The Transcription Factor GATA-6 Regulates Pathological Cardiac Hypertrophy

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Rationale: The transcriptional code that programs maladaptive cardiac hypertrophy involves the zinc finger–containing DNA binding factor GATA-4. The highly related transcription factor GATA-6 is also expressed in the adult heart, although its role in controlling the hypertrophic program is unknown.

Objective: To determine the role of GATA-6 in cardiac hypertrophy and homeostasis.

Methods and Results: Here, we performed a cardiomyocyte-specific conditional gene targeting approach for Gata6, as well as a transgenic approach to overexpress GATA-6 in the mouse heart. Deletion of Gata6-loxP with Nkx2.5-cre produced late embryonic lethality with heart defects, whereas deletion with β-myosin heavy chain-cre (βMHC-cre) produced viable adults with >95% loss of GATA-6 protein in the heart. These latter mice were subjected to pressure overload–induced hypertrophy for 2 and 6 weeks, which showed a significant reduction in cardiac hypertrophy similar to that observed Gata4 heart-specific deleted mice. Gata6-deleted mice subjected to pressure overload also developed heart failure, whereas control mice maintained proper cardiac function. Gata6-deleted mice also developed less cardiac hypertrophy following 2 weeks of angiotensin II/phenylephrine infusion. Controlled GATA-6 overexpression in the heart induced hypertrophy with aging and predisposed to greater hypertrophy with pressure overload stimulation. Combinatorial deletion of Gata4 and Gata6 from the adult heart resulted in dilated cardiomyopathy and lethality by 16 weeks of age. Mechanistically, deletion of Gata6 from the heart resulted in fundamental changes in the levels of key regulatory genes and myocyte differentiation–specific genes.

Conclusions: These results indicate that GATA-6 is both necessary and sufficient for regulating the cardiac hypertrophic response and differentiated gene expression, both alone and in coordination with GATA-4.

Key Words: hypertrophy • transcription • differentiation • heart • genetically altered mice

The myocardium can hypertrophy in response to injury, changes in workload, or increases in wall stress. In response to hypertrophic stimuli, a fundamental reprogramming occurs within the adult cardiomyocyte that results in the expression of genes encoding fetal protein isoforms. Genes such as skeletal α-actin, β-myosin heavy chain (βMHC), b-type natriuretic peptide (BNP), and atrial natriuretic factor (ANF) become highly expressed within ventricular myocytes. Neural, humoral, and intrinsic stimuli result in the activation of membrane bound receptors that in turn activate specific intracellular signaling cascades such as mitogen-activated protein kinase, protein kinase C, insulin-like growth factor-1/Akt, and the calcium-activated protein phosphatase calcineurin. These intracellular signaling cascades then modulate transcriptional regulatory proteins, altering gene expression to facilitate the growth of the heart. For example, transcription factors such as myocyte enhancer factor-2 and NFAT (nuclear factor of activated T cells) are directly activated by cytoplasmic signaling effectors, which in turn mediate hypertrophic gene expression in cardiomyocytes. Similarly, the zinc finger–containing transcription factor GATA-4 can also serve as a terminal effector of the cardiac hypertrophic response following pathological stimuli by direct phosphorylation from kinases. GATA-4 can respond to mechanical load, vasopressin infusion, or direct stretching of the ventricles in an isolated rat heart. However, the role of a closely related family member GATA-6 as a hypertrophic mediator is unknown, although GATA-4/6 are known to compensate for one another in the heart.
Non-standard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ANF</td>
<td>atrial natriuretic factor</td>
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<tr>
<td>Ang II</td>
<td>angiotensin II</td>
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<tr>
<td>BNP</td>
<td>b-type natriuretic peptide</td>
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<td>fI</td>
<td>loxP site</td>
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<tr>
<td>FS%</td>
<td>fractional shortening percentage</td>
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<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
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<td>PE</td>
<td>phenylephrine</td>
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<tr>
<td>qPCR</td>
<td>quantitative PCR</td>
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<tr>
<td>TAG</td>
<td>transverse aortic constriction</td>
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<td>VW/BW</td>
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Six GATA transcription factors have been identified in vertebrates that are parsed into 2 subclasses based on their expression patterns. GATA-1, -2, and -3 are prominently expressed in hematopoietic cell lineages, whereas GATA-4, -5, and -6 are expressed in various mesoderm and endoderm derived tissues such as heart, liver, lung, gonad, and gut. GATA factors contain a highly conserved DNA binding domain consisting of two zinc fingers that interact with the nucleotide sequence element (A/T)GATA(A/G), which has been found in the promoters of most cardiac muscle-specific genes, especially those that are altered by the hypertrophic response. GATA-4 is expressed in the adult heart, where it is thought to function as a key transcriptional regulator of numerous cardiac genes including ANF, BNP, αMHC, βMHC, and many others.

Overexpression of Gata4 in culture by adenoviral gene transfer or in the hearts of transgenic mice each induced hypertrophy indicating the sufficiency of GATA-4 in this process. Expression of a dominant negative GATA-engrailed fusion protein or antisense Gata4 mRNA each blocked GATA-4–directed transcriptional responses and features of cardiomyocyte hypertrophy induced by phenylephrine (PE) and endothelin-1 in culture. GATA-4 is also directly phosphorylated by extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase as a means of enhancing hypertrophic gene expression. In vivo, heart-specific deletion of Gata4 reduced the hypertrophic response to pressure overload stimulation and rapidly led to failure with reductions in cardiac angiogenesis. Gata4 heterozygous mice were also more susceptible to heart failure after pressure overload stimulation, and showed more cardiac injury with doxorubicin administration. Although overexpression of Gata6 in cultured neonatal myocytes was sufficient to induce a hypertrophic response, its role in controlling cardiac hypertrophy in vivo has not been investigated.

