GATA6 mutations cause human cardiac outflow tract defects by disrupting semaphorin-plexin signaling

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Congenital heart diseases (CHD) occur in nearly 1% of all live births and are the major cause of infant mortality and morbidity. Although an improved understanding of the genetic causes of CHD would provide insight into the underlying pathobiology, the genetic etiology of most CHD remains unknown. Here we show that mutations in the gene encoding the transcription factor GATA6 cause CHD characteristic of a severe form of cardiac outflow tract (OFT) defect, namely persistent truncus arteriosus (PTA). Two different *GATA6* mutations were identified by systematic genetic analysis using DNA from patients with PTA. Genes encoding the neurovascular guiding molecule semaphorin 3C (SEMA3C) and its receptor plexin A2 (PLXNA2) appear to be regulated directly by GATA6, and both GATA6 mutant proteins failed to transactivate these genes. Transgenic analysis further suggests that, in the developing heart, the expression of SEMA3C in the OFT/subpulmonary myocardium and PLXNA2 in the cardiac neural crest contributing to the OFT is dependent on GATA transcription factors. Together, our data implicate mutations in *GATA6* as genetic causes of CHD involving OFT development, as a result of the disruption of the direct regulation of semaphorin-plexin signaling.

congenital heart disease | persistent truncus arteriosus | cardiac neural crest

Congenital heart diseases (CHD) constitute a major percentage of clinically significant birth defects with an estimated prevalence of 4–10 per 1,000 live infants (1). Cardiac outflow tract (OFT) defects are estimated to account for approximately 30% of CHD (2) and usually require an intervention during the first year of life. A variety of OFT defects results from disturbance of the morphogenetic patterning of the anterior pole of the heart, which is essential for the establishment of separate systemic and pulmonary circulations in higher vertebrates. Persistent truncus arteriosus (PTA), which is attributed to missing septation of the OFT, is recognized as the most severe phenotype of OFT defect, and is often associated with an unfavorable prognosis because complete surgical repair is not always possible (3). Although an improved understanding of possible genetic causes would provide insight into the pathogenesis of CHD and allow for better assessment of disease risk, prenatal diagnosis, and critical information for disease prevention, the etiology of most CHD, including OFT defects, remains unknown because of the multifactorial nature of the diseases (4–6).

Based on animal studies, it appears that abnormal development of cardiac neural crest (CNC) cells, an ectoderm-derived cell lineage, contributes significantly to the pathology of OFT defects (7–12). During early embryogenesis, CNC cells arise from the dorsal neural tube and migrate ventrally as mesenchymal cells to populate the OFT, where they coalesce to form the aorticopulmonary septum, which divides the single truncus arteriosus (embryonic OFT) into the aorta and pulmonary artery, resulting in the establishment of separate systemic and pulmonary circulations (7, 8). A number of mouse lines in which the genes implicated in CNC development have been ablated produce offspring with OFT defects, typically PTA (9–12). More recent studies have shown that reciprocal signaling between the CNC and cells derived from the pharyngeal mesoderm (or second heart field), which give rise to OFT/subpulmonary myocardium, may also be required for OFT development (13–15).

Genes encoding members of the GATA family of zinc finger transcription factors, specifically GATA4, GATA5, and GATA6, restrict the developmental potential of multiple distinct cell lineages and regulate morphogenetic patterning in the embryo, including formation of the heart (16, 17). Null mutation of *Gata4* in mice results in embryonic lethality because of defects in cardiogenesis (18, 19). Mutations of *GATA4* in humans were identified to cause CHD characteristic of atrial and/or ventricular septal defects (20, 21), and heterozygous mutations of *Gata4* in mice recapitulate the human phenotype (22). Systemic ablation of Gata6 in mice also results in embryonic lethality (23), whereas conditional inactivation of Gata6 in the CNC results in perinatal mortality from OFT defects, typically PTA (24). These observations suggest that defects in GATA6 and its downstream target genes that regulate CNC development could be responsible for human CHD involving the OFT, however, no mutations in GATA6 have yet been reported in humans.

To develop a systematic genetic analysis for the etiology of various cardiovascular diseases, we have established a genomic bank of over 3,000 Japanese patients with cardiovascular diseases. This bank contains 21 genomes from independent Japanese patients with non-syndromic PTA and, after screening the 21 genomes, we identified two mutations of GATA6 responsible for PTA. Both mutations were reconfirmed using patients' original genomic DNA. We also showed that GATA6 directly regulated genes encoding the neurovascular guiding molecule semaphorin 3C (SEMA3C), as well as its receptor plexin A2 (PLXNA2), involved in the development of the CNC. Both GATA6 mutant proteins failed to transactivate gene expression of SEMA3C and PLXNA2, suggesting an underlying genetic and molecular basis for OFT defect.

