The del22q11.2 Candidate Gene Tbx1 Controls Regional Outflow Tract Identity and Coronary Artery Patterning

Magali Théveniau-Ruissy, Mathieu Dandonneau, Karim Mesbah, Olivier Ghez, Marie-Geneviève Mattei, Lucile Miquerol, Robert G. Kelly

Abstract—Tbx1, encoding a T-box containing transcription factor, is the major candidate gene for del22q11.2 or DiGeorge syndrome, characterized by craniofacial and cardiovascular defects including tetralogy of Fallot and common arterial trunk. Mice lacking Tbx1 have severe defects in the development of pharyngeal derivatives including cardiac progenitor cells of the second heart field that contribute to the arterial pole of the heart. The outflow tract of Tbx1 mutant embryos is short and narrow resulting in common arterial trunk. Here we show by a series of genetic crosses using transgene markers of second heart field derived myocardium and coronary endothelial cells that a subdomain of myocardium normally observed at the base of the pulmonary trunk is reduced and malpositioned in Tbx1 mutant hearts. This defect is associated with anomalous coronary artery patterning. Both right and left coronary ostia form predominantly at the right/ventral sinus in mutant hearts, proximal coronary arteries coursing across the normally coronary free ventral region of the heart. We have identified Semaphorin3c as a Tbx1-dependent gene expressed in subpulmonary myocardium. Our results implicate second heart field development in coronary artery patterning and provide new insights into the association between conotruncal defects and coronary artery anomalies. (Circ Res. 2008;103:142-148.)

Key Words: Tbx1  ■  outflow tract  ■  coronary artery patterning  ■  heart development

The significant fraction of congenital heart defects affecting the arterial pole of the heart reflects the complex events underlying formation and septation of this region of the heart. During normal development the myocardial outflow tract (OFT) forms from progenitor cells of the second heart field (SHF) situated in adjacent pharyngeal mesoderm. Addition of these cells to the heart is coordinated with that of cardiac neural crest cells and OFT endothelial cells. The cylindrical OFT is subsequently divided to generate the ascending aorta and pulmonary trunk concomitant with ventricular septation. Cardiac neural crest cells are essential for OFT septation and SHF deployment: neural crest ablation in the chick results in OFT and right ventricular hypoplasia. Direct ablation of the SHF leads to defects in heart tube extension resulting in pulmonary atresia and a failure of ventriculoarterial alignment.

Coronary arteries arise from a plexus of epicardially derived vessels that selectively invade the base of the aorta. Proximal right and left coronary arteries connect to ostia positioned at the right and left aortic sinuses facing the pulmonary trunk by a process of coalescence of endothelial strands. Anomalies in coronary artery patterning are an important component of congenital heart defects and occur in isolation and in conjunction with defects in outflow tract development. Coronary artery defects include single ostium or abnormal ostium positioning, either in the aorta or pulmonary trunk, and are a significant cause of sudden cardiac death. Avian studies have revealed that neural crest and SHF cells, in addition to epicardially derived cells, are required for normal coronary artery development. Defects in coronary artery patterning have been associated with OFT anomalies in mouse mutants for ldynein, perlecan, and Connexin43.

The del22q11.2 or DiGeorge syndrome candidate gene Tbx1 is a critical regulator of OFT development. del22q11.2 syndrome patients display craniofacial and cardiovascular defects including interrupted aortic arch, tetralogy of Fallot and common arterial trunk. Mice heterozygous for a null Tbx1 allele display a high frequency of 4th aortic arch artery defects. Absence of Tbx1 leads to underproliferation of the SHF, hypoplasia of the distal OFT, and a common ventricular outlet. Restoration of Tbx1 expression in the SHF is sufficient to rescue OFT development in a Tbx1 mutant background. Analysis of Cre lineage tracing experiments and visualization of SHF hypoplasia using an Fgf10 transgene enhancer trap line expressed in pharyngeal mesoderm and the OFT suggested that specific regions of the SHF may be particularly sensitive to loss of Tbx1.

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we report the results of a series of genetic crosses with transgene markers of OFT myocardium which reveal that a myocardioid subdomain normally associated with the base of the pulmonary trunk is reduced and malpositioned in Tbx1 mutant hearts. This defect is associated with highly anomalous coronary artery trajectories revealing critical roles for Tbx1 in the regulation of regional OFT identity and implicating SHF deployment in coronary artery patterning.

