The Tbx2(+) Primary Myocardium of the Atrioventricular Canal Forms the Atrioventricular Node and the Base of the Left Ventricle

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Abstract—The primary myocardium of the embryonic heart, including the atrioventricular canal and outflow tract, is essential for septation and valve formation. In the chamber-forming heart, the expression of the T-box transcription factor Tbx2 is restricted to the primary myocardium. To gain insight into the cellular contributions of the Tbx2\(^+\) primary myocardium to the components of the definitive heart, genetic lineage tracing was performed using a novel Tbx2\(^{Cre}\) allele. These analyses revealed that progeny of Tbx2\(^+\) cells provide an unexpectedly large contribution to the Tbx2-negative ventricles. Contrary to common assumption, we found that the embryonic left ventricle only forms the left part of the definitive ventricular septum and the apex. The atrioventricular node, but not the atrioventricular bundle, was found to derive from Tbx2\(^+\) cells. The Tbx2\(^+\) outflow tract formed the right ventricle and right part of the ventricular septum. In Tbx2-deficient embryos, the left-sided atrioventricular canal was found to prematurely differentiate to chamber myocardium and to proliferate at increased rates similar to those of chamber myocardium. As a result, the atrioventricular junction and base of the left ventricle were malformed. Together, these observations indicate that Tbx2 temporally suppresses differentiation and proliferation of primary myocardial cells. A subset of these Tbx2\(^{Cre}\)-marked cells switch off expression of Tbx2, which allows them to differentiate into chamber myocardium, to initiate proliferation, and to provide a large contribution to the ventricles. These findings imply that errors in the development of the early atrioventricular canal may affect a much larger region than previously anticipated, including the ventricular base. (Circ Res. 2009;104:1267-1274.)

Key Words: atrioventricular canal ■ lineage ■ fate ■ patterning ■ transgenic ■ Cre

The embryonic heart tube is composed of “primary” myocardium and rapidly elongates by addition of progenitor cells to its poles. During looping, specific regions in the embryonic tubular heart differentiate to chamber myocardium and expand to form the future working myocardium of the ventricles and atria. In contrast, the region in between these expanding chambers does not differentiate or expand and becomes visible as an atrioventricular constriction.\(^1\) During prenatal life, the atrioventricular canal (AVC) myocardium conducts the electric impulse between the atrial and ventricular chambers in a slow manner, reminiscent of the function of the mature atrioventricular (AV) node. This slow conducting feature allows the AVC to act as a sphincter preventing backflow from the ventricles to the atria, analogous to the AV valves. Furthermore, the primary myocardium provides the signals that initiate formation of the cushions, which subsequently will form the valves and partake in septation.\(^2,3\) The primary myocardial AVC is extensively remodeled to properly connect and align both atria and ventricles and to coordinate the formation of the fibrous insulation.\(^4\)

Given all these roles of the AVC, it may not be surprising that defects related to the AVC region, which include partial or complete AV septal defects, AV valve defects and Ebstein’s anomaly, account for a large fraction of all congenital cardiac defects.\(^5,6\) Furthermore, AVC-related defects are associated with arrhythmias including reentry tachycardia, AV nodal block, and ventricular preexcitation.\(^7\) The morphogenesis and evolving gene patterns in the developing AVC have been studied in detail.\(^1,4,8–14\) However, the actual cellular contributions of the embryonic AVC to the heart have not been assessed. Insight into these contributions to the defini-
tive heart, and the developmental molecular mechanisms that underlie formation and identity of the AVC, will aid in understanding the etiology of AVC-related defects.

