Rare *GATA5* sequence variants identified in individuals with bicuspid aortic valve

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BACKGROUND: Bicuspid aortic valve (BAV) is the most common type of congenital heart disease (CHD) and has a proposed genetic etiology. BAV is categorized by cusp fusion, with right-left (R-L) cusp fusion being associated with additional CHD, and right-noncoronary cusp (R-NC) fusion being associated with aortic valve dysfunction. Loss of murine *Gata5*, which encodes a cardiac transcription factor, results in a partially penetrant R-NC BAV, and we hypothesize that mutations in *GATA5* are associated with R-NC BAV in humans.

METHODS: A cohort of 78 BAV patients (50 with isolated BAV and 28 with associated aortic coarctation) was analyzed using Sanger sequencing to identify *GATA5* sequence variants. Biochemical assays were performed to identify functional deficits of identified sequence variants.

RESULTS: We identified two rare heterozygous nonsynonymous variants, p.Gln3Arg and p.Leu233Pro, for a frequency of 2.6% (2/78). Both individuals with nonsynonymous variants had BAV and aortic coarctation, one R-L and one R-NC subtype. Of the nonsynonymous variants, only p.Gln3Arg demonstrated decreased transcriptional activity *in vitro*.

CONCLUSION: Rare sequence variants in *GATA5* are associated with human BAV. Our findings suggest a genotype–phenotype correlation in regards to associated CHD but not cusp fusion.

Congenital heart disease (CHD) is the most common type of birth defect, with an estimated incidence of 6–19 per 1,000 live births (1). Bicuspid aortic valve (BAV) is the most common of these congenital heart malformations with an estimated prevalence of 1–2% in the general population (2). BAV can occur as an isolated anomaly or may be seen in association with other cardiovascular malformations, most commonly coarctation of the aorta (CoA). BAV is also associated with the development of aortic valvular calcification and aortic stenosis/regurgitation. Despite the high prevalence of BAV and the potential for morbidity associated with the condition, the mechanisms underlying both normal aortic valve development and the derangement of this process in the development of BAV remain poorly understood.

The normal aortic valve is composed of three cusps termed the right coronary, left coronary, and noncoronary cusps. BAV represents a spectrum of valve phenotypes in which two cusps are typically fused together. In the majority of BAV cases, fusion occurs between the right coronary and left coronary cusps (R-L). The next most commonly observed phenotype is fusion of right coronary and noncoronary cusps (R-NC). Fusion of the left coronary cusp and noncoronary cusp (L-NC) is quite rare. Interestingly, R-NC BAV has been associated with a greater incidence of aortic valve pathology than R-L BAV (3,4). Patients with R-NC BAV are more likely to develop clinically significant aortic valve stenosis and/or regurgitation than those with R-L BAV. The R-L phenotype, on the other hand, is more likely to be associated with additional cardiac malformations such as CoA, interrupted aortic arch, and hypoplastic left heart syndrome (4). Detailed analysis of animal models of R-L and R-NC BAV were found to be associated with distinct developmental derangements, abnormal outflow tract septation, and defective outflow tract cushion development, respectively, suggesting that they represent different etiologic entities (5).

BAV is considered to have a significant genetic etiologic component on the basis of observed familial clustering (6). Huntington *et al.* (7) demonstrated a 9.1% incidence of BAV in relatives of affected individuals. Linkage studies have identified disease-linked loci for BAV (8). To date, the most common reported genetic etiology of nonsyndromic BAV has been mutations in *NOTCH1* by several groups (9–12). The discovery of partially penetrant R-NC BAV in mice lacking *Gata5*, a zinc finger transcription factor expressed in the developing heart that is important for endocardial-endothelial differentiation, resulted in the recent identification of rare *GATA5* sequence variants in humans with BAV, but genotype–phenotype correlations were not studied (13,14). Here, we seek to investigate the role of *GATA5* mutations in a well-phenotyped cohort of individuals with BAV and BAV-CoA. We hypothesize that *GATA5* variants will be predominantly associated with isolated BAV and R-NC cusp fusion in humans.

