Three-Dimensional Reconstruction of the Rabbit Atrioventricular Conduction Axis by Combining Histological, Desmin, and Connexin Mapping Data

Yu-Shien Ko, MD, PhD; Hung-I Yeh, MD, PhD; Yu-Lin Ko, MD, PhD; Yu-Chun Hsu, MFA; Chin-Fen Chen, BS; Semon Wu, MS; Ying-Shiung Lee, MD; Nicholas J. Severs, PhD, DSc

Background—The 3D structure of the atrioventricular conduction axis incorporating detailed cellular and molecular composition, especially that relating to gap-junctional proteins, is still unclear, impeding mechanistic understanding of cardiac rhythmic disorders.

Methods and Results—A 3D model of the rabbit atrioventricular conduction axis was reconstructed by combining histological and immunofluorescence staining on serial sections. The exact cellular boundaries, especially those between transitional cells and atrial myocardium, were demarcated by a dense and irregular desmin-labeling pattern in conductive myocardium. The model demonstrates that the atrioventricular conduction axis is segregated into 2 connecting compartments, 1 predominantly expressing connexin45 (compact node and transitional cells) and the other predominantly coexpressing connexin43 and connexin45 (His bundle, lower nodal cells, and posterior nodal extension). The transitional zone shows unique features of spatial complexity, including a bridging bilayer structure (a deep transitional zone connecting with a superficial atrial-transitional overlay) and asymmetrical continuity (wider atrial-transitional interfaces and shorter atrial-axial distances in the hisian portion than in the ostial portion). In the latter compartment, the His bundle, lower nodal cells, and posterior nodal extension form a continual axis and longitudinal transitional-axial interface.

Conclusions—Key findings of the present study are the demonstration of a distinct anatomical border between transitional and atrial cells, connection between transitional cells and both lower nodal cells and posterior nodal extension, and distinctive connexin expression patterns in different compartments of the rabbit atrioventricular conduction axis. These features, synthesized in a novel 3D model, provide a structural framework for the interpretation of nodal function. (Circulation. 2004;109:1172-1179.)

Key Words: myocardium • immunohistochemistry • atrioventricular node • conduction

The atrioventricular conduction axis is defined as comprising the His bundle and atrioventricular node, or junction, which includes lower nodal cells, posterior nodal extension, compact node, and transitional cells. However, after more than 100 years of morphological and electrophysiological studies, several fundamental anatomical problems remain unsolved, impeding additional mechanistic investigations of this clinically important cardiac structure.

Traditional histological methods have major limitations in establishing morphological criteria for distinguishing distal compact node from His bundle and transitional cells from atrial cardiomyocytes. This has made the transitional zone, a territory recently regarded as the principal anatomical substrates of fast and slow pathways, an ambiguous region. Another key anatomical issue is the need for improved understanding of the precise 3D architecture of this region. Optical mapping during atrioventricular activation has revealed transmural reentrant circuits involving multiple transitional layers. Although earlier researchers have comprehended the spatial complexity, the transitional zone in most previous models is defined imprecisely.

A third key issue concerns the molecules associated with action potential formation and propagation. Among these molecules, the gap-junctional proteins, connexins, have been regarded as potentially key contributors to differential conductance within the atrioventricular conduction axis. Compared with those of contractile myocardium, the gap junctions within conduction myocardium are smaller, sparser, and irregularly distributed. Three main connexins, connexin40 (Cx40), Cx43, and Cx45, are expressed in the myocardium,
each conferring distinct intercellular communication properties in vitro. However, information on connexin expression in the conduction system is confined to rat, mouse, and limited studies on human; investigations in other species have been restricted to only 1 or 2 connexin types or have used potentially unreliable primary antibodies. The more comprehensive studies to date suggest that Cx45, which forms low conduction channels, is the main connexin expressed within the atrioventricular node, with Cx40 and Cx43 expressed in undetectable or at low levels. Even these data, however, are confined to the His bundle and compact node, leaving a dearth of information on the transitional zone.

