Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract

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Summary
Dysmorphogenesis of the cardiac outflow tract (OFT) causes many congenital heart defects, including those associated with DiGeorge syndrome. Genetic manipulation in the mouse and mutational analysis in patients have shown that Tbx1, a T-box transcription factor, has a key role in the pathogenesis of this syndrome. Here, we have dissected Tbx1 function during OFT development using genetically modified mice and tissue-specific deletion, and have defined a dual role for this protein in OFT morphogenesis. We show that Tbx1 regulates cell contribution to the OFT by supporting cell proliferation in the secondary heart field, a source of cells fated to the OFT. This process might be regulated in part by Fgf10, which we show for the first time to be a direct target of Tbx1 in vitro. We also show that Tbx1 expression is required in cells expressing Nkx2.5 for the formation of the aorto-pulmonary septum, which divides the aorta from the main pulmonary artery. These results explain why aortic arch patterning defects and OFT defects can occur independently in individuals with DiGeorge syndrome. Furthermore, our data link, for the first time, the function of the secondary heart field to congenital heart disease.

Key words: Tbx1, Mouse, Outflow tract, DiGeorge syndrome

Introduction
The cardiac outflow tract (OFT) forms as a simple vascular conduit that connects the embryonic right ventricle to the aortic sac, which is the site of confluence of the pharyngeal arch arteries. The embryonic OFT, which is divided into conal (proximal to the heart) and truncal (distal) segments, is soon lined by myocardial cells derived from migrating mesodermal cells. The myocardial layer of the embryonic OFT contracts and functions as a primitive valve. Septation separates the OFT into two channels, which direct the blood flow towards the aortic (systemic) and pulmonary circulation, and divides the primitive truncal valve into the aortic and pulmonary valves. Whereas the heart myocardium derives from lateral plate mesoderm precursors (the primary heart field), the OFT myocardium derives from the splanchnic mesoderm located caudal to the pharynx (the secondary heart field, or SHF) (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). The molecular mechanisms underlying induction and differentiation of myocardial precursors in the SHF are hypothesized to be similar to those of the primary heart field (Waldo et al., 2001), but the genetics of SHF function and the phenotypic consequences of SHF malfunction are uncertain. Neural crest-derived cells are also required for OFT septation and make up most of the aorto-pulmonary (AP) septum, a structure that originates from the dorsal wall of the aortic sac (Jiang et al., 2000; Kirby and Waldo, 1995; Li et al., 2000). Severe developmental defects of the OFT are tolerated during embryogenesis, provided that they do not compromise patency, but are lethal in post-natal life, when the pulmonary circulation is required for blood oxygenation. OFT developmental defects account for a high proportion of congenital heart disease cases and, consequently, are an important cause of morbidity and mortality in children.

Chromosome 22q11.2 deletion (del22q11) causes most cases of DiGeorge syndrome (DGS), velocardiofacial syndrome, and conotruncal anomaly face, and it is one of the most common genetic causes of OFT and aortic arch defects. Modeling del22q11 in mice (Jerome and Papaioannou, 2001; Lindsay et al., 1999; Lindsay et al., 2001; Merscher et al., 2001) and mutational analysis in human patients (Yagi et al., 2003), have led to the identification of Tbx1 as the major player in these syndromes. Tbx1 is required for segmentation of the embryonic pharynx, for the formation of the caudal pharyngeal arches and arch arteries, and for growth, alignment and septation of the OFT in mice. Because of the complexity of the mutant phenotype, and the close developmental relationship between the pharyngeal apparatus
and OFT, it has not been possible to establish whether Tbx1 has a specific role in OFT morphogenesis. Here we have addressed this issue using different genetic approaches in the mouse and we show that even in the presence of severe developmental abnormalities of the pharynx, the severity of the OFT phenotype can be ameliorated by a low level of Tbx1 expression. Conversely, conditional ablation of Tbx1 in the Nkx2.5 domain causes a mild pharyngeal phenotype, but it recapitulates the severe OFT phenotype observed in Tbx11/− embryos. Analysis of conditional mutants supports a dual role of Tbx1 in OFT development, one in morphogenesis of the AP septum, and one in cell proliferation in the SHF region. Reduced cell proliferation in the SHF is associated with reduced cell contribution to the OFT and, consequently, reduced number of muscle cells.

Our data provide an explanation as to why OFT defects may occur independently from other pharyngeal or aortic arch patterning defects in patients with del22q11. We propose that the separation of the aorta and pulmonary arteries requires Tbx1 in the pharyngeal endoderm, whereas proper OFT alignment and truncal valve septation requires Tbx1 function in the SHF.

