Myocardial transcription factors are modulated during pathologic cardiac hypertrophy in vivo

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Supplemental material is
available online.

Objectives: In the current study we describe and characterize a novel ovine model of biventricular hypertrophy and heart failure and evaluate the role of selected cardiac transcription factors in the regulation of cardiac gene expression during pathologic hypertrophy in vivo. The cardiac troponin T promoter is used as a model gene.

Methods and Results: Transient transfections of ovine cardiomyocytes in culture show that Sp1, transcriptional enhancer factor-1, and myocyte enhancer factor-2 activate cardiac troponin T promoter constructs. Cotransfection of Sp3 inhibits cardiac troponin T promoter activity and represses Sp1-mediated activation of the cardiac troponin T promoter. By chromatin immunoprecipitation, transcriptional enhancer factor-1, myocyte enhancer factor-2, NKX2.5, GATA-4, and Sp factors bind the cardiac troponin T promoter in vivo. To assess the role of cardiac transcription during pathologic hypertrophy, in vivo, we created surgical aorta-pulmonary shunts in utero in fetal lambs. Two weeks after spontaneous delivery, shunted lambs showed failure to thrive, significant biventricular hypertrophy, and heart failure. Shunted hearts had significant increases in myosin and cardiac troponin T protein expression. There was a shift in expression to the high-molecular-weight fetal isoforms. Transcriptional enhancer factor-1, myocyte enhancer factor-2, GATA-4, NKX2.5, and Sp1 transcription factor levels were increased in all heart chambers of shunted animals. Sp3 expression was decreased in shunted ventricles. Immunoprecipitated Sp3 was associated with significant increases in histone acetyl transferase activity and decreases in histone-deacetylase activity.

Conclusion: The shunted neonatal lamb is a valid, novel model of pathologic biventricular hypertrophy. During pathologic hypertrophy myocardial transactivators are upregulated while repressors are downregulated.

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Cardiac failure and dysfunction can result from numerous clinicopathologic conditions including hypertension, ischemic heart disease, valvular heart disease, cardiomyopathy, and congenital heart disease. The need for effective broad-based therapy for patients with heart failure far outweighs and has outpaced current therapeutic strategies. Further understanding of the complex cellular mechanisms that lead to heart failure may aid in the development of novel and effective therapies.

Abbreviations and Acronyms

ADP	= adenosine diphosphate
ChIP	= chromatin immunoprecipitation
cTnT	= cardiac troponin T
HAT	= histone acetyl transferase
HDAC	= histone deacetylase
MEF2	= myocyte enhancer factor 2
PAR	= poly(adenosine diphosphate ribose)
PARP	= poly(adenosine diphosphate ribose) polymerase
TEF-1	= transcriptional enhancer factor-1

Various pathologic signals, stressors, or biomechanical forces elicit different intracellular signaling pathways that ultimately stimulate fetal cardiac gene expression (in children and adults) and pathologic hypertrophy, an early milestone in heart failure.^{1,2} Although numerous biomechanical forces and pathologic conditions can result in myocardial dysfunction, it is hypothesized that there is a final common pathway in which transcriptional alterations in sarcomeric gene expression, common to many forms of pathologic hypertrophy (concentric, eccentric), result in contractile dysfunction of the heart. Thus, contractile dysfunction of the heart can be viewed as a transcriptional disorder.^{3,4}

Transcription factors are nuclear proteins that bind promoter regulatory elements to activate or repress gene expression. The combinatorial interactions between transcription factors and promoter elements that are required for the regulation of cardiac gene expression during normal cardiac development probably operate during pathologic cardiac remodeling and hypertrophy. NKX2.5, myocyte enhancer factor 2 (MEF2), HAND, and GATA transcription factors have been implicated in transcriptional control, pattern formation, and segmental control of cardiac development.⁵⁻⁸ Using the cardiac troponin T (cTnT) promoter as a model gene, we have shown that transcriptional enhancer factor-1 (TEF-1), MEF2, and Sp family of transcription factors also contribute to the developmental regulation of cardiac gene expression in avian embryonic cardiomyocytes.^{9,10} These myocardial transcription factors, which are important for normal growth, development, and maintenance of the cardiac phenotype, may also be implicated during induction of fetal genes and the upregulation of constitutive genes that is seen with pathophysiologic hypertrophy.^{2,11}