**Methods**

All procedures used in this study were performed as described previously; however, a full listing of the methods and materials is given in the Online Data Supplement at CircRes.ahajournals.org. Mice used in these studies were either previously published or generated with the tetracycline-inducible, cardiac-specific promoter system. Transverse aortic constriction (TAC) was induced in mice aged 8 to 12 weeks, after which mice were studied during the indicated time periods. Hearts were excised at the end of the experiment, used for morphometric measurements, and stored in formalin or snap-frozen in liquid nitrogen until further use.

**Results**

**Heart-Specific Deletion of Gata6**

Standard gene targeting of Gata6 in the mouse results in early embryonic lethality just before gastrulation, necessitating the use of conditional gene targeting with the cre-loxP system to investigate cardiogenesis and adult heart functions of this transcription factor. Here, we crossed Gata6fl/fl mice with Nkx2.5-cre “knock-in” mice and βMHC-cre transgenic mice. The Nkx2.5-cre allele, which is typically used to investigate early embryonic heart development given its early expression, resulted in efficient deletion of Gata-6 protein from the embryonic day 14.5 heart, as assessed by Western blotting, and complete embryonic lethality by birth (Online Figure I, A and B). Examination of hearts from these embryos showed prominent ventricular septal defects, irregular septal thicknesses, and some loss of trabeculae (Online Figure I, C).

Use of the βMHC-cre transgene in slightly later embryonic deletion of loxP-targeted alleles from the heart, which bypassed embryonic lethality and produced viable adults. Western blotting of protein extracts from hearts of 1-day-old Gata6fl/flβMHC-cre mice showed a near complete absence of GATA-6 protein, as did immunohistochemistry in 8-week-old hearts of the same genotype (Figure 1A and 1B). Quantitation of nuclei from hearts of Gata6fl/flβMHC-cre mice showed that deletion was achieved in more than 95% of all cells (P<0.05).

Western blotting for GATA-6 usually detects 2 isoforms of GATA-6 arising from the same gene through alternative translational start sites. However, their relative importance or potency in vivo is not known, although the shorter isoform is homologous in size with the single isoform present in other vertebrates that are parsed into 2 subclasses based on their expression patterns. Gata6fl/fl mice with MHC-cre transgenic mice showed a near complete absence of GATA-6 protein, as assessed by Western blotting, and complete embryonic lethality by birth (Online Figure I, A and B). Examination of hearts from these embryos showed prominent ventricular septal defects, irregular septal thicknesses, and some loss of trabeculae (Online Figure I, C).

We also generated Gata6fl/flβMHC-cre mice in parallel for comparison throughout our study, and remarkably, only the loss of Gata6 from the newborn heart (3 days-old) produced a reduction in total GATA DNA binding activity as measured by gel shift analysis (Figure 1C; Online Figure II; see Discussion). However, this reduction in total GATA binding activity in Gata6 heart-deleted mice may also reflect an observed significant downregulation of GATA-4 protein (Figure 1A). Finally, we also confirmed that the Gata6-loxP allele was not hypomorphic on its own, as protein expression of GATA-6 in the heart was identical to βMHC-cre control hearts (Online Figure III, A).

Although Gata6fl/flβMHC-cre mice were overtly normal well into adulthood, we did identify differences in their hearts compared with βMHC-cre and/or Gata6fl/fl mice (referred to as “control” because no differences were indentified in these 2 groups versus wild-type mice). For example, at 8 weeks of age Gata6fl/flβMHC-cre mice showed a small, albeit significant reduction in heart size compared with control mice (Figure 1D). Isolation and measurement of adult cardiomyocyte dimensions from Gata6fl/flβMHC-cre mice actually showed a significant increase in length and total area, despite the fact that these hearts are smaller (Figure 1E through 1G). This
results suggest that loss of Gata6 impacted cardiomyocyte number in the heart, and indeed, measurement of myocytic DNA synthesis during the early neonatal period by bromodeoxyuridine incorporation and phospho-histone H3 analysis showed a significant reduction (Figure 1H and 1I). These results suggest that Gata6fl/fl mice have slightly smaller hearts with fewer myocytes compared with control mice. Despite these subtle differences, cardiac function was not different at baseline up to one year of age as assessed by echocardiography (Online Table I).

Reduced Pathological Hypertrophy in Gata6 Heart-Deleted Mice

We previously showed that deletion of Gata4 from the heart resulted in less pathological cardiac hypertrophy after 4 weeks of pressure overload stimulation.15 The adult heart also expressed GATA-6, which is nearly identical to GATA-4 in the DNA binding Zn-finger domains, suggesting that if it indeed recognizes and regulates the same downstream genes, it might similarly affect the hypertrophic response. Indeed, we determined that both GATA-4 and GATA-6 protein levels are increased in pressure overload–induced hypertrophic hearts, consistent with a very prominent increase in total GATA DNA binding activity by this stimulus (Figure 2A and 2B). To begin to elucidate the functional role that GATA-6 and GATA-4 play in regulating the cardiac hypertrophic response we subjected 8-week-old Gata6fl/flβMHC-cre mice to 2 weeks of TAC stimulation. We first confirmed that the pressure overload stimulus was similar between groups (Online Figure III, B). Survival up to 6 weeks after surgery was similar between different groups (data not shown). Remarkably, Gata6fl/flβMHC-cre mice showed a significant reduction in the hypertrophic response compared to either Gata6fl/fl or βMHC-cre control groups at both the whole organ and cellular level (Figure 2C and 2D; Online Figure III, C). This defect in cardiac hypertrophy in the absence of GATA-6 also correlated with a greater sensitivity to cardiac dysfunction and heart failure, as ventricular fractional shortening was significantly reduced only in Gata6fl/flβMHC-cre mice after 2 weeks of TAC (Figure 2E). Similarly, hearts from Gata6fl/flβMHC-cre mice generally showed a greater induction of mRNA for fetal genes associated with heart failure and stress stimulation (Figure 2F through 2H). Despite the onset of heart failure and greater stress reactivity, no difference in cardiomyocyte death rates were observed between any of the surgical groups (data not shown).