Materials and Methods

Mouse mutants and breeding

The following mouse lines have been described previously: Tie2-Cre (Kisanuki et al., 2001), αMHC-Cre (Agah et al., 1997), R26R (Soriano, 1999), Tbx11+/− (Lindsay et al., 2001), Df11+/− (Lindsay et al., 1999), Nkx2.5cmt (Moses et al., 2001). Mice were genotyped using PCR as described in the original reports. The generation of the new lines Tbx1neo/w, Tbx1fen/w, and Tbx1mcm/w is described below. To induce nuclear translocation of the MerCreMer fusion protein encoded by the Tbx1mcm allele, pregnant mice were injected daily with Tamoxifen (30 mg/kg body weight) starting from E5.5. Consistent with literature data (Hayashi and McMahon, 2002), we observed that this dosage causes some embryo lethality, however, we did not observe cardiovascular defects in surviving wild-type embryos, and the expression of Tbx1 (as tested by the lacZ knock-in reporter allele) was not affected by the Tamoxifen treatment (not shown).

Cell fate mapping in Tbx1 homozygous mutant background was performed in Df1/1Tbx1mcm/R26R embryos. The Df1 deletion, which includes the Tbx1 gene, was used as the Tbx1 null allele because our Tbx1+/− allele includes a lacZ reporter gene that would have confounded the analysis. Df1/Tbx1+/− and Tbx1+/− have identical phenotype (Vitelli et al., 2002b). All mouse lines were crossed into a C57BL/6 background in the experiments described. All embryos (up to E12.5) were staged by counting the number of somites.

Generation of new mouse lines

The alleles Tbx1neo, Tbx1mcm were generated by homologous recombination in AB2 ES cells, as shown in Fig. 1A and Fig. 2A. The allele Tbx1fen was generated by transfecting a Cre recombinase expression construct containing the Tbx1neo/w ES cells. ES cells were injected into C57Bl6 blastocysts and chimera mice were crossed with C57BL/6 mice to obtain germ line transmission of the mutant alleles. Cre-induced recombination of the floxed allele causes the excision of exon 5 and generation of the Tbx1mcm allele (Fig. 1B). This deletion is predicted to cause loss of function because exon 5 encodes part of the conserved T-box domain, and because splicing between exons 4 and 6 in the mutant allele generates a frame shift from codon 169, and a stop codon after 86 codons. We could not detect exon 5 skipping in the wild-type allele using reverse-transcription polymerase chain reaction (RT-PCR) on RNA from E10.5 embryos.

The Tbx1neo allele was genotyped with primer pair Tbx1-flox_F: GGCCCTCTCTTGGCTTATG and Tbx1-flox_R: AAAGACTCTGGCCTTTT. The Tbx1mcm allele was genotyped with the primer pair Tbx1-mcm_F: GCTCACTTCAGCACATTC and Tbx1-mcm_R: CATAAGCAGAGGGTGCG.

RT-PCR

Total RNA was extracted from whole embryos at E10.5 using Trizol (Invitrogen). The concentration of RNA samples was measured using a spectrophotometer, and the concentration of all samples was adjusted to 100 ng/µl. The reverse transcription was followed by 30 cycles of PCR amplification. The location of the primers is indicated in Fig. 1A, the sequences are TTTGTGCGGTAGTACAA (forward primer) and AATCGGGCTGTATCTGTG (reverse primer).

Analysis of chimeras

The generation of Tbx1+/− mouse ES cells has been described (Vitelli et al., 2003). Tbx1+/− and Tbx1+/+ (Lindsay et al., 2001) ES cells were injected into wild-type C57BL/6 blastocysts and transferred into pseudopregnant CD1 females. Chimeric embryos were harvested at E10.5 and were stained with X-gal prior to ethanol fixation and embedding in paraaffin. Histological sections (10 µm) were counterstained with Nuclear Fast Red.

Histology, X-gal staining and immunohistochemistry

To visualize β-gal activity, we stained paraformaldehyde-fixed embryos using the X-gal substrate, according to standard procedures. Stained embryos were photographed as wholemounts and then embedded in paraaffin and cut in 10 µm histological sections. Sections were counterstained with Nuclear Fast Red. Muscle cells were identified by immunohistochemistry using an anti-α-smooth muscle actin (sma) monoclonal antibody (Clone 1A4, Sigma). Cell proliferation was assessed using a BrdU assay. Briefly, pregnant females were injected with 5 mg/100 g body weight of BrdU and sacrificed 1 hour after injection to harvest embryos. Embryos were fixed in ethanol, embedded in paraaffin and cut in 7 µm sections. BrdU incorporation was detected on histological sections using an anti-BrdU monoclonal antibody (Clone # 85-2C8, Novoceastra). The antineurofilament-M (165KD) monoclonal antibody 2H3 was developed by T. M. Jessell and J. Dodd and was obtained from the Developmental Studies Hybridoma Bank, University of Iowa.