Recent interest in “transcriptional disorders of the heart” has also implicated cardiac histone acetylation in the development of pathologic cardiac hypertrophy and heart failure. Histone acetyl transferases (HATs) are believed to acetylate histone proteins, relax chromatin, and expose “prohypertrophic” genes for activation by cardiogenic transcription factors.^{12,13} Histone deacetylases (HDAC classes I and II) have also been implicated in regulation of myocardial gene ex-

pression and cardiac hypertrophy.^{4,12,14} Class I HDACs may act as prohypertrophic agonists.¹⁵⁻¹⁷ Thus, isoform-specific inhibitors of class I HDACs and/or cardiac-specific HATs may have an important and specific inhibitory effect on the development of pathologic cardiac hypertrophy, contractile dysfunction, and heart failure.¹⁵⁻¹⁹

Recently, Kee and colleagues¹⁵ have shown that inhibition of histone deacetylation blocks cardiac hypertrophy induced by angiotensin II infusion and aortic banding. Similarly, Kong and colleagues²⁰ reported that suppression of class I and II HDACs blunts pressure-overload cardiac hypertrophy. Suppression of ventricular growth was well tolerated in terms of both clinical outcome and cardiac performance measures. Interstitial fibrosis was diminished in hearts treated with HDAC inhibitors, and collagen synthesis in isolated cardiac fibroblasts was suppressed. There was no evidence of cell death or apoptosis. Systolic function in the setting of blunted hypertrophic growth was preserved, as shown by echocardiography and hemodynamic data. The hypertrophy-associated switch of adult and fetal sarcomeric isoforms was attenuated, which likely contributed to the observed preservation of systolic function in HDAC inhibitor-treated hearts. Both these studies suggest that HDAC inhibition is a viable therapeutic strategy that holds promise in the treatment of pressure load-induced heart disease. The effects of HDAC inhibition on volume-loaded heart dysfunction are not known.

The aims of the current study are (1) to biochemically and morphologically characterize a clinically relevant model of pathologic hypertrophy and congestive heart failure and (2) to determine the changes in expression levels and posttranslational modifications of selected cardiac transcription factors. In utero placement of aorta–pulmonary arterial shunts has been reported as a model of postnatal pulmonary hypertension with increased pulmonary blood flow in lambs²¹ and is used in this study as a model of pathologic cardiac hypertrophy. Surgically created aorta–pulmonary shunts in fetal lambs produced left ventricular volume overload and right ventricular pressure overload, leading to altered contractile protein gene expression, biventricular hypertrophy, and congestive heart failure. In the shunted lamb model, pathologic cardiac remodeling and deleterious cardiac gene expression occurred in association with changes in the levels and modification states of cardiac-specific transcription factors (GATA-4, DTEF-1, Sp1, Sp3, and NKX2.5). Furthermore, we show that, functionally, TEF-1, MEF2, and Sp1 factors, which bind the cTnT promoter within the context of chromatin, transactivate the cTnT promoter in ovine cardiomyocytes, implicating them as important regulators of the pathologic hypertrophic process.

Materials and Methods**Reagents**

Media, serum, enzymes, reagents, and materials for tissue culture were purchased from the Cell Culture facility at the University of

California, San Francisco. Oligonucleotides were purchased from Operon Technologies Inc (Alameda, Calif) or Invitrogen (Carlsbad, Calif).

Plasmids and Constructs: Tissue Culture, Cell Transfections, and Reporter Gene Assays

The promoter/reporter constructs used in this study consist of 268 nucleotides of the chicken cTnT promoter upstream of the transcription initiation site (*Gallus domesticus* cardiac isoform of troponin T [TnT] gene, exon 1, and promoter sequence. *Accession# M5790*). Luciferase reporter constructs were created and subcloned into the plasmid pGL2 (Promega, Madison Wis), as previously described.⁹

