Molecular and genetic basis of congenital conotruncal heart defects

Rana, M.S.

Citation for published version (APA):
Chapter 4

Tbx1 regulates cardiac progenitor cell allocation to the poles of the heart tube


To be submitted.
Abstract

Rationale: Progenitor cells termed the second heart field (SHF) contribute new cardiomyocytes at the arterial and venous poles of the developing heart. Tbx1, the major candidate gene for the 22q11.2 deletion syndrome, has been implicated in the development of subpulmonary myocardium of the arterial pole, a substrate of common congenital heart defects (CHD). The subpulmonary myocardial lineage is related to venous pole progenitors, but the specification and segregation of these progenitors remains unclear.

Objective: To investigate whether and how Tbx1 regulates SHF segregation to both poles of the heart tube.

Methods and Results: We used gene expression profiling, genetic and Dil tracing experiments and 3D reconstruction to investigate the role of Tbx1 in the specification, segregation, and deployment of SHF progenitors. In the absence of Tbx1, anterior SHF progenitors failed to expand, leading to dorsal pericardium defects, and inability to form the subpulmonary myocardium. Progenitors acquired an anterior SHF signature and abnormally contributed to the venous pole of the heart, resulting in anomalies in the dorsal mesenchymal protrusion required for atrial and atrioventricular septation.

Conclusions: Tbx1 controls venous as well as arterial pole development by regulating the segregation of a common cardiac progenitor pool into different sublineages, providing new insights into the etiology of CHD and 22q11.2 Deletion Syndrome phenotypes.
Introduction

Genetic and environmental disturbances during cardiac morphogenesis result in a variety of congenital heart defects (CHDs), which significantly contribute to infant morbidity and mortality. Following the formation of the early heart, mesodermal cardiac progenitor cells termed the second heart field (SHF) are progressively added to both poles of the heart to contribute the major components of the heart. Even though the molecular underpinnings of SHF development have received significant attention in recent years (reviewed in), the mechanisms by which SHF cells are specified into the various cardiac structures remain to be elucidated.

Among these genes, the T-box containing transcription factor TBX1 plays a central role in regulating two of the key properties of the SHF. TBX1 induces progenitor cell proliferation through activation of FGF ligand expression in the pharyngeal region, and negatively regulates differentiation through several mechanisms including interference with BMP driven differentiation and negative regulation of Mef2c and SRF activities. Loss of Tbx1 in mice results in a reduced size of the SHF progenitor population and failure to elongate the distal outflow tract resulting in common arterial trunk. TBX1 is the major candidate gene for 22q11.2 deletion syndrome (22q11.2DS, also known as DiGeorge or Velo-cardio-facial syndrome) in man, associated with a range of conotruncal congenital heart defects including common trunk and tetralogy of Fallot. We have previously demonstrated that Tbx1 regulates regional identity in the OFT and that, at midgestation, myocardium of the inferior wall of the OFT is particularly affected in Tbx1 null embryos. This domain of the OFT has been proposed to give rise to myocardium at the base of the pulmonary trunk or subpulmonary myocardium, a region of the heart implicated in conotruncal defects such as tetralogy of Fallot. At fetal stages, loss of Tbx1 is associated with failure of the development of subpulmonary myocardium and proximal coronary artery patterning defects. The mechanisms underlying altered subpulmonary myocardial development in Tbx1 null embryos, however, remain largely unknown.

At the arterial pole, the SHF gives rise to the myocardium of the right ventricle and OFT and at the venous pole to myocardium and the precursors required for atrial and atrioventricular septation. The anterior component of the SHF (aSHF) progenitor pool can be distinguished by the expression of an Fgf10 enhancer trap transgene and the posterior SHF (pSHF) population by the expression of Tbx5. Recent genetic clonal analyses identified a common lineage giving rise to atrial myocytes and subpulmonary myocardium, but not subaortic myocardium. Dil labelling experiments analysing the contribution of the pSHF to the embryonic heart revealed an unexpected contribution to OFT myocardium. Moreover, using Cre genetic lineage tracing, anterior Hox genes, notably Hoxb1, have been shown to be expressed in a population of cells in the posterior SHF that give rise to atrial and OFT myocytes, particularly those in the inferior OFT wall. Together, these experiments suggest that a population of pSHF cells also give rise to the inferior wall, the site of future subpulmonary myocardium, of the heart affected in Tbx1 null embryos. However, the genetic and cellular mechanisms by which cardiac progenitor cells in the pSHF contribute to the arterial versus venous pole of the elongating heart tube are unknown.

Here, using transcriptional profiling, we identify a unique genetic signature of future subpulmonary myocardium. Using genetic and Dil cell tracing experiments, we demonstrate that failure of this transcriptional program in Tbx1 null embryos is due to a critical requirement for Tbx1 for the addition of cells from the caudal region of the SHF to the
arterial pole of the heart, corresponding precisely to the Dil labelled and Hoxb1-expressing subpulmonary progenitor cell population discussed above. Failure of the anterior expansion of this SHF population accounts for hypoplasia of the dorsal pericardial wall and SHF and leads to defects in dorsal mesocardial development. Furthermore, in the absence of Tbx1, failure to separate aSHF from pSHF progenitor cells leads to abnormal cell contributions at the venous pole of the heart, resulting in anomalies in the dorsal mesenchymal protrusion required for atrial and atrioventricular septation.

**Methods and Materials**

**Transgenic mouse lines**

Tbx1\(^{tm1Pa}\) (synonym: Tbx1\(^9\), Tbx1\(^{tm1Bld}\) (synonyms: Tbx1\(^{LacZ}\), Tbx1\(^{21}\), Mlc1v-nlacZ-24 (synonym: 1v-24)\(^16\), Gtrosa20\(^{tm1Sor}\) (synonym: R26R-lacZ)\(^22\), Tg(Mef2c-cre)2Blk (synonym: Mef2c\(^{Cre}\)\(^23\), Hoxb1-cre and Hoxa1-enhIII-Cre\(^20\), transgenic mice were intercrossed to harvest embryos and fetuses. Homozygosity for Tbx1 mutant alleles is also indicated as \(^{-/-}\). All mouse strains were maintained on a mixed or outbred (Bl6/CD1 or FVB/N) background. Developmental stage was determined by considering noon on the day of the appearance of a copulation plug as embryonic day (E) 0.5. Embryos and fetuses were isolated in ice-cold 1x Phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (PFA) for 1-4 hours, examined and processed for further analysis. Genomic DNA obtained from yolk sac or tail biopsies was used for genotyping by PCR, using primers described in the above-mentioned references. Animal care was in accordance with national and institutional guidelines.

**In situ hybridization and fluorescent immunohistochemistry**

Non-radioactive in situ hybridization (ISH) and fluorescent immunohistochemistry (IHC) was performed as described\(^24\)-\(^26\). Fixed embryos (4% PFA) were dehydrated, embedded in paraffin and sectioned at 10-14 \(\mu m\) for ISH and at 7 \(\mu m\) for IHC. The primary antibodies used for this study were: BrdU rat polyclonal (1:600; AbD serotec), Nkx2-5 (1:250; Santa Cruz Biotechnology), Isl1 (1:250; Neuromics), Tbx5 (1:250; Santa Cruz Biotechnology), MF20 (1:50; Hybridoma Bank), and cTnl rabbit polyclonal (1:250; Hytest Ltd.). Secondary antibodies used were Alexa 647 goat-anti-rabbit (1:250; Molecular Probes), Alexa 568 goat-anti-rabbit (1:250; Molecular Probes). Nuclei were stained using Sytox Green, Dapi, or Sytox Blue nucleic acid stain (Molecular Probes). ISH sections were observed and documented using a Leica Axiophot microscope and IHC using a Leica DM6000 microscope.

