The T-Box transcription factor Tbx5 is required for the patterning and maturation of the murine cardiac conduction system

Ivan P. G. Moskowitz1,2, Anne Pizard1,9, Vickas V. Patel13,4, Benoit G. Bruneau5,6, Jae B. Kim1, Sabina Kupershmidt7, Dan Roden8, Charles I. Berul4, Christine E. Seidman1,9 and Jonathan G. Seidman1,*

1Department of Genetics, Harvard Medical School and Howard Hughes Medical Institute, Boston, MA 02115, USA
2Department of Pathology and Cardiac Registry, Children’s Hospital and Harvard Medical School, Boston, MA 02115, USA
3Molecular Cardiology Research Center and Section of Cardiac Electrophysiology, University of Pennsylvania, Philadelphia, PA 19104, USA
4Department of Cardiology, Children’s Hospital and Department of Pediatrics, Harvard Medical School, Boston, MA 02115, USA
5Programs in Cardiovascular Research and Developmental Biology, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada
6Department of Molecular and Medical Genetics, University of Toronto, Toronto, ON M5S 1A8, Canada
7Departments of Anesthesiology and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6602, USA
8Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-6602, USA
9Division of Cardiology, Brigham and Women’s Hospital, and Howard Hughes Medical Institute, Boston, MA 02115, USA

*Author for correspondence (e-mail: seidman@genetics.med.Harvard.edu)

Accepted 4 May 2004
Development 131, 4107-4116 Published by The Company of Biologists 2004 doi:10.1242/dev.01265

Summary

We report a critical role for the T-box transcription factor Tbx5 in development and maturation of the cardiac conduction system. We find that Tbx5 is expressed throughout the central conduction system, including the atrioventricular bundle and bundle branch conduction system. Tbx5 haploinsufficiency in mice (Tbx5del/+) is expressed haploinsufficiency caused patterning defects of both the left and right ventricular bundle branches, including absence or severe abnormalities of the right bundle branch. Absence of the right bundle branch correlated with right-bundle-branch block by ECG. Deficiencies in the gap junction protein gene connexin 40 (Cx40), a downstream target of Tbx5, did not account for morphologic conduction system defects in Tbx5del/+ mice. We conclude that Tbx5 is required for Cx40-independent patterning of the cardiac conduction system, and suggest that the electrophysiologic defects in Holt–Oram syndrome reflect a developmental abnormality of the conduction system.

Key words: Cardiac, Conduction, Tbx5, Mouse

Introduction

The cardiac conduction system comprises a specialized subset of myocardial cells essential for the coordinated contraction of the multi-chambered vertebrate heart. Composed of the sinoatrial node, atrioventricular node, atrioventricular bundle and ventricular bundle branches, the central cardiac conduction system and associated peripheral Purkinje fibers have distinct electrophysiological, morphological and transcriptional profiles from the surrounding working myocardium (reviewed by Moorman et al., 1998; Gourdie et al., 2003). While some genes involved in the biologic functions of the mature conduction system have been identified, few genes required for the pattern formation of the evolutionarily conserved structure of the vertebrate cardiac conduction system, or for the differentiation of the specialized cells that comprise the conduction system, are known (Nguyen-Tran et al., 2000) (reviewed by Cheng et al., 2003).

A molecular marker of the conduction system has been reported, in a study in which selective β-galactosidase expression in the cardiac conduction system was established by placing the lacZ gene downstream of the potassium channel minK promoter (Kupershmidt et al., 1999; Kondo et al., 2003). In adult mice, β-galactosidase expression from the minK: lacZ allele (minKlacZ+/−) demarcates nuclei of cells in the mature central conduction system, including the sinoatrial node, the atrioventricular node, the atrioventricular bundle and the right and left bundle branches, from the working myocardium. In concert with in-vivo electrophysiologic techniques (Gehrmann and Berul, 2000), minKlacZ+/− mice enable detailed morphological and functional analyses of the mammalian conduction system.

We employed these tools to study the role of the transcription factor Tbx5 in the development of the cardiac conduction system. Tbx5 belongs to the T-box gene family, the members of which share a highly conserved 180-amino-acid domain required for DNA binding (Herrmann et al.,
Mutations in several human T-box genes cause dominant disorders with a variety of developmental malformations (Bamshad et al., 1997; Merscher et al., 2001). In humans, haploinsufficiency of functionally null Tbx5 mutations causes Holt–Oram syndrome (Basson et al., 1997; Li et al., 1997; Basson et al., 1999), manifest by congenital heart defects, conduction-system abnormalities and upper-limb deformities. Morphologic cardiac defects are most commonly atrial septal defects of the secundum type, although a range of structural abnormalities has been reported (Basson et al., 1997; Li et al., 1997, Basson et al., 1999). Common electrophysiologic abnormalities found in Holt–Oram syndrome include progressive atrioventricular block, bundle-branch block and sick sinus syndrome (Basson et al., 1994). Some Holt–Oram syndrome patients have electrophysiologic defects in the absence of structural heart defects (Basson et al., 1994; Newbury-Ecob et al., 1996), thereby suggesting a direct role for Tbx5 in the conduction system that is independent of this transcription factor’s function in cardiac septation.