Results

***GATA6* Mutations Were Identified in Patients with PTA.** Two *GATA6* mutations were detected and confirmed in two independent probands with PTA (Fig. 1*A*, Table 1 and Table S1). A frame shift

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The authors declare no conflict of interest.

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The sequences reported in this paper have been deposited in the GenBank database [accession nos. GATA6 (NM_005257), GATA4 (NM_002052), SEMA3C (NM_006379), PLXNA2 (NM_025179), NPPA (NM_006172), WNT2 (NM_003391), Sema3c (NM_013657), Plxna2 (NM_008882)].

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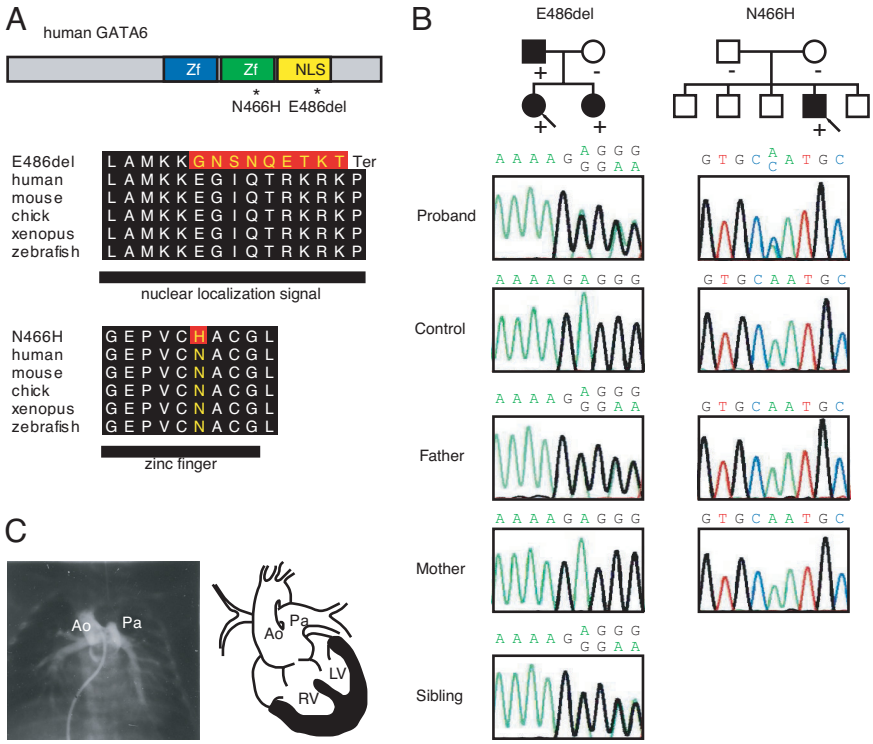

Fig. 1. Identification of GATA6 mutations in patients with persistent truncus arteriosus (PTA). (*A*) The structure of the human GATA6 gene, the position of the two mutations (asterisks), and conservation of alignment between species are shown. Zf, zinc finger; NLS, nuclear localization signal. Changes in amino acids are highlighted in red. The E486del mutation causes two nucleotide deletions, resulting in nine amino acid changes followed by P489 to termination (Ter) codon. (*B*) Pedigree indicating cardiac phenotype and the presence (+) or absence (-) of the GATA6 mutation in the family of proband A (E486del; arrow) and proband B (N466H; arrow). The probands are indicated by arrows. (circle), female; (box), male; (solid fill), with CHD. In addition, a sequence chromatogram of one frame shift mutation (E486del, proband A) and a point mutation (N466H, proband B) are shown. (*C*) A catheter angiogram of proband A reveals a single common outflow tract with no septation between the aorta (Ao) and pulmonary artery (Pa), diagnosed as PTA. A schematic diagram of PTA is shown on the right. RV, right ventricle; LV, left ventricle.

mutation (E486del) was detected in proband A. The frameshift caused changes in the amino acid sequence, converting P489 to a stop codon, disrupting the nuclear localization signal (NLS), and truncating 100 amino acids at the C terminus (Fig. 1*A*). Another was point mutation which located in the zinc finger domain in proband B (N466H in exon 4). Analysis of family members of proband A revealed that identical mutations (E486del) on *GATA6* were carried by the proband's father and sister and that both had CHD. However, proband A's mother had neither mutations of *GATA6* nor CHD (Fig. 1*B* and Table 1). No family members of probands B had CHD and genetic analysis of biological parents revealed a de novo mutation in proband B (Fig. 1*B*). Neither of the two mutant alleles was detected in 182 unrelated Japanese healthy individuals without CHD. Angiocardiograms of probands A shows feature of PTA (Fig. 1*C*). Overall, two *GATA6* mutations were identified in four individuals with CHD, three of whom were members of the same family and one unrelated case.

In this study, another nucleotide change (43G>C leading to G15R) was also identified in approximately 5% of genomic alleles from both patients and unaffected controls, thus was thought to be a single nucleotide polymorphism (SNP) of *GATA6*. None of the previously reported SNPs on *GATA6* were detected in this Japanese population (Table S1).

