RESEARCH COMMUNICATION

Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors

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GATA-family transcription factors are critical to the development of diverse tissues. In particular, GATA-4 has been implicated in formation of the vertebrate heart. As the mouse *Gata-4* knock-out is early embryonic lethal because of a defect in ventral morphogenesis, the *in vivo* function of this factor in heart development remains unresolved. To search for a requirement for *Gata4* in heart development, we created mice harboring a single amino acid replacement in GATA-4 that impairs its physical interaction with its presumptive cardiac cofactor FOG-2. *Gata4*^{ki/ki} mice die just after embryonic day (E) 12.5 exhibiting features in common with *Fog2*^{-/-} embryos as well as additional semilunar cardiac valve defects and a double-outlet right ventricle. These findings establish an intrinsic requirement for GATA-4 in heart development. We also infer that GATA-4 function is dependent on interaction with FOG-2 and, very likely, an additional FOG protein for distinct aspects of heart formation.

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Transcriptional activity of the GATA-factors is modulated through interaction with nuclear proteins, including zinc finger proteins of the Kruppel and FOG/U-shaped families, general coactivators (p300 and CBP), the myocardial-expressed protein Nkx2.5, and NF-AT3 (Durocher and Nemer 1998; Mackay and Crossley 1998; Blobel 2000; Molkentin 2000). Whereas the specificity and

in vivo functional relevance of many of these interactions are incompletely defined, the association of GATA-1 with FOG-1 has been examined in detail. FOG-1 interacts with GATA-1 in hematopoietic cells and regulates the ability of GATA-1 to promote terminal differentiation of erythroid cells and megakaryocytes (Tsang et al. 1997). Mutation of specific residues within the conserved N-terminal zinc finger of GATA-1, such as V205G, disrupts binding to FOG-1, preserves DNA-binding properties of GATA-1, and renders GATA-1 unable to promote terminal differentiation of red blood cells (Crispino et al. 1999). Furthermore, mutation of Val 205 in humans leads to congenital dyserythropoietic anemia and thrombocytopenia (Nichols et al. 2000). Taken together, these findings demonstrate that direct physical association of GATA-1 and FOG-1 is essential for GATA-1's roles in transcription and, critical for the experiments reported herein, identifies a specific residue of the N finger that mediates cofactor interaction.

GATA-4, GATA-5, and GATA-6, nonhematopoietic expressed factors, are implicated in development of heart, endoderm, and intestinal epithelia, where they are expressed in an overlapping and dynamic fashion (Morrisey et al. 1997; Bossard and Zaret 1998; Gao et al. 1998; Charron and Nemer 1999; Koutsourakis et al. 1999; Parmacek and Leiden 1999; Molkentin 2000). GATA-4 has been extensively studied in the context of heart development, as it is present in precardiac splanchnic mesoderm and binds to and activates promoters and enhancers of numerous myocardial-expressed genes (Charron et al. 1999). In its absence, mouse embryos die by E7.0–9.5, with failure of ventral morphogenesis leading to cardiac bifida (Kuo et al. 1997; Molkentin et al. 1997). The death of the *Gata4* null embryos before formation of a heart tube precludes analysis of the role of this factor in later cardiac organogenesis.

The second member of the FOG protein family, FOG-2, is expressed in cardiac and nervous system tissues and interacts with the N fingers of all GATA factors, including those expressed within the developing heart (GATA-4, GATA-5, and GATA-6; Lu et al. 1999; Svensson et al. 1999; Tevosian et al. 1999). As such, it is a candidate cofactor for these GATA factors in the heart. Mouse embryos lacking *Fog2* die of heart failure between E12.5 and E15.5 (Svensson et al. 2000; Tevosian et al. 2000). *Fog2*^{-/-} hearts exhibit a constellation of defects, such as overriding aorta, subpulmonic stenosis, and subaortic ventricular septal defect (VSD), seen in the human congenital malformation Tetralogy of Fallot. In addition, despite formation of an intact epicardial layer and expression of epicardium-specific genes, initiation of coronary vasculature fails to take place. This is evidenced by the absence of induction of markers of coronary vessel development, including ICAM-2 and Flk-1 in *Fog2*-deficient hearts. These defects in coronary vasculature formation are secondary to lack of *Fog2* expression specifically in myocardium, as demonstrated by transgenic rescue (Tevosian et al. 2000).