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RESULTS

Patient Population and Identification of GATA5 Sequence Variants

Of the 78 patients with BAV, the majority had isolated BAV (n = 50) while the remaining had associated CoA (Table 1). In our study population, patients with BAV and CoA were more likely to have a R-L type of BAV vs. a R-NC type of BAV, while R-L and R-NC phenotypes were equally common in isolated BAV (P value = 0.036). Of the 78 cases, 18 were considered familial as they had additional family members with a left ventricular outflow tract malformation (five individuals had two affected family members reported). Cardiac phenotypes of affected family members are available in **Supplementary Table S1** online.

We screened for mutations by DNA sequencing of the coding regions of *GATA5* in the 78 individuals with BAV. Analysis of the sequencing data revealed two variants that predicted nonsynonymous amino acid substitutions, p.Gln3Arg and p.Leu233Pro (Table 2). Both variations were identified in Caucasian males. The p.Gln3Arg variation was present as a heterozygous change in one individual with BAV-CoA and

Table 1. Cardiac phenotype of study population

	BAV	BAV-CoA	Overall
R-L	26 (33%)	20 (26%)	46 (59%)
R-NC	23 (29.5%)	7 (9%)	30 (38.5%)
L-NC	1 (1%)	1 (1%)	2 (2.5%)
Overall	50 (64%)	28 (36%)	

BAV, bicuspid aortic valve (isolated); BAV-CoA, bicuspid aortic valve with coarctation of the aorta; L-NC, fusion of left coronary cusp and noncoronary cusp; R-L, fusion of right coronary cusp and left coronary cusp; R-NC, fusion of right coronary cusp and noncoronary cusp.

Table 2. Rare nonsynonymous sequence variants identified in patients with BAV

Nucleotide change	Amino acid change	BAV phenotype	Allele frequency in patients	Allele frequency in all EVS controls	Allele frequency in EA EVS controls	Allele frequency in 1000G controls	PolyPhen2 analysis (score)	SIFT analysis (score)
c.8A>G	p.Gln3Arg	BAV-CoA, R-L	0.006	0.003	0.004	0.004	Predicted damaging (0.891)	Predicted damaging (0.001)
c.698T>C	p.Leu233Pro	BAV-CoA, R-NC	0.006	0.001	0.001	0.003	Predicted damaging (0.723)	Predicted damaging (0.005)

BAV, bicuspid aortic valve; BAV-CoA, bicuspid aortic valve with coarctation of the aorta; EA, European American; EVS, Exome Variant Server; R-L, fusion of right coronary cusp and left coronary cusp; R-NC, fusion of right coronary cusp and noncoronary cusp; 1000G, 1000 Genomes.