Mice lacking a functional Tbx5 allele (Tbx5<del/+>) were constructed to understand how Tbx5 haploinsufficiency disturbs limb and cardiac development (Bruneau et al., 2000). Briefly, 6-limb ECGs with a right precordial lead were obtained using 25-gauge subcutaneous electrodes in unanesthetized newborn mice. Adult mice were lightly anesthetized with pentobarbital (0.033 mg/kg IP) and 6-limb lead ECGs with a right precordial lead were obtained using 25-gauge subcutaneous electrodes. For in vivo electrophysiology studies in adult mice, a jugular vein cutdown was performed and an octapolar 2-French electrode catheter (CIBer mouse-EP; NuMED, Inc.) was placed in the right atrium and ventricle under electrogram guidance to confirm catheter position. Recording of atrioventricular bundle potentials was confirmed by the presence of a triphasic signal on one of the distal bipolar electrograms, and was accomplished using simultaneous multielectrodes and persistent catheter manipulation (Maguire et al., 2000).

**Electrophysiology study**

In-vivo electrophysiologic studies were performed in all adult mice using standard pacing protocols to assess atrial and ventricular conduction, refractoriness and arrhythmia inducibility (Maguire et al., 2000). ECG channels were filtered between 0.5 and 250 Hz and intracardiac electrograms filtered between 5 and 400 Hz. Signals were displayed on an oscilloscope and simultaneously recorded through an A-D converter at a digitization rate of 2 kHz (MacLab Systems, Inc.) for offline analysis. ECG intervals were measured in 6-limb leads and a right precordial lead by two independent observers, blinded to genotype.

**β-galactosidase (lacZ) activity**

Dissected hearts were fixed at 4°C in 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) for 1 hour. Following a PBS rinse, hearts were permeabilized in 0.01% sodium deoxycholate, 0.02% NP-40, and 2 mmol/L MgCl2 in 0.1 mol/L PBS for 30 minutes. Following a PBS rinse, hearts were stained in permeabilization solution plus 1 mg/ml X-Gal, 5 mmol/L potassium ferrocyanide, and 5 mmol/L ferrocyanide at 37°C overnight. Hearts were washed at least three times with PBS and post-fixed and stored in 4% paraformaldehyde at 4°C.

**In-situ hybridization**

Whole-mount in-situ hybridization was performed as previously described (Bruneau et al., 2001). To insure probe access to the entire heart, the systemic and pulmonary veins were opened and the ventricular apex was removed. In-situ hybridization was performed on slide sections with the following modifications. Newborn hearts were dissected, washed once in PBS, fixed overnight in 4% paraformaldehyde in PBS and either stored at room temperature in ethanol 70% or immediately paraffin embedded and sectioned. In-situ hybridization on paraffin wax tissue sectioned at 5 μm was performed using radioactive Tbx5 probe (Bruneau et al., 2001) labeled with 35S-UTP according to previously described protocol (Sibony et al., 1995).

**Materials and methods**

**Animals**

Creation of Tbx5 and Cx40 knockout mice has been previously described (Simon et al., 1998; Bruneau et al., 2001). All mice were analyzed as 129 SV inbred. All protocols conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care and the Children’s Hospital Animal Care and Use Committee.

**ECGs**

Surface ECGs were recorded from unanesthetized Tbx5<del/+> newborn mice (n=14) and compared with those for littermate wild-type mice (n=22). Surface ECG recordings and complete in-vivo electrophysiologic studies (EPS) were recorded from 14-week-old adult Tbx5<del/+> mice (n=21) and compared with those for wild-type littermate mice (n=13). Surface ECG recordings were also recorded from 14-week-old adult Cx40<−/−/minKlacZ/+ and Tbx5<del/+>/minKlacZ/+ mice (n=19) and compared with those for age-matched wild-type mice (n=14).

Protocols for the surface ECG and in-vivo mouse electrophysiology studies are previously described (Berul et al., 1996; Maguire et al., 2000). Briefly, 6-limb ECGs with a right precordial lead were obtained using 25-gauge subcutaneous electrodes in unanesthetized newborn mice. Adult mice were lightly anesthetized with pentobarbital (0.033 mg/kg IP) and 6-limb lead ECGs with a right precordial lead were obtained using 25-gauge subcutaneous electrodes. For in vivo electrophysiology studies in adult mice, a jugular vein cutdown was performed and an octapolar 2-French electrode catheter (CIBer mouse-EP; NuMED, Inc.) was placed in the right atrium and ventricle under electrogram guidance to confirm catheter position. Recording of atrioventricular bundle potentials was confirmed by the presence of a triphasic signal on one of the distal bipolar electrograms, and was accomplished using simultaneous multielectrodes and persistent catheter manipulation (Maguire et al., 2000).
Results

Tbx5 is expressed in the developing cardiac conduction system

The murine conduction system was visualized in mice heterozygous for the mink: lacZ allele (minKlacZ/+ ) after staining cardiac tissue with X-gal to detect β-galactosidase activity (Fig. 1A,B). Heterozygous minKlacZ/+ mice have no morphologic or physiologic abnormalities (Kuperschmidt et al., 1999) and β-galactosidase activity demarcates cells of the mature and developing conduction system. The atrioventricular junction was directly viewed after removing both atria (Fig. 1B) and two rings of staining were observed, one surrounding the tricuspid annulus and the other surrounding the mitral annulus. This circumferential pattern is consistent with classic histological and more recent molecular descriptions of the developing atrioventricular conduction system in the vertebrate heart (Wenink 1976; Wessels et al., 1992; Coppen et al., 1999; Davis et al., 2001).