During and after differentiation of ventricular and atrial chamber myocardium, T-box transcription factor Tbx2 is expressed in the primary myocardium of the AVC, inner curvatures, and outflow tract but not in the developing chambers.\(^\text{15-18}\) Tbx2 suppresses chamber-specific gene expression in the AVC.\(^\text{16,18}\) These properties make Tbx2 an attractive tool to assess the contributions of the primary myocardial AVC to the heart and the mechanisms underlying its formation. We generated a Tbx2\(^{\text{Cre}}\) allele and used it for genetic lineage tracing, a powerful method that combines tracing the fate of cells and monitoring specific aspects of their changing phenotype.\(^\text{19}\) Furthermore, we analyzed the AV junction in Tbx2-deficient (Tbx2\(^{\text{Z/EG}}\)) embryos.

### Materials and Methods

An expanded Material and Methods section is available in the online data supplement at http://circres.ahajournals.org.

#### Transgenic Mice

The generation of the Tbx2\(^{\text{Cre}}\) line is described in Figure I in the online data supplement. Briefly, 5.9 kb of upstream and 2.2 kb of downstream Tbx2 129/SvJ genomic fragments were amplified by PCR and ligated to an Frt-flanked Cre-polyA-PGK-neo cassette replacing the first five codons of the Tbx2-coding region in exon 1. The PGK-neo cassette was removed, and the Tbx2\(^{\text{Cre}}\) allele was maintained on an FVB background. Animal care was in accordance with national and institutional guidelines.

#### Results

### The Origin of the Atrioventricular Canal

To define the fate of the Tbx2\(^{\text{Cre}}\) primary myocardium of the embryonic AVC, we generated transgenic mice expressing Cre under control of the Tbx2 locus. Heterozygous Tbx2\(^{\text{Cre/+}}\) mice are healthy and fertile. We first confirmed that Cre recapitulated the pattern of Tbx2 expression (Online Figure I).

In all tissues examined, the expression pattern of Tbx2 and Cre were identical. We next crossed Tbx2\(^{\text{Cre/+}}\) mice with R26R or Z/EG mice. In Tbx2\(^{\text{Cre/+};R26R}\) (or Tbx2\(^{\text{Cre/+};Z/EG}\)) embryos, cells that express Cre undergo irreversible recombination of the R26R (or Z/EG) allele on which these cells and their progeny produce β-galactosidase (or enhanced green fluorescent protein [EGFP]), allowing assessment of the fate of Tbx2\(^{\text{+}}\) cells. At embryonic day (E)9.25, the assessment of β-galactosidase was highly similar to the pattern of Tbx2 (Cre), indicating that recombination precisely follows Tbx2 expression. Recombination and subsequent expression of the reporter gene following Cre expression is delayed,\(^\text{19}\) explaining the absence of β-galactosidase activity from the outflow tract, which has just initiated Tbx2/Cre expression at this stage.

Tbx2 was first expressed in the limbs of the cardiac crescent. Expression was not detected in the fused cranial part (Figure 1). At E8 and E8.5, Tbx2 was expressed in the inflow tract of the heart but not in the embryonic ventricle, which is commonly thought to give rise to the left ventricle (Figure 1). At E9 to E9.5, expression was confined to the AVC and septum transversum region and became visible in the outflow tract (Figure 2A). Tbx2 expression was absent from the ventricular and atrial region, in line with previous observations.\(^\text{15,17,18}\) In Tbx2\(^{\text{Cre/+};R26R}\) (or Tbx2\(^{\text{Cre/+};Z/EG}\)) embryos, first cardiac recombination was observed in the inflow tract of E8 to E8.5 hearts (Figure 1), colocalizing with Tbx2 expression. Subsequently, at E9.25, recombination was observed in the AVC, whereas the ventricle and the just-formed atria remained free of recombination (Figure 2A). These data suggest that the E9 to E9.5 AVC is represented at E7.5 by the limbs of the first heart field-derived cardiac crescent. The spatiotemporal pattern of the cGataa-enhancer transgene supports this notion.\(^\text{10}\) Furthermore, these data show that the E8 to E8.5 inflow tract population gives rise to the E9 to E9.5 AVC and indicate that the atrial auricles are recruited from Tbx2-negative precursors at the dorsal side (Figure 1, open arrow).