Hearts were harvested from neonatal lambs and cardiomyocyte cultures established by standard techniques.¹⁰ The differentiation state of cultured cardiomyocytes was determined by light microscopy, presence of beating cardiomyocytes, immunocytochemistry for MF20, and Western blotting for myosins, cTnT, GATA-4, MEF2, and NKX2.5. Plasmid constructs were transfected according to the manufacturer's protocol (Effectene Transfection kit; Qiagen, Valencia, Calif) with minor modifications.⁹ Reporter gene activity was determined with the Dual Luciferase reporter assay (DLR-Promega, Madison, Wis). Firefly light intensity was read on a Luminometer TD-20/20, DLR Ready (Turner Designs Instrument, Sunnyvale, Calif). Cotransfection with 20 ng phRL-TK reporter was used to standardize for transfection variability. Cotransfection efficiency of Sp, MEF2, and DTEF-1 expression constructs was verified with pSV- β -galactosidase (Promega, Madison Wis) and/or pAAV-GFP staining. Reporter gene activity was expressed as mean $\pm$ standard deviation. Statistical comparisons were performed by paired *t* tests.

Nuclear Extracts

Cardiac muscle was harvested from 2-week-old lambs (control and shunted) at 4°C. Nuclear extracts were prepared by standard methods (Active Motif, Inc, Carlsbad, Calif).

Western Blot Analysis and Densitometry

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 μ g) on a 10% gel and followed by transfer to a polyvinylidene difluoride membrane (Amersham Biosciences, GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Membranes were then blocked and incubated with antibody as previously described.⁹ 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 loaded quantity.

Surgical Preparations and Care: Ewes

Pregnant, mixed-breed Western ewes (135–140 days' gestation; term = 145 days) were operated on under sterile conditions. Through a left lateral fetal thoracotomy, an 8.0-mm polytetrafluoroethylene vascular graft (2-mm length; W. L. Gore & Associates, Inc, Flagstaff, Ariz) was sewn between the ascending aorta and main pulmonary artery. All lambs were spontaneously delivered and were instrumented as previously described.²¹

Heart Tissue Preparation

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

Determination of DNA Content

DNA content was determined by standard methods (Genomic DNA mini-prep; Bay Gene Inc, San Francisco, Calif). Values were normalized to gram of cardiac muscle.

Statistical Analysis

Comparisons between shunt and age-matched controls were made by an unpaired *t* test.

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

Results

In Utero Placement of Aorta–Pulmonary Shunts Results in Growth Failure, Four-chamber Cardiomegaly, and Biventricular Hypertrophy

Shunted lambs showed clinical evidence of failure to thrive. Compared with control animals, shunted lambs were tachypneic and demonstrated less activity and lower tolerance to stress after 1 week of age. By 2 weeks of age, shunted lambs had significantly slower weight gain than controls. The hearts of shunted lambs were significantly larger than those of controls (Figure 1, Table 1, and Figure E1). All four chambers showed substantial increases in mass without significant increases in DNA content, suggesting that hypertrophy and not hyperplasia was the predominant stress response. The left ventricle was dilated and showed evidence of eccentric hypertrophy, while the right ventricle was predominantly thick-walled with evidence of concentric hypertrophy.

Hemodynamics of 2-week-old Shunted Lambs

Table 1 summarizes the hemodynamics of shunted versus control lambs at 2 weeks of age. Shunted animals had significantly higher mean pulmonary artery pressures with a mean pulmonary/systemic flow ratio of 2.9:1. Left and right

Figure 1. Gross pathology of shunted hearts at 2 weeks of age. *Left panel*, Photograph of shunted heart. *Right panel*, Photograph of control heart.

atrial pressures were significantly higher in shunted animals.

Sarcomeric Protein and Myocardial Transcription Factor Levels Are Altered During Pathophysiologic Shunting in the Neonatal Ovine Heart

Western blots of shunted (n = 5) and control (n = 5) hearts were analyzed by band densitometry. The hearts of 2-week-old shunted lambs showed significant increases in myosin and cTnT expression (Figure 2 and Figure 3, A and B). Fetal, high-molecular-weight isoforms of cTnT were up-regulated, consistent with the activation of a pathologic hypertrophic program (Figure 2). The increase in sarcomeric protein expression and associated fetal isoforms was seen in all four chambers. Associated with the increase in sarcomeric proteins, a variety of cardiac transcription factors known to govern cardiac gene expression during em-