***GATA6* Mutations Cause Defects in Nuclear Localization and the Transactivation Ability of the GATA6 Protein.** To assess the impact of the two mutations on the structural and functional properties of GATA6, site-directed mutagenesis was performed on human *GATA6* cDNA cloned in a FLAG-tagged expression vector. Western blot analyses showed that the GATA6-E486del mutant protein was approximately 15 kDa smaller than the wild-type and N466H mutant proteins (Fig. 2*A*). Immunostaining of FLAG-tagged GATA6 after transfection of the expression vector into COS-1 cells showed that N466H mutant protein was located in the nuclei in a pattern similar to that seen for the wild-type protein (Fig. 2*B*). In contrast, the GATA6-E486del mutant exhibited an abnormal localization pattern in the nucleus, probably as a result of disruption of the NLS (Fig. 2*B*). In cotransfection luciferase assays driven by the GATA6-dependent cardiac promoters, *NPPA* (25) and *WNT2* (26), both GATA6-E486del and GATA6-N466H proteins were

Table 1. Characteristics of individuals with *GATA6* mutations and congenital heart disease

Patient	Sex	Nucleotide change (amino acid change)	Location	Phenotypes of CHD	Syndrome
Proband A	F	1456–1457delGA, (E486del)	Exon5,NLS	PTA, ASD	None
Father of Proband A	M	1456–1457delGA, (E486del)	Exon5,NLS	PS (dysplastic)	None
Sister of Proband A	F	1456–1457delGA, (E486del)	Exon5,NLS	PS, ASD, PDA	None
Proband B	F	1396A > C, (N466H)	Exon4, Zf	PTA	None

Zf, zinc finger; NLS, nuclear localization signal; CHD, congenital heart disease; PTA, persistent truncus arteriosus; ASD, atrial septal defect; PS, pulmonary stenosis; PDA, patent ductus arteriosus.

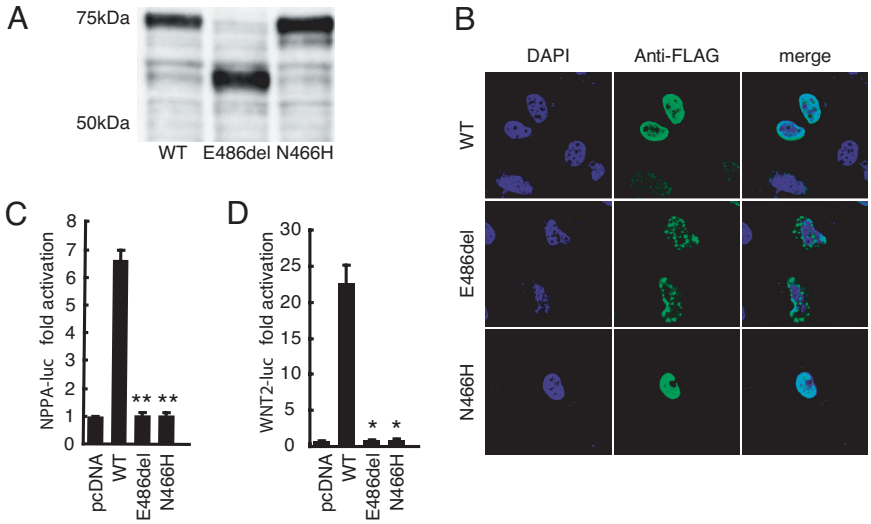

Fig. 2. Molecular size, nuclear localization and transactivation ability of GATA6 mutant proteins. (*A*) Western blot analyses show that the band of the FLAG-tagged E486del mutant GATA6 protein is approximately 15 kDa smaller than the FLAG-tagged wild-type (WT) and N466H mutant proteins. (*B*) Nuclear localization of FLAG-tagged GATA6 wild-type (WT) and mutant (E486del and N466H) proteins, shown in green. Purple staining (DAPI) indicates the nucleus. The E486del mutant shows an abnormal localization pattern. (*C* and *D*) Relative luciferase activity in HeLa cells transfected with wild-type GATA6 (WT) or GATA6 mutant (E486del or N466H) expression constructs and *NPPA-luc* (*C*) *or WNT2-luc* (*D*). Both mutants are unable to activate the transcription of each reporter construct (*C*: WT vs. E486del, $P = 0.0023$; WT vs. N466H, $P = 0.0016$, $n = 3$; *D*: E486del, $P = 0.013$; N466H, $P = 0.013$; two-tailed unpaired *t* test, $n = 3$). *, $P < 0.05$; **, $P < 0.01$ compared with WT.

unable to activate transcription of either promoter (Fig. 2*C* and *D*). These in vitro functional analyses demonstrated that each mutation may lead to a functional disturbance of the GATA6 protein and may affect the regulation of its downstream target genes during embryogenesis.