**Tbx2\(^{\text{Cre/+}}\) Atrioventricular Canal Lineage Provides a Large Contribution to the Left Ventricle**

Until E9.0 to E9.5, the recombination pattern in the AVC of Tbx2\(^{\text{Cre/+};R26R}\) embryos was highly similar to the expression pattern of Tbx2 (Cre), with coinciding expression borders between the AVC and ventricle. At E10.5, however, the pattern of recombination in Tbx2\(^{\text{Cre/+};R26R}\) (and Tbx2\(^{\text{Cre/+};Z/EG}\)) embryos was discordant with that of Tbx2 expression. Tbx2 (Cre) was confined to the AVC myocardium, whereas β-galactosidase or EGFP expression was found, in addition, in a large portion of the working myocardium of the base of the left ventricle contiguous with the AVC (Figure 2A).

Between E10.5 and E17.5, Cre (Tbx2) expression was always confined to a small AVC region. The Egfp expression domain, however, had expanded across most of the ventricular free wall (Online Figure II). These data indicate that Tbx2\(^{\text{+}}\) progeny of the AVC has formed most of the left ventricular free wall. In contrast, the apex and left part of the ventricular septum remained free from recombination, indicating that the recombination-free embryonic left ventricle at E8 and E9 to E9.5 (Figures 1 and 2A), generally assumed to form the complete definitive left ventricle.,\(^\text{20,21}\) only forms the
left side of the ventricular septum and apex in the definitive heart (Figure 3A and 3B and Online Figure II).

To confirm that the AVC contributes to the left ventricle, we performed DiI labeling of the AVC at E9.5 and cultured the embryos for 24 hours. Of 20 successfully labeled embryos, 11 developed normally. After 24 hours of culture, the label was found in the left ventricular compartment of 9 embryos and in the AVC of 2 embryos (Figure 2C and Online Figure III). Several of the labels placed in the AVC close to the left ventricle formed stripes from the AVC into the ventricle, whereas a label placed in the middle of the AVC did not convincingly incorporate into the ventricle, and a label placed at the AVC-left ventricular border did not form a stripe but was found in the ventricle in its entirety (Figure 2C and Online Figure III). These data suggest that AVC cells expand and that part of the progeny has formed ventricle, essentially confirming the findings of the genetic lineage analysis. Using a mouse line in which \textit{Egfp} expression mimics expression of chamber marker \textit{Nppa}, we found that on serial sections of an E10.5 heart the \textit{Egfp} mRNA was present in ventricular cells up to the border with the left AVC, whereas EGFP protein was detected in the ventricle but not in the cells bordering the AVC. Because it takes at least several hours for protein to accumulate after initiation of mRNA transcription, this experiment suggests that these mRNA positive but protein-negative ventricular cells have differentiated from \textit{Egfp} AVC cells very recently (Online Figure IV).

**Origin of the Components of the AV Conduction System**

The mechanism of development of the AV node and AV bundle has been unclear. Analysis of \textit{Tbx2\textsuperscript{Cre}\textsuperscript{+};Z/EG} fetuses at later stages of development revealed complete labeling of the AV node and AV ring bundle, indicating that these structures derive from the \textit{Tbx2\textsuperscript{+}} AVC myocardium (Figure 3C). In contrast, the AV bundle, the proximal right bundle branch, and left bundle branches were free of recombination (Figure 3D), indicating that in contrast to the AV node, the AV bundle and branches are not derived from the \textit{Tbx2\textsuperscript{+}} AVC, even though a myocardial continuity (physical connection) is present throughout development. These findings indicate that the precursors of these AV conduction...
system components segregate already very early in development and have been exposed to distinctive regulatory signals.

