GATA-6 regulates semaphorin 3C and is required in cardiac neural crest for cardiovascular morphogenesis

John J. Lepore, Patricia A. Mericko, Lan Cheng, Min Min Lu, Edward E. Morrisey, and Michael S. Parmacek

Molecular Cardiology Research Center and the Penn Cardiovascular Institute, University of Pennsylvania, Philadelphia, Pennsylvania, USA.

GATA transcription factors play critical roles in restricting cell lineage differentiation during development. Here, we show that conditional inactivation of GATA-6 in VSMCs results in perinatal mortality from a spectrum of cardiovascular defects, including interrupted aortic arch and persistent truncus arteriosus. Inactivation of GATA-6 in neural crest recapitulates these abnormalities, demonstrating a cell-autonomous requirement for GATA-6 in neural crest–derived SMCs. Surprisingly, the observed defects do not result from impaired SMC differentiation but rather are associated with severely attenuated expression of semaphorin 3C, a signaling molecule critical for both neuronal and vascular patterning. Thus, the primary function of GATA-6 during cardiovascular development is to regulate morphogenetic patterning of the cardiac outflow tract and aortic arch. These findings provide new insights into the conserved functions of the GATA-4, -5, and -6 subfamily members and identify *GATA-6* and GATA-6–regulated genes as candidates involved in the pathogenesis of congenital heart disease.

Introduction

In higher vertebrates, the circulatory system has evolved to facilitate perfusion and exchange of oxygen and metabolites in dual systemic and pulmonary circulations that function in series. During embryogenesis, morphogenetic patterning of these dual circulations occurs through a complex process in which the pharyngeal arch arteries and cardiac outflow tract are sequentially repatterned, ultimately giving rise to the pulmonary artery, which arises from the right ventricle and perfuses the lungs, and to the aorta, which arises from the left ventricle and perfuses the systemic circulation. Defects in patterning and morphogenesis in this vascular remodeling program cause common forms of congenital heart disease observed in humans (reviewed in ref. 1).

Elegant quail-chick chimera experiments and chick embryo tissue ablation studies revealed that the cardiac neural crest plays a critical role in patterning the vertebrate vascular system (2). During early embryogenesis (E8.0–9.0 in the mouse), an ectodermal cell population, the cardiac neural crest, arises from the dorsal neural tube and migrates ventrally to populate the aortic arch arteries and cardiac outflow tract. These neural crest–derived cells respond to poorly characterized developmental cues to differentiate into VSMCs that populate the proximal aortic arch to the level of the ductus arteriosus, the pulmonary trunk, the ductus arteriosus, the carotid arteries, and the proximal subclavian arteries. In addition, 2 columns of cardiac neural crest cells migrate into the cardiac outflow tract where they fuse to form the aorticopulmonary septum and divide the single truncus arteriosus into a separate pulmonary artery and aorta.

Mutagenesis studies in mice have shown that defects in multiple signaling pathways that converge upon cardiac neural crest cells result in morphogenetic abnormalities of the aortic arch and cardiac outflow tract. The primary mechanism responsible for some of these defects is failure of cardiac neural crest cells to differentiate into SMCs whereas in other cases the defects result from impaired migration or survival of neural crest–derived cells (reviewed in ref. 3). Targeted inactivation of semaphorin 3C, a secreted class 3 semaphorin expressed in and adjacent to cardiac neural crest cells, causes impaired migration of neural crest cells to the developing cardiac outflow tract, resulting in interruption of the aortic arch and persistent truncus arteriosus (4, 5). It has been proposed that semaphorin 3C acts as a guidance molecule, regulating migration of neural crest cells that express semaphorin receptors such as plexin A2. However, the molecular mechanisms that regulate semaphorin 3C signaling and the role of cell-intrinsic expression of semaphorin C in cardiac neural crest cells are not well understood.

GATA-6, a member of the GATA family of zinc finger transcription factors, is abundantly expressed in VSMCs during murine embryonic and postnatal development (6). However, the function of GATA-6 in VSMCs remains unclear. Several studies have suggested that GATA-6 might play a role in maintaining the contractile VSMC phenotype by activating SMC-restricted genes and by inhibiting SMC proliferation (7–9). In this regard, it is noteworthy that other GATA family members have been shown to restrict the developmental potential of specific cell lineages during embryonic and postnatal development (reviewed in ref. 10). However, the following findings suggest that the function of GATA-6 must be more complex than simple promotion of a contractile SMC phenotype: (a) GATA-6 is not expressed in all SMCs (6); (b) GATA-6 is expressed in proliferating VSMCs during embryonic development (6); (c) most SMC-restricted transcriptional regulatory elements lack functionally important GATA-binding sites; and (d) forced

Nonstandard abbreviations used: Ad, adenovirus; BAC, bacterial artificial chromosome; Cre, Cre recombinase; *Dab2*, *disabled homolog 2*; neo, neomycin phosphotransferase II.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **116**:929–939 (2006). doi:10.1172/JCI27363.