We also conducted a parallel study in Gata6fl/flβMHC-cre mice subjected to 2 weeks of TAC stimulation (not previously done at this time point) to directly assess the potential difference in magnitude of effect versus Gata6 deletion. Gata4fl/flβMHC-cre mice also showed a significant reduction in the hypertrophic response at both the organ and cellular levels after 2 weeks of TAC, although the reduction was similar to that observed in Gata6fl/flβMHC-cre mice (Figure 3A and 3B). Gata4fl/flβMHC-cre mice also showed a similar reduction in ventricular performance by echocardiography after 2 weeks of TAC to that observed in Gata6fl/flβMHC-cre mice (Figure 3C). These results begin to suggest that GATA-4 and

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Figure 1. Baseline characterization of Gata6-deleted hearts. A, Western blot for GATA-6 and GATA-4 in wild-type (Gata6fl/fl) and Gata6-deleted hearts from 1-day-old mice. Arrows indicate the long and short isoforms of GATA-6. B, Immunohistochemistry for GATA-6 (green), wheat germ agglutinin (red, membranes), and TO-PRO 3 (blue, nuclei) from hearts of 8-week-old control and Gata6-deleted mice. Original magnification, ×400. C, Gel-shift assay for total GATA binding activity from 1-day-old heart lysates from control (Gata4fl/fl and Gata6fl/fl) or MHC-cre, Gata4-deleted (Gata4fl/flβMHC-cre), and Gata6-deleted (Gata6fl/flβMHC-cre) mice. D, Ventricular weight to body weight (VW/BW) ratio in 8-week-old mice. E, Adult cardiomyocytes isolated from 8-week-old wild-type and Gata6-deleted hearts stained for α-actinin (red). Original magnification, ×600. F, Length measurements from isolated adult cardiomyocytes, 3 mice per group, were analyzed, and total number of cells is shown in the bars. G, Total surface area from isolated adult cardiomyocytes, 3 mice per group, were analyzed, and total number of cells analyzed is shown in the bars. H, Bromodeoxyuridine (BrdU) positively stained nuclei per field from control and Gata6-deleted hearts from 3-day-old mice. The number of mice or cells analyzed is shown in the bars of each graph. *P<0.05 vs control (for all graphs).
GATA-6 may have redundant functions in controlling the cardiac hypertrophic response.

The results discussed above were obtained in mice subjected to pressure overload stimulation, but it was also of interest to determine whether loss of GATA-6 from the heart impacted hypertrophy attributable to neuroendocrine-like stimuli. To this end we implanted osmotic minipumps in young adult mice for 2 weeks containing angiotensin II/PE (Ang II/PE). As expected, the combination of Ang II/PE induced hypertrophic growth of hearts from control mice, although Gata6fl/fl/H9252MHC-cre mice showed significantly less heart growth (Figure 4A). As with TAC stimulation, this defect in hypertrophic growth in Gata6fl/fl/H9252MHC-cre mice also correlated with a significant reduction in cardiac function as assessed by fractional shortening and more ventricular dilation (Figure 5B and 5C). Similarly, hearts from Gata6fl/fl/H9252MHC-cre mice showed a greater induction of mRNA for fetal genes associated with heart failure and stress stimulation after Ang II/PE infusion, compared to hearts from control mice (Figure 4D through 4F). Taken together, these results indicate that loss of GATA-6 from the adult heart compromises the hypertrophic program to pathological stimulation, rendering the heart more susceptible to dysfunction.

**Gata6 Heart-Deleted Mice Are More Susceptible to Heart Failure**

The observation of reduced ventricular performance in Gata6fl/fl/H9252MHC-cre mice after only 2 weeks of TAC suggested a predisposition to heart failure. To examine this effect in more detail, we subjected Gata6fl/fl/H9252MHC-cre mice to 6 weeks of TAC stimulation (Online Table II). These mice continued to show reduced ventricular performance at 2, 4, and 6 weeks after induction of TAC, compared with no significant reduction in βMHC-cre or Gata6fl/flTAC. Gata6fl/fl/H9252MHC-cre mice also correlated with a significant reduction in cardiac function as assessed by fractional shortening and more ventricular dilation (Figure 5B and 5C). Similarly, hearts from Gata6fl/fl/H9252MHC-cre mice showed a greater induction of mRNA for fetal genes associated with heart failure and stress stimulation after Ang II/PE infusion, compared to hearts from control mice (Figure 4D through 4F). Taken together, these results indicate that loss of GATA-6 from the adult heart compromises the hypertrophic program to pathological stimulation, rendering the heart more susceptible to dysfunction.