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Results and Discussion

We sought to establish an intrinsic role for GATA-4 in heart development by generating mice harboring a knock-in mutation that cripples its interaction with FOG-2 or other FOG-factors. Residue 217 of GATA-4, which corresponds to Val 205 of GATA-1, was changed to glycine by gene targeting in embryonic stem (ES) cells. This residue faces away from DNA (Fig. 1A) and lies within the GATA-N finger: FOG interface. Substitution with glycine disrupts interaction with either FOG-1 or FOG-2 and leaves DNA-binding properties of GATA factors unperturbed. Targeted mutation of murine *Gata4* was accomplished with the construct depicted in Figure 1B. A floxed neomycin resistance cassette was incorporated into an intron downstream of the exon containing V217. ES cells harboring both the V217G mutation and the floxed neomycin cassette were injected into host blastocysts to generate chimeras. *Gata4*^{+/ki} mice appeared normal. No liveborn homozygotes resulted from interbreeding of heterozygous offspring, indicating the *Gata4*^{ki/ki} embryos are embryonic lethal. Southern blotting of timed matings (Fig. 1C) demonstrated that *Gata4*^{ki/ki} embryos die between E11.5 and E13.5. Their gross appearance was remarkably similar to that of *Fog2*^{-/-} embryos (Svensson et al. 2000; Tevosian et al.

2000). *Gata4*^{ki/ki} embryos are pale and edematous, compared with wild-type littermates, and peripheral hemorrhage is often observed (Fig. 2A,B). The neomycin cassette was removed in some strains by interbreeding with a Cre-recombinase-deleter strain (Mao et al. 1999). No differences in phenotype were observed between strains containing or lacking the neomycin cassette. Furthermore, heterozygotes were born at the expected frequency and displayed no detectable phenotype, arguing that the GATA-4 mutant protein does not act in a dominant negative manner.

Wild-type and the *GATA-4*^{ki/ki} embryonic hearts at E12.5 were examined in serial sections, cut in the transverse plane, and inspected from the cephalic to the caudal aspect of the specimens (Fig. 2C–H). *Gata4*^{ki/ki} hearts revealed a double-outlet right ventricle where both great arteries arise from the right ventricle (Fig. 2F). The ventricular septal defect is the only outlet for the left ventricle (Fig. 2F). In addition, the endocardial cushion cells of both the pulmonary and aortic outflow tracts appear more numerous than in wild-type embryos. Furthermore, robust mitotic activity is evident in these areas, and the pulmonary and aortic outflow tracts comingle in some planes of section. In wild-type embryos, however, the pulmonary and aortic outflow tracts display distinct endocardial cushions (Fig. 2E). Right and left atria of the

Figure 1. Targeting the GATA4-FOG-2 interaction in mice. (A) Structure of the N finger of chicken GATA-1 modeled with DNA. The essential valine is highlighted in red in both the illustration and in the sequence alignment of the murine factors, GATA-1 (V205) and GATA-4 (V217), shown below. Note the high conservation of the residues within the N finger of the two GATA proteins. (B) Partial restriction map of the murine *Gata4* wild-type locus (top), the *Gata4* knock-in targeting vector (middle), and targeted homologous recombination before excision of the selection cassette (bottom). The targeting construct contains the HSV-tk and neomycin resistance (*neo*^R) genes under the control of the mouse phosphoglycerate kinase (PGK) promoter. Homologous recombination results in replacement of wild-type *Gata4* with genomic DNA harboring a substitution of valine to glycine at position 217 in the N finger of GATA-4, as well as the incorporation of neomycin cassette. *Gata4* coding exons are shown as empty boxes, whereas the exon used as a probe used for Southern blot analysis is highlighted by a black box. S, *SacI*; E, *EcoRV*; B, *BglII*; N, *NotI*. (C) Southern blot analysis of ES cell DNA and mouse tail DNA (left panel) showing the presence of heterozygous mutant animals (ki/+). Analysis of E12.5 embryos resulting from an intercross of *Gata4* knock-in heterozygotes (ki/+), demonstrating the presence of all expected genotypes (right panel). The wild-type allele (WT) generated a 3.8-kb band after digestion of genomic DNA with *BglII*. In contrast, the knock-in mutated allele (Ki) generated a much larger fragment because of the replacement of the intronic *BglII* site with *NotI*.