RNA in situ hybridization

RNA in situ hybridization experiments, with radioactive or non-radioactive probes, were performed on sectioned or wholemount embryos, respectively, according to published protocols (Albrecht et al., 1997). Labeled probes (sense and antisense) were prepared by reverse transcription of DNA clones in the presence of digoxigenin-labeled UTP (Roche) or 35 S-UTP (ICN). A45UTP (Roche) or 35 S-UTP (ICN). A labeled probe, transcript was detected using the probe described (Giguere et al., 1990).

Luciferase assay

A mouse Tbx1 cDNA (Lindsay et al., 2001) (Accession # AF326960) was fused with a c-myc tag and cloned into the pCDNA3.1 expression vector. The Fgf10Luc and Fgf10MutLuc constructs, and the luciferase assay procedure have been described (Agarwal et al., 2003). Constructs were transfected in COS-7 cells and data were normalized to a co-transfected β-Gal expression vector. Experiments were performed in duplicate and repeated three times.

Results

Outflow tract development is Tbx1-dosage dependent

Tbx1+− animals have severe OFT abnormalities including complete lack of septation and abnormal alignment of the
truncus arteriosus with the ventricles (Jerome and Papaioannou, 2001; Vitelli et al., 2002b). These abnormalities may be secondary to the severe defects of the pharyngeal apparatus associated with Tbx1 loss of function or may be because of a specific role of Tbx1 in OFT development. To begin addressing this issue, we have generated a new allelic series of the Tbx1 gene, as shown in Fig. 1A,B. We tested in vivo whether a Tbx1 neo allele, which carries a PGKneo cassette inserted into intron 5, functions as a hypomorphic allele.

Tbx1neo/+ fetuses were examined at E18.5 (n=36) and 42% had the same aortic arch patterning defects observed in Tbx1+/– animals (not shown). We extracted RNA from Tbx1 neo/neo and from Tbx1 Tbx1–/– embryos and performed RT-PCR using primers on exons flanking the PGKneo cassette insertion (Fig. 1A). Results show that the Tbx1neo allele transcribes a low amount of Tbx1 RNA (Fig. 1C). Tbx1 Tbx1–/– embryos were grossly indistinguishable from Tbx1 Tbx1–/– embryos, and show the same cardiovascular phenotype. However, none of the five Tbx1 neo/– embryos examined at E18.5 had cleft palate, a defect observed in all the Tbx1 Tbx1–/– embryos examined in this genetic background (Vitelli et al., 2002a). Analysis of the cardiovascular phenotype revealed that out of 14 Tbx1 neo/neo fetuses examined, eight had an alignment defect of the truncus identical to that observed in Tbx1 Tbx1–/– embryos (Fig. 1F,F'), whereas six had correct alignment (Fig. 1E,E') as the truncus communicates directly with both ventricles and overrides a subvalvular ventricular septal defect (VSD). Improved OFT alignment was also evident at E11.5 (Fig. 1G-I). However, the pharyngeal phenotypes in Tbx1 neo/neo and Tbx1 Tbx1–/– embryos were essentially identical at E10.5 (not shown). Thus the OFT phenotype resulting from loss of Tbx1 function can be partially rescued even in the presence of severe pharyngeal abnormalities.

Tbx1 is not required in myocytes or endothelial cells of the OFT

Despite the severity of the OFT phenotype in Tbx1 Tbx1–/– mutants, Tbx1 gene expression in the OFT is modest (Vitelli et al., 2002b). Tbx1 is expressed in endothelial cells (from E11.5) and in a subpopulation of α-sma-positive cells of the outer wall of the OFT (Vitelli et al., 2002b). Endothelial cells in the conal OFT, transform into mesenchymal cells of the outflow ridges (van den Hoff et al., 1999), thus, these cells might be important for septation and valvulogenesis. Therefore, we asked whether Tbx1 is required by endothelial cells for conal septation. To this end, we used mice carrying a Tbx1 conditional, floxed allele, generated as shown in Fig. 1B. Tbx1flox/flox mice were viable and fertile, and were crossed with Tbx1 Tbx1–/–; Tie2:Cre mice. Tie2:Cre transgenic mice express Cre in endothelial cells and their precursors (Kisanuki et al., 2001), including endothelial cells of the OFT that also express Tbx1 (not shown). Tbx1 Tbx1flox–; Tie2:Cre fetuses were analyzed at E18.5 (n=8), but none presented with cardiovascular abnormalities other than aortic arch defects of Tbx1 RNA (Fig. 1C).
associated with Tbx1 haploinsufficiency (not shown). Next, we asked whether Tbx1 expressed in muscle cells of the OFT is required for OFT growth and morphogenesis. To this end, we crossed Tbx1+/−; αMHC:Cre mice with Tbx1flox/flox mice. Tbx1flox/flox; αMHC:Cre fetuses at E18.5 (n=6) had normal cardiovascular phenotype, apart from the aortic arch abnormalities characteristic of Tbx1 haploinsufficiency (not shown).

