Canonical Wnt Signaling Regulates Atrioventricular Junction Programming and Electrophysiological Properties

Benjamin S. Gillers, Aditi Chipunkar, Haytham Aly, Tomas Valenta, Konrad Basler, Vincent M. Christoffels, Igor R. Efimov, Bastiaan J. Boukens, Stacey Rentschler

Rationale: Proper patterning of the atrioventricular canal (AVC) is essential for delay of electrical impulses between atria and ventricles, and defects in AVC maturation can result in congenital heart disease.

Objective: To determine the role of canonical Wnt signaling in the myocardium during AVC development.

Methods and Results: We used a novel allele of $\beta$-catenin that preserves $\beta$-catenin’s cell adhesive functions but disrupts canonical Wnt signaling, allowing us to probe the effects of Wnt loss of function independently. We show that the loss of canonical Wnt signaling in the myocardium results in tricuspid atresia with hypoplastic right ventricle associated with the loss of AVC myocardium. In contrast, ectopic activation of Wnt signaling was sufficient to induce formation of ectopic AV junction-like tissue as assessed by morphology, gene expression, and electrophysiological criteria. Aberrant AVC development can lead to ventricular pre-excitation, a characteristic feature of Wolff–Parkinson–White syndrome. We demonstrate that postnatal activation of Notch signaling downregulates canonical Wnt targets within the AV junction. Stabilization of $\beta$-catenin protein levels can rescue Notch-mediated ventricular pre-excitation and dysregulated ion channel gene expression.

Conclusions: Our data demonstrate that myocardial canonical Wnt signaling is an important regulator of AVC maturation and electric programming upstream of Tbx3. Our data further suggest that ventricular pre-excitation may require both morphological patterning defects, as well as myocardial lineage reprogramming, to allow robust conduction across accessory pathway tissue. (Circ Res. 2015;116:398-406. DOI: 10.1161/CIRCRESAHA.116.304731.)

Key Words: arrhythmias, cardiac $\bullet$ arrhythmogenic cardiomyopathy $\bullet$ Notch signaling pathway $\bullet$ septal defects $\bullet$ tricuspid atresia $\bullet$ ventricular pre-excitation $\bullet$ Wnt signaling pathway

Proper patterning of the atrioventricular canal (AVC) is necessary for diverse processes within the developing heart, including alignment of cardiac chambers, AV valve formation, and delay of the electrical impulse between the atria and ventricles to allow for sequential activation and contraction of atria and ventricles. At later developmental stages, much of the embryonic AVC canal myocardium regresses, such that in the mature heart the AV node is the only remaining myocardial connection between atria and ventricles.12 Elegant lineage tracing experiments in the chick demonstrated that AV nodal cells derive from the cardiomyocyte lineage, which was confirmed more recently in a mammalian model using genetic lineage tracing tools.14 Coincident with these processes, epicardially derived cells undergo epicardial epithelial-to-mesenchymal transformation, migrate into the region of the AV junction, and contribute to formation of the annulus fibrosus, an electrically insulating plane of cardiac fibroblasts between the atria and ventricles.16 Perturbations in AVC maturation give rise to a wide spectrum of congenital heart defects ranging from structural defects, including AV septal defects, tricuspid atresia, and Ebstein anomaly, to arrhythmias such as AV reentrant tachycardia, AV nodal block, and ventricular pre-excitation. Given that similar genetic pathways regulate both the morphology and the electrophysiology of the AVC, it is not surprising that patients with structural AVC defects often have arrhythmias. For example, patients with Ebstein anomaly, characterized by malpositioning of the septal leaflet of the tricuspid valve, also frequently display ventricular pre-excitation.

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Wnt signaling regulates many aspects of cardiac development.7–12 Loss of Wnt2 in inflow tract mesoderm results in a phenotype resembling complete common AVC.13 Additional studies in zebrafish have implicated myocardial Wnt signaling in the
regulation of BMP4 and Tbx2b expression within the AVC.14 Therefore, we speculate that perturbations of Wnt signaling may be associated with congenital heart defects and arrhythmias involving the AV junction. Wolff–Parkinson–White (WPW) syndrome, a common disorder characterized by ventricular pre-excitation and palpitations, results from accessory AV pathways in the heart bypassing the slow-conducting AV node. Dual AV electrical connections with different conduction velocities and refractory periods can lead to AV reentrant tachycardias, syncope, and sudden cardiac death. Although WPW syndrome has been well described clinically and effective ablation therapies have been developed, there is still much to be learned about the developmental mechanisms underlying its etiology.

Our recent work has shown that activation of Notch signaling within murine myocardium results in accessory AV pathway formation and ventricular pre-excitation, similar to WPW syndrome.15 Notch-mediated pre-excitation was associated with blurring of the boundary between the AVC and ventricle during late fetal development, as well as electrical reprogramming of ventricular cardiomyocytes.15,16 Therefore, WPW syndrome may be considered a disorder of altered AVC programming and maturation. Given that Wnt and Notch signaling coregulate many aspects of cardiac development, in this study, we sought to decipher a coordinated role for Wnt and Notch signaling during AVC morphogenesis and myocardial cellular electrical programming.