Tbx5 expression was assessed by whole-mount in-situ hybridization in wild-type mouse hearts. Previous work demonstrating high levels of Tbx5 expression in both atria and the left ventricle and low levels in the right ventricle was confirmed (data not shown) (Bruneau et al., 1999; Hatcher et al., 2000). Abundant Tbx5 expression was also observed in rings surrounding both the tricuspid annulus and mitral annulus in the atrioventricular canal (Fig. 1C). Tbx5 expression appeared to co-localize with minK expression in both these rings (cf. Fig. 1B,C). No expression was apparent in the atrioventricular valves.

To clarify the relationship between conduction and Tbx5-expressing cells, Tbx5 expression was examined at higher resolution in histologic sections. The conduction system was identified by visualizing β-galactosidase activity in sections from minKlacZ/+ mouse hearts (Fig. 1E) or connexin 40 expression in sections from wild-type mouse hearts (Fig. 1G). Connexin 40 expression was comparable to that previously described (Coppen et al., 2003). In newborn minKlacZ/+ mouse hearts, β-galactosidase activity was present in the atrioventricular bundle (arrow), and the ventricular bundle branches (arrowheads). Scale bar: 100 μm. In-situ hybridization with a Tbx5 probe in wild-type hearts (Fig. 1F) demonstrated expression in the atrioventricular bundle (arrow) and bundle branch (arrowheads) conduction system. In-situ hybridization with a connexin 40 probe (G) and Tbx5 probe (H) in sequential sections from the same wild-type heart demonstrates overlapping expression in the atrioventricular bundle (arrow) and bundle branch (arrowheads) conduction system.

Tbx5 haploinsufficiency prevents atrioventricular canal conduction system maturation

Given Tbx5 expression in the central conduction system of newborn mice and ECG abnormalities in Tbx5del/+ mice (Bruneau et al., 2001), we hypothesized that Tbx5 haploinsufficiency might affect conduction system...
Fig. 2. Conduction system maturation failure in Tbx5<sup>del/+</sup> mice. The atrioventricular canal conduction system in minKlacZ/+ (A,B) and Tbx5<sup>del/+minKlacZ/+</sup> (C,D) hearts was studied in newborn (A,C) and adult (B,D) mice. The atria were removed and the atrioventricular canal was viewed from the superior/posterior, with the tricuspid annulus and mitral annulus on the right and the mitral annulus on the left. Rings of specialized conduction cells observed in the atrioventricular canal of newborn minKlacZ/+ mouse hearts (A) matured into a well-defined atrioventricular node (arrow) and atrioventricular bundle in adult minKlacZ/+ mouse hearts (B). (A) Scale bar: 200 μm. (B) Scale bar: 800 μm. Rings of specialized conduction cells in the atrioventricular canal of newborn Tbx5<sup>del/+minKlacZ/+</sup> mouse hearts (C) failed to mature into a discrete atrioventricular node or atrioventricular bundle in adult Tbx5<sup>del/+minKlacZ/+</sup> mouse hearts (D). Instead the neonatal pattern (rings of specialized conduction tissue) was maintained. Arrow denotes expected location of the atrioventricular node.

development. Hearts from compound heterozygous Tbx5<sup>del/+minKlacZ/+</sup> (see Materials and methods) and minKlacZ/+ mice were compared after X-gal staining. At the atrioventricular junction, hearts from newborn minKlacZ/+ (Fig. 2A) and Tbx5<sup>del/+minKlacZ/+</sup> (Fig. 2C) mice demonstrated similar rings of specialized conduction tissue surrounding both the tricuspid annulus and mitral annulus. This same pattern of β-galactosidase activity was observed in all minKlacZ/+ (n=25) and Tbx5<sup>del/+minKlacZ/+</sup> (n=22) newborn mouse hearts.

Expression of β-galactosidase in hearts from 14-week-old adult minKlacZ/+ mice revealed a mature conduction system with a strikingly different structure than that observed in the newborn mouse (cf. Fig. 2A,B). Adult minKlacZ/+ hearts no longer showed the atrioventricular rings found in newborns but instead exhibited a more restricted expression of β-galactosidase activity consolidated in the atrioventricular node and atrioventricular bundle. The striking morphologic changes in the conduction system between newborn and adult mice indicate that postnatal development of the electrophysiologic system is essential for establishment of the mature structures found in adults. By contrast, adult Tbx5<sup>del/+minKlacZ/+</sup> mice (Fig. 2D) did not demonstrate restricted expression of β-galactosidase activity. There was no localized staining of the atrioventricular node and bundle, and instead there was a persistent expression of β-galactosidase activity in both atrioventricular rings, which remarkably resembled the pattern observed in hearts from newborn mice (Fig. 2C).