One approach that may contribute to progress in these areas involves combined morphological and molecular mapping with accurate 3D modeling. To this end, we have reconstructed such a model by serial sectioning of the rabbit atrioventricular conduction axis, a system well suited to this approach by virtue of its size and previous thorough morphological and functional investigations. Key elements of our study involve integration of histochemistry with desmin labeling to define exact cellular boundaries between conductive and contractile cardiomyocytes and analysis of connexin distribution within the 3D model.

Methods

Isolation and Sectioning of Rabbit Atrioventricular Conduction Axis

Ten New Zealand White rabbits (3 kg body weight; Taiwan Livestock Research Institute [TLRI], Taiwan, ROC) were anesthetized by intraperitoneal pentobarbital injection (50 mg/kg). After hearts were dissected out and inner right cardiac chambers exposed, a rectangular area, extending 2 to 3 mm beyond the borders of the triangle of Koch, was cut out and cryoembedded. The tissue blocks were sectioned serially in a plane perpendicular to the long axis of the triangle of Koch with a thickness of 16 μm. C. Terminology of orthogonal plans for atrioventricular conduction axis. Bar=2 mm.

Immunohistochemistry for Desmin and Connexins

The anti-desmin antibody used was a mouse monoclonal antibody (Sigma: 1:100). The anti-connexin antibodies included a mouse monoclonal anti-Cx43 antibody (Chemicon; 1:1000) and a panel of well-characterized affinity-purified polyclonal antibodies against Cx40 (V15K/GP318, raised in guinea pig; 1:50), Cx45 (Rat9, raised in rat; 1:50), and, for double-labeling, Cx43 (Chk, raised in chicken; 1:50). The procedures for single and double immunolabeling and for recording with conventional fluorescence microscopy and confocal laser scanning microscopy (Leica TCS SP2-MP system) have been described previously. Specificity of the anti-connexin antibodies was confirmed by specific labeling patterns in rabbit sinoatrial nodal tissues.

Figure 2. Distinct desmin fluorescence immunolabeling patterns differentiating contractile and conductive cardiomyocytes. In ventricular cardiomyocytes, desmin localized along Z lines and intercalated disks, presenting as a typical transverse pattern (A). In the transitional zone (B, H&E staining), desmin signals were much stronger and distributed irregularly within cytoplasm (C), specifying exact cellular boundaries from neighboring atrial cardiomyocytes (D). The box in B indicates the corresponding area in C, and the star in C the corresponding area in D. IAS indicates interatrial septum; TC, transitional cells; and IVS, interventricular septum. Bar=10 μm in A and D. Bar=200 μm in B. Bar=80 μm in C.
Image Quantification
Confocal images of Cx43 and Cx45 fluorescent signals were analyzed as described previously. In brief, for each anatomical component, 6 46.5/H11003 46.5/H9262 m2 images were randomly selected from 3 rabbit samples, and numbers and areas of gap-junctional spots in each image were quantified using Scion Image software (Scion Corporation).

Three-Dimensional Reconstruction of Atrioventricular Conduction Axis
Thirty-three sets of consecutive sections were used, with set intervals of ~120 μm and a total thickness of ~4 mm. For each set, the contour of every anatomical component was determined by histological characteristics with H&E staining. However, the exact boundary between transitional cells and atrial cardiomyocytes could not be determined histologically. However, cardiomyocytes in the atrioventricular conductive axis were readily distinguished from contractile myocardium by desmin immunolabeling (Figure 2). Desmin labeling in contractile cardiomyocytes appeared decorated delicately along Z lines and intercalated disks as a typical transverse pattern (Figure 2A), whereas that in conductive cardiomyocytes was prominent and distributed irregularly within cyto-