GATA6 Directly Regulates SEMA3C and PLXNA2 Through a Consensus GATA-Binding Site, and *GATA6* Mutations Attenuate Transcriptional Activity of SEMA3C and PLXNA2. A recent study showed that specific ablation of Gata6 in the CNC of mice resulted in OFT defects (24). In that Gata6 mutant mouse, the neurovascular guiding molecule Sema3c (27) was downregulated in the developing OFT, together with Plxna2 (28), a receptor subunit for class 3 or 6 semaphorins (10, 11, 29). It has also been reported that genetic ablation of Sema3c or Plxna2 causes PTA in mice (9, 10). Taking these observations into consideration, we hypothesized that SEMA3C and PLXNA2 are direct downstream targets of GATA6 and that both factors are implicated in the OFT defect associated with *GATA6* mutations in humans.

First, we subcloned the 3.2-kb human *SEMA3C* promoter sequence into a luciferase reporter construct (Fig. 3*A*; *SEMA3C-luc*), based on the previously described mouse *SEMA3C* promoter sequence (24), and identified a consensus binding site for GATA transcription factors (30) within intron 1 of *SEMA3C*, labeled Site1, which is well-conserved across species (Fig. 3*A*). In promoter activation assays, specific mutation of Site1 (Fig. 3*A*; ΔSite1-*luc*) resulted in a significant decrease in transcriptional activation of the *SEMA3C* promoter by GATA6 (Fig. 3*B*), suggesting that GATA6 may regulate *SEMA3C* directly through Site1.

Next, using VISTA analyses (http://genome.lbl.gov/vista/), we searched the 5′-untranslated region (UTR) sequence of human *PLXNA2* for well-conserved GATA-binding sites between humans and mice. We identified Site2 as a conserved consensus binding site for GATA transcription factors right in front of exon 1 in the *PLXNA2* 5′-UTR and subcloned the putative *PLXNA2* promoters into the luciferase reporter construct in the context of the 1-kb DNA fragment (Fig. 3*A*; *PLXNA2-luc*). Specific mutation of Site2 (Fig. 3*A*; ΔSite2-*luc*) resulted in a significant decrease in luciferase activity (Fig. 3*C*), suggesting that Site2 is essential for the activation of *PLXNA2* by GATA6.

In cotransfection luciferase assays driven by *SEMA3C* and *PLXNA2* promoters, both E486del and N466H mutants showed complete loss of transcriptional activity of the *SEMA3C* and *PLXNA2* promoters compared with the wild-type GATA6 (Fig. 3*D* and *E*), consistent with our results for the *NPPA* and *WNT2* promoters. Moreover, the E486del mutant possessed dominant negative activity on the *SEMA3C* and *PLXNA2* promoter when mixed with wild-type GATA6, whereas the N466H mutant exhibited no significant interaction with wild-type GATA6 (Fig. S1). The electromobility shift assay showed that wild-type GATA6 binds to Site1 on the *SEMA3C* promoter and to Site2 on the *PLXNA2* promoter, whereas the E486del mutant could not bind efficiently to Site1 or Site2 and the N466H mutant could not bind efficiently to Site1 (Fig. 3*F* and *G*). These results suggest that SEMA3C and PLXNA2 are directly regulated by GATA6 in humans through consensus binding sites on their enhancer elements and that each of the *GATA6* mutations disturbs semaphorin-plexin signaling to varying degrees, resulting in abnormal development of the OFT.

Separable GATA-Dependent Enhancers Direct Tissue-Specific Expression of Sema3c and Plxna2 During Cardiovascular Development. During murine cardiovascular development, Sema3c is expressed in the OFT/subpulmonary myocardium and pharyngeal arch arteries (9, 24, 31), whereas Plxna2 is expressed in the CNC derivatives (10). We established a transgenic mouse system to test in vivo whether Sema3c and Plxna2 expression is directly dependent on GATA transcription factors during OFT development. A 4.7-kb mouse *Sema3c* promoter sequence corresponding to the human *SEMA3C* promoter sequence was subcloned into a *lacZ* reporter construct (Fig. 4*A*). Five of five transgenic mice at embryonic day (E) 11.5 harboring the *Sema3c* promoter-*lacZ* transgene (Fig. 4*A*; *Sema3c-lacZ*) showed X-gal staining in the OFT/subpulmonary myocardium and right ventricle, as well as in the neural tube, somites, and limb buds (Fig. 4*B–E*), consistent with the endogenous expression pattern of Sema3c (9, 24, 31, 32). At E11.5, *lacZ* expression in the cardiovascular system, including the OFT region, was abolished in four of four independent transgenic embryos harboring the transgene with the Site1 mutation (Fig. 4*A*; ΔSite1-*lacZ*), although *lacZ* expression was preserved in the extracardiac tissues of these embryos (Fig. 4*F–I*).