The AV and outflow tract cushions and derived valves showed little recombination (Online Figure II and Figure 3A). In contrast, the mediastinal atrial myocardium, atrial septum, and the dorsal mesenchymal protrusion mesenchyme and derived myocardium, which is an important component of the AV mesenchymal complex,24,25 show extensive recombination. Indeed, Tbx2 is expressed in these structures and their progenitors in the posterior second heart field (Figures 1 and 3A).

Cells of the second heart field and early outflow tract are known to form the right ventricle, with contributions to the ventricular septum, depending on the marker used.20,26–28 In accordance, the definitive outflow tract and right ventricle, including the right part of the ventricular septum, were found to be labeled (Figure 3A), indicating that these cells derive from the Tbx2+ embryonic outflow tract and anterior heart field (Figure 2A). Interestingly, the boundary between the left, nonrecombined ventricular septum myocytes and the recombined, presumably embryonic outflow tract-derived, myocytes, coincides with the previously defined clonal growth and (trans) gene expression boundary,29 suggesting the presence of a regulatory and lineage boundary within the ventricle septum that may be important for its morphogenesis and function.

**Tbx2 Is Required to Suppress Chamber Differentiation and Proliferation in the Left Atrioventricular Canal**

Previously, Tbx2 was shown to be necessary and sufficient to suppress chamber genes Nppa and Cx40 in the AVC.16–18 To further explore the role of Tbx2 in the specification of the AVC, we analyzed the hearts of Tbx2Cre/Cre mice that were maintained on an FVB background. Tbx2 protein expression was detectable in AVC of wild-type embryos (Figure 2B), whereas expression was diminished in Tbx2Cre/Cre embryos (data not shown). Furthermore, all Tbx2Cre/Cre embryos recovered after E14 (n = 17) were found to have a bilateral hindlimb-specific duplication of digit IV, consistent with previous observations in another Tbx2-deficient (Tbx2tm1Pa) strain.18 These data indicate that our Tbx2Cre allele is a null allele and that Tbx2Cre/Cre mice are deficient for Tbx2. Nevertheless, we found that Nppa and Cx40 were not ectopically expressed in the AVC in E9.5 Tbx2Cre/Cre hearts (Online Figure V) and that all embryos formed normal AV cushions, which contrasts to the findings by Harrelson et al with the Tbx2Cre/Cre strain that was maintained on a mixed 129/C57/ICR background.18 These data indicate that on the FVB background, Tbx2 deficiency causes a less severe phenotype.

We next defined whether the AVC was specified correctly. Bmp2, which is required for Tbx2 expression and specification of the AVC,3 is normally expressed in the AVC of E9.5 mutants, indicating that the AVC-specifying pathway is not affected (Figure 4A). Cre was expressed in the AVC of mutants, which indicates that Tbx2 is not required to regulate its own expression (Figure 4B). Expression of chamber-specific genes was absent from the AVC in E9.5 mutants, yet we noted ectopic expression of Cx40 and Nppa specifically in the Cre− left AVC from E10.5 onwards, whereas the right AVC was unaffected (Figure 4B and Online Figure VI). Cx40 was not expressed in the Tbx3+ part of the left AVC (Online Figure VII). These observations suggest functional redundancy with Tbx3, a highly related T-box transcription factor in the AVC,30–32 Tbx2 is expressed at higher levels in the left AVC, whereas Tbx3 expression is more prominent in the right AVC,14–18 providing a possible explanation for the left-sided defect in Tbx2-deficient mice. The cell cycle–related markers Mycn, Ccna2, (Cyclin A2), and Cdkn1c (P57), which are normally highly expressed in the developing chamber myocardium, were ectopically expressed in the left AVC (Figure 4B). Furthermore, the left AVC myocardial wall acquired aberrant trabecular morphology with pockets. Interestingly, these pockets extended into the base of the left ventricular wall (Figure 4C).