**β-galactosidase activity detection**

Detection of β-galactosidase activity on whole embryos to analyze LacZ reporter gene expression in transgenic embryos was performed as described\(^16\). Embryos were analyzed and photographed using a Zeiss StereoLumar microscope and a Leica MZ FLIII microscope connected to a Leica DFC320 photocamera.
Dil Injection and embryo culture

*Mlc1v-nLacZ-24* embryos on an *Tbx1*−/− or wildtype background ranging from 4 to 8 somite stages were collected, transferred to Hank’s solution and labelled by injection with the long-chain carbocyanine lipophilic dye Dil (Molecular Probes, D3911) as described19. Dil was injected into the caudal part of the dorsal pericardial wall (t=0 hours). Labelled embryos were cultured for 18 hours in 75% rat serum, 25% T6 medium, 5% CO₂, 20% O₂ and 75% N₂ in rolling bottles, and harvested for fate map analysis (t=18 hours). A total of 51 wildtype and *Tbx1*−/− and 19 *Tbx1*−/− mouse embryos were used for Dil labelling and fate map analysis. The position of Dil labels at t=18 was scored in 5 zones along the anterior-posterior axis between the arterial pole (region A) and venous pole (region E). Embryos were photographed using a Zeiss StereoLumar fluorescence microscope and a selection of embryos was analyzed further by cryosectioning.

Quantitative analysis and visualization of proliferation rate

The proliferation rate of cells in mouse embryos was determined as described26 using the mitotic S-phase marker Bromodeoxy-Uridine (BrdU; Sigma nr. B5002) and the cell division marker Ki67 (Dako). Mouse embryos ranging from somite stage 0-18 were exposed to BrdU by culturing in 0.05 mg BrdU / ml medium (DMEM (Invitrogen), pH 7.4, supplemented with 10% fetal calf serum) for 1 hour, washed in PBS (1x) and fixed in 4% PFA (PBS 1x) prior to IHC analysis. Embryos ranging from embryonic day 9 and older were exposed to BrdU peritoneally by injecting pregnant mice with 50 mg BrdU / kg (10 mg BrdU / ml 0.9% NaCl solution) as described before27. After 1 hour of BrdU exposure, the pregnant mice were sacrificed by cervical dislocation and the embryos were isolated in ice-cold PBS (1x), fixed in 4% PFA, staged according to known morphological features28 and processed for fluorescent IHC.

3D visualisation of gene expression

Acquisition and 3D reconstruction of labeling indices using AMIRA (version 3.5.2; TGS Template Graphics Software, http://www.tgs.com) has been performed using a previously described method29, which demonstrated a confidence interval (CI) of 95% at most 0.5±0.075 for nearly all voxels in embryonic chicken hearts. Reconstructions of embryonic hearts ranging from E8.25 to E10 were generated in triplicate per stage and genotype. Factor correction30 was used to remove systematic bias due to variability in BrdU exposure between embryos and experiments, while conserving biological differences between genotypes and heart regions.

Morphometry of developing anatomical landmarks

To evaluate the distance between the arterial and venous pole, we generated and compared 3D reconstructions, in which we measured the Euclidean distance using the AMIRA 3D measurement tool31 (in triplicate). Student’s t-test was used to correct for stage-related variability.
Microarray-based gene expression analysis of outflow tract tissue

Inferior and superior OFT regions were dissected from CD1 mouse embryos at E10.5. Regions from six littermate embryos were combined. The screen was performed with biological triplicates from inferior and superior OFT regions collected from three pregnant females. Total RNA was processed for use on Affymetrix 430 2.0 mouse array after one round of amplification. The samples were homogenized and RNA extracted using Trizol before cleaning using the RNeasy cleanup kit (QiAgen). RNA samples were transferred to the IGBMC (Strasbourg, France; http://www-microarrays.u-strasbg.fr/) where RNA quality was verified using an Agilent 2100 Bioanalyzer. Microarray hybridization and analysis were performed following Affymetrix protocols. Microarray data was analyzed using Limma and Affy packages from Bioconductor32. Two lists of significantly regionalized genes were generated and filtered for p value <0.05. Selected genes found to be enriched in the inferior OFT were subjected to further analysis by qRT-PCR and in situ hybridization.

Quantitative real-time PCR

For validation of the microarray results, amplified RNA from the inferior and superior regions was reverse transcribed using iScript (BioRad). Real-time PCR assays were performed using SYBR Green conditions on the IQ5 BioRad cycler with 200ng of cDNA and gene specific primers. Reactions were performed in quadruplicate. Normalization was performed using HPRT. Primer details are available on request.

Results

Future subpulmonary myocardium is characterized by the expression of regulators of neural crest development and migration

Despite its clinical importance as a hotspot of congenital heart defects, the genetic networks regulating subpulmonary myocardium development are poorly understood. The subpulmonary myocardium of the heart has been shown to be regulated by Tbx113. In order to identify transcriptional features of the inferior OFT wall that are potentially important during subpulmonary myocardium development, we performed microarray-based gene expression profiling of the inferior and superior wall of the E10.5 OFT (iOFT and sOFT, respectively). Using an Affymetrix 430 2.0 mouse array, we found that the majority of gene transcripts expressed in the outflow tract at this stage accumulate equivalently in the iOFT and sOFT (Figure 1A, Volcano plot). However, 211 probes corresponding to 181 genes were found to be particularly enriched in the iOFT with log fold changes of >0.8 (p<0.05 based on biological triplicates), including 25 genes detected by two or more probe sets. To discriminate between highly enriched and moderately or marginally enriched transcripts in the iOFT, we focused on a selection of 18 genes with fold changes of >1.6 (set as cut-off value; Table 1). These include Sema3c, encoding a Semaphorin ligand required for neural crest influx into the OFT13, a gene previously shown to be enriched in this part of the heart as a result of its location adjacent to the integration site of the y96-Myf5-nlacZ-16 (96-16) enhancer trap transgene on chromosome 513. This transgene is expressed in subpulmonary myocardium of the fetal heart and its SHF precursors, including the inferior OFT wall at
midgestation. In addition to Sema3c, the top 18 enriched transcripts included genes encoding EFHD1, involved in neural differentiation, Barx1 and Dlx2, transcription factors important for the development of neural crest-derived mesenchyme and Nrp2, a receptor protein with roles in cardiovascular development which binds with high affinity to Sema3c. Enrichment of selected genes in the iOFT was validated by comparing the relative expression levels in the sOFT (normalized at 1.00) with the iOFT using quantitative RT-PCR (Figure 1B). Together, these findings demonstrate that future subpulmonary myocardial cells in the iOFT have a gene expression profile distinct from that of sOFT myocardium. This genetic repertoire includes signalling molecules and transcription factors involved in neural crest development that may influence NC-influx to facilitate OFT septation and SHF development.