Materials and Methods

Mice
Tbx1+/− mice carrying the null allele Tbx1m<sup>op</sup> were genotyped by PCR analysis of tail and yolk sac DNA. The yMyf5-nlacZ-96-16 and Myf5-nlacZ-A17-T55 transgenes have been previously described.27,28 Connexin40<sup>Gal</sup> and Sema3c<sup>−/−</sup> mice were genotyped as described.29,30 Mice were maintained on a mixed genetic background. Mouse care and procedures were in accordance with institutional and national guidelines.

Reporter Gene Analysis and Histology
Embryos were dated taking E0.5 as the day of the vaginal plug, fixed in 4% paraformaldehyde (PFA), rinsed in 1xPBS, and incubated in X-gal solution at 37°C for 2 hours to overnight as described.22 GFP activity was scored under a Zeiss Labor stereomicroscope. 10 µm paraffin or cryostat sections were counterstained with hematoxylin and eosin.

Scanning Electron Microscopy
E18.5 hearts were fixed in 4% PFA. Atria and great arteries were removed to visualize aortic and pulmonary valves. A standard protocol was followed consisting of further fixation in 2.5% glutaraldehyde, dehydration in a graded alcohol series, and drying with hexamethyldisilazane. Samples were coated with 30 nm of gold and examined in a Leica S440 scanning electron microscope at 20kV.

Immunohistochemistry
Details of immunohistochemistry techniques are provided in the online Data Supplement (available online at http://circres.ahajournals.org). Primary antibodies used were rat anti–platelet endothelial cell adhesion molecule (PECAM) clone CD31 (PharMingen) diluted 1:50 to 1:100, mouse monoclonal antiactinin Clone 1A4 (Sigma) diluted 1:500, mouse monoclonal anti–smooth muscle actin (SMA) Clone EA-53 (Sigma) diluted 1:500, mouse monoclonal antiaclonin Clone EA-53 (Sigma) diluted 1:500, mouse monoclonal antiaclonin Clone EA-53 (Sigma) diluted 1:500. Whole-mount immunohistochemistry on E18.5 hearts was carried out using anti–SMA-FITC antibody (Sigma) diluted 1:500.

DAF-2DA Labeling
DAF-2DA has been found to be an early marker of smooth muscle at the arterial pole of the zebrafish and chick heart.31 Embryonic hearts were transferred into RPMI culture medium supplemented with 10 µmol/L of DAF-2DA (Sigma) and maintained in a 5% CO<sub>2</sub> incubator at 37°C for 4 hour. Hearts were washed in PBS and fluorescence scored under a Zeiss dissecting microscope (Lumar) using a GFP filter.

In Situ Hybridization
Whole-mount in situ hybridization was carried out as described.22 The antisense Sema3c riboprobe was synthesized from a plasmid kindly provided by Dr Fanny Mann (IBDML, Marseilles, France).30

Fluorescence In Situ Hybridization
Fluorescence in situ hybridization was carried out according to standard techniques as detailed in the online Data Supplement.

Results
Tbx1 is required within the SHF for myocardial progenitor cells to proliferate normally; as a result the distal OFT of Tbx1<sup>−/−</sup> embryos is shorter and narrower than in control embryos at E10.5 (Figure 1A through 1D).20–24 To investigate this further we analyzed Tbx1<sup>−/−</sup> embryos carrying the 96-16 transgene, normally expressed in the inferior (or caudal) wall of the OFT at embryonic day (E) 10.5 and subsequently in myocardium at the base of the pulmonary trunk.27 The strong asymmetrical expression domain of 96-16 in the inferior OFT wall is lost in mutant embryos (Figure 1A through 1D); however, β-galactosidase positive cells are distributed around the circumference of the proximal region of the mutant OFT (Figure 1B and 1D). In control hearts, the 96-16 transgene is expressed in a small number of cells in the superior (or cranial) wall of the proximal OFT at E10.5 and in myocardium around the base of the aorta at E14.5 (Figure 1C and 1E).27 At E14.5 low-level and patchy transgene expression was observed around the entire undivided ventricular outlet of Tbx1<sup>−/−</sup> hearts (Figure 1F). However, a domain of higher expression in the dorsal/left side of the common outlet likely identifies a residual and malpositioned myocardial domain with subpulmonary identity (Figure 1F).

