**Rationale:** Growth and remodeling of the pharyngeal arch arteries are vital for the development of a mature great vessel system. Dysmorphogenesis of the fourth arch arteries can result in interruption of the aortic arch type B, typically found in DiGeorge syndrome. Tbx1 haploinsufficient embryos, which model DiGeorge syndrome, display fourth arch artery defects during formation of the vessels. Recovery from such defects is a documented yet unexplained phenotype in Tbx1 haploinsufficiency.

**Objective:** To understand the nature of fourth arch artery growth recovery in Tbx1 haploinsufficiency and its underlying genetic control.

**Methods and Results:** We categorized vessel phenotypes of Tbx1 heterozygotes as hypoplastic or aplastic at the conclusion of pharyngeal artery formation and compared these against the frequency of vessel defects scored at the end of great vessel development. The frequency of hypoplastic vessels decreased during embryogenesis, whereas no reduction of vessel aplasia was seen, implying recovery is attributable to remodeling of hypoplastic vessels. We showed that Smad7, an inhibitory Smad within the transforming growth factor-β pathway, is regulated by Tbx1, is required for arch artery remodeling, and genetically interacts with Tbx1 in this process. Tbx1 and Tbx1;Smad7 haploinsufficiency affected several remodeling processes; however, concurrent haploinsufficiency particularly impacted on the earliest stage of vascular smooth muscle cell vessel coverage and subsequent fibronectin deposition. Conditional reconstitution of Smad7 with a Tbx1Cre driver indicated that the interaction between the 2 genes is cell autonomous.

**Conclusions:** Tbx1 acts upstream of Smad7 controlling vascular smooth muscle and extracellular matrix investment of the fourth arch artery. (Circ Res. 2013;112:90-102.)

**Key Words:** DiGeorge syndrome  ■ great vessel morphogenesis  ■ pharyngeal arch artery  ■ Smad7  ■ Tbx1

DiGeorge syndrome or chromosome 22q11 deletion syndrome (22q11DS) is among the most common genetic causes of congenital heart disease. The clinical manifestations of 22q11DS arise from maldevelopment of the pharyngeal apparatus, a vertebrate-specific, transient structure that forms in the lateral side of the embryo. Five sets of arteries form within the pharyngeal arches, serving as the embryonic connection between the cardiac outflow tract and the paired dorsal aorta. Over the course of mammalian development, this symmetrical configuration remodels into a mature, unilateral, left-sided system. The first and second arch arteries become vasculature of the head, the third pair forms the common carotid arteries whereas the fourth arch arteries contribute to the distal part of the aortic arch (left fourth) and both the brachiocephalic artery and a proximal part of the right subclavian artery (right fourth). The right sixth arch artery and the carotid ducts (dorsal aortae segments connecting the third and fourth arch arteries) regress completely and the left sixth arch artery forms the ductus arteriosus and the proximal parts of the pulmonary arteries. The seventh intersegmental arteries (caudal branches of the dorsal aorta) remodel into parts of the subclavian arteries. This extensive reconfiguration process initiates at around E11.5 in the mouse and by E13.5 it is completed for all branchial arch arteries and major vessels. Fourth arch artery derivative dysmorphogenesis is of major clinical significance to 22q11DS because the resulting interruption of the aortic arch type B is lethal without surgical intervention.

Heterozygosity of the T-box transcription factor TBX1 is considered the major determinant of 22q11DS. Tbx1+/− mutations in mouse phenocopy aspects of the syndrome, including fourth-specific arch artery defects. The pharyngeal artery defects present with high penetrance at E10.5, but a notable recovery takes place through the remodeling of the
vasculature reducing defect frequency by the conclusion of great vessel development, as originally reported in the Tbx1 haploinsufficient deletion Df/1 and ≥4 subsequent independent studies of Tbx1 heterozygotes.14–18 Despite this, the nature of the rescue is poorly understood, and little is known concerning the molecular players involved.

The pharyngeal arteries form after local differentiation of mesodermal into endothelial cells, which coalesce to establish a primitive vessel.19 Neural crest cells (NCCs) migrate to these endothelial tubes and differentiate in situ into vascular smooth muscle cells (VSMCs) in response to endothelial cues,20 and eventually form a sheet surrounding the primitive vessels providing support and stabilization to the arch arteries.4,21 Although VSMCs have various origins, NCCs are the exclusive source of smooth muscle in the pharyngeal arteries.7,12 Differentiated VSMCs proliferate, and subsequently secrete and organize extracellular matrix (ECM) molecules within the vessel wall as well as provide the vascular tone. The transforming growth factor-β (TGFβ) superfamily members play pivotal roles in SMC differentiation,22 proliferation,23 and ECM molecule synthesis and organization.24–26 Remodeling of the pharyngeal arteries, which follows the formation of the primitive vessels, requires programmed asymmetrical expansion, regression, persistence, and change of relative position of the different vascular segments. Despite the detailed description of the different stages of remodeling of the great vessels4 and identification of remodeling defects in mouse mutants, the impact of these complex processes on the Tbx1 haploinsufficiency recovery remains elusive. It is clear that genetic background has an influence,29 and reduction of retinoic acid synthesis accelerates the recovery but has no effect on the final number of recovered vessels.30

Here, a Tbx1 haploinsufficiency model was used to study the remodeling of the fourth arch artery in both normal and compromised vessels and to describe the arterial growth recovery phenomenon seen in Tbx1 heterozygotes during these stages. From previously identified potential Tbx1-interacting genes31 we selected Smad7, an inhibitor of the TGFβ/bone morphogenetic protein (BMP) pathway, to examine a possible genetic interaction with Tbx1 in great vessel development. We show that Tbx1 acts upstream of Smad7 in the early gestation embryo and that Smad7 is primarily required for pharyngeal artery remodeling, rather than formation. Double heterozygosity for Tbx1 and Smad7 impeded great vessel remodeling by affecting the fourth arch artery defect recovery. This was attributable to initially compromised NCC-derived VSMC coverage of the fourth arch arteries, which affected the subsequent production of ECM components. Using conditional rescue of Smad7 expression, we additionally showed that the requirement of Smad7 during recovery is autonomous to the Tbx1 lineage.

### Methods

#### Mice

Animal maintenance, husbandry, and procedures were done in accordance with British Home Office regulations. Mouse strains are detailed in the Online Data Supplement.