**Methods**

Expanded Methods are presented in the Online Data Supplement. Whole mount Xgal staining, Masson’s Trichrome, Oil-Red-O, and immunohistochemistry were performed as described.13,17 Reverse transcription quantitative polymerase chain reaction was performed as described.16 ECGs were performed as described.11 Optical mapping was performed as described.18

**Results**

**Canonical Wnt Signaling Is Required in the Myocardium for AVC Programming**

We sought to determine whether canonical Wnt signaling is active within AVC myocardium throughout development by assessing the expression pattern of the direct Wnt target Axin2 through use of Axin2*Cre/Le reporter mice.16 Axin2 is expressed within AVC myocardium as early as E10.5 and continues to be expressed within myocytes of the lower rim of the atria and AV node, as well as in AV valve endocardium and mesenchyme, during early postnatal life (Figure 1A–1H). Given the diversity of Wnt ligands, genetic manipulation of canonical Wnt signaling is often probed by manipulating the key transcriptional effector β-catenin. Because β-catenin also plays an important role in cell adhesion, results from this manipulation may not reflect the role of Wnt signaling specifically. Therefore, we used a β-catenin allele that lacks both N- and C-terminal transcriptional outputs, while cell adhesion is preserved (Ctnnb1dm).20 We placed the mutant β-catenin allele over a conditional knockout allele under the control of different Cre drivers to allow for tissue-specific deletion within cardiomyocytes (Ctnnb1dm/fl; hereafter referred to as Wnt LOF). In Cre-expressing cells that undergo recombination, the mutant allele is the only remaining source of β-catenin protein. Indeed, complete absence of β-catenin within the Mlc2v+/− expression domain results in a different phenotype when compared with isolated loss of canonical Wnt signaling in this region (Online Table I).

Wnt LOF mice (αMHC-Cre) were born in Mendelian ratios; however, by postnatal day 1 there was uniform lethality, presumably because of the observed cardiac defects (Online Table II). Initial AVC programming at E10.5 to E12.5 is normal as evidenced by grossly normal cardiac morphography (Figure 1I, 1M, 1Q, 1J, 1N, 1R, and 1V) and normal expression of Tbx3 and Tbx20 in the AVC (data not shown). By E14.5, Wnt LOF mice begin to exhibit overt structural defects, including an abnormal tricuspid valve with hypoplastic right ventricle (RV), and malalignment of the interventricular septum (Figure 1K, 1O, 1S, and 1W; data not shown). By birth, Wnt LOF mice have a range of structural heart defects, including an atretic tricuspid valve with hypoplastic RV observed in all embryos, while ventricular and atrial septal defects are also highly penetrant (Figure 1L, 1P, 1T, and 1X; Online Figure I). Echocardiograms at late fetal stages demonstrate diminished blood flow between right atrium and RV in Wnt LOF embryos, which is associated with a malformed tricuspid annulus (Online Movie I and II).

To determine whether the observed effects of loss of canonical Wnt signaling on the AVC are stage dependent, we inhibited canonical Wnt signaling using Tbx2+/-; Tbx2 has been shown to be active within the AVC by E912 and is downstream of Wnt signaling in zebrafish AVC specification.14 AVC morphology is grossly normal and Tbx3 expression is preserved in Wnt LOF (Tbx2+/-) embryos at E10.5 (Online Figure II), again suggesting that canonical Wnt signaling plays a role in maintenance of the AVC phenotype.

To address the possibility that the primary role of β-catenin is in forming functional ventricles, which in turn are required for normal AVC patterning, we assessed whether AV valve defects occur in Mlc2v+/−; Ctnnb1dm/fl or Mlc2v+/−; Ctnnb1fl mice. Recombination with Mlc2v+/−; Ctnnb1dm mice results in normal AV valve formation (Online Figure III). In contrast to loss of Wnt signaling, complete loss of β-catenin within the Mlc2v+/− expression domain leads to defective right ventricular development while formation of the tricuspid valve is normal (Online Figure IV). Therefore, the tricuspid atresia observed in Wnt LOF (αMHC-Cre) mice is likely a primary defect due to loss of canonical Wnt signaling within the AVC expression domain and not secondary to ventricular defects.

Previous reports have demonstrated that mice harboring a deletion of Tbx20 in early AVC myocardium fail to maintain the AVC myocardial phenotype, as evidenced by a loss of Bmp2 and Tbx3 expression at subsequent gestational time points.22 Although the expression of Tbx3 appears normal at E12.5, Wnt LOF (αMHC-Cre) mice exhibit subtle defects in gene expression along the right side of the AVC by E16.5, including decreased expression of Bmp2 (Figure 2A–2D). There is progressive loss of AVC myocardium throughout...
A VC myocardium is virtually absent (Figure Online Movies I and II, Figure I, and Tables I and II. 

See also P, Q, L, and T, respectively. Scale bars, 125 μm to 500 μm.

Valve is completely absent in Wnt LOF mice (P, Q, and L), whereas a hypoplastic right heart can already be seen in Wnt LOF embryos. By E18.5, the tricuspid valve is also corresponded to E, F, and H, and is 100 μm. Scale bar in D is 50 μm. DAPI indicates 4',6-diamidino-2-phenylindole.

Figure 2. Progressive loss of atrioventricular canal (AVC) myocardium in aMHC-Cre;Ctnnb1fl/fl (Wnt LOF) embryos. A to G, Immunohistochemistry (IHC) for Tbx3 (AVC myocardium) and peristin (a marker of fibroblasts undergoing epithelial-to-mesenchymal transition) demonstrate a grossly normal AVC structural organization at E12.5 in Wnt LOF when compared with control (A–C). D, In situ hybridization demonstrates decreased Bmp2 expression in Wnt LOF when compared with control (D) at E16.5. E–H, By E19.5, IHC reveals a near absence of Tbx3/

Connexin 40 AVC myocardium in Wnt LOF mice when compared with controls (E–H). White outlines denote the AVC myocardium in control hearts, white asterisks denote the region between the right atrium and right ventricle where AVC myocardium is absent in Wnt LOF embryos. The sections shown in G and H are serial to E and F.

