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GATA5 interacts with GATA4 and GATA6 in outflow tract development

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ABSTRACT

Members of the GATA family of transcription factors are critical regulators of heart development and mutations in 2 of them, *GATA4* and *GATA6* are associated with outflow tract and septal defects in human. The heart expresses 3 GATA factors, GATA4, 5 and 6 in a partially overlapping pattern. Here, we report that compound *Gata4*/*Gata5* and *Gata5*/*Gata6* mutants die embryonically or perinatally due to severe congenital heart defects. Almost all $Gata4^{+/-}Gata5^{+/-}$ mutant embryos have double outlet right ventricles (DORV), large ventricular septal defects (VSD) as well as hypertrophied mitral and tricuspid valves. Only 25% of double compound *Gata4*/*Gata5* heterozygotes survive to adulthood and these mice have aortic stenosis. Compound loss of a *Gata5* and a *Gata6* allele also leads to DORVs associated with subaortic VSDs. Expression of several transcription factors important for endocardial and myocardial cell differentiation, such as Tbx20, Mef2c, Hey1 and Hand2, was reduced in compound heterozygote embryos. These findings suggest the existence of important genetic interactions between Gata5 and the 2 other cardiac GATA factors in endocardial cushion formation and outflow tract morphogenesis. The data identify *GATA5* as a potential genetic modifier of congenital heart disease and provide insight for elucidating the genetic basis of an important class of human birth defects.

Introduction

Congenital heart disease (CHD) is estimated to occur at a prevalence of 1–2% of live births and is the leading cause of death in the first year of life (Pierpont et al., 2007). Abnormal development of the outflow tract (OFT) accounts for about 12–14% of all CHDs, leading to malformations such as persistent truncus arteriosus (PTA), tetralogy of Fallot (TOF), double outlet right ventricle (DORV) and transposition of the great arteries (TGA) (Hoffman and Kaplan, 2002). Significant efforts have been deployed over the last decade to elucidate the cell and molecular mechanisms involved in CHD. Several lines of evidence suggest that CHD is heritable (Insley, 1987), but to date, only a few human genes have been linked to CHDs. Many of those are developmental regulatory genes. For example, mutations in *NKX2.5* are associated with cases of TOF and atrial septal defects (ASD) while mutations in the *TBX5* gene cause the Holt–Oram syndrome, an autosomal dominant disease with varying cardiac defects (Basson et al., 1997; Goldmuntz et al., 2001; Li et al., 1997; Schott et al., 1998). Moreover, mutations in *GATA4* have been associated with atrial and/or ventricular septal defects, TOF and PTA (Garg et al., 2003; Nemer et al., 2006; Rajagopal et al., 2007). Interestingly, heterozygous mutations of *Gata4*, *Nkx2.5 and Tbx5* in mice recapitulate the human phenotype (Biben et al., 2000; Bruneau et al., 2001; Rajagopal et al., 2007; Winston et al., 2010). Recently, mutations in the human *GATA6* gene have been associated with PTA and TOF (Kodo et al., 2009; S.C. Lin et al., 2010; Maitra et al., 2010). What has emerged from combined human and mouse genetic studies is that mutations in different genes can lead to similar cardiac defects while mutations in the same gene can lead to varying defects. The complexity of CHD is evident at both genetic and cellular levels, as multiple lineages contribute to proper heart development. The first heart field contributes to the formation of the left ventricle, the atrioventricular canal and both atrial chambers (Buckingham et al., 2005). Whereas, the secondary heart field (SHF) contributes to the formation of the right ventricle and the OFT (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Remodeling of the OFT into the distinct vessels of the aorta and pulmonary trunk requires complex interactions between the myocardium, the endocardium and cardiac neural crest cells (NCC) (Hutson and Kirby, 2007). Consequently, impaired development of the OFT results in conotruncal defects.

Members of the GATA family of transcription factors play important roles in differentiation, proliferation and survival of different cell types. In the heart, 3 GATA factors are present in a partially overlapping pattern (Molkentin, 2000; Nemer and Nemer, 2003; Patient and McGhee, 2002). Gata4 is highly expressed in myocytes, endocardial cells and epicardial cells of the heart. Embryos lacking *Gata4* die by E8.5 because of defects in ventral migration causing cardia bifida (Kuo et al., 1997; Molkentin et al., 1997). Analysis of rescued $Gata4^{-/-}$ embryos revealed cardiac defects including disrupted heart looping, absence of endocardial cushion formation, lack of a proepicardial