Tbx1 is expressed in precursors of OFT cells and its loss of function reduces cell contribution to the OFT

Because Tbx1 is not required in resident, differentiated OFT cells, we asked whether Tbx1 may be expressed in progenitors of these cells. Therefore, we analyzed the fate of Tbx1-expressing cells by generating and establishing in mice an allele of Tbx1, named Tbx1mcm, in which a Tamoxifen-inducible Cre construct (Sohal et al., 2001; Verrou et al., 1999) is driven by the endogenous regulatory elements of Tbx1 (Fig. 2A). The insertion point of the Cre construct is identical to the one we used for a lacZ reporter construct shown to recapitulate the developmental expression of Tbx1 (Lindsay et al., 2001; Vitelli et al., 2002b). We then crossed Tbx1mcm+/ mice with the R26R reporter line (Soriano, 1999) to evaluate Cre-induced recombination. Because the Cre construct that we used encodes a Tamoxifen-inducible recombinase, Tbx1mcm+/R26R embryos from mothers that had not been injected with Tamoxifen showed no blue cells upon X-gal staining (n=13, not shown).

The cell fate of Tbx1-expressing cells was studied using Tbx1mcm+/;R26R embryos where the pregnant mothers had been treated with daily injections of Tamoxifen from E5.5. Hereafter we refer to these embryos, stained with X-gal, as Tbx1-tracing embryos. The staining intensity was variable but, overall, Tbx1 tracing resulted in a pattern of X-gal staining that was very similar to Tbx1 gene expression (Fig. 2B,C), at the stages tested (E9.5, E10.5 and E12.5), with two exceptions. First, the ectoderm lining the lateral aspect of the pharyngeal region showed extensive staining (Fig. 2C, red arrowheads), probably because of transient Tbx1 expression in the ectoderm before E9.5 (E.A.L., unpublished). Second, in the OFT (Fig. 2E-J), Tbx1 tracing showed extensive contribution to the myocardial wall and endothelium of the OFT, and some contribution to the myocardium of the right ventricle (Fig. 2F,HJ) (five embryos analyzed at E9.5, seven at E10.5, and four at E12.5). In contrast, Tbx1 expression in the OFT was much more restricted (Fig. 2E,G1), indicating that Tbx1 is expressed transiently in a substantial number of progenitors of cells fated to the OFT. Contribution of Tbx1-traced cells was observed at all stages tested but was relatively low at E9.5 (earliest stage analyzed is 25 somite), suggesting that although this contribution must start before E9.5, it appears more substantial between E9.5 and E10.5.

Next, we asked whether Tbx1 function is required for the contribution of Tbx1-traced cells to the OFT. We performed cell fate mapping in a Tbx1 null background and observed reduced number of Tbx1-traced cells in the OFT (Fig. 2D,L,M). However, this reduction could be attributed in part to the overall size reduction of the OFT in Tbx1 null mutants. Tbx1-traced cells were observed in the SHF region of Tbx1 homozygous mutant embryos (n=4) (Fig. 2M arrowhead, compare with lacZ knock-in K and cell fate mapping in heterozygous background, L’). Thus, Tbx1 loss of function reduces but does not prevent the contribution of Tbx1-traced cells to the OFT and SHF.