AV junctions, we expressed a stabilized form of β-catenin within a mosaic pattern in developing ventricular myocardium (Mlc2αCre, Ctnnb1fl/fl, Wnt GOF).23 Wnt GOF mice are viable into adulthood and exhibit ectopic AV constrictions with coronary vessels, adipocytes, and fibroblasts throughout both ventricles (Figure 3A–3C). Although subepicardial adipocytes are specific for the AV junction and are not found elsewhere throughout the ventricle in control hearts (Figure 3D), they are found prominently within Wnt GOF ventricles (Figure 3E).

Smooth muscle cells, vasculature, and fibroblasts comprising the annulus fibrosus within the AV region are derived, at least in part, from epicardial-derived cells that migrate into the AV junction from E14 until birth.6,24,25 Because altered Wnt signaling has been postulated to cause differentiation of a precursor cell into adipocyte-like cells,26 we sought to determine whether Wnt GOF results in ectopic fibro-fatty regions via cell autonomous or non-cell autonomous effects in our model system. Lineage tracing (Mlc2αCre; Ctnnb1fl/fl; R26RtdTomato) reveals that ectopic fibroblasts and adipocytes are not primarily derived from Cre-expressing myocytes, consistent with a model where myocardial-derived signals influence the migration and differentiation of nonmyocardial cells, perhaps including multipotent lineage tracing (Mlc2αCre; Ctnnb1fl/fl; R26RtdTomato).

midgestation, such that by E19.5 the Tbx3+/Connexin 40- AVC myocardium is virtually absent (Figure 2E–2H).

Ectopic Wnt Activation Induces an AV Junction–Like Phenotype Within the Ventrices

In addition to its unique gene expression profile, the murine AV junction has a well-defined structural organization characterized by a myocardial constriction containing subepicardial coronary vessels and adipocytes, and an insulating layer of fibroblasts comprising the annulus fibrosus. To determine whether canonical Wnt signaling is sufficient to ectopically induce

Figure 1. Loss of myocardial canonical Wnt signaling results in congenital heart defects. A to D, Developmental time course of Axin2lacZ expression as assessed by Xgal staining denotes active canonical Wnt signaling in the region of the atrioventricular canal (AVC) in hearts imaged from the posterior view (white arrows). E to H, Histological sections of hearts from A to D reveal Axin2lacZ expression within AVC myocardium (black arrows). I–X, Trichrome staining of representative sections from aMHC-Cre; Ctnnb1fl/fl (Wnt LOF) mice show normal development at E10.5 (I, M, Q, and U) and E12.5 (J, N, R, and V) when compared with littermate controls. By E14.5, the tricuspid valve is underdeveloped and the right ventricle is hypoplastic in Wnt LOF mice (K, O, S, and W). The red asterisks denotes a similar size opening between right atrium and right ventricle in control and Wnt LOF mice (O and W), whereas a hypoplastic right heart can already be seen in Wnt LOF embryos. By E18.5, the tricuspid valve is completely absent in Wnt LOF mice (L, P, T, and X). The red arrow in X denotes the region of the atrial valve. M to P. Higher magnification images from boxed regions in I to L, respectively. U to X, Higher magnification images from boxed regions in Q to T, respectively. Scale bars 500 μm in A to H, I to L, and Q to T. Scale bars, 125 μm in M to P and U to X. See also Online Movies I and II, Figure I, and Tables I and II.
Figure 3 Continued. Immunohistochemistry of E14.5 embryos demonstrates ectopic Tbx3 and periostin near the ventricular apex in Wnt GOF mice when compared with littermate controls (white line demarcates the apex in both genotypes, n=3). Scale bars 500 μm in A and C, scale bar for G corresponds to G–I and is 50 μm, scale bar in L corresponds to L–Q and is 100 μm. Data are represented as mean±SEM. Group comparison was performed using a Student unpaired 2-tailed t test. *P<0.05.

Ectopic Wnt Activation Programs Ventricular Myocytes to Adopt an AVC Electrical Phenotype

Defining characteristics of AVC and AV nodal tissue are slow conduction velocity, which enables sequential atrial and ventricular chamber activation and contraction, and decremental conduction. The more frequently AV nodal tissue is stimulated the slower it conducts, and this decremental conduction property of the AV node prevents rapid atrial impulses from conducting to the ventricles in cases of rapid atrial rhythms, such as atrial fibrillation. Conduction velocity is determined by the ion channel characteristics and physical properties of the myocytes and is closely related to the maximum upstroke velocity of the depolarization phase of the action potential (determined by the fast Na+ current) and to the degree of cell–cell coupling. The AV node and AV junction have a relative absence of Scn5a expression, which encodes the major cardiac sodium channel Na1.5, as well as an absence of the high conductance gap junction isoforms connexin 43 and Cx43, which results in a slower conduction velocity when compared with atrial and ventricular chamber myocardium.

To determine whether the observed decrease in Scn5a and Cx43 expression within the ventricles in Wnt GOF mice affects cardiac conduction, we performed optical mapping on adult mice. In sinus rhythm, the PR interval and QRS duration were significantly prolonged in Wnt GOF mice when compared with littermate controls. (Figure 4A and 4B; Online Figure V). Prolongation of the QRS complex suggests that conduction velocity through the His-Purkinje system or ventricular myocardium is slowed. Total epicardial activation time is significantly prolonged in Wnt GOF mice (Figure 4C–4E; Online Movies III and IV), and because epicardial activation of the RV occurs after the end of the QRS complex in both control and Wnt GOF mice, QRS complex prolongation does not entirely reflect the severity of conduction slowing.27 To directly measure ventricular conduction velocity, we performed programmed electrical stimulation of the epicardial surface of the LV and RV. Epicardial conduction velocity was significantly decreased in...
both ventricles of Wnt activated mice, but strikingly, the RV was more severely affected (Figure 4F–4K; Online Movies V–VIII). One Wnt GOF mouse had an electrically inexcitable RV at baseline, whereas 2 other mice have a markedly decreased conduction velocity at baseline and became inexcitable at cycle lengths shorter than 125 ms (Figure 4K), consistent with long refractory periods, a characteristic of AVC and AV nodal tissue.