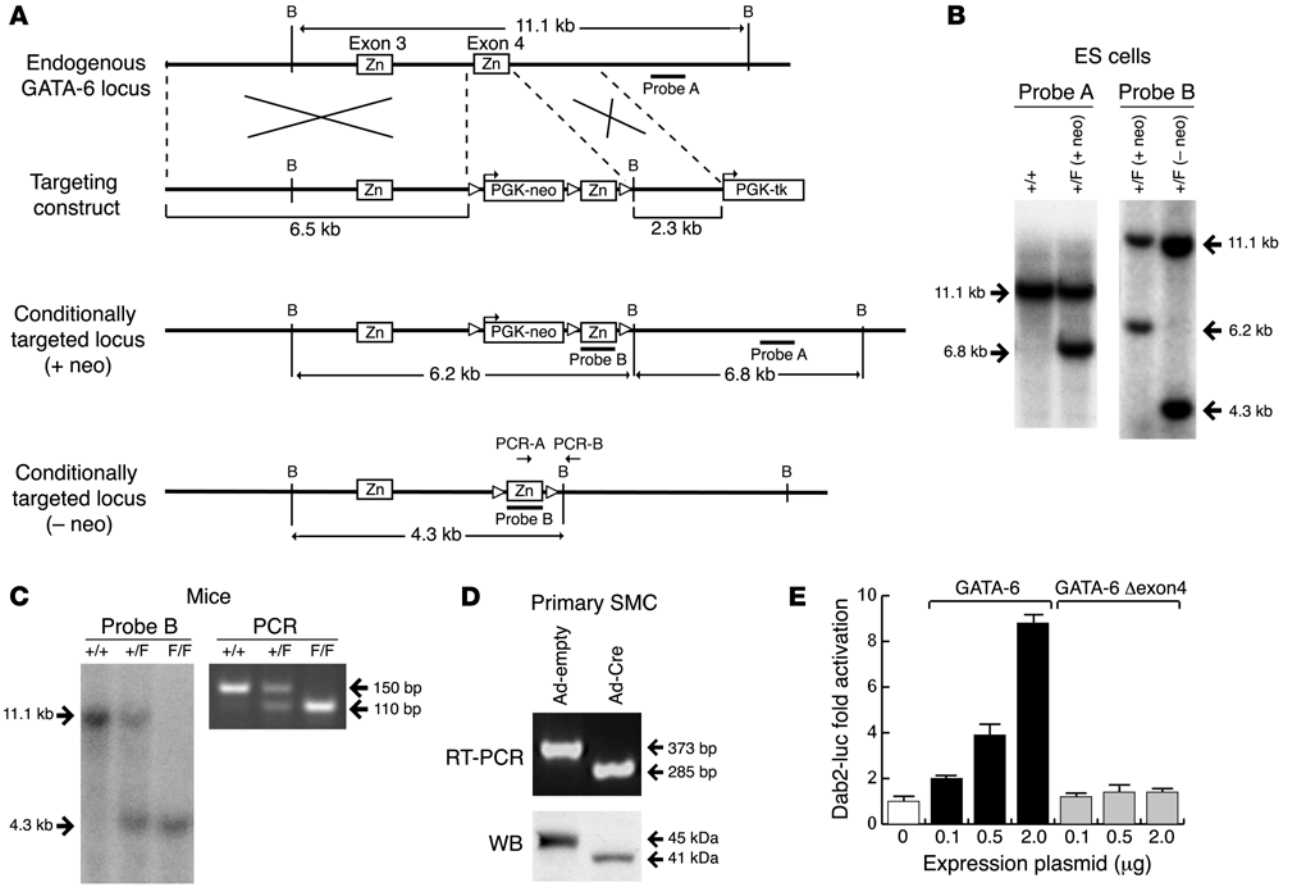

Figure 1

Conditional targeting of murine *GATA-6*. (**A**, top panel) *GATA-6* locus containing exons 3 and 4 (rectangles) and the targeting construct containing phosphoglycerate kinase–regulated (PGK-regulated) *neo* and HSV thymidine kinase (*tk*) genes. loxP sites (triangles) flank *neo* and exon 4 encoding the C-terminal zinc finger (Zn) DNA-binding domain. B, *BamHI*. (Middle panel) Conditionally targeted *GATA-6* allele. (Bottom panel) Targeted allele following selective *neo* deletion. (**B**, left) Southern blotting (probe A) of targeted ES cells identifies wild-type (11.1 kb) and conditionally targeted (6.8 kb) alleles. (Right) Southern blotting (probe B) of ES cells following Cre transfection identifies wild-type (11.1 kb) and conditionally targeted alleles with *neo* (6.2 kb) and with selective *neo* deletion (4.3 kb). (**C**) Genotyping of wild-type (+/+), heterozygous (+/F), and homozygous (F/F) conditionally targeted mice. (Left) Southern blotting (probe B) identifies wild-type (11.1 kb) and conditionally targeted (4.3 kb) alleles. (Right) PCR using primers PCR-A and PCR-B identifies products corresponding to wild-type (150 bp) and targeted (110 bp) alleles. (**D**) Analysis of primary *GATA-6*$^{F/F}$ aortic SMCs infected with Ad-empty or Ad-Cre. RT-PCR identifies products corresponding to wild-type GATA-6 (373 bp) and GATA-6 following exon 4 deletion (285 bp). Western blotting (WB) identifies full-length (45 kDa) and truncated (41 kDa) GATA-6 proteins. (**E**) Activation of the GATA-dependent *Dab2*-LUC reporter. NIH3T3 cells were transiently transfected with 100 ng of the *Dab2*-LUC reporter and with 0.1–2.0 μg of either pcDNA3–GATA-6 or pcDNA3–GATA-6–Δexon4. The reporter was activated by expression of increasing amounts of wild-type GATA-6, but not by expression of the truncated GATA-6–Δexon4 protein.

expression of myocardin activates serum response factor–dependent SMC genes in wild-type as well as in GATA-6–deficient ES cells (11). In support of this view, an unbiased screen of genes regulated by GATA-6 in VSMCs demonstrated that GATA-6 regulates genes encoding growth factors and their receptors and proteins involved in cell-cell and cell-matrix interactions but not SMC-restricted cytoskeletal and contractile proteins (11).

Mice harboring a null mutation in *GATA-6* exhibit a block in differentiation of the visceral endoderm and lethality at E6.5 (12), precluding assessment of the function of GATA-6 in the cardiovascular system. In the studies described in this report, we employed tissue-specific gene targeting to selectively inactivate GATA-6 in VSMCs and in the cardiac neural crest. These studies revealed a critical, cell-autonomous role for GATA-6 in neural crest–derived SMCs. We show that GATA-6 functions in these cells not to mediate SMC differentiation, but rather to regulate morphogenetic patterning of the aortic arch and cardiac outflow tract, at least in part by transcriptional regulation of *semaphorin 3C*.

Results

Generation of ES cells and mice with conditional targeting of GATA-6. To conditionally inactivate *GATA-6*, we created a modified allele in which exon 4 is flanked by loxP sites. We targeted exon 4 because it encodes the carboxyterminal zinc finger DNA-binding domain that is required for DNA binding and transcriptional activity of GATA factors. Hence, deletion of *GATA-6* exon 4 is predicted to produce a functional null allele (13, 14). Correctly targeted ES cell clones (Figure 1A) and targeted ES cell clones in which the neomycin phosphotransferase II (neo) was selectively deleted by transient transfection of Cre recombinase (Cre) (Figure 1, A and B)

Table 1
Genotype distribution of embryonic and perinatal mortality following *SM22Cre*- and *Wnt1Cre*-mediated conditional deletion of *GATA-6*

Genotype	E9.5–E15.5	E18.5	P1	2 wk
SM22Cre Cross				
SMCre^{-}G6$^{+/F}$	78	60	31	20
SMCre^{-}G6$^{F/F}$	76	52	24	25
SMCre^{+}G6$^{+/F}$	70	50	30	22
SMCre^{+}G6$^{F/F}$	69 (24%)	35 (18%)	2 (2%)	0
Wnt1Cre Cross				
Wnt1Cre^{-}G6$^{+/F}$	17	27	16	–
Wnt1Cre^{-}G6$^{F/F}$	14	27	18	–
Wnt1Cre^{+}G6$^{+/F}$	12	29	10	–
Wnt1Cre^{+}G6$^{F/F}$	18 (29%)	23 (22%)	0	–

were identified by Southern blotting. The conditionally targeted *GATA-6* allele was transmitted through the germline (Figure 1C). Homozygous *GATA-6$^{F/F}$* mice were viable and fertile and exhibited no cardiovascular abnormalities. RT-PCR performed on mRNA isolated from *GATA-6$^{F/F}$* primary aortic SMCs infected with control adenovirus (Ad-empty) or adenovirus expressing Cre (Ad-Cre) produced 373-bp and 285-bp products, respectively (Figure 1D). DNA sequence analysis verified that the PCR products corresponded to the wild-type GATA-6 cDNA and to the expected GATA-6 cDNA in which exon 4 sequences are deleted, respectively (data not shown). These data demonstrate that transcription of the *GATA-6* gene from the conditionally targeted locus is not impaired and that Cre-mediated deletion of the floxed exon 4 occurs as designed.

Western blotting of protein extracts from *GATA-6$^{F/F}$* primary aortic SMCs infected with Ad-empty or Ad-Cre identified 45 kDa and 41 kDa proteins, respectively, consistent with the expected molecular weights of full-length GATA-6 and GATA-6 lacking the amino acids encoded by exon 4 (Figure 1D). The demonstration of a truncated protein lacking the C-terminal zinc finger in conditionally targeted cells suggested the possibility that the observed phenotype might be hypomorphic. To determine whether the truncated GATA-6 protein (GATA-6–Δexon4) is functionally null, as predicted, we examined the capacity of pcDNA3–GATA-6–Δexon4 to transactivate a luciferase reporter plasmid containing the GATA-dependent *disabled homolog 2* (*Dab2*) promoter (15). As anticipated, the reporter plasmid was activated by cotransfection of increasing concentrations of pcDNA3–GATA-6 but not by cotransfection of similar concentrations of pcDNA3–GATA-6–Δexon4 (Figure 1E). In similar experiments, pcDNA3–GATA-6–Δexon4 also failed to transactivate the GATA-dependent *aquaporin-5* promoter (16) and the *semaphorin 3C* promoter (data not shown). Moreover, mice homozygous for germline deletion of exon 4 exhibited early embryonic lethality (data not shown), recapitulating the phenotype we previously observed in *GATA-6$^{-/-}$* mice (12). In addition, foregut endoderm–specific deletion of *GATA-6* in *GATA-6$^{F/F}$* mice results in loss of lung development (E.E. Morrisey, unpublished observation), which phenocopies tetraploid rescue of *GATA-6$^{-/-}$* mice (17). Taken together, these findings argue strongly that the observed cardiovascular phenotype in conditionally targeted *GATA-6* mice is null, not hypomorphic.

Conditional deletion of GATA-6 in VSMCs results in perinatal lethality. To investigate the function of GATA-6 in VSMCs in vivo, *GATA-6$^{F/F}$* mice were interbred with transgenic mice expressing Cre under the transcriptional control of the *SM22α* promoter. We reported recently that *SM22Cre* transgenic mice promote highly efficient Cre-mediated recombination as early as E9.5 in neural crest–derived SMCs, including those populating the third, fourth, and sixth pharyngeal arch arteries and in their derivatives; in mesoderm-derived SMCs, including those populating the paired dorsal aortae; and in cardiac myocytes (11). Offspring of *SMCre^{+}GATA-6$^{+/F}$* mice intercrossed with *GATA-6$^{F/F}$* mice failed to produce the anticipated Mendelian ratio of inheritance (Table 1). Of 67 offspring analyzed at 2 weeks of age, no viable mice were observed that carried both the *SM22Cre* allele and 2 copies of the targeted *GATA-6* allele (*SMCre^{+}GATA-6$^{F/F}$* mice). In contrast, analysis of 293 embryos up to age E15.5 revealed the expected Mendelian ratio of *SMCre^{+}GATA-6$^{F/F}$* embryos (Table 1). A slight reduction in the expected number of *SMCre^{+}GATA-6$^{F/F}$* mice was observed at E18.5. However, all *SMCre^{+}GATA-6$^{F/F}$* mice died between E18.5 and P2 (Table 1).

