supply the myocardium. Following the surgical procedure, the graft was palpated through the abdomen on a daily basis and given a subjective grade corresponding to the strength of cardiac impulse. Controls were non-transplanted donor hearts from age- and sex-matched FVB/N mice or isografts subjected to only a single round of transplant surgery into FVB/N recipients and harvested at either 15 or 26 days after transplant. Explanted heart grafts were visualized under a dissection microscope and trimmed to remove the atria and coronary vessels. Trimmed ventricles were frozen in liquid nitrogen, ground to powder while submerged under liquid nitrogen using a mortar and pestle, and extracted for either non-denaturing electrophoretic mobility shift assays (EMSA) or denaturing sodium dodecyl sulphate polyacrylamide gel electroporesis (SDS-PAGE) as described below.

Immunohistochemical methods

The anti-YB-1 rabbit polyclonal antibodies (anti-YB1 M85–110 and anti-YB1 M276–302) were developed in the laboratory of Robert J Kelm, Jr, as described previously.[34–36] Commercial polyclonal antibodies specific for amino-terminal (Y-0396, amino acid residues 137–150) and carboxyl-terminal (Y-0271, amino acid residues 307–324) segments of YB-1 were obtained from Sigma-Aldrich, St Louis, MO, USA. Immunohistochemistry was performed on 4-μ thick, paraffin-embedded sections from 4% paraformaldehyde-fixed isografts or non-transplanted donor hearts.[12] Sections were de-paraffinized, rehydrated, blocked with 2% goat serum, 0.1% bovine serum albumin and 0.05% Tween 20™ in PBS (phosphate-buffered saline; blocking solution) and incubated for 90 min at 37°C with the primary antibodies at a concentration of 2 μg/mL in blocking solution. After washing, sections were incubated for 30 min with a horseradish peroxidase (HRP)-conjugated, goat antirabbit secondary antibody and a Vectastain™ protocol was followed for color development using a 3,3′-diaminobenzidine (DAB) peroxidase kit (Vector Laboratories, Burlingame, CA, USA). To detect SMαA, HRP-conjugated antihuman SMαA mouse monoclonal (clone 1A4; Dako Cytomation California, Carpiteria, CA, USA) was diluted 1:100 prior to use. Dual localization of two antigens was accomplished by incubating tissue sections with a second antibody after completing the detection protocol for the first antibody and washing the sections in Tris-buffered saline (TBS), pH 7.4. To distinguish between the two antibody reaction products, a nickel solution was added to the second color development reagent which produced a blue-gray precipitate that contrasted with the red-brown reaction product obtained using the first developing solution without nickel ions. Sections were viewed on a Zeiss Axioscope™ 40 microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) using 10×, 20× and 40× brightfield objectives and color images digitally recorded using Zeiss MRGrab™ 1.0 software.

Isolation of cardiomyocytes and cardiac fibroblasts from adult rat, adult mouse and neonatal mouse hearts

Highly enriched preparations (>95% pure) of rat ventricular cardiomyocytes were isolated from adult female rats (Sprague-Dawley-Ivanovas, 300–350 g) by retrograde perfusion of hearts with collagenase type 2 (281 units/mL; Worthington Biochemical Corporation, Freehold, NJ, USA) according to the method reported by Eppenberger-Eberhardt *et al.*[16] as described previously.[1] Cardiomyocytes were maintained at 37°C in a humidified atmosphere of 95% air (21% molecular oxygen) and 5% carbon dioxide on 1% laminin-coated, 100 mm tissue culture dishes (Upstate, Lake Placid, NY, USA) using M199 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 10 μmol/L cytosine arabinoside. Medium was changed every third day over the course of a 15-day observation period. For isolation of mouse heart cells, trimmed ventricles were removed under sterile conditions from multiple 6-day-old neonatal mice or single 8-to-10-week-old adult DBA/2 mice and processed as described previously to obtain culture preparations enriched for either cardiomyocytes or cardiac fibroblasts.[2] Washed cells were collected by centrifugation and stored at −80°C for SDS-PAGE and immunoblot analysis. Embryonic AKR-2B mouse fibroblasts were maintained in McCoys 5A medium supplemented with 5% FBS and penicillin-streptomycin-Fungizone™ as described previously[37,38] and induced to differentiate into SMαA-positive myofibroblasts by treatment with recombinant human TGF-β1 (2–5 ng/mL; R&D Systems, Minneapolis, MN, USA). Cardiac fibroblasts were isolated as described previously[39] and maintained in complete DMEM supplemented with 10% FBS and antibiotics using a tissue culture incubator at 37°C containing a humidified atmosphere of 5% carbon dioxide/95% air (21% molecular oxygen). When cultivated on plastic substrates in the presence of serum at normal levels of molecular oxygen (21%), rat or mouse cardiac fibroblasts spontaneously differentiate into SMαA-positive myofibroblasts as described in our

previous report.[39] For some fibroblast preparations, TGF-β1 was added at 2–5 ng/mL to augment myofibroblast differentiation efficiency and SMαA expression.

Collection of human endomyocardial biopsy specimens

Right ventricular septal endomyocardial biopsy retrieval from participating patients providing informed written consent was performed in accordance with Institutional Review Board-approved protocols under aseptic conditions in the cardiac catheterization laboratory in the Ross Heart Hospital, The Ohio State University Medical Center, OH, USA. For patients enrolled in this study, post-transplant biopsy retrieval usually is performed every 3 months from years 1 to 2, every 6 months from years 2 to 5, and annually thereafter. The right internal jugular vein is the site of entry although, if occluded, the left internal jugular or femoral veins are alternate routes. After local instillation of 1% lidocaine, the vein was entered with an 18 gauge Cournand needle, the guidewire positioned under fluoroscopy, and an 8 French (Fr) introducer placed for passage of the 7 Fr bioptome instrument. Samples were immediately placed in liquid nitrogen and pulverized to powder form under liquid nitrogen. Some of the biopsies used in this analysis were collected from long-term transplant patients who returned to the clinic for their annual evaluation of graft function. For all these patients, at least one year elapsed since their last biopsy. Therefore, the data are not likely to reflect processes related to the formation of granulation tissue at earlier sites of biopsy retrieval. Only tissue samples showing viable myocardial tissue were retained for our analysis and samples with visible scar tissue were not included in our study. A second cohort of patients contributed four-sample sets of serial biopsies collected over a 0.7-to-210-week post-transplant interval for immunoblot evaluation of YB-1 expression and CSD phosphorylation as described below.

Electrophoretic mobility shift assays

Mouse cMFB or pulverized ventricles from pooled 6–13-day-old neonatal DBA/2 mice, adult donor DBA/2 mice and DBA/2 isografts and allografts harvested 30 days after transplant were used to prepare protein extracts for use in EMSA with a ^{32}P-labeled, single-strand DNA oligonucleotide probe (D_R) containing the YB-1-binding site derived from the MCAT motif in the SMαA promoter, as described previously.[40,41] DNA sequence-specific binding of YB-1 variants was demonstrated for samples from neonatal mouse hearts using a second but similar-size, single-strand DNA oligonucleotide probe called D_F that does not bind YB-1 protein (see below for EMSA oligonucleotide probe sequence information). EMSA reaction mixtures were incubated for 30 min at ambient temperature before electrophoresis on 5% non-denaturing polyacrylamide gels. Autoradiograms prepared from dried EMSA gels were quantified using laser densitometry (ImageQuant software; GE Healthcare Biosciences, Piscataway, NJ, USA).