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**Figure 2. GATA-6 is required for pressure overload–induced cardiac hypertrophy.** A, Western blot for GATA-4 and GATA-6 from cardiac nuclear protein extracts from sham- and TAC-operated wild-type mice. Lamin A/C was used as a loading control. B, Gel-shift assay for total GATA binding activity in sham- and TAC-operated adult, wild-type mice after 2 weeks of pressure overload. *P<0.05 vs sham; †P<0.05 vs βMHC-cre and Gata6fl/flTAC. C, DNA-microarray slides showing expression of ANF mRNA in sham- and TAC-operated mice. *P<0.05 vs sham; †P<0.05 vs control (Gata4fl/fl) TAC. D, Cardiomyocyte cell surface area measured from histological sections. *P<0.05 vs sham; †P<0.05 vs βMHC-cre and Gata6fl/flTAC. E, Cardiac fractional shortening percentage (FS%) from sham- and TAC-operated mice after 2 weeks of pressure overload, measured by M-mode echocardiography. *P<0.05 vs βMHC-cre and Gata6fl/flTAC. Number of mice analyzed is shown in the bars. F, TaqMan qPCR for expression of ANF mRNA in sham- and TAC-operated mice. *P<0.05 vs sham; †P<0.05 vs control TAC. G, TaqMan qPCR for expression of αMHC mRNA in sham- and TAC-operated mice. *P<0.05 vs βMHC-cre and Gata6fl/fl sham; †P<0.05 vs ∆MHC-cre and Gata6fl/fl TAC. H, TaqMan qPCR for expression of skeletal α-actin mRNA in sham- and TAC-operated mice. *P<0.05 vs βMHC-cre and Gata6fl/flTAC.

**Figure 3. GATA-4 is required for pressure overload–induced cardiac hypertrophy.** A, VW/BW from sham- and TAC-operated adult mice after 2 weeks of pressure overload. *P<0.05 vs sham; †P<0.05 vs control (Gata4fl/fl) TAC. B, Cardiomyocyte cell surface area measured from histological sections. *P<0.05 vs sham; †P<0.05 vs control TAC. C, Cardiac FS% from sham- and TAC-operated mice after 2 weeks of pressure overload. †P<0.05 vs control TAC. Number of mice analyzed is shown in the bars.
hemodynamics with a Millar catheter showed a trend toward a baseline reduction in Gata6fl/flMHC-cre mice, with a significant reduction after TAC stimulation compared with no deficit in control mice (Figure 5D). Indeed, Gata6fl/flMHC-cre mice showed greater increases in left ventricular wall stress compared with control mice after 6 weeks of TAC, as well as significant elevations in fibrotic content in the heart by hydroxy-proline quantitation (Figure 5E and 5F). Interestingly, after 6 weeks of TAC stimulation the significant decrease in organ-level cardiac hypertrophy observed at 2 weeks was lost (Figure 5G). However, hearts from Gata6fl/flMHC-cre mice showed a robust dilatory response as measured by echocardiography and histological methods (Figure 5H and 5I). Increases in heart weight during heart failure can occur through a dilatory process associated with only an addition of sarcomeres in series.23 Indeed, measurement of cellular hypertrophy still showed a significant reduction in myocyte cross-sectional surface areas in hearts from Gata6fl/flMHC-cre mice after 6 weeks of TAC, suggesting that cellular hypertrophy was still inhibited in the absence of GATA-6 (Figure 5J).

Overexpression of GATA-6 Enhances Cardiac Hypertrophy

We have previously shown that Gata4 overexpression in the heart with the αMHC promoter produced baseline hypertro-

Figure 4. GATA6 is required for the Ang II/PE induced cardiac hypertrophy response. A, VW/BW after 2 weeks of vehicle (Veh) or Ang II/PE infusion using osmotic minipumps. *P<0.05 vs Gata6fl/fl vehicle; †P<0.05 vs Gata6fl/fl Ang II/PE. B, Cardiac FS% from vehicle- and Ang II/PE-infused mice. *P<0.05 vs Gata6fl/fl Ang II/PE. C, Left ventricular end diastolic dimension (LVED) after 2 weeks of vehicle or Ang II/PE infusion. *P<0.05 vs Gata6fl/fl Ang II/PE. D, TaqMan qPCR for expression of ANF mRNA in vehicle- and Ang II/PE-infused mice. *P<0.05 vs Gata6fl/fl Ang II/PE.

Figure 5. Gata6-deleted mice are susceptible to heart failure. A, Cardiac FS% at 0, 2, 4, and 6 weeks of TAC stimulation in control (αMHC-cre and Gata6fl/flMHC-cre) and Gata6fl/flMHC-cre mice. *P<0.05 vs control (αMHC-cre). B, Lung weight to body weight ratio (LV/BW) 6 weeks after TAC stimulation. *P<0.05 vs control. C, Left ventricular end-diastolic pressure (LVEDP) from sham- and TAC-operated animals 6 weeks after surgery, measured by invasive hemodynamics. *P<0.05 vs Sham; †P<0.05 vs control TAC. D, Contractility (+dP/dt) by invasive hemodynamics 6 weeks after a sham or TAC procedure. *P<0.05 vs Gata6fl/flMHC-cre sham; †P<0.05 vs control TAC. E, Left ventricular free wall stress from control and Gata6-deleted mice 6 weeks after TAC. *P<0.05 vs control. F, Hydroxy-proline content as a measure of fibrosis from sham- and TAC-operated hearts 6 weeks after surgery. *P<0.05 vs sham; †P<0.05 vs control TAC. G, VW/BW 6 weeks after TAC in the indicated groups of mice. H, Left ventricular end diastolic dimension (LVED) 6 weeks after TAC, measured by echocardiography. *P<0.05 vs control. I, Hematoxylin/eosin-stained heart sections from sham- and TAC-operated mice 6 weeks after surgery from control and Gata6fl/flMHC-cre mice. Original magnification, ×0.75. J, Cardiomyocyte surface areas measured from cardiac histological sections 6 weeks after TAC. *P<0.05 vs control. The number of animals measured is shown in the bars of the various graphs.
to model the known increase of GATA-6 protein in the heart following TAC stimulation (Figure 2A), here we generated heart-specific GATA-6 expressing transgenic mice using the tet-inducible system (Figure 6A). We generated 3 lines of transgenic mice that were classified as high (8.5-fold), medium (5.5-fold), and low (3.5-fold) overexpression based on Western blotting (Figure 6B). The low and medium overexpressing lines approximated the known increase in GATA-6 protein observed after TAC stimulation. At 8 weeks of age, none of the 3 lines of mice showed cardiac hypertrophy, although by 40 weeks of age, the medium and high overexpressing lines showed an increase in heart weight normalized to body weight (Figure 6C and 6D). This profile of cardiac hypertrophy in GATA-6 transgenic mice is reminiscent of data obtained in GATA-4 transgenic mice in which hypertension was not observed until 6 months of age. More importantly, TAC stimulation for 2 weeks in 8-week-old GATA-6 transgenic mice showed enhanced cardiac hypertrophy at the organ and cellular level in medium and high expressing lines (Figure 6E and 6F). Echocardiographic assessment of cardiac dimensions and function showed no significant changes between the different groups after TAC (Online Table III). Although wall dimensions were higher in the transgenic mice after TAC, this did not reach statistical significance. Taken together, these results indicate that overexpression of GATA-6 in the heart can enhance cardiac hypertrophy, although it did not appear to worsen heart disease.