Cardiac outflow tract defects in SMCre^{+}GATA-6$^{F/F}$ mutant embryos. To determine the cause of perinatal lethality, *SMCre^{+}GATA-6$^{F/F}$* embryos were characterized at E18.5. Visual inspection, histological analysis, and vascular casting with opaque material injected into the left ventricle revealed aortic arch patterning defects and/or cardiac outflow tract abnormalities in all 11 mutant embryos examined (Table 2 and Figure 2). Cardiac outflow tract abnormalities included persistent truncus arteriosus in 7 embryos and double-outlet right ventricle in 4 embryos. All 11 embryos exhibited membranous ventricular septal defects. Aortic arch patterning defects included interrupted aortic arch in 7 embryos, hypoplastic aortic arch in 7 embryos, retroesophageal right subclavian artery in 6 embryos, and absent ductus arteriosus in 1 embryo. Consistent with these defects, several newborn mice were observed to be cyanotic and to expire shortly after birth, and postmortem analyses revealed cardiac outflow tract defects similar to those observed in E18.5 embryos. Together, these data demonstrate that GATA-6 is required for proper patterning of the aortic arch arteries and septation of the cardiac outflow tract as well as for neonatal survival. Because the *SMCre* transgene promotes Cre-mediated recombination in both VSMCs and cardiac myocytes (11), these data support the conclusion that the observed phenotype of *SMCre^{+}GATA-6$^{F/F}$* embryos resulted from mutation of *GATA-6* in either VSMCs or in cardiac myocytes.

The cardiovascular malformations observed recapitulated common forms of congenital heart disease that have been attributed

Table 2
Summary of phenotypes of E18.5 embryos following *SM22Cre*-mediated conditional deletion of GATA-6

Phenotype	Number
Persistent truncus arteriosus	7/11
Double-outlet right ventricle	4/11
Membranous ventricular septal defect	11/11
Interrupted aortic arch	7/11
Retroesophageal right subclavian artery	6/11
Hypoplastic aortic arch	7/11
Absent ductus arteriosus	1/11

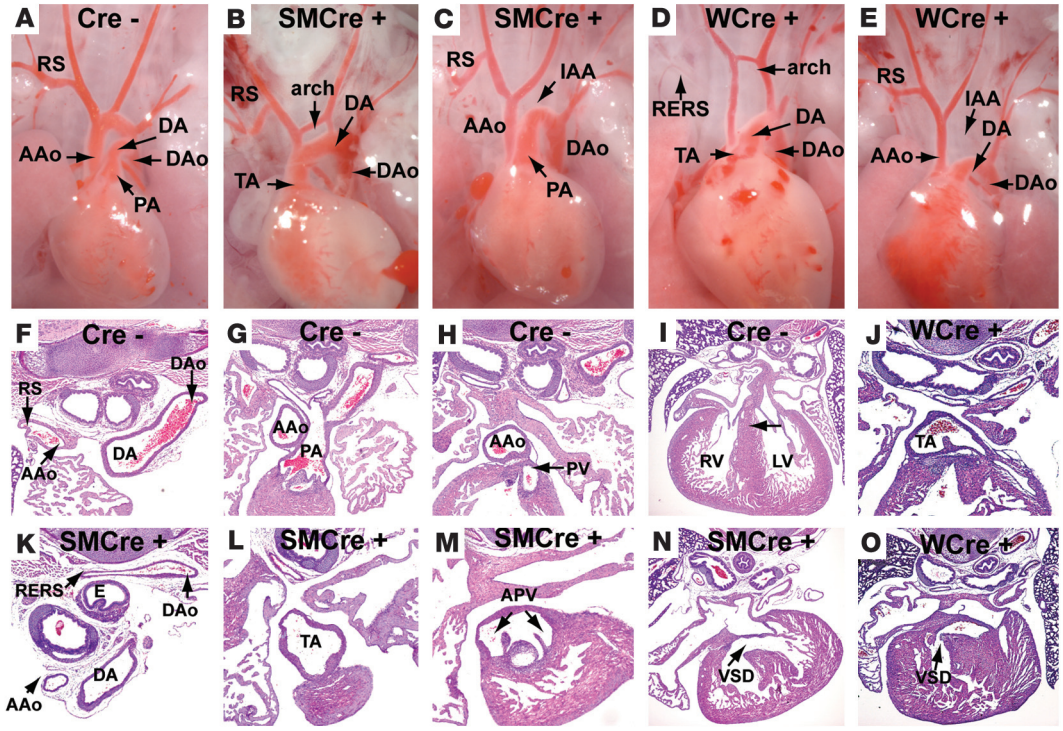

Figure 2

Cardiovascular abnormalities produced by conditional *GATA-6* deletion. Aortic arch patterning and cardiac outflow tract septation were examined in E18.5 *GATA-6*$^{F/F}$ (Cre–; **A** and **F**–**I**), *SMCre*$^{+}$*GATA-6*$^{F/F}$ (SMCre+; **B**, **C**, and **K**–**N**), and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ (WCre+; **D**, **E**, **J**, and **O**) embryos using polymer vascular casting (**A**–**E**) and H&E staining (**F**–**O**). (**A**) In normal aortic arch patterning, the ascending aorta (AAo) and pulmonary artery (PA) are distinct, septated vessels. The ductus arteriosus (DA) is patent and connects the PA to the proximal descending aorta (DAo). The right subclavian artery (RS) branches from the AAo. (**B**) SMCre^{+} embryo demonstrating truncus arteriosus (TA) and hypoplastic aortic arch (arch). (**C**) SMCre^{+} embryo demonstrating interrupted aortic arch (IAA). (**D**) WCre^{+} embryo demonstrating TA, hypoplastic arch, and retroesophageal right subclavian artery (RERS). (**E**) WCre^{+} embryo demonstrating IAA. (**F**–**I**) Serial histological sections through the heart and great vessels of a normal embryo. (**F**) The DA connects the PA to the DAo. The RS branches from the AAo. (**G** and **H**) The AAo and PA are distinct, septated vessels. The pulmonary valve (PV) is shown. (**I**) An intact ventricular septum (arrow) separates the right and left ventricles. (**K**–**N**) Corresponding serial sections from a SMCre^{+} embryo. (**K**) A RERS branches from the DAo and travels posterior to the esophagus (E). (**L**–**N**) There is single outflow tract vessel, or TA; a single, common aorticopulmonary valve (APV); and a membranous ventricular septal defect (VSD). (**J** and **O**) Representative WntCre^{+} embryo exhibiting TA and VSD. Original magnification, ×20 (**A**–**E**); ×40 (**F**–**O**).

to defects in cardiac neural crest (1). In situ hybridization studies demonstrated that GATA-6 is abundantly expressed in neural crest derivatives during embryonic development. In wild-type embryos, GATA-6 mRNA is observed as early as E9.5 in the cardiac outflow tract (Figure 3A), which is thought to be populated by SMCs derived from both cardiac neural crest and the secondary heart field (18–20). At E9.5, GATA-6 is also expressed in cardiac myocytes in the atrium, bulbous cordis, and left ventricle (Figure 3B). At E11.5, GATA-6 is expressed in the SMC layers (Figure 3C) of the ascending aorta and pulmonary artery (Figure 3C). At E11.5, GATA-6 is also expressed in regions of the conotruncal endocardial cushions that contain columns of migrating cardiac neural crest cells that are required for outflow tract septation (3, 5) (Figure 3D) and in the cuff of cardiac myocytes at the base of the outflow tract (Figure 3D). Of note, expression of GATA-6 mRNA was observed from the proximal myocardial cuff through the proximal ascending aorta and pulmonary artery, suggesting that GATA-6 is expressed in SMCs derived from both the cardiac neural crest and secondary heart fields (18–20). As anticipated, in situ hybridization analyses, performed with a cRNA probe corresponding to exon 4 of the *GATA-6* gene, demonstrated that *GATA-6* gene expression in the myocardium and cardiac outflow tract was essentially abolished in E11.5 *SMCre*$^{+}$*GATA-6*$^{F/F}$ embryos compared with control embryos (Figure 3, E and F). Subsequently, at E12.5, GATA-6 was expressed abundantly in VSMCs of the aorta, pulmonary artery, and ductus arteriosus (Figure 3G), in cardiac myocytes in the atria and left and right ventricles (Figure 3G), and in the conotruncal endocardial cushions (Figure 3G). Together, these studies demonstrate that GATA-6 is abundantly expressed in SMCs derived from the neural crest and secondary heart field that populate the great vessels and in the conotruncal cushions. In addition, GATA-6 is expressed in the myocardium and in mesodermally derived VSMCs. Therefore, because *SM22Cre* mice promote Cre-mediated recombination in SMCs derived from lateral mesoderm in cardiac myocytes as well as in neural crest–derived SMCs (21), the observed cardiovascular abnormalities in *SMCre*$^{+}$*GATA-6*$^{F/F}$ embryos could result from cell autonomous defects in either neural crest or nonneural crest derivatives populating the cardiac outflow tract and major arteries.

