The role of Tbx2 in the development of the atrioventricular canal and conduction system of the heart. "Making the beat go on and on"
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Chapter 5

Defective Tbx2-dependent patterning of the atrioventricular canal myocardium causes accessory pathway formation in mice

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Abstract

Ventricular preexcitation, a feature of Wolff-Parkinson-White syndrome, is caused by accessory myocardial pathways that bypass the annulus fibrosus. This condition increases the risk of atrioventricular tachycardia and, in the presence of atrial fibrillation, sudden death. The developmental mechanisms underlying accessory pathway formation are poorly understood, but are thought to involve primarily malformation of the annulus fibrosus. Before birth, however, slowly conducting atrioventricular myocardium causes a functional atrioventricular activation delay in the absence of the annulus fibrosis. This myocardium remains present after birth, suggesting that disturbed development of atrioventricular canal myocardium may be primarily involved in the formation of rapidly conducting accessory pathways. We show that myocardium-specific inactivation of Tbx2, a transcription factor essential for atrioventricular canal patterning, leads to the formation of fast-conducting accessory pathways, malformation of the annulus fibrosus and ventricular preexcitation in mice. The accessory pathways ectopically express proteins required for fast conduction (Cx40, Cx43 and Scn5a/Nav1.5). Additional inactivation of Cx30.2, a subunit for gap-junctions with low conductance expressed in the atrioventricular canal and unaffected by loss of Tbx2, did not affect the functionality of the accessory pathways. Our results suggest that malformation of the annulus fibrosus and preexcitation arise from disturbed development of the atrioventricular myocardium.
Introduction

In the heart, the atrial and ventricular muscle masses are electrically insulated from each other by the annulus fibrosus. Normally, the only muscular connection that crosses this insulation is formed by the atrioventricular (AV) node and AV bundle. In the general population, 1-3 in 1000 individuals have accessory myocardial pathways that bypass the insulation, known as bundles of Kent.1,2 These accessory connections may lead to preexcitation of the ventricle, circus movement tachycardia and even life-threatening ventricular fibrillation in the presence of atrial fibrillation, as seen in Wolff-Parkinson-White (WPW) syndrome patients.3-5 The mechanism by which these accessory myocardial connections develop, and the molecular properties of these connections are largely unknown.

Accessory myocardial connections that can lead to preexcitation are commonly thought to result from malformation of the annulus fibrosus.6-9 However, during development, the AV canal myocardium causes an adequate AV delay to allow synchronized alternating contraction of the atria and ventricles10 in the absence of an annulus fibrosus. Remnant strands of this AV myocardium have still been observed in normal hearts around and after birth, which disappear when the annulus fibrosus is fully formed.6,11 Usually, these strands do not lead to preexcitation, indicating they maintain slow conduction properties of the AV myocardium. Furthermore, in the adult heart, after formation of the annulus fibrosus has been completed, slow-conducting AV canal-type myocardium remains present around the orifices of the mitral and tricuspid valve.12-14 Together, these data suggest that AV canal myocardium plays a central role in the AV delay, and that defects in AV canal myocardium could underlie formation of functional accessory pathways.

The AV canal myocardium is specified early in cardiac development. Bone morphogenetic protein 2 (Bmp2) is expressed in the AV canal myocardium progenitors in the early heart tube where it stimulates the expression of Tbx2,15 a T-box factor required for the development of the AV canal.16-18 Repressors Tbx3 and Msx2, Notch signaling, and Hey transcription factors further establish the AV canal phenotype.19-22 Other factors involved in formation and regulation of gene expression of the AV canal and AV node include the more broadly expressed transcription factors Nkx2-5, Gata4 and Tbx5, which interact or compete with the localized repressors.16,23-27 To explore the possible role of the AV canal myocardium in the formation of accessory connections, we studied morphology and function of mice in which Tbx2 was specifically inactivated in selected tissues. Our results indicate that defective patterning and gene regulation within the AV canal myocardium may lead to malformation of the annulus fibrosus, to formation of accessory AV connections and ventricular preexcitation.
Methods

Transgenic mice
The \( Tbx2^{tm1.1(cre)Vmc} \) (synonyms: \( Tbx2^{Cre} \), \( Tbx2^{fl} \), \( Myh6-Cre \), \( Tie2-Cre \), \( Wnt1-Cre \), and \( Cx30.2^{lacZ} \) alleles have been described previously.\(^{18,28-32}\) All mice were held on FVB/N background, except for \( Cx30.2^{lacZ} \), which was held on C57BL/6 background. Experiments with \( Tbx2^{Cre/+};Cx30.2^{lacZ/+} \) double transgenic mice were performed in mixed FVB/N;C57BL/6 background. Animal care was in accordance with national and institutional guidelines.

Human embryos
Human embryos were collected from medically induced abortions performed for social reasons at the Gynaecology Department of the Tartu University Hospital, Estonia. Collection and use of the human embryonic material for research presented here were approved by the Medical Ethics Committees of the Universities of Tartu, Estonia, and Amsterdam, the Netherlands. Subsequent processing has been previously described.\(^{33}\)

BrdU assay
Pregnant females were injected intraperitoneally with 50 mg of 5′-bromo-2′deoxyuridine (BrdU) / kg bodyweight (Sigma B5002) dissolved in 0.9% NaCl. After 1 hour of BrdU exposure the mice were killed by cervical dislocation. The embryos were isolated on ice-cold PBS and further processed for immunohistochemistry.