**Tbx1-dependency of future subpulmonary myocardium**

Loss of Tbx1 has been shown to compromise y96-Myf5-nlacZ-16 transgene expression in the OFT and SHF, suggesting that Tbx1 preferentially affects subpulmonary myocardial precursors rather than subaortic myocardial precursors during OFT development. To further validate the expression of genes enriched in the iOFT and to test Tbx1-dependency, we performed non-radioactive whole-mount in situ hybridization using riboprobes directed against mRNA for a selection of iOFT-enriched genes (Barx1, Dlx2, Sema3c and Nrp2) in E9.5-10.5 wildtype and Tbx1−/− embryos. In wildtype embryos, transcripts for these genes were enriched in the iOFT, consistent with their identification in our microarray screen, in addition to extracardiac expression sites, for example pharyngeal arch mesenchyme (Barx1 and Dlx2; Figure 1C and Supplementary Figure 1). The expression of Barx1, Dlx2, Sema3c and Nrp2 was greatly reduced in the iOFT of Tbx1−/− embryos (Figure 1D). Note that a Sema3c expression domain in the superior proximal OFT is maintained in Tbx1−/− hearts. These findings suggest that Tbx1 is required in subpulmonary myocardial progenitor cells to activate a specific gene program and/or to regulate the addition of future subpulmonary myocardium to the OFT during heart tube extension, suggested by the distal OFT hypoplasia displayed by Tbx1−/− embryos at midgestation.

To test the hypothesis that the addition of subpulmonary myocardial precursors to the OFT is significantly reduced in Tbx1 nulls, we performed genetic lineage tracing experiments using a Hoxb1ires-Cre allele recently reported to be expressed in subpulmonary and atrial myocardial progenitor cells. Hoxb1ires-Cre;Tbx1−/− mice were crossed with R26R-lacZ;Tbx1−/− reporter mice to analyze the contribution of Hoxb1 expressing myocardial precursors to the OFT. Hoxb1ires-Cre;R26R-lacZ genetic tracing revealed that fewer Hoxb1 expressing progenitor cells contribute to the Tbx1 null OFT at E10.5 compared to the wildtype situation (Figure 1E), consistent with failure of addition of an entire genetic lineage to the OFT of Tbx1 null hearts. Midsagittal sections confirmed that the narrow distal OFT in Tbx1 null embryos is negative for cells derived from the Hoxb1 lineage with the exception of a small number of positive cells in the inferior wall of the distal OFT (Figure 1F).
**Table 1.** Probe sets enriched more than 1.6x in the iOFT

<table>
<thead>
<tr>
<th>Probe</th>
<th>Gene</th>
<th>Gene symbol</th>
<th>Fold enrichment (log 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1448507_at</td>
<td>EF hand domain containing 1</td>
<td>EFHD1</td>
<td>1.505</td>
</tr>
<tr>
<td>1423342_at</td>
<td>BarH-like homeobox 1</td>
<td>barx1</td>
<td>1.427</td>
</tr>
<tr>
<td>1420715_a_at</td>
<td>peroxisome proliferator activated receptor gamma</td>
<td>PPARG</td>
<td>1.331</td>
</tr>
<tr>
<td>1456778_at</td>
<td>neuropilin 2</td>
<td>NRP2</td>
<td>1.273</td>
</tr>
<tr>
<td>1436998_at</td>
<td>ankyrin repeat domain 43</td>
<td>ANKRD43</td>
<td>1.235</td>
</tr>
<tr>
<td>1419028_at</td>
<td>cyclic AMP-regulated phosphoprotein, 21</td>
<td>ARPP21</td>
<td>1.210</td>
</tr>
<tr>
<td>1434188_at</td>
<td>solute carrier family 16, member 12</td>
<td>SLC16A12</td>
<td>1.174</td>
</tr>
<tr>
<td>1417812_a_at</td>
<td>laminin, beta 3</td>
<td>Lamb3</td>
<td>1.169</td>
</tr>
<tr>
<td>1426528_at</td>
<td>neuropilin 2</td>
<td>NRP2</td>
<td>1.133</td>
</tr>
<tr>
<td>1420696_at</td>
<td>Semaphorin 3C</td>
<td>SEMA3C</td>
<td>0.983</td>
</tr>
<tr>
<td>1419606_a_at</td>
<td>troponin T1, skeletal, slow</td>
<td>TNNT1</td>
<td>0.951</td>
</tr>
<tr>
<td>1420667_at</td>
<td>double C2, beta</td>
<td>DOC2B</td>
<td>0.943</td>
</tr>
<tr>
<td>1450606_at</td>
<td>phenylethanalamine-N-methyltransferase</td>
<td>Pnmnt</td>
<td>0.937</td>
</tr>
<tr>
<td>1433549_at</td>
<td>neuropilin 2</td>
<td>NRP2</td>
<td>0.901</td>
</tr>
<tr>
<td>1429348_at</td>
<td>Semaphorin 3C</td>
<td>SEMA3C</td>
<td>0.893</td>
</tr>
<tr>
<td>1416561_at</td>
<td>glutamic acid decarboxylase 1</td>
<td>Gad1</td>
<td>0.875</td>
</tr>
<tr>
<td>1422573_at</td>
<td>adenosine monophosphate deaminase 3</td>
<td>AMPD3</td>
<td>0.864</td>
</tr>
<tr>
<td>1432466_a_at</td>
<td>apolipoprotein E</td>
<td>APOE</td>
<td>0.859</td>
</tr>
<tr>
<td>1443036_at</td>
<td>zinc finger protein 804A</td>
<td>Zfp804a</td>
<td>0.849</td>
</tr>
<tr>
<td>1448877_at</td>
<td>distal-less homeobox 2</td>
<td>Dlx2</td>
<td>0.830</td>
</tr>
<tr>
<td>1427020_at</td>
<td>scavenger receptor class A, member 3</td>
<td>SCARA3</td>
<td>0.810</td>
</tr>
<tr>
<td>1424248_at</td>
<td>cyclic AMP-regulated phosphoprotein, 21</td>
<td>ARPP21</td>
<td>0.801</td>
</tr>
</tbody>
</table>

At fetal stages, *Hoxb1*\(^{RES-Cre, R26R}\) embryos express β-galactosidase at the ventral myocardial base of the pulmonary trunk of the heart; few cardiomyocytes derived from *Hoxb1* expressing progenitor cells were observed in the ventral region of *Tbx1* null hearts at E14.5, consistent with failure of subpulmonary myocardial development (Figure 1F). In contrast, *Hoxb1* expressing progenitor cells contributed to atrial and venous pole myocardium of *Tbx1*\(^{-/-}\) hearts similarly to the situation in wildtype embryos (Figure 1F). A second anterior *Hox* gene, *Hoxa1*, has also been shown to be expressed in the subpulmonary myocardial lineage\(^ {20}\). We also investigated the distribution of cells derived from *Hoxa1* expressing progenitor cells using a *Hoxa1-enhIII* derived Cre line and *R26R* conditional reporter gene (Supplementary Figure 1). Whereas in wildtype hearts this Cre line resulted in labelling in future subpulmonary myocardium in the midgestation OFT and myocardium at the base of the pulmonary trunk in fetal hearts, no labelled cells were observed in the OFT or common arterial trunk of *Tbx1*\(^{-/-}\) hearts (Supplementary Figure 1). Decreased expression of the 96-16 transgene and IOFT specific genes as well as hypoplasia of the distal OFT of *Tbx1*\(^{-/-}\) embryos are therefore most likely the result of abnormal development and compromised deployment of subpulmonary myocardial precursor cells to the elongating OFT.
Figure 1. Transcriptional analysis and Tbx1-dependence of the future subpulmonary myocardial lineage. A, Volcano plot of the results of a comparative microarray screen showing that transcripts for a minority of genes expressed in the OFT are significantly enriched in the iOFT or sOFT wall. B, Quantitative RT-PCR validation of 5 genes enriched in the iOFT (Pparγ, Barx1, Dlx2, Sema3c, Npn2) and 1 in the sOFT (Raldh2). C, Left hand views of E10.5 wildtype embryos after whole-mount in situ hybridization with Barx1, Dlx2, Sema3c and Nrp2 riboprobes showing transcript enrichment in the iOFT wall. Note Sema3c transcripts also accumulate in the proximal sOFT. D, Left hand views of E10.5 Tbx1−/− embryos after in situ hybridization with Barx1, Dlx2, Sema3c and Nrp2 riboprobes showing loss of iOFT wall transcript accumulation; note maintenance of Sema3c transcripts in the proximal sOFT. E, E10.5 hearts from Hoxb1-Cre;R26R embryos stained with X-gal showing the contribution of the Hoxb1 genetic lineage to the OFT. Note that while a large part of the circumference of the OFT is labelled in wildtype embryos few labelled cells are observed in the OFT of Tbx1−/− hearts. F, Left panels: mid-sagittal sections after X-gal staining showing failure of the Hoxb1 genetic lineage to contribute to the Tbx1−/− OFT. Right panels: ventral views of the ventricular outlets at E14.5 showing that the Hoxb1 genetic lineage gives rise to ventral subpulmonary myocardium in wildtype embryos but is not observed in the ventral region of the common arterial trunk in Tbx1−/− hearts. avc, atrioventricular canal; iOFT, inferior outflow tract; la, left atrium; lv, left ventricle; oft, outflow tract; sOFT, superior outflow tract.