#### Intracardiac Ink Injection, In Situ RNA Hybridization, and Quantitative Real-Time Polymerase Chain Reaction

Standard methods were followed, which are detailed in the Online Data Supplement.

#### Immunohistochemistry and Histology

Immunohistochemistry was performed on optimal cutting temperature compound-embedded sections using standard protocols or on paraffin-embedded sections as previously described.12 The sections used for pSmad1/5/8 and pSmad2/3 staining were processed according to the TSA tetramethylrhodamine system protocol (NEL702001KT, Perkin Elmer LAS). Primary antibodies were incubated overnight. Alexa Fluor-conjugated secondary antibodies (Molecular Probes) were used at 1:500. Sections were counterstained using Dapi (1:25,000, Molecular Probes). Images were acquired using either a Zeiss AxiosImager with ApoTome or a Zeiss LSM 710 confocal microscope equipped with argon and helium neon lasers. Primary antibodies and methods are detailed in the Online Data Supplement.

#### Chromatin Immunoprecipitation

Twelve E8.5 embryos, 4 E9.5 embryos, or 2 E10.5 embryos were pooled for each chromatin immunoprecipitation (ChIp) experiment, which was performed using 10 mg of TBX1 antibody (ab18530, Abcam) and 10 mg of rabbit IgG antibody (ab46540, Abcam) as negative control, as previously described.32 Briefly, the embryos were enzymatically dissociated to a single-cell suspension, fixed in 1% paraformaldehyde, and neutralized in 0.125 mol/L glycine. The cells were then washed in PBS with protease inhibitors and lysed in lysis buffer containing SDS and protease inhibitors. The chromatin was sheared into 200- to 600-bp long fragments, diluted 1:10 in dilution buffer, precleared in protein A agarose beads (Millipore) and incubated with the respective antibody overnight. The immunocomplexes were captured by protein A agarose beads (Millipore) and washed extensively before the bound DNA fragments were eluted in elution buffer. Crosslinking of the input and immunoprecipitated DNA was reversed at 65°C for 4 hours, followed by treatment with 100 mg/mL proteinase K at 45°C for 1 hour. The DNA was subsequently purified using the QIAquick polymerase chain reaction (PCR) purification kit (Qiagen). Equal volumes of input, IgG, and TBX1 immunoprecipitated DNA were used for quantitative real-time PCR (q-PCR) analysis.

#### Statistical Analysis

One-tailed Fisher exact test was used to compare defect frequencies between the different genotypes. The t test was used to compare expression levels in q-PCR analyses. Mann-Whitney nonparametric test was used to compare smooth muscle and proliferating cells among the different genotypes. Groups were considered significantly different when P<0.05, unless indicated otherwise. The single (*) and double (**) asterisks represent P<0.05, and P<0.01, respectively. All experiments have been performed on ≥3 independent sets of embryos for each condition.

#### Results

**Smad7 Is Expressed in Overlapping Domains With Tbx1**

Previous work by us used microarray analysis of fluorescence-activated cell sorter-sorted cells to enrich for cell autonomous...
alterations of gene expression after Tbx1 loss of function at E9.5. Smad7 was among the candidate Tbx1 targets. Whole-mount in situ hybridization was used to determine Smad7 expression during embryonic development. At E8.5, Smad7 was detected in the surface ectoderm and was ubiquitously expressed in the pharyngeal region by E9.5 (Figure 1). Between E10.5 and E12.5, Smad7 became restricted to the ectoderm again (Figure 1). The expression pattern of Smad7 matched the dynamic distribution of Tbx1 RNA during embryogenesis. As shown previously, at E8.5, Tbx1 was highly expressed in the pharyngeal epithelia and mesoderm, a day later, at E9.5, it became confined in the epithelia of the caudal pharyngeal system and the mesodermal cores, and by E10.5 it was no longer expressed in pharyngeal tissues (Figure 1). Smad7 expression was diminished in Tbx1 knockout embryos at E9.5, whereas it colocalized with Tbx1-lineage traced cells (Tbx1Cre;R26RYFP) in caudal pharyngeal tissues at the same stage (Figure 1).

**Tbx1 Occupies Binding Elements on the Smad7 Gene at E9.5**

To date, Tbx1 is known to bind to VEGFR3, Wnt5A, Fgf8, and Fgf10. Seven evolutionary conserved T-box binding elements were identified within the gene and promoter sequence (see Experimental Procedures in Online Data Supplement). Of the 7, 6 were examined for Tbx1 binding using ChIP (Figure 2). At E9.5, of the 6 sites showed statistically significant Tbx1 binding by q-PCR of the immuno-precipitated material (ChIP analyzed by q-PCR; Figure 2). To determine whether Tbx1 acts at a specific or multiple developmental stages on Smad7, we also examined binding at E8.5 and E10.5. By ChIP analyzed by q-PCR, we found that at E8.5 there was statistically significant binding of Tbx1 on all 4 of the T-box binding elements on Smad7 that showed positive binding at E9.5 (Figure 2). At E10.5, only 2 of the 4 sites displayed statistically significant binding (Figure 2).

**Smad7 Is Required for Great Vessel Development**

An embryonic stem cell line carrying a gene-trapped Smad7 allele was obtained from BayGenomics (MGI; Smad7Gt(YHC053)Byg) and injected in mouse blastocysts. Germline transmission was achieved and efficient knockdown of the gene confirmed by q-PCR (data not shown). Genotyping specific primers were designed for the trapped allele. This gene-trap model (Smad7^-^GT here onward) was used to address potential roles of Smad7 in cardiovascular morphogenesis. Chen and colleagues have here onward) was used to address potential roles of Smad7 produced embryos with outflow tract, ventricular septum, ventricular compaction, and cardiac function defects. Of the 24 (12.5%) homozygous gene-trap embryos also displayed a cleft palate.