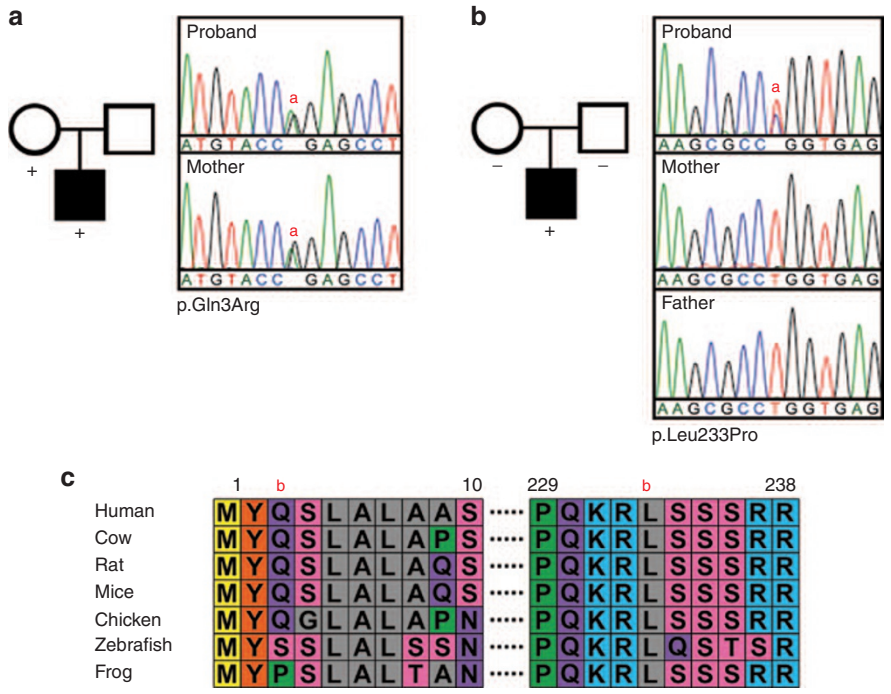

Figure 1. Rare nonsynonymous sequence variants identified in subjects with bicuspid aortic valve. (**a**) Family pedigree demonstrating p.Gln3Arg is inherited from unaffected mother and sequence chromatogram showing A to G transition which predicts the nonsynonymous amino acid substitution, p.Gln3Arg. (**b**) Family pedigree, which demonstrates p.Leu233Pro is a *de novo* mutation, and sequence chromatogram shows heterozygous T to C transition, predicting nonsynonymous amino acid substitution, p.Leu233Pro, in proband only. In pedigrees shown in (**a, b**), unshaded shapes indicate no known cardiac phenotype (no echocardiograms available), while shaded shapes indicate affected individuals; squares represent males while circles are females; +, presence of mutation; -, absence of mutation; [a] indicates nucleotide variant position; [b] indicates amino acid variant position. (**c**) Alignment of human GATA5 protein sequence with orthologs from multiple species. The NCBI GenBank accession numbers that were utilized for the alignment are as follows: Human-NP_536721.1, Cow-NP_001029393.1, Rat-NP_001019487.1, Mice-NP_032119.2, Chicken-NP_990752.1, Zebrafish-NP_571310.2, and Frog-NP_001081962.1.

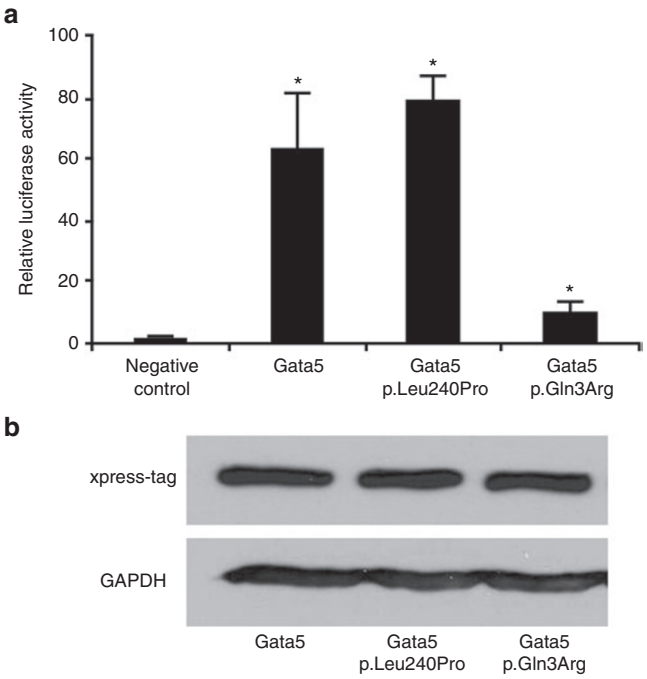

Figure 2. *In vitro* functional analysis of GATA5 sequence variations. (**a**) Transactivation assays in HeLa cells transfected with 100 ng of murine Gata5 p.Leu240Pro and murine Gata5 p.Gln3Arg along with cotransfection of ANF-luciferase reporter and *Renilla* control reporter (pRL-SV40 plasmid). The p.Gln3Arg protein demonstrated reduced activation ability as compared to wild-type Gata5 while p.Leu240Pro activation ability was unchanged. Luciferase activity is normalized to *Renilla*. Experiments were performed in triplicate, and means and standard deviations are shown. *$P < 0.05$. (**b**) Immunoblotting demonstrates equal expression of Gata5 p.Leu240Pro and p.Gln3Arg when compared to wild type. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

R-L cusp fusion phenotype and was inherited from the subject's mother, who is reportedly unaffected but did not undergo echocardiographic evaluation and therefore could have unrecognized BAV (**Figure 1a**). This individual also had a history of inguinal hernia. The mutated amino acid lies within a known transcriptional activation domain of *GATA5* and is highly conserved (**Figure 1c**). The p.Gln3Arg variation is predicted to be damaging by *in silico* analysis using both PolyPhen2 and SIFT algorithms. This variation occurs at an allele frequency of 0.003 in the Exome Variant Server control population and 0.004 in the 1000 Genomes control population (**Table 2**).