Preparation of native protein extracts for nucleic acid-binding assays

Cultured adult rat cardiomyocytes were washed twice with Dulbecco's PBS, scraped into fresh PBS, sedimented at 3000 rpm, washed once more in PBS, and extracted directly in eight packed-cell volumes of ice-cold, low-salt buffer containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 20 mmol/L KCl, 0.2 mmol/L ethylenediaminetetraacetic acid (EDTA), protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA), 0.2 mmol/L phenylmethyl sulfonylfluoride (PMSF) and 0.5 mmol/L dithiothreitol (DTT) using a Dounce homogenizer with a low-clearance pestle. An expanded extraction protocol was required for processing of mouse and human heart tissue samples to maximize the yield of total cellular protein. Human endomyocardial biopsy tissue powder or pulverized, biopsy-sized fragments of normal non-transplanted mouse left ventricular tissue were suspended in eight volumes (80 uL) of ice-cold, low-salt, hypotonic cardiomyocyte extraction buffer (20 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, protease inhibitor cocktail, 0.2 mmol/L PMSF and 0.5 mmol/L DTT), allowed to swell for 20 min on ice, and then processed with 100 strokes delivered using a small Dounce homogenizer with a low-clearance pestle. One and a half volumes of high salt buffer (20 mmol/L HEPES, pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 1.2 mol/L KCl, 0.2 mmol/L EDTA, protease inhibitor cocktail, 0.2 mmol/L PMSF and 0.5 mmol/L DTT) was added followed by gentle rocking for 30 min at 4°C. Extracts were clarified by centrifugation at 49,000 rpm for 1 h at 4°C and the supernatants transferred to a ultrafiltration device (Microcon Ultracel YM-10; Millipore Corporation, Billerica, MA, USA) and centrifuged at 14,000 rpm for 1 h at 4°C to exchange the extraction buffer with 200 uL of low salt buffer containing 20 mmol/L HEPES, pH 7.9, 25% glycerol, 1.5 mmol/L $MgCl_2$, 20 mmol/L KCl, 0.2 mmol/L EDTA, protease inhibitor cocktail, 0.2 mmol/L PMSF and 0.5 mmol/L DTT. Extracts were assayed for protein concentration using a reducing agent-compatible version of the BCA (bicinchoninic acid) colorimetric method, aliquoted, and stored at −80°C for use in DNA- or mRNA-binding assays. Each human biopsy specimen or mouse heart tissue fragment (used for control purposes) had a wet weight of slightly <10 mg and the protein concentration of the final extract usually was about 4–5 μg/μL. Several synthetic biotinylated oligonucleotide probes were used in the nucleic acid-binding study: (1) a pyrimidine-rich, 32-base segment of single-strand DNA encompassing the reverse strand of the 5′-flanking region of the mouse SMαA gene located between −195 and −164 (referred to as D_R: 5′-ccctcgtcttgtctccttacgtcaccttctct-3′) and previously shown to bind YB-1 with high affinity;[35,40,41] (2) the purine-rich forward-strand complement to D_R called D_F that does not bind YB-1 and serves as a negative control for DNA-binding specificity (D_F: 5′-agagaaggtgcgtaaggagacaagacgaggg-3′; (3) the mRNA coding element located in exon 3 of SMαA mRNA (CE-RNA: 5′-gggaguaaugguuggaaugggccaaaaaga-3′) previously shown to bind YB-1 and Pur proteins[36]; and (4) its mutant counterpart

(CE-RNAmut: 5′-uugaguaaugguuuuccguggccaaccaga-3′). Underlined sequences denote regions changed by mutation. Reaction mixtures containing protein extracts (100 μg of protein) and biotinylated oligonucleotides (100 pmol; Integrated DNA Technologies, Coralville, IA,USA) were incubated in a buffer containing poly (dI–dC), 10 mmol/L Tris, pH 7.5, 50 mmol/L NaCl, 0.5 mmol/L DTT, 0.5 mmol/L EDTA, 0.2 mmol/L PMSF and 4% glycerol. Protein–biotinylated DNA complexes were captured on streptavidin-immobilized paramagnetic particles (Promega, Madison, WI, USA; 0.6 mL/reaction, 30 min incubation) as described previously.[37,38] After washing four times with buffer containing 25 mmol/L Tris-HCl, pH 7.5, 1 mmol/L EDTA and 100 mmol/L NaCl, bound protein was eluted using 2X protein denaturing buffer containing β-mercaptoethanol and analyzed by SDS-PAGE and immunoblotting procedures.

Reducing SDS-PAGE and immunoblotting methods

Isolated, washed and sedimented cardiomyocytes, cardiac fibroblasts and AKR-2B myofibroblasts, as well as frozen tissue powders derived from pulverized (1) ventricles from donor mouse hearts, isografts, two-hit isografts and allografts; (2) ventricles from 4-, 6-, 9- and 13-day-old neonatal mice and adult mice; or (3) human endomyocardial biopsies were denatured by boiling in SDS-PAGE sample buffer for 5 min.[2] Mouse and human heart tissue samples were additionally subjected to brief, alternating cycles of sonication and boiling in SDS sample buffer to completely disrupt the material prior to electrophoresis. Aliquots containing 5–20 μg of protein were size-fractionated by SDS-PAGE on 10% polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schull, Keene, NH, USA) for 90 min at 300 mA in 25 mmol/L Tris-HCl, 192 mmol/L glycine and 20% (v/v) methanol. After overnight blocking at 4°C, blots were incubated with one of the several anti-YB-1 antibodies as noted above at 1:1000 overnight at 4°C, washed in TBS containing Tween 20™ (0.05% v/v) (TBST) and incubated with peroxidase-conjugated goat or donkey antirabbit secondary antibody (1:2500) for 60 min. Some immunoblots prepared from human heart biopsies were also reacted with an antibody specific for phosphorylated serine 102 present in an YB-1 CSD peptide antigen (C34A2; 1:1000; Cell Signaling, Danvers, MA, USA). Bound antibodies were visualized by chemiluminescence (ECL™; Amersham Biosciences, Arlington Heights, IL, USA) and imaged onto Biomax™ film (Eastman Kodak, Rochester, NY, USA). For detection of SMαA, membranes were incubated with HRP-conjugated 1A4 (1:100) in TBST. Following a 60–90 min incubation at ambient temperature with gentle mixing, the membrane was washed 2–3 times in TBST and developed using reagents provided in a DAB-peroxidase Vectastain™ kit (Vector Laboratories). Quantitation of immunoblot band density was performed using laser densitometry (ImageQuant software; GE Healthcare Biosciences) and normalized to values for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein as detected by anti-GAPDH antibody. The Pearson correlation coefficient for two normally distributed variables was calculated from optical density determinations for immunoblots depicting total p50 YB-1 and SMαA protein levels in human endomyocardial biopsy extracts. Analysis of variance (ANOVA) was used to evaluate differences between the mean densitometric values for protein expression on immunoblots with statistical significance set to $P \leq 0.05$.

Results

In an earlier study, EMSA of DNA:protein complexes from adult mouse cardiomyocytes using non-denaturing polyacrylamide gels revealed expression of a high-mobility form of YB-1.[2] The observed fast migration of native YB-1 expressed in adult cardiomyocytes that do not normally express SMαA protein was consistent with a small molecular size but distinctly different from a more slowly migrating form of native YB-1 expressed in SMαA-positive neonatal cardiomyocytes and cMFB.[2] Comparative EMSA revealed that a similar relationship exists between the electrophoretic mobility of native YB-1 and level of SMαA expression in the context of cardiac transplant. A slow-migrating form of native YB-1 was more abundant on EMSA gels prepared from chronically rejected, SMαA-enriched allografts with proportionally less expression of a faster-migrating variant that was the predominant form of YB-1 in native donor hearts (Figure 1, upper panel). Based on densitometric evaluation of each gel-shift band, fast-migrating YB-1 in donor hearts (fYB-1) was less abundant and/or had reduced affinity for the DNA probe compared with the slow-mobility form (sYB-1) present in allografts. These observations suggested that chronic-rejection pathobiology was associated with changes in the apparent size as well as availability and/or nucleic acid-binding capability of the cardiac YB-1 protein.