Neural crest–specific deletion of GATA-6 causes cardiac outflow tract defects. To determine whether the aortic arch patterning and out-

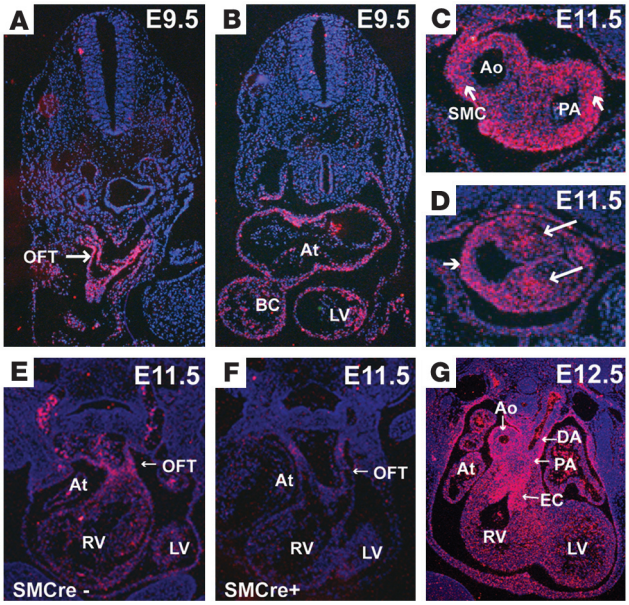

Figure 3
Expression of GATA-6 in cardiac neural crest derivatives. GATA-6 expression was examined by in situ hybridization in wild-type, *SM22Cre⁻GATA-6$^{F/F}$* (SMCre⁻), and *SM22Cre⁺GATA-6$^{F/F}$* (SMCre⁺) embryos. (**A** and **B**) In wild-type embryos at E9.5, GATA-6 is expressed in the cardiac outflow tract (OFT), atria (At), bulbus cordis (BC), and left ventricle. (**C** and **D**) In wild-type embryos at E11.5, GATA-6 is expressed in the SMCs (**C**, arrows) that populate the vascular wall of the AAo and PA, in the conotruncal endocardial cushions in the regions populated by migrating neural crest cells (**D**, long arrows) and in the cuff of cardiac myocytes at the base of the outflow tract (**D**, short arrow). (**E** and **F**) In situ hybridization studies with a GATA-6 exon 4 probe demonstrate GATA-6 expression in the OFT, At, and right and left ventricles that is markedly attenuated in E11.5 SMCre⁺ embryos (**F**) as compared with SMCre⁻ embryos (**E**). (**G**) In wild-type embryos at E12.5, GATA-6 is abundantly expressed in neural crest–derived SMCs populating the aorta (Ao), PA, and DA; in cardiac myocytes in the At, right ventricle, and left ventricle; and in the endocardial cushions (EC). Original magnification, ×100 (**A**, **B**, and **E**–**G**); ×200 (**C** and **D**).

flow tract septation defects observed in *GATA-6* mutant embryos resulted from a cell-intrinsic defect in neural crest–derived cells, *GATA-6$^{F/F}$* mice were interbred with *Wnt1Cre* transgenic mice (18). These mice generated highly efficient Cre-mediated recombination in the vast majority of neural crest–derived SMCs populating pharyngeal arch arteries 3, 4, and 6 and the truncus arteriosus beginning as early as E9.5. By E11.5, they also produced Cre-mediated recombination in cardiac neural crest cells invading the conotruncal cushions and the aorticopulmonary septum. However, in contrast to *SM22Cre* mice, *Wnt1Cre* mice did not produce Cre-mediated recombination in cardiac myocytes or in mesoderm-derived SMCs. Remarkably, the phenotype of *Wnt1Cre⁺GATA-6$^{F/F}$* embryos recapitulated that of *SMCre⁺GATA-6$^{F/F}$* mutant embryos. These embryos survived to E18.5 in slightly less than the expected Mendelian ratio (Table 1), but no *Wnt1Cre⁺GATA-6$^{F/F}$* mice survived past P1. E18.5 *Wnt1Cre⁺GATA-6$^{F/F}$* embryos demonstrated a spectrum of aortic arch patterning and cardiac outflow tract septation defects that were not distinguishable from those observed in *SMCre⁺GATA-6$^{F/F}$* embryos (Figure 2). Hence, GATA-6 is required in neural crest–derived SMCs for proper cardiovascular morphogenesis.

Effect of GATA-6 deletion on SMC specification and differentiation. Previous studies suggested that GATA-6 regulates SMC differentiation. In theory, failure of cardiac neural crest cells to differentiate into SMCs could contribute to the observed phenotype. However, no evidence of impaired SMC differentiation was observed following deletion of *GATA-6* in either *SMCre⁺GATA-6$^{F/F}$* or *Wnt1Cre⁺GATA-6$^{F/F}$* embryos. In a representative E11.5 *SMCre⁺GATA-6$^{F/F}$* embryo (Figure 4B), the pharyngeal arch arteries were present and morphologically normal and exhibited expression of SMα-actin that was similar to that observed in a control *SMCre⁻GATA-6$^{F/F}$* embryo (Figure 4A). At E12.5, major malformations of the aortic arch and outflow tract septation were observed. As shown in an E12.5 *SMCre⁺GATA-6$^{F/F}$* embryo exhibiting aortopulmonary window (Figure 4, D and F), the distal aortopulmonary septum as well as the vascular walls of the aorta and the pulmonary artery contained morphologically normal SMC layers and exhibited SMα-actin immunostaining that was indistinguishable from that observed in the corresponding vessels of control embryos (Figure 4, C and E). Similarly, at E18.5, the morphology and expression of SMα-actin of the great vessels in mutant (Figure 4, H and J) and control embryos (Figure 4, G and I) were indistinguishable. Moreover, deletion of *GATA-6* in *GATA-6$^{F/F}$* primary aortic SMCs infected with Ad-Cre did not significantly alter expression of SM22α, SMα-actin, smooth muscle myosin heavy chain, or calponin h1, which are markers of the contractile SMC phenotype (Figure 4K).

Together, these data indicate that the aortic arch patterning defects observed in *SMCre⁺GATA-6$^{F/F}$* or *Wnt1Cre⁺GATA-6$^{F/F}$* mutant embryos did not result from impaired differentiation of neural crest cells into SMCs and suggest that the patterning defects were not related to impaired survival of neural crest–derived SMCs. Importantly, *Wnt1Cre*-mediated recombination occurred in the cardiac neural crest prior to differentiation of neural crest cells into VSMCs, directly supporting the conclusion that GATA-6 is not required for specification or differentiation of neural crest–derived SMCs. In addition, since the aortic arch patterning defects noted in older embryos were not caused by failure of formation of the pharyngeal arch arteries (Figure 4, A and B), these data strongly suggest that the patterning defects observed resulted from lack of 1 or more instructive signals required for appropriate remodeling of the pharyngeal arch arteries and cardiac outflow tract.

Regulation of semaphorin 3C expression by GATA-6 in cardiac neural crest cells. The observed phenotype of interrupted aortic arch and persistent truncus arteriosus recapitulated that observed in *semaphorin 3C* null mice (4, 5). Moreover, in an unbiased screen of genes regulated by GATA-6 in VSMCs, we previously observed that *semaphorin 3C* gene expression (EST BM390322) was downregulated by a dominant-negative GATA-6 protein (11). Therefore, expression of the *semaphorin 3C* gene was assessed by in situ hybridization analysis in *SM22Cre⁺GATA-6$^{F/F}$* and *Wnt1Cre⁺GATA-6$^{F/F}$* embryos.