The p.Leu233Pro variation was present as a heterozygous change in one individual with BAV-CoA and R-NC cusp fusion phenotype. The p.Leu233Pro variation was a *de novo* change as it was not identified in either of the proband's parents (**Figure 1b**). This individual also had a history of type 1 diabetes mellitus and syringomelia. Of note, there is a remote family history (maternal second cousin) of CoA. The affected amino acid occurs within the proposed nuclear localization signal and was also highly conserved (**Figure 1c**). The p.Leu233Pro variation is predicted damaging by *in silico* analysis using both PolyPhen2 and SIFT algorithms and is present at an allele frequency of 0.001 in the Exome Variant Server control population and 0.003 in the 1000 Genomes control population (**Table 2**).

Two rare synonymous changes at p.Ser42Ser (c.123G>A) and p.Leu226Leu (c.678C>T) were also identified and are detailed in **Supplementary Table S2** online. The p.Ser42Ser variation was present as a heterozygous change in one Caucasian male with isolated BAV and R-NC cusp fusion phenotype. The p.Leu226Leu variation was present as a heterozygous change in one Caucasian male with isolated BAV and R-L cusp fusion phenotype. This individual has a family history of BAV in a maternal cousin (no sample available for testing). The p.Leu226Leu (c.678C>T) change occurs at an allele frequency of 0.0003 in the Exome Variant Server control population, whereas the p.Ser42Ser (c.123G>A) change is not reported in this control cohort. Neither variant was reported in the 1000 Genomes control population.

In Vitro Functional Analysis of Gata5 Sequence Variants

To determine if the amino acid substitutions in GATA5 resulted in functional abnormalities, we tested murine mutant proteins analogous to the two human nonsynonymous variants found in our cohort using *in vitro* transfection assays. We generated mutant expression vectors in the highly conserved murine *Gata5* gene for the human p.Gln3Arg and p.Leu233Pro mutations (murine p.Gln3Arg and p.Leu240Pro) and performed reporter assays using the Gata-dependent cardiac enhancer, atrial natriuretic factor (ANF), upstream of a luciferase reporter. Transfection of wild-type Gata5 demonstrated a 60-fold increase in the activation of the ANF promoter as compared to negative control (**Figure 2a**). While the p.Leu240Pro variant showed similar activation of the ANF-luciferase reporter as compared to wild type, the p.Gln3Arg variant had significantly decreased transactivational activity (10-fold) (**Figure 2a**). The Gata5 p.Leu240Pro and p.Gln3Arg mutant proteins were equally expressed in HeLa cells (**Figure 2b**).

DISCUSSION

Here, we identified two rare sequence variants that predict nonsynonymous amino acid substitutions in *GATA5*, for an estimated prevalence of 2.6% in our BAV cohort (2/78). Both nonsynonymous variants were found in individuals with BAV occurring with CoA, altered evolutionarily conserved amino acid residues, and are putatively disease causing based on *in silico* analysis. The p.Leu233Pro variation was a *de novo* change, while the p.Gln3Arg variation was identified in a presumed unaffected mother. The p.Gln3Arg mutant protein demonstrated decreased transcriptional activity in functional assays. In summary, these findings support a role for rare *GATA5* sequence variants in the etiology of a minority of human BAV.

Multiple studies have established a role for rare sequence variants of cardiac transcription factors in the development of congenital heart defects in humans. Murine studies by Laforest *et al.* (14) suggested a role for *GATA5* in human BAV when they demonstrated that *Gata5*-null mice had partially penetrant, R-NC BAV. Furthermore, they showed that endocardial-specific loss of Gata5 recapitulated the *Gata5*-null phenotype. *Gata5*, similar to its related transcription factors *Gata4* and *Gata6*, is expressed in the developing heart but this expression

is not maintained in adult cardiovascular structures, unlike *Gata4* and *Gata6* (ref. (15)). Murine studies demonstrated that *Gata5* mRNA is present in the precardiac mesoderm and atrial and ventricular myocardium in the earliest stages of cardiac differentiation (15). At later stages of heart morphogenesis, Gata5 is predominantly expressed in the developing endocardium and endocardial cushions of the outflow tract and atrioventricular cushions, and this expression diminishes during the late stages of fetal development (14,16).