**Postnatal Notch Activation Downregulates Canonical Wnt Signaling and Reprograms AV Junction Myocytes Into Chamber-Like Myocardium**

We previously described a completely penetrant genetic model of ventricular pre-excitation that models WPW syndrome via activation of Notch signaling in a subset of embryonic AV canal (AVC) and ventricular myocardium (Mlc2vCre/+; Ctnnb1fl(ex3)/+; Wnt GOF (bottom) mice).15 Notch-activated mice have a prolonged QRS complex when compared with control littersmates (9.2±0.4 vs 12.6±1.2 ms; n=5 each genotype). C and D. Reconstructed electrical activation pattern from optical mapping experiment during sinus rhythm in control (C) and Wnt GOF (D) mice. E. Total epicardial activation time is significantly prolonged in Wnt GOF mice (4.4±0.4 vs 11.5±1.0 ms; n=4 each genotype). F to I. Representative electrical activation pattern of the left ventricle (LV) and right ventricle (RV) during epicardial stimulation in control (F and G) and Wnt GOF (H and I) mice. J, LV longitudinal conduction velocity of Wnt GOF mice was slower during stimulation at each cycle length, and the difference between the 2 genotypes became larger at faster pacing rates (111 and 100 ms cycle lengths; n=4). K, RV longitudinal conduction velocity of Wnt GOF mice was also slower and was more severely decreased than in the LV. One Wnt GOF mutant had an electrically inexcitable RV when paced at 143 ms cycle length, whereas 2 others had markedly decreased conduction velocity and became inexcitable at pacing rates above 125 ms cycle interval. This is consistent with decremental conduction, a property of AVC and AV nodal tissue. Note the different time scales between genotypes.

Data are represented as means±SEM. Group comparison for conduction velocity was performed using a Student unpaired 2-tailed t test at each cycle length. Group comparison for inexcitability was performed using a χ² test without Yates correction. *P<0.05.

**Figure 4.** Ectopic Wnt activation programs ventricular myocytes to adopt an atrioventricular canal (AVC) electrical phenotype. A, Representative surface ECG from control (top) and Mlc2vCre/+; Ctnnb1fl(ex3)/+; Wnt GOF (bottom) mice. B, Wnt GOF mice have a prolonged QRS complex when compared with control littersmates (9.2±0.4 vs 12.6±1.2 ms; n=5 each genotype). C and D. Reconstructed electrical activation pattern from optical mapping experiment during sinus rhythm in control (C) and Wnt GOF (D) mice. E. Total epicardial activation time is significantly prolonged in Wnt GOF mice (4.4±0.4 vs 11.5±1.0 ms; n=4 each genotype). F to I. Representative electrical activation pattern of the left ventricle (LV) and right ventricle (RV) during epicardial stimulation in control (F and G) and Wnt GOF (H and I) mice. J, LV longitudinal conduction velocity of Wnt GOF mice was slower during stimulation at each cycle length, and the difference between the 2 genotypes became larger at faster pacing rates (111 and 100 ms cycle lengths; n=4). K, RV longitudinal conduction velocity of Wnt GOF mice was also slower and was more severely decreased than in the LV. One Wnt GOF mutant had an electrically inexcitable RV when paced at 143 ms cycle length, whereas 2 others had markedly decreased conduction velocity and became inexcitable at pacing rates above 125 ms cycle interval. This is consistent with decremental conduction, a property of AVC and AV nodal tissue. Note the different time scales between genotypes.

Data are represented as means±SEM. Group comparison for conduction velocity was performed using a Student unpaired 2-tailed t test at each cycle length. Group comparison for inexcitability was performed using a χ² test without Yates correction. *P<0.05.

**Inhibition of Canonical Wnt Signaling Is Required, But Not Sufficient, for Ventricular Pre-Excitation**

Ventricular pre-excitation can arise secondary to perturbations of several developmental pathways that regulate AVC morphogenesis, so we speculated that the loss of myocardial Wnt signaling may predispose mice to ventricular pre-excitation. Whereas activation of Notch signaling within the Mlc2v expression domain is sufficient to induce ventricular pre-excitation, mice harboring loss of canonical Wnt signaling within the same expression domain do not develop ventricular pre-excitation (Online Figure VI). However, Wnt LOF mice (Mlc2vCre) upregulate Scn5a, which encodes one of the major molecular
Axin2Lacz/+ myocardium and tricuspid valve mesenchyme at P3. Notch GOF downregulates C not affect expression in the valve mesenchyme.