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organ and hypoplastic ventricular myocardium (Watt et al., 2004). Several studies were performed to further investigate the role of Gata4 in endocardial or myocardial cell development. Inactivation of *Gata4* in endothelial cells causes embryonic lethality by E12.5 due to failure to promote endocardial cushion formation and remodeling (Rivera-Feliciano et al., 2006). Early myocardial specific deletion of *Gata4* results in myocardial thinning and hypoplastic endocardial cushions (Zeisberg et al., 2005). Moreover, haploinsufficiency of *Gata4* has been associated with cardiac defects including common atrioventricular canal, DORV and hypoplastic ventricular myocardium (Pu et al., 2004). Gata6 is expressed in myocytes but also in neural crest as well as endocardial and vascular smooth muscle cells (VSMC) (Nemer and Nemer, 2003). Inactivation of *Gata6* specifically in neural crest cells is sufficient to cause PTAs and lethality by E18.5-P2, revealing a role for GATA6 in the patterning of the OFT and aortic arch (Lepore et al., 2006). Loss of both *Gata4* and *Gata6* in mice leads to acardia, suggesting that genetic interactions between these factors are essential for the onset and/or maintenance of cardiogenesis (Zhao et al., 2008). Interestingly, mice with compound heterozygous mutation in *Gata4* and *Gata6* die embryonically around E13.5 due to vascular defects; in addition, these mice display PTA, evidence of failed OFT septation as well as myocardial thinning. These results are indicative of functional interactions between Gata4 and 6 in cardiac and vascular development (Xin et al., 2006). In contrast to GATA4 and 6, GATA5 expression is more restricted to endocardial cells and endocardial cushions of the OFT and atrioventricular canal during heart development. The dynamic expression of GATA5 in endocardial cells suggests a specific function for this transcription factor in endocardial development. Consistent with this, *faust* (which encode gata5) mutants in zebrafish have *cardia bifida* and lack endocardial cells (Reiter et al., 1999). In addition, downregulation of *Gata5* in an *in vitro* model of endocardial differentiation inhibits terminal differentiation and expression of endocardial differentiation markers (Nemer and Nemer, 2002). An important role for GATA5 in endocardial differentiation is further supported by recent findings showing that lack of *Gata5* in mice leads to bicuspid aortic valve formation (Laforest et al., 2011). Thus, all three cardiac GATA factors appear to play important functions in endocardial cushion development and/or outflow tract morphogenesis.

Because expression of the three cardiac GATA factors partially overlaps and since they can bind similar DNA regulatory elements and activate common target promoters through heterotypic interactions (Charron et al., 1999; Nemer and Nemer, 2003), we tested whether Gata5 might genetically interact with Gata4 and Gata6 in OFT development. Here we show that $Gata4^{+/-}Gata5^{+/-}$ and $Gata5^{+/-}Gata6^{+/-}$ double heterozygote mice die embryonically or perinatally due in large part to profound defects of OFT development including DORVs and VSDs. The results raise the possibility that subtle alterations in the level or activity of any 2 cardiac GATA factors might lead to human congenital heart defects.

Materials and methods

Animals

Mice handling and experimentation were performed in accordance with institutional guidelines. All protocols were approved by the institutional animal care committees.

Mice heterozygous for *Gata4*, *Gata5* and *Gata6* were generated and genotyped as previously described (Aries et al., 2004; Koutsourakis et al., 1999; Laforest et al., 2011). All lines were maintained in the C57/BL6 background. To generate double heterozygotes, mice heterozygous for *Gata4* or *Gata6* were mated to *Gata5* heterozygote mice and pregnant mothers or newborn litters were sacrificed at various embryonic timepoints and postnatal timepoints. The morning a vaginal plug was observed was defined as embryonic day (E) 0.5.

Histology

Whole embryos were fixed in 4% paraformaldehyde at 4 °C overnight, dehydrated through graded ethanol series, embedded in paraffin and sectioned at 4-μm intervals. Masson's trichrome staining was carried out on heart sections using standard procedures to visualize defects.

Cell count

Image J software was used to count the number of myocytes, endocardial cells and cushions cells in three different sections of 3–4 different heart samples for each genotype. The different cell types are easily distinguished morphologically in embryonic hearts.

Echocardiography

Transthoracic echocardiography was performed as described before (Aries et al., 2004) using a VisualSonics Vevo 770 ultrasound system with a RMV 707 30 MHz transducer. Doppler and M-mode imaging was obtained from 70 days old mice. Groups of 3–6 mice from different litters were used for the M-mode measurements and Doppler readings at 70 days.

Quantitative polymerase chain reaction (Q-PCR)

Total RNA was isolated from whole hearts at E12.5 with TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. cDNAs were generated from 2 μg of total RNA using the Omniscript RT kit (Qiagen). Q-PCR was performed with cDNA diluted 1/100 using the Qiagen Q-PCR kit. Briefly, DNA template and 400 nM oligonucleotides were used at an annealing temperature of 58 °C using the Quantitect SYBR green PCR kit (QIAGEN) in an MX3500 real-time PCR machine (Stratagene, La Jolla, CA). Mean gene expression was corrected by GAPDH and calculated from wildtype and double heterozygote embryos ($n=3$–6 per group). Primers used were as previously reported (Laforest et al., 2011).

Statistical analysis

Values are presented as mean ± S.E.M. and n refers to the number of mice per group. For comparison of multiple groups, a one-way ANOVA statistical test was performed. A Student's two-tailed t-test was then performed to confirm statistical significance between two groups. Statistical significance was considered as $P<0.05$.

Results

Reduced viability of $Gata4^{+/-}Gata5^{+/-}$ embryos

Both Gata4 and Gata5 are expressed in the endocardial cushions at the same embryonic stages (between E9 and E12). We investigated possible *in vivo* interactions between them in heart development by crossing mice heterozygous for either a *Gata5* or a *Gata4* allele. According to Mendelian transmission, equal ratios of wildtypes, *Gata4* heterozygotes, *Gata5* heterozygotes and *Gata4*/*Gata5* double heterozygotes were expected. However, at weaning, $Gata4^{+/-}Gata5^{+/-}$ double heterozygotes (*G4*/*G5* hets) were obtained at far lower frequency than expected (6% *vs* 25%) (Fig. 1A). Analysis of embryos from timed matings suggested high perinatal lethality of *G4*/*G5* hets, although decreased viability was evident starting at E14.5 (Fig. 1A). Visual inspection of the $Gata4^{+/-}Gata5^{+/-}$ heterozygotes at E11.5 revealed no gross abnormalities but by E15.5, it was evident that the $Gata4^{+/-}Gata5^{+/-}$ heterozygotes were smaller (Figs. 1B–K). To determine if this phenotype was caused by growth retardation, the number of somites was calculated at E10.5; no significant changes were observed among all