Differential responses of the right ventricle to abnormal loading conditions in vivo: Possible pathophysiologic mechanisms

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Objective: The right ventricle (RV) demonstrates differential adaptations in response to pressure versus volume loading, a phenomenon that may be important in the management of children and adults with congenital heart disease (CHD). The purpose of this study is to elucidate possible transcriptional mechanisms of the RV response to pressure versus volume loading in vivo.

Methods: Fetal lambs had aortopulmonary shunting or pulmonary artery (PA) banding. Four weeks after spontaneous delivery, ovine hearts were evaluated for hemodynamic changes and changes in expression of sarcomeric gene proteins and transcriptional factors. Western blot densitometry and chromatin immunoprecipitation were applied using standard techniques. Transactivation assays were performed using transient transfections in Schneider's *Drosophila* line 2 cells in culture.

Results: After PA banding, the RV pressure increased from 36 ± 4 mm Hg (n = 4) to 96 ± 8 mm Hg (n = 4, $P < .05$). The RVs of shunted and banded animals showed significant increases in the expression levels and promoter binding of activators myocyte enhancer factor 2, GATA-4, Nkx2.5, transcriptional enhancer factor 1, and specificity protein (Sp) 1. The transcriptional repressor Sp3 was downregulated in shunted animals, but its expression was increased paradoxically in the RV of the PA band group. Immunoprecipitation of Sp3 showed posttranslational modification to the acetylated isoform. In transient transfections of Schneider's *Drosophila* line 2 cells, acetylation of Sp3 converted it from a transcriptional repressor to an activator.

Conclusions: Posttranslational modifications of the transcriptional repressor Sp3, by acetylation, may be an important mechanism in the differential response of the RV to abnormal loading conditions. Sp3 may serve as a biomarker for RV failure for various heart defects in children and adults with CHD. These findings may have therapeutic implications in the management of right heart failure. (J Thorac Cardiovasc Surg 2013;145:1335-44)

The transcriptional regulation of cardiac differentiation, hypertrophy, and heart failure has important implications for the development of cell-based or pharmacologic treatment of myocardial disease.[1,2] We have shown previously in a shunted fetal lamb model of congenital heart disease and pathologic hypertrophy that transcription factors are modulated in a predictable way.[3,4] In this model, sarcomeric gene expression follows a pathologic hypertrophic program that recapitulates fetal expression patterns and represents an early milestone of heart failure. Myocardial transactivators are upregulated whereas repressors are downregulated. It has been postulated that a final common pathway to heart failure exists at a transcriptional level.[5-8] This transcriptional disorder in the heart may be a target for pharmacotherapy of cardiac hypertrophy and dysfunction.

In the current study, we sought to extend our observations and validate the hypothesis of transcriptional convergence on the road to cardiac hypertrophy and heart failure. We have developed a clinically relevant model of right ventricular pressure load in lambs by placing pulmonary artery (PA) bands in utero. The increased afterload causes severe right ventricular hypertrophy. The regulation of transcription factors in this model confirms many of our observations in the ovine model of cardiac volume loading. However, there appears to be a divergence in the regulation and posttranslational modifications of specificity protein (Sp) 3, a member of the Sp1 family of transcription factors, which is critical to the assembly of the basal transcriptional complex as well as comprehensive regulation of the network of cardiac gene expression.

Right ventricular function is critical to the outcomes of patients with congenital heart disease, especially those with chronic left-to-right shunting (patent ductus arteriosus, atrial septal defect, ventricular septal defect, AV canal, and so on), right heart valvular insufficiency (repaired tetralogy of Fallot with pulmonary insufficiency, tricuspid regurgitation, Ebstein's anomaly), single-ventricle disorders, congenitally corrected transposition of the great arteries, atrially repaired D-transposition of the great arteries, and

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Abbreviations and Acronyms

BNP = brain natriuretic peptide
HAT = histone acetyl transferase
HDAC = histone deacetylase
LV = left ventricle
PA = pulmonary artery
RII = receptor type II
RV = right ventricle
SD = standard deviation
SL2 = Schneider's *Drosophila* line 2
Sp = specificity protein
TSA = trichostatin A

chronic right ventricular outflow tract obstruction. Characterization of the differential transcriptional profiles in right ventricle (RV) dysfunction resulting from pressure versus volume loading may have important mechanistic implications for the identification of relevant biomarkers as well as the treatment of the RV in congenital heart disease.[9] We report an ovine model of RV pressure loading, and show that Sp3—a zinc finger protein essential for normal cardiac development—is expressed differentially in volume versus pressure-loaded RVs and, in turn, may regulate gene expression differentially during pathologic hypertrophy.

METHODS

Reagents, Constructs and Reporter Gene Assays, Cell Culture, Transfection, and Transactivation Assays

Reagents, media, enzymes, and sera for tissue culture were purchased from the Cell Culture Facility at the University of California (San Francisco, Calif). Oligonucleotides were purchased from Operon Biotechnologies, Inc (Alameda, Calif) or Invitrogen (Carlsbad, Calif).

The promoter/reporter constructs used in this study consist of fragments of the cardiac troponin T promoter and brain natriuretic peptide (BNP) promoter as reported previously.[3,4] Luciferase reporter constructs were created and subcloned into the plasmid pGL2 (Promega, Madison, Wis) as described described.[3,4]

Neonatal lamb cardiomyocyte or Schneider's *Drosophila* line 2 (SL2) cell cultures were performed using established standard techniques.[3,4] Plasmid constructs were transfected according to the manufacturer's protocol (Effectene Transfection Kit; Qiagen, Inc, Valencia, Calif) with minor modifications. Reporter gene activity was determined using the dual-luciferase reporter assay (DLR; Promega). Light intensity assays were read on a luminometer TD-20/20 DLR ready (Turner Designs Instruments, Sunnyvale, Calif). Cotransfection with 20 ng phRL-TK reporter was used to standardize for transfection variability. Cotransfection efficiency was verified with pSV-β-galactosidase (Promega) and/or pAAV-GFP staining. Reporter gene activity was expressed as the mean $\pm$ the standard deviation (SD). Statistical comparisons were performed by paired *t* tests.