Taken together, the genetic rescue experiments demonstrate that Scn5a expression. Ventricular Notch activation upregulates direct Notch targets and downregulates Wnt targets Axin2 and Gata6, as measured by reverse transcription quantitative polymerase chain reaction. Important regulators of the AV junction, including Tbx3, Tbx20, Bmp2, and periostin are also downregulated (n=8 each genotype). B, Histological sections of Axin2Lacz/+ hearts show active canonical Wnt signaling in AV junctional myocardium and tricuspid valve mesenchyme at P3. Notch GOF downregulates Axin2 expression in the AV junctional myocardium but does not affect expression in the valve mesenchyme. C–E, Immunohistochemistry (IHC) demonstrates decreased Tbx3+ AV junctional myocardium in Notch GOF mice when compared with littermate controls (C and D). IHC demonstrates ectopic Na1,5 in Notch GOF AV junctional myocardium, whereas Na1,5 is absent within the AV junctional myocardium in littermate controls (E). F, Membrane localization of Na1,5 is shown in control atria for comparison. Data are expressed as mean±SEM. Group comparison was performed using a Student unpaired 2-tailed P test. *P<0.05; **P<0.001. Scale bars in B and C are 100 μm. Scale bar in C corresponds to C and D. Scale bar in E corresponds to E and F and is 10 μm. Black arrows in B denote AV junctional myocardium, and stars in B denote the tricuspid valve. White lines in D demarcate the AV junctional myocardium. Arrow in D denotes the sparse Tbx3+ region in Notch GOF. Sections in D represent a magnified region corresponding to the white box in C. Sections in E are serial to C. RA indicates right atrium; RV, right ventricle; and TV, tricuspid valve.

Determinants of cardiac conduction velocity, demonstrating a role for Wnt signaling in ion channel homeostasis (Figure 6A).

Although loss of Wnt is not sufficient for developing pre-excitation, we asked whether downregulation of Wnt signaling is required for Notch-mediated ventricular pre-excitation. Whereas accessory pathway formation and ventricular pre-excitation are completely penetrant by 4 weeks of age in Notch-activated mice, one quarter of mice where β-catenin is stabilized have a complete rescue of the phenotype, including both rescue of accessory pathways (Figure 6B–6D) and normalization of the PR interval (Figure 6E–6G; Online Figure VII). In the remainder of the mice, there is a partial rescue of the PR interval (Online Figure VII). In addition to regulating AVC morphology, Wnt and Notch cooperatively regulate ion channel gene expression. Ventricular Notch activation upregulates Scn5a expression, whereas ectopic Wnt activation downregulates Scn5a expression, consistent with programming to an AV junction-like phenotype. In addition to rescue of accessory pathway formation and PR interval, Wnt activation can also partially rescue the Notch-mediated effects on Scn5a expression (Figure 6H).

Taken together, the genetic rescue experiments demonstrate that Notch-mediated effects giving rise to ventricular pre-excitation are mediated, at least in part, via downregulation of canonical Wnt signaling. Our data also suggest that canonical Wnt and Notch signals cooperatively regulate AVC morphology and cardiac electrical programming, and perturbations of both processes may be required for ventricular pre-excitation.

Discussion

Global loss of Wnt2, which signals primarily via the canonical Wnt pathway, has been shown previously to result in a phenotype resembling complete common AVC in humans. The developmental basis for this defect is thought to be a failure of expansion of mesodermal progenitors within the posterior cardiac pole, resulting in subsequent defects in myocardial proliferation and differentiation. In the current study, we specifically inhibit canonical Wnt signaling within the myocardium at later developmental stages. Although the AVC myocardium is initially programmed, Wnt LOF mice fail to maintain the AVC phenotype along the right AVC, whereas left-sided structures are less affected. Loss of AVC myocardium is associated with other structural anomalies, including tricuspid atresia, a common form of congenital heart disease that accounts for ≈1% of congenital heart defects. 29 The developmental basis for tricuspid atresia is not well understood, but it has been previously associated with perturbations in either Notch or Gata signaling. 29,30 In our model, tricuspid atresia...
is completely penetrant and presents at later stages of cushion maturation than previously described, providing a useful model for further dissection of the pathogenesis of this disease. Because canonical Wnt signaling is known to regulate expansion of second heart field–derived structures, the primarily right-sided ventricular hypoplasia in Wnt LOF mice is not entirely surprising. It is interesting to note that the RV begins to become hypoplastic at stages before severe right-sided AV valvar abnormalities (Figure 1). In children with hypoplastic ventricles, the underdeveloped ventricle is thought to arise secondary to a lack of blood flow during embryogenesis,31 but our data suggest that perhaps both valvular and ventricular hypoplastic defects may be primary defects regulated by similar genetic pathways.

Notch GOF reprograms AVC myocardium, at least in part, through downregulation of canonical Wnt signaling (Figure 6). Because Wnt LOF alters expression of important ion channel genes, including Scn5a, but does not give rise to accessory pathways or ventricular pre-excitation, myocardial Notch signaling likely regulates other signaling pathways in addition to Wnt to mediate this effect (see model, Figure 7). Notch GOF mice develop accessory pathways throughout the annulus, with a preponderance of right-sided accessory pathways and a relative sparing of the left annulus.32 Interestingly, accessory pathways in Tbx2−/− mice are exclusively left sided, which has been attributed to redundancy with Tbx3 expression along the right AVC.32 Because hypomorphic alleles of Tbx3 may also result in ventricular pre-excitation,33 our model is consistent with a role for Notch and Wnt-mediated regulation of Tbx3 as a contributing factor in the pathogenesis of pre-excitation syndrome, whereas the relatively preserved expression of Tbx2 (Figures 3K and 5A) could potentially explain the predominantly right-sided accessory pathways in Notch GOF mice.