A role for *GATA5* in human BAV was first supported by Padang *et al.* (13) who identified four rare, nonsynonymous sequence variants among a cohort of 100 patients with BAV, including the p.Gln3Arg variant identified in our cohort. This study had comparable findings to our similar-sized cohort of 78 patients. Not surprisingly, Foffa *et al.* (9) did not identify rare, nonsynonymous *GATA5* variants in their small cohort of 11 patients with familial BAV. The significance of these rare variants in disease causation remains unclear. The data supporting an etiologic link between p.Gln3Arg variants are strengthened by functional abnormalities in *in vitro* assays and the similar association by Padang *et al.* While the data supporting the p.Leu233Pro variation is less clear, its potential contribution is strengthened by its *de novo* inheritance, predicted functional effects by *in silico* analysis, and low allele frequency. As the p.Leu233Pro nonsynonymous amino acid substitution is located within the zinc finger domain, which is important for protein-protein interactions, the lack of functional effect in our *in vitro* transactivation assays may not be that surprising. Similar to other groups, we identified rare synonymous variants identified in our cohort (8,13). Of note, the c.678C>T (Leu226Leu) variant has been previously reported in human BAV but has a low allele frequency in reference genomes (8). The possible contribution of synonymous variants to disease states such as BAV remains poorly understood. The exceedingly low allele frequencies of these changes in large control cohorts suggest a potential role in BAV, but further investigation is required. In summary, the finding of *GATA5* rare sequence variants in humans with BAV suggests that they are contributing to disease but it remains unclear if additional genetic or environmental factors are required to cause the cardiac defect.

Our studies support an etiologic role for *GATA5* mutations in BAV, but genotype–phenotype correlations remain unclear. Our data are similar to Padang *et al.* who identified rare *GATA5* sequence variants in individuals with R-L or indeterminate BAV and suggest that the cusp fusion in humans is not similar to the murine findings. Where associated cardiac defects are concerned, our data suggest a link between *GATA5* and BAV occurring with CoA, whereas Padang *et al.* did not identify any rare, nonsynonymous variants in their subcohort of patients with BAV and coarctation. Our largely pediatric cohort limited the ability to explore genotype–phenotype correlations involving advanced valvular dysfunction and/or aortopathy. Interestingly, *GATA5* variants have been reported in patients with tetralogy of Fallot, ventricular septal defects, and familial atrial fibrillation, but further studies are needed to investigate a potential role for *GATA5* in isolated cases of CoA (17–19).

To date, *NOTCH1* and *GATA5* remain the only genes associated with isolated human BAV, but our study along with others demonstrates that variants in these genes are not involved in the majority of cases of BAV. Multiple additional candidate genes for BAV have been investigated in both human cohorts and murine models. Mutations in *KCNJ2* have been linked to Andersen syndrome, which can present with BAV among other cardiovascular malformations, but no role has been identified in isolated BAV (20). Similarly, *ACTA2* has been shown via linkage analysis to be associated with BAV occurring with aortic aneurysm (21). Murine models have suggested roles for *Alk2, Nkx2.5*, and *Nos3* in BAV formation, but there have been no reported mutations of these genes in cases of human BAV (22–25). Additional investigation is required in the BAV population as there may be roles for *GATA4* and *GATA6*, mutations in which have been associated with other types of human CHD (26–28).

In conclusion, this study supports a role for *GATA5* variants in human BAV, though it remains uncertain if such variants are directly causal or serving as critical genetic modifiers. While our findings did not support the hypothesized genotype–phenotype correlation between *GATA5* and cusp fusion morphology, we did find that *GATA5* variants may distinguish between isolated BAV and BAV occurring with aortic coarctation, consistent with a role for *GATA5* in endothelial-endocardial differentiation (29). Studies with larger populations are needed to further elucidate the relationship of *GATA5* variants with both isolated BAV and BAV-CoA. These findings further emphasize the complex, heterogeneous genetic basis of BAV as well as the need to explore new candidate genes in large cohort studies.