Figure 3. Cx43 expression in the atrioventricular conduction axis. Boxes in the H&E images of A (the His bundle level, His), E (the compact node level, CN), and H (the transitional zone level, TC) indicate the corresponding areas in the desmin-labeling images of B, F, and I; those in B, F, and I indicate the corresponding areas in the desmin-labeling image of C and the Cx43-labeling images of D, G, and J. K, Sectioning levels of A, E, and H. A through D, Differential desmin and Cx43 labeling patterns within His bundle and ventricular myocardium (IVS). E through G, Cx43-positive lower nodal cells (LNC) contact directly with both Cx43-negative mid-nodal cells (MNC) and circumferential transitional cells (CTC). H through J, Contact between Cx43-negative transitional cells and Cx43-positive posterior nodal extension (PNE). G and J, Faint green is background tissue autofluorescence; Cx43 signals are red. Bar=500 μm in A, E, and H. Bar=200 μm in B, F, and I. Bar=50 μm in C, D, G, and J. Bar=2 mm in K.

Statistical Analysis
Quantitative data from the above studies are presented as mean±SD and analyzed statistically by ANOVA, with statistical significance defined as P<0.05.

Results
Identification of Rabbit Atrioventricular Conduction Axis by H&E Staining and Desmin Immunolabeling
The main components of the rabbit atrioventricular conduction axis were identified by typical histological characteristics with H&E staining. However, the exact boundary between transitional cells and atrial cardiomyocytes could not be determined histologically.

However, cardiomyocytes in the atrioventricular conduction axis were readily distinguished from contractile myocardium by desmin immunolabeling (Figure 2). Desmin labeling in contractile cardiomyocytes appeared decorated delicately along Z lines and intercalated disks as a typical transverse pattern (Figure 2A), whereas that in conductive cardiomyocytes was prominent and distributed irregularly within cyto-
plasm. Combining histochemical staining and desmin immunolabeling in consecutive sections thus made it possible to specify the exact cellular boundaries of the atrioventricular conductive axis, in particular that bordering the atrial myocardium (Figures 2B through 2D).

**Connexin Expression in Rabbit Atrioventricular Conduction Axis**

Cx40 signal, although prominent in vascular endothelium, was barely detectable or of very low levels in the atrioventricular conduction axis (data not shown). For Cx43 (Figure 3) and Cx45 (data not shown), both were clearly expressed in the His bundle, lower nodal cells, and posterior nodal extension, and Cx45 was the main connexin detected in the compact node and transitional cells. In contrast to the typical rosette arrangement between contractile cardiomyocytes, the punctate connexin signals between conductive cardiomyocytes were distributed diffusely and irregularly.

Quantitative analysis of immunofluorescent signals showed that Cx43 expression was high in the His bundle and lower nodal cells and decreased progressively toward the ostial end of posterior nodal extension (Figure 4). Total gap-junctional areas within the His bundle were significantly greater than those within the hisian and ostial portions of posterior nodal extension (Figure 4). Total gap-junctional sizes of the hisian end of posterior nodal extension were significantly greater than those at the ostial end (P<0.05). For Cx45, expression was sparse in the His bundle and showed the highest levels in the compact node (P<0.001). For the remaining zones analyzed, the Cx45
expression levels appeared of approximately equal abundance ($P>0.05$) and were lower than those in the compact node ($P<0.001$, Figure 4). In general, Cx43 signals, where present, were more prominent and abundant than Cx45 signals, and total gap-junctional labeling was higher in the His bundle, lower nodal cells, and posterior nodal extension than in the compact node and transitional cells.

Double labeling of desmin and Cx43 was performed to evaluate the boundary between atrial cardiomyocytes and transitional cells (Figure 5). At the junctional area, multiple cell columns from each side appeared to protrude into each other’s territories in interdigitating fashion. The desmin-defined cellular borders between the 2 cell types were distinct, and Cx43 signals were apparent between atrial cardiomyocytes but absent from the transitional zone and on contacting surfaces of these 2 cell types.