**Deletion of Gata6 From the Heart Alters Homeostatic Gene Expression**

To begin to understand the transcriptional regulatory mechanisms whereby loss of GATA-6 from the heart leads to heart failure, dilation, and less cellular hypertrophy, we performed an analysis of global gene expression in hearts from 9-week-old Gata6fl/flβMHC-cre mice. We identified 21 genes that were significantly altered in these hearts compared with hearts from Gata6fl/fl and βMHC-cre mice. A select group of these genes that had mechanistic implications in the hypertrophy or failure response were confirmed by quantitative (q)PCR (Figure 7). For example, deletion of GATA-6 protein from the heart resulted in upregulation of βMHC, Lrrcc1, Lamc2, BNP, and downregulation of Adamts3, Pde1c, and Egf (Figure 7A through 7G). BNP and βMHC genes are activated by stress to the heart, although both also contain important GATA DNA binding sites in their promoters. Pde1c is a major dual specificity cAMP and cGMP diesterase implicated in cardiac contractility, whereas Egf has been implicated in heart failure through development of cardiotoxicity after trastuzumab treatment (epidermal growth factor receptor blocker) in breast cancer patients. In addition, we found a number of upregulated and downregulated genes for which the importance in the heart is less clear, although they are clearly central regulatory proteins. For example, the centrosomal protein Lrrcc1 is required for spindle pole integrity during cell division, and Adams3 encodes a protein that contains both metalloproteinase domains and thrombospondin motifs. These results suggest that loss of GATA-6 from the heart dramatically alters the expression of diverse regulatory genes that could impact heart failure propensity and the hypertrophic response.

**Combined Deletion of Gata4 and Gata6 Results in Heart Failure and Death**

To further investigate the issue of redundancy between GATA-4 and GATA-6 in the adult heart, we generated mice with combined deletion of both Gata4 and Gata6 using the βMHC-cre transgene, which surprisingly produced offspring at expected Mendelian frequencies (data not shown). However, these mice began to show signs of heart failure, and by 16 weeks of age, all Gata4fl/flGata6fl/flβMHC-cre mice had died (Figure 8A). Examination of mice and hearts just before this lethality time point showed overt heart failure by echocardiography with extremely thin myocardial walls and dilation (Figure 8B and 8D). Echocardiographic assessment of cardiac function showed significantly reduced fractional shortening at 12 weeks of age (Figure 8C). Although these experiments do not prove redundancy, they do suggest that GATA-4 and GATA-6 may have an additive effect in the adult heart.

**Discussion**

In the early developing embryo, Gata4 and Gata6 were shown to be of critical importance for the establishment of the...
entire cardiac gene program, because loss of both factors, but not either alone, results in acardia. This result demonstrates the importance of GATA transcription factors as necessary regulators of cardiac differentiation-specific gene expression; hence, it was not entirely unexpected when GATA-4 was shown to be necessary for cardiac hypertrophy in the adult heart, as this process requires re-establishment of the fetal gene program. Although almost nothing is known about the role of GATA-6 in regulating hypertrophy or differentiation specific gene expression in the adult heart, GATA-4 and GATA-6 are each capable of inducing hypertrophy when overexpressed in neonatal rat cardiomyocytes, suggesting for the first time that GATA-6 might function similar to GATA-4 in this respect. Indeed, GATA-4 can functionally and physically interact with GATA-6 in activation of the ANF and BNP promoters. Although GATA-4 and GATA-6 have been shown to positively regulate BNP and MYH promoters in cultured cardiomyocytes, we actually observed upregulation of BNP and βMHC expression in the absence of GATA-6. It is likely that gene regulation in vivo is distinctly different from conditions that occur with transfection of minimal promoters and co-overexpression of GATA-4 and/or GATA-6 in cultured cells. Also, these minimal promoter constructs that were transfected into neonatal myocytes could also easily lack inhibitory GATA binding sites or other negative regulatory sites that can bind secondary transcription factors induced by GATA-4/6.