**Smad7 Haploinsufficiency Abrogates Recovery of Great Vessel Morphogenesis in Tbx1 Heterozygotes**

A potential genetic interaction between Tbx1 and Smad7 was addressed by examining great vessel development in doubly heterozygous embryos. At E11.5, Tbx1;Smad7 heterozygotes displayed the same frequency of arch artery defects as Tbx1 heterozygotes (63% against 65%, \( P=0.58 \), Table 1; Online Figure I). Consistent with the literature, at E15.5, a stage by which the great vessels have completely developed, single heterozygotes for Tbx1 partially recovered from the defects such that 29% had arch artery abnormalities. However, at the same stage, Tbx1;Smad7 heterozygotes failed to recover with great vessel defects in 68% (\( P=0.02 \), Table 1; Online Figure I). There was no statistical difference between the double heterozygous populations at the different time points (\( P=0.47 \)). We observed that the penetrance of the aplatotic fourth arch artery defects in Tbx1^-^lacZ embryos at E11.5 (24%) was comparable with the penetrance of the great vessel anomalies scored at E15.5 (29%, \( P=0.5 \)). We rationalized that the aplatotic vessels observed in E11.5 Tbx1^-^lacZ embryos are responsible for the late great vessel phenotype and that hypoplastic vessels are capable of recovery. However, in the Tbx1^-^lacZ;Smad7^-^GT embryos, the 38% of aplatotic vessels observed at E11.5 could not provide a basis for the 68% of fourth-specific arch artery defects scored at E15.5 (\( P=0.04 \)). This suggests that the Tbx1^-^Smad7 haploinsufficiency interaction interfered with the recovery of hypoplastic vessels.

**The Tbx1 Haploinsufficiency Phenotype**

We hypothesized that recovery of the Tbx1 haploinsufficient arch artery phenotype could be attributable to successful incorporation of hypoplastic (thin patent) but not aplatotic (nonpatent) vessels to the mature configuration. We therefore examined Tbx1 heterozygotes throughout arch artery remodeling (E11.5-E13.5) and distinguished between hypoplastic and aplatotic vessel phenotypes (see Experimental Procedures in Online Data Supplement), to determine their respective frequencies. Consistent with the literature, at E11.5, the end of arch artery formation, we observed defects in 21 of 29 Tbx1^-^lacZ embryos (71%). Of these 21, 12 (41%) were aplatotic vessels and 9 (31%) were hypoplastic. At E12.5, of a total of 30 embryos, 16 (53%) had vessel defects, which included 5 cases of patent right carotid duct. By that stage, the right carotid duct had completely regressed in wild-type littermates. Of the 16, aside from the 5 embryos with patent right carotid duct, the remainder (11, 37%) presented with aplatotic vessels. By the end of great vessel development, at E13.5, 14 of 30 embryos (46%) presented with fourth-related abnormal great vessel configurations. Consistent with our hypothesis, we noted a sharp decrease in hypoplastic vessel frequency (31%, 0%, and 0%, respectively) and comparable values in aplatotic vessel frequency (41%, 37%, and 46%, respectively) during the stages examined (Figure 3; Table 2).
NC Migration, VSMC Differentiation, and Subsequent Proliferation in Tbx1<sup>+/lacZ</sup> Versus Tbx1<sup>+/lacZ; Smad7<sup>+/GT</sup></sup> Embryos

We and others have shown that Tbx1 heterozygotes display aberrant cardiac NCC migration trajectories. Whole-mount in situ hybridizations for Sox10, an NCC marker that labels the cardiac crest among other populations revealed no gross difference in NCC migration between the Tbx1 and Tbx1;Smad7 heterozygotes, and the latter displayed aberrant migration trajectories comparable with the Tbx1 heterozygous phenotype (Online Figure II). To assess whether the NCCs that do reach the arch arteries eventually differentiated into VSMCs, we compared the SMC component of the fourth arch arteries of Tbx1 heterozygotes, Tbx1;Smad7 heterozygotes and wild-type controls. At E10.5, we observed that the number of SM22-positive cells surrounding the fourth arch arteries was significantly reduced in double heterozygotes compared with both the Tbx1 heterozygotes and the wild-type controls.
controls (Figure 4). This correlated with gaps in the SM22-positive cell coverage of the vessel walls. Tbx1 heterozygote vessels also displayed a reduction of SM22-positive cells compared with the wild-type controls, but not so severely as in double heterozygotes (Figure 4). At E11.5, although the VSMC component was reduced in depth/thickness in the hypoplastic vessels versus wild types, discontinuities of VSMC coverage were no longer observed in either Tbx1 or Tbx1;Smad7 heterozygotes (Figure 4). A difference between the 2 mutant groups at this stage was identified by serial sectioning along the anteroposterior axis which revealed that hypoplastic vessels within Tbx1;Smad7 heterozygous embryos had a smaller cross-sectional area than Tbx1 heterozygotes, whereas some also displayed irregular lumen shapes (Figure 4). At E12.5, very few hypoplastic vessels were detected in Tbx1 heterozygotes, and only 1 in Tbx1;Smad7 heterozygotes, which was a case of severe hypoplasia.

We next considered whether the ability of the pharyngeal arch arteries to recover in Tbx1 heterozygotes, versus Tbx1;Smad7 heterozygotes, was a function of altered cell proliferation, apoptosis, or a non-NCC contribution to the VSMC layer. The ratio of proliferating cells surrounding the fourth arch arteries at E10.5 was examined using bromodeoxyuridine incorporation as a marker of proliferation. Increased overall proliferation was observed in the pharyngeal system in both mutants. We analyzed 3 sets of 3 wild-type, single (Tbx1) and double heterozygote embryos and compared the percentage of proliferating SM22-positive VSMCs surrounding the fourth pharyngeal arch arteries of each genotype. There was no uniform increase in the proportion of proliferating VSMCs in the vessels of mutant genotypes, although a statistically significant increase was observed in 1 of 6 vessels examined in the Tbx1 single and Tbx1;Smad7 heterozygote groups, versus wild-type controls (Figure 4). During normal remodeling of the great vessels, the right sixth arch artery as well as the carotid ducts regress, a process initiated by apoptosis in the mesenchyme surrounding each vessel. An apoptosis assay using activated Caspase3 at E11.5 showed increased numbers of apoptotic cells surrounding both sixth arch arteries in the Tbx1 and Tbx1;Smad7 heterozygote vessels compared with controls, but there was no difference between the 2 mutant genotypes (data not shown). Using the Mesp1Cre mesodermal cell lineage-tracing model we examined whether mesodermal cells could aberrantly differentiate into smooth muscle in Tbx1 heterozygotes, to compensate for the loss of NCC-derived VSMCs. However, in both wild-type (Mesp1Cre:R26R-YFP) and Tbx1 heterozygotes...
Table 1. Arch Artery Defect Frequencies in Tbx1;Smad7 Heterozygous Embryos at E11.5 and E15.5 and Tbx1Cre;Smad7 Heterozygous Embryos at E15.5