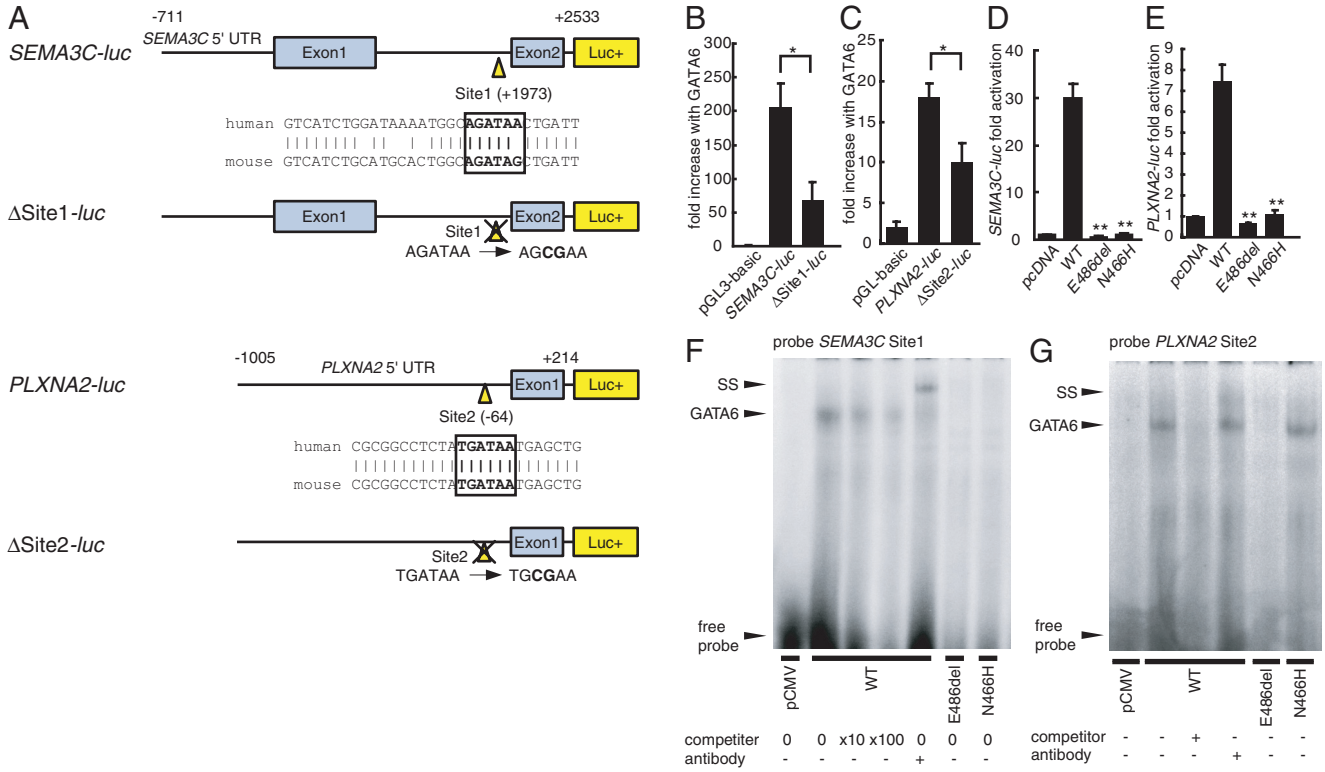

Fig. 3. GATA6 mutations attenuate the direct regulation of semaphorin 3C (SEMA3C) and its receptor plexin A2 (PLXNA2) by GATA6. (*A*) Structure of *SEMA3C-luc* and *PLXNA2-luc* with a consensus GATA-binding site (Site1 and Site2). and structure of *SEMA3C-luc* with site-directed mutagenesis for Site1 (ΔSite1-*luc*) and *PLXNA2-luc* with site-directed mutagenesis for Site2 (ΔSite2-luc) are shown. (*B* and *C*) Fold increase in relative luciferase activity directed by *SEMA3C* promoter-*luc* constructs or *PLXNA2* promoter-*luc* constructs in HeLa cells cotransfected with GATA6-pcDNA. The Site1 or the Site2 mutation results in a significant decrease in activation by GATA6 compared with controls (*B*; *SEMA3C-luc* vs. ΔSite1-*luc*, $P = 0.027$; $n = 3$; *C*; *PLXNA2-luc* vs. ΔSite2-*luc*, $P = 0.037$, $n = 5$). *, $P < 0.05$. (*D* and *E*) Relative luciferase activity in HeLa cells transfected with pcDNA, GATA6 wild-type (WT) or each mutant (E486del or N466H) expression construct and *SEMA3C-luc* or *PLXNA2-luc*. Both mutants display a significant decrease in activation of each promoter compared with WT (*D*; E486del, $P = 0.0028$; N466H, $P = 0.0030$; two-tailed unpaired *t* test, $n = 4$; *E*; E486del, $P = 0.0031$; N466H, $P = 0.0030$, $n = 3$). **, $P < 0.01$ compared with WT. (*F*) Binding of each GATA6 protein for Site1. GATA6 WT can bind Site1, but the E486del and N466H mutants cannot. (*G*) Binding of each GATA6 protein for Site2. GATA6 WT and N466H mutant can bind Site2, but the E486del mutant cannot. SS, super shift.