Immunohistochemistry and in situ hybridization
Embryos were fixed in 4% formaldehyde, embedded in paraplast and sectioned at 7-8 μm for immunohistochemistry and at 10-14 μm for in situ hybridization. In situ hybridization was performed according to a previously described method.\(^{34}\) Probes have been described previously.\(^{18,35-37}\) Rehydration, unmasking, blocking and washing steps were performed according to the protocol of the tetramethylrhodamine based amplification kit (Perkin Elmer). Primary antibodies used for mouse sections were: cTnI rabbit polyclonal (1:250; Hytest Ltd); Tbx3 goat polyclonal (1:500; Santa Cruz Biotechnology); Cx40 mouse monoclonal (1:250; US Biological); Cx43 mouse monoclonal (1:250; BD Transduction); Scn5a rabbit polyclonal (1:250; Alemone labs); Nkx2.5 goat polyclonal (1:250; Santa Cruz Biotechnology); Hcn4 Goat polyclonal (1:250 Santa Cruz Biotechnology); BrdU rat polyclonal (1:600; AbD serotec), Cx30.2 rabbit polyclonal (1:200; gift from K. Willecke). For apoptosis detection we used the Cleaved Caspase 3 antibody (rabbit polyclonal, 1:250; Cell Signaling Technology). Primary antibodies used for human sections were: Tbx2 mouse monoclonal (1:100; gift from Colin Godin), TBX3 goat polyclonal (1:250 Santa Cruz), CX40 rabbit polyclonal (1:250; Santa Cruz). Secondary antibodies when using amplification were: Biotinylated donkey-anti-goat (1:250; Jackson Immunology); biotinylated goat-anti-rabbit (1:250; DAKO); biotinylated goat-anti-
mouse (1:250; DAKO). For visualization without the amplification step, secondary antibodies coupled to an Alexa fluorescent (1:250; Invitrogen) were used.

3D reconstruction
Image acquisition, processing and subsequent 3D reconstruction was performed according to a previously described method.\textsuperscript{38} Serial sections were stained for \textit{cTnI} (labeling all cardiomyocytes) and \textit{Cx40} and reconstructed.

Preparation of the hearts and recording of electrograms and optical action potentials

\textit{Adult hearts}
Electrocardiograms were recorded for a period of 5 minutes during 1.5 \% isoflurane anesthesia. Signals were averaged after which RR, PQ, QRS, QT and QTc were calculated. For the local recordings mice were anesthetized by an intraperitoneal injection of pentobarbital, after which the heart was excised, cannulated, mounted on a Langendorff perfusion set-up, and perfused at 37\textdegree C with Tyrode’s solution (in mmol/L) 128 NaCl, 4.7 KCl, 1.45 CaCl\textsubscript{2}, 0.6 MgCl\textsubscript{2}, 27 NaHCO\textsubscript{3}, 0.4 NaH\textsubscript{2}PO\textsubscript{4}, and 11 glucose (pH maintained at 7.4 by equilibration with a mixture of 95\% O\textsubscript{2} and 5\% CO\textsubscript{2}). After that, the hearts were incubated in 10 ml Tyrode’s solution containing 15 \textmu M Di-4 ANEPPS and subsequently placed in a optical mapping setup. Excitation light was provided by a 5 Watt power LED (filtered 510 +/- 20 nm). Fluorescence (filtered > 610nm) was transmitted through a tandem lens system on CMOS sensor (100 x 100 elements, MICAM Ultima). Activation patterns were measured during sinus rhythm, ventricular and atrial pacing at a basic cycle length of 120 ms, (twice the diastolic stimulation threshold). The effective refractory period of the atrioventricular node was determined by atrial pacing and reducing the coupling interval of a premature stimulus (after trains of 10 stimuli at basic cycle length 120 ms) in steps of 5 ms until activation of the ventricle failed. Optical action potentials were analyzed with custom software.

\textit{Fetal hearts}
The hearts where removed from the embryo and incubated for 5 minutes with Tyrode’s solution containing 5 \textmu M Di-4 ANEPPS at 37 \textdegree C. After incubation fetal hearts were superfused with Tyrode’s solution and placed on the stage of an inverted microscope set-up for recording optical signals.

Statistics
Group comparisons were performed using ANOVA. Values are given as mean +/- SEM. Genotype and phenotype frequencies were tested with a Chi-square test. A P-value of 0.05 was considered statistically significant.
Chapter 5

Results

*Tbx2*-deficiency does not affect the AV conduction axis, but causes formation of a myocardial connection in the left atrioventricular junction

Heterozygotes containing the *Tbx2*Cre allele on an FVB/N background are viable, fertile, and display no obvious phenotypic abnormalities. *Tbx2*<sup>Cre/Tbx2*Cre</sup> mutants (*Tbx2*-/-) develop cleft palate and were not recovered after birth. Tbx2<sup>-/-</sup> fetuses collected at E14.5 and E17.5 from heterozygous intercross matings were present at less than expected on Mendelian inheritance (Supplemental Figure 1C). Fetal death of *Tbx2*- fetuses was less severe than found previously for another *Tbx2*-null allele on the mixed C57BL/6/129/ICR background. Surviving E14.5 and E17.5 fetuses were of normal size and were used for further analysis. We assessed whether chamber-specific genes involved in conduction of the electrical impulse are ectopically expressed in the AV canal of *Tbx2*-/- fetuses. In wild type fetuses, *Cx40* and *Cx43* were not expressed in the AV canal myocardium at E14.5 and E17.5. In contrast, *Cx40* and *Cx43* were ectopically expressed in the left side of the AV canal myocardium in all *Tbx2*-/- littermates (n=18; Figure 1A and Supplemental Figure 1A). The right side of the AV canal myocardium was not affected in mutants (Supplemental Figure 1B). The right side of the AV canal is presumably to a larger extent under control of *Tbx3* compared to the left side. The redundant function of *Tbx3* is illustrated by the ectopic expression of *Cx40* in the epicardial side of the left AV canal, complementary to that of *Tbx3* at the endocardial side (Supplemental Figure 1A). *Scn5a*, encoding the major cardiac sodium channel (Nav1.5) that is required for fast conduction, was expressed in the AV junction of *Tbx2*-/- but not in wild type animals. Heterozygous *Tbx2* mutants were not different from wild type (data not shown).

In the *Tbx2*-/- fetus, the compact AV node (*Hcn4* positive) and AV bundle (*Cx40*-positive) were not affected (Figure 1C and 1D) and were not in contact with the aberrantly formed *Cx40*-positive and *Cx43*-positive myocardial connection. Three-dimensional reconstructions of a E17.5 wild type and a *Tbx2*-/- fetus (Figure 1B) show the *Cx40*-positive pathway in *Tbx2*-/- that is not connected to the AV conduction axis (Figure 1B).