The T55 transgene is expressed in the superior OFT wall and base of the aorta in a complementary pattern to that of the 96-16 transgene (Figure 2).28 We observed that the T55 negative (96-16 positive) OFT domain was reduced in Tbx1<sup>−/−</sup> hearts at midgestation (Figure 2A and 2B). At later developmental stages transgene expression was observed in the ventral region of Tbx1<sup>−/−</sup> hearts, normally comprising T55 negative subpulmonary myocardium (Figure 2C through 2F). A domain of T55 negative myocardium was invariably

Figure 1. 96-16 transgene expression in Tbx1 mutant hearts. A and C, Ventral and right views of an E10.5 heart showing β-galactosidase expression in the inferior OFT wall (arrow). Note a small number of positive cells in the superior OFT wall (arrowhead). Inset in C, Section through the OFT, superior to the top. B and D, Ventral and right views of an E10.5 Tbx1<sup>−/−</sup> heart showing a reduced number of 96-16 positive cells evenly distributed around the OFT. Inset in D, Section through the OFT at the level of the arrowhead. E, Superior view of an E14.5 96-16 heart showing strong expression in myocardium at the base of the pulmonary trunk (PT, arrow) and a narrow ring of expression at the base of the aorta (Ao, arrowhead). F, Superior view of a Tbx1<sup>−/−</sup> heart showing a patchy distribution of 96-16 positive cells around the common ventricular outlet with a region of stronger expression in the dorsal/left domain (arrowhead).
observed on the left side of the common ventricular outlet (Figure 2D). This result supports our observations with the 96-16 transgene and suggests that a residual myocardial domain with subpulmonary identity is maintained and malpositioned in Tbx1 mutant hearts.

We subsequently examined OFT cushion and outlet valve structure in Tbx1 mutant hearts. At E12.5 2 major and 2 intercalated cushions could be scored in 8/12 mutant hearts (Figure 3A through 3D). The major cushions fail to spiral around one another consistent with lack of rotation of the Tbx1−/− OFT (supplemental Figure I).23 In addition, the mutant cushions were hypoplastic compared to controls, the leftward positioned (pulmonary) intercalated cushion being severely affected (4/12) or absent (4/12) in a fraction of mutant hearts (Figure 3C and 3D). Three outlet valve leaflets were observed in 34/39 Tbx1−/− hearts analyzed at E18.5 by histology and scanning electron microscopy (Figure 3E through 3H); 5 hearts were observed with only 2 valve leaflets. In contrast, 4 outlet valve leaflets have been reported in Tbx1 mutant hearts homozygous for a Tbx1loxZ allele,23 suggesting that cushion and valve leaflet development may be subject to variation arising from differences between Tbx1 mutant alleles or genetic backgrounds.

The distribution of coronary arteries in Tbx1−/− hearts was investigated using a Connexin40-eGFP allele expressed in coronary endothelial cells.20 Whole mount visualization of Cx40-eGFP expression revealed a spectrum of anomalous coronary artery trajectories in Tbx1−/− hearts at E18.5 (Table; Figure 4E through 4L). In control hearts left and right coronary arteries are observed along the lateral sides of the ventricles, the first of a series of branches being the conal artery from the right (and occasionally the left) and circumflex artery from the left (Figure 4A through 4D). In 28/34 (82%) Tbx1−/− hearts the proximal left coronary artery