Nuclear Extracts, Western Blot Analysis, and Densitometry

Cardiac muscle was harvested from 4-week-old lambs (control, shunt, PA band) at 4°C and was prepared by standard methods (Active Motif, Inc, Carlsbad, Calif).

TABLE 1. Heart mass in 4-week-old lambs

Cardiac chamber	PA Band			Shunt		
	Control	PA Band	*P* value	Control	Shunt	*P* value
RA	2.2 ± 0.4	4.3 ± 0.4	.003	2.9 ± 1.9	5.8 ± 1.6	.001
LA	1.7 ± 0.4	3.5 ± 0.7	.002	1.8 ± 0.3	8 ± 2	.001
RV	10.8 ± 0.7	29.7 ± 2.6	.007	12.6 ± 3	25.5 ± 7.7	.003
LV	22 ± 2.5	31 ± 4	.07	20 ± 1.8	33 ± 5	.003
Septum	9.5 ± 2.7	17.8 ± 4.3	.001	9.5 ± 3	18 ± 2	.0002
Total	44 ± 5	104 ± 30	.001	36 ± 6.5	113 ± 34	.0001

Values in grams are represented as mean ± standard deviation. *PA*, Pulmonary artery; *RA*, right atrium; *LA*, left atrium; *RV*, right ventricle; *LV*, left ventricle.

Total protein concentration was quantitated with the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, Calif). An equal amount of protein was loaded in each lane for Western blots. Sodium dodecylsulfate–polyacrylamide gel electrophoresis was used to separate nuclear proteins (10 μg) on a 10% gel and was followed by transfer to a polyvinylidene difluoride membrane (Amersham Biosciences, GE Healthcare Bio-Sciences Corporation, Piscataway, NJ). Membranes were then blocked and incubated with antibody as described previously.[4] Reactive bands were visualized with the SuperSignal West Femto Maximum Sensitivity Substrate Kit (Pierce, Rockford, Ill) and Kodak 440CF image station (Kodak, New Haven, Conn). The image was selected and analyzed with the public domain program NIH Image, and band intensity was quantified. Parallel sodium dodecylsulfate gels and immunoblots of β-actin and glyceraldehyde-3-phosphate dehydrogenase controls were performed to verify sample integrity and loading quantity.

Surgical Preparations and Care: Ewes

Pregnant, mixed-breed Western ewes (gestation range, 135-140 days; term, 145 days) were operated under sterile conditions. Through a left lateral fetal thoracotomy, an 8.0-mm polytetrafluoroethylene vascular graft (length, 2 mm; W.L. Gore & Associates, Inc, Flagstaff, Ariz) was sewn between the ascending aorta and main PA as described previously.[10] Pressure loading of the RV was generated by banding the main PA 75% of its circumference. All lambs were delivered spontaneously and were instrumented as previously described.[3,4]

Heart Tissue Preparation

Whole heart and chamber-specific weights were measured in all shunted, banded, and control specimens. Samples of right atrium, RV, left atrium, left ventricle (LV), and ventricular septum were excised, weighed, and used to make fresh nuclear preparations. Whole tissue and nuclear preparations were snap-frozen in liquid nitrogen. Samples were stored at −70°C until used for analysis.

Immunoprecipitation

Right ventricle and LV nuclear extracts were prepared from PA banded sheep hearts, and an equal amount of protein was used in each experiment. Nuclear extracts were preincubated with protein A/G-Sepharose for 3 hours at 4°C according to the manufacturer's instructions (Pierce crosslink IP Kit; Thermo Scientific, Rockford Ill). The beads were collected by centrifugation at 5000*g* for 2 minutes at 4°C. The supernatants were transferred to new tubes and were then incubated with either anti-Sp3 or anti-Sp1 antibodies with rotation overnight at 4°C.

Histone Acetyl Transferase and Histone Deacetylase Activity Assays

The histone acetyl transferase (HAT) activity and inhibition of histone deacetylase (HDAC) activity was performed using HAT (Enzo Life Sciences, Inc, New York, NY) and HDAC (Upstate Biotechnology, Lake Placid, NY) colorimetric detection assay kits.

TABLE 2. Hemodynamics

Shunted	Control (n = 5)	Shunted (n = 6)
MAP	75 ± 6	70 ± 8
RAP	4.5 ± 2	2.9 ± 0.8
LAP	5.4 ± 2	6.4 ± 3
PAP	17 ± 2	22 ± 2*
PA banded	**Control (n = 4)**	**Banded (n = 4)**
MAP	77 ± 7	66 ± 9
RAP	5.2 ± 2	9.4 ± 5
LAP	4.9 ± 2	5.9 ± 3
PAP	32 ± 16	27 ± 10
RVSP	36 ± 4	96 ± 8*
PROXPA, Sys	38 ± 8	82 ± 13*
DISTALPA, Sys	31 ± 6	27 ± 10

Values are in millimeters of mercury, mean ± standard deviation. *MAP*, Mean arterial pressure; *RAP*, right atrial pressure; *LAP*, left atrial pressure; *PAP*, pulmonary artery pressure; *RVSP*, right ventricular systolic pressure; *PROXPA*, proximal pulmonary artery pressure; *Sys*, systolic; *DISTALPA*, distal pulmonary artery pressure. *$P < .05$.