Tbx1 deficiency affects the anterior movement of caudal SHF cells from somite stage 7 onwards

In order to further investigate the failure of Hoxb1 and Hoxa1 expressing subpulmonary myocardial progenitor cells to contribute to the OFT of Tbx1−/− embryos, we decided to
determine the fate of posterior SHF cells in wildtype and \( Tbx1^{+/−} \) embryos using Dil tracing experiments at somite stages 4-6 and at 7-8 (designated time point t=0; Figure 2A). E8.5 embryos were generated from \( Tbx1^{+/−} \) intercrosses and cells labelled with Dil in the dorsal pericardial wall at the level of the posterior limit of arterial pole progenitor cells, as determined by expression of an aSHF marker, the Fgf10 enhancer trap transgene \( Mlc1v-nlacZ-24 \) (Figure 2A). After labelling, embryos were cultured in rolling bottles for 18 hours (t=18; Figure 2B) and the anterior limit of Dil labelled cells was scored with respect to 5 zones along the anterior-posterior axis between the arterial and venous poles: zone A being close to the arterial pole and zone E close to the venous pole (Figure 2C). In wildtype and \( Tbx1^{+/−} \) embryos, the majority of labelling events resulted in labelled cells proximal to the arterial pole of the heart (zones A-C) as well as labelled cells in the distal OFT (Figure 2D). These findings are consistent with recent work showing a contribution of the pSHF to both poles of the heart tube\(^4\). The results obtained for \( Tbx1 \) null embryos that were labelled prior to the 7-somite stage were indistinguishable from those for wildtype and \( Tbx1^{+/−} \) control embryos (Figure 2D). The majority of these labelling events resulted in the most anteriorly positioned labelled cells being close to the arterial pole of the heart and in the distal outflow tract (Figure 2D and Supplementary Figure 2). In contrast, in \( Tbx1^{−/−} \) embryos with 7 or more somites, labelled cells failed to contribute to zones A-C or the distal OFT and in these embryos the most anteriorly labelled cells were found close to the inflow region of the heart (zones D and E) and in the dorsal atrial wall (Figure 2D and Supplementary Figure 2). These results suggest that, in the absence of \( Tbx1 \), the movement of cells in the dorsal pericardial wall towards the arterial pole fails from 7-somite stage onwards. Consequently, progenitor cells that would normally contribute to subpulmonary myocardium appear to remain in the posterior region of the \( Tbx1^{−/−} \) SHF.

![Image](image.jpg)

**Figure 2.** Failure of anterior displacement of cells in the posterior SHF of \( Tbx1 \) mutant embryos. A, Site of Dil labelling at E8.5 in the posterior region of the SHF prior to embryo culture and on transverse section after X-gal staining of a \( 1v-24^{+/−} \) embryo dissected immediately after labelling (t=0). B, Left hand views of embryos harvested after 18 hours of embryo culture (t=18) showing the localisation of Dil labelled cells close to the outflow tract (left) or venous pole of the heart (right). C, The anterior limit of labelled cells was scored with respect to 5 regions (A-E) between the arterial and venous poles (left); bar graph showing the % of wildtype and \( Tbx1^{+/−} \) embryos (green) and \( Tbx1^{−/−} \) (red) embryos showing labelled cells in regions A-C (close to the arterial pole) and regions D and E (close to the venous pole). D, Breakdown of the data based on the number of somites at the time of labelling reveals that prior to 7 somites (left) \( Tbx1^{−/−} \) embryos behave indistinguishably from wildtype and \( Tbx1^{+/−} \) embryos (left). In contrast, labelled cells in \( Tbx1^{−/−} \) embryos fail to show anterior displacement towards regions A-C after the 7 somite stage (right). avc, atrioventricular canal; fg, foregut; h, heart; ia, left atrium; lv, left ventricle; of, outflow tract; ss, somite stage.
**Tbx1-deficiency affects the expansion of the SHF progenitor pool and the proliferation rate in the left anterior SHF**

The dorsal pericardial wall of the mouse heart has recently been shown to be a highly proliferative structure and Tbx1 has been shown to play a pro-proliferative role in cardiac progenitor cells. We next investigated how failure of the future subpulmonary myocardial progenitor pool to progress towards the arterial pole of the elongating heart tube in Tbx1−/− embryos impacted on proliferation in the dorsal pericardial wall. We evaluated the spatiotemporal requirement for Tbx1 during anterior SHF expansion by quantifying the fraction of proliferating 1v-24+ cells in the dorsal pericardial wall using BrdU incorporation in embryonic day (E) 8-8.25 or somite stage (ss) 6-10 wildtype and Tbx1-deficient embryos (n=3 per stage and genotype). In stage E8 (ss6-7) and E8.25 (ss8-10) wildtype embryos, the percentage of BrdU+ cells in the anterior SHF was estimated to range from 50 to 75% (Figure 3A). The BrdU+ fraction was found to decrease in a caudo-cranial gradient, to 0-25% adjacent to the OFT (Figure 2D, blue domain), presumably reflecting differentiation of SHF into OFT myocardium. In 6-7 somite stage Tbx1−/− embryos (n=3), the percentage of BrdU+ cells in the left 1v24 expressing domain was decreased (Figure 3A, blue domain, arrow), but not in 8-10 somite embryos, in which the left SHF was only moderately affected. In addition to the proliferation defect, the distance between the left and right 1v-24 cardiac precursors in the dorsal pericardial wall of Tbx1 null embryos was found to be greater than that in wildtype embryos, suggesting abnormal dorsal mesocardial development (Figure 3A, asterisks). These early proliferation defects potentially underlie the reduced pool of SHF cells observed at E9.5 and suggest that Tbx1 plays a pivotal role in maintaining the cardiac progenitor cell pool in the dorsal pericardial wall and consequently SHF deployment to the arterial pole.