To ascertain whether Tbx5 had functional, as well as morphological, roles in atrioventricular conduction system maturation, we evaluated the consequences of Tbx5 haploinsufficiency using surface ECG monitoring and in-vivo electrophysiology. Surface ECGs were recorded from unanesthetized, newborn animals and lightly anesthetized adult animals. The PQ interval, which detects the time for electrical propagation from sinoatrial node to ventricular myocardium, is significantly longer in both newborn and adult Tbx5<sup>del/+</sup> mice compared with age-matched wild-type mice. The PQ interval is not significantly different between newborn Tbx5<sup>del/+</sup> and wild-type mice, but was significantly longer in adult Tbx5<sup>del/+</sup> than in adult wild-type mice. Note that the PQ interval of wild-type mice shortens with age, whereas the PQ interval of Tbx5<sup>del/+</sup> mice fails to undergo physiologic shortening in the postnatal period. The QRS interval is significantly longer in both newborn and adult Tbx5<sup>del/+</sup> mice compared with age-matched wild-type mice.

Comparison of PQ intervals in neonatal and adult wild-type and Tbx5<sup>del/+</sup> mice (Table 1) demonstrates that the time required for electrical propagation from sinoatrial node to ventricular myocardium (Fig. 3A) normally decreases with maturation, even though the signal must travel further in the larger adult heart. In wild-type mice, the PQ interval becomes significantly shorter between newborn and adult animals. The AH interval, which detects the time for electrical conduction from the sinoatrial node to the atrioventricular node, is also prolonged in wild-type mice compared with age-matched adult animals (Fig. 3A). The AH interval is significantly longer in both newborn and adult Tbx5<sup>del/+</sup> mice compared with age-matched wild-type mice. The AH interval is significantly longer in Tbx5<sup>del/+</sup> mice than in wild-type mice. The AH interval is significantly longer in Tbx5<sup>del/+</sup> mouse hearts no matter what the type of atrial septal defect (primum or secundum) altered the PQ interval of the wild-type (<41.9±1.0 ms; <0.01) (Fig. 3B, Table 1). Neither the presence of the minKlacZ allele nor the type of atrial septal defect (primum or secundum) altered the PQ interval of the wild-type or Tbx5<sup>del/+</sup> newborn mice (ASD primum 50.7±5.6 ms vs. ASD secundum 52.5±5.3 ms; <0.01).

Table 1. Conduction intervals from wild-type and Tbx5<sup>del/+</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>Wild-type adult</th>
<th>Tbx5&lt;sup&gt;del/+&lt;/sup&gt; adult</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQ interval</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>48.0±7.6</td>
<td>41.9±1.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adult</td>
<td>50.7±5.8</td>
<td>49.1±4.6</td>
<td>&lt;0.46</td>
</tr>
<tr>
<td>AH interval</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Newborn</td>
<td>37.0±1.5</td>
<td>42.3±6.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Adult</td>
<td>11.5±1.5</td>
<td>10.3±2.0</td>
<td>&lt;0.69</td>
</tr>
</tbody>
</table>

† Wild-type PQ interval: Tbx5<sup>del/+</sup> PQ interval.
‡ Wild-type QRS interval: Tbx5<sup>del/+</sup> QRS interval.
§ The AH and HV interval and P-wave duration were compared in adult wild-type and Tbx5<sup>del/+</sup> mice. The HV interval and P-wave duration are not significantly different between wild-type and Tbx5<sup>del/+</sup> adult mice. However, the AH interval is significantly longer in Tbx5<sup>del/+</sup> mice than in wild-type adult mice.
the newborn and adult periods (48.0±7.6 ms vs. 41.9±1.0 ms; P<0.001) (Fig. 3B, Table 1). However, in Tbx5<sup>del/+</sup> mice, the PQ interval does not change significantly between the newborn and adult periods (50.7±5.8 ms vs. 49.1±4.6 ms; P>0.46) (Fig. 3B, Table 1), suggesting that a failure of normal maturation accounts for the prolonged PR interval in adult Tbx5<sup>del/+</sup> mice.