For the HAT assay, 20 μg of nuclear extracts or 200 ng of the Sp1 and Sp3 immunoprecipitated proteins were plated in 96-well plates and incubated with the reaction substrates for 60 minutes at 37°C, following the manufacturers' protocols. The reaction mix was analyzed in a plate reader (Spectramax M2; Molecular Devices, Sunnyvale, Calif). The histone acetyl transferase activity is expressed as the relative absorbance 440 nm OD value. The HDAC Activity Colorimetric Assay kit (Upstate Biotechnology) was used according to the manufacturer's protocol. Briefly, 10 μL (20 μg) of nuclear protein or 200 ng of Sp1 or Sp3 immunoprecipitated proteins was diluted in a final volume of 85 μL of ddH_2O and placed in a 96-well plate. A total of 10 μL of the HDAC assay substrate was added to each well and was mixed thoroughly. The mixture was incubated at 37°C for 1 hour. A total of 20 μL of the diluted activator solution was added to each well and was mixed thoroughly, and the plate was then incubated at room temperature for 20 minutes. Samples were analyzed in a plate reader (Spectramax M2; Molecular Devices) at 405 nm. Results are expressed as mean ± SD for 4 separate experiments.

Statistical Analysis

Comparisons between shunt and age-matched controls were made by appropriate *t* testing.

All protocols and procedures were approved by the Committee on Animal Research of the University of California, San Francisco. All animals were euthanized by appropriate methods as described in the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

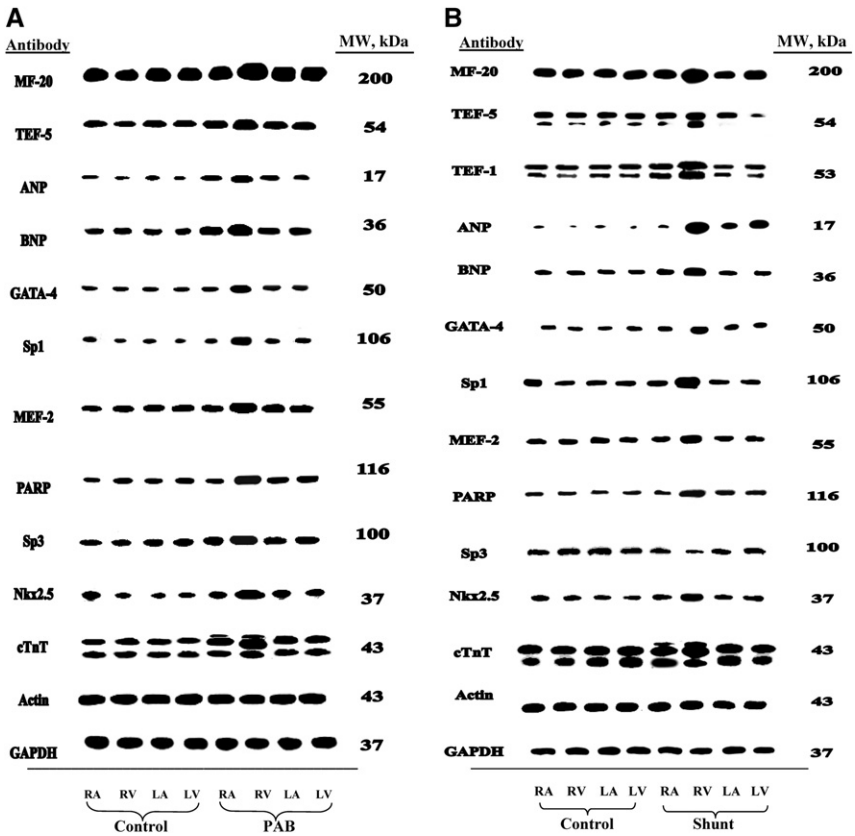

FIGURE 1. Characteristic Western blots of myocardium from 4-week-old banded lambs versus controls (A) and shunted lambs versus controls (B). Expression of sarcomeric myosins and cTnT is upregulated. ANP and BNP, markers of stretch or hypertrophy, are also upregulated. The transactivators Sp1, MEF-2, Nkx2.5, and GATA-4 are increased whereas Sp3 expression is enhanced in banded hearts and repressed in the shunted RV. The antibody used for Western blot analysis is shown on the *left*. *RA*, Right atrium; *RV*, right ventricle; *LA*, left atrium; *LV*, left ventricle; *MW*, molecular weight; *TEF*, transcriptional enhancer factor; *ANP*, atrial natriuretic peptide; *BNP*, brain natriuretic peptide; *Sp*, specificity protein; *MEF*, myocyte enhancer factor; *PARP*, poly-(adenosine diphosphate) ribose polymerase; *cTnT*, cardiac troponin T; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