Consistent with prior studies (4, 5), semaphorin 3C was expressed abundantly in neural crest–derived SMCs in the vascular wall of the aorta, pulmonary artery, and ductus arteriosus in control E12.5 *SMCre⁻GATA-6$^{F/F}$* embryos (Figure 5A) as well as in

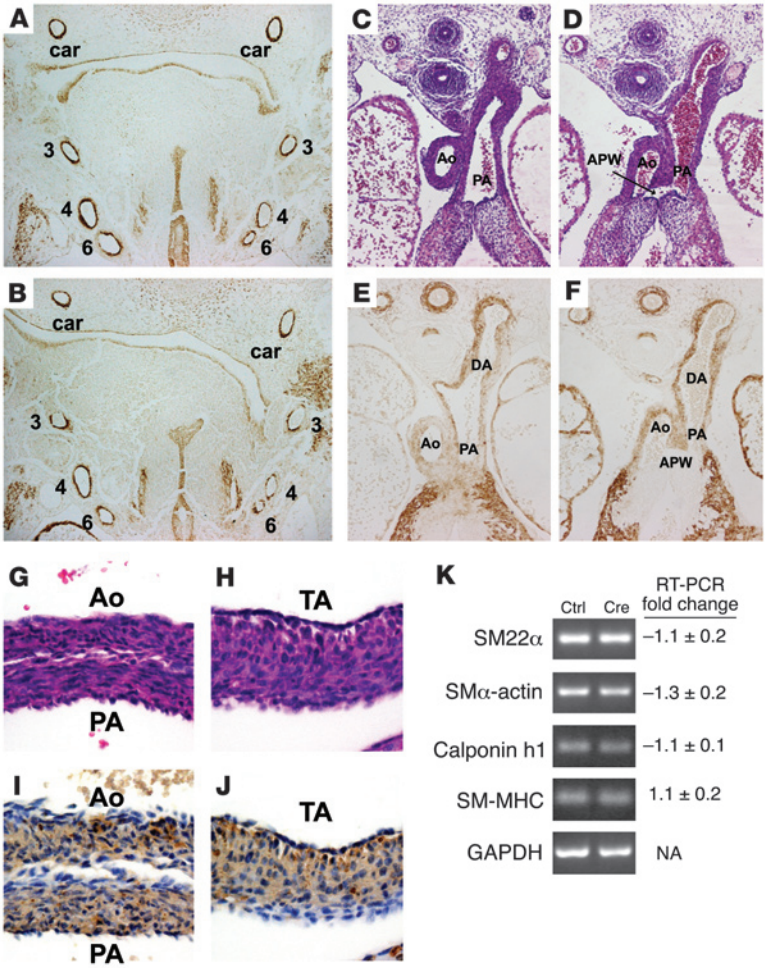

Figure 4
Effect of GATA-6 deletion on neural crest–derived SMC differentiation. (**A**–**J**) H&E staining (**C**, **D**, **G**, and **H**) and SMα-actin (SMA) immunohistochemistry (**A**, **B**, **E**, **F**, **I**, and **J**) of *SM22Cre⁻GATA-6$^{F/F}$* (SMCre⁻) and *SM22Cre⁺GATA-6$^{F/F}$* (SMCre⁺) embryos. Original magnification, ×100 (**A**–**F**); ×200 (**G**–**J**). (**A** and **B**) Frontal section through the pharyngeal arches in an E11.5 SMCre⁺ embryo (**B**) demonstrates normal formation of pharyngeal arches 3, 4, and 6 and SMA immunostaining in SMCs of the pharyngeal arch arteries and carotid arteries (car) similar to that in a normal SM22Cre⁻ embryo (**A**). (**C**–**F**) In a representative E12.5 SMCre⁺ embryo (**D** and **F**), despite the presence of aortopulmonary window (APW), the structure of the vessel wall and the presence of SMA immunostaining in neural crest–derived SMCs in the vascular wall of the aorta and PA are not different compared with a normal SMCre⁻ embryo (**C** and **E**). (**G**–**J**) Cross sections through the vascular walls of Ao and PA of an E18.5 SM22Cre⁻ embryo (**G** and **I**) and the vascular wall of the truncus arteriosus (TA) of a SM22Cre⁺ embryo (**H** and **J**) demonstrate similar structure of the vessel wall and presence of SMA immunostaining neural crest–derived SMCs. (**K**) Quantitative RT-PCR of primary *GATA-6$^{F/F}$* aortic SMCs infected with Ad-empty (Ctrl) or Ad-Cre (Cre) demonstrates that GATA-6 deletion does not change expression of mRNA encoding SMC-restricted cytoskeletal and contractile proteins. The ethidium-stained products amplified at the cycle threshold for each message and for control GAPDH are shown with the fold change in expression.

a cuff of myocardial cells at the base of the cardiac outflow tract (Figure 5, A and B, arrows). However, semaphorin 3C expression was markedly decreased in SMCs in the walls of the aorta and pulmonary artery in *SM22Cre⁺GATA-6$^{F/F}$* embryos (Figure 5B) as well as in *Wnt1Cre⁺GATA-6$^{F/F}$* embryos (data not shown) whereas myocardial expression of semaphorin 3C was not significantly changed (Figure 5B). In contrast, expression of plexin A2, a marker of the neural crest, was not decreased in SMCs populating the aorta and pulmonary artery in *SM22Cre⁺GATA-6$^{F/F}$* embryos (Figure 5, C and D) or in *Wnt1Cre⁺GATA-6$^{F/F}$* embryos (data not shown). These findings suggest that plexin A2–expressing cardiac neural crest cells correctly migrate into the great vessels and differentiate into SMCs, but that these neural crest–derived SMCs exhibit a specific block in *semaphorin 3C* gene expression.

In addition to populating the tunica media of the great arteries, cardiac neural crest cells also migrate as 2 columns of cells that invade the cardiac outflow tract and populate the conotruncal endocardial cushions where they play a critical role in mediating conotruncal septation, which occurs at approximately E11.5 in mice (3, 5). In cross sections through the developing cardiac outflow tract in E11.5 *Wnt1Cre⁻GATA-6$^{F/F}$* embryos, 2 distinct populations of plexin A2–expressing cells, which likely represent the 2 columns of invading cardiac neural crest cells, were identified by in situ hybridization with probes for semaphorin 3C and plexin A2, respectively (Figure 5, E and G). In contrast, neither plexin A2– nor semaphorin 3C–expressing cells were observed in the conotruncal cushions in *Wnt1Cre⁺GATA-6$^{F/F}$* embryos (Figure 5, F and H) or in *SM22Cre⁺GATA-6$^{F/F}$* embryos (data not shown). These findings are consistent with a model in which migration of cardiac neural crest cells into the conotruncal cushions is impaired and suggests that absence of cardiac neural crest cells in these tissues may contribute to failure of conotruncal septation. Thus, *Wnt1Cre⁺GATA-6$^{F/F}$* and *SM22Cre⁺GATA-6$^{F/F}$* embryos exhibit both impaired expression of semaphorin 3C in SMCs populating the great vessels and impaired migration of plexin A2–expressing cells, which most likely represent neural crest derivatives, into the conotruncal cushions.

Transcriptional regulation of semaphorin 3C by GATA-6. Together with prior studies suggesting that *semaphorin 3C* gene expression is regulated by GATA-6 in vitro, the observation that semaphorin 3C expression is markedly decreased in SMCs of the great vessels suggested that *semaphorin 3C* might be a direct transcriptional target of GATA-6 in neural crest–derived SMCs. Comparison of the mouse and human *semaphorin 3C* genes by VISTA analysis (http://genome.lbl.gov/vista/index.shtml) revealed regions of greater than 75% sequence identity in the promoter region as well as in the first intron (Figure 5I). Within or adjacent to these regions of sequence homology, there are 4 putative GATA-binding sites (Figure 5I) that are conserved in the mouse, rat, and human *semaphorin 3C* genes, and each species also contains multiple addi-