<table>
<thead>
<tr>
<th>Embryos</th>
<th>Tbx1&lt;sup&gt;+/−&lt;/sup&gt;;Smad7&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Smad7&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Tbx1&lt;sup&gt;+/Δc&lt;/sup&gt;</th>
<th>Tbx1&lt;sup&gt;+/Δc&lt;/sup&gt;;Smad7&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>E11.5 Total embryos</td>
<td>19</td>
<td>13</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>Normal</td>
<td>19</td>
<td>12</td>
<td>6</td>
<td>9</td>
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<td>1 (8%)</td>
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<td>L4th PAA</td>
<td>–</td>
<td>–</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>R4th PAA</td>
<td>–</td>
<td>–</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>L4th and R4th PAA</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Vessel patency Th-P</td>
<td>–</td>
<td>–</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>NP</td>
<td>–</td>
<td>1 (8%)</td>
<td>4 (24%)</td>
<td>9 (38%)</td>
</tr>
<tr>
<td>E15.5 Total embryos</td>
<td>14</td>
<td>30</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>Normal</td>
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<td>Abnormal</td>
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<td>1 (3%)</td>
<td>5 (29%)</td>
<td>13 (68%)</td>
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<td>L4th PAA</td>
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<td>–</td>
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<td>2</td>
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<tr>
<td>R4th PAA</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>8</td>
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<tr>
<td>L4th and R4th PAA</td>
<td>–</td>
<td>–</td>
<td>3</td>
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</table>

Pharyngeal arch/great vessel defects and their developmental origins in Tbx1<sup>+/−</sup> and Tbx1<sup>+/Δc</sup>;Smad7<sup>−/−</sup> embryos at E11.5 and E15.5 and in Tbx1<sup>+/Δc</sup> and Tbx1<sup>+/Δc</sup>;Smad7<sup>−/−</sup> embryos at E15.5 compared with wild-type controls. The numbers indicate individual embryos whereas the respective frequencies are in brackets. NP indicates nonpatent; PAA, pharyngeal arch artery; Th-P, thin patent; and WT, wild type.

(Mesp1Cre;R26RYFP;Tbx1<sup>−/−</sup>), the mesodermal progenitors formed only the endoderm of the arch arteries (Online Figure II). Using the Wnt1Cre NCC lineage-tracing model, we established that all the SM22-positive cells were double positive for yellow fluorescent protein in Wnt1Cre;R26RYFP;Tbx1<sup>−/−</sup> embryos (Online Figure II).

ECM Deposition/Organization of the Fourth Arch Arteries Does Not Occur Normally in Tbx1<sup>−/−</sup>;Smad7<sup>+</sup>/<sup>GT</sup> Versus Tbx1<sup>−/−</sup> Embryos

TGFB-Smad signaling has a well-established role in ECM production, so we examined this process in our model through a range of cell matrix and adhesion markers. Fibronectin expression appeared diminished in hypoplastic vessels of Tbx1;Smad7 heterozygous embryos compared with both hypoplastic and normal vessels of Tbx1 heterozygous and wild-type control embryos (Figure 5). Collagen IV expression also displayed abnormal patterning; however, it was equivalently diminished in hypoplastic vessels of Tbx1 and Tbx1;Smad7 heterozygous embryos compared with wild-type controls. The expression of laminin and β1-integrin appeared unaffected in hypoplastic vessels of either genotype compared with wild-type controls, when allowance is made for the fact that these vessels are, by definition, smaller (Figure 5). Elastin deposition was also examined, but expression was not detectable around the arch arteries at E11.5 (data not shown); it was therefore concluded that deficiency of elastin cannot play a role in Tbx1;Smad7 heterozygote vessel collapse.

Smad7 Is Required in the Tbx1 Lineage During Arch Artery Recovery

We restored Smad7 expression in the gene-trap animals using a Tbx1Cre driver. This driver is a knock-in allele, and the resulting Tbx1 heterozygosity had a somewhat more penetrant phenotype than Tbx1<sup>−/−</sup> at the stages examined. At E15.5, we observed the same frequency of great vessel defects in the Tbx1<sup>+/−</sup> and the Tbx1<sup>+/−</sup>;Smad7<sup>−/−</sup> embryos (47% against 52%, P = 0.5; Table 1), suggesting that recovery of the Tbx1 heterozygous arch artery defect occurred normally in both populations.

Smad7 Regulates Great Vessel Remodeling Via the TGFB Pathway

Smad7 is a potent inhibitor of both branches of the TGFB/BMP pathway. Activation of these pathways was examined by staining for the appropriate phosphorylated protein complex. Smad7<sup>−/−</sup> GT embryos were compared with wild types at E9.5 and E10.5, when Smad7 is most highly expressed and when the fourth arch artery has formed, respectively. We observed a moderate increase of pSmad2/3 levels in Smad7<sup>−/−</sup> GT embryos compared with wild-type controls at E9.5, which was more prominent at E10.5, whereas no obvious differences were seen in pSmad1/5/8 levels at either stage (Figure 6). By examining pSmad2/3 and pSmad1/5/8 in E10.5 Tbx1<sup>−/−</sup> and Tbx1<sup>−/−</sup>;Smad7<sup>−/−</sup> GT embryos, with parallel assessment of their arch artery VSMC component, we observed a similar pattern. There was an increase of pSmad2/3 levels in Tbx1<sup>−/−</sup>;Smad7<sup>−/−</sup> GT embryos.
compared with Tbx1+/lacZ embryos (Figure 6). No differences were observed among the different genotypes in pSmad1/5/8 levels (data not shown).