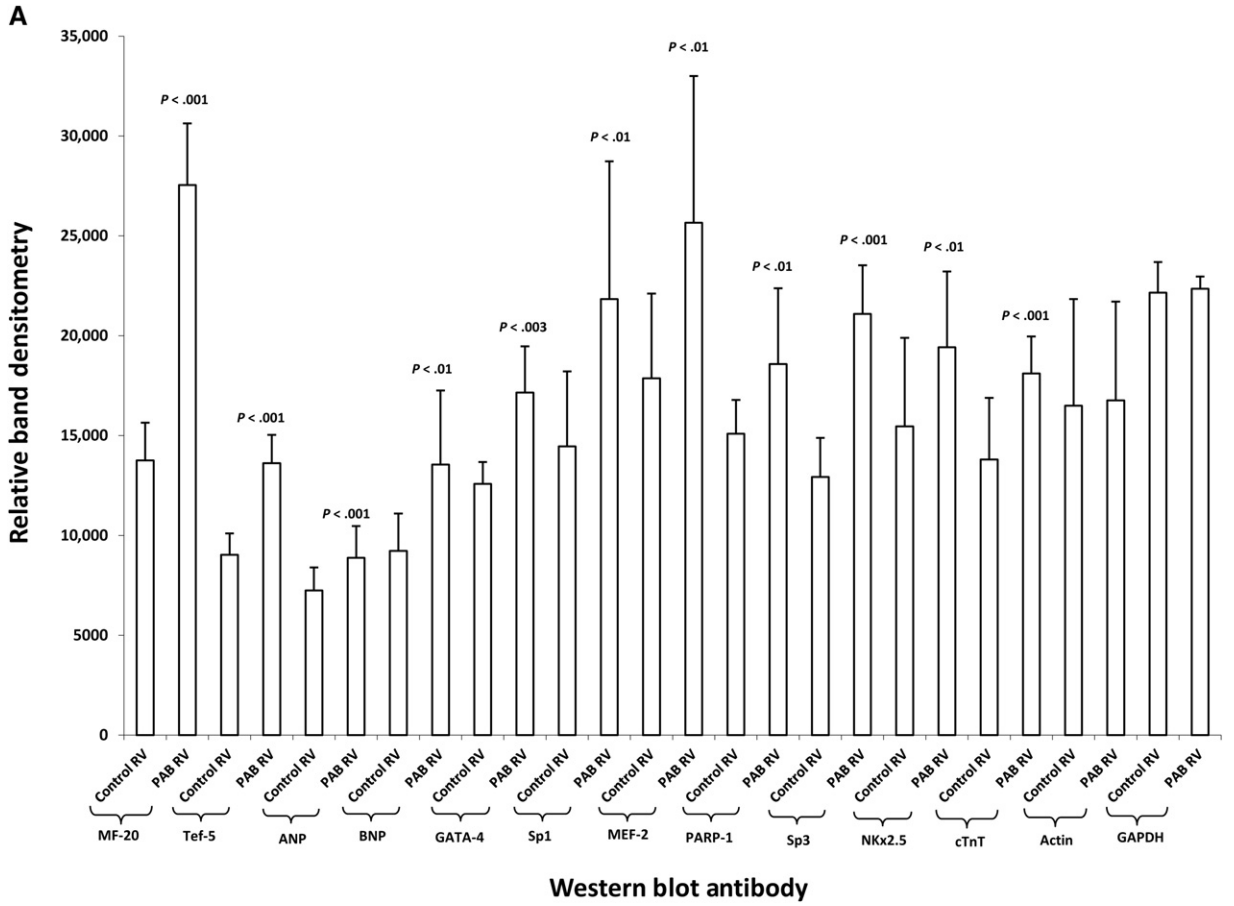

FIGURE 2. Densitometry of Western blot bands in banded RVs (n = 4) versus controls (n = 4) (A) and shunted RVs (n = 6) versus controls (n = 5) (B). Relative densitometry is on the vertical axis. Antibody used for control versus banded (A) or shunted (B) specimens is on the horizontal axis. Bar graphs with variations and *P* values comparing control with test RV are shown. Actin and GAPDH controls show no difference. Sarcomeric proteins and transcription factor expression is increased significantly for all tested proteins in the band model, including Sp3. In the shunted RV model, Sp3 expression is reduced significantly. *RV*, Right ventricle; *PAB*, Pulmonary artery banding; *TEF*, transcriptional enhancer factor; *ANP*, atrial natriuretic peptide; *BNP*, brain natriuretic peptide; *Sp*, specificity protein; *MEF*, myocyte enhancer factor; *PARP*, poly-(adenosine diphosphate) ribose polymerase; *cTnT*, cardiac troponin T; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

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RESULTS

Pulmonary Artery Banding Produces Right Ventricular Hypertrophy

Four-week-old lambs that had in utero banding of the PA showed a significant increase in total cardiac mass compared with control animals (104 ± 30 g vs 44 ± 5 g, $P = .001$; Table 1). The increase in total cardiac mass was comparable with that seen in shunted lambs of the same age (113 ± 34 g vs 36 ± 7 g, $P = .001$). Significant increases were seen for all chambers and the septum. The increase in mass of the LV in banded lambs was not statistically significant (Table 1). Banded animals developed systemic RV systolic pressures, with a peak systolic gradient ranging from 25 to 60 mm Hg (Table 2). The increased RV pressure in banded lambs was significantly higher than in controls, the PA pressure proximal to the band in banded lambs was significantly higher than the proximal PA pressure in controls, and in the PA band group, the proximal PA pressure was significantly higher than the PA pressure distal to the band (Table 2). Four-week-old shunted animals had hemodynamics similar to our previously published results for 2-week-old lambs.[3,4]

Right Ventricle Pressure Overload Results in Increased Expression of Sarcomeric Proteins and Myocardial Transcription Factors

Tissue homogenates of myocardium derived from sheep that had PA banding or aortopulmonary shunt insertion were used for Western blot analysis (Figure 1, *A* and *B*, respectively). In both PA-banded and shunted hearts, MF-20 was upregulated significantly in the RV. There was an associated increase in cardiac troponin T expression in the RVs of both banded and shunted lambs. The