In-vivo electrophysiology studies were performed in adult animals to localize the functional deficit in Tbx5<sup>del/+</sup> mice to the atrium, atrioventricular node, or the atrioventricular bundle. The AH interval (representing conduction propagation through the atrium and the atrioventricular node) and the HV interval (representing conduction propagation through the atrioventricular bundle and proximal bundle branches) were measured separately (Fig. 3A). The HV interval of adult Tbx5<sup>del/+</sup> mice and wild-type mice were not significantly different (10.3±2.0 ms vs. 11.5±1.5 ms; P>0.69; n=6). However, the AH interval of adult Tbx5<sup>del/+</sup> mice was significantly longer than the AH interval of adult wild-type mice (42.3±6.0 ms vs. 37.0±1.5 ms; P<0.001; n=6), demonstrating a functional deficit of atrial or atrioventricular node conduction in adult Tbx5<sup>del/+</sup> mice. To distinguish between these, propagation of the electrical signal in the atria (atrial depolarization or P-wave) was directly measured by analyzing P-wave duration. P-wave duration was not significantly different between adult Tbx5<sup>del/+</sup> mice and wild-type mice (15.9±2.5 ms vs. 15.0±1.1 ms; P>0.36; n=12). Taken together, the normal P-wave duration and AH interval prolongation suggests that the PQ prolongation in Tbx5<sup>del/+</sup> mice is the result of a defect of the atrioventricular node or its connection with the atria or atrioventricular bundle.

**Tbx5 haploinsufficiency causes atrioventricular bundle and bundle branch conduction system patterning defects**

We used β-galactosidase activity in minK<sup>lacZ/+</sup> mice to characterize the morphology of the postnatal ventricular conduction system, including the atrioventricular bundle and bundle branches. The left bundle branch, lying on the left side of the interventricular septum, was identified in all newborn minK<sup>lacZ/+</sup> mouse hearts (n=25), as a broad sheet of cells with β-galactosidase activity (Fig. 4B, arrowhead). This pattern was consistent with classic histological descriptions of the left bundle branch in young mouse hearts (Fig. 4B) (Lev and Thaemert, 1973). In all adult minK<sup>lacZ/+</sup> mouse hearts (n=18), β-galactosidase activity was more discretely concentrated into a bundle branch fascicle. In all adult minK<sup>lacZ/+</sup> mouse hearts (n=18), β-galactosidase activity also demarcated a well-defined atrioventricular bundle, located on the crest of the interventricular septum (Fig. 4C, red arrowhead).

The effect of Tbx5 haploinsufficiency on atrioventricular bundle and left ventricular bundle branch morphology was evaluated in Tbx5<sup>del/+</sup>/minK<sup>lacZ/+</sup> mice (Fig. 4). In all newborn Tbx5<sup>del/+</sup>/minK<sup>lacZ/+</sup> hearts (n=22), β-galactosidase activity in the left ventricle was indistinguishable from that in newborn minK<sup>lacZ/+</sup> hearts (Fig. 4D). By contrast, in adult Tbx5<sup>del/+</sup>/minK<sup>lacZ/+</sup> hearts, β-galactosidase activity (Fig. 4E) was present in a broader sheet of cells, exhibiting a pattern reminiscent of the immature left bundle branch conduction system. Absence of a consolidated, discrete band of cells in the left ventricular bundle branch was found in all adult Tbx5<sup>del/+</sup>/minK<sup>lacZ/+</sup> hearts examined (n=15). Furthermore, the
The atrioventricular bundle appeared foreshortened in all adult Tbx5del/+ hearts examined (n=15).

The right bundle branch, present on the surface of the right side of the interventricular septum, had a different structure than that of the left bundle branch (Fig. 4F-H). In newborn minKlacZ/+ mouse hearts (n=25), β-galactosidase activity identified a loose bundle of cells adjacent to the septal band and anterior papillary muscle of the right ventricle, consistent with classic histological descriptions (Lev and Thaemert, 1973) of the right bundle branch (Fig. 4G). The right bundle branch in adult minKlacZ/+ mouse hearts (Fig. 4H) was more well-defined than that in newborn mice, displaying a highly stereotyped pattern in all 18 hearts examined that was remarkably analogous to that of the adult human right bundle branch.
Severe patterning defects of the right bundle branch were observed in newborn and adult Tbx5<sup>del/+minKlacZ/+</sup> mice. In all newborn Tbx5<sup>del/+minKlacZ/+</sup> mouse hearts studied (n=22), there was a paucity of cells with β-galactosidase activity in the right ventricle. The most severe cases (10/22 hearts) had complete absence of a discrete right bundle branch on the right ventricular septal surface (Fig. 4I). In less severe cases (12/22 hearts), the right bundle branch was foreshortened and failed to associate with the anterior papillary muscle (data not shown).

As in neonates, adult Tbx5<sup>del/+minKlacZ/+</sup> mouse hearts exhibited markedly abnormal right bundle branches. In 15 hearts examined, there was a marked paucity of β-galactosidase-expressing cells on the right ventricular septal surface. In 8 of 15 hearts, the right bundle branch was entirely missing, and only a few dispersed cells with β-galactosidase activity could be identified on the right ventricular septal surface (Fig. 4J). In the remaining mutant hearts (n=7), a foreshortened right bundle branch was present (not shown).