Discussion

Fourth arch artery dysmorphogenesis has major clinical implications as it can result in aberrant origin of the right subclavian artery or in interruption of the aortic arch type B, which is lethal without surgical intervention. This phenotype has been associated with lack of TBX1 in DiGeorge syndrome patients\textsuperscript{9–11,42,43} and animal models.\textsuperscript{12,13} Despite this, to date only 2 genes have been described as directly regulated by Tbx1.\textsuperscript{34, 35} To investigate downstream effectors of Tbx1, we previously undertook a microarray approach designed to identify cell autonomously regulated genes by Tbx1.\textsuperscript{31} Of the list of potential Tbx1 interactors, we selected Smad7, a TGFβ/BMP pathway inhibitor gene. By in situ hybridization we demonstrated that Smad7 showed a more time and tissue restricted expression profile than Tbx1. Smad7 RNA was detectable from E8.5 and up to midgestation it was found predominantly in the ectoderm. A peak of expression was observed at E9.5 when Smad7 was almost ubiquitously expressed in the pharyngeal region. Tbx1 is expressed earlier, starting from E7.5 in the head and splanchnic mesoderm.\textsuperscript{44,45} Between E8.5 and E9.75 Tbx1 is still active in the mesoderm but expression is also detected in the pharyngeal epithelia.\textsuperscript{13,40,45} At no developmental stage is Tbx1 ever expressed in the neural crest–derived mesenchyme.\textsuperscript{13,40,44–46} After comparing the expression profiles of the 2 genes, we hypothesized that the Tbx1–Smad7 interaction takes place around E9.5. We used a Tbx1 lineage-tracing model to show colocalization of the 2 proteins at E9.5, by detecting Smad7
and the yellow fluorescent protein produced by the activity of the Tbx1Cre driver. Furthermore, Smad7 expression was diminished in Tbx1 knockout embryos at E9.5. To address a direct interaction between Tbx1 and Smad7, we examined direct binding of the Tbx1 protein to evolutionary conserved T-box binding elements found within the Smad7 gene and regulatory sequence by ChIP analyzed by q-PCR. Of the 6 potential binding sites, we identified positive interaction in 4, the first within the promoter, the second in the first intron, and the third and fourth within intron 3 of Smad7. This interaction was evident from E8.5, peaked at E9.5, and by E10.5 only 2 of the 4 sites showed positive interaction, consistent with expression (this article) and microarray data.31

A targeted Smad7 mutation is shown to cause arrhythmia, outflow tract, and ventricular septum defects in mice.38 To date, however, Smad7 has not been invoked as a regulator of pharyngeal artery development. Using a gene-trap allele for Smad7, we showed that homozygous knockdown of the gene primarily caused fourth-related arch artery defects, which increased in penetrance during remodeling of the great vessels. Haploinsufficiency of Smad7 did not affect arch artery development, but concurrent haploinsufficiency of Smad7 and Tbx1 led to impaired great vessel remodeling. At E11.5, Tbx1;Smad7 heterozygotes presented with a frequency of fourth arch artery abnormalities comparable with the Tbx1 heterozygote. By the end of cardiovascular development, at E15.5, the Tbx1 heterozygotes displayed reduced penetrance of fourth arch artery defects compared with the earlier stage, consistent with previous studies.14–17 The double heterozygotes did not show similarly reduced penetrance in great vessel defects, which were exclusively fourth arch-derived and presented with comparable frequency as scored at E11.5. This suggests that diminished levels of Smad7 compromise the recovery from arch artery defects observed in Tbx1 haploinsufficient embryos.

In an effort to study the mechanisms involved in this process, we examined the different elements of arch artery remodeling in our mutants. Vessel growth failure,18,40 poor NCC differentiation into VSMCs,18,47 as well as abnormal NCC migration14,39,40 have been proposed as factors possibly underlying the fourth arch-derived great vessel defects in models of Tbx1 haploinsufficiency (2 of these were engineered chromosomal deletions affecting additional genes). Aberrant neural crest migration trajectories have also been observed in a series of Tbx1 hypomorph mutations, correlating with progressive reduction of Tbx1 mRNA levels and with increasing penetrance of fourth arch artery defects.14 We first examined NCC contribution to the developing pharyngeal arteries of the Tbx1 and Tbx1;Smad7 heterozygotes, compared with wild-type controls, but detected no notable difference between the 2 mutant genotypes. We concluded that Smad7 does not participate in this process because doubly heterozygous embryos did not show an exacerbation of the Tbx1-related neural crest phenotype. To examine whether the NCC that do reach the arch arteries effectively differentiate from VSMCs, we quantified the proportion of SMCs around Tbx1;Smad7 heterozygotes and the wild-type controls, whereas Tbx1 heterozygotes displayed mildly diminished SMC vessel investment compared with controls. By E11.5, the vessel coverage with SM22-positive cells did not display discontinuities in either genotype, indicating that VSMC differentiation occurred in all mutants, although all hypoplastic vessels still had a thinner sheet of SM22-positive cells compared with controls. The lack of VSMC discontinuity in Tbx1;Smad7 heterozygous embryos at E11.5 is likely to be attributable to a moderate compensatory increase in proliferation, in the context of a smaller vessel surface area requiring coverage. Indeed, we detected an increase in the proportion of actively proliferating SMCs in 1 of the 3 Tbx1;Smad7 heterozygotes examined at E10.5. We additionally assessed mesenchymal apoptosis at E11.5 among the different genotypes, and although there was some increase in the apoptotic cells in both Tbx1 and Tbx1;Smad7 heterozygotes versus wild-type controls, we did not detect any differences between the mutant genotypes. The difference we identified between Tbx1 and Tbx1;Smad7 heterozygotes

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Table 2. Arch Artery Defect Frequencies in Tbx1 Heterozygotes Between E11.5 and E13.5

<table>
<thead>
<tr>
<th></th>
<th>Tbx1+/+ (WT)</th>
<th>Tbx1+/-hod1</th>
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<tbody>
<tr>
<td>E11.5 Total embryos</td>
<td>36</td>
<td>29</td>
</tr>
<tr>
<td>Normal</td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td>Abnormal</td>
<td>0 (10%)</td>
<td>21 (71%)</td>
</tr>
<tr>
<td>L4th PAA</td>
<td>5 (17%)</td>
<td>4 (46%)</td>
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Pharyngeal arch artery/great vessel defects and their developmental origins in Tbx1+/hod1 embryos at E11.5 (beginning of remodeling), E12.5 (during remodeling), and E13.5 (end of remodeling) compared with wild-type controls. The numbers indicate individual embryos, whereas the respective frequencies are in brackets. *Five embryos displayed patent right carotid duct. N.B. the cases of bilateral defects were scored as “Th-P” if Th-P/Th-P and “NP” if Th-P/Th-P or NP/Th-P. NP indicates nonpatent; PAA, pharyngeal arch artery; Th-P, thin patent; and WT, wild type.
reflected abnormalities in vessel structure at E11.5. The hypoplastic vessels within Tbx1;Smad7 heterozygous embryos had a smaller cross-sectional area than Tbx1 heterozygotes, whereas some also displayed irregular lumen shapes. Embryos were examined at E12.5, but by then the Tbx1;Smad7 heterozygotes presented almost exclusively with aplastic vascular fourth arch derivatives, whereas very few hypoplastic vessels were detected in Tbx1 heterozygotes.