Tbx1 is required in Nkx2.5-expressing cells

The data presented so far support the hypothesis that the primary role of Tbx1 in OFT morphogenesis is performed in progenitors of cells contributing to the OFT. The induction of naïve mesodermal cells in the SHF to an OFT myocardial fate is thought to use a molecular circuitry similar to that used in the primary heart field (Waldo et al., 2001). Consistent with this view, Nkx2.5 is expressed in the SHF (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001) and it is required for the development of the OFT (Lyons et al., 1995; Tanaka et al., 1999). The OFT of Nkx2.5+/− embryos is not canalized (Lyons et al., 1995; Tanaka et al., 1999) (M.M., unpublished), which is an earlier and more severe defect than that of Tbx1−/− animals. Hence, we hypothesize that Tbx1 function in OFT progenitors is downstream to Nkx2.5 function, and that deletion of Tbx1 in cells expressing Nkx2.5 is sufficient to recapitulate the OFT abnormalities found in Tbx1−/− mutants. To test this hypothesis, we crossed Nkx2.5Cre/+ mice, which carry a Cre gene inserted into the Nkx2.5 gene (Moses et al., 2001), with Tbx1+/− animals, and then crossed Nkx2.5Cre/+;Tbx1−/− mice with Tbx1flox/flox mice. The external appearance of E18.5 Nkx2.5Cre/+;Tbx1flox/flox embryos (hereafter referred to as conditional mutants) was indistinguishable from that of normal littermates, in particular, they did not have the characteristic external ear defects or cleft palate observed in Tbx1−/− embryos (not shown). The thymus was clearly visible but small, with the lobes widely separated (Fig. 3A,B). The cardiovascular phenotype included truncus arteriosus of the same type as that seen in Tbx1−/− animals (complete lack of septation and a single four-leaflet truncal valve communicating exclusively with the right ventricle, and a large infundibular VSD) (Fig. 3C-E). The aortic arch phenotype differed from the Tbx1−/− phenotype because the 6th pharyngeal arch arteries (PAAs) formed, persisted and connected the outflow to the descending aorta (on the left) and to the right subclavian artery (on the right) (Fig. 3D-E). In contrast, in Tbx1−/− embryos the 3rd, 4th and 6th PAAs do not form (Vitelli et al., 2002b). Thus, conditional mutants recapitulate the OFT phenotype but not the aortic arch patterning phenotype of Tbx1−/− mutants, which is considerably milder in conditional mutants.

Because Nkx2.5Cre is a loss of function allele (Moses et al., 2001), and because Nkx2.5 has been shown to interact with another member of the T-box family of proteins, Tbx5 (Bruneau et al., 2001), we tested whether Tbx1 and Nkx2.5 may interact genetically. However, double heterozygous Nkx2.5Cre/+;Tbx1−/− embryos at E18.5 were viable (n=11) and presented with abnormalities attributable to haploinsufficiency of each gene (not shown), as both Tbx1 (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001) and Nkx2.5 (Biben et al., 2000) are haploinsufficient. These results indicate that the OFT phenotype observed in conditional mutants is not because of genetic interaction between Tbx1 and Nkx2.5 but deletion of Tbx1 in the Nkx2.5 expression domain.

Conditional deletion of Tbx1 causes ablation of the AP septum

Conditional mutants have normal 3rd and 6th PAAs but have
no 4th PAAs, as visualized by intracardiac ink injection (n=3) (Fig. 3F,G). This pattern is also seen in Tbx1+/– embryos, however, the distance between the 3rd and 6th PAAs is smaller than in wild-type or Tbx1+/– embryos, suggesting malformations of the 4th pharyngeal arch and aortic sac. Histological sections showed that the 4th pharyngeal arches of conditional mutants were small and the 4th PAAs were very hypoplastic or undetectable (Fig. 4A’,B’). Consistent with hypoplasia of the 4th pharyngeal arch, we observed a reduced number of neural crest-derived cells migrating through the 4th arch, but a normal pattern in the 3rd and 6th arches of conditional mutants, as tested by Crabp1 in situ hybridization (Fig. 4C-F). Immunohistochemistry with an anti-neurofilament M antibody showed the presence of an apparently normal vagus nerve bundle in the 4th arch, suggesting normal differentiation of neural crest-derived cells in this arch (Fig. 4A,A’,B,B’).
Nkx2.5cre induces recombination in a portion of the pharyngeal endoderm that expresses Tbx1 in the 4th pouch, around the 4th arch, and 3rd pouch (Fig. 4G,H). These overlaps correlate well with small 4th arch and small thymus, which is derived from the 3rd pharyngeal pouches. The other structures of the pharyngeal apparatus were normal. Conditional mutants have no separation between the aorta and pulmonary trunk (Fig. 3D,D'), suggesting a defect of the AP septum. Normally, this structure forms around E10.5 from the dorsal wall of the aortic sac, between the confluence of the 4th and 6th PAAs, but it is absent in conditional mutants (Fig. 4I,J), suggesting a morphogenetic defect of the aortic sac. Nkx2.5cre-induced recombination and Tbx1 expression overlap in the endodermal lining of the aortic sac, providing a regional correlation with the morphogenetic defect (Fig. 4K,L). Alternatively, the AP septum abnormality could be secondary to reduced neural crest migration through the 4th arch. However, such reduction appears to be modest, and the AP septum is also contributed by neural crest cells of the 3rd and 6th pharyngeal arches (Phillips et al., 1987), which migrate normally in conditional mutants (arrows in Fig. 4C,D).