Size modulation of cardiac YB-1 was also apparent when denatured proteins were evaluated on SDS-polyacrylamide gels (Figure 1, middle and lower panels). Non-transplanted adult DBA/2 mouse hearts expressed two YB-1 size variants but were predominantly enriched for a 60-kDa YB-1 variant (p60), whereas DBA/2 to C57BL/6 allografts exhibiting typical chronic rejection histopathology and transplant-associated expression of fetal SMαA[2,12] were enriched for a YB-1 polypeptide that migrated in the vicinity of the 50-kDa size marker. The migration of this protein species in mouse and human samples typically was equal to or slightly faster than the 50-kDa size marker on SDS gels. For consistency with earlier literature reports that also describe the variable electrophoretic behavior of SDS-denatured YB-1, we will refer to this form of YB-1 in our study as ‘p50’ based on its co-migration with authentic SDS-denatured recombinant mouse YB-1. The level of p60 was approximately equal to p50 in syngeneic DBA/2 to DBA/2 heart grafts (isografts), suggesting that the apparent shift in YB-1 polypeptide size after cardiac transplant was not directly linked to an adaptive alloimmune response by the host. Rather, the apparent size shift of YB-1 seemed to be initiated by an aspect of transplantation protocol that was common to both isografts and allografts, such as peri-transplant surgical trauma and/or associated I/R injury. Notably, interstitial and perivascular fibrosis and coronary vasculopathy that was observed only in allografts seemed necessary to significantly augment p50 YB-1 expression

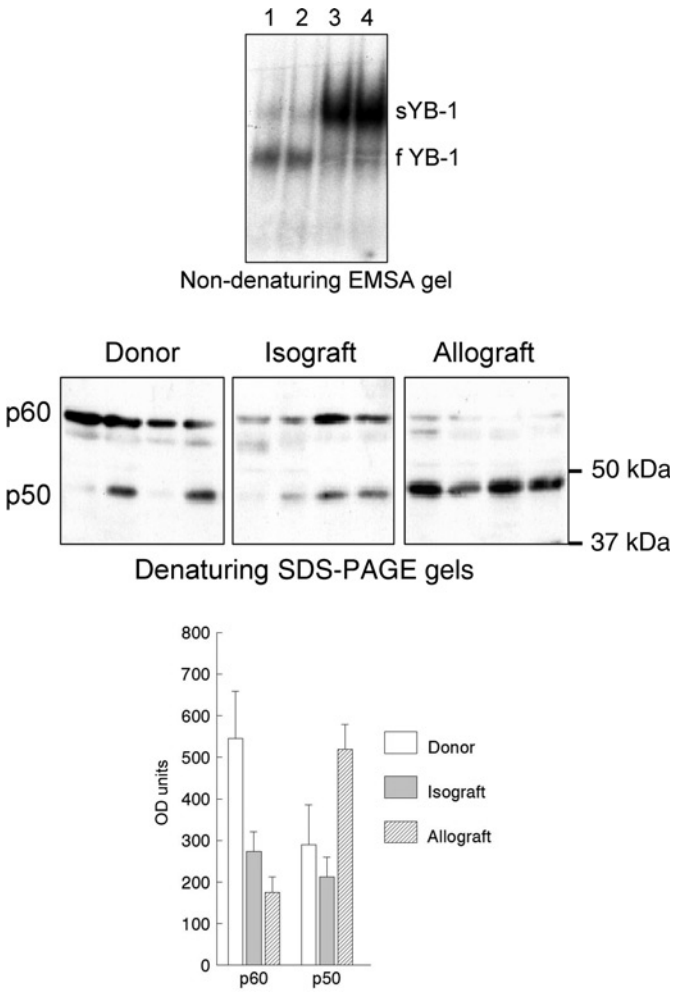

Figure 1 Y-box binding protein-1 (YB-1) molecular size heterogeneity was observed in protein extracts prepared from mouse cardiac allografts using both non-denaturing and denaturing buffer conditions. The upper panel depicts an electrophoretic mobility shift assay (EMSA) autoradiograph showing binding of the radiolabeled, single-stranded DNA probe to non-denatured extracts prepared from donor hearts (lanes 1 and 2) or allografts harvested 60 days after transplant (lanes 3 and 4). A fast mobility form of YB-1 (fYB-1) was noted in donor hearts whereas a slower migrating form was observed in allografts (sYB-1). The middle panel depicts YB-1 immunoblots (anti-YB1 polyclonal antibody M85-110) prepared following sodium dodecyl sulphate (SDS)-denaturing polyacrylamide gel electrophoresis (PAGE) showing that the p60 form of YB-1 was the major species present in donor hearts with replacement by p50 evident after allotransplant (allograft). Transplanted syngeneic hearts (isograft) contained mostly p60 with smaller amounts of p50 detected in some grafts. Four different hearts were evaluated for each category. Molecular size markers are noted on the right. Quantitation of YB-1 size variants observed after SDS-PAGE and immunoblot analysis of donor hearts, isografts and allografts are shown in the lower panel. The *y*-axis indicates densitometry unit values

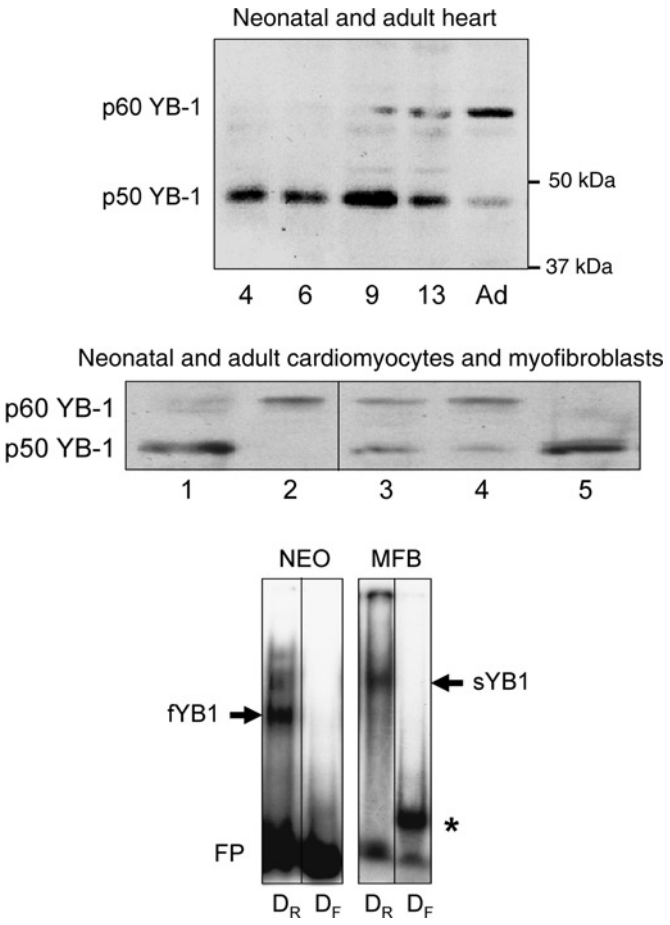

Figure 2 Developmental regulation of Y-box binding protein-1 (YB-1) molecular size was noted in mouse cardiac tissue and isolated cells. The upper panel depicts an immunoblot (anti-YB1 polyclonal antibody M85-110) prepared after sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showing that accumulation of p60 in the immature mouse heart commenced about 9 days after birth and was the major form of YB-1 in the adult heart (Ad). The numbers refer to days after birth. Molecular weight markers noted on the right. The middle panel shows that p60 is the dominant YB-1 variant in isolated adult mouse cardiomyocytes (lane 2), although co-expression of p60 and p50 was observed in two independent preparations of neonatal cardiomyocytes (lanes 3 and 4). Transforming growth factor β1 (TGF-β1)-activated AKR-2B mouse embryonic myofibroblasts (lane 1) and mouse cardiac myofibroblasts (cMFB) cultivated in 21% molecular oxygen (lane 5) are both known to express abundant smooth muscle α-actin (SMαA) protein (not shown) and appear highly enriched for p50 YB-1. The lower panel depicts an electrophoretic mobility shift assay (EMSA) autoradiograph illustrating that non-denatured fYB-1 and sYB-1 are enriched in protein extracts from neonatal mouse heart (NEO) and cMFB, respectively, and that both bind sequence specifically to a DNA probe (D_R) harboring an authentic YB-1-binding site from the SMαA promoter but not the complementary strand (D_F) that instead binds Pur proteins (*) which assist YB-1 in regulating SMαA gene represssion

after cardiac transplant. YB-1 size heterogeneity on denaturing and non-denaturing gels was not mouse-strain-specific because it also was observed in donor FVB/N heart grafts transplanted into recipient C57BL/6 mice.