Next, we subcloned a 1.2-kb mouse *Plxna2* promoter sequence corresponding to the human *PLXNA2* promoter in a *lacZ* reporter construct (Fig. 4*J*; *Plxna2-lacZ*). In mouse embryos at E12.5, the *Plxna2* promoter-*lacZ* transgene was sufficient for *lacZ* expression in the truncus arteriosus and pharyngeal arch arteries, as well as in the neural tube, somites, and limb buds in four of five independent embryos (Fig. 4*K*–*N*), consistent with the endogenous expression pattern of Plxna2 (10, 33). It is of note that the expression pattern of *lacZ* consistently identified CNC migration (Fig. 4*M* and *N*). Mutation of the GATA binding site in the *Plxna2* promoter (Fig. 4*J*; ΔSite2-*lacZ*) abolished *lacZ* expression in the OFT region in five of five independent transgenic embryos analyzed at E12.5 (Fig. 4*O*–*R*). These results indicate that the 5′ promoter/enhancer DNA sequences of *Sema3c* and *Plxna2* analyzed in the present study may be essential and sufficient for the expression of these genes in cardiovascular development. These results, together with those of the in vitro studies, indicate that the expression of Sema3c and Plxna2 may be regulated directly through the binding of GATA transcription factors, especially GATA6, to the conserved GATA consensus sites in their promoter/enhancer sequences during OFT development, suggesting a possible underlying molecular mechanism for OFT defects resulting from *GATA6* mutations.

Discussion

Here we report the identification and characterization of two mutations of *GATA6* (9.5%) in our series of 21 Japanese patients with PTA. Both mutations disrupted the transcriptional activity of the GATA6 protein on downstream target genes involved in the development of the OFT. We also confirmed that the expression of *SEMA3C* and *PLXNA2* in the developing OFT was regulated directly through the consensus GATA binding sites well conserved between human and mouse, in vitro and in vivo. Mutant GATA6 proteins failed to transactivate *SEMA3C* and *PLXNA2*, and mutation of the GATA sites on enhancer elements of *Sema3c* and *Plxna2* abolished their activity, specifically in the OFT/subpulmonary myocardium and CNC derivatives in the OFT region, respectively, suggesting that mutations of *GATA6* cause specific forms of human OFT defects, or PTA.

The identification and characterization of mutations of GATA6 in the present study, together with results of previous animal studies (9–11, 24, 29), suggest a model for OFT development. In this model, GATA6 promotes the expression of both SEMA3C in the OFT/subpulmonary myocardium and PLXNA2 in the CNC. During migration of the CNC from the dorsal neural tube to the OFT, SEMA3C may act as a CNC attractant through SEMA3C-PLXNA2 or plexin D1-neuropilin1 signaling (11, 29). Conversely, class 6 semaphorins (e.g., Sema6a or 6b), expressed in the lateral pharyngeal arch, may moderate CNC migration through cell-cell and/or cell–matrix adhesion by acting as repellents via binding with the PLXNA2-neuropilin1 complex (29). We hypothesize that mutations of *GATA6* may lead to attenuation of the proper influx of CNC into the OFT, thus resulting in PTA as a result of disturbances to semaphorin–plexin signaling between the OFT/subpulmonary myocardium and the CNC in the developing OFT. This hypothesis is supported by results from previous animal studies that showed that null mutations of *Sema3c* or *Plxna2* in mice resulted in PTA (9,