Conditional deletion of Tbx1 reduces cell proliferation in the splanchnic mesoderm

Immunohistochemistry with an anti-α-sma antibody showed a thinner and discontinuous immunoreaction in the OFT myocardial layer of conditional mutants (Fig. 5A-C,A'–C'). The growth of the myocardial layer of the OFT relies mainly on cell migration from the SHF because cell proliferation activity is modest in the OFT. Thus, a possible explanation for reduced contribution of cells to the OFT is reduced proliferation in the SHF. We performed a BrdU assay in embryos with 32 and 29 somites, and results showed that the mitotic index in the SHF and adjacent splanchnic mesoderm of conditional mutants is reduced by 18% and 19%, respectively, compared with somite-matched wild-type embryos (Table 1). In contrast, we found no significant difference in the myocardial OFT and in tissues where there is no overlap between Tbx1 expression and Nkx2.5cre-induced recombination (Table 1). This result is consistent with overlap between Tbx1 expression and Nkx2.5cre-driven recombination in the SHF region (Fig. 6D,G).

Chimera analyses support a cell non-autonomous role of Tbx1 in the SHF

The Tbx1 role in cell proliferation could be produced through a cell-autonomous mechanism or through activation of an extracellular signaling system (cell non-autonomous mechanism). Our cell fate mapping data show that loss of function of Tbx1 causes overall reduction of cell contribution to the OFT, not restricted to Tbx1-traced cells, suggesting a cell non-autonomous mechanism. To further investigate this issue, we asked whether Tbx1–/– cells may have a proliferation disadvantage in populating the SHF when competing with wild-type cells, in a chimeric context. Therefore, we generated Tbx1–/–:Tbx1+/+ chimeric embryos as previously described (Vitelli et al., 2003). In this experimental approach, mutant cells that have a transcriptionally active (but non-functional) Tbx1 gene will be blue upon X-gal staining, because they express a lacZ reporter gene inserted into the Tbx1 locus. Results showed that Tbx1–/– blue cells are not excluded from the SHF of chimeras (n=10) and produce an X-gal staining pattern similar to the one observed in Tbx1+/–:Tbx1+/+ chimeras (n=8).

Fig. 3. Phenotype of conditional mutants Nkx2.5Cre+/+;Tbx1floxed–.

(A,B) Thymic hypoplasia in a conditional mutant (B) compared with wild-type embryo (A) at E18.5. Asterisks indicate thymic lobes.

(C-E) Intracardiac phenotype in wild-type (C), conditional (D) and Tbx1–/– (E) mutants. Embryos in D and E have essentially identical phenotypes. C–E' aortic arch and great artery patterning in wild-type (C'), conditional (D') and Tbx1–/– (E') mutants at E18.5.

(F,G) Lateral views of ink-injected Tbx1+/– (F) and conditional mutant (G) embryos at E10.5. Note the absence of the 4th pharyngeal arch artery and reduced distance between the 3rd and 6th in the conditional mutant embryo. ao: aorta; rec and lcc: right and left common carotid artery; rsa and Isa: right and left subclavian artery; pa: pulmonary arteries; pt: pulmonary trunk; RV: right ventricle; T: truncus arteriosus; VSD: ventricular septum defect. Scale bars: 1 mm in A–E and C–E'; 200 μm in F,G.
(Fig. 6A-B) and lacZ knock-in germ line mutants (Fig. 2K,K′). These results provide further support to the hypothesis that Tbx1 is not required cell-autonomously for SHF cell proliferation.

Table 1. Results of BrdU assay in conditional mutants and somite-matched controls

<table>
<thead>
<tr>
<th>Embryo (somites)</th>
<th>Tissue</th>
<th>Total cell number</th>
<th>BrdU +</th>
<th>Mitotic index</th>
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<tr>
<td>Control 1 (29)</td>
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<td>1011</td>
<td>488</td>
<td>0.48</td>
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<td></td>
<td>OFT myocardium</td>
<td>1187</td>
<td>117</td>
<td>0.099</td>
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<td></td>
<td>Brain</td>
<td>1175</td>
<td>650</td>
<td>0.55</td>
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<tr>
<td>Conditional mutant 1 (29)</td>
<td>Splanchnic mesoderm</td>
<td>917</td>
<td>360</td>
<td>0.39 (P=0.00007)</td>
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<tr>
<td></td>
<td>OFT myocardium</td>
<td>1232</td>
<td>107</td>
<td>0.087 (P=0.32)</td>
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<tr>
<td></td>
<td>Brain</td>
<td>1142</td>
<td>640</td>
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<tr>
<td>Control 2 (32)</td>
<td>Splanchnic mesoderm</td>
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<td>652</td>
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<tr>
<td></td>
<td>OFT myocardium</td>
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<td></td>
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<td>0.58</td>
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<tr>
<td>Conditional mutant 2 (32)</td>
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<td>306</td>
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<tr>
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<td>OFT myocardium</td>
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<td>0.085 (P=0.31)</td>
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<td></td>
<td>Somites</td>
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<td>702</td>
<td>0.60 (P=0.32)</td>
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</table>