SDS-PAGE analysis indicated that postnatal development of the mouse ventricle was associated with replacement of p50 YB-1 with the p60 variant (Figure 2). Although p50 was the predominant form of YB-1 detected in whole ventricle extracts prepared from 4- to 6-day-old neonatal mice, by 13 days after birth, the proportion of p60 YB-1 increased substantially and was the major YB-1 size variant detected in the adult mouse ventricle (Figure 2, upper panel). To confirm that the YB-1 size heterogeneity observed in the developing ventricle was specifically associated with cardiomyocytes rather than cardiac fibroblasts, we isolated each cell type from neonatal and adult hearts for SDS-PAGE analysis. As shown in the middle panel of Figure 2, neonatal cardiomyocytes previously shown to express SMαA protein[37] expressed p60 YB-1 as well as a p50 variant that co-migrated with the sole form of YB-1

expressed in SMαA-positive cMFB and TGF-β1-activated mouse embryo myofibroblasts.[38] In contrast, SMαA-negative cardiomyocytes freshly isolated from adult mouse hearts were highly enriched for the p60 YB-1 variant. EMSA data presented in Figure 2 (lower panel) indicated that fYB-1 and sYB-1, as identified in non-denatured protein extracts from mouse heart grafts (Figure 1), were also present in extracts from neonatal mouse heart and cMFB, respectively. Both forms of non-denatured YB-1 bound sequence specifically to a DNA probe containing the authentic YB-1-binding site from the SMαA promoter (D_R) but not the complementary strand (D_F) that instead binds Pur α and Pur β, two closely related single-strand DNA-binding proteins that assist YB-1 in SMαA gene repression.[35,37]

The finding that p60 YB-1 was specifically expressed in normal adult cardiomyocytes, and less abundant in SMαA-positive neonatal cardiomyocytes and myofibroblasts, implied that diminished expression of p60 (or reduced detection of anti-YB-1 antibody epitopes specific to this variant) coupled with replacement by the p50 form of YB-1 may be functionally related to *de novo* activation of SMαA gene expression after cardiac transplant. Many published studies on YB-1 center on its functional behavior in the cell nucleus where it regulates the transcription activity of numerous genes associated with cell and tissue adaptation to metabolic stress. However, recent studies also indicate a potential role for cytosolic YB-1 in mRNA translation control of genes required for stress response,[21,27,28] epithelial-to-mesenchymal transitions during embryonic development,[42–44] and cancer metastasis.[44–46] In accord with cytosolic localization and possible post-transcriptional regulatory function in cardiomyocytes, we observed that p50 YB-1 was localized in heart allografts adjacent to cardiac intercalated discs (Figure 3a). The intercalated disc (ID) is a membrane junctional bridge between electrically coupled cardiomyocytes that is highly enriched for connexin 43-enriched gap junctions and F-actin-linked fascia adherentes adhesion complexes.[47] Junctional ID regions were also reported to be enriched for ribosomes,[48] as were the cardiac subsarcolemmal and actin filament/I-band regions. Moreover, IDs in chronically rejected, human cardiac allografts reacted strongly with an antibody specific for the phosphorylated form of ribosomal protein S6.[49] We previously reported that p50 YB-1 binds a purine-rich sequence in exon 3 of SMαA mRNA and regulates its translational efficiency during TGF-β1-induced myofibroblast differentiation.[20,36,50] Localization of YB-1 at ribosome-enriched IDs after cardiac transplant, therefore, may be potentially important in the post-transcriptional deployment of newly synthesized fetal SMαA mRNA in stress-injured cardiomyocytes. To further characterize YB-1 expression and subcellular distribution in the transplanted heart, we utilized a syngeneic graft model that amplifies interstitial fibrosis and cardiomyocyte biomechanical stress without instigating adaptive alloimmune responses in the host.[1,7] This 'two-hit' model utilizes two successive rounds of isograft transplantation surgery that specifically enhances signaling due to mechanical trauma, thrombosis, peri-transplant I/R injury and TGF-β1 receptor activation.[1] Importantly, alloantigen-reactive monocytes, macrophages and T-cells do not infiltrate two-hit isografts. This reduces immune-cell release of proinflammatory interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) that could inhibit TGF-β1 signaling and obscure post-transplant evaluation of YB-1, which is exported from the nucleus in a TGF-β1-dependent manner.[19,20,22] Immunohistochemical analysis of two-hit heart grafts revealed accumulation of YB-1 protein within 3 days that was highly localized at cardiac IDs by day 11 after second transplant (Figures 3b–d). YB-1 in two-hit heart grafts also was frequently detected in the form of punctate deposits localized proximal to the ID and perinuclear regions of cardiomyocytes (Figure 3d, arrows). Importantly, immunoblot analysis revealed that biochemical changes in YB-1 expression after cardiac retransplant resembled that seen in allografts. Two-hit isografts showed significant depletion of the p60 YB-1 variant and a large increase in p50 expression within 3 days after second transplant (Figure 3h). YB-1 was not readily detected in non-transplanted donor mouse hearts using the available anti-YB-1 antibodies (Figure 3g). Moreover, YB-1 deposition at cardiac IDs was not observed in hearts subjected to only a single round of transplant surgery (data not shown). Comparison of Figures 3e and f shows that *de novo* expression of fetal SMαA protein was not observed in one-hit isografts but required two rounds of transplant surgery consistent with the development of chronic rejection-like histopathology only in the two-hit model.

To determine if the increased expression of p50 YB-1 after transplant was associated with the activation of fetal SMαA gene expression specifically in the cardiomyocyte population, we examined YB-1 size heterogeneity in perfusion-isolated adult rat cardiomyocytes. As previously reported,[1] freshly isolated adult rat cardiomyocytes are rod-shaped and do not express SMαA but acquire this ability during phenotypic modulation into polymorphic, neonatal-like cells over a 14-day culture period in serum-containing medium (Figure 4). There was a lag in the accumulation of SMαA protein in cultured adult cardiomyocytes with little accumulation noted during the first 3 days after perfusion isolation. In contrast, immunoblot analysis revealed that enhanced expression of p50 YB-1 was evident within 24 h after isolation and increased more than six-fold over the 14-day observation period (Figure 4). The level of p60 YB-1 was low but relatively stable in cultured cardiomyocytes, although a small molecular-size shift was noted within the first 3 days after isolation producing p60 doublet bands. Notably, SMαA-positive cMFB contained only the p50 YB-1 variant (Figure 4, lane cMFB).

YB-1 is a known component of mRNA-protective stress granules in a variety of cell types.[23,51–53] We reasoned that YB-1 may perform a similar task in transplant-injured cardiomyocytes by stabilizing nascent SMαA transcripts, and perhaps other mRNAs encoding essential fetal or stress-response proteins, during transit to polyribosome-enriched regions of the sarcoplasm. The deposition of YB-1 proximal to cardiac IDs and ribosomes in mouse heart grafts implied that this protein may chaperone fetal mRNA needed for stress remodeling after cardiac transplant. As shown in the