Here, we showed for the first time, that in addition to GATA-4, GATA-6 protein expression is dramatically enhanced in mouse hearts subjected to pressure overload stimulation, which correlated with a dramatic increase in total GATA DNA binding activity. It was intriguing that deletion of Gata6 from the neonatal mouse heart, but not Gata4, severely reduced total GATA DNA binding activity assayed with a canonical GATA binding site from the βMHC promoter. This suggests that GATA-6 protein levels may be higher than GATA-4 in the postnatal heart, although the overall transcriptional potency of both proteins may differ such that GATA-4 may still be just as critical. In addition, we found a significant reduction in GATA-4 protein in the Gata6-deleted hearts, which could contribute to the reduced DNA binding activity. Intriguingly, this result hints at a...
positive regulatory relationship between GATA-6 and GATA-4 in the adult heart.

We modeled the observed increase in GATA-6 protein levels after pressure overload stimulation using a transgenic approach. The low and medium expressing line approximated this increase in endogenous GATA-6 with hypertrophy, suggesting that our transgenic approach closely model this known increase. Finally, and perhaps more importantly, deletion of Gata6 from the mouse heart significantly reduced the hypertrophic growth response, both after TAC and after stimulation with Ang II/PE, together suggesting that GATA-6 functions as a necessary and sufficient mediator of adult cardiac hypertrophic growth.

That GATA-6 appears to be equally important to GATA-4 in regulating the cardiac hypertrophic response might suggest a degree of functional redundancy between these 2 transcription factors. Indeed, we previously demonstrated that Gata4+/−Gata6+/− (double heterozygotes) are not viable and perish during midgestation, whereas single heterozygotes for either gene were viable.9 In the adult heart, the phenotype of reduced cardiac hypertrophy after 2 weeks of TAC was nearly identical between Gata4 and Gata6 heart-deleted mice, and each succumbed to heart failure and reductions in cardiac function to a roughly similar extent. Moreover, GATA-4- and GATA-6-overexpressing transgenic mice each developed similar levels of spontaneous hypertrophy at about the same time in middle adulthood.13 Finally, deletion of both Gata4 and Gata6 specifically from the heart resulted in spontaneous heart failure and death by the age of 16 weeks. Although these 'collective' observations suggest functional redundancy between GATA-4 and GATA-6, some differences were noted that might suggest unique functions for each. First, Gata4fl/fl/Gata6−/− mice showed spontaneous deterioration of cardiac function with aging, whereas Gata4fl/+Gata6−/− mice showed more preserved cardiac function up to 1 year of age, as assessed by echocardiography (although invasive hemodynamics showed a trend toward reduced baseline function with Gata6 deletion). Similarly, we found a slight but significant reduction in heart size at baseline in Gata6-deleted mice resulting from decreased cardiomyocyte content in the heart, whereas we did not observe this effect in Gata4-deleted mice. However, Pu and colleagues did observe a decrease in myocyte proliferation in the hearts of Gata4 heart-specific hypomorphic mice (their targeted allele had partial expression), suggesting that there could still be functional similarity between GATA-4 and GATA-6 with respect to control of cell number in the heart.29 Thus, our working hypothesis is that GATA-4 and GATA-6 are roughly equivalent in effect in the heart, such that each of the 4 alleles functions in a dosage-dependent manner. However, it will be important to generate combinations of Gata4 and Gata6 allelic deletions, as well as conduct rescue approaches whereby the GATA-4 transgene is crossed into the Gata6 heart-specific deleted background, and vice versa, before we can unequivocally determine whether these 2 factors are completely redundant in function in the heart or if some unique regulatory actions persist between them.

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Disclosures
None.

References

**Novelty and Significance**

**What Is Known?**

- GATA-6 can induce hypertrophy in cultured cardiomyocytes.
- After pressure overload, GATA binding activity increases in the murine heart.
- GATA-4 has been shown to be both required and sufficient for cardiac hypertrophy in vivo.

**What New Information Does This Article Contribute?**

- We provide the first description of cardiac-specific deletion of both GATA-4 and GATA-6 from the adult heart, which results in spontaneous dilated cardiomyopathy.

This study was designed to evaluate the importance of GATA-6 in vivo for cardiac hypertrophy and compensation. We studied both GATA-6 overexpression and deletion as effectors of pressure overload–induced hypertrophy. We also studied the role of GATA-6 deletion in neurohormonal-induced hypertrophy. Finally, we also studied GATA-4 deletion as a comparison and both GATA-4 and GATA-6 deletion to begin to address the possible redundancy between these transcription factors. Our results show the requirement of GATA-6 for cardiac hypertrophy and compensation in response to pressure overload and neurohormonal stimulation. In addition, we show the both GATA-4 and GATA-6 are required to maintain normal cardiac function suggesting they may act redundantly.
The Transcription Factor GATA-6 Regulates Pathological Cardiac Hypertrophy
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SUPPLEMENTAL MATERIAL

Supplemental Materials and Methods

Mice

The generation of Gata6 loxP-targeted (fl) mice, in which exon 2 was flanked by loxP sites to allow tissue specific deletion, was previously described.\(^1\) Mice harboring the Gata6fl/fl alleles were crossed with mice expressing Cre recombinase under control of the endogenous Nkx2.5 locus,\(^2\) or the βMHC promoter.\(^3\) For embryonic studies Gata6fl/fl littermates were compared against Gata6fl/fl\(^{Nkx2.5-cre}\) embryo’s. For adult studies, Gata6fl/fl littermates and βMHC-cre transgenic mice were independently generated from the same back-cross used to generate Gata6fl/fl\(^{MHC-cre}\) mice. The Gata4fl/fl and Gata4fl/fl\(^{MHC-cre}\) mice were described previously.\(^3\) Gata6 overexpressing transgenic mice were generated with the tet-inducible αMHC promoter expression vector system described previously.\(^4\) This system requires crossing with a tetracycline transactivator (tTA) transgenic line as described previously.\(^3\) All experiments involving animals were approved by the Institutional Animal Care and Use Committee at Cincinnati Children’s Hospital Medical Center.