To verify the severe bundle-branch-patterning defects observed in Tbx5<sup>del/+minKlacZ/+</sup> mouse hearts, conduction system morphology in Tbx5<sup>del/+</sup> newborn mouse hearts was also evaluated by in-situ hybridization of connexin 40 expression on sagittal sections. In wild-type newborn mouse hearts (n=6), connexin 40 expression uniformly marked the atrioventricular bundle and left and right bundle branches (Fig. 4L). In Tbx5<sup>del/+</sup> newborn mouse hearts (n=6), connexin 40 was also observed in the atrioventricular bundle and left bundle branch (Fig. 4M). However, no right bundle branch could be identified in 3/5 Tbx5<sup>del/+</sup> newborn hearts (Fig. 4M). In these cases, a right bundle branch was absent even from the region adjacent to the membranous septum, where the right bundle branch normally exits the atrioventricular bundle to enter the right ventricle (Fig. 4M).

To assess the functional consequences of the profound bundle branch morphologic abnormalities in Tbx5<sup>del/+</sup> mice, ventricular conduction was evaluated using surface ECG analysis. Previous studies using single lead Holter monitoring revealed no ventricular conduction differences between wild-type and Tbx5<sup>del/+</sup> mice (Bruneau et al., 2001). Using 6-lead ECGs, we found that the QRS interval, produced by depolarization and activation of the ventricular myocardium (Surawicz and Knilans, 2001a), was significantly longer in Tbx5<sup>del/+</sup> mice than that in wild-type mice. QRS prolongation occurred both in neonates (12.7±2.6 ms vs. 9.8±1.5 ms; P<0.001) and in adults (16.5±1.0 ms vs. 13.7±1.3 ms; P<0.005) (Fig. 3B; Table 1B). Neither the minKlacZ allele nor the type of septal defect (primum or secundum) had a significant effect on the QRS interval of the wild-type or Tbx5<sup>del/+</sup> neonatal or adult mice (ASD primum 4/4 vs. ASD secundum 5/7; P-value not significant).

To determine whether the developmental failure of a morphologic right bundle branch correlated with the ECG finding of right-bundle-branch block, structure and function were assessed in 10 Tbx5<sup>del/+</sup> mice. All (n=4) mice with morphologic absence of the right bundle branch demonstrated right-bundle-branch block by precordial ECG analysis. By contrast, only 2/6 mice with a morphologically visible right bundle branch demonstrated a right-bundle-branch block on ECG.

Normal conduction system patterning in mice lacking Cx40, a Tbx5-target gene

Previous studies demonstrated that Cx40, which encodes a gap junction protein required for normal conduction system function, is a gene target of Tbx5. Adult Tbx5<sup>del/+</sup> mice express only 10% of normal levels of Cx40 transcripts, and Tbx5<sup>del/+</sup> mice have prolonged PQ intervals, prolonged QRS intervals, and right-bundle-branch block. The atrioventricular node, atrioventricular bundle, left bundle branch and right bundle branch identified by β-galactosidase activity were indistinguishable from that observed in littermate Cx40<sup>+/+</sup> mice (Tamaddon et al., 2000; van Rijen et al., 2001).

To test whether Cx40 insufficiency accounted for the morphologic and electrophysiologic defects in the conduction system of Tbx5<sup>del/+</sup> mice, we studied Cx40<sup>−/−</sup> mice (Simon et al., 1998). The morphology of the conduction system was analyzed in compound Cx40<sup>−/−</sup>/minKlacZ/+ mouse by evaluating β-galactosidase activity in adult hearts (Fig. 5). Precordial ECG analyses confirmed previously described electrophysiologic abnormalities: 19/19 Cx40<sup>−/−</sup> mice had a prolonged PR interval and 15/19 had a right-bundle-branch block (data not shown). Morphologic analyses were performed in mice with the most severe ECG abnormalities, those with prolonged PR interval and right-bundle-branch block. The atrioventricular node, atrioventricular bundle, left bundle branch and right bundle branch identified by β-galactosidase activity were well-formed and normally patterned in all Cx40<sup>−/−</sup>/minKlacZ/+ mice (n=10) (Fig. 5A-F). Both the amount and the distribution of β-galactosidase activity were indistinguishable from that observed in Cx40<sup>+/+</sup>lacZ/+ mice (Fig. 5).

Discussion

Our findings demonstrate a critical role for Tbx5 in development of the specialized electrophysiologic system of the heart. Tbx5 is expressed in the central conduction system including the atrioventricular node, atrioventricular bundle and ventricular bundle branches. In the atrioventricular canal, Tbx5 has specific roles in postnatal morphologic and functional maturation of the atrioventricular node and atrioventricular
restricted to the endocardial surface of the left ventricle. By contrast, Tbx5 levels in the atrioventricular bundle and bundle branches (Fig. 1) are maintained at birth, and levels in these conduction system structures are higher than in the surrounding ventricular myocardium (Fig. 1). Tbx5 is also known to activate the promoters of genes encoding the gap junction proteins Cx40 and ANF (Brunet et al., 2001), two molecules that distinguish adult electrophysiologic cells from working myocardial cells of the ventricle (Houweling et al., 2002; Coppen et al., 2003). The temporal and spatial pattern of Tbx5 expression and transcriptional activity is consistent with a role for this transcription factor in specification of the conduction system cells.