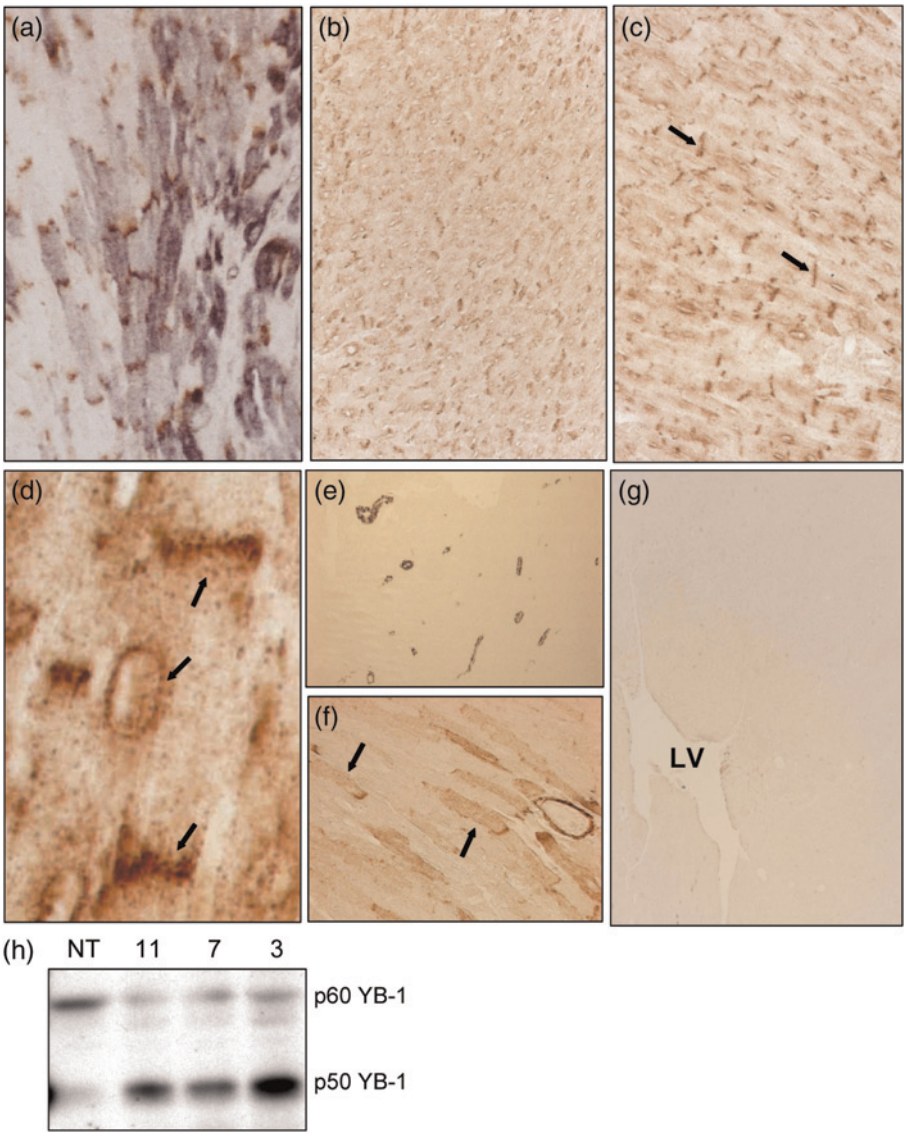

Figure 3 Expression and localization of Y-box binding protein-1 (YB-1) after murine cardiac transplant. An antibody specific for the YB-1 cold-shock domain (anti-YB1 polyclonal antibody M85-110) revealed deposition of YB-1 protein (red-brown) at cardiac intercalated discs (IDs) in an accepted allograft 60 days after heterotopic transplant (a). Double-labeling with an anti-smooth muscle α-actin (SMαA) antibody revealed that cardiomyocyte sarcomeres in allografts also contained fetal SMαA protein (blue-purple), although its distribution did not always correlate with YB-1 deposition at IDs which seemed more consistently reactive with the anti-YB-1 antibody. Panels (b–d) show YB-1 distribution in two-hit isografts at day 3 (b) and day 11 (c and d) using an antibody specific for the C-terminus of the YB-1 polypeptide (Y-0271, amino acid residues 307–324) that only reacts with p50 YB-1. Although p50 YB-1-enriched IDs were noted at day 3, reactivity of these structures with the anti-YB-1 antibody was more pronounced by day 11 (arrows, (c)). Punctate YB-1 deposits were observed in the peri-nuclear and ID regions of cardiomyocytes in two-hit, day-11 hearts when viewed at high magnification (arrows, (d)). Panels (e–f) depict one- and two-hit isografts, respectively, reacted with the anti-SMαA antibody. Only heart grafts subjected to two rounds of transplant surgery contained SMαA-positive sarcomeres (arrows, (f)), although SMαA-positive microvascular structures were evident in both types of grafts. Non-transplanted donor heart tissue did not react with the p50-specific anti-YB-1 antibody (g). LV designates the left ventricular luminal space. Objective magnification: (a–c) and (e–g), 10×; (d) 40×. Panel (h) shows that two-hit isografts accumulated substantial amounts of p50 YB-1 within 3 days after second transplant. The numbers above the immunoblot (anti-YB1 polyclonal antibody M85-110) refer to days after second transplant. Lane NT depicts a non-transplanted donor heart containing mostly p60 YB-1

upper panel of Figure 5, the interaction of p50 YB-1 with fetal SMαA mRNA increased 10-fold in adult rat ventricular cardiomyocytes over a 14-day culture period. Although expressed at approximately the same level as p50 in freshly isolated cardiomyocytes on day 0 of the study, p60 did not bind SMαA mRNA. These observations suggested that the interaction of YB-1 with fetal mRNA transcripts in cardiomyocytes was under developmental control and linked to accumulation of p50 YB-1. The p50 form of YB-1 showed a preference for the exon 3 sequence of SMαA mRNA in comparative binding analysis using single-stranded DNA and RNA probes harboring YB-1-binding elements in both native and mutant sequence context (Figure 5, lower panel). The binding preference for mRNA over DNA was consistent with the extranuclear localization of p50 YB-1 at cardiac IDs after transplant.

An analysis of endomyocardial biopsies collected from heart transplant patients over a 17-year post-transplant surveillance period revealed informative trends regarding the expression of YB-1 and SMαA in accepted allografts.

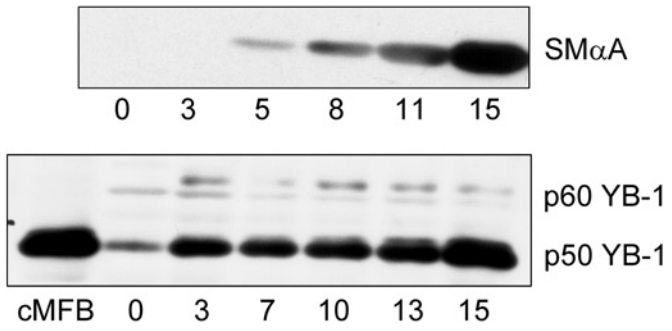

Figure 4 Immunoblots depicting expression of fetal smooth muscle α-actin (SMαA) and Y-box binding protein-1 (YB-1) proteins in isolated adult rat cardiomyocytes. The upper panel shows that accumulation of fetal SMαA commenced within 5 days after isolation and cultivation in serum-containing culture medium. In contrast, rapid accumulation of p50 YB-1 in these cells was noted within 3 days after isolation (lower panel). The numbers below the immunoblots refer to days in culture. Primary culture preparations of cardiac myofibroblasts (cMFB) maintained in 21% molecular oxygen expressed only p50 YB-1

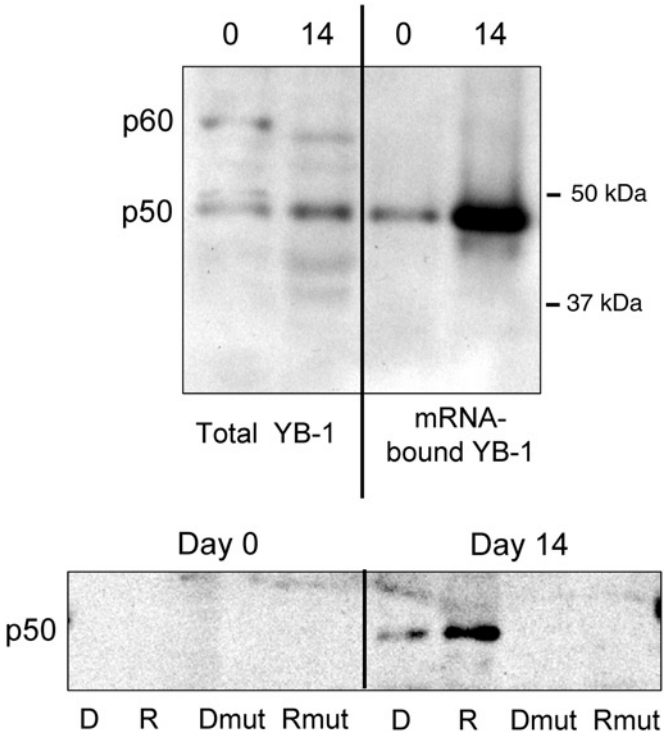

Figure 5 Single-strand nucleic acid-binding properties of rat cardiomyocyte Y-box binding protein-1 (YB-1) size variants. The upper panel shows that both p60 and p50 are expressed in freshly isolated cardiomyocytes at day 0 but the proportion of p50 increases after 14 days in culture. Smooth muscle α-actin (SMαA) mRNA binding was notably higher for p50 YB-1 compared with p60 and markedly elevated in cardiomyocytes cultivated for 14 days compared with freshly isolated cells. The lower panel shows that p50 binding to SMαA mRNA noted in the 14-day cardiomyocytes (lane R) is sequence-specific and absent if the exon 3 YB-1-binding site is mutated (Rmut). Moreover, p50 YB-1 shows a preference for mRNA (lane R) compared with single-stranded DNA (lane D)