Table. Proximal Coronary Trajectories and Ostia Position in Tbx1−/− Hearts

<table>
<thead>
<tr>
<th>No.</th>
<th>%</th>
<th>Examples</th>
</tr>
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<tr>
<td>Total</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Left coronary artery crosses ventrally</td>
<td>28/28</td>
<td>4E–4L; IIE–IIH</td>
</tr>
<tr>
<td>Left coronary artery crosses dorsally</td>
<td>2/6</td>
<td>III–III</td>
</tr>
<tr>
<td>Left and dorsal cusps noncoronary</td>
<td>16/47</td>
<td>5B–5D, 5F–5H</td>
</tr>
<tr>
<td>Left and right ostia over right cusp</td>
<td>14/41</td>
<td>4E–4G, 4K, 5B, 5D, 5F</td>
</tr>
<tr>
<td>Left ostium over left cusp</td>
<td>4/12</td>
<td>III–III</td>
</tr>
<tr>
<td>Left ostium over dorsal cusp</td>
<td>2/6</td>
<td>...</td>
</tr>
<tr>
<td>Right ostium over dorsal cusp</td>
<td>4/12</td>
<td>...</td>
</tr>
<tr>
<td>Common right and left ostium over right cusp</td>
<td>9/26</td>
<td>4H–4J, 4L, 5C, 5G, 5H</td>
</tr>
<tr>
<td>Common right and left ostium over dorsal cusp</td>
<td>1/3</td>
<td>...</td>
</tr>
<tr>
<td>Independent conal ostium</td>
<td>5/15</td>
<td>4G, 4K, 5D</td>
</tr>
<tr>
<td>Independent circumflex ostium</td>
<td>6/18</td>
<td>4H, 4L, 5C</td>
</tr>
<tr>
<td>Additional independent artery</td>
<td>7/21</td>
<td>...</td>
</tr>
</tbody>
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1 No. of E18.5 hearts analyzed by cryosection.  
2 Also observed in 1/6 Tbx1−/− aortas.  
3 Also observed in control aortas.  
4 See figure panels for examples.
Figure 4. Coronary artery patterning in Tbx1<sup>+</sup>- hearts at E18.5. A–D, Cx40-eGFP expression in a wild-type heart in ventral (A), right (B), left (C), and superior (D) whole-mount views. Cx40-eGFP is expressed in atrial myocardium and coronary artery endothelial cells, identifying right (R) and left (L) coronary arteries and their branches, including conal (C) and circumflex (Cx) arteries. E–I, K, and L, Connexin40-eGFP expression in Tbx1<sup>+</sup> hearts showing anomalous coronary artery patterning on the ventral surface of the heart. E, Left coronary artery connecting to the common outlet adjacent to the right coronary artery; F, as in E with a separate circumflex artery; G, as in E with a separate conal artery; H, convergence of right and left proximal coronary stems and independent circumflex artery. I and J, Tbx1<sup>+</sup> hearts with common proximal coronary stems revealed by Cx40-eGFP expression (I) and smooth muscle actin FITC-coupled immuno-fluorescence (J). K and L, Superior views showing the proximal coronary stems of the hearts in G and H.

courses abnormally across the ventral region of the heart, connecting with the common ventricular outlet close to the right coronary ostium (Figure 4E through 4G and 4K) or merging with the right coronary artery in a single ostium (Figure 4H through 4I and 4L). Additional coronary arteries were observed in Tbx1<sup>+</sup> hearts including circumflex (Figure 4F, 4H, and 4L) or conal (Figure 4G and 4K) arteries with independent circumflex artery. In 16/34 (47%) hearts with common proximal coronary stems revealed by Cx40-eGFP expression and their branches, including conal (C) and circumflex (Cx) arteries. E–I, K, and L, Connexin40-eGFP expression in Tbx1<sup>+</sup> hearts showing anomalous coronary artery patterning on the ventral surface of the heart. E, Left coronary artery connecting to the common outlet adjacent to the right coronary artery; F, as in E with a separate circumflex artery; G, as in E with a separate conal artery; H, convergence of right and left proximal coronary stems and independent circumflex artery. I and J, Tbx1<sup>+</sup> hearts with common proximal coronary stems revealed by Cx40-eGFP expression (I) and smooth muscle actin FITC-coupled immuno-fluorescence (J). K and L, Superior views showing the proximal coronary stems of the hearts in G and H.

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Figure 5. Coronary artery patterning in Tbx1<sup>+/−</sup> hearts at E18.5. Cryostat sections of Tbx1<sup>+/−</sup> (A and E) and Tbx1<sup>−/−</sup> (B–D, F–H) hearts showing coronary arteries revealed by Cx40-eGFP expression (A–F), PECAM staining (D, G, H), and smooth muscle actin FITC-coupled (G and H) or uncoupled (E and F) immunofluorescence. A, In Tbx1<sup>+/−</sup> hearts right (R) and left (L) ostia are positioned over the right and left aortic valve leaflets. B, Right and left ostia positioned over the right/ventral valve leaflet of a Tbx1<sup>−/−</sup> heart. C, Common left and right ostium (arrow) and independent circumflex artery. D, Independent conal artery over the right/ventral valve leaflet. E–H, Smooth muscle is associated normally with coronary arteries in Tbx1<sup>−/−</sup> hearts. G and H, Unique ostium (arrow) over the rightward valve leaflet giving rise to all coronary arteries. Ao indicates aorta; PT, pulmonary trunk. Scale bars A–F, G, and H, 200 μm.