These developmental time points are consistent with a recovery of hypoplastic vessels in Tbx1+/lacZ embryos by E12.5, with recovered vessels being scored as normal from this point onward. By contrast, in Tbx1;Smad7 heterozygotes collapse of hypoplastic vessels would result in their being scored as aplastic. We sought to study this recovery process that has been reported by ≥4 independent studies in Tbx1 haploinsufficiency.14–17 We distinguished between thin and nonpatent...
vessels in Tbx1 heterozygotes and observed that the frequency of aplastic vessels scored during formation of the pharyngeal arteries was essentially the same as the frequency of great vessel defects scored once the mature great vessel architecture had emerged. We hypothesized that the recovery of the fourth arch artery phenotype is attributable to the efficient incorporation of hypoplastic vessels to the adult configuration. In Tbx1 heterozygotes, we observed a consistent decrease in the occurrence of hypoplastic vessels, but a relatively fixed frequency of aplastic vessels throughout the remodeling stages. We noticed that the stage during which the hypoplastic arteries recover or collapse (E12.5) in Tbx1 haploinsufficient embryos coincides with a normally occurring reduction of cellularity around the great vessels. During development, the pharyngeal arteries form within a mesenchymal matrix, but this recedes over time and by E15.5, the great vessels stem from the heart and lead to vessels of the body without external tissue support. This event signifies the requirement for intrinsic stability of the vessel. A factor widely responsible for the structural support of the vascular bed is the ECM, so we examined ECM deposition around the fourth arch arteries in the different genotypes. We hypothesized that during recovery, hypoplastic vessels in Tbx1 heterozygotes are strengthened by SMC expansion and ECM support. Conversely, in Tbx1;Smad7 heterozygotes the initial lack of VSMCs at E10.5 shown as delayed SMC coverage of the vessel and the subsequent insufficient contribution to ECM support mean that hypoplastic vessels cannot react to the increasing pressure of blood flow. A range of ECM components were examined across normal and hypoplastic vessels in Tbx1 and Tbx1;Smad7 heterozygotes compared with wild-type controls. Collagen IV expression was reduced in hypoplastic vessels of both mutant genotypes, whereas no difference in laminin or β1 integrin expression was detected in either Tbx1 or Tbx1;Smad7 heterozygotes compared with wild-type controls. Interestingly, fibronectin coverage was markedly reduced in Tbx1;Smad7 haploinsufficient hypoplastic vessels compared with both Tbx1 heterozygotes and wild-type controls. Molin and colleagues have previously shown aberrant TGFβ/Smad signaling in the media of hypoplastic, atretic, or interrupted aortic arches (fourth arch artery derivative) in Tgfβ2-deficient mice. This was associated with severely reduced fibronectin expression. Given that Smad7 inhibits TGFβ signaling, reduced expression of fibronectin and other phenotypes similar to those observed in Tgfβ2-deficient mice appears counterintuitive. However, several different aspects of embryogenesis are similarly affected by up- and downregulation of the same pathway. For example, Keyte and Hutson, in a review of the role of neural crest in cardiac congenital anomalies, state “Thus, a common theme emerges: too much or not enough signaling in a particular pathway (eg, TGFβ/BMP, FGF, retinoic acid) results in similar cardiovascular defects by perturbing the development of the NCCs as they migrate from the neural tube, into the pharynx and eventually the heart.” Furthermore, TBX1 gain of function mutations can result in similar phenotypes as 22q11DS.11 As a specific example within the TGFβ pathway, knockdown of SMAD7 using small interfering RNA was recently shown to be sufficient to inhibit TGFβ2 driven expression of laminin and fibronectin in human trabecular meshwork cells. We identified that the Tbx1;Smad7 interaction interferes with the TGFβ pathway, as shown by excessive levels of the phosphorylated protein complex of this branch (pSmad2/3) and unaltered levels of the respective BMP-specific phosphorylated protein complex (pSmad1/5/8). The overexpression of pSmad2/3 correlated with arch artery defects in Tbx1;Smad7 heterozygotes compared with Tbx1 heterozygotes at E10.5. Similarly, Smad71/2−/− embryos showed increased levels of pSmad2/3 in the pharyngeal region at both E9.5 and E10.5, compared with wild-type controls. Smad7 interacts with this branch of the pathway in the atriocavitary cushion and ventricular endocardium of mice homozygous for a deletion in the MH2 domain of the protein.

Last, to demonstrate in vivo that the altered vascular remodeling in Tbx1;Smad7 heterozygotes was because of a cell autonomous interaction between Tbx1 and Smad7, we conditionally restored Smad7 expression using a Tbx1Cre driver. With this knock-in and, therefore, loss-of-function allele of Tbx1, we rescued the failure of the fourth arch artery recovery phenotype seen in double heterozygotes, indicating that Smad7 is required in the Tbx1 lineage for recovery of the fourth arch artery phenotype.
To conclude, we have shown that the widely quoted recovery of the Tbx1 haploinsufficient fourth arch artery phenotype occurs through the successful incorporation of hypoplastic vessels to the adult vasculature. Furthermore, we have identified a novel effector of Tbx1 that is required for pharyngeal artery remodeling and hence suggest that Tbx1 has roles beyond the arch artery formation stage, as previously proposed. We have delineated the successive steps that describe great vessel development and shown that Smad7-dependent compensatory mechanisms within the Tbx1 cell lineage allow normal growth and remodeling of the initially hypoplastic vessels in Tbx1 heterozygotes. Tbx1/Smad7 heterozygotes have a diminished early coverage of VSMCs that recovers in time, likely by increased cell proliferation. However, we propose that this results in thinner walled, narrower vessels and a late elaboration of the fibronectin-rich vessel support structure, ultimately leading to vessel collapse. There are several cardiovascular disorders described with loss of vessel architecture linked to TGFβ superfamily members through mutations in animal models. Increased TGFβ signaling has also been associated with excessive ECM synthesis in different contexts, such as fibrotic disease or atherosclerosis. Here, we describe a novel pathway that places a T-box transcription factor upstream of a TGFβ/BMP signaling inhibitor and is required for great vessel development during mammalian embryogenesis.