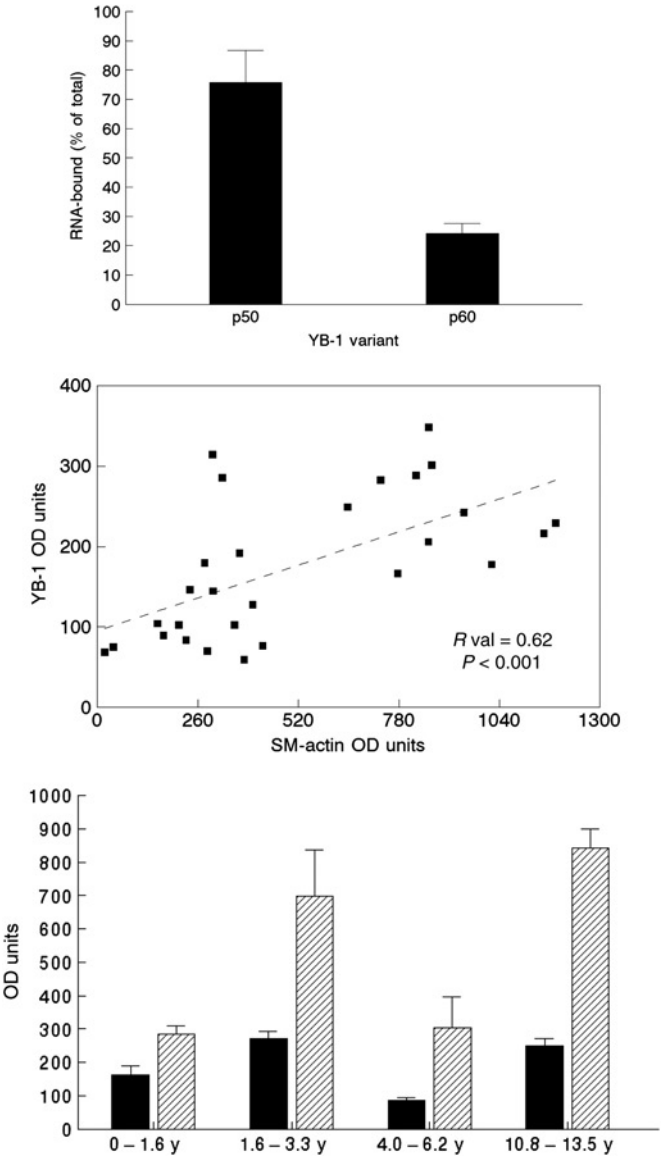

Figure 6 Analysis of Y-box binding protein-1 (YB-1) RNA-binding activity and expression in human endomyocardial biopsies. RNA-affinity binding assays using extracts prepared from pooled endomyocardial biopsies indicated that smooth muscle α-actin (SMαA) mRNA bound more p50 YB-1 compared with p60 (top panel). Individual biopsies collected from cardiac transplant patients over a 14-year post-transplant observation period were evaluated for both p50 YB-1 (anti YB-1 polyclonal antibody 85–110) and SMαA expression by immunoblot analysis (middle panel). Correlation analysis revealed significant concordance between levels of the two proteins in the biopsy collection (R value = 0.62, $P < 0.001$). The bottom panel shows coordinate increases in p50 YB-1 (solid bars) and SMαA (hatched bars) protein levels during the first 3 years after transplant with a notable reduction in expression of both proteins by year 6. However, a significant rebound in protein expression was observed during the 11–14-year post-transplant interval ($P < 0.01$). The y-axis scale indicates densitometry unit values that were normalized to glyceraldehyde-3-phosphate dehydrogenase levels to adjust for possible sample-loading differences

RNA-binding assays using extracts prepared from pooled endomyocardial biopsies indicated that the exon 3 sequence of SMαA mRNA bound more p50 YB-1 compared with p60 (Figure 6, top panel) even though both YB-1 size variants were detectable in biopsy-protein extracts (see below). Preferential interaction of SMαA mRNA with p50 YB-1 was observed regardless of whether samples were collected within 5 years after transplant or from long-term heart grafts 9–17 years after transplant. There was significant statistical correlation between the levels of p50 YB-1 and SMαA protein expression in 28 individual biopsies collected at

various times between year 1 and 13.5 after cardiac transplant (Figure 6, middle panel). For many of the patients providing tissue samples, at least one year had elapsed since their last biopsy. Therefore the data depicted in Figure 6 are unlikely to reflect processes related to the formation of myofibroblast-enriched granulation tissue at earlier sites of biopsy retrieval. Only tissue samples showing viable endomyocardial tissue were retained for analysis and samples showing fibrous scar were not included in the study. Given these considerations, we feel that the data reflects long-term processes directly relevant to endomyocardial remodeling and not simply an acute wound healing response due to prior biopsy. Data presented in the bottom panel of Figure 6 shows that the relative expression of p50 YB-1 in biopsies from accepted human allografts seemed to be linked to time-after-transplant and varied concordantly with expression of SMαA protein. Expression of p50 YB-1 in biopsies increased during the first 3–4 years after transplant but then subsided in most samples by year 6 only to re-emerge after the 10-year post-transplant interval. Notably, the mean levels of YB-1 and SMαA protein expression in biopsies collected from patients more than 10 years after transplant were significantly higher ($P < 0.01$) than those observed during the 4.0–6.2-year interval (Figure 6, bottom panel).

Using two antibodies that were specific for peptide antigens derived from the YB-1 cold-shock domain, we discovered that the p60 form of YB-1 demonstrated electrophoretic microheterogeneity and was specifically phosphorylated at serine 102 within the YB-1 CSD but only in human myocardial tissue and not mouse. Temporal expression of p50 and p60 YB-1 size variants was assessed in serial biopsies collected from cardiac transplant patients over a 1-week to 4-year post-transplant interval (Figure 7). Compared with the single p60 band detected in SDS extracts prepared from native mouse hearts, two or three closely migrating bands were variably detected on immunoblots prepared from SDS extracts of human endomyocardial biopsies. The human variant referred to as p60b co-migrated with the single p60 species detected in mouse heart. The relative amount of each p60 electrophoretic species varied between serial biopsies collected from individual patients with no obvious temporal relationship noted between p50 and p60 expression in each four-sample series. Interestingly, two human cardiac p60 variants (p60b and p60c) consistently reacted with an antibody that was highly specific for the phosphoserine 102 modification within the YB-1 CSD previously linked to serine and threonine protein kinase B (AKt)-mediated nuclear localization of YB-1 in proliferating tumor cells. The p60c band was more reactive with the anti-YB-1 phosphoserine 102 antibody and exhibited variable intensity within each four-biopsy series, whereas phosphorylated p60b was more prevalent in late time points rather than earlier biopsies collected in each heart-graft series. The observed reactivity with a peptide-specific anti-YB-1 phosphoserine 102 antibody suggested, at least in human heart samples, that multiple polypeptides migrating with an apparent molecular weight of around 60 kDa most likely represented different SDS conformers of authentic YB-1 phosphoprotein. Of note, p50 was not detected in phosphoserine 102-modified form in any heart tissue samples from either mouse or human.