Recently, broadly expressed Gata factors together with local Bmp signaling has been demonstrated to be a sufficient regulatory switch to establish AVC specificity during heart development, as well as to regulate expression of a Tbx3 enhancer.34,35 Ectopic activation of canonical Wnt signaling within the ventricular myocardium is sufficient to program ectopic AV junction–like regions within the ventricles and upregulate Tbx3 expression, whereas Bmp2 and Tbx2 are unaffected (Figure 3K). Future work will determine whether Gata and Bmp signaling program the AVC together in a linear pathway with Wnt signaling or whether they represent alternate mechanisms to activate Tbx3 expression and program the AVC phenotype.

Given that genetic mutations giving rise to ventricular pre-excitation affect AVC patterning as well as myocardial lineage programming, we propose that alterations in both of these developmental processes might be required for pre-excitation to...
manifest. The mechanism whereby altered myocardial Wnt signals regulate migration and differentiation of nonmyocyte populations, perhaps including multipotent epicardial-derived cells known to give rise to the annulus fibrosus,36 will be an area of future investigation. Pre-excitation syndromes have a variable risk of sudden cardiac death, which is primarily related to the refractory period of the accessory pathway. It is intriguing to speculate that the same transcriptional networks regulating AV junction programming and expression of Scn5a may also regulate the accessory pathway effective refractory period, thereby governing the risk of sudden death in WPW syndrome and perhaps in arrhythmia syndromes more broadly. Indeed, activating mutations in Scn5a have been postulated to increase cardiomyocyte excitability,17 whereas Scn5a+/− mice have an increased effective refractory period perhaps secondary to the changes in excitability.18

Alterations in canonical Wnt pathway activity secondary to mutations in plakoglobin have been suggested to result in differentiation of a precursor cell into adipocyte-like cells in association with arrhythmogenic cardiomyopathy, a disease characterized by subepicardial fibro-fatty deposition preferentially affecting the RV.28,39,40 It has been recently shown that activation of canonical Wnt signaling may rescue a zebrafish model of arrhythmogenic cardiomyopathy.41 Wnt GOF mice bear a striking resemblance to arrhythmogenic cardiomyopathy including fibro-fatty deposition and slowed conduction velocity affecting the RV more than the LV (Figures 3B and 3C and 4). Therefore, our results are generally consistent with a role for altered canonical Wnt signaling in arrhythmogenic cardiomyopathy. However, we detect fibro-fatty deposition with Wnt signaling regulators of cardiac conduction.42–44 Indeed, we observe myocyte transdifferentiation into adipocytes (Figure 3F–3I). Genetic variants associated with prolongation of conduction intervals are most commonly found in noncoding regions of the genome and are traits associated with arrhythmias such as atrial fibrillation and sudden death. Future work will address the possibility that a subset of these risk-associated genetic variants affect regulatory elements that modulate Notch and Wnt-mediated expression of ion channels and predispose an individual to disease.

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Disclosures
None.

References
Novelty and Significance

What Is Known?
- Defects in the gene regulatory networks that pattern the embryonic atrioventricular (AV) canal are often associated with ventricular pre-excitation.
- Ecstatic Notch activation produces a murine model of ventricular pre-excitation that resembles Wolff–Parkinson–White syndrome.

What New Information Does This Article Contribute?
- Inhibition of canonical Wnt signaling within the myocardium results in tricuspid atresia associated with the loss of AV junction myocardium.
- Ecstatic activation of canonical Wnt signaling in the ventricular myocardium is sufficient to program an AV junction phenotype that phenotypically resembles arrhythmogenic cardiomyopathy.
- Genetic rescue experiments demonstrate that Notch-mediated effects giving rise to ventricular pre-excitation are mediated, in part, via downregulation of canonical Wnt signaling.

Structural and electrical disorders involving the AV junction represent some of the most common forms of congenital heart disease. A better understanding of the gene regulatory networks underlying AV canal programming and maturation could lead to improved diagnostic and therapeutic options for patients. In this article, we describe a coordinated role for Wnt and Notch signaling in regulating both the structural and electric properties of AV junction myocardium. Based on our results, we propose that both structural and electrical alterations of the AV junction might be required for ventricular pre-excitation. In addition, several independent loci identified in genome-wide association studies suggest that Wnt and Notch signaling may be regulators of cardiac conduction. In support of this, we observe that Wnt mutant mice have prolonged conduction intervals, a trait that is often associated with arrhythmias such as atrial fibrillation and sudden cardiac death. Future work will address the possibility that a subset of genetic variants associated with arrhythmias may involve regulatory elements that modulate Wnt and Notch feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol. 2002;22:1184–1193.


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Detailed methods

Mice

Mlc2vCre \(^1\), αMHC-Cre \(^2\), αMHCrtTA \(^3\), tetO-NICD \(^4\), Axin2\(^LacZ\) \(^5\), Ctnnb1\(^1\) \(^6\), Ctnnb1\(^dm\) \(^7\), Ctnnb1\(^\text{fl(ex3)}\) \(^8\), NICD \(^9\), Tbx2\(^\text{Cre}10\) and R26\(^\text{TdTomato}11\) mice have been described previously, and were maintained on a mixed genetic background. For experiments involving conditional gene expression, timed pregnancies were determined and induction of gene expression was accomplished with doxycycline chow (BioServ 200 mg/kg) during the stated timepoints. αMHCrtTA and tetO-NICD littermates fed doxycycline were used for comparison in all conditional gene expression experiments unless otherwise noted. Littermate controls were used for all experiments. All animal protocols were approved by the Animal Studies Committee at Washington University.