The etiology of this novel aspect of the Tbx1 mutant phenotype was analyzed by investigation of the distribution of the coronary vascular plexus at E12.5, before arteri-alization. Coronary ostia formation results from selective maintenance of endothelial connections between the coronary plexus and ascending aorta.7–11 PECAM staining revealed an abnormal distribution of the coronary plexus in Tbx1<sup>+/−</sup> hearts (Figure 5E through 5H).

The 96-16 transgene is expressed in the heart as the result of an integration site position effect. Using fluorescent in situ hybridization we mapped the integration site to chromosome

side of the common ventricular outlet. In 23/34 investigated hearts both right and left proximal coronary arteries were connected above the right and slightly ventrally positioned valve leaflet (Figure 5B through 5D). The left coronary artery crossed the ventral region of the heart to form either a distinct ostium (14/34 hearts; Figure 5B) or a direct connection with the right coronary stem resulting in a single ostium positioned above the right/ventral valve leaflet (9/34 hearts; Figure 5C, 5G, and 5H). Independent origins of the circumflex artery (Figure 5C) or conal artery (Figure 5D) were observed, occasionally positioned over the left or noncoronary valve leaflets. In 3/5 Tbx1<sup>−/−</sup> hearts with a bicuspid valve leaflet the left coronary artery was connected to ostia over the ventrally positioned cusp (data not shown). Endothelial (PECAM, Cx40-eGFP) and smooth muscle (SMA, DAF-2DA) markers were normally associated with coronary arteries in Tbx1<sup>−/−</sup> hearts (Figure 5E through 5H).

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The 96-16 transgene is expressed in the heart as the result of an integration site position effect. Using fluorescent in situ hybridization we mapped the integration site to chromosome
control hearts (Figure 7D and 7E), Sema3c transcript accumulation was severely reduced or absent in Tbx1−/− hearts (Figure 7F). Sema3c encodes a molecule involved in chemorepulsion and chemotraction that may potentially contribute to the maintenance of a coronary artery free zone in subpulmonary myocardium. Analysis of Sema3c−/− hearts revealed that proximal coronary arteries do not course across the ventral region of mutant hearts but connect laterally to the base of the aorta or common ventricular outlet (7/7 hearts; Figure 7G and 7H); 4 valve leaflets were observed in Sema3c−/− hearts with common arterial trunk (Figure 7I). We conclude that, despite restricted expression and Tbx1-dependence, Sema3c is not a critical downstream mediator of the Tbx1−/− coronary artery phenotype.

### Discussion

Loss of Tbx1 results in hypoplasia of the SHF leading to a short and narrow embryonic OFT and a common ventricular outlet. Our analysis of 2 transgenes expressed in complementary subdomains of the embryonic OFT reveals differential Tbx1 sensitivity in that the inferior OFT wall and subpulmonary myocardial domain are particularly Tbx1-dependent. The similar results observed with the 2 transgenic lines analyzed, coupled with the decrease in distal OFT length and diameter, suggest that this phenotype corresponds to a reduction in cell number, although transcriptional changes cannot be excluded. Persistence of a residual region with subpulmonary identity on the left side of the common outlet, together with our analysis of cushion morphology, supports the suggestion of Vitelli and colleagues that there is a failure of OFT rotation in Tbx1−/− hearts.