**Loss of Tbx1 impacts on dorsal mesocardium development**

Our proliferation analysis in E8-8.25 (ss6-10) Tbx1−/− embryos suggested early roles for Tbx1 during dorsal mesocardial development. We therefore evaluated dorsal pericardial wall development between E8.5-9.5, stages at which the dorsal mesocardium has ruptured and the embryonic heart remains attached to the body only through the arterial and venous poles. In E8.5 wildtype embryos, the fusion line of the coelomic wall was clearly visible (Figure 3B), which also points to the ruptured dorsal mesocardium initially connecting the lateral margins of the embryonic bowl-shaped heart. In Tbx1-deficient embryos, the fusion line was not apparent until E9.25 (Figure 3B), and also appeared to connect a smaller part of the wall when compared with wildtype embryos (Figure 3B, E9.5 timepoints), suggesting a delay in dorsal mesocardium formation and rupture.
Abnormal rupture of the dorsal mesocardium in Tbx1-deficient embryos was found to decreased the distance between the IFT and OFT (Figure 3B, C). To elucidate how this would impact IFT and OFT development, we generated and analyzed 3D reconstructions of E8.5-10 wildtype and Tbx1 null mutant hearts, using cTnl or MF20 as a myocardial marker (Figure 3C). Wildtype E8.5 hearts were still partially connected to the dorsal mesocardium (Figure 3C, black arrow, compare with green arrow, illustrating separated myocardium). In contrast, E8.5 Tbx1-deficient hearts had yet to detach from the dorsal coelomic wall (Figure 3C, black arrow), which did occur from stage E9,25 onwards. Furthermore, the distance between the IFT and OFT lumen was reduced in Tbx1⁺/⁻ hearts (compare double-arrowed bar in Figures 3C). The distance of the IFT-OFT was determined by comparing 3D reconstructions of E9.5-10 stage hearts and measuring the Euclidean distance (in triplicate) using the AMIRA 3D measurement tool. We found a difference of approximately 65 µm in Tbx1 null hearts.
compared with wildtype hearts (Figure 3D, n=4 per stage). Collectively, these observations indicate that loss of Tbx1 delays the formation and rupture of the dorsal mesocardium and, consequently, reduces the distance separating the IFT and OFT. These defects may result from failure of expansion of subpulmonary myocardial progenitor cells towards the arterial pole of the heart.

Fluorescent immunohistochemical examination of transverse sections at the level of the most cranial part of the pulmonary ridges (indicated by red interrupted line in Figures 3C) revealed that the cells most adjacent to the caudal dorsal wall (known as the pulmonary pit) displayed increased expression of the myocardial marker cardiac Troponin I (cTnI) in Tbx1+/− embryos (Figure 3E, circled). In contrast to wildtype embryos, this region displayed morphological signs of what at earlier stages would be identified as a dorsal mesocardium, connecting the heart and dorsal pericardial wall (Figure 3E). This phenotype reflects premature and possibly ongoing differentiation of mesodermal cardiac progenitors, consistent with reported ectopic differentiation within the dorsal pericardial wall of mutant embryos39,7.

In E9.5 wildtype embryos, Nkx2-5 is expressed in cardiomyocytes and in the most anterior or posterior (differentiating) SHF cells (Figure 3E, arrowheads), separated by Nkx2-5-negative SHF cells in the dorsal wall (Figure 3E). We found that in Tbx+/− embryos Nkx2-5+ cells in the dorsal pericardial wall were found throughout the SHF of Tbx+/− embryos (Figure 3E), consistent with juxtaposition of differentiated cells at the poles of the heart tube. To facilitate a more accurate identification of differentiating SHF cells, we generated 3D reconstructions of cTnI+ cells in the heart and dorsal pericardial wall at stage E9.5 (n=4). We detected a small subset of cTnI+ SHF cells bordering OFT myocardium (Figure 3E, red area, arrowheads) and none adjacent to the venous pole (Figure 3E, dashed line, black arrow) in wildtype embryos. Interestingly, cTnI+ cells present in Tbx+/− embryos extended from the OFT to the venous pole (Figure 3E, right panels, white arrow), overlapping with the Nkx2-5+ domain and consistent with premature differentiation of progenitor cells in the Tbx1+/− dorsal pericardial wall.

Gene expression changes and misspecification of SHF cells in Tbx1 null mutants

Considering the anomalous development of the dorsal pericardial wall and failure of expansion of future subpulmonary myocardial cells in the absence of Tbx1, we explored whether Tbx1 deficiency affects the genetic profile of cells in anterior and posterior SHF domains. The expression of genes in anterior SHF and posterior SHF domains of the dorsal pericardial wall was evaluated in wildtype and Tbx+/− embryos at E8.25-9.5 by fluorescent immunohistochemistry on transverse and sagittal sections. At E8.25, β-galactosidase under control of the 1v-24 transgene was robustly expressed in anterior SHF cells, as described before16,42. Tbx5 is known to be required for posterior SHF development and venous pole morphogenesis43,17. β-galactosidase+ cells were not observed in the Tbx5+ IFT of wildtype embryos at these stages (Figure 4A). In contrast, in E8.25 Tbx+/− embryos, we observed ectopic 1v-24-driven β-galactosidase expression in the Tbx5+ venous pole (Figure 4A). We generated three-dimensional reconstructions of E8.25 hearts to thoroughly examine morphology and co-localization of protein distribution. In contrast to wildtype embryos, 1v-24-driven β-galactosidase+ myocardium extended towards the venous pole of Tbx1+/−.
embryos, where Tbx5 expression was now absent. In addition, a small portion of β-galactosidase+ myocardium overlapped with Tbx5+ atrial cells (Figure 4B).

At E9.5 1v24 expressing cells are restricted to the anterior and lateral regions of the dorsal pericardial wall in continuity with the outflow tract (Figure 4C), whereas Tbx5 is expressed in the posterior medial region of the dorsal pericardial wall in continuity with venous pole and atrial myocardium (Figure 4C). The 1v-24 transgene and Tbx5 thus identify distinct and reciprocal anterior/lateral and posterior/medial transcriptional domains within the dorsal pericardial wall. Loss of Tbx1 resulted in gene expression changes in the dorsal pericardial wall and perturbation of the border between these domains, as also observed at E8.25, by which β-galactosidase now colocalized with Tbx5+ SHF cells (Figure 4D). Three-dimensional analysis of the E9.5 dorsal pericardial wall revealed that the Tbx5+ posterior SHF domain is now oriented dorsally to the Tbx1+/‐ heart instead of caudally (Figure 4D, red), compared to wildtype littermates (Figure 4D), consistent with the abnormal juxtaposition of the cardiac poles documented above. Moreover, in contrast to wildtype embryos, a significant population of 1v-24-driven β-galactosidase+ cells in the right-lateral side of the dorsal pericardial wall were found to colocalize with Tbx5 expressing cells (Figure 4D). Similar results were obtained after in situ expression analysis of Osr1, a Tbx5 target gene expressed in the pSHF 17, in wildtype and Tbx1+/‐ embryos (not shown).