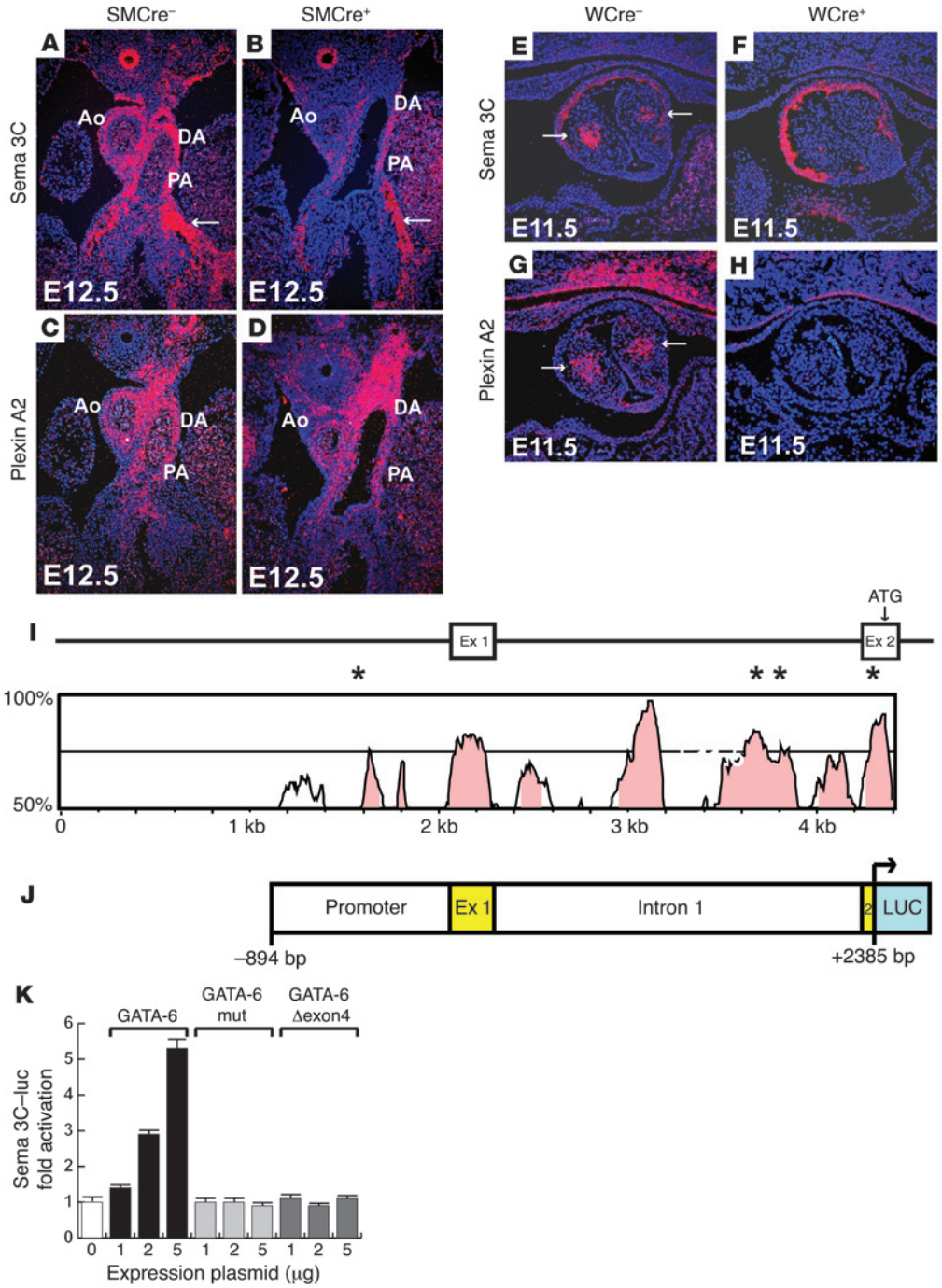

Figure 5

Transcriptional regulation of *semaphorin 3C* (sema 3C) by GATA-6. (**A**–**H**) Semaphorin 3C and plexin A2 in situ hybridization. In E12.5 *SM22Cre⁻ GATA-6$^{F/F}$* (SMCre⁻) embryos, semaphorin 3C (**A**) and plexin A2 (**C**) are abundantly expressed in neural crest–derived SMCs populating the aorta, PA, DA, and cuff of surrounding myocardial cells (arrows). In SMCre⁺ embryos, SMC expression of semaphorin 3C (**B**) is markedly reduced whereas plexin A2 expression (**D**) is not. Semaphorin 3C– (**E**) and plexin A2– (**G**) expressing neural crest cells are identified within the conotruncal endocardial cushions (arrows) in E11.5 *Wnt1Cre⁻GATA-6$^{F/F}$* (WntCre⁻) embryos, but semaphorin 3C and plexin A2 expression is not observed in WCre⁺ embryos (**F** and **H**, respectively). Original magnification, ×100 (**A**–**D**); ×200 (**E**–**H**). (**I**) VISTA comparison of murine and human *semaphorin 3C* proximal promoter, exon 1 (ex 1), and exon 2. The *x* and *y* axes indicate sequence length (kb) and percentage of homology (≥ 75%, pink), respectively. GATA-binding sites conserved in mouse, rat, and human sequence are indicated by asterisks. (**J**) Schematic of the *sema3C*-LUC reporter construct containing the 0.9-kb proximal promoter, exon 1, intron 1, and 35 bp of exon 2 upstream of firefly luciferase (LUC). (**K**) Activation of the *sema3C*-LUC reporter by GATA-6. NIH3T3 cells were transiently transfected with 100 ng of *sema3C*-LUC and with 1–5 μg of expression plasmid encoding wild-type GATA-6, GATA-6 containing zinc finger mutations abrogating DNA binding (GATA-6 mut), or GATA-6 lacking sequences encoded by exon 4 (GATA-6 Δexon4). The reporter was activated by expression of wild-type GATA-6 but not by expression of GATA-6 mut or GATA-6 Δexon4.

tional GATA sites in this region (ENSEMBL and NCBI databases, http://www.ensembl.org/indix.html and http://www.ncbi.nih.gov/BLAST, respectively). To determine whether *semaphorin 3C* gene expression is directly regulated by GATA-6, the capacity of GATA-6 to transactivate a luciferase reporter plasmid under the transcriptional control of the *semaphorin 3C* promoter and first intron sequences (Figure 5J) was assessed. Remarkably, cotransfection of increasing concentrations of the pcDNA3–GATA-6 expression plasmid resulted in stepwise activation of the *sema3C*-LUC reporter plasmid, demonstrating that the *semaphorin 3C* promoter is activated by GATA-6 (Figure 5K). In contrast, cotransfection with pcDNA3–GATA-6–mut or pcDNA3–GATA-6–Δexon4 did not increase luciferase activity, suggesting that transcriptional activation of the *sema3C*-LUC reporter is dependent on zinc finger–mediated binding of GATA-6 to DNA (Figure 5K).

Discussion

Members of the GATA family of zinc finger transcription factors restrict the developmental potential of multiple distinct cell lineages and regulate morphogenetic patterning in the embryo required for organogenesis (reviewed in ref. 10). Members of the GATA-1, -2, and -3 subfamily are expressed in distinct hematopoietic cell lineages, where they regulate cell differentiation and survival. In contrast, relatively little is understood about GATA-4, -5, and -6, in part because of their complex developmentally regulated expression in multiple embryologically distinct cell lineages and tissues (10). In the studies described in this report, we used tissue-specific gene targeting to identify a novel, cell-autonomous function of GATA-6 in neural crest–derived SMCs. We show that GATA-6 functions in these cells, not to regulate differentiation of SMCs from neural crest precursors, but rather to regulate aortic arch patterning and cardiac outflow tract septation, at least in part through regulation of *semaphorin 3C* gene expression.

These data suggest strongly that regulation of morphogenetic patterning in the embryo may be a conserved ancient function of the closely related GATA-4, -5, and -6 subfamily. GATA-4–deficient embryos die between E8.5 and E10.5 and display severe disruption of the ventral body plan as well as failure of formation of the linear heart tube (22, 23). Similarly, mice harboring either SMC-restricted or neural crest–restricted deletion of GATA-6 also exhibit defects in morphogenetic patterning of the ventral body plan, though these defects occur later in development and are restricted to the cardiac outflow tract and great arteries. As was the case with *GATA-4* mutant mice, a block in (smooth) muscle cell lineage differentiation was not observed in *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ mice or *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ mice. Taken together, these data suggest strongly that a primary function of the closely related transcription factors GATA-4 and GATA-6 during embryonic development is regulation of morphogenetic patterning of the ventral body plan, rather than cell lineage differentiation.

Previous studies suggested that GATA-6 promotes differentiation of the SMC lineage and the contractile SMC phenotype (7–9). In theory, a block in differentiation of SMCs from neural crest precursors could explain the observed patterning defects in conditionally targeted *GATA-6* embryos. For example, recent studies have shown that the serum response factor cofactor, myocardin-related transcription factor B, is required in cardiac neural crest cells for activation of genes encoding SMC-restricted contractile proteins and for proper morphogenesis of the cardiac outflow tract (24). Therefore, it was surprising that no abnormalities in SMC differentiation were observed in *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ or *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ mice despite marked abnormalities in aortic arch patterning. In addition, the dorsal aorta and femoral arteries of *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ mice were histologically indistinguishable from those of control mice (J.J. Lepore and M.S. Parmacek, unpublished observations) despite the fact that the *SM22*α promoter drives high-level recombination in the SMCs populating these vessels (21). Consistent with these findings, deletion of GATA-6 in primary mouse aortic SMCs derived from *GATA-6*$^{F/F}$ mice did not alter expression of genes encoding multiple SMC-restricted contractile proteins (Figure 4K). Importantly, *Wnt1Cre*-mediated recombination occurs in neural crest precursors prior to their differentiation into SMCs (18). As such, the finding of differentiated SMCs in *Wnt1CreGATA-6*$^{F/F}$ embryos directly demonstrates that GATA-6 is not required for specification or differentiation of neural crest–derived SMCs.