Histology and Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections with antibodies recognizing Tbx3 (sc-17871, Santa Cruz), connexin 40 (CX40-A, Alpha Diagnostic International), Na,1.5 (AS-005, alomone labs), CD31 (DIA-310, dianova), and periostin (Ab14041, Abcam). Secondary antibody-fluorescent conjugates included anti-rabbit Alexa 568 (Invitrogen), anti-goat Alexa 488 (Invitrogen), and for connexin 40, signal amplification was performed using anti-rabbit ImmPRESS (MP-7401, Vector Laboratories) with TSA (SAT702001, Perkin Elmer). Histology, immunohistochemistry, and whole-mount Xgal images were analyzed using Adobe Photoshop. Control and mutant images were treated identically in all cases where brightness and contrast were altered.

In situ hybridization

In situ hybridization for Bmp2 was performed as described previously \(^{12}\). Digoxigenin-labeled probes were detected using an antidigoxigenin-alkaline phosphatase conjugate (Roche) and visualized with an enzyme catalyzed color reaction.

Reverse Transcription-Quantitative Polymerase Chain Reaction

Total RNA was isolated from atria/AV or ventricles using Trizol (Invitrogen) and DNase treated using TURBO DNA-free DNase Treatment Kit (Ambion). First-strand cDNA was synthesized using a high Capacity cDNA Reverse Transcription kit (Applied Biosystems). Gene expression was assayed using the Power SYBR Green PCR Master Mix (Applied Biosystems) with primers listed in the attached Table and quantified using the StepOne Plus Real-Time PCR system or ViiA™ 7 qRT-PCR systm (Applied Biosystems). Relative fold changes were calculated using the comparative threshold cycle methods (2^{ΔΔCt}).

Optical Mapping

Optical mapping was performed as previously described \(^{13}\). Briefly, mice were anesthetized with a ketamine/xylose cocktail (ketamine, 80 mg/kg bodyweight; xylazine, 10 mg/kg bodyweight) and heparinized (100 units) by intraperitoneal injection. Hearts were excised and mounted on a Langendorff set-up and perfused at 37°C with Tyrode’s solution ((in mmol/L) 128.2 NaCl, 4.7 KCl, 1.19 Na H2PO4, 1.05 MgCl2, 1.3 CaCl2, 20.0 NaHCO3, and 11.1 glucose, pH maintained at 7.4 by equilibration with a
mixture of 95% O₂ and 5% CO₂). To record optical action potentials, a bolus injection of 10 mM Di-4-Anepps was administered with 15 mM blebbistatin to prevent motion artifacts. Optical signals were processed using MATLAB software. All optical signals were spatially binned (5x5 pixels), filtered using a 0-100Hz finite impulse response filter, and normalized. Activation times were defined at $dV_m/dt_{max}$. Simultaneous recording of a pseudo-electrocardiogram using electrograms placed 5 mm from the heart was performed, and the QRS duration was determined according to previously described methodology ¹⁴.

Statistical Analysis.
All data are expressed as means ± standard error (SEM). Statistical analyses were performed using student unpaired $t$ tests or one-way ANOVA followed by post-hoc Tukey’s test. Significant differences are indicated by *$P<0.05$, **$P<0.005$. 
Online Figure 1. Wnt LOF mice exhibit septal defects. Trichrome staining of αMhc-Cre; Clnnb1<sup>innh</sup> demonstrates prominent septal defects at E17.5 (C) and P0 (D) when compared with littermate controls (A and B respectively). Panel C demonstrates a membranous ventricular septal defect, while panel D demonstrates a muscular ventricular septal defect. Scale bar corresponds to A-D and is 500 μm.
Online Figure II. Canonical Wnt signaling is not required for early AVC maintenance. Gross morphology is normal in Tbx2<sup>Cre</sup>;Ctnnb<sup>1<sub>ΔN</sub></sup> embryos at E10.5 when compared with littermate controls (A) and Tbx3 expression is preserved (B, C). Scale bar in B corresponds to B, C and scale bars in A, B are 100 μm. Sections in B, C are magnified regions corresponding to the white box in A.
Online Figure III. Deletion of β-catenin within the AV Junction myocardium leads to tricuspid valve defects.

(A,B) In Mic2vCre; Ctnnb1flox/flox the tricuspid valve (B) is grossly similar to that in control (A) as assessed by Trichrome staining. Lineage tracing analysis demonstrates that Mic2vCre is expressed in a subset of ventricular myocytes, while only very sparsely within the AV junction as assessed in Mic2vCre; R26RtdTomato hearts (C,D). (E-H) In αMhc-Cre; Ctnnb1flox/flox mice where recombination occurs throughout the entire AV junction (G,H), the tricuspid valve is atretic (F compared to E). Black arrow in F denotes region of atretic tricuspid valve. White arrows in C, G denote the AV junction. Scale bar in A corresponds to A,B,E,F, scale bar in C corresponds to C,D,G,H and both are 100 µm.
Online Figure IV. Complete loss of β-catenin within the ventricles leads to abnormal right ventricular development with normal tricuspid valve formation. Trichrome staining at E16.5 shows a malformed RV in Mic2v^{Cre+};Ctnnb1^{f/f} when compared with control (A) and IHC staining for CD31 delineates grossly normal tricuspid valve morphology in Mic2v^{Cre+};Ctnnb1^{f/f} when compared with control (B,C). Scale bar in A is 500 μm. Scale bar in B corresponds to B,C and is 100 μm.
Online Figure V. PR interval and QRS interval prolongation in Wnt GOF mice.
(A) The PR interval is prolonged in Wnt GOF mice (46.0 ± 2.6 ms) when compared with littermate controls (32.5 ± 0.3 ms) as measured by surface ECG. (B) The QRS interval is prolonged in Wnt GOF mice (11.7 ± 0.8 ms) when compared with littermate controls (9.1 ± 0.4 ms) as measured by surface ECG. n=4, *p<0.005, **p<0.05. Data are expressed as mean ± SEM. Group comparison was performed using a Student’s unpaired 2-tailed t-test.
Online Figure VI. Wnt LOF is not sufficient for the development of ventricular preexcitation.