We tested the hypothesis that posterior SHF cells may be misspecified and give rise to venous pole structures in Tbx1 null embryos. Analysis of the expression domain of the anterior SHF-specific 1v24 transgene in E17.5 Tbx1 null mutant fetuses, revealed that 1v-24-driven β-galactosidase+ cells were ectopically expressed in the mediastinal or primary atrial septum (Figure 4E, arrow), in sharp contrast to littermate controls. Interestingly this ectopic atrial expression domain appears to be in continuity with transgene expressing cells in the dorsal wall of the common arterial trunk (Figure 4E). We complemented this analysis by performing genetic lineage experiments using the Mef2c-AHF-Cre transgene that is expressed in the anterior SHF and genetically labels cells in the arterial pole of the heart, as well as a small population of cells at the venous pole. Tbx1+/‐;Mef2c-Cre+ cells were traced using the Cre-recombinogenic lacZ reporter allele R26R-lacZ. Analysis of the Mef2c-Cre fate map in E10.5 Tbx1 null embryos (n=3) showed an increase in the accumulation of β-galactosidase in atrial myocardium (Figure 4F), compared with littermate controls, suggesting an expanded contribution of the Mef2c-AHF-Cre lineage to the venous pole of Tbx1+/‐ hearts. Together these results indicate that, in the absence of Tbx1, a subpopulation of pSHF cells normally contributing to the arterial pole expresses remains in the region of the pSHF and contributes ectopically to posterior cardiac structures.
Figure 4. Altered gene expression and genetic lineages in the anterior and posterior SHF of Tbx1+/− embryos. A, immunofluorescence on transverse sections at E8.5 showing overlap between 1v-24-driven β-galactosidase and TBX5 labelling at the venous pole in Tbx1+/− embryos. B, 3D reconstruction showing altered TBX5 and 1v-24 transgene expression domains in the region of the venous pole of Tbx1+/− embryos at E8.5; note the region of overlap (green). C, Left two panels: sagittal sections through E9.5 embryos carrying the iv2-4 transgene after in situ hybridization with riboprobes detecting lacZ and Tbx5 transcripts. Note the loss of a sharp boundary between 1v-24 and Tbx5 expressing cells in the dorsal pericardial wall of Tbx1+/− compared to Tbx1+/+ embryos. Right panel: Immunofluorescence on transverse sections showing overlap between 1v-24 expressing and TBX5 positive cells in the right venous pole. D, 3D reconstruction showing altered TBX5 and 1v-24 transgene expression domains in the region of the venous pole of Tbx1+/− embryos at E9.5; note the expanded region of overlap in the right venous pole (pink). E, Right lateral views of E17.5 X-gal stained hearts showing ectopic 1v-24 expressing cells in the region of the primary atrial septum (pas; arrow) of Tbx1+/− compared to Tbx1+/+ hearts. Note the arc of expression continuous with X-gal labelled cells in the dorsal wall of the common arterial trunk. F, Lateral and frontal views of X-gal positive cells in Mef2c-Cre;R26R hearts at E10.5 showing increased atrial recombination (arrow) on a Tbx1-deficient background. fg, foregut; la, left atrium; lv, left ventricle; pas, primary atrial septum; ra, right atrium; rv, right ventricle.
Abnormal development of the dorsal mesenchymal protrusion in Tbx1-deficient embryos

Considering the ectopic 1v24 expressing cells in the primary atrial septum of Tbx1\(^+/^-\) fetuses (Figure 4E), we sought to elucidate whether atrial septal precursors are affected by this anomaly, resulting in septal abnormalities. Developmentally, atrial septum formation is contingent on mesenchymal cells derived from endocardium and from an extracardiac structure known as the dorsal mesenchymal protrusion (DMP)\(^{34-46}\). The DMP is a SHF derivative that forms on the dorsal side of the common atrium, where the dorsal mesocardium persists at the venous pole, and contributes to the atroventricular mesenchymal complex\(^{45, 46}\). To test whether Tbx1-deficiency affects DMP development, we immunohistochemically analyzed Nkx2-5 and Tbx5 distribution in E10 wildtype and Tbx1\(^-/-\) embryos. In wildtype embryos, the Tbx5\(^+\) DMP was positioned ventrally to the developing lungbuds (Supplementary Figure 3)\(^{17, 47}\) and caudally to the Nkx2-5\(^+\) dorsal mesocardium, from where the DMP will protrude the atrial cavity at subsequent stages. Although loss of Tbx1 did not overtly affect the formation of DMP precursors, the orientation of the dorsal mesocardium relative to the venous pole was abnormal (Supplementary Figure 3), which could be expected to affect the mode of DMP entry into the heart. These findings suggest that the dorsal mesocardium defects observed at earlier stages might affect DMP morphogenesis and migration.

We next tested the requirement for Tbx1 in DMP precursors and the genetic fate map of DMP cells on a Tbx1\(^+/^-\) background. The Mef2c-Cre SHF line has been shown to contribute the DMP\(^{15, 17}\). We thus analyzed E10.5 Tbx1\(^+/-;\)Mef2c-Cre;R26R and Tbx1\(^-/-;\)Mef2c-Cre;R26R embryos (n=3 per genotype) and compared \(\beta\)-galactosidase-positive cells with the expression domain of LIM homeodomain transcription factor Isl1, which is expressed in SHF cells and in the DMP\(^{45}\). The wildtype DMP is comprised of Isl1 expressing cells (Figure 5A), all of which colocalized with \(\beta\)-galactosidase-positive mesenchyme, as expected. In Tbx1-deficient embryos, however, both the Isl1-positive and \(\beta\)-galactosidase-positive domain had expanded, while regions expressing neither protein were also observed (Figure 5A) revealing a heterogeneous genetic profile. These observations suggest that Tbx1 is required in DMP precursors for proper cell fate specification, as such impacting on DMP differentiation and atrial septum morphogenesis.

During atrial septation, the primary atrial septum (PAS) protrudes into common atrium towards the superior and inferior endocardial cushions to fuse\(^{15}\). As the Mef2c-Cre-positive lineage also contributes to the PAS, we deployed our genetic fate mapping approach to test whether this contribution is perturbed in the absence of Tbx1. The majority of the PAS as well as the mediastinal portion of atrial myocardium in E10.5 Tbx1\(^+/-;\)Mef2c-Cre;R26R embryos expressed \(\beta\)-galactosidase and Isl1 (Figure 5B, cranial and caudal sister sections). Interestingly, similar to our observations in the DMP, the PAS in Tbx1\(^+/-;\)Mef2c-Cre;R26R embryos displayed a heterogeneous gene expression profile, with \(\beta\)-galactosidase-positive and negative cells, which did not necessarily co-express Isl1 (Figure 5B). Moreover, a small portion of PAS cells expressed neither \(\beta\)-galactosidase or Isl1 (arrow in Figure 5B), suggestive of heterogeneous cellular contributions that could underlie disturbed development of cells normally contributing to atrial or atroventricular septation.
Figure 5. Venous pole development in $Tbx1^{+/−}$ hearts. A, Transverse sections at the level of the dorsal mesenchymal protrusion after immunofluorescence showing the distribution of cTnI, ISL1 and β-galactosidase in $Mef2c$-Cre;$R26R$ hearts at E10.5. Note the altered angle of the dorsal mesenchymal protrusion and broader distribution of β-galactosidase positive cells in $Tbx1^{+/−}$ compared to $Tbx1^{+/+}$ hearts. B, Transverse sections at the level of the forming primary atrial septum after immunofluorescence showing the distribution of ISL1 and β-galactosidase in $Mef2c$-Cre;$R26R$ hearts at E10.5. Note the reduced size and more heterogeneous cellular composition of the forming septal structure in $Tbx1^{+/−}$ compared to $Tbx1^{+/+}$ hearts. dmp; dorsal mesenchymal protrusion; la, left atrium; ra, right atrium.