It is noteworthy that Kirby and colleagues have shown in avian species that a band of SMCs in the proximal cardiac outflow tract is not derived from the cardiac neural crest, but instead is derived from a population of SMCs arising in the secondary heart field that migrate to populate the outflow tract (19, 20). As such, subtle differences in the phenotype of *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ embryos with respect to the rostral-to-caudal level of the observed outflow tract defects might have been expected. However, comparison of the outflow tract pathologies in *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ embryos failed to reveal consistent differences (Table 2, Figure 2, and data not shown). Because a spectrum of outflow tract defects were observed in both *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ embryos, subtle differences in phenotype cannot be excluded. Alternatively, the migrating neural crest cells may provide a required instructive signal to the SMCs derived from the secondary heart field required for their migration, proliferation, survival, and/or differentiation. If so, this would explain why the phenotype of *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ embryos might appear similar or identical.

How then does GATA-6 regulate morphogenetic patterning of the cardiac outflow tract and great arteries? Aorticopulmonary septation and pharyngeal arch remodeling require precisely orchestrated cell-cell signaling. Previous studies demonstrated that mutations in genes encoding secreted growth factors, including Fgf8, semaphorin 3C, endothelin-1, and VEGF, provide critical cues for survival, proliferation, migration, and/or differentiation of cardiac neural crest cells that are required for cardiac outflow tract formation and aortic arch patterning (reviewed in ref. 3). In these studies, the specific cell type secreting and transducing the developmental signal has been difficult to define because neural crest cells can receive inductive signals from adjacent tissues and paracrine signals from adjacent neural crest cells as well as autocrine signals. GATA-6 is expressed abundantly in neural crest–derived SMCs populating the developing aortic arch but is also expressed in the surrounding pharyngeal endoderm and in the myocardium, potentially complicating analysis of the cell autonomous functions of GATA-6. However, by interbreeding conditionally targeted *GATA-6* mice with either SMC-restricted *SM22Cre* mice or with neural crest–restricted *Wnt1Cre* mice, our studies demonstrated an unequivocal, critical, cell autonomous function of GATA-6 in neural crest–derived SMCs required for cardiovascular patterning.

An unbiased microarray screen of putative GATA-6–regulated gene products in VSMCs identified *semaphorin 3C* (11) as a can-

didate gene that might be responsible for the observed morphogenetic defects in conditionally targeted *GATA-6* mice. Consistent with this hypothesis, dramatically downregulated and/or absent expression of semaphorin 3C was observed in neural crest–derived SMCs populating the cardiac outflow tract and major arteries in *SM22Cre*$^{+}$*GATA-6*$^{F/F}$ and *Wnt1Cre*$^{+}$*GATA-6*$^{F/F}$ mice. The observation that the phenotype observed in *semaphorin 3C*–null mice recapitulates the phenotype observed in conditionally targeted *GATA-6* mice suggests strongly that the defects in morphogenetic patterning of the cardiac outflow tract and great arteries were due, at least in part, to downregulation of semaphorin 3C in neural crest–derived SMCs. The identification of multiple evolutionarily conserved GATA-binding sites in the *semaphorin 3C* promoter and the demonstration that forced expression of GATA-6 transactivates the *semaphorin 3C* promoter strongly suggest a molecular model in which GATA-6 directly activates *semaphorin 3C* transcription in neural crest–derived SMCs, although additional in vivo experiments will be required to definitively determine this.

Semaphorin 3C is a member of the semaphorin family of secreted factors that are known to play important roles in regulating neuronal as well as vascular patterning (4, 5). During murine embryonic development, semaphorin 3C is expressed in the mesenchyme surrounding the developing aortic arch arteries and in a cuff of cardiac myocytes at the base of the cardiac outflow tract. Our studies as well as prior reports (4, 5) demonstrate that semaphorin 3C is also expressed within neural crest–derived SMCs. These cells also express neuropilin 1 and plexin A2, the components of a potential heterodimeric semaphorin 3C receptor (5). Semaphorin 3C–deficient mice exhibit impaired migration of neural crest cells into the developing outflow tract, where it has been hypothesized that loss of semaphorin 3C expression by adjacent tissues is responsible for the impairment of neural crest migration. The finding that initial formation of the pharyngeal arch arteries occurred normally following deletion of GATA-6 in neural crest cells, but that subsequent aortic arch patterning defects closely phenocopied those observed in semaphorin 3C-deficient mice, suggests that expression of semaphorin 3C within neural crest–derived SMCs populating the aortic arch arteries may provide an instructive signal for a later stage of pharyngeal arch remodeling. Consistent with this model, it has been suggested that 1 potential cell-autonomous function of semaphorin 3C in neural crest cells may be to regulate responsiveness to semaphorin 3C expressed by adjacent cells, as has been described in neurons that both respond to and express axon guidance molecules (4). Alternatively, semaphorin 3C produced in neural crest cells may mediate paracrine signaling to adjacent endothelial cells in which it has been shown that the semaphorin receptor plexin D1 is required for aortic arch patterning (25). It is also possible that additional secondary effects on endothelial cells and/or cardiac myocytes may have contributed to the observed cardiovascular phenotype.

Deletion of *GATA-6* in neural crest cells also resulted in an absence of detectable semaphorin 3C– or plexin A2–expressing cardiac neural crest cells within the conotruncal endocardial cushions that contribute to aorticopulmonary septation. This finding is consistent with the impairment in neural crest cell migration into the proximal cardiac outflow tract observed in semaphorin 3C–deficient mice (4). It was hypothesized that impaired neural crest migration into the outflow tract in *semaphorin 3C*–null mutants was caused by loss of an attractive signal by semaphorin 3C secreted by the myocardial cuff (4). However, semaphorin 3C expression in the myocardial cuff was not significantly decreased in our studies, suggesting that cell-autonomous expression of semaphorin 3C in neural crest–derived cells is required either for migration into the proximal cardiac outflow tract or for survival of these cells within the endocardial cushions.

The demonstration that expression of GATA-6 in neural crest–derived SMCs is required for angiogenic patterning of the pharyngeal arch arteries into the mature pulmonary and systemic circulations and for neural crest–mediated conotruncal septation is directly relevant to understanding the molecular pathogenesis of common forms of congenital heart disease observed in humans. Persistent truncus arteriosus and a spectrum of interrupted aortic arch defects are observed in infants born with life-threatening cyanotic heart disease (reviewed in ref. 1). If not surgically corrected, these conditions are invariably lethal. Anatomic ablation studies and cell fate mapping studies have revealed the central role of the cardiac neural crest in the pathogenesis of these clinical syndromes (reviewed in ref. 3). Our studies extend these observations at a molecular level by identifying a novel, GATA-6–dependent, neural crest–restricted transcriptional program that is required for aortic arch patterning and by defining a cell-autonomous requirement for semaphorin 3C expression in neural crest–derived SMCs. Moreover, these data identity *GATA-6* and GATA-6–regulated genes as novel candidates that may be responsible for some cases of congenital heart disease observed in humans. Further elucidation of the signaling pathways that lie downstream of GATA-6 in the cardiac neural crest will provide additional insights into the molecular basis of these commonly observed pathologies.