To assess whether the patterns of Tbx2 and Cx40 are conserved in human, we analyzed the expression of these proteins in a human fetus of Carnegie stage 14 (comparable to mouse E11.5). As in mouse, TBX2 and TBX3 are expressed in the AV canal myocardium, whereas CX40 is strictly absent from the AV canal myocardium but expressed in the atrial and ventricular myocardium (Supplemental Figure 2).

Next, we investigated the formation of the annulus fibrosus in *Tbx2*-/- embryos. In wild type embryos of E14.5, when annulus fibrosus formation is in progress, a narrow myocardial AV junction connected the atria with the ventricles. At E17.5, the atria and ventricles where almost completely separated by the annulus fibrosus, although many small myocardial connections were still observed. In *Tbx2*-/- animals at E17.5, however, a myocardial connection in the left AV junction was formed. The size and morphology of the connection varied between animals.
Figure 1. In the left side of the atrioventricular (AV) canal of Tbx2−/− fetuses working myocardial genes are ectopically expressed and connect the left atrium with the left ventricle while the AV node and AV bundle are unaffected. Panel A, C and D show in situ hybridization analyses of serial sections wild type (left) and Tbx2−/− fetuses (right) at E17.5. (A) In wild type the left AV canal myocardium does not express Cx40 and Cx43. Tbx2−/− ectopically expressed Cx40 and Cx43 in the left AV canal (arrowheads). Furthermore, the AV canal in Tbx2−/− fetuses was broader. (B) 3-Dimensional reconstructions of the heart of wild type (left) and Tbx2−/− (right) fetuses at E17.5. Green represents all Cx40-positive myocardium and grey represents Cx40-negative myocardium. In Tbx2−/− fetuses a Cx40-positive myocardial connection formed through the left AV canal. (C) In the upper panels, cTnl reveals the myocardium. The lower panels show the AV node based on Hcn4 expression and location. The AV node is not affected in Tbx2−/− fetuses. (D) In the upper panel, cTnl reveals all myocardium. The lower panels show the AV bundle based on Cx40 expression and location. The AV bundle is not affected in Tbx2−/− fetuses. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle; avb, atrioventricular bundle; avn, atrioventricular node; cs, coronary sinus; lsh, left sinus horn.
Non-myocardial cells intermingled with the persisting myocardial connection, but did not form a complete separation. The right AV junction developed normally.

In Tbx2−/− animals, the sulcus mesenchyme is normally formed at the right side of the AV canal, while it is not formed at the left side. The proliferation rate of the epicardial sulcus was found to be similar between wild type (0.36 +/- 0.019) and Tbx2−/− animals (0.38 +/- 0.036) (Figure 2C,D). Previously we established an increased proliferation rate in the left AV canal of Tbx2−/− at E9.5.18 At E11.5 the epicardial side of the left atrioventricular canal myocardium of

(Figure 2A,B). In Tbx2−/− fetuses working myocardial proteins are ectopically expressed. The proliferation rate in the epicardium is not different between wild type and Tbx2−/− fetuses. (A) Immunohistochemical analyses of serial section of E17.5 wild type and two Tbx2−/− fetuses (f1,f2). In wild type Cx30.2, is expressed in the AV canal complementary to Cx40. In Tbx2−/− fetuses Cx40 is expressed ectopically in the AV canal, and Cx30.2 is still expressed in the AV canal myocardium. Notice the variable size and morphology of the aberrant myocardial connection. (B) Schematical representation of the expression profiles of connexins in the left AV canal myocardium in wild type and Tbx2−/− fetuses. Note that Cx40 expression withdraws from the compact myocardium, however Cx43 remains present. (C) Immunohistochemical analyses of BrdU incorporation in epicardial cells at the left side of the AV canal in a wild type (upper) and Tbx2−/− fetus (lower). The right panel is a magnification of the area within the white squares in the left panel. (D) A bar graph representing the proliferation rate based on BrdU incorporation in the myocardium of the left ventricle and in the epicardium at the right and left side of the AV canal. la, left atrium; lv, left ventricle; avc, atrioventricular canal; mv, mitral valve; sm, sulcus mesenchyme; lavc, left atrioventricular canal; r AVC, right atrioventricular canal.

Figure 2. In the left side of atrioventricular (AV) canal of Tbx2−/− fetuses working myocardial proteins are ectopically expressed. The proliferation rate in the epicardium is not different between wild type and Tbx2−/− fetuses. (A) Immunohistochemical analyses of serial section of E17.5 wild type and two Tbx2−/− fetuses (f1,f2). In wild type Cx30.2, is expressed in the AV canal complementary to Cx40. In Tbx2−/− fetuses Cx40 is expressed ectopically in the AV canal, and Cx30.2 is still expressed in the AV canal myocardium. Notice the variable size and morphology of the aberrant myocardial connection. (B) Schematical representation of the expression profiles of connexins in the left AV canal myocardium in wild type and Tbx2−/− fetuses. Note that Cx40 expression withdraws from the compact myocardium, however Cx43 remains present. (C) Immunohistochemical analyses of BrdU incorporation in epicardial cells at the left side of the AV canal in a wild type (upper) and Tbx2−/− fetus (lower). The right panel is a magnification of the area within the white squares in the left panel. (D) A bar graph representing the proliferation rate based on BrdU incorporation in the myocardium of the left ventricle and in the epicardium at the right and left side of the AV canal. la, left atrium; lv, left ventricle; avc, atrioventricular canal; mv, mitral valve; sm, sulcus mesenchyme; lavc, left atrioventricular canal; r AVC, right atrioventricular canal.
the Tbx2−/− animals had an increased proliferation profile compared to the wild type (Figure 2C). Neither wild type (n=3) nor Tbx2−/− embryos (n=3) showed apoptosis in the right AV junction myocardium and epicardium at E11.5 (data not shown). These data suggest that the annulus fibrosus is malformed due to altered migration or dysmorphogenesis of the sulcus mesenchyme.