TABLE 1. General hemodynamics in 2-week-old lambs

	Control lambs	Shunted lambs
Mean arterial blood pressure (mm Hg)	65 ± 7	55 ± 7
Pulmonary arterial pressure (mm Hg)	15 ± 2	22 ± 4*
Left pulmonary blood flow (mL · kg ⁻¹ · min ⁻¹)	60 ± 21	157 ± 28*
Heart rate (beats/min)	171 ± 24	174 ± 25
Left atrial pressure (mm Hg)	3 ± 1.7	7 ± 3.4*
Right atrial pressure (mm Hg)	2.2 ± 1.4	4.8 ± 3.1*
Pulmonary/systemic blood flow	1.00	2.91 ± 01*
Right ventricular weight (g)	11 ± 2	21 ± 5*
Left ventricular weight (g)	19 ± 4	31 ± 7*
Total cardiac weight (g)	49 ± 6	80 ± 8*

Values are means ± SD; n = 6 shunted lambs and 7 twin or age-matched controls. *P < .05 shunt versus control lambs; weight comparisons were performed for 5 control and 5 shunted lambs.

Figure 2. Characteristic Western blot of sarcomeric proteins and selected transcription factors in 2-week-old control and shunted lambs. Antibody used for Western blot analysis is listed on left. MF20 (antimyosin) and cTnT levels are increased in shunted animals, consistent with the hypertrophy and increased mass seen on gross pathologic examination. Fetal/embryonic isoforms of cTnT are expressed in the hypertrophied, shunted hearts. There are associated increases in DTEF-1, MEF2 (not shown), GATA4, NKX2.5, and Sp1 levels and concomitant decreases in Sp3 levels within the cardiac chambers (see Figure 5). β -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control proteins are comparable in shunted and control specimens. RA, Right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; Sept, interventricular septal muscle; MW (kDa), molecular weight in kilo-Daltons.

bryogenesis, growth, and development were upregulated (Figures 2 and 3). TEF-1 factors, which regulate muscle gene promoters (eg, cTnT, α - and β -myosin heavy chains, tropomyosins, β -acetylcholine receptor, and α -actin genes) through muscle CAT sites, were significantly upregulated in the right side of the heart (Figure 3, C). There was a trend to increased expression in the left atrium and left ventricle. Both GATA-4 and NKX2.5 levels were significantly in-

creased in all four chambers (Figure 3, D and E). The magnitude of the increase in NKX2.5 levels, although statistically significant, was small (Figure 3, E). Densitometric analysis of Sp factor expression showed that Sp1, a transcriptional activator of cardiac promoters, was increased in all four chambers; Sp3, a transcriptional repressor of cTnT promoter constructs, was significantly downregulated during pathologic hypertrophy.

Immunohistochemistry was performed to determine the cell type and compartment within which transcription factors were localized (Figures E2, E3, and E4). Previous studies have shown that MEF2, GATA-4, and NKX2.5 predictably localize to the nucleus of myocytes and act as transcriptional activators.^{5-7, 22-25} TEF-1, Sp1, and Sp3 are expressed within the myocyte population of the ovine heart and localize to the nucleus. By chromatin immunoprecipitation, DTEF-1, MEF2, GATA-4, NKX2.5, Sp1, Sp3, and poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) bind the ovine cTnT promoter “in vivo,” within the context of chromatin (Figure E5). Furthermore, anti-PAR and pan-acetyl antibody immunoprecipitated chromatin from cardiomyocytes that yielded a positive chromatin immunoprecipitation (ChIP) assay for cTnT binding, implicating that these epigenetic modifications (poly-ADP-ribosylation and acetylation) were present in the transcriptional proteins that bound the cTnT promoter in vivo. Co-immunoprecipitation experiments with MEF2 and neonatal cardiomyocytes confirmed that Sp factors, PARP, NKX2.5, and DTEF-1 directly interacted to potentially form a transcriptional complex (data not shown).

Transcriptional Effects on cTnT Promoter Activity

Having shown that selected cardiac transcription factors are modulated during pathologic hypertrophy and bind the cTnT promoter (and are associated with epigenetic modifications of acetylation and poly-ADP-ribosylation), in vivo, we sought to determine their transactivating effects. Expression constructs of DTEF-1A and B, MEF2C, Sp1, and Sp3 were cotransfected with cTnT promoter constructs in primary neonatal sheep cardiomyocytes in culture (Figure 4). Relative activity was indexed to a reporter gene driven by thymidine kinase to control for transfection variability. Both