Figure 2. Heart defects in *Gata4* mutant (*ki/ki*) embryos. (A,B) Wild-type (A) and mutant (B) embryos at E13.5 showing edema and peripheral hemorrhaging in a mutant. (C,D) Transverse sections through wild-type (C) and mutant (D) hearts at E13.5 at the level of the atrioventricular (AV) junction show enlarged atria, thin myocardium, and the absence of a ventricular septum. Original magnification, 40 $\times$. (E,F) Transverse sections of wild-type (E) and mutant (F) hearts at the level of the aortic and pulmonary outflow tracts. *Gata4*^{ki/ki} hearts have a double outlet right ventricle, in which all blood exits the heart into both great arteries, the pulmonary artery and the aorta. The left ventricle, which normally delivers blood to the aorta, fails to communicate with an artery in the mutant. Also note the apparent increase in cellularity of both outflow tracts and semilunar valves in the mutant. Original magnification, 400 $\times$. (G,H) Transverse sections of wild-type (G) and mutant (H) hearts at the level of the AV junction. *Gata4*^{ki/ki} hearts form a common AV valve that is situated between the left and right ventricles. For comparison, the mitral (MV) and tricuspid (TV) valves of the wild-type heart are indicated by arrowheads. Original magnification, 100 $\times$.

mutant are massively dilated and freely communicate (Fig. 2D,H), and a common atrioventricular valve opens into the center of a large ventricular cavity, which lacks a ventricular septum (Fig. 2H). In contrast to a wild-type heart, in which two distinct atrioventricular valves (the tricuspid and mitral valves; Fig. 2G) develop in continuity with the septum and with the aortic valve, respectively (Fig. 2E), the common AV valve in the mutant heart has no fibrous continuity with the aortic valve. In addition, *Gata4*^{ki/ki} myocardium appears thin (cf. Fig. 2G,H). These abnormalities were consistently seen in the *GATA-4*^{ki/ki} embryos. Similarly, complete penetrance was also observed in *FOG-2*^{-/-} embryos (Tevoesian et al. 2000).

To validate that the observed phenotype results from expression of a qualitatively different GATA-4 protein rather than from an altered pattern, or level, of expression, we examined GATA-4 protein expression. Wild-type and *Gata4*^{ki/ki} E12.5 hearts were sectioned in the sagittal plane and immunostained for expression of GATA-4 (Fig. 3A,B). At this stage, GATA-4 protein is present in both endocardium and myocardium of developing atria and ventricles. Immunostaining is strongest in surface endothelial cells and mesenchymal cells of the endocardial cushion tissues, which develop into the semilunar (Figs. 3B,F) and atrioventricular valves (Fig. 3D,H). GATA-4 protein is also present in the embryonic

liver and gut epithelium in both wild-type and mutant embryos, as expected (Fig. 3A,E; Arceci et al. 1993). Taken together, these studies demonstrate that *GATA-4*^{ki} protein is expressed comparably to wild-type protein.

In several respects, the morphology of *Gata4*^{ki/ki} hearts resembles that seen of *Fog2*^{-/-} embryos. A distinctive feature of *Fog2*^{-/-} hearts is the absence of coronary vasculature (Tevoesian et al. 2000). To examine the status of their coronary vasculature, *Gata4*^{ki/ki} hearts were immunostained for two endothelial cell markers. Flk-1, the receptor for vascular endothelial growth factor (VEGF), is not detectable in *Gata4*^{ki/ki} hearts, though lung tissue stains intensely (Fig. 4A). Similarly, abundance of the intracellular adhesion molecule-2 (ICAM-2) is reduced in *Gata4*^{ki/ki} hearts; note the absence of the developing capillary plexus (Fig. 4B, arrows). These findings are indistinguishable from those reported previously for *Fog2*^{-/-} hearts. As such, they provide strong evidence that *FOG-2* serves as a transcriptional cofactor for GATA-4 in myocardium as an essential step in the initiation of coronary vascular development.