Preexcitation of the ventricles and retrograde activation of the atria in Tbx2−/− mice

In all E14.5 wild type (n=5) and Tbx2+/− fetuses (n=6), the left ventricle was activated from apex to base within 2 ms, after an AV delay of 67 +/- 18 ms during sinus rhythm. However, we observed a functional accessory pathway in 6 of 14 Tbx2−/− fetuses. 5 of 14 Tbx2−/− fetuses showed retrograde activation of the atria after a normal AV delay. In 1 of 14 Tbx2−/− fetuses we observed that the ventricular myocardium was completely activated via the accessory pathway during sinus rhythm, after an AV delay of only 8 ms (Figure 3, Supplemental Figure 3 for movies). The location of the earliest ventricular activation coincided with the location of the accessory pathway as shown in the 3D-reconstruction (Figure 1B). The ventricular activation pattern and

![Diagram of activation patterns](image)

**Figure 3.** Typical example of an activation pattern in a wild type (left) and Tbx2−/− (middle and right) heart at E14.5. In the wild type (left), activation starts in the atria, and after a delay of 50 ms the ventricles are activated within 3 ms after the first moment of activation of the apex of the left ventricle. In the middle, an activation pattern of a Tbx2−/− heart is shown. The activation starts in the atria and after a normal atrioventricular delay the ventricles are activated from apex to base, after which the atria are activated for the second time via the left side. The right panels show an example of ventricular preexcitation in a Tbx2−/− heart. The activation starts in the atria after which the base of the left ventricle is activated with an atrioventricular delay of 8 ms. Complete activation of both the left and right ventricle is within 15 ms. ra, right atrium; la, left atrium; lv, left ventricle; rv, right ventricle; t, time; ms, millisecond.
total excitation time of the atria and ventricles (Figure 3) indicated that the AV conduction axis (AV node and bundle) was not involved in activation of the ventricular myocardium in this heart. Nevertheless, the AV conduction axis appeared to be intact because this heart also showed periods of normal AV conduction where the ventricular myocardium was activated from the apex to base with an AV delay of 51 ms.

At E17.5, the average AV conduction delay in wild type (n=5) and heterozygous (n=6) mice were not significantly different (72 +/- 1.3 ms and 56.1 +/- 4.7 ms, respectively) respectively. In 6 of 13 of the Tbx2⁻/⁻ fetuses we found a functional, conducting accessory pathway (Table 1). One of these fetuses showed complete activation of the ventricle via the accessory pathway during sinus rhythm with an AV-delay of 8.5 ms, and 5 fetuses showed normal activation of the ventricles with subsequently retrograde activation of the left atrium. During ventricular stimulation the ventricle to atrium conduction delay in Tbx2⁻/⁻ fetuses was 16.5 +/- 5.5 ms and in wild type 92 +/- 48 ms (p < 0.05). We tested whether delaying AV nodal conduction would reveal concealed pathways. However, administration of adenosine did result in AV block suggesting absence of functional accessory pathway (n=3).

**Figure 4.** Genes typical for the AV canal and genes typical for the working myocardium are simultaneously expressed in the left side of the atrioventricular (AV) canal of Tbx2⁻/⁻ fetuses. Images of in situ hybridization analyses in sections of wild type (A,B) and Myh6-Cre;Tbx2⁻/⁻ (C,D). B and D are magnifications of the black squares in, respectively, panel A and C. (A,C) cTnI labels all myocardium. (B) In wild type fetuses, the AV canal myocardium did not express Cx40 and Scn5a, genes associated with fast conduction. The AV canal myocardium did express typical AV canal genes associated with slow conduction (Cacna1g, Cacna2d2), automaticity (Hcn4) and AV conduction system maturation (Id2). (D) In Tbx2⁻/⁻ fetuses Cx40 and Scn5a are ectopically expressed in the left AV canal myocardium. The AV canal specific genes are still expressed and even found in the left ventricular wall in some cases (red arrowheads). la, left atrium; lv, left ventricle.
The AV canal-specific gene program, including Cx30.2 expression, is not affected in Tbx2−/− fetuses

Although all Tbx2−/− examined formed left-sided AV connections expressing working myocardial genes and had a malformed annulus fibrosus, only a subpopulation of the Tbx2−/− fetuses showed a conducting accessory pathway. A possible explanation for the discrepancy is that intrinsic factors in the myocardium that are independent of Tbx2 influence conduction in the AV junction. Cx30.2 is a connexin subunit with low conductance which is expressed in the AV node where it decelerates conduction and depends on Gata factors and Tbx5 for its expression in the AV canal. Co-immunostaining for Cx40 and Cx30.2 revealed that Cx30.2 was expressed in the AV canal myocardium of the fetal heart complementary to Cx40 (Figure 2A). In Tbx2−/− hearts, Cx30.2 remained expressed in the AV junction that now ectopically co-expressed Cx40. Expression of AV canal-specific genes Hcn4, Cacna2d2, Cacna1g (Cav3.1) and Id2 was also maintained in the malformed left AV canal of Tbx2−/− fetuses (Figure 4). Our findings indicate that regulation of these AV canal genes, including Cx30.2, is independent of Tbx2.

To test whether maintained Cx30.2 expression contributed to the AV delay in mutants we generated double mutants for Tbx2 and Cx30.2 by intercrossing Tbx2+/Cre;Cx30.2+/LacZ transgenic mice. AV conduction time and activation pattern were assessed by optical mapping of fetal hearts at E14.5 (n=11). The fraction of Tbx2 mutants with functional bundles was not affected by the presence or absence of Cx30.2 (Table 1).