isoforms of DTEF-1, A and B, transactivated the 268cTnT promoter approximately 3.2-fold and 3.6-fold above baseline, respectively (268cTnT = 16 ± 3 relative light units vs 268cTnT + DTEF1A = 51 ± 7 relative units; 268cTnT + DTEF-1B = 58 ± 6 relative units, $n = 5$ transfections per construct, $P < .01$). Cotransfection of MEF2C with the 268cTnT promoter construct resulted in a 4.3-fold increase in baseline reporter gene activity (268cTnT + MEF2C = 70 ± 6 relative units, $n = 5$, $P < .01$). Cotransfection of both MEF2C and DTEF-1B expression constructs produced a 5.7-fold increase in reporter gene activity (92 ± 8 relative units, $n = 5$, $P < .01$). Sp1 acted as a transcriptional activator of –268cTnT promoter constructs that were transiently transfected in ovine cardiomyocytes in culture, producing a 2.2-fold increase in baseline reporter gene activity (36 ± 6 relative units, $n = 5$, $P < .01$). Sp3 repressed baseline –268cTnT promoter activity by over 50% and inhibited Sp1-mediated transcriptional activation of the promoter (Figure 4).

Discussion

In utero placement of aorta–pulmonary shunts produces pathologic biventricular hypertrophy and clinical congestive heart failure in neonatal lambs. There are associated increases in constitutively expressed sarcomeric proteins and induction of fetal isoforms of contractile proteins in all four cardiac chambers. In this model, there is upregulation of myocardial transcriptional activators and downregulation of repressors. Furthermore, cardiac transcription factors also undergo changes in interactions with cardiac histone acetylating and deacetylating enzymes, as well as ADP-ribose polymerases, each of which are potential pharmacologic targets of PARP, HDAC, and HAT inhibitors.^{4,26}

In Vivo Model of Pathologic Cardiac Hypertrophy

Murine models of cardiac hypertrophy are favorable because the mouse is amenable to transgenic manipulation, allowing for the identification of molecular mechanisms of pathophysiologic processes. Aortic banding or constriction in the mouse, which has a heart rate of 500 beats/min and chamber dimensions in the millimeter range, is a commonly

Figure 3. Bar graphs of densitometric analysis of selected myocardial transcription factors during pathologic biventricular hypertrophy in neonatal lambs. Bands seen on Western blots of myocardial specimens from hearts of control ($n = 5$) and shunted ($n = 5$) neonatal lamb were analyzed by quantitative densitometry. Myosins and cardiac troponin protein expression are increased in all four chambers. TEF-1 proteins are primarily increased in the right side of the heart whereas NKX 2.5, GATA-4, and Sp1 are upregulated and Sp3 is downregulated in all four cardiac chambers. A, Myosin expression detected with MF20 antibody. B, Cardiac troponin T (*cTnT*). C, TEF-1 proteins. D, GATA-4. E, NKX2.5. F, Sp1. G, Sp3. β -actin (H) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (I) were used as controls. RA, Right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; Septum, interventricular septal muscle.

Figure 4. Luciferase reporter gene activity driven by –268cTnT promoter constructs that are transiently transfected in primary cultures of neonatal ovine cardiomyocytes. Baseline –268cTnT relative activity is increased when Sp1, DTEF-1A or B, and MEF2C are cotransfected. Cotransfection of Sp3 results in repression of baseline cTnT promoter activity. Sp3 inhibits Sp1-mediated gene activation. MEF2 and DTEF-1 have a synergistic effect on reporter gene activity. *Horizontal axis*, Cotransfected expression constructs. *Vertical axis*, Relative reporter gene activity indexed to TK-driven reporter to control for transfection variability. Cells alone, pGL2 basic vector, and pcDNA4HisMax expression constructs have undetectable activity (not shown). Anti-luciferase staining of cardiomyocyte cultures shows reporter gene expression restricted to myocyte population.

used animal model for the study of pathologic hypertrophy. Echocardiographic or hemodynamic analysis of murine models, although well established, requires special equipment and expertise. Compared with mouse models of pathologic hypertrophy, the ovine model has some advantages. It is amenable to standard echocardiographic and catheterization techniques and is not limited to the study of only pressure loading of the left side of the heart, but can be used to study other biomechanical stressors including volume loading of the heart. Furthermore, pathologic hypertrophy can be generated in either the left or right ventricle, or both, thus allowing for the determination of common or asymmetric transcriptional responses of either side of the heart.