**Tbx5 is required for maturation of the atrioventricular canal conduction system**

Development of the mature conduction system requires both specification of cells with electrophysiologic functions and morphologic patterning of the central and peripheral electrophysiologic components. Organization of specialized electrophysiologic cells into distinct components of the mature conduction system appears to occur in distinct temporal steps. Some cells in the central conduction system appear to be specified early in development, since node-like pacemaker activity is evident by the linear-heart-tube stage (Kamino et al., 1981). Differentiation of the fast-conducting atrioventricular bundle and bundle branches occurs later in embryogenesis (Delorme et al., 1995; Sedmera et al., 2003). Analysis of minKlacZ/+ mice (Fig. 2A,B) extends the temporal sequence for central conduction system development into the postnatal period. At birth, rings of conduction tissue persist around both the tricuspid annulus and mitral annulus. Consolidation of electrophysiologic cells into a discrete atrioventricular node, atrioventricular bundle and bundle branches, the pattern of a mature conduction system, is a postnatal process that in the mouse is completed by week 14.

Although the rings of specialized electrophysiologic tissue are similar in newborn minKlacZ/+ mice and Tbx5del/+ mice, postnatal maturation of the atrioventricular canal conduction system fails to occur in Tbx5del/+ mice. Adult Tbx5del/+ mice maintain a neonatal pattern of specialized atrioventricular rings around both the tricuspid annulus and mitral annulus. The failure of morphologic atrioventricular canal maturation correlates with functional immaturity of the atrioventricular conduction system: absence of the normal age-dependent decrease of the PQ interval results in age-dependent atrioventricular block in Tbx5del/+ mice (Fig. 3).

In-vivo electrophysiology localized the anatomic source of the PQ prolongation in adult Tbx5del/+ mice. The PQ interval encompasses electrical conduction within the atrial musculature, atrioventricular node, atrioventricular bundle and proximal bundle branches (Fig. 3A). The normal HV interval in Tbx5del/+ mice suggests that the atrioventricular bundle has normal function, despite appearing physically foreshortened (Table 1, Fig. 4). The prolonged AH interval in Tbx5del/+ mice placed the functional defect in the atrial myocardium or atrioventricular node. Furthermore, a normal P-wave duration, indicative of atrial depolarization, ruled out a functional deficit within the atrial myocardium (Table 1). From these findings, we infer that the prolonged PQ interval in Tbx5del/+ mice is the result of a maturation failure of the atrioventricular node or its connection with the atria or atrioventricular bundle. This finding

---

**Fig. 5.** Normal conduction system morphology in Cx40+/− mice. β-Galactosidase expression in minKlacZ/+ (A,C,E) and Cx40+/−/minKlacZ/+ (B,D,F) mouse hearts. In the atrioventricular canal, a well-defined atrioventricular node (arrow) and atrioventricular bundle were observed in both minKlacZ/+ (A) and Cx40+/−/minKlacZ/+ (B) mouse hearts. The atria were removed and the atrioventricular canal was viewed from the superior/posterior, with the tricuspid annulus on the right and the mitral annulus on the left. A well-formed left bundle branch was found in both minKlacZ/+ (C) and Cx40+/−/minKlacZ/+ (D) mouse hearts. The left ventricular free wall and mitral valve were removed and the left interventricular septum viewed from the left. A well-formed right bundle branch was present in minKlacZ/+ (E) and Cx40+/−/minKlacZ/+ (F) mouse hearts. The right ventricular free wall and tricuspid valve were removed and the right interventricular septum viewed from the right. Comparable β-galactosidase expression and morphology were demonstrated in 12/12 minKlacZ/+ and 10/10 Cx40+/−/minKlacZ/+ mice.

bundle. In the ventricular conduction system, Tbx5 is required for morphologic maturation of the atrioventricular bundle and left bundle branch and is essential for the patterning and function of the right bundle branch.

**Tbx5 expression and specification of central conduction system cells**

Early in embryogenesis, Tbx5 is expressed throughout the cardiac crescent, but becomes restricted during formation of the linear heart tube. High levels persist in the atria, but Tbx5 levels in the primordial ventricles decrease throughout gestation, and prior to birth Tbx5 expression is largely...
Development and disease

could also reflect a deficit in one of the distinct subpopulations of cells within the atrioventricular node (Coppen et al., 2003).

Progressive atrioventricular block is found in human Holt–Oram syndrome. Conduction system disease is unusual at birth, but first or second-degree atrioventricular block occurs commonly in adult patients. The progressive onset of atrioventricular canal conduction dysfunction in humans and mice with Tbx5 haploinsufficiency suggests that the same postnatal Tbx5-dependent processes are required for the maturation of the atrioventricular canal conduction system in mice and humans.