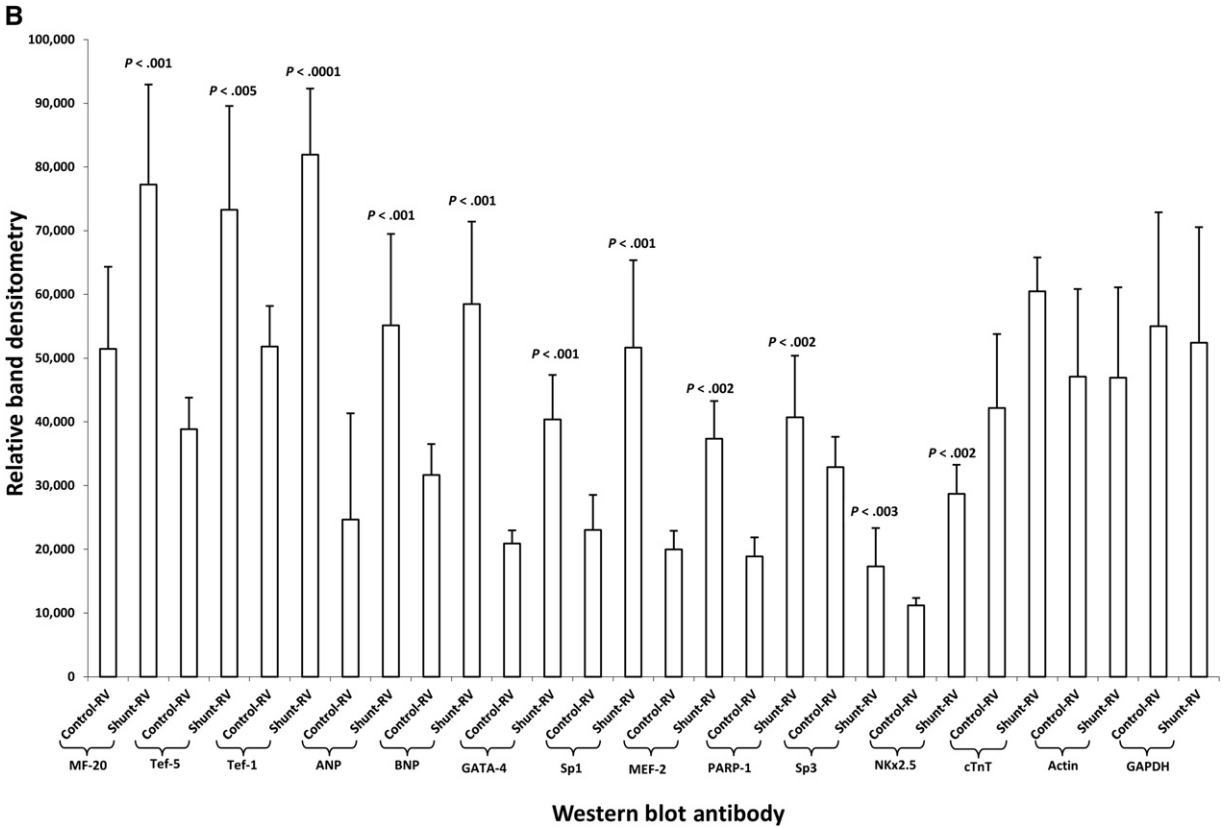

FIGURE 2. (*continued*).

upregulation of cTnT expression was associated with expression of high-molecular weight isoforms, which characterize the early fetal program. Relevant cardiac transcription factors, including GATA-4, transcriptional enhancer factor 5, atrial natriuretic peptide, BNP, myocyte enhancer factor 2, poly-(adenosine diphosphate) ribose polymerase, Nkx2.5, and Sp1, were all upregulated in the ventricles of both shunted and banded lambs. In the shunted animals, Sp3 expression in the RV was suppressed; in contrast, the expression of Sp3 in banded sheep hearts was significantly increased. Quantitative analysis of sarcomeric protein and myocardial transcription factor expression is summarized in Figure 2. Relative band densitometry was performed to compare expression levels quantitatively (banded, n = 4; controls, n = 4). Densitometric analysis confirms that the upregulation in sarcomeric protein and transcription factor levels in the RVs of PA-banded (Figure 2, *A*) and shunted lambs (Figure 2, *B*) compared with controls was statistically significant. There was no significant difference in actin or glyceraldehyde 3-phosphate dehydrogenase expression levels in shunted or banded versus control RVs. By densitometry, Sp3 levels were significantly lower in shunted RVs compared with controls, and were upregulated significantly in banded RVs compared with controls.

Sp3 is Upregulated and Acetylated, and Demonstrates Increased Promoter Binding in the RV Subject to Pressure Loading

We postulated that Sp3 may undergo the posttranslational modification of acetylation. Sp3 expression and acetylation in the shunted RV is repressed. Nuclear protein from the RV of 4-week-old PA-banded and control lambs were immunoprecipitated with Sp3 and then run on a Western blot. Screening of the Western blots with either Sp3 or pan-acetyl antibody confirmed that Sp3 expression and acetylation were upregulated in the RV of banded animals (Figure 3, *A*). Furthermore, lower molecular weight isoforms of Sp3 were translated in banded RVs. Relative band densitometry confirmed that the differences in expression and acetylation of Sp3 in banded RVs compared with controls are significant (Figure 3, *B*). We sought to determine the interactions between Sp3 and histone acetylating and deacetylating nuclear proteins in control and banded RVs (Figure 3, *C*). Immunoprecipitates of Sp3 derived from RV nuclear extracts from banded and control lambs were analyzed by colorimetry. The interaction of Sp3 with HAT was upregulated significantly in the RVs of banded versus control lambs. Sp3 association with HDAC was diminished significantly in the RV of banded lambs compared with controls.