The ovine model used in this study, that is, in utero placement of a large aorta-pulmonary shunt in fetal lambs, produces significant left-to-right shunting and increases in pulmonary blood flow and pulmonary hypertension. The left ventricular myocardium is stressed by persistent and significant volume loading, while the right ventricle eventually experiences pressure loading. This model is clinically relevant and mimics some congenital heart defects, such as patent ductus arteriosus, large ventricular septal defects, and aorta-pulmonary windows. Furthermore, volume loading of the left ventricle is also clinically seen with valvular heart disease such as aortic and mitral insufficiency. Right ventricular pressure overload is also commonly seen clinically,

as in pulmonary hypertension, tetralogy of Fallot, and pulmonary stenosis. Last, the model mimics the surgically created aorta–pulmonary shunts used for the management of single ventricle anomalies with inadequate pulmonary blood flow.

The central features of the cardiomyocyte hypertrophic response include increases in contractile protein content, induction of contractile protein isoforms, and expression of embryonic markers.¹¹ The observed protein biochemical changes in this model are consistent with expected changes in sarcomeric protein expression. There is an upregulation of constitutively expressed genes (myosins), as well as induction of fetal contractile and noncontractile cardiac genes (high-molecular-weight troponin isoforms and atrial natriuretic factor/brain natriuretic peptide [data not shown]). Proto-oncogenes and other members of the immediate-early gene programs, also known to be activated during hypertrophy, were not evaluated in this study, nor were regulators of cell survival and apoptosis.¹¹

The finding that TEF-1, NKX2.5, Sp1, and GATA factors are upregulated during the cardiac hypertrophic response is consistent with their chromatin binding and transactivating properties. Likewise, downregulation of Sp3 during pathologic hypertrophy is consistent with its inhibitory effects on cTnT promoter activity. These data suggest that the increase in sarcomeric proteins and induction of embryonic cardiac genes seen during pathologic hypertrophy are preceded and regulated by increases in myocardial transactivators, decreases in cardiac gene repressors, and induction of posttranslational modifications that modulate transcription factor function. These data do not provide direct evidence that TEF-1, MEF2, NKX2.5, GATA-4, and Sp1 upregulation and Sp3 downregulation, *in vivo*, are mechanistically responsible, in part, for the changes in sarcomeric protein expression that are seen during pathologic cardiac hypertrophy in the ovine model. Further studies are warranted to investigate these important mechanisms.

The TEF-1 family of transcription factors has been implicated in muscle gene regulation.²⁷ We have previously cloned and characterized DTEF-1, a cardiac-enriched family member that regulates cardiac gene expression and is expressed in the early embryo.^{9,10} During volume loading of the left ventricle and pressure loading of the right ventricle, TEF-1 polypeptides are upregulated to a greater degree in the right side of the heart. TEF-1 upregulation during biomechanical stress may therefore be an asymmetric, chamber-specific response or may act as a pressure-responsive rather than volume-responsive transactivator. Poly-ADP-ribose polymerase, a known coactivator of TEF-1,²⁸ has been implicated in myocardial cell hypoxic-ischemic injury and cardiomyocyte survival. In this model, we have also found that DTEF-1 becomes heavily

ADP-ribosylated (data not shown), a process amenable to inhibition and potential target for pharmacotherapy of heart failure.²⁹

The Sp family of transcription factors is important in the assembly of the basal transcriptional machinery, potentially acting as a central and widespread regulatory network in pathologic hypertrophy. We have previously shown that Sp1 and Sp3 have counterregulatory functions in avian myocytes.³⁰ Although Sp3 expression is downregulated during pathologic hypertrophy, we have found that Sp3-associated HAT activity is increased and HDAC activity is significantly decreased (Figures E6 and E7). Hence, acetylation of Sp3 may convert it from a repressor to a transcriptional activator during the induction of pathologic cardiac hypertrophy and heart failure.