METHODS

Study Population

The study cohort was comprised of 78 unrelated individuals (59 males and 19 females) with BAV who underwent care at Nationwide Children's Hospital. Subjects ranged in age from newborn to 38 y old at time of enrollment (mean = 11 y). All subjects had clinical echocardiograms performed at Nationwide Children's Hospital with images adequate to identify cusp fusion morphology. The majority of individuals were of Caucasian ethnicity, with one African-American, one Asian, and three Hispanic individuals. Fifty of the 78 subjects (64%) had isolated BAV, while the remainder had BAV with CoA. Forty-six subjects (59%) had R-L cusp fusion, while 39% had R-NC fusion and only 2% had L-NC fusion. Thirty-three subjects (42%) had some degree of aortic stenosis at the time of enrollment; none had aortic insufficiency. The participating subjects were prospectively recruited from June 2004 to June 2011 as part of a larger study studying the genetic contributors to left ventricular outflow tract malformations. Informed consent was obtained under a protocol approved by the Institutional Review Board at Nationwide Children's Hospital; parental consent was obtained for all study participants less than 18 y of age at enrollment with assent provided by all patients between 9–17 y old at enrollment. Medical records were reviewed to identify noncardiac congenital malformations and assess for known genetic syndromes. Subjects with known chromosomal abnormalities were excluded from the analysis. Parent samples, when possible, were also obtained under the same Institutional Review Board protocol. Genomic DNA was isolated from blood or saliva samples using the 5 PRIME DNA extraction kit (Fisher, Pittsburgh, PA).

Sequencing of GATA5 and Variant Analysis

The six exons of the coding region of *GATA5* (Genbank Accession Number NM_080473) were sequenced bidirectionally via Sanger sequencing. Reference populations from the Exome Variant Server

and 1000 Genomes Project were used as controls and therefore limited phenotype information was available in regards to subclinical cardiac anomalies (30,31). When rare variants were identified in our cohort and parent DNA was available, parent samples were also sequenced. Sequencing primers are available upon request. *In silico* analysis was performed using the Polyphen 2 and SIFT algorithms.

Plasmid Construction and Site-Directed Mutagenesis

The mouse *Gata5* expression vector was generously provided by Dr. Solway and Dr. Chen (32). The c.8A>G and c.719T>C point mutations were introduced into the plasmid containing the mouse *Gata5* cDNA (Genbank Accession Number NM_008093.2) to generate the mouse *Gata5* p.Gln3Arg and p.Leu240Pro mutant expression vectors using the QuickChange II Site-Directed Mutagenesis Kit following the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). The mutation was verified by direct sequencing. A tagged expression vector pcDNA-HisB-Gata5, which also contains an Xpress epitope tag sequence (DLYDDDDK), was also generated for the full-length murine wild type and mutant Gata5 cDNAs. These constructs were generated using PCR amplification from the original untagged expression vector using the following primers: 5′-AATT*GAATTC*CAAGCTCGCGCGGGGAAAA-3′ and 5′-AATT*CTCGAG*GTGGTGACAGTTTCCTGAGC-3′ (EcoRI and XhoI sites are underlined). The PCR fragments were digested with EcoRI and XhoI and cloned into the EcoRI-XhoI sites of pcDNA3.1-HisB (Invitrogen, Grand Island, NY).

Immunoblotting

After transfection of wild type and mutant Gata5 tagged expression constructs into HeLa cells, 20 μg of cell lysate was loaded per lane and separated using 10% sodium dodecyl sulfate–polyacrylamide gels, and transferred to immune-blot polyvinylidene difluoride membranes (Bio-rad, Hercules, CA). After blocking with 5% nonfat milk in PBST, the membrane was probed with primary monoclonal mouse anti-Xpress (1:1,000, R910-25; Life Technologies, Grand Island, NY) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1:5,000, NB300-221; Novus Biologicals, Littleton, CO). The membrane was further probed with horseradish peroxidase–conjugated horse anti-mouse IgG (PI-2000).

Transactivation Assay

The luciferase reporter studies were performed as described previously (26). Briefly, HeLa cells were transiently transfected with 100-ng ANF-luciferase reporter plus 50 ng pRL-SV40 plasmid, in combination with 100 ng of wild-type *Gata5*, *Gata5* p.Gln3Arg, or *Gata5* p.Leu240Pro plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luciferase activity was measured 48 h after transfection using the Dual-Glo Luciferase Assay (Promega, Madison, WI) according to the manufacturer's protocol. Mean luciferase activity was calculated after normalization to *Renilla*. Three independent experiments were performed in triplicate.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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