Acknowledgments

We thank Jennifer Sutherland and Nelo Popal for technical support; Dr Karen McCue for critical reading of the manuscript; and Debby Mustafa and Kyle O’Sullivan for animal facility coordination.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- DiGeorge syndrome is attributed to TBX1 mutations in humans and could be modeled in mice that lack 1 copy of the Tbx1 gene (haploinsufficient mice).
- Embryos lacking 1 Tbx1 copy fail to grow a specific artery (the fourth arch artery), although a proportion manage to recover this vessel by late gestation.

**What New Information Does This Article Contribute?**

- The fourth arch artery defect could be rescued if Tbx1 haploinsufficient embryos have a primitive small artery; rescue is not possible if the early vessel is completely absent.
- Smad7 is downstream of Tbx1 and is required for the formation of great vessels and for the rescue phenomenon noted above.
- The Tbx1:Smad7 interaction affects extracellular matrix ensheathment of pharyngeal arteries.

Tbx1 haploinsufficient embryos present with an unexplained recovery of arch artery defects during development. We distinguish between hypoplastic and aplastic artery phenotypes at the conclusion of fourth arch artery formation and show that in Tbx1 heterozygotes the frequency of hypoplastic vessels decreased during embryogenesis, whereas no reduction in the frequency of vessel aplasia was observed. This suggests that hypoplastic vessels can eventually catch up with normal venous development. We also show that Tbx1 genetically interacts with Smad7, an inhibitory Smad within the transforming growth factor-β pathway, which is required for arch artery remodeling. Tbx1 protein was present at T-BOX binding sites within the Smad7 locus. Finally, we demonstrate that the interaction between the 2 genes is part of the pathway that regulates extracellular matrix deposition on the primitive pharyngeal vessels. Studies of mice with targeted mutations of transforming growth factor-β signaling components have revealed several instances where loss of normal vessel architecture is a major feature. Of interest, both increased and decreased transforming growth factor-β signaling produce overlapping phenotypes, indicating that exquisite control of the pathway is necessary for normal development. This work describes a novel pathway that places a T-box transcription factor upstream of a transforming growth factor-β/bone morphogenetic protein (BMP) signaling inhibitor that is required for great vessel development during mammalian embryogenesis.
Tbx1 Genetically Interacts With the Transforming Growth Factor-β/Bone Morphogenetic Protein Inhibitor Smad7 During Great Vessel Remodeling

Irinna Papangeli and Peter J. Scambler

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**SUPPLEMENTAL MATERIAL**

*Tbx1* genetically interacts with the TGFβ/BMP inhibitor Smad7 during great vessel remodeling

Irina Papangeli¹ PhD and Peter J. Scambler¹,² MD

¹Molecular Medicine Unit, UCL Institute of Child Health, London WC1N 1EH, UK  
²Corresponding author: p.scambler@ucl.ac.uk, Phone: +44-207-905-2635

**Detailed Methods**

**Mice**

The mouse lines used were: Tbx1<sup>1<sub>lacZ</sub></sup> (MGI; Tbx1<sup>1<sub>im1Blbd</sub></sup>, Lindsay et al., 2001), Tbx1<sup>Cre</sup> (MGI; Tbx1<sup>tm6(cre)Bld</sup> ¹), Smad7<sup>GT</sup> (MGI; Smad7<sup>YHC053JByg</sup>, Baygenomics), Wnt1Cre (MGI; Tg(Wnt1-cre)11Rth, ²), Mesp1Cre (MGI; Mesp1<sup>tm2(cre)Ysa</sup> ³), R26RYFP (MGI; Gt(ROSA)26Sor<sup>1<sub>im1(EYFP)Cos</sub></sup> ⁴). All lines were maintained on a C57Bl/6J background. CD1 mice were used as wild type controls for gene expression profile analysis. The genotyping primers designed and used for the Smad7 wild type and gene-trap alleles were as follows, Smad7-F 5’-GCTCCAGATGTTCAGTTGC-3’, Smad7-WT-R 5’-CTCTTTAGAAAGAGTGGTGCC-3’, Smad7-GT-R 5’-CATACAGTCCTCTTCATCCATGCTG-3’. Animal maintenance, husbandry and procedures were done in accordance with British Home Office regulations.

**T-Box binding Elements on the Smad7 gene and regulatory sequence**

Conservation of Tbx5 binding sites or the T-half site ² in human mouse and rat genomic sequences of Smad7, including the 10kb upstream and downstream sequence was examined by sequence alignment using the Multiple sequence local alignment and visualization tool (MULAN, http://mulan.dcode.org/). The user defined consensus sequence search for the T-half site was performed using the TCACAM sequence, whereby M = A or C. Six Tbx5 binding sites and two T-half sites were identified. One of the T-half sites overlapped with one of the Tbx5 binding sites. Of the seven sites in total, six were taken forward for analysis, because the 6<sup>th</sup> Tbx5 binding site was within an A/T rich sequence, which could not be amplified. The sequences were as follows, TBE 1: TCCTTTAGAAAGAGTGGTGCC, TBE 2: TGAGGTGTGTGG, TBE 3: CAGGTGTGAA, TBE 4: GAACACCTTA, TBE 5: ATGGGTGTTATC, TBE 6: ATGGGTGTTATC, TBE 7: TCACAM.

**Intraperitoneal Injections**

BrdU labeling reagent (Invitrogen) at 1ml of reagent per 100g body weight was injected intraperitoneally into pregnant mice, and embryos were collected 4hrs later, at E10.5.

**Intracardiac Ink Injection**

Embryos were fixed in 4% paraformaldehyde or 10% neutral buffered formalin overnight at 4°C. The outflow tract (E11.5) or cardiac ventricles (E12.5-E15.5) of the embryos were injected with India ink (Pelican) through a pulled glass capillary as previously described ⁶.

**In situ hybridization**

Whole-mount RNA in situ hybridization was performed using digoxigenin-labelled probes for *Tbx1, Smad7*, and *Sox10* as previously described (Wilkinson, 1992). *Tbx1* plasmid was a gift from Albert Basson, *Smad7* plasmid was a gift from Christine Mummery, *Sox10* plasmid was a gift from Christiana Ruhrberg.