Histone acetylation has been implicated in relaxing chromatin and globally exposing genes for transcription. Removal of acetyl groups is presumed to result in chromatin compaction, making some promoters inaccessible for transcriptional activation. Inhibition of HDAC activity, and particularly class I HDACs, has ironically been associated with attenuation of cardiac growth and hypertrophy *in vitro* and *in vivo*. The exact mechanism(s) by which HDAC inhibition attenuates hypertrophy is not entirely known. Class II HDACs are antihypertrophic, whereas class I HDACs may stimulate cardiac growth and sarcomeric genes. Selective HDAC or HAT inhibitors are being actively developed and are in trials for the treatment of cancer. As such, identification of the specific HATs or HDACs associated with hypertrophy-responsive transcription factors, such as GATA-4, DTEF-1, or Sp1/3, (1) may allow for the identification of the transcriptional mechanisms of pathologic hypertrophy and (2) may allow for the use of isoform-specific HAT/HDAC inhibitors to target pathologic hypertrophy and contractile dysfunction of the heart.

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Discussion

Dr Robert C. Robbins (Stanford, Calif). You went through it very quickly, but this is a tour de force; technically it is a very difficult model and you are to be congratulated for getting the animals through this. Also impressive is the sophisticated use of molecular genetics that you used here to help answer important questions. Certainly heart failure is an important public health issue. Finally, the experimental therapeutic approach to try to identify potential targets for developing small molecules or other strategies to treat heart failure is impressive.

I have just a couple of comments and questions for you.

GATA-4 and NKx2.5 are things that we are looking at as embryonic stem cells differentiate into cardiac myocytes. I thought it was interesting that you targeted these particular transcription factors. You mentioned the isoform shift of cardiac myosin in your manuscript, and a lot of work has been done by Jeff Robbins in this area. I would like to note that you mentioned cardiac myosin. Because it is so important in allograft rejection and autoimmune disease, can you comment on what you found with cardiac myosin specifically?

Dr Azakie. We used the MF20 antibody, which selects for myosins. The purpose of looking at changes in sarcomeric gene expression was to show that, in fact, there was biochemical hypertrophy. We did not look specifically for different myosin isoforms. What we wanted to show is that despite no change in DNA content, there was an upregulation in myosin proteins. The purpose in evaluating cTnT expression was to show that, in fact, an embryonic gene expression pattern induced a marker of the development of pathologic hypertrophy. We also looked at serum brain natriuretic peptide and found that it was significantly upregulated in shunted animals. We did not look at isoform-specific expression of different myosins.

Dr Robbins. You hit on other things that I was going to ask you about. Have you thought about using microarrays? I've been impressed that often this is a fishing expedition, but it can often direct you to other things. You have done it the old-fashioned way. You have picked a couple of things and gone after them in the classic sense. Have you thought about using microarrays to look for other targets? We have recently discovered a gene termed APLN in heart failure patients who had ventricular assist devices. I wonder whether you could get more targets if you would consider using microarray techniques.

Dr Azakie. We thought about using microarrays, but there are no good ovine arrays available now. They are in preparation. The main issue that my colleagues and collaborators have had with that approach is that it does not always necessarily provide a mechanistic answer toward the switch to a hypertrophic program.

I think the main message here resided in the posttranslational modifications and the functional assays that we performed. Neonatal ovine cardiomyocytes were cultured and cotransfected with cardiac promoters and expression constructs that encoded different transcription factors. We found that TEF-1 and Sp1 were transcriptional activators, whereas Sp3 was a repressor. GATA-4 and NKX2.5 were selected because they are very well-known transcriptional activators that are important for development and really set a standard.

The next level was to look at the posttranslational modifications that occurred, and specifically acetylation and ADP-ribosylation. Cardiac histone acetylation has been implicated in pathologic cardiac growth, and currently there are HDAC inhibitors that are being developed for use in cancer therapy in ongoing clinical trials.

Class I HDAC inhibitors have recently been shown to suppress the development of hypertrophy in a rodent model where the aorta was banded or angiotensin was infused.

The answer to the question, in short, is that we were interested in trying to determine the mechanisms by which the hypertrophic transcriptional program was initiated and that is why we did not do microarrays.

Dr Robbins. You mentioned in your manuscript about models of pressure overload to the left ventricle that are much easier to do, such as constriction of the ascending aorta in transgenic mice. This is very specific to the previous point. You are to be congratulated for focusing in and testing the specific hypothesis, but I wonder whether this is too narrow an area. I understand that your interest is in surgery for congenital heart disease, but how do you think that these data would translate into more generalizable models of ischemic cardiomyopathy or rapid pacing, other models of heart failure?

Finally, how do you choose the next step? Do you try to block Sp1 or do you try to increase Sp3 in pharmacologic methods?

