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 aortopulmonary 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 asplexin 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
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)
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 DNA and to the expected GATA-6 DNA 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–Δ exon4) is functionally null, as predicted, we examined the capacity of pcDNA3–GATA-6–Δ exon4 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–Δ exon4 (Figure 1E). In similar experiments, pcDNA3–GATA-6–Δ exon4 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. Morrissey, 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.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>E9.5–E15.5</th>
<th>E18.5</th>
<th>P1</th>
<th>2 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM22Cre Cross</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM2Cre G6+/F/F</td>
<td>78</td>
<td>60</td>
<td>31</td>
<td>20</td>
</tr>
<tr>
<td>SM2Cre G6+/F/F</td>
<td>76</td>
<td>52</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>SM2Cre G6+/F/F</td>
<td>70</td>
<td>50</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>SM2Cre G6+/F/F</td>
<td>69 (24%)</td>
<td>35 (18%)</td>
<td>2 (2%)</td>
<td>0</td>
</tr>
<tr>
<td>Wnt1Cre Cross</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wnt1Cre G6+/F/F</td>
<td>17</td>
<td>27</td>
<td>16</td>
<td>–</td>
</tr>
<tr>
<td>Wnt1Cre G6+/F/F</td>
<td>14</td>
<td>27</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>Wnt1Cre G6+/F/F</td>
<td>12</td>
<td>29</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Wnt1Cre G6+/F/F</td>
<td>18 (29%)</td>
<td>23 (22%)</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

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 SM2Cre 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 (SM2Cre 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 in their derivatives; in mesoderm-derived SMCs, myocytes (11). Offspring of 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 DNA and to the expected GATA-6 DNA 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–Δ exon4) is functionally null, as predicted, we examined the capacity of pcDNA3–GATA-6–Δ exon4 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–Δ exon4 (Figure 1E). In similar experiments, pcDNA3–GATA-6–Δ exon4 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. Morrissey, 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.

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

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Number</th>
</tr>
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<tbody>
<tr>
<td>Persistent truncus arteriosus</td>
<td>7/11</td>
</tr>
<tr>
<td>Double-outlet right ventricle</td>
<td>4/11</td>
</tr>
<tr>
<td>Membranous ventricular septal defect</td>
<td>11/11</td>
</tr>
<tr>
<td>Interrupted aortic arch</td>
<td>7/11</td>
</tr>
<tr>
<td>Retroesophageal right subclavian artery</td>
<td>6/11</td>
</tr>
<tr>
<td>Hypoplastic aortic arch</td>
<td>7/11</td>
</tr>
<tr>
<td>Absent ductus arteriosus</td>
<td>1/11</td>
</tr>
</tbody>
</table>
derived from both cardiac neural crest and the secondary heart field are required for outflow tract septation (3, 5) (Figure 3D) and in that contain columns of migrating cardiac neural crest cells that also expressed in regions of the conotruncal endocardial cushions 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. Of note, expression of GATA-6 mRNA was observed from the proximal myocardial cuff through the proximal ascending aorta (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 abundantly expressed in cardiac myocytes as well as in neural crest–derived SMCs (21), the observed cardiovascular abnormalities in SMCre-GATA-6+/− 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-
flow tract septation defects observed in GATA-6 mutant embryos resulted from a cell-intrinsic defect in neural crest–derived cells, GATA-6<sup>F/F</sup> 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 SM2Cre mice, Wnt1Cre mice did not produce Cre-mediated recombination in cardiac myocytes or in mesoderm-derived SMCs. Remarkably, the phenotype of Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> embryos recapitulated that of SM2Cre<sup>GATA-6<sup>F/F</sup></sup> mutant embryos. These embryos survived to E18.5 in slightly less than the expected Mendelian ratio (Table 1), but no Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> mice survived past P1. E18.5 Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> embryos demonstrated a spectrum of aortic arch patterning and cardiac outflow tract septation defects that were not distinguishable from those observed in SM2Cre<sup>GATA-6<sup>F/F</sup></sup> 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 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> or Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> embryos. In a representative E11.5 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> 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 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> embryo (Figure 4A). At E12.5, major malformations of the aortic arch and outflow tract septation were observed. As shown in an E12.5 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> 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<sup>F/F</sup> 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 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> or Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> 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<sup>GATA-6<sup>F/F</sup></sup> and Wnt1Cre<sup>GATA-6<sup>F/F</sup></sup> 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 SM2Cre<sup>GATA-6<sup>F/F</sup></sup> embryos (Figure 5A) as well as in
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-6F/F embryos (Figure 5B) as well as in Wnt1Cre’GATA-6F/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-6F/F embryos (Figure 5, C and D) or in Wnt1Cre’GATA-6F/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-6F/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-6F/F embryos (Figure 5, F and H) or in SM22Cre’GATA-6F/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-6F/F and SM22Cre’GATA-6F/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 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+/− (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/index.html and http://www.ncbi.nlm.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 hemato poetic 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-1, -2, and -3 subfamily. In this context, it is notable 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-6ΔF/F and Wnt1Cre/GATA-6ΔF/F embryos relative 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-6ΔF/F and Wnt1Cre/GATA-6ΔF/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-6ΔF/F and Wnt1Cre/GATA-6ΔF/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-6ΔF/F and Wnt1Cre/GATA-6ΔF/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-
GATA-6 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 circulation 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 identify 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′-GGGCCTCGGGATAGCTGGTGCAGAGA3′ and 5′-CTCCGATTTGTGCTTCAAG-3′. DNA from G418-resistant ES clones was analyzed by Southern blot analysis after BamHI digestion with a probe corresponding to exon 4 encoding the carboxyterminal zinc finger GATA-6 DNA-binding domain was amplified using primers 5′-AGATCTGTGATGTACATATAAGCCATTA-3′ and 5′-GGATCCGATAGCTGGTGCAGAGA-3′. DNA from G418-resistant ES 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 1A 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 1A 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+/− mice were interbred to generate homozy-

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gous conditionally targeted GATA-6 mice (GATA-6+/− mice). Genotype was determined by Southern blotting (probe B) or by PCR using primers A (′-AGTTTATAAGACAGAATCCAAATTG-3′) and B (′-CTCCTTATGG-GCTGAAGCTGCTG-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+/− mice were interbred with −2.8-kb SM22Cre transgenic mice (21). To delete GATA-6 specifically in neural crest derivatives, GATA-6+/− mice were interbred with Wnt1Cre kindly provided by A. McMahon (Harvard University, Cambridge, Massachusetts, USA) (18). Matings were performed between male compound heterozygous 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 Supercript RT kit (Invitrogen Corp.). To amplify the region surrounding exon 4, PCR was performed using primers 5′-GGCTCCATCCAGCCGCAT- GTGG-3′ and 5′-AAATGAGAGTGTACAGGGGACAGAC-3′, corresponding to sequences encoded by exons 3 and 5 of mouse GATA-6 (22). To amplify the full-length cDNA produced following deletion of exon 4, PCR was performed using primers 5′-GGATCCATGCAGCCGCATG- GTGG-3′ and 5′-AAATGAGAGTGTACAGGGGACAGAC-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+/− 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://bapac.chori.org/) containing the mouse semaphorin 3C gene using primer pairs 5′-GTACCCCCATCCTCCGCGCCAGAATCTTGACAGG-3′ and 5′-ACCGCTTCTTTTCAAGGTCCTGTCTCCTGCC-3′ as well as 5′-CTCG- GACCTGACCTGGCGAATCTTGACAGG-3′ and 5′-AACTTTCTCTC-3′.