The inferior OFT wall may be derived from the caudal pharyngeal region, which is severely hypoplastic in Tbx1−/− embryos. Lineage tracing experiments using a Cre transgene under control of a Tbx1 regulatory element suggested that Tbx1 is not expressed in the entire SHF but in future subpulmonary myocardium. Recent analysis of a Tbx1 Cre allele support this conclusion and reveal a lineage derived from Tbx1-expressing cells in the inferior wall of the embryonic OFT. The lack of regional markers has precluded analysis of the impact of loss of Tbx1 on different domains of OFT myocardium. Our phenotypic analysis of Tbx1−/− hearts provides evidence for a mutant phenotype consistent with the lineage studies. Together, these results suggest an explanation for the pulmonary atresia associated with tetralogy of Fallot frequently observed in del22q11.2 syndrome patients. Interestingly, in avian embryos laser ablation of the SHF results in

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**Figure 6.** PECAM immunostaining in Tbx1 mutant hearts. Whole-mount views of E12.5 hearts after PECAM staining to reveal the coronary artery endothelial plexus in wild-type (A, C, E) and Tbx1−/− (B, D, F) hearts. A and B, Ventral views; C and D, right lateral views; E and F, left lateral views. The PECAM positive vascular plexus does not cover the ventral pulmonary trunk of wild-type hearts (arrowheads) but invades the ventral region of the Tbx1−/− ventricular outlet (arrows).

**Figure 7.** Evaluation of Semaphorin3c as a 96-16 candidate gene. A, Fluorescent in situ hybridization with a lacZ probe to metaphase chromosomes identifies the 96-16 transgene integration site on chromosome 5 A3–B1. B and C, In situ hybridization showing Sema3c transcripts in the E10.5 heart (B) and in myocardium at the base of the pulmonary trunk (PT) but not aorta (Ao) at E12.5 (C), in a similar distribution to the 96-16 transgene. D–F, Ventral views showing Sema3c transcripts in subpulmonary myocardium of E12.5 Tbx1+/+ and Tbx1−/− control hearts but not Tbx1−/− hearts (arrows in D–F). G and H, Superior views of Sema3c+/− (G) and Sema3c−/− (H) hearts labeled with anti-SMA-FITC to reveal smooth muscle cells at the base of the great arteries and in proximal coronary arteries. Note that coronary arteries (L, left; R, right) do not cross the ventral region of the Sema3c−/− heart. I, Cryosection through the common ventricular outlet of a Sema3c+/− heart labeled with anti–α-actinin antibody (red) to detect myocardium and PECAM (green) to detect endothelial cells, showing 4 valve leaflets (asterisks) and lateralized coronary arteries. Scale bar: 200 μm.
pulmonary hypoplasia or atresia. In the most severely affected cases 3 outflow valve leaflets were detected, similar to the situation in Tbx1<sup>−/−</sup> hearts. Thus experimental and genetic reduction of the SHF converge on a pulmonary hypoplasia phenotype.

A distinguishing feature of the aorta versus pulmonary trunk is the presence of coronary ostia. Coronary arteries form from a plexus of endothelial cells of epicardial origin that selectively invade the base of the aorta. Although the molecular and cellular mechanisms underlying this selective invasion remain unknown, multiple cell types are involved in this process, including epicardially-derived, cardiac neural crest, and SHF-derived cells, ablation of which in avians leads to coronary artery patterning defects. Abnormal coronary artery patterning has been reported in mice mutant for lrdynein, Connexin43, and Perlecan; in each case the defects are associated with specific OFT anomalies including defects associated with laterality, infundibular pouches, or transposition of the great arteries. Tbx1<sup>−/−</sup> mice provide a model for investigation of coronary artery patterning in common arterial trunk. In the majority of Tbx1<sup>−/−</sup> hearts both right and left coronary arteries connect to ostia over the rightward/ventrally positioned valve leaflet and proximal coronary arteries course across the ventral, normally coronary free, region of Tbx1<sup>−/−</sup> hearts. At E12.5, before arterialization, the plexus of epicardially derived vessels is abnormally distributed in the ventral region of Tbx1 mutant hearts. Recently it has been shown that 5% of normal C57BL/6 mice have a single coronary ostium; however, the elevated incidence of failure of a left ostium in Tbx1<sup>−/−</sup> hearts and the normal coronary trajectories observed in control hearts suggest that the coronary patterns observed in the present study result directly or indirectly from loss of Tbx1. We favor the hypothesis that persistence of a residual and malpositioned coronary-refractory myocardial domain results in failure of development of a coronary ostium on the left side of the common outlet. Tbx1 may therefore play an indirect role in coronary patterning by controlling the contribution and positioning of a coronary-refractory subset of cardiac progenitor cells during OFT formation. Abnormal development of this myocardial region is likely to impact on formation of the underlying intercalated cushion resulting in a tricuspid common outlet valve. Alternatively, loss of Tbx1 may impact directly on coronary artery development through abnormal SHF-neural crest interactions or defective arterial pole epicardial or smooth muscle development. Cells of the SHF have recently been shown to give rise to smooth muscle at the base of the great arteries, suggesting that there may be a direct SHF contribution to coronary artery development. Future experiments will distinguish between these potential mechanisms.