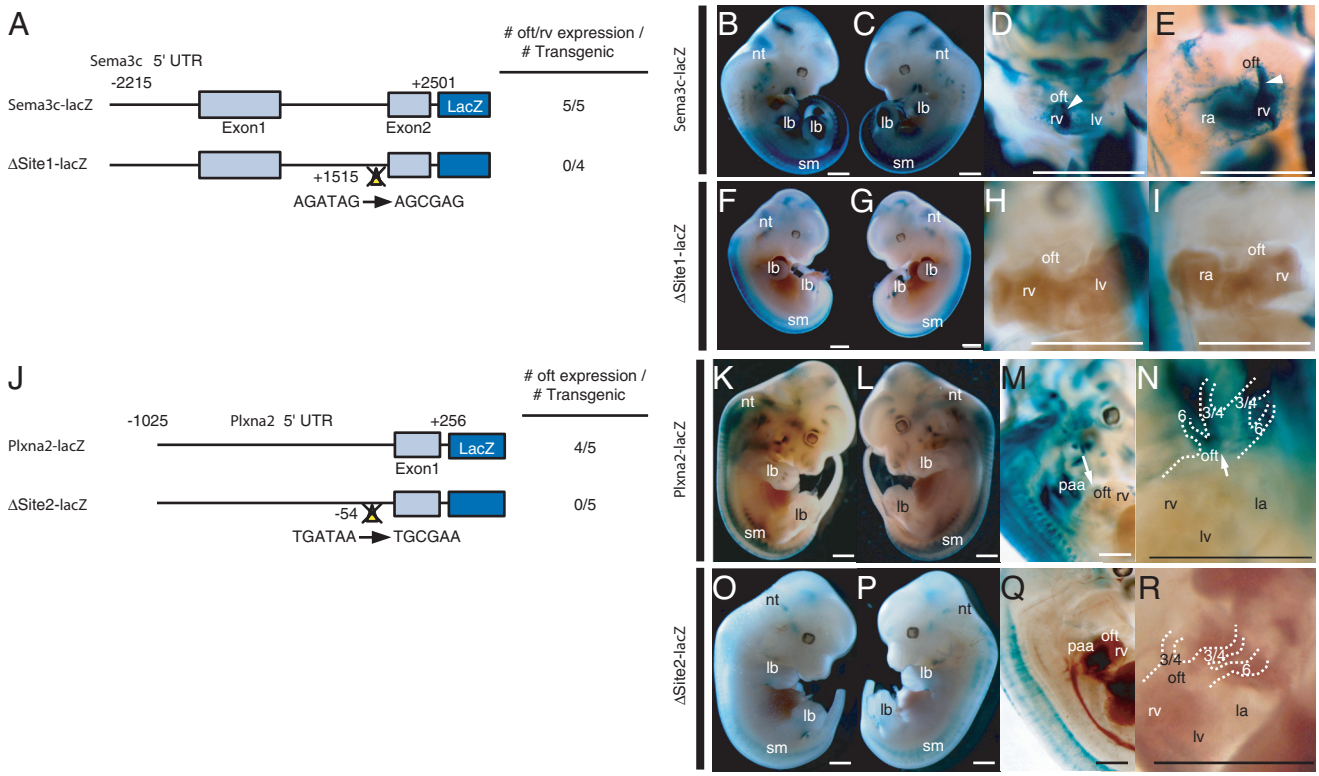

Fig. 4. GATA *cis*-elements in the *Sema3c* and *Plxna2* enhancer/promoter are essential for their expression in outflow tract development. (*A*) Schematic diagram of the mouse 4.7-kb *Sema3c* promoter-*lacZ* reporter plasmid with or without site-directed mutagenesis of Site1 (ΔSite1-*lacZ*). The number of transgenic embryos analyzed is indicated. (*B* and *C*) Embryonic day (E) 11.5 embryos harboring the *Sema3c-lacZ* transgene. (*D* and *E*) Clearing of the embryo revealed *lacZ*-positive cells migrating into the outflow tract (oft) and subpulmonary myocardium (arrowheads), and the right ventricle (rv) shown in the higher-magnification image of the heart. (*F* and *G*) E11.5 embryo harboring the ΔSite1-*lacZ* transgene. (*H* and *I*) Clearing of the embryo showed no *lacZ*-positive cells in the oft and rv, shown in the higher-magnification image of the heart. (*J*) Schematic diagram of a mouse 1-kb *Plxna2* promoter-*lacZ* reporter plasmid with or without site-directed mutagenesis of Site2 (ΔSite2-*lacZ*). The number of transgenic embryos analyzed is indicated. (*K*,*L*) E12.5 embryo harboring the *Plxna2-lacZ* transgene. (*M*) Clearing of embryos revealed *lacZ*-positive cells in the oft and pharyngeal arch arteries (paa). The white arrow indicates the truncus arteriosus. (*N*) Higher magnification of the heart. The white arrow indicates the truncus arteriosus. (*O* and *P*) E12.5 embryo harboring the ΔSite2-*lacZ* transgene. (*Q*) Clearing of embryos revealed no *lacZ* expression in the oft. (*R*) Higher magnification of the heart. (Scale bars, 1 mm.) sm, somites; lb, limb bud; nt, dorsal neural tube; ra, right atrium; lv, left ventricle; la, left atrium; 3/4, paa 3 and 4; 6, paa 6.

10). Downregulation of both ligand and receptor genes, as demonstrated in the present study, may enhance the disturbance of GATA6-centered regulation of target genes in the OFT region in humans. Screening for mutations of *SEMA3C* and *PLXNA2* may provide further evidence of the involvement of semaphorin-plexin signaling in human OFT defects.