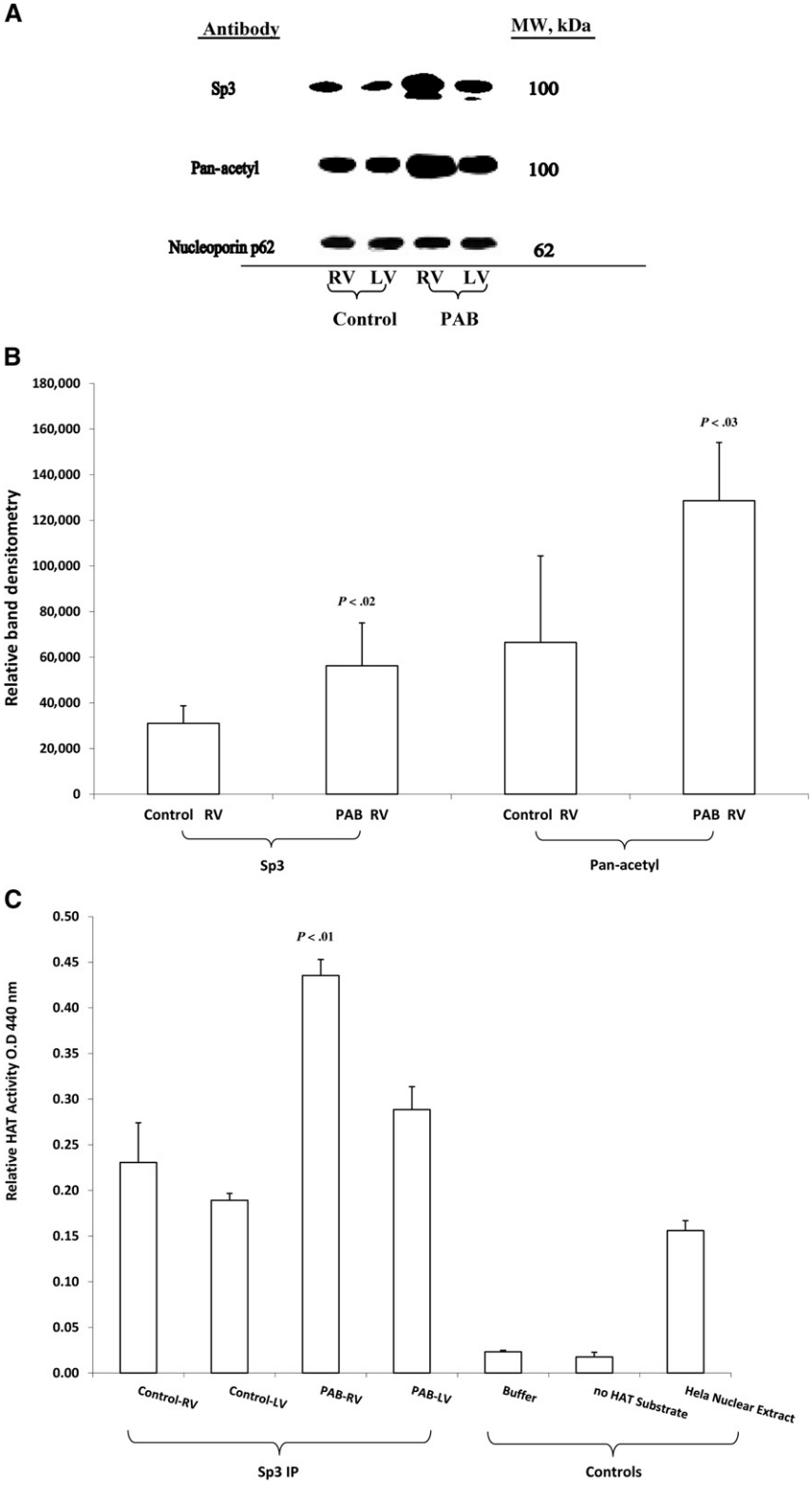

FIGURE 3. A, Nuclear protein derived from control or banded (PAB) lamb myocardium that is immunoprecipitated with Sp3 was screened by Western blot analysis using antibody to Sp3 or acetyl groups. Sp3 expression is increased in banded hearts; lower molecular weight *(MW)* isoforms are also translated. The degree of acetylation of Sp3 in RVs is increased. Antibody is listed on the *left*. Nucleoporin p62 is the control antibody. B, Bar graphs of densitometric analysis of Western blots shown in (A) (banded, n = 4; controls, n = 4). C and D Immunoprecipitates of cardiac nuclear protein using Sp3 antibody were assayed for associated histone acetyl transferase (C) or HDAC (D) activity. Histone acetyl transferase activity associated with Sp3 is upregulated

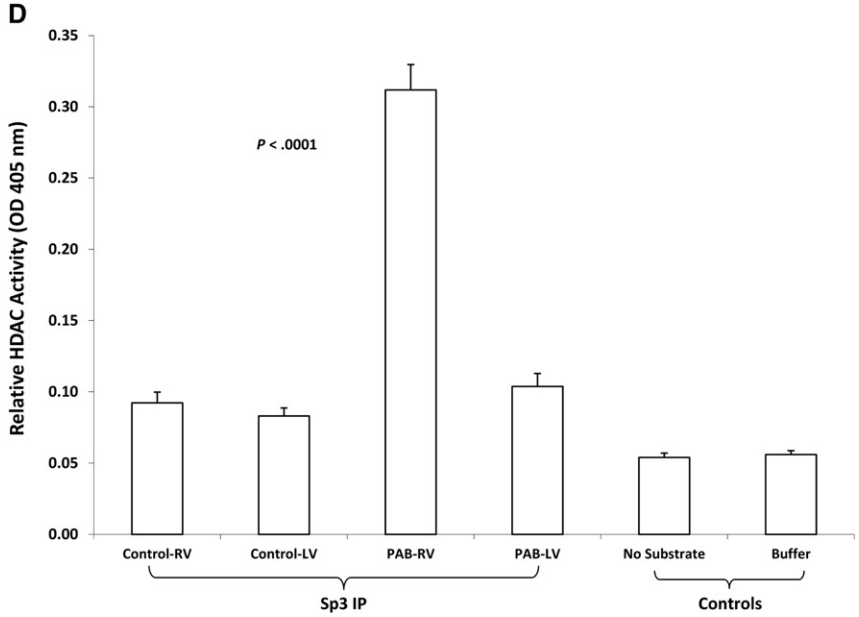

FIGURE 3. (*continued*).

Having determined that Sp3 expression and acetylation of Sp3 is increased in the RVs, we sought to determine whether Sp3 binding to cardiac promoters changed with pressure loading. Chromatin immunoprecipitation of the cardiac troponin T and BNP promoters was performed with Sp3 antibody. Figure 4 shows that the Sp3 nuclear protein has qualitatively increased binding to the BNP and cTnT promoter in the RV of banded hearts (n = 4) compared with controls (n = 4). By relative band densitometry, Sp3 binding affinity to the cTnT promoter more than doubled in the RVs of banded hearts (818 ± 49 relative OD units [banded] vs 405 ± 84 OD units [control]; n = 4 each, $P = .005$). Although the increase in binding of Sp3 to the BNP promoter in banded RVs was increased slightly (609 ± 45 relative OD units [control] vs 708 ± 20 relative OD units [PA banded]; n = 4, $P = .002$), the difference was significant.