Table 1. Presence of a functional accessory pathway

<table>
<thead>
<tr>
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<th>E14.5</th>
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<td></td>
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<td>6 (n=13)</td>
<td>-</td>
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<tr>
<td>Tbx2−/−;Cx30.2+/+</td>
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<td>-</td>
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<td>7 (n=11)</td>
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<tr>
<td>FVB/N</td>
<td></td>
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<tr>
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<td>0 (n=5)</td>
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<tr>
<td>Myh6-Cre;Tbx2fl/fl</td>
<td>-</td>
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Myocardium-specific inactivation of Tbx2 results in accessory pathways and defective annulus fibrosus formation

Tbx2 is expressed in the AV canal myocardium and in the AV cushion mesenchyme, which is implicated in annulus fibrosus formation. Furthermore, Tbx2 is expressed in the neural crest which has been associated with the formation of the conduction system, in the second heart field, and the dorsal mesenchymal protrusion-derived cells that contribute to the AV septation process. We assessed the function of Tbx2 in these different cell lineages by inactivating Tbx2 specifically in either endocardium and derived cushion mesenchyme, the neural crest, or the second heart field and dorsal mesenchymal protrusion-derived cells by crossing Tbx2fl/fl mice with, respectively, the Tie2-Cre;Tbx2+/-, Wnt1-Cre;Tbx2+/- or the Mef2c-AHF-Cre;Tbx2+/- mice. Tie2-Cre recombines all endothelia, endocardium and derived mesenchyme of the AV cushions. However, lineage analyses revealed that the annulus fibrosus does not receive contributions of these cells (and data not shown). Wnt1-Cre labels all neural crest including the innervations of the heart. Wnt1-Cre-derived cellular contributions to the AV canal region are very limited. In all three lines examined the AV canal myocardium was normally patterned and the sulcus mesenchyme, the precursor of the annulus fibrosus, was normally formed (Supplemental Figure 4). Tie2-Cre;Tbx2fl/fl and Mef2c-AHF-Cre;Tbx2fl/fl mice are viable and AV conduction was not altered in adult mice (data not shown). Wnt1-Cre;Tbx2fl/fl mice die at birth due to cleft palate and were measured at E17.5. Also in these mutants AV conduction was not altered (data not shown).

Next, Tbx2 was specifically inactivated in the myocardium by crossing Tbx2fl/fl with Myh6-Cre;Tbx2+/- mice. At E10.5, Tbx2 protein was not found in the AV canal myocardium of Myh6-Cre;Tbx2fl/fl mutants, but was still observed in the AV cushions (Supplemental Figure 5A). The left AV junction ectopically expressed Cx40, whereas Tbx3 was still expressed robustly at the luminal side of the left AV canal (Supplemental Figure 5A). The cardiac phenotype of Myh6-Cre;Tbx2fl/fl and the Tbx2-/- mice is similar. To study annulus fibrosus formation, we assessed the patterning of the epicardial cells in the Myh6-Cre;Tbx2fl/fl at E12.5 by examining markers that are expressed in the epicardial cells and in the epicardial-derived sulcus mesenchyme, which include Periostin (Pstn), Collagen 3a1 (Coll3a1), Tbx18 and Wilms tumor 1 homolog (Wt1). In E12.5 wild type hearts, annulus fibrosus formation was ongoing. In the AV sulcus, epicardial-derived mesenchymal cells were found that express Pstn, Coll3a1 and Wt1. In Myh6-Cre;Tbx2fl/fl fetuses, the sulcus mesenchyme at the left AV junction was not formed (Figure 5A). Furthermore, Coll3a1 and Wt1 expression was decreased in the AV sulcus and Tbx18 expression appeared diminished (Figure 5B). This indicates that Tbx2 function in the AV myocardium influences the formation of AV sulcus mesenchyme, and that the formation of the annulus fibrosus is not properly initiated.

Myh6-Cre;Tbx2fl/fl animals survived after birth at lower frequency than expected on the basis of Mendelian inheritance (p<0.05, Table 1). This could be due to variations in the degree of recombination of Tbx2 or in viability. However, recombination by Myh6-Cre in the AV canal
is complete by E10.5, and a fraction of the Tbx2 mice also survived until late fetal stages, suggesting that the survival of the Myh6-Cre;Tbx2 animals can be attributed to variability in phenotype. All adult Myh6-Cre;Tbx2 animals studied (n=7) had persisting myocardial strands that connected the left atrium with the left ventricle and that expressed Cx40 and Cx43 (Figure 6J). The myocardial connections varied in size from small strands to a relatively thick connection. More importantly, the accessory connections were always found at the left and caudal side of the AV junction and did not affect the compact AV node nor AV bundle (Figure 5A-G). In both wild type and Myh6-Cre;Tbx2 mice, Cx30.2 expression was no longer detectable in the AV junction, but was detectable in the AV node (Supplemental Figure 5B).

Electrocardiogram (ECG) recordings revealed that 4 out of 8 mice with myocardium-specific inactivation of Tbx2 had a normal PR interval (34+/-4.0) (Table 2). The other 4 mice showed a severely shortened PR interval with a broadened QRS complex and initial slurring consistent with preexcitation (Figure 6H). The total activation time of the atria and ventricles with preexcitation is less than the normal AV delay. Therefore, the AV node and AV bundle complex are not used to activate (part of) the ventricle. Hence, these QRS complexes are not a

**Figure 5.** Absence of Tbx2 in the myocardium of the atrioventricular canal leads to absence of epicardial derived mesenchyme and abnormal epicardial patterning. (A) In situ hybridization analyses of E12.5 Tbx2 and Myh6-Cre;Tbx2 embryos. When Tbx2 is inactivated within the myocardium Cx40 is ectopically expressed in the shortened and broadened atrioventricular canal myocardium. Furthermore, the accumulation of epicardial derived mesenchyme in the left atrioventricular sulcus is lost (black arrowheads). (B) Tbx2 in the atrioventricular canal myocardium is required for correct patterning of the epicardium and the accumulation of epicardial derived mesenchyme in the atrioventricular sulcus. Images of in situ hybridization analyses of E12.5 Tbx2 and Myh6-Cre;Tbx2 embryos. In Tbx2 embryos the epicardium derived mesenchyme accumulates specifically in the AV sulcus and invaginate in between the atria and ventricles. Coll3a1, Tbx18 and Wt1 are expressed in the epicardium and epicardial derived mesenchyme that also expressed Pstn. In the Myh6-Cre;Tbx2 embryos the epicardial derived mesenchyme fails to accumulate in the atrioventricular sulcus and the examined genes are aberrantly expressed (red arrowheads). la, left atrium; lv, left ventricle.
representation of a fusion of normal activation and preexcitation via the accessory bundle. We therefore prefer not to describe this initial deflection as a delta wave.