It is of note that *GATA6* mutations identified in humans were associated with PTA, in contrast with *GATA4* mutations, which are commonly associated with atrial and/or ventricular septal defects (20–22). This result suggests that GATA6 may play a dominant role in OFT development, although Gata6 has been reported to have a redundant role with Gata4 during cardiogenesis (25, 34–36). It is known that there is a significant association between the DiGeorge/22q11.2 deletion syndrome and PTA (37), and *TBX1* on chromosome 22q11.2 has been proposed as a major genetic determinant of the clinical features of this syndrome (38–41). The clinical phenotype of DiGeorge/22q11.2 deletion syndrome is extensive and includes a characteristic facial appearance, thymic hypoplasia, cleft palate, hypoparathyroidism, developmental and behavioral problems, and many other extracardiac disorders in addition to OFT defects (42, 43). Commonly, the clinical phenotype of individuals with *GATA6* mutations involves the heart, but is distinct from that of DiGeorge/22q11.2 deletion syndrome and, rather, manifests as non-syndromic CHD. To date, no molecular link has been demonstrated between GATA6 and TBX1. Interestingly, a recent study showed that the expression of *Sema3c* in the OFT was downregulated in mouse embryos deficient for *Tbx1* (31), suggesting that GATA6 may share, at least in part, a common molecular pathway with TBX1 during OFT development (31).

The results of the present study demonstrate that both GATA6-E486del and GATA6-N466H mutants are unable to activate the transcription of the reporter genes examined. The GATA6-E486del mutation disrupted the NLS, was unable to bind to the proper GATA binding sites, and exerted a dominant negative effect on wild-type GATA6 protein. Conversely, the N466H mutant retained DNA binding activity for Site2 in the *PLXNA2* promoter, but lost DNA binding to Site1 in the *SEMA3C* promoter. This suggests that the N466H mutant is an altered specificity mutant, consistent with the role of Asn-466 in sequence-specific DNA binding. Asn-466 in the C-terminal zinc finger domain is highly conserved among members of the GATA family (Fig. S2*A*) (44) and plays a key role in forming the core zinc module that is essential for recognition of the GATA binding site, as well as for protein interaction (Fig. S2*B*) (45). The N466H mutation probably disrupts the core zinc module, causing not only undesirable DNA binding, but also failed interactions with numerous transcriptional modulators, resulting in dysregulation of GATA6-dependent genes. Such a dominant negative effect and altered specificity may be the putative effects of heterozygous mutations in human GATA6 that cause the OFT defects, although cardiac malformations were not found in mice heterozygous for a null Gata6 allele or exhibiting haploinsufficiency of Gata6 (35). Alternatively, heart development in humans may be more sensitive to subtle genetic abnormalities than that in mice, as suggested previously (4–6). In humans, disturbances in co-

activation with other modulators or secondary factors may affect the threshold of gene expression necessary for normal heart development.

We believe that both mutations affect development of CNC with such underlying molecular mechanism, resulting in the OFT defect. However, it is not clear why family members of proband A had pulmonary stenosis instead of PTA. Recently, it is reported that pulmonary valves might be derived from CNC (46), and pulmonary stenosis/obstruction was caused by abnormal development of CNC in some animal models (47, 48). Unknown second modifiers, epigenetic factors, and/or environmental factors might account for the phenotypic variability within the family. Further mutation screens and functional assays for GATA6 in patients with various forms of CHD would reveal the impact of GATA6 mutations on CHD and more precise genotype-phenotype correlations.

In conclusion, our results implicate mutations in *GATA6* as a genetic cause of human CHD through disruption of its direct regulation of semaphorin-plexin signaling involving the CNC in the pathogenesis of OFT defects. The GATA6-centered regulatory mechanism during cardiogenesis provides insight into the etiology of CHD.

Materials and Methods

The complete methods are described in detail in the *SI Materials and Methods*.

Mutation Analysis and Clinical Evaluation of Patients. Establishment of genomic bank with cell lines, extraction of genomic DNA samples were reported previously (21, 41). All exons and flanking introns of *GATA6* were PCR amplified and sequenced using direct, bidirectional sequencing; a detailed procedure is given in *SI Materials and Methods*. Identified mutations were confirmed by using patients' original genomic DNA extracted from peripheral blood leukocytes. Primer sequences are given in Table S2. Phenotype data for the affected individuals and their family members were obtained from detailed clinical evaluations based on echocardiogram, cardiac catheterization, and/or surgical findings, and are summarized in Table 1. Clinical evaluations and genetic studies of the patients and their families were approved by the Internal Ethics Committee of Tokyo Women's Medical University, and were undertaken only after informed consent had been obtained.

Statistics. For luciferase assays, all experiments were performed at least in triplicate and data are reported as normalized relative light units (fold activation) together with the SEM. For promoter activity assays, all experiments were performed at least in triplicate and data are reported as the ratio of normalized relative light units for coexpression with GATA6 to that with mock (pcDNA3.1). Error bars show the SEM. Data were analyzed by two-tailed unpaired *t* test. A *P* value of 0.05 or less was considered significant.

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