To determine the transactivating effects of Sp3, we performed transient transfections of the luciferase reporter gene driven by the cardiac troponin T promoter in primary cultures of SL2 cells that lack endogenous Sp factors (Figure 5). The baseline activity of the −268cTnT promoter is repressed by cotransfection of Sp3 expression constructs. Western blots of cell lysates of cultured SL2 cells transfected with Sp3 expression constructs confirm that the Sp3 protein is translated. Addition of trichostatin A (TSA), an HDAC inhibitor, to SL2 cells cotransfected with cTnT reporter constructs and Sp3 expression vectors results in acetylation of Sp3 in the SL2 system. −268cTnT Promoter activity is superactivated significantly to levels beyond the superactivating effects of TSA alone. By mutational analysis, the activating effects of acetylated Sp3 are dependent on an intact, wild-type GC box—the cTnT promoter binding site for Sp factors. Mutation of the GC box led to substantial reduction of promoter activity that was not responsive to cotransfection of Sp1 or Sp3, or the addition of TSA.

DISCUSSION

In the current study, we report an ovine model of RV pressure loading by in utero PA banding. The RV in this model develops significant hypertrophy, increased mass, regression to the fetal cardiac gene program, and upregulation of cardiac myosins and markers of hypertrophy (BNP). Cardiac muscle transcription factors (myocyte enhancer factor 2, Nkx2.5, GATA-4, transcriptional enhancer factors, and Sp1) known to regulate cardiac differentiation and development are upregulated in a way comparable with that seen in shunted ventricles.[3,4] Sp3, a transcriptional repressor, is regulated differentially. It is downregulated in volume-loaded ventricles, and paradoxically upregulated in pressure-loaded ventricles where it is also converted to a transactivator through the posttranslational modification of acetylation. The differential responses of the RV to

significantly in banded lambs (C) whereas HDAC association with Sp3 is reduced significantly in banded RVs (n = 4) compared with controls (n = 4) (D). *PAB*, Pulmonary artery banding; *HDAC*, histone deacetylase; *RV*, right ventricle; *LV*, left ventricle; *Hela*, Hela nuclear extracts; *HAT*, histone acetyl transferase; *Sp*, specificity protein.

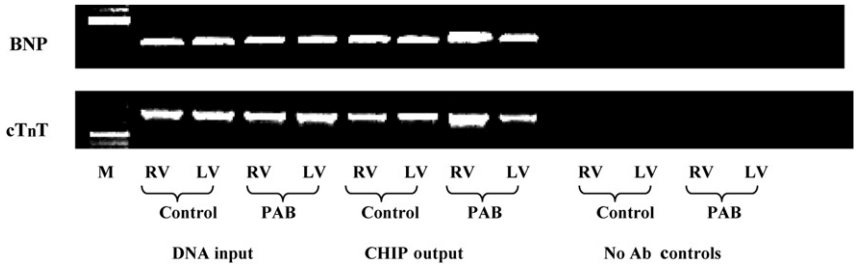

FIGURE 4. Sp3 binding to cTnT and BNP promoters is enhanced in the RV after pulmonary artery banding. Chromatin immunoprecipitation from control or banded (PAB) lamb right and left ventricles. DNA prepared from ventricular chromatin (input DNA) was complexed using Sp3 and eluted (output DNA). Input DNA and CHIP output DNA were used for polymerase chain reactions (*PCRs*) using primers specific for the BNP and cTnT promoters. Promoter segments derived from PCR are labeled on the *left*. *RV*, Right ventricle; *LV*, left ventricle; *PAB*, pulmonary artery banding; *Ab*, antibody; *BNP*, brain natriuretic peptide; *cTnT*, cardiac troponin T.

volume versus pressure load may, in part, be regulated by Sp3 transactivation pathways. Sp3 may serve as a clinical biomarker for differential RV responses to variable loading conditions, a prognostic indicator of severity or type of RV dysfunction, or may represent an important transcriptional pathway for targeted pharmacotherapy of heart failure.

Bartelds and colleagues[9] recently reported on the differential responses of the RV to variable loading conditions in a murine model. Right ventricle pressure loading was created by PA banding whereas volume overload was generated by aortocaval shunts. Pressure load appeared to be more harmful than volume loading because RV failure developed more rapidly. They postulate that, in addition to the hemodynamic and pathophysiologic differences, the type of loading may also induce different molecular signaling patterns that define the RV response and dysfunction. We propose that Sp3 may play an important role in the maladaptive, differential responses of RVs to abnormal or variable loading conditions. Sp3 is required for normal cardiac development and plays a critical role in myocardial differentiation. Mice lacking the zinc finger transcription factor Sp3 die in utero. Sp3 null hearts have defective looping and develop severe cardiac malformations. We have shown previously that Sp3 inhibits cardiac promoter activity and