Optical mapping of adult Myh6-Cre;Tbx2fl/fl hearts in a Langendorff set-up during preexcitation, showed that the ventricular myocardium was completely activated without the involvement of the AV nodal conduction axis (Figure 6I). Moreover, all Myh6-Cre;Tbx2fl/fl adult hearts that were investigated showed a shorter AV delay than in wild type (13 +/- 2 ms vs 52.4 +/- 8.9 ms). In some hearts, atrial echo beats could be induced after premature stimulation (Supplemental Figure 6). In none of the adult Myh6-Cre;Tbx2fl/fl mice arrhythmias could be induced. Administration of sodium channel blocker Ajmaline converted ventricular preexcitation into normal AV conduction (n=3).

### Table 2. ECG characteristics of adult mice

<table>
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<tr>
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<th>wild type (n=4)</th>
<th>Myh6-Cre;Tbx2fl/fl normal (n=4)</th>
<th>Myh6-Cre;Tbx2fl/fl preexcitation (n=4)</th>
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<tr>
<td>hr (bpm)</td>
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<td>426.9±49.1</td>
<td>435.3 ± 23.4</td>
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<td>9.2±0.4*</td>
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<td>qrs (ms)</td>
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<td>qt (ms)</td>
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<td>48.3±15.0</td>
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<td>qt̅e (ms)</td>
<td>36.7±11.7</td>
<td>40.6±3.1</td>
<td>39.3±11.2</td>
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</tbody>
</table>

*p<0.05

### Discussion

In this study we show that global and myocardium-specific inactivation of Tbx2 causes formation of accessory AV bundles and preexcitation of the ventricles. The significance of our findings is that erroneous patterning in part of the embryonic AV canal myocardium can be the primary event in the formation of accessory pathways leading to preexcitation and to malformation of the insulating annulus fibrosus.

The cardiac Tbx2 mutant provides a paradigm for the developmental mechanisms that can lead to accessory pathways

Both Tbx2 mutant mice and patients with preexcitation have myocardial accessory pathways that express Cx43. Therefore, we propose that the formation of a fast-conducting muscular connection between the atrium and the ventricle that bypasses the AV conduction axis in the Tbx2 mutants and in patients with preexcitation, can share a similar underlying developmental mechanism. However, the phenotypic outcome of these pathways differs between mice and human.
Figure 6. Myocardial specific deletion of Tbx2 leads to formation of accessory pathway that express Cx43 and causes ventricular preexcitation without involvement of the atrioventricular (AV) conduction axis. (A-D) Images of sections of a representative adult Myh6-Cre;Tbx2<sup>fl/fl</sup>. The location of each section is shown in the scheme in panel (E). (E) The affected area is always located in at the left and caudal side of the AV junction, while the AV conduction axis remains intact (F, G) Magnifications of the area within the black squares in, respectively, (C) and (D). (H) The electrocardiogram shows ventricular preexcitation in a Myh6-Cre;Tbx2<sup>fl/fl</sup> mouse (lower) and normal AV delay in a wild type (upper). (I) Reconstructed activation pattern of a representative wild type (upper) and Myh6-Cre;Tbx2<sup>fl/fl</sup> (lower) adult mouse. In wild type after an AV delay of 34 ms the ventricle is activated from apex to base. In Myh6-Cre;Tbx2<sup>fl/fl</sup> the ventricle is activated from base to apex after an AV delay of 9 ms. (J) Images of serial sections in Tbx2<sup>fl/fl</sup> and Myh6-Cre;Tbx2<sup>fl/fl</sup>. In Myh6-Cre;Tbx2<sup>fl/fl</sup> Cx43 is expressed in the accessory myocardial connection. la, left atrium; lv, left ventricle; vs, ventricular septum; avn, atrioventricular node; lavc, left atrioventricular canal; avb, atrioventricular bundle; avr, atrioventricular ring; ine, inferior nodal extension.
Firstly, there is an essential difference in preexcitation between patients and preexcitation in mouse models. In contrast to patients with preexcitation, in which a delta wave is often seen, mouse models with preexcitation show activation patterns that indicate complete activation of the ventricle without involvement of the AV conduction system.\(^{44-49}\) In mice the AV delay is relatively long compared to the total activation time of the ventricles. Therefore, a fusion complex as seen in human is less likely to occur, especially when conduction via an accessory pathway is fast. Indeed, in our model, the sum of the activation delay in the accessory pathway and the total ventricular activation time was shorter than the normal AV delay. These data indicate that in mice the ventricle is completely activated through the accessory pathways.

Secondly, in mice the AV node has a relatively long refractory period compared to the conduction time along the reentrant circuit. Therefore, it is difficult to induce AV nodal reentrant tachycardia in mice. However, we were able to induce an atrial echo beat via administration of a sodium channel blocker (Ajmaline) (Supplemental Figure 6).

Thirdly, the pathways in \(Tbx2\) mutants were variable in size, and always located at the left-posterior (caudal) side of the AV junction. In patients with preexcitation, the pathways are often relatively smaller and can be located at any position in the AV junction, although there is a preference for the left and posterior location as well.\(^{50}\) In addition, the accessory bundles in humans are at the epicardial side of the annulus fibrosus.

Taken together, although the physiological outcome differs between patients with preexcitation and \(Tbx2\) mutant mice, we conclude that the underlying developmental mechanisms of accessory pathway formation are similar, making the \(Tbx2\) mutant model a valuable tool to study and understand these molecular mechanisms of accessory bundle formation.

Although an accessory connection was formed in all \(Tbx2^{-/}\) fetuses that expressed Cx40, Cx43 and Scn5a, not all \(Tbx2^{-/}\) fetuses had a conducting accessory pathway (Table 1). We

![Figure 7](image.png)

**Figure 7.** Model of the transcriptional regulatory network in the myocardium of the atrioventricular canal that depends on \(Tbx2\) for correct patterning of this myocardium, formation of the annulus fibrosus, and generation of an atrioventricular delay. Genetic or epigenetic mechanisms and environmental factors that affect the \(Bmp2-Alk3-Tbx2-Notch\) regulatory network may lead to the formation of accessory atrioventricular bundles and underlines the close relationship between the embryonic atrioventricular canal and the development of the atrioventricular conduction system.
hypothesized that other AV specific genes may have modulated accessory pathway formation. However, the presence or absence of Cx30.2 did not influence the percentage of mutant fetuses with functional accessory pathways (Table 1). Alternatively, the size and fiber direction of the accessory bundle may vary between mutants. This can affect conduction through these accessory pathway through a mechanism known as current-to-load mismatch, in which small myocardial strands are not able to activate a large myocardial area.\textsuperscript{51} Indeed, upon blocking the sodium current we enhanced the intrinsic current-to-load mismatch and were able to convert preexcitation into retrograde atrial activation or even normal AV conduction in the adult \textit{Myh6-Cre;Tbx2\textsuperscript{fl/fl}} mice. This indicates that variation in the occurrence of current-to-load mismatch determines whether an accessory pathway causes preexcitation or not.