Dr Azakie. This model is interesting because it mimics certain congenital heart defects, but the model can be tailored to produce pressure loading of the left ventricle.

With regard to other more common causes of congestive heart failure, I think that our hypothesis, and the hypotheses of others, is that although there are different types of biomechanical stressors, like pressure overload, volume overload, and ischemia-reperfusion injury, which stimulate different intracellular signaling pathways, we believe that ultimately there is a transcriptional reprogramming that develops within the nucleus of the cardiac myocyte and so really represents a final common pathway.

Finally, with regard to the next step for this line of research, I think our plan will be to use selective class I HDAC inhibitors as well as selective HAT inhibitors in determining whether or not those agents can either reverse the (volume-loaded) hypertrophic process or prevent the development of heart failure.

Dr Ludwig K. von Segesser (*Lausanne, Switzerland*). Did you consider going the other way around and closing the shunt to see how your molecular patterns precede or follow the morphology?

Dr Azakie. That's a very good question. We did not do that. Maybe doing that would be an interesting way to support the mechanisms that we are trying to infer here. It's a very good question and good suggestion. Thank you.

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Appendix E1: Chromatin Immunoprecipitation Supplement

Method: Chromatin Immunoprecipitation (ChIP)

All ChIP assays were performed on the basis of the protocols of the chromatin immunoprecipitation assay kit (Upstate, Lake Placid, NY) [Azakie, 2005—AJP]. Five micrograms of primary antibody against DTEF-1, GATA-4, MEF2, NKX2.5, Sp1, Sp3, PARP, or PAR was added to a 500 μ L chromatin sample for the immunoprecipitation reaction. ChIP dilution buffer and preimmune rabbit serum were used as controls for nonspecific interactions and DNA contamination. Anti- β -actin and anti-cTnT antibodies were used as negative controls.

Polymerase Chain Reaction

After DNA purification, samples were subjected to polymerase chain reaction with primers designed for the chick heart cTnT promoter (cTnT-268) as follows: upper primer: 5'-GCTGGCTG-GCTTGTGTCA-3'; lower primer: 5'-CTTGGGGGGCAGAG-GCTTT-3'.

The primers used for polymerase chain reaction were designed by a primer analysis software (Oligo 6.8; Molecular Biology Insights, Inc, Cascade, Colo). The amplified polymerase chain reaction product is 265 bp.

Figure E1. Shunted hearts showed 4-chamber cardiomegaly.

Figure E2. Immunohistochemistry of sheep myocardium double-stained with MF20 (red) antibody and Sp1 antibody (green immunofluorescence).

Figure E3. Immunohistochemistry of sheep myocardium double-stained with MF20 (red) antibody and Sp3 antibody (green immunofluorescence).

Figure E4. Immunohistochemistry of sheep myocardium double-stained with MF20 (red) antibody and DTEF-1 antibody (green immunofluorescence).

Figure E5. Transcriptional complexes bind the cTnT promoter in vivo. Polymerase chain reaction using primers specific for cTnT promoter on DNA purified after chromatin immunoprecipitation. After cross-linking and sonication, chromatin complexes from neonatal sheep heart nuclear preparations were purified. Chromatin was incubated with antibodies to DTEF-1, MEF2, GATA-4, NKx2.5, Sp1, Sp3, poly-ADP-ribose polymerase (PARP), poly-ADP-ribose (PAR), β -actin, cTnT, and preimmune serum. Separation of immune complexes by elution from protein A/G agarose beads was followed by reversal of protein-DNA crosslinks and DNA purification. *Lane A* depicts chromatin input DNA plus antibody. *Lanes A-E* include the ChIP "output" DNA for polymerase chain reactions. Immunoprecipitation of chromatin preparations was performed with antibodies listed on the left-sided column. *Lane F* contains ChIP buffer as the "DNA source." *Lane G* contains the control, "input DNA" to verify the quality of nuclear preparations. a, Output DNA; b, input DNA; c, polymerase chain reaction buffer and primers.

Figure E6. Histone acetyl transferase (HAT) activity associated with immunoprecipitated Sp3 is upregulated during pathologic cardiac hypertrophy.

Figure E7. Histone deacetylase activity associated with immunoprecipitated Sp3 is decreased during pathologic hypertrophy.