P values refer to comparison with respective controls, and are calculated with a \( \chi^2 \)-square test.

**Fgf10 is a direct target of Tbx1 in a tissue culture system**

The above results suggest that Tbx1 may activate an extracellular signaling system that regulates cell proliferation.
in the SHF. To date, no transcriptional target of Tbx1 has been reported, but it has been shown that Fgf10 expression is reduced in the SHF of Tbx1<sup>–/–</sup> mutants, by in situ hybridization (Kochilas et al., 2002; Vitelli et al., 2002c). It has been shown that FGF signaling can induce cell proliferation in SHF explants (Waldo et al., 2001), so Fgf10 might mediate, at least in part, a cell non-autonomous function of Tbx1 in this region. Recently, it has been shown that Tbx5 can activate Fgf10 through a conserved T-box binding element (TBE) located in the 5′ region of the Fgf10 gene (Agarwal et al., 2003). Therefore, we asked whether Tbx1 could also activate the Fgf10 gene via this TBE. To this end, we co-transfected into COS-7 cells a Tbx1 expression vector and an Fgf10 promoter-luciferase construct. We observed up to 20-fold activation of luciferase activity in repeated experiments, but when we co-transfected the Tbx1 expression vector with an Fgf10 promoter construct carrying a mutant TBE (Agarwal et al., 2003), we observed no activation (Fig. 6C), demonstrating that this binding site is required for activation. For comparison, the same experiment was performed with a Tbx5 expression vector with similar results, raising the intriguing possibility that different T-box transcription factors may share target genes. In situ hybridization on tissue sections of wild-type embryos showed overlap between Tbx1 and Fgf10 expression in the SHF region (Fig. 6D,E). In addition, Fgf10 expression in this region is reduced or abolished in conditional mutants (Fig. 6F), consistent with overlap with Nkx2.5<sup>Cre</sup>-driven recombination (Fig. 6G). These results identify Fgf10 as a candidate mediator of the Tbx1 cell non-autonomous role in cell proliferation in the SHF.

**Discussion**

**Del22q11 causes a broad range of phenotypic abnormalities, many related to developmental defects of the pharyngeal apparatus.** Mouse studies have attributed these abnormalities to haploinsufficiency of Tbx1, a T-box transcription factor, included in the del22q11 region. Recently, mutational analyses have found TBX1 gene mutations in patients with a ‘pharyngeal’ phenotype essentially identical to that associated with del22q11 (Yagi et al., 2003), demonstrating that Tbx1 has similar developmental roles in human and mouse. A cardinal feature of del22q11/DGS is congenital heart disease, which includes aortic arch/great artery patterning defects and OFT defects. Tbx1<sup>+/–</sup> mice have mainly aortic arch patterning defects, whereas Tbx1<sup>–/–</sup> embryos have severe OFT abnormalities, but they also have severe developmental defects of the entire pharyngeal apparatus. In this study we asked whether the OFT phenotype in Tbx1<sup>–/–</sup> embryos is merely a consequence of pharyngeal apparatus abnormalities, and, if not, what are the specific roles of Tbx1 in OFT morphogenesis.

**Tbx1 has direct roles in OFT morphogenesis**

Our data show that Tbx1<sup>neo/neo</sup> animals present with severe abnormalities of the pharyngeal apparatus, including loss of the 3rd-6th pairs of pharyngeal arches and arteries, as in Tbx1<sup>–/–</sup> embryos. However, the OFT phenotype is milder in approximately half of the Tbx1<sup>neo/neo</sup> embryos. Thus, the OFT mutant phenotype is partially independent of the pharyngeal phenotype. Conversely, Nkx2.5<sup>Cre</sup>-induced somatic deletion of Tbx1 recapitulates the OFT phenotype of Tbx1<sup>–/–</sup> animals, even though the abnormalities of the pharyngeal apparatus are much milder. Overall, these data clearly indicate that Tbx1 has specific functions in OFT morphogenesis.