Tbx5 and the ventricular conduction system

Tbx5 is also required for normal patterning and function of the proximal ventricular conduction system, the atrioventricular bundle and the left and right bundle branches. In adult Tbx5 del/+ mouse hearts, each of these components demonstrates a morphologic patterning defect (Fig. 4). The fast-conducting ventricular conduction system also demonstrates a functional deficit of the right bundle branch but not the left bundle branch (Table 1). We conclude that the patterning of the right bundle branch is sufficiently disrupted to cause malfunction, whereas patterning of the left bundle branch is not. Furthermore, deficits in the right bundle branch are visible at birth (Fig. 4). These findings define a role for Tbx5 in the development of the right bundle branch and suggest a substantial role in the differentiation of the components of the fast-conducting ventricular conduction system, including the atrioventricular bundle. Distinct differences in these components of the central conduction system in newborn Tbx5 del/+ mouse hearts suggest that development of the atrioventricular node, atrioventricular bundle and bundle branches have distinct molecular requirements. These data are consistent with a model in which regional components of the conduction system are specified independently and are then assembled into a continuous network.

Complete absence or a diminutive right bundle branch was found in all Tbx5 del/+ mice, indicating that normal levels of the transcription factor are essential for genesis of this structure. As in the atrioventricular canal, the morphologic deficits of the bundle-branch conduction system were mirrored by functional defects. In particular, there was a close correlation between the severity of the right-bundle-branch morphology defects and functional right-bundle-branch block in Tbx5 del/+ mice. All mice with morphologic absence of the right bundle branch had right-bundle-branch block by precordial ECG, whereas few mice with a visible right bundle branch demonstrated right-bundle-branch block. These data suggest that a functional electrophysiologic deficit in Tbx5 del/+ mice occurred as a consequence of an underlying primary, maldeveloped morphology in the conduction system.

Tbx5 haploinsufficiency directly disrupts the central conduction system

Several lines of evidence suggest that malformation of the central cardiac conduction system in Tbx5 del/+ mice occurs independent of cardiac structural defects. First, despite an intact ventricular septum, all Tbx5 del/+ mice had malformations in the ventricular conduction system, usually affecting both the right and left bundle branches. Second, there was no relationship between the specific type of ASD in Tbx5 del/+ mice and conduction system abnormalities. The presence of a secundum or primum ASD did not correlate with the severity of morphologic defects in the central conduction system, and no statistically significant differential effect was observed on the PQ interval, QRS interval or likelihood of right-bundle-branch block (Table 1). We conclude that Tbx5 has a direct role in conduction system development independent of its role in structural heart development. Furthermore, the finding that Tbx5 is expressed at high levels in conduction system cells suggests that its conduction system requirement may be cell-autonomous.

Cx40, a transcriptional target of Tbx5 that encodes a gap junction protein required for normal electrophysiologic function of the heart, was considered a potential cause for the patterning defects evident in the central conduction system of Tbx5 del/+ mice. Like Tbx5 del/+ mice, Cx40−/− mice demonstrate prolonged PQ intervals, prolonged QRS intervals, and in some cases right-bundle-branch block (data not shown) (Kirchhoff et al., 1998; Simon et al., 1998; Tamaddon et al., 2000). The degree to which the decrement in Cx40 transcription in Tbx5 del/+ mice accounts for the functional conduction system abnormalities in Tbx5 del/+ mice remains unclear. Our recent findings demonstrate the critical importance of even limited Cx40 expression in Tbx5 del/+ mice: whereas Tbx5 del/+ mice usually live to adulthood, Tbx5 del/+ /Cx40−/− mice die in utero (A.P., unpublished).

Cx40 deficiency does not, however, explain the morphologic abnormalities of the central conduction system found in Tbx5 del/+ mice. Normal morphology of the atrioventricular node, atrioventricular bundle and bundle branches was present in all adult Cx40−/− mice, indicating that this gap junction protein is not required for the morphologic maturation or patterning of the central conduction system. These findings implicate yet unidentified genes downstream of Tbx5 in the patterning of the conduction system.

Our results delineate several distinct roles for Tbx5 in conduction system development. Early in cardiac development, the temporal and spatial expression of Tbx5 is compatible with a role in specification of cells in the conduction system. Tbx5-dependent expression of Cx40, and presumably other molecules that are required for the critical electrophysiologic properties of these cells, supports this hypothesis. Tbx5 directs the expression of genes (e.g. Cx40) in the mature conduction system, after the primitive AV node, left bundle branch and right bundle branch have assumed their adult structures, which may account for why some Holt-Oram patients and Tbx5 del/+ mice evolve conduction system disease with age. In addition to regulating gene transcription in the conduction system, Tbx5, but not Cx40, is critical for conduction system pattern formation. Normal morphology of the atrioventricular canal components and ventricular components of central conduction system are dependent on physiologic levels of this factor. Tbx5 is the first gene to be implicated in the pattern formation and the developmental maturation of the centralized cardiac conduction system. A link between a patterning abnormality of the developing conduction system and a functional abnormality of the mature conduction system is demonstrated.

We wish to thank Dr David Paul for the use of connexin 40 knockout mice and Michael Peterson for excellent technical assistance. This work was supported by funding from the Howard Hughes Medical Institute. L.P.G.M. was supported by a Howard Hughes Medical Institute Physician Postdoctoral Fellowship.
References