(A,B) Representative surface EKG from control (A) and Ml/c2v<sup>Cmt<sup>Cm<sup>/Wnt<sup>LOF adult mice (B), demonstrating a normal PR interval. (C) There is no difference in the PR interval between control and Wnt GOF mice (n=8 each genotype). Data are expressed as mean ± SEM. Group comparison was performed using a Student’s unpaired 2-tailed t-test.
Online Figure VII. Canonical Wnt signaling rescues Notch-mediated ventricular preexcitation. (A) Representative surface EKG traces demonstrate a shortened PR interval and widened QRS in Notch GOF mice, indicative of ventricular preexcitation. Activation of canonical Wnt signaling completely rescues the PR interval in 1/4 Notch GOF+Wnt GOF mice. (B) Measurement of PR interval reveals partial rescue in the remainder of Notch GOF+Wnt GOF mice (32.7 ± 3.2 ms in control, 1.5 ± 1.5 ms in Notch GOF, 12.3 ± 3.2 ms in Notch GOF+Wnt GOF). Data is expressed as ± SEM. Group comparison was performed using a one-way ANOVA followed by a post-hoc Tukey's test. p<0.05, n>20.
### Online Table I. Survival Difference at Birth Between β-catenin Null and Wnt Signaling Mutant Alleles.

*Mlc2v\(^{Cre/+}\) Ctnnb\(^{1^{-/+}}\) mice*, which retain one copy of β-catenin deficient in canonical Wnt signaling but with preserved cell adhesion, are represented in Mendelian ratios at birth. Complete loss of β-catenin in the myocardium is embryonic lethal since no *Mlc2v\(^{Cre/+}\); Ctnnb\(^{1^{-/ff}}\)* pups are recovered at birth. Group analysis was performed with a Chi squared test.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed</th>
<th>Expected</th>
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</thead>
<tbody>
<tr>
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<tr>
<td><em>Mlc2v(^{Cre/+})</em></td>
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<td>6/48</td>
</tr>
<tr>
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</tr>
<tr>
<td><em>Ctnnb(^{1^{-/dm+}})</em></td>
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</tr>
<tr>
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<td><em>Mlc2v(^{Cre/+}); Ctnnb(^{1^{-/dm+}})</em></td>
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*Mlc2v\(^{Cre/+}\); Ctnnb\(^{1^{-/dm+}}\)* mice were crossed with *Ctnnb\(^{1^{-/ff}}\) mice.*
<table>
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MHC-Cre; Ctnnb1^fl/+ mice were crossed with Ctnnb1^fl/fl mice, *p<0.05 when compared with expected.

**Online Table II. Myocardial Wnt LOF results in perinatal lethality.**

While MHC-Cre; Ctnnb1^fl/dm (Wnt LOF) mice are found in Mendelian ratios at birth, they uniformly die prior to postnatal day 1. Group analysis was performed with a Chi squared test.
Online Table III. Wnt heterozygosity sensitizes Notch GOF mice to lethality before 3 weeks of age

Mice with loss of one allele of β-catenin (Mlc2v<sup>Cre<sup>+</sup>; Ctnnb1<sup>fl/+</sup>) or myocardial Notch activation (Mlc2v<sup>Cre<sup>+</sup>; NICD) are represented in Mendelian ratios; however, Mlc2v<sup>Cre<sup>+</sup>; NICD; Ctnnb1<sup>fl/+</sup> have decreased survival at three weeks. Group analysis was performed with a Chi squared test.
### SYBR Green Primers

<table>
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<tr>
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### Taqman Assay IDs

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Supplemental References


13. Laughner JI, Ng FS, Sulkin MS, Arthur RM, Efimov IR. Processing and analysis of cardiac optical mapping data obtained with potentiometric dyes. \textit{Am J Physiol Heart Circ Physiol}. 2012;303:H753-765

Movie Legends

**Online Movie I. Echocardiogram of control heart at E18.5.**
Echocardiogram of E18.5 control mouse shows distinct blood flow across both the tricuspid and mitral valves.

**Online Movie II. Echocardiogram of Wnt LOF heart at E18.5**
Echocardiogram of E18.5 Wnt LOF mouse shows distinct blood flow across the mitral valve, while no distinct flow can be seen between right atrium and right ventricle, likely due to the atretic tricuspid valve.

**Online Movie III. Optical Mapping of control heart in sinus rhythm**
Optical mapping of control heart in sinus rhythm shows normal epicardial activation time.

**Online Movie IV. Optical Mapping of Wnt GOF heart in sinus rhythm**
Optical mapping of Wnt GOF heart in sinus rhythm shows prolonged LV and RV activation time.

**Online Movie V. Optical Mapping of control LV after epicardial stimulation**
Optical mapping of control LV after epicardial stimulation shows normal conduction velocity.

**Online Movie VI. Optical Mapping of control RV after epicardial stimulation**
Optical mapping of control RV after epicardial stimulation shows normal conduction velocity.

**Online Movie VII. Optical Mapping of Wnt GOF LV after epicardial stimulation**
Optical mapping of Wnt GOF LV after epicardial stimulation shows decreased conduction velocity.

**Online Movie VIII. Optical Mapping of Wnt GOF RV after epicardial stimulation**
Optical mapping of Wnt GOF RV after epicardial stimulation shows markedly decreased conduction velocity and heterogeneous conduction.