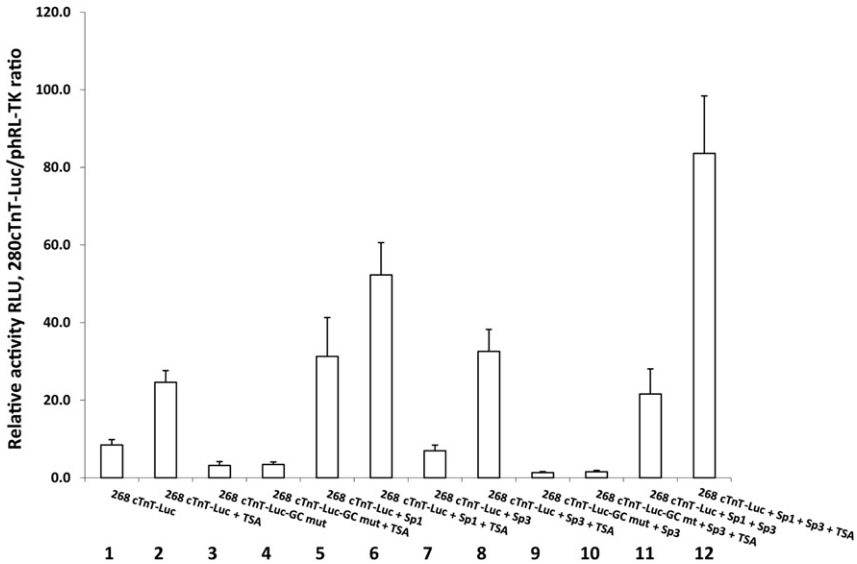

FIGURE 5. Reporter gene activity driven by −268cTnT promoter constructs transfected in cultured SL2 cells that lack endogenous Sp1/Sp3. Baseline promoter activity (*lane 1*) is enhanced by Sp1 (*lane 5*) but repressed by Sp3 (*lane 7*). Sp3 also inhibits Sp1-mediated superactivation of the promoter (*lane 11*). Addition of TSA results in increased baseline promoter activity (*lane 2*), as well as Sp1-mediated promoter activation (*lane 6*). In the presence of TSA, Sp3 switches from a repressor to an activator and enhances promoter activity (*lane 8*) to levels greater than with TSA alone. Sp3 repression of Sp1-mediated superactivation (*lane 11*) of the promoter is reversed with the addition of TSA (*lane 12*). TSA and Sp1/Sp3 effects are mediated through and dependent on the GC box, which when mutated results in significant depression of promoter activity despite the addition of coactivators (Sp1, TSA; *lanes 3, 4, 9, and 10*). Western blots of all lysates confirm that Sp1 and Sp3 are expressed when transfected into SL2 cells, and are acetylated when TSA is added to the culture (data not shown). *GC-mut*, Mutated GC box in 268cTnT promoter; *TK*, thymidine kinase; *TSA*, trichostatin A, histone deacetylase inhibitor; *RLU*, relative light unit; *cTnT*, cardiac troponin T; *Sp*, specificity protein.

represses Sp1-mediated activation of the cTnT promoter.[4] In the volume-loaded heart, inhibitory Sp3 is downregulated, consistent with derepression of the pathologic hypertrophic program. Sp3 is expressed differentially in the pressure-loaded RV, where it is paradoxically upregulated. The differential regulation of Sp3 may have mechanistic implications for the differential response of the RV to variable, abnormal loading conditions. Braun and associates[11] have shown that Sp3 is regulated by acetylation, the implications of which may explain is dual role in promoter inhibition versus Activation. During pressure loading, the interaction of Sp3 with HAT is upregulated whereas interaction with HDACs is diminished, favoring modification of the protein by acetylation. Acetylated Sp3 is a transcriptional activator. We show that acetylation of Sp3 with TSA results in superactivation of the cTnT promoter and that the effect is dependent on the GC box found in the proximal portion of the cTnT promoter. We have shown previously that the GC box is a binding site for the Sp family of nuclear proteins and is essential for cTnT activity.[4] These findings are consistent with the report from Ammanamanchi and coworkers,[12] which showed that the HDAC inhibitor TSA induces acetylation of Sp3, which acts as a transcriptional activator of transforming growth factor-β receptor type II (RII). Promoter mutational analysis indicated the TSA response is mediated through a GC box located on the RII promoter, which was identified previously as an Sp1/Sp3-binding site that was critical for RII promoter activity.[12]

Chromatin remodeling is an important mechanism of transcriptional regulation. Histone acetylation results in decondensation of chromatin, transforming it into a loosely organized structure. Relaxed chromatin allows for exposure of gene promoters and transcriptional activation, and can be brought about by inhibition of HDAC or activation of HAT. Conversely, deacetylation of chromatin transforms it into a less transcriptionally active, tightly packed structure. Widespread inhibition of HDACs does not always induce sweeping transactivation of gene promoters.[13,14] Trichostatin A may induce an initial, brief increase in histone acetylation, chromatin loosening, and transcriptional activation; however, hyperacetylation is often a transient phenomenon. The addition of the HDAC inhibitor TSA to SL2 cells in culture transfected with the cTnT promoter constructs resulted in increased promoter activity. In the presence of TSA, cotransfection of Sp3 did not suppress promoter activity, but further enhanced both baseline and Sp1-mediated promoter activation of reporter gene expression. The effect may not only be a result of the conversion of Sp3 to an activator by acetylation, but also may relate to enhanced recruitment and binding of Sp3 and Sp1 to promoter sites as shown by chromatin immunoprecipitation. The ability of Sp factors to mediate the effects of TSA has been shown in a number of other systems including bcl-2, Na+/H+ exchange and p21 genes.[15-17] Hence, TSA-induced activation of the cTnT promoter may extend beyond acetylation of chromatin or transcription factors, and include enhanced recruitment of Sp factors (and probably ribonucleic acid polymerase II) to target gene promoters.[13]

The RV is different phylogenetically and ontogenetically from the LV, having different embryologic origins, and distinct physiologic and structural characteristics. Preliminary data in the ovine model, however, suggest that the LV response to pressure versus volume loading is regulated in a manner similar to the RV. We have generated an animal model of supravalvar aortic stenosis and LV pressure loading in lambs. The regulation of Sp3 expression and acetylation patterns is similar to that seen in the RV, inferring a basic role for Sp3 in the maladaptive responses of cardiac muscle in general. Further characterization of the LV transcriptional response to abnormal loading conditions in ovine models is in progress. Models that use banding (or shunting) in very late gestation represent an acute pressure/volume load shortly prior to birth that has limitations in terms of extrapolating the data to congenital heart defects in which the hemodynamic lesion may be present through most of gestation. Future areas of investigation will also include the characterization of the transcriptional response to other clinically relevant conditions such as milder forms of PS, or combined PS and PI, and the therapeutic effects of TSA and other HDAC inhibitors on maladaptive myocardial hypertrophy.

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