Western blotting, gel shift assay, and hydroxy-proline assay

Western blot analysis of mouse neonatal heart homogenates was performed as previously described.\(^5\) Adult hearts were first fractionated to enrich for nuclear proteins before western blotting. Antibodies used were GATA-4 (C-20) from Santa Cruz Biotechnologies, GATA4 (AF2606) and GATA6 (AF1500) from R&D systems and lamin A/C (2032) from Cell Signaling Technology.

Gel shift assays were performed as previously described.\(^6\) Briefly, neonatal mouse heart homogenates (30 µg) were incubated in gel shift buffer (12 mM HEPES, pH 7.9, 4 mM Tris, pH 7.9, 50 mM KCl, 12% glycerol, 1.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.7 µg/ml pepstatin) with 0.5 µg poly (dI-dC) and \(^{32}\)P-labeled GATA binding site in the αMHC promoter\(^6\) for 20 min at room temperature followed by non-denaturing gel electrophoresis. As a negative control, we used the same probe with mutated GATA sites.\(^6\) Hydroxy-proline content to assay for fibrosis was performed as previously described.\(^3\)

Cardiomyocyte isolation, immunolocalization studies and myocyte proliferation
Adult cardiomyocytes were isolated as previously published. Following isolation, cardiomyocytes were attached to laminin coated glass coverslips. Immunocytochemistry was performed with α-actinin for sarcomeric staining (Sigma), counterstained with ALEXA Fluor 568 anti-mouse secondary antibody (Molecular probes). Antibody incubations were performed at 4°C for at least 1 hour and cells were washed 3 times between antibody incubations with PBS containing 0.1% Tween. After the last wash, the coverslip was inverted and mounted with Vectashield (Vector laboratories). Immunohistochemistry was performed on sectioned paraffin embedded hearts as described previously. Sections were also stained with wheat germ agglutinin (WGA)-TRITC (Sigma) to show myocyte membranes in histological sections. Cell surface area was assessed in at least 3 animals per group with at least 3 randomly taken sections per heart and at least 100 myocytes were counted per animal. To assess myocyte DNA synthesis in vivo, 3-day old pups were injected with BrdU (i.p.) at 10 mg/ml. Four hours later, hearts were excised and embedded in OCT, sectioned and stained with anti-BrdU (Invitrogen) or anti-phospho serine-10-histone H3 antibody (Cell Signaling Technologies). At least 3 animals per genotype were assessed and at least 3 randomly taken sections per heart were quantified. We quantified a total of at least 1000 nuclei per heart for BrdU and at least 4500 nuclei per heart for the phospho-serine-10-histone H3 stainings.

Pressure overload, osmotic minipumps, echocardiography and invasive hemodynamics for cardiac function

All mice were anesthetized with 2% Isoflurane by inhalation. Echocardiography was performed in M-mode using a Hewlett Packard SONOS 5500 instrument equipped with a 15 MHz transducer as described previously. Invasive hemodynamics was performed using the closed chest approach by cannulating the right carotid artery with a Millar pressure transducing catheter placed through the aorta and into the left ventricle. Recordings were made using a Millar MPVS-400 integrated with ADinstruments Powerlab technology and further analyzed using Labchart software. Cardiac hypertrophy was induced by either transverse aortic constriction (TAC) to produce pressure overload as previously described, or by using Alzet osmotic minipumps (Durect corp.) that continuously infused angiotensin II (432 µg/kg/d) and phenylephrine (100 mg/kg/d) for 2 weeks.

mRNA expression analysis
RNA was extracted from snap-frozen ventricles of 9-week old mice after homogenization using Trizol (Invitrogen) and a glass Dounce homogenizer. mRNA was processed for hybridization on Affymetrix mouse set ST1.0 chips for gene expression profiling according to the manufacturer’s protocols. Genes with significant expression differences between βMHC-cre (2 samples), Gata6fl/fl (2 samples) and Gata6fl/flβMHC-cre (3 samples) were selected with a Student’s t test with a p value cutoff of 0.05 and an average fold change greater than 1.5 as described previously. Selected gene differences were confirmed by real-time qPCR using SYBR green (Applied Biosystems). Reactivation of the fetal gene program was measured using RNA extracted with a fibrous tissue kit according to supplied protocol (Qiagen). Individual Taqman gene expression assays were ordered from Applied Biosystems. Quantified mRNA expression was normalized to GAPDH and PPIA and expressed relative to the control.

Statistics

All results are presented as mean ± SEM. Statistical analyses were performed in Microsoft Excel and Prism using unpaired t-test for 2 groups, single condition, 1-way ANOVA with Tukey post-test analysis for multiple groups, single condition and 2-way ANOVA with Bonferroni post-test analysis comparing each group to every other group for 2 or more groups, more than 1 condition. Survival curves were compared using a log-rank test. A p value of <0.05 was considered significant.