Discussion

In this study, we have demonstrated that $Tbx1$ regulates the segregation of second heart field cardiac progenitor cells to the poles of the elongating heart tube. In particular, we find that $Tbx1$ is required for the anterior contribution of a population of cells from the posterior SHF that normally gives rise to future subpulmonary myocardium. Furthermore, in the absence of $Tbx1$, these cells remain in the pSHF resulting in anomalous development of the dorsal mesenchymal protrusion. By identifying a role for $Tbx1$ in progenitor cell segregation our study provides new insights into DiGeorge syndrome phenotypes and the etiology of congenital heart defects. Furthermore, our results have implications for the mechanisms by
which cells are directed to divergent cellular fates from common progenitor pools during organogenesis.

The transcriptional profile of Tbx1-dependent future subpulmonary myocardium

Previous work has demonstrated that loss of Tbx1 interferes with the development of subpulmonary myocardial precursors and with the migration of cardiac NC cells to the developing OFT. The clinical significance of subpulmonary myocardial precursors is reflected by the high incidence of congenital heart defects affecting subpulmonary myocardial cells, notably tetralogy of Fallot and pulmonary atresia. Here, we show that future subpulmonary myocardium displays a distinct transcriptional profile in the iOFT wall, which is characterized by the enriched expression of genes many of which play pivotal roles in neuronal and neural crest development, migration and chemotaxis. These include genes encoding EFDH1, required for neurite outgrowth during neuron projection development, Barx1 and Dlx1, required for the development of neural crest-derived mesenchyme during craniofacial morphogenesis and Sema3c, a signalling molecule required for axonal guidance and cardiac neural crest influx into the outflow tract. Sema3c was previously shown to be enriched in subpulmonary myocardium through its identification as a gene adjacent to the integration site of the enhancer trap transgene expressed in this region of the heart. The presence of two probe sets detecting Sema3c in the top 18 enriched probe sets as well as 25 genes detected by two or more probe sets in the top 155 provide strong support for the effectiveness of our microarray screen. Furthermore, transcripts encoding Nrp2, a coreceptor of Sema3c, also had two hits among the top 18 enriched probe sets. PPARG has also been shown to impact on neural crest cell fate in zebrafish embryos. Thus many of the genes enriched in the iOFT are required for or have the potential to facilitate the influx of cardiac NC cells into the OFT and contribute to OFT septation. In agreement with this role, our in situ hybridization and genetic lineage analysis indicate that expression of these genes in the iOFT wall is severely reduced or absent in Tbx1 mutant embryos in which the OFT fails to divide. Together, our findings highlight that the development and addition of the transcriptionally distinct future subpulmonary myocardium to the OFT is Tbx1-dependent, and suggest that decreased levels of proteins normally enriched in the developing iOFT could contribute to the etiology of human conotruncal congenital heart defects including tetralogy of Fallot and common arterial trunk. These iOFT enriched transcripts thus identify new candidate genes for conotruncal congenital heart disease. In support of such a conclusion Barx1 has recently been identified as a candidate gene in tetralogy of Fallot based on a systematic screen for rare polymorphisms in human patients.

The role of Tbx1 in segregating SHF cell contributions to the poles of the heart tube

Considering the impact that Tbx1 deficiency has on subpulmonary myocardium, we explored the early effects of Tbx1 deficiency on SHF expansion and deployment, and identified novel aspects of Tbx1 function in the SHF. Recent studies have addressed the origin of subpulmonary myocardium in the mouse embryo. Experiments based on retrospective clonal analysis, genetic tracing and Dil labelling have revealed that cells in the posterior
region of the SHF in the dorsal pericardial wall contribute not only to the venous pole but also to future subpulmonary myocardium at the arterial pole of the heart. Our results identify Tbx1 as a regulator of this process and demonstrate that an entire genetic lineage derived from Hoxb1 and Hoxa1 expressing progenitor cells fails to contribute or contributes minimally to the short narrow outflow tract of Tbx1 mutant embryos. Hox gene function is required for anterior-posterior patterning of the pharyngeal region and SHF. Hypoplasia of the caudal pharyngeal region characterises the Tbx1 mutant phenotype and many of the structures derived from this part of the embryo are affected in 22q11.2DS patients, haploinsufficient for a multigene deletion including TBX1. In the mouse, pharyngeal hypoplasia becomes apparent during E8.5 when bilateral pharyngeal arches develop in an anterior-posterior sequence. In Tbx1 mutant embryos only the first and second arches develop and caudal arch morphogenesis fails. Our observation that anterior displacement of progenitor cells from the caudal region of the SHF fails from the 7 somite stage (E8.5) is coincident with the onset of caudal pharyngeal arch morphogenesis, suggesting that formation of the caudal arches is intrinsically coupled with anterior expansion of cells from the pSHF. Furthermore, the time at which loss of Tbx1 impacts on segregation of arterial pole progenitors from the pSHF is consistent with elegant data defining the temporal requirement for Tbx1 in different developmental processes. Baldini and colleagues used a conditional Tbx1 allele and inducible Cre recombinase to reveal that Tbx1 is required from mid-E8.5 for conotruncal development. Our results now provide a mechanistic explanation for why this timepoint is critical. Finally, given the Tbx1-dependent contribution of a population of Hoxb1 and Hoxa1 expressing progenitor cells from the pSHF to the OFT, future experiments will investigate whether Hox transcription factors function co-operatively with Tbx1 in subpulmonary myocardial progenitor cells.

Failure of the anterior expansion of cells from the pSHF in the absence of Tbx1 impacts on dorsal pericardial wall and early heart development prior to OFT morphogenesis. Indeed the dorsal pericardial wall in mutant embryos is foreshortened as evidenced by a reduced 1v-24 expressing cell population in Tbx1−/− embryos and consistent with the proliferative role ascribed to TBX1. Quantitative evaluation of proliferation using three-dimensional reconstructions of BrdU-incorporation in the developing E8.25 1v-24 dorsal pericardial wall furthermore revealed that the left caudal SHF was slightly more affected than the right side. Pitx2c, encoding a bicoid-like homeobox transcription factor, is expressed in the left SHF and iOFT and is involved in regulating the left-sided SHF contribution to the heart. Loss of Pitx2c in mice causes conotruncal anomalies as well as defects in atrial and atroventricular septation. Furthermore, left venous pole structures such as the left atrial myocardium and pulmonary vein have been shown to be clonally related to pulmonary myocardium, in agreement with the fact that both arterial and venous pole defects have been observed in mouse embryos lacking Pitx2c. Interestingly, the proliferation rate in the left SHF seems to be maintained by a TBX1-binding enhancer element located in the Pitx2 gene. The proliferative differences we observed in the left SHF of Tbx1−/− embryos suggest that this population of SHF progenitor cells is particularly Tbx1-dependent.

Failed anterior expansion of cells from the pSHF also impacts on early heart tube formation by delaying dorsal mesocardial development and rupture. Our results demonstrate that loss of Tbx1 delays the detachment of the heart from the coelomic wall, and thus the closure of the developing heart, which now occurs at E9.25 rather than E8.5. An abnormal dorsal mesocardium-like connection was observed near the pulmonary pit, and an expanded Nkh2-5 expression domain and a cTnl domain connecting IFT and OFT are
observed, consistent with premature differentiation of cells within the SHF in the absence of Tbx1. Furthermore, analysis of the expression profiles of the 1v-24 transgene, Tbx5 and Osr1 suggests that in mutant embryos the normal boundary between the aSHF and pSHF has broken down. In contrast to wildtype littermates, a significant part of the anterior SHF in Tbx1−/− embryos co-expresses 1v-24 and Tbx5, consistent with elevated Tbx5 levels in the dorsal pericardial wall of Tbx1 null mutant reported by Liao and colleagues.