Methods

Generation and characterization of mice with a conditionally targeted GATA-6 allele. The targeting vector was generated in a modified pPNT plasmid containing the pGH-*neo*-poly(A) and phosphoglycerate kinase–*tk*–poly(A) [PGK-*tk*-poly(A)] cassettes for positive and negative selection, respectively, as described (12). The 6.5-kb and 2.3-kb *GATA-6* genomic subfragments were amplified from SV129 mouse genomic DNA using primers 5′-GCGGCCGCGATAGCTTGTGCAGAGA-3′and 5′-CTCGAGTTTCTATCTTCAA-3′; and 5′-GGATCCGTAATGTGACCAGTCAGTTC-3′and 5′-GGTACCCCATGGCCCAACTATACAA-3′, respectively. As schematically depicted in Figure 1A, the 6.5-kb fragment was subcloned into the *NotI/XhoI*, and the 2.3-kb fragment was subcloned into the *BamHI/KpnI* sites of pPNT. A third genomic fragment containing exon 4 encoding the carboxyterminal zinc finger GATA-6 DNA-binding domain was amplified using primers 5′-AGATCTGTTGATGTACATATAAGCCATTA-3′ and 5′-GGATCCATAACTTCGTATAGCATACATTATACGAAGTTATGAGCAGCTTGAATTCGC-3′ and subcloned into the *BamHI* site of pPNT. The conditional targeting construct was linearized with *NotI* and electroporated into R1 ES cells as described (12). DNA from G418-resistant ES cell clones was analyzed by Southern blot analysis after *BamHI* digestion with a radiolabeled probe derived from genomic sequences located 3′ of the targeting vector (Figure 1, A and B). To selectively delete the *neo* from the targeted GATA-6 locus, targeted ES cells were transiently transfected with the pMC-Cre plasmid (26). ES cell clones with selective *neo* deletion were identified by Southern blot analysis of *BamHI*-digested DNA with a probe corresponding to exon 4 (Figure 1, A and B) and were microinjected into C57BL/6 donor blastocysts as described (12). The resulting male chimeras were mated with C57BL/6 females, and agouti offspring were genotyped by Southern blot analysis using *BamHI* digested DNA and probe B (Figure 1C). Heterozygous *GATA-6*$^{+/F}$ mice were interbred to generate homozy-

gous conditionally targeted *GATA-6* mice (*GATA-6$^{F/F}$ mice*). Genotype was determined by Southern blotting (probe B) or by PCR using primers A (5′-AGTTATAAGACAGAATCAGCAAATG-3′) and B (5′-CTCCTTAGTG-GCTGAACTGACTGG-3′) (Figure 1, A and C). All animal experimentation was performed under NIH guidelines, with approval by the University of Pennsylvania Institutional Animal Care and Use Committee, in the University of Pennsylvania Animal Care Facility.

To delete *GATA-6* selectively in VSMCs, *GATA-6$^{F/F}$* mice were interbred with –2.8-kb *SM22αCre* transgenic mice (21). To delete *GATA-6* specifically in neural crest derivatives, *GATA-6$^{F/F}$* mice were interbred with *Wnt1Cre* kindly provided by A. McMahon (Harvard University, Cambridge, Massachusetts, USA) (18). Matings were performed between male compound heterozygous *SMCre^{+}GATA-6$^{+/F}$* or *Wnt1-Cre^{+}GATA-6$^{+/F}$* mice and female *GATA-6$^{F/F}$* mice.

Isolation and characterization of primary mouse aortic SMCs with conditional deletion of GATA-6. Primary SMCs were isolated from the aortae of 4-week-old *GATA-6$^{F/F}$* mice using the aortic explant technique (11) and were infected (200 MOI) with Ad-empty or Ad-Cre (University of Pennsylvania Adenoviral Vector Core). After 48 hours, total RNA and protein were isolated with Trizol (Invitrogen Corp.). cDNA was prepared using the Superscript RT kit (Invitrogen Corp.). To amplify the region surrounding exon 4, PCR was performed using primers 5′-GGCTCCATCCAGACGCCACT-GTGG-3′and 5′-AAGTAGGAGTCATAGGGACAGAGC-3′, corresponding to sequences encoded by exons 3 and 5 of mouse *GATA-6*, respectively. To amplify the full-length cDNA produced following deletion of exon 4, PCR was performed using primers 5′-GGATCCATGTACCAGACCCTC-GCCGCCCTGTCC-3′ and 5′-CTCGAGTCAGACCAGGGCCAGAGCA-CACCAAG-3′. The resulting PCR products were cloned and analyzed by DNA sequencing. Quantitative RT-PCR of SMC-restricted gene expression was performed using the DNA Engine Opticon 2 Real Time Detection System (Bio-Rad) as described (11, 27). Western blotting was performed using GATA-6 polyclonal antibody (AF1700; R&D Systems) and previously described methods (12).

Plasmids and transient cotransfection analyses. pcDNA3–GATA-6 encoding full-length mouse GATA-6 cDNA and pcDNA3–GATA-6–mut containing mutations changing amino acids 293–294, cysteine and alanine, to serine and arginine (eliminating DNA binding) were previously described (11). pcDNA3–GATA-6–Δexon4 containing GATA-6 lacking amino acids encoded by exon 4 was generated by subcloning the full-length RT-PCR product amplified from Ad-Cre–infected *GATA-6$^{F/F}$* SMCs into pcDNA3 (Invitrogen Corp.). The *Dab2*-LUC luciferase reporter plasmid was previously described (15). The *sema3C*-LUC luciferase reporter plasmid contains a 3.2-kb genomic fragment from the mouse *semaphorin 3C* gene consisting of 0.9 kb of promoter sequence, exon 1, intron 1, and the first 35 bp of exon 2 cloned upstream of firefly luciferase (Figure 5J). The construct was generated using the bacterial artificial chromosome (BAC) recombineering method (28). Briefly, 0.5-kb bands corresponding to the 5′ and 3′ ends of the genomic fragment were generated by PCR from BAC RP23-20H6 (CHORI BACPAC Resource Center, http://bacpac.chori.org/) containing the mouse *semaphorin 3C* gene using primer pairs 5′-GTACCCCATCTCAGGCAGGCGAATCTTGCAACG-3′ and 5′-ACGCGTTTTCAAGAATGCTGTTTCCAATGGTCC-3′ as well as 5′-CTC-GAGCATGCACTGGCAGATAGCTGATTGG-3′ and 5′-AAGCTTCTTCT-GATTTGGGAATTAATATCCAAGG-3′ and were cloned into the *KpnI/MluI* and *XhoI/HindIII* sites of pGL3-Basic (Promega), respectively. The plasmid was linearized with *NheI* and electroporated into competent EL350 bacteria containing the BAC, and transformants were screened for ampicillin resistance. NIH3T3 cells (1×10^5) were cotransfected with 100 ng of either the *Dab2*-LUC or the *sema3C*-LUC reporter plasmid; with 0.1 μg to 5.0 μg of either pDNA3–GATA-6, pcDNA3–GATA-6–mut, or pcDNA3–GATA-6–Δexon4; and with 50 ng of the pRL-CMV reference plasmid (Promega) using FuGENE 6 (Roche Diagnostics Corp.) as described (11, 27). Luciferase activity was measured and normalized for transfection efficiency using the Dual Luciferase Assay System (Promega). Data are reported as mean normalized relative light units (fold activation) ± SEM, as described (11).

Histology, immunohistochemistry, and in situ hybridization studies. Histology, H&E staining, SMα-actin immunohistochemistry, and in situ hybridization analyses were performed as described (24). The GATA-6, semaphorin 3C, and plexin A2 antisense cRNA in situ probes were previously described (5, 6). An in situ probe to detect RNA sequences encoded by exon 4 of the mouse *GATA-6* gene was generated by PCR from the mouse GATA-6 cDNA using primers 5′-GTGTCTATTCCAAGGGCTGTCATCG-3′ and 5′-TAATACGACTCACTATAGGGGTGTCTATTCCAAGGGCTGT-CATCG-3′ and T7 polymerase.

Vascular polymer casting. E18.5 embryos were euthanized, the anterior chest wall was dissected away, and the atria and thymus were removed. The left ventricle was then injected with polymer casting material (07349; Polysciences Inc.) to produce a polymer arteriogram of the vascular system as described (5).

Statistics. For luciferase assays, all experiments were performed in triplicate and data are reported as normalized relative light units (fold activation) ± SEM. For the quantitative RT-PCR experiments, cycle threshold values were converted to relative gene expression levels using the $2^{-\Delta\Delta C(t)}$ method as described in reference 27 and are expressed as fold change ± SEM.

Acknowledgments

The authors thank Jonathan Epstein for helpful comments and suggestions and critical reading of the manuscript and Diane Zhou, Xiaohong Zhu, and Maozhen Zhang for expert technical assistance. This work was supported by an allocation from the Commonwealth of Pennsylvania and by grants from the Mary L. Smith Charitable Lead Trust (to J.J. Lepore) and the NIH (K08-HL075520 to J.J. Lepore and PO-1-HL075380 to E.E. Morrisey and M.S. Parmacek). E.E. Morrisey is an Established Investigator of the American Heart Association.

Received for publication November 8, 2005, and accepted in revised form January 31, 2006.

Address correspondence to: Michael S. Parmacek, Division of Cardiovascular Medicine, University of Pennsylvania School of Medicine, 9123 Founders Pavilion, 3400 Spruce St., Philadelphia, Pennsylvania 19104, USA. Phone: (215) 662-3140; Fax: (215) 349-8017; E-mail: michael.parmacek@uphs.upenn.edu.

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