**Tbx1 regulates cell contribution to the OFT**

Tbx1 is expressed in progenitors as well as in differentiated, resident myocytes and endothelial cells of the OFT. However, our conditional deletion experiments indicate that Tbx1 function is not required in myocardial or endothelial cells. Instead, cell fate mapping experiments indicate that Tbx1 regulates, but is not required for contribution of myocytes, and possibly other cell types, to the OFT. Most probably, Tbx1 does not directly regulate cell proliferation of progenitor cells because in chimeras, Tbx1<sup>–/–</sup> cells do not have a proliferative disadvantage in the SHF. Thus, the Tbx1 role in regulating cell contribution to the OFT will probably be cell non-autonomous. Fgf10 is a candidate mediator of this function because its expression in the SHF is abolished in Tbx1<sup>–/–</sup>, Tbx1<sup>neo/neo</sup>, and conditional mutants, and Tbx1 can directly activate the Fgf10 promoter in a tissue culture assay. Interestingly though, Fgf10<sup>–/–</sup> animals do not have OFT defects, raising the question as to whether Tbx1 also activates other Fgf genes or other extracellular signaling systems critical for OFT morphogenesis. Alternatively, Fgf10<sup>–/–</sup> animals may not have OFT defects because other Fgf genes compensate for the loss of Fgf10, as proposed recently (Kelly and Buckingham, 2002).
Tbx1 has at least two roles in OFT morphogenesis

Consistent with previously discussed data, the OFT of Tbx1 conditional mutants have reduced numbers of α-sma-positive cells. This correlates well with a significant downregulation of cell proliferation in the SHF. In contrast, the proliferation of cells of the myocardial layer of the OFT is not affected in these mutants. Hence, the most probable explanation for the lower number of α-sma-positive cells in the OFT is a reduced supply from a pool of precursors, secondary to reduced cell proliferation. It is also possible that Tbx1 is required to specify a subpopulation of OFT myocyte precursors because the α-sma phenotype is more obvious in the region where truncal valve septation is occurring (Fig. 5B,B’). This is an intriguing observation because this cell population may have a specialized, patterning role in truncal valve septation. Interestingly, the Drosophila homologue of Tbx1, Org-1, is expressed in a subpopulation of visceral muscle cell precursors, the so-called pioneer cells, which have patterning activity (Lee et al., 2003).

Our data show that Tbx1 has an additional role in OFT morphogenesis because it is required for the formation of the AP septum in Tbx1+/−, Tbx1neo/neo, and conditional mutants. Because the AP septum is mainly contributed by neural crest-derived cells, it is possible that this phenotype is caused, at least in part, by the observed reduced population of migrating neural crest-derived cells in the 4th pharyngeal arch of conditional mutants. However, this reduction appears modest, and the conotruncal ridges, which are also populated by a substantial number of neural crest cells, are only slightly reduced in size. Therefore, we hypothesize that AP septum aplasia in conditional mutants is because of defective morphogenesis of the aortic sac. This morphogenetic defect is consistent with the pharyngeal segmentation defect observed in Tbx1−/− mutants. It has been proposed that the Tbx1 role in pharyngeal segmentation may be related to its expression in the pharyngeal endoderm (Baldini, 2002; Lindsay, 2001). The AP septum phenotype in conditional mutants correlates well with overlap of Tbx1 and Nkx2.5Creinduced recombination in the pharyngeal endoderm and wall of the aortic sac.

SHF function and congenital heart disease

Presumably, an as yet unknown signal induces naïve splanchnic mesodermal cells to an OFT myocardial fate. One of the consequences of this signal is the expression of Nkx2.5, possibly one of the first markers of specification of this cell population. Nkx2.5 is required for the formation of a canalized OFT conduit (Lyons et al., 1995; Tanaka et al., 1999) (M.M., unpublished). However, proper morphogenesis of the OFT requires sustained contribution of specified cells throughout an extended period of time (approximately between E9.0 and E11.0) because resident OFT myocytes have a low proliferative capacity at this stage. We propose that the function of Tbx1 is
to maintain cell contribution to the OFT at a sufficiently high level to support growth and remodeling of the OFT (Fig. 6H). This function may be directed towards the entire population of myocardial precursors, or perhaps more probable, towards a specific sub-population. Whatever the target cell population may be, the effect of Tbx1 on cell proliferation is cell non-autonomous.

Our data provide the first evidence that a genetic defect related to human congenital heart disease affects directly the function of the SHF. Our data do not allow us to determine exactly what type of defect is caused by SHF malfunction. Therefore, it is very probable that the definition of genetic pathways regulating SHF function will lead to other genes involved in congenital heart disease. The identification of the role of Tbx1 in the SHF is the first step in this direction.

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