Tbx2 has been implicated in the regulation of the cell cycle and senescence in various cell types, including fibroblasts and certain tumors.33 A role of Tbx2 in regulation of proliferation in the heart was not observed.18 We defined regional prolif-
crem. A and B, Three-dimensional reconstruction of pro-

valves, the AV node, AV ring, and, possibly, the AV

the atria, the myocardium that supports the developing AV

embryonic AVC gives rise to the lower rim (or vestibules) of

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lineage relations and aberrant expression of Cre could not be
distinguished.

Targeting of Cre to the endogenous Tbx2 locus ensured

selective expression in the Tbx2+ primary (nonchamber) myocardium, including the AVC, after initiation of chamber differentiation.15–18 The Tbx2Cre fate map revealed that the vestibules of the atria, the myocardium that supports the developing AV valves, the AV node, and AV ring are derived from the embryonic AVC, confirming the previously proposed fates. However, we observed an unexpectedly large part of the left ventricular wall to be derived from the embryonic Tbx2+ AVC, whereas the embryonic ventricle, generally assumed to be the precursor compartment of the definitive left ventricle,20,21,27 only gave rise to the apex, left ventricular septum, and AV bundle. We confirmed the physical relation between the E9.5 AVC and E10.5 left ventricular wall (base) by Dil labeling. Fate map studies in chicken have indicated that cells of the primitive inlet contributed to the base of the left ventricle.58 Because the myocardium lining the AV cushions was labeled, these studies, in retrospect, indicated ventricularization of the embryonic AVC region during development, in line with our data. The myocardium in the AVC of E10.5 mutants was aberrantly trabecularized, containing pockets. At E12.5, larger pockets were seen in the AVC extending into the left ventricular free wall (Figure 4C), suggesting that the early AVC myocardial region contributes to the left ventricular free wall. Early mis specification of the AVC may therefore lead to left ventricular wall defects.59

Previous studies suggested that AV node and AV bundle (bundle of His) develop in structural continuity2 from an embryonic myocardial population that has a specific expression profile in common,1,9,14,39,40 indicative of early specification of a progenitor population. However, other functional and genetic studies indicated that the AV node and AV bundle may develop as separate modules.23,30,41 We found that the AV bundle was derived from the Tbx2-negative embryonic ventricle, without any contributions from the Tbx2+ AVC, suggesting early segregation between the precursors of the AV node and AV bundle.

Discussion

Origin and Fate of the Tbx2+ AV Myocardium

Previous histological and genetic studies suggested that the

embryonic AVC gives rise to the lower rim (or vestibules) of

the atria, the myocardium that supports the developing AV

valves, the AV node, AV ring, and, possibly, the AV

bundle.4,8–14,35 However, these studies did not provide fate maps because during development cells differentiate and change their phenotype, thereby switching the (trans)gene marker on or off. Consequently, the relation between the embryonic AVC and the definitive AV node and other structures has remained unclear.1,8,23,36,37 The cGata6-