**Developmental mechanisms of AV accessory pathway formation**

Several mechanisms for accessory pathway formation have been put forward and discussed previously.\textsuperscript{52} 1) malformation of the annulus fibrosis,\textsuperscript{6-9} 2) acquired during fetal or postnatal life,\textsuperscript{39} 3) misregulation of AV canal myocardium. Our study supports the latter explanation for the formation of functional accessory pathways.

During development, the AV canal provides an adequate AV delay to allow synchronized alternating contraction of the atria and ventricles.\textsuperscript{10} Remnant strands of this myocardium have still been observed in normal hearts around and after birth.\textsuperscript{11} Furthermore, in the adult heart, after formation of the annulus fibrosus has been completed, slow-conducting AV canal-type myocardium remains present around the orifices of the mitral and tricuspid valve.\textsuperscript{12-14} Defects in the annulus fibrosus alone may therefore not automatically lead to preexcitation. Our study helps to reconcile these observations. Myocardium-specific inactivation of \textit{Tbx2} caused erroneous gene expression and morphogenesis of part of the embryonic AV canal. As a result, AV myocardium was malformed and acquired fast-conducting properties, and annulus fibrosus formation was affected, which led to preexcitation. The myocardial pathways were seen to develop mainly at the epicardial side, whereas the medial/endocardial side was largely unaffected, likely due to the redundant function of Tbx3, a functional homologue of Tbx2\textsuperscript{18} (Supplemental Figure 1A).

The malformation of the annulus fibrosus can be caused by disturbed epicardial ingestion\textsuperscript{39} at the level of the AV sulcus due to dysmorphogenesis of the AV canal myocardium in \textit{Tbx2} mutants. On the other hand, the epicardium-derived mesenchymal material, which is formed before ingestion, was not formed at the left side in myocardium-specific \textit{Tbx2} mutants. Furthermore, the patterning of the epicardium was disrupted as shown by altered expression of marker genes. These findings indicate the existence of a \textit{Tbx2}-dependent signal in the AV canal myocardium is involved in the formation of the annulus fibrosus.

**Disruption of the AV canal regulatory network underlies accessory bundle formation**

Our study indicates disturbed AV canal development is linked with formation of accessory pathways and preexcitation. Several genes have been implicated in AV canal development.
Bmp2-mediated signaling, essential for AV canal specification, can be detected as early as E8 in the precursors of the AV canal. Here, it activates Tbx2, which subsequently, in concert with Tbx3 and Msx2, suppresses chamber-specific gene expression and proliferation. Notch activity and Hey1/2 (Hesr1/2) in the adjacent developing chamber myocardium restrict the Bmp2-Tbx2 pathway to the developing AV canal. Other components that have been implicated in AV canal (AV node) development include Nkx2.5, Tbx20, Tbx5, Gata4 and Foxn4. We propose that genetic or epigenetic mechanisms and environmental factors that affect these and other components of the regulatory network for AV canal development may cause dysmorphogenesis of the AV canal myocardium, acquisition of fast-conducting properties, and malformation of the annulus fibrosus (Figure 7). Consistently, micro-deletions of BMP2 and Notch ligand JAGGED1 have been associated with ventricular preexcitation as part of a syndrome of congenital defects. Furthermore, deletion of the Alk3 receptor (Bmpr1a, activated by, among others, Bmp2) in the AV canal myocardium in mice results in accessory pathways and AV nodal defects.

Mutations in LAMP260 and PRKAG2 are linked to the formation of accessory pathways. In these glycogen storage diseases it has been proposed that glycogen toxicity causes annulus fibrosus thinning that underlies formation of accessory pathways. However, in a cardiomyocyte-specific overexpression model of mutated PRKAG, accessory pathways only developed when the mutated PRKAG was overexpressed during development. When overexpression started in adulthood, glycogen storage disease and conduction system degeneration occurred, but accessory pathways did not develop, suggesting that PRKAG may play a role in AV canal development. Nevertheless, there is not an apparent link between PRKAG and the AV canal regulatory network.

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**Supplemental Figure 1.** Images of *in situ* hybridization in serial sections of wild type and *Tbx2*^-/-^ embryos of the (A) left and (B) right atrioventricular canal (both E14.5). In wild type the myocardium of the atrioventricular canal expressed *Tbx2* and *Tbx3*, whereas *Cx40* and *Cx43* are complementarily expressed in the working myocardium of the atria and ventricles. In *Tbx2*^-/-^ embryos the left atrioventricular canal myocardium has broadened and shortened. In addition, *Cx40* and *Cx43* are ectopically expressed in the atrioventricular canal myocardium complementary to the remaining *Tbx3* expression. The right atrioventricular canal is unaffected in the mutants. Arrowheads point to the atrioventricular canal myocardium. (C) Expected (based on Mendelian inheritance) and observed numbers of *Tbx2*^-/-^ fetuses (E14.5 and E17.5) and adult *Myh6-Cre;Tbx2*^fl/fl^. *la*, left atrium; *lv*, left ventricle; *ra*, right atrium; *rv*, right ventricle.