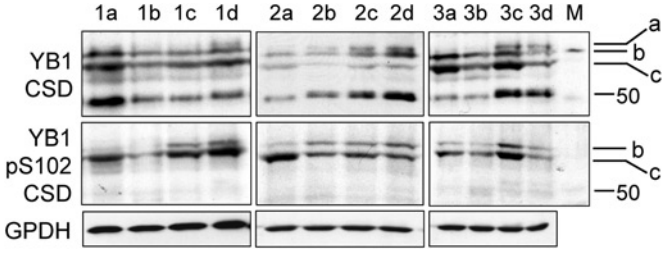

Figure 7 Immunoblots depicting expression of polypeptides in human endomyocardial biopsy extracts that bind antibodies specific for the Y-box binding protein-1 (YB-1) cold-shock domain (CSD). Shown are immunoblots prepared from four serial biopsies collected from three different cardiac transplant patients over a 0.7-to-210-week post-transplant observation period. Each four-sample series was probed with antibodies specific for the YB-1 CSD (YB1 CSD, anti-YB1 polyclonal antibody M85-110), the YB-1 CSD containing a phosphorylated serine residue at position 102 (YB1 pS102CSD), and glyceraldehyde-3-phosphate dehydrogenase (GPDH) to document equivalent protein loading. Lane markers denote samples collected from patient I at 0.7, 14, 41 and 82 weeks after transplant (lanes 1a–1d, respectively); patient II at 3, 23, 45 and 107 weeks (lanes 2a–2d, respectively); patient III at 93, 113, 145 and 210 weeks (lanes 3a–3d, respectively). Lane M is an aliquot of mouse heart extract showing the position of the p60 YB-1 size marker. Of the three p60 YB-1 size variants detected using the YB1 CSD antibody (labeled a, b, c), only p60b and p60c reacted with the phosphoserine 102 antibody. Although p50 expression was detected in all biopsy specimens (p50), this YB-1 size variant did not react with the anti-phosphoserine 102 antibody

Discussion

Cardiac allograft dysfunction is a slowly evolving, poorly understood pathogenic process that resembles unresolved wound healing.[2,3,54] Pro-fibrotic TGF-β1 and thrombin are activated during the early peri-transplant period and stimulate cardiac myofibroblast differentiation.[20] Scar-forming myofibroblasts may damage the coronary arterial vasa vasorum leading to accelerated transplant vasculopathy.[2,12,55,56] Moreover, biomechanical stress due to interstitial collagen may reactivate fetal contractile protein gene expression in graft cardiomyocytes via angiotensin II and TGF-β1 signaling that activates mitogen-activated protein kinase kinase 1 (MEK1)/extracellular signal-regulated protein kinase 1 and 2 (Erk1,2) and phosphatidyl inositol 3-kinase (PI3K)/Akt kinases needed for hypertrophic growth and cellular survival.[57,58] Reactivation of fetal contractile protein genes such as *acta2* encoding the SMαA protein is a key feature of biomechanical stress-induced cardiomyocyte hypertrophy in the native heart as demonstrated in rodent models of left-heart failure caused by aortic banding and hemodynamic pressure overload.[15,59–61] Studies on transgenic heart grafts programmed to express a green fluorescent protein (GFP) reporter gene driven by the mouse SMαA promoter revealed that cardiomyocytes surrounded by scar tissue were highly GFP-positive, suggestive of biomechanical stress-specific reactivation of fetal SMαA gene transcription.[1] Using transgenic mice that develop severe interstitial fibrosis due to over-expression of renin, Smithies and coworkers[62] observed significantly more expression of the fetal β-myosin heavy chain protein in cardiomyocytes located proximal to scar tissue compared with distal myocytes.

In this report, we show that the YB-1 stress-response protein accumulates in the sarcoplasm of adult cardiomyocytes in parallel with enhanced expression of fetal SMαA protein. Induction of YB-1 was evident in isolated cardiomyocytes that were induced to de-differentiate either in serum-containing medium or *in situ* following two rounds of cardiac transplant designed to amplify alloantigen-independent, pro-fibrotic TGF-β1 signaling.[1] The localization of p50 YB-1 at intercalated discs coupled with its ability to bind SMαA mRNA, F-actin[63] and microtubules[64] suggests that this protein could mediate transport of newly transcribed mRNAs to polysomes as well as reprogram protein synthesis needed for myocyte remodeling and adaptation to cardiac biomechanical stress. YB-1 has the ability to bind SMαA mRNA transcripts via a purine-rich sequence site located in exon 3 that is in proximity to the AUG translation start codon based on secondary-structure models (Willis and Strauch, unpublished data).

An intriguing observation was the apparent size shift of the YB-1 polypeptide in fibrotic heart grafts and during cardiomyocyte *in vitro* de-differentiation. YB-1 has a theoretical molecular mass of 35,924 Da although its electrophoretic behavior is quite complex owing to the presence of proline- and glutamine-enriched N- and C-terminal domains that place it in a class of prion-like, natively disordered proteins typically observed in various forms of senile dementia.[65] In the presence of SDS, YB-1 can form elongated structures with uncharacteristically large hydrodynamic radii that might explain anomalous electrophoretic migration on denaturing gels.[66] We do not know the molecular basis for YB-1 size heterogeneity in developing or transplanted hearts. Heart muscle contains a single YB-1 mRNA transcript[67] and while YB-1 pseudogenes have been identified in mammalian DNA, none seem to be expressed *in vivo*.[68] ATP production during normal cardiac development is linked to a well-known shift after birth from carbohydrate-based metabolism to oxidation of fatty acids.[69] Mitochondrial electron transport is associated with transient yet significant production of reactive oxidant species (ROS) that may be more prevalent in adult cardiomyocytes that utilize fatty acids for ATP production compared with glycogen-fueled neonatal myocytes. This pro-oxidant milieu could provide the underlying biochemical basis for the specific accumulation of p60 YB-1 during mouse heart development either through direct modification of YB-1 or indirectly via an associated ROS-regulated enzymatic process. Conversely, reverse-metabolic reprogramming may occur when freshly isolated adult cardiomyocytes are maintained in a glucose-rich culture medium that could explain the loss of adult-type p60 and gain of fetal-type p50 YB-1. ROS may serve as local signaling agents in the adult heart compared with the more hypoxic conditions encountered in fetal heart or heart grafts with impaired coronary flow due to transplant arteriosclerosis.

The cardiac CSD protein referred to as ZONAB, zonula occludens-1-associated nucleic acid-binding protein (dbpA/YB-2), reportedly migrates at around 60 kDa on SDS gels.[70] Immunoblot analysis revealed ZONAB expression in native and transplanted mouse hearts but the antibody-reactive bands did not co-migrate with the novel p60 YB-1 variant (David and Strauch, unpublished data). On the contrary, multiple anti-YB-1 polyclonal antibodies reacted with both p60 and p50. One antibody was specific for a 25-amino acid segment of the CSD (amino acids 85–110) representing an ancient protein sequence with a β-barrel spatial structure[65] that is identical in all YB-1 orthologs.[23] Another antibody was specific for a highly conserved, arginine-rich segment of YB-1 encompassing amino acids 137–150 within the N-terminal half of the protein adjacent to the carboxyl-end of the CSD. Both the CSD- and N-terminal-specific anti-YB-1 antibodies recognized p50 and p60 variants, with the CSD-specific antibody showing reproducibly higher reactivity with both bands. Of the two antibodies specific for the highly conserved C-terminal region of YB-1, one that was specific for a basic, arginine/proline-rich segment encompassing amino acids 276–302 reacted weakly with p60 but strongly with p50. Similarly, a C-terminal-specific antibody prepared using an acidic, glutamic/aspartic acid/proline-rich peptide corresponding to the last 17 amino acids of the YB-1 polypeptide chain reacted well with p50 but failed to detect p60. The reactivity of p50 with C-terminal antibodies rules out proteolytic processing of a larger YB-1 polypeptide chain as proposed in thrombin-injured endothelial cells.[71] Importantly, an antibody developed against a CSD-peptide antigen harboring a phosphorylated form of serine 102 reacted with two of the three closely migrating p60 variants but not the single p50 band detected in serial endomyocardial biopsies collected from heart transplant patients. Given that four of the five anti-YB-1 antibodies reacted with p60 size variants, we feel it is reasonable to conclude that p60 corresponds to an authentic form of YB-1 that is phosphorylated within the CSD in a species-specific manner in the adult heart. Failure to detect phosphorylated p60 in extracts prepared from whole mouse ventricle compared with human biopsy samples collected specifically from the right ventricle septal wall will require additional investigation but could be explained by a protein dilution effect if expression of phosphorylated p60 YB-1 is normally restricted to the endocardium.