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ing chambers retains the slow-proliferating primary phenotype and forms the AVC, inner curvature, and outflow tract (Figure 6A), which largely disappear toward birth. Our present findings indicate that the boundaries between the early compartments are dynamic and that part of the primary AVC and outflow tract myocardium of the early tube undergoes differentiation to chamber myocardium. Therefore, the compartments delineated in the early heart tube do not represent the definitive compartments. Instead, shifts in the boundaries between primary and chamber myocardium, at the expense of primary myocardium, contribute to the expanding (ballooning) ventricular chambers. Consistently, the primary outflow tract was found to “disappear” mainly through continuous differentiation to ventricular myocardium, with integration of cells into the right ventricular chamber.27 We propose that slowly proliferating $Tbx2^+\$ primary myocardial AVC, inner curvature, and outflow tract cells are marked by $Tbx2-Cre$. Subsequently, primary myocardial cells (or their daughters) at the border with the ventricular domain, switch off $Tbx2$, which, in turn, results in differentiation into ventricular myocardium and an increase in proliferation rate. As a consequence of the latter, a small AVC contribution results into a large ventricular accretion. Expression of $Tbx2$ diminishes in the border zone (shaded), resulting in patchy recombination. Colored dots and stripes represent an interpretation of the behavior of DiI labels in the AVC region. Dashed lines indicate lineage relations. For abbreviations, see the legend to Figures 2 and 3.
Our data suggest that Tbx2 is expressed strongly in the developing AVC, steeply tapering off toward the embryonic ventricle. Previous data indicated that repressor Tbx2 and activator Tbx5 compete for binding site occupation and Nkx2–5 interaction, suggesting that high expression of Tbx2, as found in the future AVC, is sufficient to repress chamber differentiation and allow formation of AVC, whereas low Tbx2 expression, as found in the border zone between the future AVC and ventricle, may not be sufficient to suppress chamber differentiation. Hence, it is likely that the robustly recombined cell population in the ventricular base is derived from daughter cells of the embryonic AVC, whereas the zone of patchy recombination found more toward the apex is derived from daughter cells of the Tbx2-low border zone (Figure 6B).

A pitfall of Cre-mediated lineage analyses is the discrepancy in sensitivity between the Cre-reporter (eg, R26R, Z/EG) and the methods to detect expression of Cre (or the Cre-driving gene). Thus, one may perceive a tissue to be Cre (or driver gene)-negative, whereas it expresses sufficient Cre to recombine the reporter. As a result, the tissue may be misinterpreted as being derived from another Cre+ tissue.46

In the present study, putative undetected Cre (Tbx2) expression in the ventricle may have caused the activation of the reporter gene in the ventricular base, leading to an incorrect interpretation. The small zone of graded Tbx2 expression between the early AVC and ventricle may be particularly prone to this effect, because in this area, the perceived Cre/Tbx2 expression gradient may be smaller (or larger) than the zone of recombination (Figure 6B), leading to an over- or underestimation of the contribution of the Tbx2+ AVC to the ventricle. Hence, the genetic fate map of the Tbx2+ primary myocardium, and especially the extent of the contribution, should be interpreted with care. Nevertheless, several arguments favor our interpretation that progeny of the early Tbx2+ primary myocardium differentiated to Tbx2-negative chamber myocardium, including the discrepancy in Cre expression and recombination only after E9 to E9.5, the DiI label in the AVC traced to the ventricle, the discrepancy between Nppa/Egfp mRNA and protein in the E10.5 ventricular base, and AVC-derived defects in the ventricular base in Tbx2 mutants.

Atroventricular Conduction System Formation: Patterning and Growth or Recruitment?

Retrospective clonal analyses in chicken have shown that single cell–derived clones in the AV conduction system, comprising the AV node, AV ring bundle, and AV bundle always extended into the adjacent working myocardium. Because the myocardium of the forming conduction system proliferates much less compared to the adjacent (chamber) myocytes, the AV conduction system components were proposed to be formed by inductive recruitment of myocytes to an initial conduction system framework. An alternative hypothesis is that the primary myocardium of the embryonic heart is patterned early in development either to form the chamber myocardium or to remain primary. The primary cells in the AV region subsequently form the mature AV conduction components by slow growth, and not by recruiting adjacent (chamber) myocytes.1,7,12 Here, we found that AVC cells labeled between E8 and E9.5 by Tbx2-driven Cre (corresponding to the early conduction system framework) give rise to the definitive AV node and AV ring bundle and the adjacent working myocardium. Hence, our data suggest that the clonal analysis in chicken may have revealed recruitment (differentiation) of working myocardium from the early AV conduction system precursors rather than the other way around. The quantitative 3D reconstructions of BrdUrd incorporation demonstrate that these AV myocardial cells do proliferate significantly, arguing that their increase in number is sufficient to generate the AV conduction system components and provide cells for the chambers.

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Disclosures

None.

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