Of numerous myocardial-expressed genes assayed in *Fog2*^{-/-} hearts, only the basic-helix-loop helix *eHand* and *dHand* genes were altered in their expression on loss of *Fog2* (Tevoesian et al. 2000). To assess this phenotype in *Gata4*^{ki/ki} hearts, the expression of *Hand* genes was examined by RNA in situ hybridization. Expression of

Figure 3. Expression of *Gata4* in the heart. Sagittal sections of wild-type (A–D) and *Gata4*^{ki/ki} (E–H) embryos at E12.5 were stained with an α-GATA-4 antibody. Both wild-type and mutant hearts display similar staining within the semilunar and AV valve cells. Note the staining of outflow tracts in both the wild-type and the mutant heart. At, atrium; AV, atrioventricular; V, ventricle. Original magnification, A,E,G, 40×; B,D,F,H, 400×; C, 100×.

eHand is reduced in the myocardium of *Gata4*^{ki/ki} hearts relative to that seen in wild-type hearts (Fig. 4C). Interestingly, whereas the expression of *eHand* in the outer curvature of the ventricles is reduced (Fig. 4C, white ar-

rows), its expression in the outflow tract is increased (Fig. 4C, dark arrows). This increased staining likely results from the increased cellularity within this region of the *Gata-4* mutant hearts and was not observed in *Fog2*-

Figure 4. Aberrant expression of coronary vessel and myocardial transcripts. (A) Staining of E12.5 wild-type (+/+) and mutant (ki/ki) hearts with an α-Flk-1 antibody (dorsal view). Staining of the mutant is vastly reduced within the heart, but lung tissue stained with equal intensity. (B) Immunostaining of wild-type and mutant hearts at E12.5 using the α-ICAM-2 antibody (dorsal view). Note the absence of a well-developed vascular tree in the mutant heart. (C) Whole-mount RNA in situ staining of *eHand* in E11.5 hearts. *eHand* expression is down-regulated in the outer myocardial layer (white arrows), whereas there is more intense staining in the outflow tract of the mutant (dark arrows). Note that the direction of the outflow tract relative to the heart is altered in the mutant, consistent with the pathological findings.

deficient hearts (Fig. 2F). In contrast, the expression of *dHand* is only marginally reduced in the mutant heart (data not shown).

Our data point to essential roles for the GATA-4:FOG-2 interaction in heart morphogenesis and coronary vascular development. The power of our analysis rests on the exquisite specificity of the knock-in mutation within the N finger of GATA-4. The residue we have chosen to modify is required for physical interaction with FOG-like proteins and does not influence the DNA-binding specificity of the GATA-protein (Crispino et al. 1999). Although displaying many similar features, *Gata4*^{ki/ki} hearts are distinguished from *Fog2*^{-/-} hearts, however, by the presence of a double-outlet right ventricle and defects in the semilunar valves and outflow tracts. As immunostaining confirmed that GATA-4 is expressed at wild-type levels in the semilunar valves of the *Gata4* mutant heart, it is likely that another FOG, or FOG-like protein, that functions as a cofactor for GATA-4 in transcription is expressed in these valve cells. Though high-level expression of the only other known vertebrate FOG-like factor, FOG-1, has not previously been observed by *in situ* RNA hybridization, FOG-1 transcripts are present at low levels in Northern blots of total heart RNA (A.P. Tsang and S.H. Orkin, unpubl.). Thus, it is quite possible that disruption of the physical interaction between GATA-4 and FOG-1, or a novel, undefined FOG protein, is responsible for impaired development of the semilunar valves and the appearance of a double-outlet right ventricle. Given the profound effects of mutation of either GATA-4 or FOG-2 proteins on heart morphogenesis in mice, it is worth considering their potential relevance to human congenital heart defects, such as the Tetralogy of Fallot or the double-outlet right ventricle (Walters et al. 2000). Whereas mutation of several genes, such as *Jmj* (Jumanji), *Sox4*, and *Egfr/Shp2*, give rise to the double-outlet right ventricle defect in mice (Chen et al. 1996; Ya et al. 1998; Lee et al. 2000), and defects in other genes for transcription factors, such as FOG-2, NF1, neurotrophin 3, and RXR α , result in all or a subset of the Tetralogy of Fallot (Jacks et al. 1994; Donovan et al. 1996; Gruber et al. 1996; Lee et al. 1997; Tevosian et al. 2000), the consistent and combined phenotype seen in the *Gata4*^{ki/ki} mice is unique. Although it is sometimes difficult to distinguish between double-outlet right ventricle with associated pulmonary stenosis and the Tetralogy of Fallot, it is clear that the defects in the *GATA-4*^{ki/ki} hearts are different in the outflow tracts than those observed in *Fog2*-deficient embryos.