**Supplemental Figure 2.** Images of immunohistochemical analyses in sections of human embryonic hearts at Carnegie stage 14 (33 dpf) (comparable to mouse E11.5). TBX2 and TBX3 are both expressed in the atrioventricular (AV) canal myocardium. CX40 is expressed in the working myocardium of the atria and ventricles and not in the AV canal myocardium. This suggests a similar role for TBX2 and TBX3 in human and mice to maintain the AV canal myocardial phenotype by repression of working myocardial genes. *la*, left atrium; *lv*, left ventricle.
Supplemental Figure 4. *In situ* hybridization images of serial sections of wild type and after specific inactivation of Tbx2 in (A) endothelial derived tissue (Tie2-Cre), (B) anterior secondary heart field and dorsal mesenchymal protrusion derived tissue (Mef2c-AHF-Cre), and (C) neural crest derived tissue (Wnt-Cre). The atrioventricular canal patterning and morphology was unaffected in all three lineage-specific knock-outs. The boxes indicate the area that is enlarged in the related sections. Black arrowheads indicate the atrioventricular canal myocardium. Red arrowheads indicate the epicardium and epicardium derived mesenchyme. ra, right atrium; rv, right ventricle; la, left atrium; ra, right atrium; pv, pulmonary vein; lsh, left sinus horn.

Supplemental Figure 5. Myocardial specific deletion of Tbx2 results in ectopic expression of working myocardial proteins in the left atrioventricular (AV) canal myocardium. (A) Immunohistochemical analyses in serial sections of Tbx2fl/fl and Myh6-Cre;Tbx2fl/fl embryos at E10.5. In Tbx2fl/fl embryos Tbx2 protein is expressed in the AV canal myocardium, the AV cushions and the body wall (white arrowhead). Tbx3 is expressed in the AV canal myocardium and Cx40 is expressed in the atria and ventricles. In the Myh6-Cre;Tbx2fl/fl littermate Tbx2 is expressed in the AV cushions and body wall but is lost from the AV canal myocardium (yellow arrowhead). Cx40 is ectopically expressed in the left AV canal complementarily to the remaining Tbx3 (red arrowhead). (B) Images of immunohistochemical analyses in serial section of a 3 months old Myh6-Cre;Tbx2fl/fl mouse. The central conduction system is demarcated by Tbx3 and Hcn4 expression. The atrioventricular bundle is also Cx40-positive while the atrioventricular node does not express Cx40. The left panel reveals that Cx30.2 is expressed in the atrioventricular node but not in the atrioventricular bundle. This suggests that the atrioventricular conduction axis is intact in Myh6-Cre;Tbx2fl/fl mice. ra, right atrium; la, left atrium; rv, right ventricle; avcs, atrioventricular cushion; avn, atrioventricular node; avb, atrioventricular bundle; vs, ventricular septum.
Supplemental Figure 6. An induced atrial echo beat in an adult mouse heart with a myocardial specific deletion of Tbx2. This heart showed ventricular preexcitation during sinus rhythm (not shown) and atrial stimulation (S1) and premature stimulation. After premature stimulation (S2) in the presence of Ajmaline ventricular activation failed. This means that both the AV node and the accessory pathway were not able to activate the ventricles. After a third stimulation (S3) the ventricle was activated after a normal AV delay followed by atrial activation. We hypothesize that the accessory pathway was activated during the first premature stimulus (S2), but was not able to activate the ventricles because of current-to-load mismatch, while the AV node was still refractory. After a second premature stimulus (S3) the accessory bundle is refractory while the AV node is able to propagate the impulse to the ventricle (note the apex to base activation pattern) followed by a retrograde activation of the atria via the accessory bundle. La, left atrium, Lv, left ventricle, acc bundle, accessory bundle.
Addendum

In this chapter accessory myocardial pathways that can lead to preexcitation have been referred to as bundles of Kent. Although the name ‘bundle of Kent’ is frequently used for bundles that lead to ventricular preexcitation or AV re-entrant tachycardia, especially in the clinical setting, historically this is not correct. In 1893, Kent suggested that there were multiple muscular pathways crossing the atrioventricular junctions that were responsible for normal atrioventricular conduction. Kent was wrong with his observations, since the structures he observed were never shown crossing the plane of atrioventricular insulation. In reality, they are the remnants of the atrioventricular ring tissue that exist in the developing heart. It is now recognised that, rarely, the nodes can give rise to muscular connections that form the substrate for so-called Mahaim pre-excitation. In fact, Öhnell was the first to describe fast-conducting accessory myocardial connections within the fibro-fatty tissue of the atrioventricular sulcus. So as to avoid confusion and to correct a historically wrong association, Anderson and co-workers proposed that the name ‘bundle of Kent’ should not be used anymore.

In addition, we indicated that it is commonly thought that accessory atrioventricular pathways are the mere result of malformation of the annulus fibrosus due to incomplete separation of the atria and ventricles. We used the term annulus fibrosus to indicate the entire atrioventricular insulating plane comprising both the annulus fibrosus in the strict sense and the fibro-fatty sulcus tissue. In the myocardial Tbx2 knock-out model we clearly showed that the accessory atrioventricular pathways are derived from the myocardium of the atrioventricular canal and are localized in the entire insulating plane, i.e. both in the insulating fibrous ring and in the fibro-fatty atrioventricular sulcus tissue. Therefore, we proposed that the isolation of the atria from the ventricles was impaired and suggested that loss of expression of Tbx2 in the atrioventricular canal myocardium results in malformation of the annulus fibrosus. However, from the anatomical stance, the annulus fibrosus is solely the fibrous part, while the fibro-fatty part is not. Nevertheless, the larger part of the fibrous part of the plane of insulation in our mouse-model was grossly intact. As the mice analyzed represented a surviving subgroup, the relative intact fibrous part of the plane of insulation might have been a requirement for the affected animals to survive. For example, the fibrous part could be required for the support and proper function of the valvular apparatus. Interestingly, in patients with preexcitation due to a left-sided atrioventricular accessory pathway, the fibrous part of the plane of insulation was found to be intact and the accessory pathway was found within the fibro-fatty part of the plane of insulation (Figure), a striking similarity with our mouse model.
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Figure. An example of a left sided atrioventricular accessory pathway (arrows) that crosses the plane of insulation through the fibro-fatty tissue, in a patient with ventricular preexcitation. Left panel overview, right panel a detail of the same picture. ef, epicardial fibro-fatty tissue; am, atrial muscle; vm, ventricular muscle; mv, mitral valve; ca, coronary artery. From: Becker, et al, 1978).