As a first step toward the identification of genes differentially expressed between subaortic and subpulmonary myocardium, localization of the integration site of the 96–16 transgene led to the observation that Sema3c is expressed in the inferior OFT wall and subpulmonary myocardium. Sema3c is a member of the Semaphorin family of signaling molecules which effect axonal growth cone guidance and vascular patterning through plexin and neuropilin corecep-

tors; Sema3c is required for neural crest influx and endothelial cell function during OFT septation. Given the Tbx1-dependent expression pattern of Sema3c, and the chemorepulsive/attractive role of this molecule, Sema3c is a candidate mediator of the coronary-free nature of subpulmonary myocardium. However, investigation of coronary artery configuration in Sema3c<sup>−/−</sup> hearts shows that coronary arteries are lateralized and do not cross the ventral region of the heart. Thus, despite credentials as a candidate gene, Sema3c is not the critical downstream mediator of the Tbx1<sup>−/−</sup> coronary artery phenotype. Semaphorin signaling may nevertheless play a role in coronary artery patterning, possibly by repelling coronary endothelial cells through redundant ligand sources.

Several studies have documented coronary artery trajectories in human hearts with a common ventricular outlet, revealing a wide range of proximal coronary artery defects. In general it has been found that the right coronary artery connects with the rightward positioned valve leaflet and the left coronary artery connects dorsally. In contrast, the left coronary artery of Tbx1<sup>−/−</sup> mice commonly traverses the ventral part of the heart to connect over the right valve leaflet. Ongoing analysis will determine whether there is a specific coronary artery configuration associated with common arterial trunk in del22q11.2 patients.

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**Disclosures**

None.

**References**


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Expanded Materials and Methods

Immunohistochemistry
Embryos were fixed in 4% PAF for 20 min to overnight, depending on developmental stage, and prepared for cryosectioning by incubation in 30% sucrose PBS solution and inclusion in OCT and freezing. 10µm sections were collected on SuperFrost Plus slides (Menzel-Glaser) and either counterstained with 1% Eosine or processed for immunochemistry as follows: slides were incubated in PBS for 5 min and permeabilized for 10 min in PBS supplemented with 0.2% Triton-X100. After 3 PBS washes, sections were incubated for 1 hr in a PBS blocking solution containing 2% bovine serum albumin and 0.05% saponin. Subsequently, sections were incubated overnight at 4°C with primary antibodies in blocking solution. Sections were washed in PBS and incubated for 45 min at room temperature (RT) with secondary antibodies. Rat antibodies were detected either with a secondary antibody conjugated with FITC (Interchim Jackson) or anti-rat Alexa Fluor 546 (Invitrogen Molecular Probe) at 1:200 dilution. Mouse monoclonal antibodies were detected with a secondary antibody conjugated with CY3 (Interchim Jackson) at 1:500 dilution. After 3 washes in PBS, slides were mounted in Mowiol. Immunoreactions were examined with an Axiophot II Zeiss microscope.

Whole-mount immunochemistry
Embryonic hearts were dissected in PBS and atria and distal regions of the great arteries removed prior to 45 min to 2 hr fixation in 4% PAF at 4°C depending on developmental stage. E12.5 hearts were used for anti-PECAM immunochemistry
using AP-conjugated antibody detection. E18.5 hearts were processed differently for a direct detection assay using anti-SMA-FITC antibody (Sigma). After fixation, the hearts were washed 3 times 1 hr in PBS before an overnight incubation in saturation buffer (PBSMT: PBS supplemented with 3% instant skim milk and 0.1% triton-X100). Subsequently, samples were incubated for 2-3 days with the antibody diluted 1:500 in PBSMT and processed for extensive washes in PBS supplemented with 0.1% Triton-X100 (5 times 1 hr with an additional overnight wash). All procedures were performed under continuous gentle shaking at 4°C. Fluorescence activity was scored using a Zeiss dissecting microscope (Lumar) with a GFP filter.