Through the use of an altered specificity mutant, we demonstrate that GATA-4 very likely requires both FOG-2 and an additional FOG, or FOG-like protein, as cofactors for distinct aspects of heart development. Interaction with FOG-2 is essential for the initiation of coronary vasculature and for some morphogenetic events, whereas interaction with a distinct FOG protein appears to be required for formation of cardiac valves. Our results are surprising in that two other GATA-factors, GATA-5 and GATA-6, are also expressed in myo-

cardium, and indirect data have suggested that they might compensate for the absence of GATA-4. For example, previous studies show that GATA-4 is dispensable for terminal differentiation of cardiomyocytes and that *Gata4*^{-/-} ES cells contribute to all layers of the heart. In these experiments, it has been suggested that GATA-5 or GATA-6 functionally replace GATA-4 (Narita et al. 1996). It is possible that proper expression of the *GATA-4*^{ki/ki} protein, as distinguished from the absence of GATA-4 in the knock-out situation, precludes compensation by other GATA factors. Indeed, immunostaining with an α -GATA-6 antibody demonstrated that GATA-6 expression, though similar to that of GATA-4, is not up-regulated in the *Gata4*^{ki/ki} hearts (data not shown). In addition, staining with α -GATA-5 antibody revealed a normal pattern in *Gata4*^{ki/ki} hearts (data not shown). As GATA-5 is no longer expressed within the ventricles of the heart at E12.5, it is unlikely that it would compensate for the absence of functional GATA-4 (Morrissey et al. 1997).

Our findings implicate GATA-4 as the principal GATA factor relevant to heart morphogenesis and coronary vasculature development and as the primary partner for FOG proteins in the heart. This represents the second example of transcriptional regulation involving GATA-FOG protein complexes and argues for their broad involvement as key regulators of multiple developmental pathways.

Materials and methods

Targeted mutagenesis of the murine *Gata4* gene

An 8.2-kb *EcoRV*-*SacI* fragment of murine *Gata4* genomic DNA containing the N finger of GATA-4 was subcloned into pBluescript II KS (+/-) phagemid (Stratagene). By site-directed mutagenesis, Val 217 was changed to glycine (the codon GGC was changed to GTC; GeneEditor, Promega). An intronic *BglII* site was changed to a *NotI* site to facilitate introduction of a floxed neomycin expression cassette. HSV-tk was cloned into a *SalI* site 5' of the homology region. The targeting construct was linearized with *PvuI* and electroporated into TL1 ES cells. The *Gata4* gene from two independently generated, targeted ES clones was amplified by PCR, sequenced, and found to be correctly mutated in one clone. This clone was injected into C57BL/6 blastocysts to generate chimeras. Genotyping was done thereafter by Southern blot analysis, as described in the legend of Figure 1C.

Histological analysis

Mouse embryos were isolated from *Gata4* heterozygous knock-in matings between E10.5 and 13.5. Embryos were fixed in Bouin's, dehydrated into increasing concentrations of ethanol, transferred into xylene, and sectioned in paraffin at 6 μ m and stained with hematoxylin and eosin.

Immunohistochemistry

Murine tissue used for immunohistochemistry was fixed in 10% formalin overnight, processed, and paraffin embedded using standard histologic techniques. Tissue sections 4 μ thick were dewaxed in xylene then rehydrated by passage through graded alcohol solutions. Sections were immersed in 10 mmole/L citrate buffer (pH 6.0) in a thermoresistant container and heated in a microwave oven (800 W, General Electric) at 199°F for 30 min. A rabbit polyclonal antibody to GATA-4 (sc-9053, Santa Cruz Biotechnology) was used at a 1:50 dilution and detected by the Rabbit DAKO Envision Plus System, Peroxidase DAB (Dako). Negative controls were performed by substituting the primary antibody with species- and isotype-matched, nonimmune immunoglobulins. Additional controls included omission of the primary antibody as well as substitution of the primary antibody of interest with one of differing specificity. Whole-mount staining of hearts with the α -Flk-1 and α -ICAM-2 antibodies (ob-

tained from Pharmingen) was performed as described (Tevosian et al. 2000).

In situ hybridization analysis

Whole-mount hybridization was performed using riboprobes labeled with digoxigenin-UTP as described (Tevosian et al. 2000).

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