Interaction of p50 YB-1 with SMαA mRNA increased 10-fold during de-differentiation of perfusion-isolated adult rat ventricular cardiomyocytes. Accumulation of p50 also occurred in two-hit heart grafts that showed substantial YB-1 compartmentalization in subcellular regions known to contain polyribosomes. YB-1 is known to form mRNA-containing stress granules in other cell types.[32,51,52] We suspect that it has a similar function in ventricular myocytes where it might bind and stabilize SMαA transcripts during transport to polyribosomes to augment fetal SMαA protein synthesis during periods of biomechanical stress. Stress injury-activated formation of YB-1 ribonucleoprotein complexes may hasten delivery of SMαA mRNA payloads to polyribosomes for high-output biosynthesis of G-actin monomers that can be quickly added to the ID-anchored, fast-assembly ends of F-actin. The localization of YB-1 at cardiac intercalated discs in allografts was often proximal to SMαA-containing filamentous structures in two-hit heart grafts, suggestive of the coupling of mRNA translation with actin filament polymerization. Interestingly, many YB-1-enriched IDs did not contain associated SMαA actin

filaments, implying that there may be a lag between deployment of cytosolic p50 YB-1 to IDs and up-regulation of SMαA protein expression. Studies on isolated cardiomyocytes presented in Figure 4 revealed a similar temporal delay between accumulation of p50 YB-1 and SMαA proteins. Importantly, the phosphorylated form of the S6 ribosomal protein (S6RP) has been localized at IDs in human cardiac allografts.[49] S6RP is a marker for active protein synthesis required for graft rejection in human heart transplant recipients. The subcellular localization of YB-1 at cardiac intercalated disks in fibrotic heart grafts is remarkably similar to the distribution of the S6RP, which is known to be regulated by the PI3K/Akt/mTOR (mammalian target of rapamycin) pro-survival signaling that controls mRNA translation in stressed cells.[26,72,73]

YB-1 ubiquitination and proteosome-mediated turnover is inhibited by mRNA,[74–76] implying that RNA-bound p50 may be preferentially stabilized after cardiac transplant at the expense of p60 which does not bind mRNA and thus may be targeted for protein turnover perhaps by serine 102 phosphorylation. SMαA mRNA transcripts and p50 YB-1 may be mutually beneficial in that the presence of one enhances stability of the other. This molecular mechanism may have evolved in cardiac muscle to efficiently couple gene transcription in the nucleus with transcript protection during transit to cytosolic polyribosomes. This process could assure adequate production of fetal gene protein needed for the compensatory response of cardiac muscle to abnormal biomechanical stress. Enhanced stability of cytosolic, mRNA-bound p50 YB-1 in stressed cardiomyocytes may also be related to its ability to bind actin thin filaments in muscle either directly[63] or via its high-affinity interaction with cytosolic Purα and Purβ proteins.[77] Protein biosynthesis and quality control may also be governed by structural interactions between YB-1 and a number of other cardiac proteins, including the cardiac ankyrin repeat domain 1 factor also known as CARP, titin, desmin, calsequestrin, myopalladin, and the MuRF1/2 (muscle-specific RING finger protein 1 and 2) E3 ubiquitin ligases.[78] As recently reviewed by Mikhailov and Torrado[78], these proteins demonstrate dynamic combinatorial interplay via shared ankyrin repeat and coiled coil domains, resulting in the formation of specific gene regulatory complexes capable of shuttling between the nucleus and sarcoplasm as part of a cardiomyocyte biomechanical stress-response circuit in the developing and injured heart.

Our analysis of biopsies from individual transplant patients grouped according to time-after-transplant revealed potentially important trends in YB-1 expression and function. The resurgence of YB-1 and SMαA expression 10 years after cardiac transplant was especially intriguing because these late-stage grafts typically are at high risk for allograft dysfunction due to perivascular and interstitial fibrosis as well as accelerated coronary vasculopathy.[3,5,79] Although not providing a comprehensive view of the full spectrum of disease, the biopsy data nevertheless suggested an early post-transplant period up to around year 3 to 4 when YB-1 and SMαA expression was relatively unsettled, perhaps reflecting patient-to-patient variation in graft surgical healing and clinical condition. Thereafter, there was a two-year period when YB-1 and fetal SMαA expression in all biopsies seemed to be uniformly reduced. As observed in isolated rat cardiomyocytes, p60 YB-1 in human biopsies did not bind mRNA especially well, whereas expression of mRNA-binding p50 correlated reasonably well with expression of the SMαA cardiac hypertrophy marker. The most likely physiological benefit provided by YB-1 interaction with SMαA mRNA is that altered cell–cell and cell–matrix contacts following transplant causes mechanical unloading and cytoskeleton remodeling that requires new contractile protein isoforms to adjust cardiomyocyte contraction and enhance cell survival. Although SMαA is a useful indicator for extreme biomechanical stress in rodent models of cardiac hypertrophy, its utility in evaluating human cardiac transplant disease is unexplored. We previously showed that SMαA-containing arterioles were present in histological preparations of endomyocardial biopsies but the relative abundance of these microvessels did not markedly change over the three-year post-transplant observation period, indicating that their protein contribution mostly likely would be invariant across the population of biopsy samples used in the current analysis.[79] Indeed, the observed coordinately cyclical changes in SMαA and p50 YB-1 protein levels was suggestive of a rather dynamic process. The biochemical analysis we performed cannot discriminate between SMαA expression in reprogrammed cardiomyocytes from that associated with episodes of cardiac myofibroblast differentiation, endocardium-to-mesenchymal transition, or microvascular remodeling that may be ongoing activities especially in long-term heart grafts. However, YB-1 may contribute to SMαA regulation in multiple cell types and tissues because of its demonstrated role in myofibroblast differentiation[19,20] and newly found function as a paracrine factor for epithelial-to-mesenchymal transition.[42,43] Analysis of human samples is ongoing and technical refinements may clarify the precise cellular origins and functional relationships between YB-1 and dysfunctional cardiac remodeling, as well as allow purification of phosphoserine-102 modified human YB-1 for detailed proteomic and protein turnover analysis.

In summary, YB-1 may govern cellular responses in the transplanted heart through its ability to bind and protect injury-activated mRNA transcripts that are prioritized for translation to assure graft adaptation during periods of metabolic and biomechanical stress. Besides fetal SMαA mRNA, we have confirmed that YB-1 can also bind homologous sequences in mRNAs encoding mouse and human forms of β-myosin heavy, α-myosin heavy chain, skeletal α-actin, VEGF-A and VEGF-B (Willis and Strauch, unpublished data). These observations imply that YB-1 accumulation in stressed cardiac muscle may have a global role in cardioprotection by functioning as chaperone proteins for newly transcribed fetal mRNAs during their passage from the nucleus to sites of protein synthesis located at cardiac IDs. Validating these ideas in the context of heart graft pathophysiology will provide much needed insight concerning the sequence of molecular events that lead to allograft dysfunction, as well as enable development of therapeutic interventions that improve heart graft performance and long-term survival.

Author contributions: JJD and SVS contributed an equal effort in performing the studies described in this study.

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All the authors participated in the design, interpretation of the studies, analysis of the data and review of the final manuscript. JJD, SVS, AZ, WLW and ARS conducted the experiments; RJK provided essential antibody reagents and editorial assistance with scientific content and production of the manuscript; CVL secured patient consent with assistance from JJD and performed collection of the human heart graft biopsy specimens; and ARS wrote the manuscript. This study was supported by NIH NHLBI grants HL 070294, HL 085109 (ARS), HL 054281 (RJK), and an American Heart Association Grant-in-Aid 09GRNT2170060 (RJK).

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