What is the fate of pSHF cells that fail to contribute to the arterial pole in Tbx1−/− embryos? Dil labelling experiments revealed that after the 7 somite stage labelled pSHF cells remain in the pool of venous pole progenitor cells in Tbx1 mutant embryos and also contribute to venous pole myocardium. This observation is supported and extended by our finding that arterial pole markers such as the 1v-24 enhancer trap and Mef2c-AHF-Cre transgenes are abnormally activated or expanded in the venous pole of Tbx1 mutant hearts, in particular in the region of mediastinal myocardium. This conclusion is based on 3D-reconstructions from early stages of heart development when 1v-24 positive cells contribute along with Tbx5 positive cells to atrial myocardium across the dorsal mesocardium and is associated with abnormal cell contributions and development of the dorsal mesenchymal protrusion (DMP) as discussed below. In the absence of Tbx1, therefore, failure of anterior movement of pSHF cells is associated with an apparent fate change from OFT myocardium to that of atrial or mediastinal myocardium. Tbx1 thus regulates divergent fate generation from a common progenitor pool in the pSHF.

**Tbx1 promotes DMP specification and morphogenesis**

As most 22q11.2DS patients display anomalies of the great arteries, the role of the Tbx1 during SHF and arterial pole development has been vigorously explored over the years. Tbx1 fate mapping studies revealed that venous pole structures, including atrial myocardium, septum primum and the dorsal mesenchymal protrusion (DMP) are partly derived from a Tbx1Cre-positive posterior SHF lineage. Nevertheless, Tbx1 has never been attributed a major role during venous pole development.

The DMP is a pSHF-derived extracardiac mesenchymal structure, which muscularizes to give rise to the floor of the oval fossa. Development of the DMP is intimately related to the dorsal mesocardium, which borders the pulmonary pit from where the DMP protrudes into the common atrium. SHF fate map studies have demonstrated the contribution of the Mef2c-AHF-Cre and Isl1-Cre lineage to the DMP and development of the dorsal mesenchymal protrusion is perturbed in embryos mutant for regulators of pSHF deployment, including components of the WNT and BMP signalling pathways, resulting in AVSD. We found that, even though the dorsal mesocardium was aberrantly positioned, the DMP still protrudes from the dorsal aspect of the heart into the atrial chamber in Tbx1 null embryos. Whereas Isl1 and the Mef2c-Cre-positive lineages colocalize in the wildtype DMP, loss of Tbx1 results in the presence of a mixed population of Isl1-positive and Mef2c-Cre-derived cells. TBX1 has been reported to negatively regulate Mef2c expression in the SHF, which could contribute to the molecular heterogeneity we observe in the DMP and PAS. AVSD is only rarely observed in 22q11.2DS patients in which conotruncal congenital heart defects dominate. Our findings suggest an explanation for occasional venous pole defects in 22q11.2DS patients and provide the first mechanistic insights into how cardiac progenitor cells are segregated to the cardiac poles during early heart development.
Perspectives

Recent work has considerably expanded our developmental and molecular insights into the transformation of an unseptated OFT into separated intrapericardial arterial trunks. The observation that *Tbx1* mutation results in failure of pSHF cells to expand anteriorly and support OFT development contributes to our understanding of the complex morphogenic processes and intricate balance between numerous molecular networks during SHF diversification, deployment and differentiation. Future work will investigate the molecular mechanisms by which *Tbx1* regulates divergent cell fates from a common progenitor cell population in the SHF.

Acknowledgements

We thank Niels Hofschreuder and Mrigendra Subba for technical assistance.

Funding

This work was supported by the European Commission under the FP7 Integrated Project CardioGeNet (HEALTH-2007-B-223463 to V.M.C. and R.G.K.), the Fondation pour la Recherche Médicale (Equipe FRM 2011 to R.G.K.), Agence National pour la Recherche (R.G.K.) and the Netherlands Organization for Scientific Research (Vidi grant 864.05.006 to V.M.C. and Mosaic grant 017.004.040 to M.S.R.).
Supplementary Figure 1. Regionalised OFT expression of iOFT enriched genes identified by microarray screen and Hoxa1-enhIII-Cre;R26R genetic lineage in wildtype and Tbx1-/- embryos at E10.5 and E13.5. A-C, Ventral views of E10.5 wildtype mouse embryos after whole-mount in situ hybridization with Barx1, Dlx2 and Nrp2 riboprobes with the hearts removed up to most of the OFT, showing transcript accumulation in the iOFT wall. D-F, In situ hybridisation showing Barx1, Dlx2 and Nrp2 enrichment in the iOFT wall on transverse sections through E10.5 wildtype embryos. G, Hoxa1-enhIII-Cre;R26R lineage in wildtype and Tbx1-/- mouse embryos, revealing loss of X-gal staining in subpulmonary myocardial precursors. dpw, dorsal pericardial wall; fg, foregut; la, left atrium; oft, outflow tract; ra, right atrium.
Supplementary Figure 2. Examples of Dil labelling in the arterial and venous poles. Transverse sections and whole mount views of embryos harvested after 18 hours of embryo culture (t=18) showing Dil labelled cells close to the outflow tract (upper lane) or venous pole of the heart (lower lane). avc, atrioventricular canal; dpw, dorsal pericardial wall; fg, foregut; la, left atrium; lsv, left sinus venosus; oft, outflow tract; ra, right atrium.

Supplementary Figure 3. Morphological changes of the Tbx5 DMP in Tbx1-deficient embryos. A, Sagittal section of an E9.5 wildtype mouse embryos showing the location of the DMP (arrowhead) population relative to the left atrium (la) and developing lungbuds (lb). B, Abnormal positioning of the DMP in Tbx1−/− embryos (arrow). dmp; dorsal mesenchymal protrusion; la, left atrium; lb, lungbud; lv, left ventricle; oft, outflow tract.
References

4. Vincent SD, Buckingham ME. How to make a heart: The origin and regulation of cardiac progenitor cells. *Current topics in developmental biology*. 2010;90:1-41
15. Briggs LE, Kakarla J, Wessels A. The pathogenesis of atrial and ventriculoatrial septal defects with special emphasis on the role of the dorsal mesenchymal protrusion. *Differentiation; research in biological diversity*. 2012;84:117-1322
19. Domínguez JM, Meilhac SM, Bland YS, Buckingham ME, Brown NA. Asymmetric fate of the posterior part of the second heart field results in unexpected left/right contributions to both poles of the heart. *Circulation research*. 2012;111:1323-1335


31. de Boer BA VF, van den Berg G, Ruijter JM. Measurement of the shortest path length: Distance estimation within the 3d borders of a tissue of interest. *Image Anal Stereol.* 2010;29:53-60


40. Xu H, Morishima M, Wylie JN, Schwartz RJ, Bruneau BG, Lindsay EA, Baldini A. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development*. 2004;131:3217-3227


45. Snarr BS, O’Neal JL, Chintalapudi MR, Wirrig EE, Phelps AL, Kubalak SW, Wessels A. Isl1 expression at the venous pole identifies a novel role for the second heart field in cardiac development. *Circulation research*. 2007;101:971-974


49. Tomina M, Tomooka Y. Novel genes cloned from a neuronal cell line newly established from a cerebellum of an adult p53(-/-) mouse. *Biological and biophysical research communications*. 2002;297:473-479