**Quantitative real time PCR**

Total RNA was isolated from whole embryos using the Trizol reagent (Invitrogen), according to the supplier’s instructions and reverse-transcribed using Superscript III RT (Invitrogen). Real-time qRT–PCR analysis was performed on an ABI 7900 Sequence Detector (Applied Biosystems) using SYBR Green (QuantitectTM SYBR Green PCR Kit, Qiagen). Data were normalized to *Gapdh* expression. Fold-changes in gene expression were determined by the 2<sup>−ΔΔCT</sup> method ⁷ and are presented relative to levels in wild type embryos.

**Immunohistochemistry and Histology**

Embryos were fixed in 4% paraformaldehyde (for Hematoxylin/Eosin) or 10% neutral buffered formalin (for IHC) overnight at 4°C, dehydrated and embedded in paraffin, prior to sectioning at 8µm. Sections for IHC were kept at 4°C. Embryos were fixed in 4% paraformaldehyde for 1hr at 4°C and equilibrated in 30% sucrose in PBS overnight prior to embedding in OCT and sectioning at 8-10µm. Primary antibody concentrations were: Smad7 (1/200; HPA028897, Sigma Aldrich), phospho-Smad1
(Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428) (1/100; 9511, Cell Signaling), phospho-
Smad2 (Ser465/467)/Smad3 (Ser423/425) (1/100; 9510, Cell Signaling), GFP (for OCT 1/1000;
ab13970, Abcam) GFP (for paraffin 1/200; ab290, Abcam), SM22 (1/200; ab14106, Abcam), BrdU
(1/100; ab6326, Abcam), Endomucin (1/50; sc-65495, Santa Cruz), Fibronectin (1/200; ab23750,
Abcam), Collagen IV (1/200; 2150, AbD Serotec), β1 integrin (1/200; MAB1997, Chemicon),
Laminin (1/200; ab11575, Abcam). BrdU-positive nuclei were detected by treating sections with 2N
HCl for 1hr at 37°C to denature the DNA and neutralizing in 0.1M sodium borate pH 8.5 twice for
5min before incubating with the anti-BrdU antibody. Owing to the destruction of cellular antigens
resulting from acid treatment, these steps were performed after the incubation with antibody to SM22.

**Scoring Systems**

Vessels were scored as “Th-P” where there was unilateral or bilateral hypoplasia, whereas vessels
were categorized as “NP” where there was unilateral or bilateral aplasia as well as where one side was
hypoplastic and the contralateral one was aplastic.

SM22 and BrdU positive cells were assessed by cell counts in the circumference of the vessel through
serial sections (n = 3 sections per vessel). The proportion of total smooth muscle cells was expressed
as the mean ratio of SM22-positive cells, from three sections, divided by the total number of cells
counted. The proportion of proliferating smooth muscle cells was expressed as the mean ratio of
SM22/BrdU-double positive cells divided by the SM22-positive cells.
### Supplemental Tables

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### Online Table I. Arch artery defect frequencies in Smad7 gene-trap embryos at E11.5 and E15.5.

Pharyngeal arch artery/great vessel defects and their developmental origins in Smad7+/-GT and Smad7-/-GT embryos at E11.5 and E15.5 compared to wild type controls. The numbers indicate individual embryos while the respective frequencies are in brackets. *both 6ths regressing. PAA, pharyngeal arch artery; WT, wild type.
Supplemental Figures

Online Figure I. Arch artery defects in Smad7 gene-trap and Tbx1;Smad7 heterozygous embryos at E11.5 and E15.5.

Intracardiac ink injection of E11.5 (A) and E15.5 (B) Smad7<sup>GT/GT</sup> embryos, compared to control embryos. (A) Side views of the pharyngeal arch arteries of a control embryo and Smad7<sup>GT/GT</sup> embryos presenting with hypoplastic left fourth or aplastic right fourth arch artery. (B) Frontal view of a control embryo and Tbx1<sup>+/lacZ</sup>;Smad7<sup>+/GT</sup> embryos presenting with HAA, RAA or cervical RSCA. (C) Side views of the pharyngeal arch arteries of a control embryo and Tbx1<sup>+/lacZ</sup>;Smad7<sup>+/GT</sup> embryos presenting with hypoplastic left fourth or bilateral aplastic fourth arch arteries. (D) Frontal view of a control embryo and Tbx1<sup>+/lacZ</sup>;Smad7<sup>+/GT</sup> embryos presenting with no RSCA, retroesophageal RSCA, cervical RSCA, HAA or IAA-B with cervical RSCA. Latin numerals (III, IV and VI) indicate the respective arch arteries; red asterisks indicate abnormal configurations due to fourth arch artery defects. HAA, high aortic arch; IAA-B, interrupted aortic arch type B; LCC, left common carotid; LSCA, left subclavian artery; RAA, right sided aortic arch; RCC, right common carotid; RSCA, right subclavian artery. Scale bar represents 500µm.
Online Figure II. NCC migration patterns in \textit{Tbx1} and \textit{Tbx1;Smad7} heterozygous embryos and NC and mesoderm cell contribution to the fourth arch artery in \textit{Tbx1} heterozygotes.

(A) Whole mount in situ hybridization at E10.5 for \textit{Sox10} on \textit{Tbx1}\textsuperscript{+/lacZ} and \textit{Tbx1}\textsuperscript{+/lacZ;Smad7\textsuperscript{+/GT}} embryos compared to a control embryo. (B) Coronal pharyngeal region sections of immunofluorescent staining at E11.5 for YFP (red) on \textit{Mesp1Cre;R26RYFP} and \textit{Mesp1Cre;R26RYFP; Tbx1\textsuperscript{+-lacZ}} embryos.

(C-E) Coronal pharyngeal region sections at the level of the fourth arch artery of immunofluorescent staining at E11.5 for YFP (red) (C, D), SM22 (green) (C, E) and endomucin (magenta) (D, E) on a \textit{Wnt1Cre;R26RYFP; Tbx1\textsuperscript{+-lacZ}} embryo. Latin numerals (III, IV and VI) indicate the lumen of the pharyngeal arch arteries; Arabic numerals (1-6) indicate NCC streams entering the respective arches; Red asterisks show the aberrant migration trajectories; White asterisk shows aplastic fourth arch artery; OV, otic vesicle. Scale bar represents 500µm in (A), 50µm in (B-E).
Supplemental references