### Three-Dimensional Reconstruction of Rabbit Atrioventricular Conduction Axis

A 3D model of the rabbit atrioventricular conduction axis is illustrated in Figure 6. Inspection of the model shows that the predominantly Cx43-Cx45 and the Cx45 expressing compartments are readily discriminated spatially. In the former, the His bundle connects ostially to lower nodal cells and then to posterior nodal extension, forming a continual axis alongside the tricuspid annulus. In the latter, the transitional zone constitutes the major territory. Although parts of the transitional cells in the hisian portion connect to the compact node and subsequently to lower nodal cells and the His bundle, other circumferential transitional cells bypass the compact node and link directly to lower nodal cells and those in the ostial portion contact directly to posterior nodal extension (Figures 6A and 6B). Quantitative analysis revealed that in the hisian portion of the transitional zone, the atrial-transitional interfaces were significantly wider ($P<0.05$) and the atrial-axial distances shorter ($P<0.001$) than those in the ostial portion (Figure 7).

The complexity of the transitional zone revealed by the present model includes bridging and anatomical dead-end features of the atrial-transitional overlay, a superficial subendocardial tissue overlaying the main deep portion of atrioventricular conduction axis (Figure 6). Immunolabeling demonstrated that the atrial-transitional overlay was mainly composed of transitional cells, with varying amounts of atrial myocardium occupying the atrial portion. The atrial-transitional overlay mostly remained isolated from the deep transitional layer, with connection only noted at atrial, hisian, and ostial edges, bridging the hisian and ostial portions of the transitional zone. Another unique feature of the atrial-transitional overlay was the anatomically dead-end nature at its valvular edge and hisian protrusions, where distal transitional cells appeared embedded within connective tissue in disjointed fashion.

### Discussion

In the present study, by combining histological staining, desmin and connexin immunolabeling, and spatial reconstruction, we have built a comprehensive 3D model of the rabbit atrioventricular conduction axis, which would not have been possible by implementing these methods individually. In particular, the immunolabeling of desmin, a muscle-specific intermediate filament expressed in conductive myocardium at higher levels and with more disorganized arrangement than in contractile myocardium,\(^{23}\) has permitted clear demarcation of the atrioventricular conduction axis and unambiguous delineation of the transitional zone, a key element in this novel model.

The present model demonstrates that the transitional cells, rather than being intermediate between midnodal cells and atrial cardiomyocytes, constitute a distinct cell population as defined by desmin and connexin expression, and that the transitional zone is the most prominent and spatially complex part of the atrioventricular conduction axis. The transitional zone contains a deep layer and a superficial subendocardial atrial-transitional overlay, with connection apparent at atrial, hisian, and ostial edges. The spatial complexity of the transitional zone has been noted in earlier morphological studies\(^{2}\) and accords with the multilayer conduction patterns revealed by microelectrode and optical mapping studies,\(^{9,29}\) suggesting a possible role in the linkage of hisian fast and ostial slow pathways.\(^{21}\)
An additional key aspect of the spatial complexity of the transitional zone revealed by the present model is asymmetrical continuity, with the hisian portion possessing wider atrial-transitional interfaces and shorter atrial-axial distances than the ostial portion. The continuity and asymmetry of the transitional zone support the optical mapping interpretation that multiple atrial inputs are from functional, but not histological, nondiscrete pathways involving different quantities.
of transitional cells, with traditional division of transitional cell groups being arbitrary.

The present finding of a similar connexin profile and clear spatial continuity suggests that the His bundle, lower nodal cells, and posterior nodal extension constitute a continual axis. This finding complements earlier and recent morphological and electrophysiological studies that showed that posterior nodal extension is made of slow-conducting cells, connects with transitional cells, provides a direct connection to lower nodal cells and His bundle, and is involved in slow-fast reentry. Besides direct connection between the ostial portion of transitional cells with posterior nodal extension, the present results also show that whereas some of the transitional cells in the hisian portion connect to the compact node and subsequently to lower nodal cells, others, i.e., the circumferential transitional cells, bypass the compact node and link directly to lower nodal cells. This finding accords with earlier morphological and recent functional studies.