Fluorescence in situ hybridization

Metaphase spreads were prepared from male mice carrying the 96-16 transgene. Concanavalin A-stimulated splenic cells were cultured at 37°C for 72 h with 5-bromodeoxyuridine added for the final 6 hr of culture (60 mg/ml of medium) to ensure chromosomal R-banding. The lacZ probe was biotinylated by nick translation with biotin-16-dUTP and hybridization to chromosome spreads performed using standard protocols. Biotin-labeled DNA was mixed with hybridization solution at a final concentration of 50 mg/ml and used at 200 ng per slide. Before hybridization, the labeled probe was annealed with a 200-fold excess amount of murine Cot-1 DNA (GIBCO-BRL) (for 45 min at 37°C) in order to compete aspecific repetitive sequences. The hybridized probe was detected by fluorescent isothiocyanate-conjugated avidin (Vector laboratories). Chromosomes were counterstained with propidium iodide diluted in antifade solution pH 11. A total of 50 metaphase cells were analyzed and 100% showed a large and unique fluorescent signal in the proximal region of chromosome 5, localizing the transgene to 5 A3-B1.
Supplemental figure legends

Supplemental Figure I. Outflow tract cushion development in Tbx1 mutant embryos. Transverse (A, C, D, F) and sagittal (B, E) sections through the OFT region of Tbx1+/− (A-C) and Tbx1−/− (D-F) hearts at E10.5 (A, D), E11.5 (B, E) and E12.5 (C, F). Sections from 96-16 transgenic embryos are stained with X-gal (A, D). OFT cushions (asterisks) are hypoplastic and fail to spiral around one another in Tbx1−/− compared to Tbx1+/− embryos. Note the reduction in 96-16 positive cells in the Tbx1−/− heart in D (arrow).

Supplemental Figure II. Additional examples of coronary artery anomalies in Tbx1−/− hearts at E18.5. SMA-FITC immunostaining of a wild-type heart in ventral (A), right (B), left (C) and superior (D) whole-mount views. SMA-FITC labels coronary artery smooth muscle cells, identifying right (R) and left (L) coronary arteries and their branches, including conal (C) and circumflex (Cx) arteries. (E-H) SMA-FITC immunostaining of a Tbx1−/− heart with a rightward positioned proximal left coronary artery and normal right coronary artery. (I-P) Two cases of rare variant Tbx1−/− phenotypes where the left coronary artery does not course over the ventral surface of the mutant heart: (I-L) SMA-FITC immunostaining of a Tbx1−/− heart in which the proximal left coronary artery passes dorsal to the common ventricular outlet to converge with the right coronary stem; an independent conal artery is also observed. (M-P) DAF-2DA staining of a Tbx1−/− heart in which the left coronary artery connects over the leftward positioned valve leaflet.
Supplemental Figure III. Coronary artery patterning is normal in $Tbx1^{+/+}$ hearts. (A-D) $Cx40$-$eGFP$ expression in an E18.5 $Tbx1^{+/+}$ heart in ventral (A), right (B), left (C) and superior (D) whole-mount views. $Cx40$-$eGFP$ expression reveals normally positioned right (R) and left (L) coronary arteries and their branches (compare with Fig 4A-D and supplemental Figure IIA-D). (E-F) $Cx40$-$eGFP$ expression in an adult wildtype heart in ventral (E) and superior (F) views. (G, H) A similar coronary artery distribution is observed in an adult $Tbx1^{+/+}$ heart.

Supplemental Figure IV. Coronary artery patterning is normal in $Connexin40$ mutant hearts. (A-C) $Cx40$-$eGFP$ expression in an E18.5 $Tbx1^{+/+} Cx40^{eGFP/+}$ heart in ventral (A), right (B), and left (C) whole-mount views showing right (R), left (L), conal (C) and circumflex (Cx) arteries. (D-F) $Cx40$-$eGFP$ expression in a representative E18.5 $Tbx1^{+/+} Cx40^{eGFP/eGFP}$ heart showing a normal coronary artery distribution.