References


Supplemental Figure I. Developmental phenotype of Gata6fl/flNkx2.5-cre mice. A. Western blot for GATA-6 and GATA-4 from cardiac protein lysates from embryo's at day 14.5. B. Survival curve of wildtype and Gata6-deleted embryo's. Time is indicated in embryonic developmental day after conception. C. Immunohistochemistry of control and Gata6fl/flNkx2.5-cre embryo's at E16.5 showing ventricular septum defect (arrow) and thinned (arrowhead) septum in Gata6-deleted embryos.
Supplemental Figure II. Gel shift for total GATA DNA binding activity in the adult heart of Gata6 and Gata4 heart-specific KO mice. The data show that loss of GATA-6 from the heart reduces total GATA DNA binding activity better than loss of GATA-4. Control samples are from newborn hearts and rat ventricular myocytes with and without infection with AdGATA-6 adenovirus as a migration marker of GATA-6 on the gel shift. A probe only negative control is also shown where the GATA binding sites are mutated. Arrows indicate specific GATA binding activity.
Supplemental Figure III.  

A, Western blot for GATA-6 and GATA-4 of nuclear extracts from βMHC-Cre and Gata6fl/fl shows equal GATA-6 expression (i.e. the loxP allele is not hypomorphic). B, Doppler echocardiography 1 day after TAC surgery to measure pressure gradients across the constriction shows equal gradients between Gata6fl/fl and Gata6fl/flβMHC-Cre. C, 1 week after induction of pressure overload in the same mice shown in B, Gata6fl/fl mice again show significantly higher ventricular/body weight ratio’s than Gata6fl/flβMHC-Cre mice (* p<0.05).
### Supplemental Tables

#### Supplemental table I. Assessment of cardiac dimensions and function of 1 year-old mice of indicated genotypes, measured by M-mode echocardiography.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of mice</th>
<th>IVS (mm)</th>
<th>LVPW (mm)</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>FS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Cre</td>
<td>13</td>
<td>0.94±0.03</td>
<td>1.00±0.02</td>
<td>4.08±0.13</td>
<td>2.76±0.15</td>
<td>32.7±1.7</td>
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<td>G6fl/fl</td>
<td>17</td>
<td>0.95±0.03</td>
<td>1.01±0.02</td>
<td>3.93±0.09</td>
<td>2.63±0.08</td>
<td>33.3±0.8</td>
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<tr>
<td>G6fl/flβ-Cre</td>
<td>8</td>
<td>0.93±0.05</td>
<td>0.96±0.03</td>
<td>4.21±0.12</td>
<td>2.86±0.13</td>
<td>32.2±1.5</td>
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</table>

Abbreviations: IVS, interventricular septum; LVPW, left ventricular posterior wall; LVED, left ventricular end diastolic dimension; LVES, left ventricular end systolic dimension; FS, fractional shortening.

#### Supplemental table II. Assessment of cardiac dimensions and function up to 6 weeks after TAC, measured by M-Mode echocardiography, *p<0.05 G6fl/flβ-Cre vs β-Cre at the same time point, †p<0.05 G6fl/flβ-Cre vs G6fl/fl, ‡p<0.05 G6fl/fl vs β-Cre.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Week</th>
<th>IVS (mm)</th>
<th>LVPW (mm)</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>FS (%)</th>
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<td>β-Cre</td>
<td>0</td>
<td>0.79±0.02</td>
<td>0.92±0.02</td>
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<td>33.1±0.7</td>
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<td>(N=16)</td>
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<td>1.14±0.04</td>
<td>1.28±0.04</td>
<td>3.41±0.10</td>
<td>2.26±0.08</td>
<td>33.9±0.9</td>
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<tr>
<td></td>
<td>4</td>
<td>1.15±0.05</td>
<td>1.22±0.04</td>
<td>3.78±0.08</td>
<td>2.67±0.08</td>
<td>29.4±1.3</td>
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<tr>
<td></td>
<td>6</td>
<td>1.18±0.05</td>
<td>1.25±0.04</td>
<td>3.81±0.10</td>
<td>2.72±0.08</td>
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<td>G6fl/fl</td>
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<td>0.83±0.04</td>
<td>0.96±0.05</td>
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<td>37.3±1.0</td>
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<td>(N=9)</td>
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<td>1.01±0.06</td>
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<td>0.98±0.05‡</td>
<td>1.19±0.06</td>
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<td>2.78±0.14</td>
<td>28.2±1.2</td>
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<td>33.5±0.8</td>
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<tr>
<td>(N=13)</td>
<td>2</td>
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<tr>
<td></td>
<td>4</td>
<td>0.87±0.04*</td>
<td>1.01±0.04*†</td>
<td>4.26±0.16*</td>
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<tr>
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<td>6</td>
<td>0.85±0.05*</td>
<td>1.04±0.04*†</td>
<td>4.37±0.18*</td>
<td>3.54±0.19*†</td>
<td>19.4±1.4*†</td>
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Abbreviations: IVS, intraventricular septum; LVPW, left ventricular posterior wall; LVED, left ventricular end diastolic dimension; LVES, left ventricular end systolic dimension; FS, fractional shortening.
Supplemental table III. Assessment of cardiac dimensions and function of control and GATA-6 overexpressing mice after Sham and TAC surgery, measured by M-mode echocardiography.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>treatment</th>
<th>Number of mice</th>
<th>IVS (mm)</th>
<th>LVPW (mm)</th>
<th>LVED (mm)</th>
<th>LVES (mm)</th>
<th>FS (%)</th>
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<tbody>
<tr>
<td>TTA</td>
<td>Sham</td>
<td>11</td>
<td>0.85±0.03</td>
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<td>2.30±0.10</td>
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<td>TAC</td>
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<td>36.6±1.1</td>
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<td>40.0±0.9</td>
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<td>38.4±1.2</td>
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<td>Sham</td>
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<tr>
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</table>

Abbreviations: IVS, intraventricular septum; LVPW, left ventricular posterior wall; LVED, left ventricular end diastolic dimension; LVES, left ventricular end systolic dimension; FS, fractional shortening.