The connexin expression profile demonstrated in the present study differs from previous reports on rabbit, using the same monoclonal Cx43 antibody as that used in midnodal cells and circumferential transitional cells of the subendocardial layer but neglected the deep transitional layer and, again, lower nodal cells. These disparities may in part arise from use of different primary antibodies and in part from lack of combined histological and myocardial staining to provide an interpretative framework. In the present study, to verify our findings, we applied 2 well-characterized anti-Cx43 antibodies, neither of which was raised in rabbit, and both led to the same conclusions. We used Cx43-desmin double labeling and traced the labeling through consecutive serial sections to take account of all conductive cardiomyocytes within these areas to exclude any Cx43-gap junctions from the desmin-negative fibroblasts and to differentiate the Cx43-positive lower nodal cells from the Cx43-negative midnodal and transitional cells.

However, the functional significance of the connexin expression pattern in the present model should be interpreted critically. Although the relative Cx43 expression in the His bundle/lower nodal cell/posterior nodal extension axis correlates with the conduction velocities derived from microelectrode and optical mapping studies, that of Cx45 expression in compact node and transitional cells appears inversely related to the conduction properties of electrically defined N (nodal) and AN (atrioventricular) cells. Although connexin compositions and quantities of gap junctions are important contributors to intercellular communication properties, direct correlation of immunocytochemically determined connexin phenotype with conduction velocity is not always apparent as multiple factors come into play (eg, regulation of channel gating, state of connexin phosphorylation, and coexpression of other connexins to create heteromeric channels of different properties). Additional combined morphological, molecular, and functional investigations are necessary to explore the roles of gap-junctional communication in the conduction properties of the rabbit atrioventricular conduction axis.

In conclusion, the unique morphological and molecular features demonstrated in the present 3D model reveal how key constituent structures in the rabbit atrioventricular conduction axis are interconnected. The atrial myocardium, which expresses Cx43 and Cx45 (and possibly low quantities of Cx40), contacts with the predominantly Cx45-expressing transitional cells through a continuous and asymmetrical atrial-transitional interface, which is wider hisianly and narrower ostially. Some of the hisian portion of the transitional cells is connected to the compact node, which also mainly expresses Cx45. Compact node and transitional cells, both ostial and hisian, then link directly to the predominantly Cx43-Cx45-expressing posterior nodal extension/lower nodal cell/His bundle axis, with the horizontal atrial-axial distances longer ostially and shorter hisianly. Finally, there is a superficial subendocardial atrial-transitional overlay that contacts proximally atrial myocardium and bridges the hisian and ostial portions of transitional zone. The spatial connection framework provided by the present study may contribute to establishment of an unambiguous and comprehensive morphological and molecular basis for additional functional investigations.

Acknowledgments

This work was supported by Republic of China (Taiwan) National Science Council Grants (NSC-89-2314-B-182A-176, NSC-90-2314-B-182A-052, NSC-91-2314-B-182A-061, and NSC-92-2314-B-182A-175) and Chang Gung Memorial Hospital Grants (CMRP-1010 and CMRP-G32095).

References


Three-Dimensional Reconstruction of the Rabbit Atrioventricular Conduction Axis by Combining Histological, Desmin, and Connexin Mapping Data
Yu-Shien Ko, Hung-I Yeh, Yu-Lin Ko, Yu-Chun Hsu, Chin-Fen Chen, Semon Wu, Ying-Shiung Lee and Nicholas J. Severs

_Circulation_. 2004;109:1172-1179; originally published online February 9, 2004; doi: 10.1161/01.CIR.0000117233.57190.BD
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/9/1172

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/