Hemodynamics Is a Key Epigenetic Factor in Development of the Cardiac Conduction System

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Abstract—The His-Purkinje system (HPS) is a network of conduction cells responsible for coordinating the contraction of the ventricles. Earlier studies using bipolar electrodes indicated that the functional maturation of the HPS in the chick embryo is marked by a topological shift in the sequence of activation of the ventricle. Namely, at around the completion of septation, an immature base-to-apex sequence of ventricular activation was reported to convert to the apex-to-base pattern characteristic of the mature heart. Previously, we have proposed that hemodynamics and/or mechanical conditioning may be key epigenetic factors in development of the HPS. We thus hypothesized that the timing of the topological shift marking maturation of the conduction system is sensitive to variation in hemodynamic load. Spatiotemporal patterns of ventricular activation (as revealed by high-speed imaging of fluorescent voltage-sensitive dye) were mapped in chick hearts over normal development, and following procedures previously characterized as causing increased (conotruncal banding, CTB) or reduced (left atrial ligation, LAL) hemodynamic loading of the embryonic heart. The results revealed that the timing of the shift to mature activation displays striking plasticity. CTB led to precocious emergence of mature HPS function relative to controls whereas LAL was associated with delayed conversion to apical initiation. The results from our study indicate a critical role for biophysical factors in differentiation of specialized cardiac tissues and provide the basis of a new model for studies of the molecular mechanisms involved in induction and patterning of the HPS in vivo. (Circ Res. 2003;93:77-85.)

Key Words: chick embryo • His-Purkinje system • heart development • optical mapping
of excitation spread. Near the completion of ventricular septation in the embryonic chick heart, a distinct shift in the topology of ventricular activation emerges. The immature base-to-apex sequence of left ventricular activation undergoes an apparent reversal, altering rapidly to an apex-to-base pattern. This shift marks the emergence of mature “apex-first” epicardial breakthrough near the termini of the right and left bundle branches of the HPS.

Previously, we hypothesized a role for biophysical factors in differentiation of specialized cardiac tissues; either from effects of physical conditioning or through hemodynamic-induced molecular signaling cascades. We decided to test these assumptions on previously characterized in vivo models in which hemodynamic loading of the ventricle was either increased or decreased. This analysis revealed that the timing of emergence of apex-first activation shows a striking dependence on hemodynamic load; conversion from the immature to the mature pattern of ventricular activation was accelerated by increased loading and delayed by decreased load. The results from this study confirm the key importance of biophysical forces in the differentiation of the HPS in vivo.

Materials and Methods

Egg Incubation and Embryo Staging

Fertilized White Leghorn chicken eggs (ISE, Newberry, SC) were incubated until the end of a 38°C forced-draft incubator to Hamburger-Hamilton stages (HH) 16 to 36. These stages were selected to cover normal ventricular activation development from immature looping to mature four-chamber heart.

Conotruncal Banding (CTB) and Left Atrial Ligation (LAL)

The embryonic surgical procedures were performed essentially as described. For details, see the expanded Materials and Methods section in the data supplement, available online at http://www.circresaha.org. Since the surviving sham-operated embryos from both procedures appeared normal and did not differ from intact embryos in HH stage or other measured parameters, they were pooled together as controls. Typical phenotype of hearts exposed to CTB or LAL was evaluated macroscopically as described previously: only hearts with clearly developed morphological phenotype were used for further analysis.

Optical Mapping

Optical mapping was performed essentially as described in detail previously. For details, see the expanded Materials and Methods section in the data supplement, available online at http://www.circresaha.org.

Immunohistochemistry

After the recordings, the isolated control or experimental hearts were photographed, fixed in Dent’s fixative (80% methanol/20% DMSO), processed into paraffin, and serially sectioned at 8 μm for immunohistochemical detection of early markers of ventricular conduction system (PSA-NCAM, clone 5A5, fresh ascites fluid, from Developmental Studies Hybridoma bank and α-myosin heavy chain, clone 169-1A6, shown to stain developing ventricular conduction system in the avian heart in a manner described by Sanders et al. Imaging was carried out on Leica TCS SP2 AOBS confocal microscope.

Cell Birth-Dating Study With Tritiated Thymidine

See the expanded Materials and Methods section in the data supplement, available online at http://www.circresaha.org.

Statistical Analysis

For statistical analysis of treatment effects, we used Pearson’s χ² test. A value of P<0.05 was considered significant.

Results

Optical Mapping of the Embryonic Chick Heart Confirms the Occurrence of a Distinct Shift in Topology of Ventricular Activation at Septation

Earlier studies using bipolar electrodes indicated that the functional maturation of the HPS in the chick embryo is marked by a distinctive shift in the sequence of activation at the ventricular epicardial surface. Our initial goal was to use optical mapping and voltage-sensitive dye to confirm the occurrence of this shift and to characterize the overall changes in ventricular activation pattern that accompanied it. We thus studied patterns of ventricular activation during chick embryonic development from HH 16 to 36. In the chick heart in anterior view, the right ventricle comprises the substantive ventricular part. Thus, when studying left ventricular activation transition, dorsal view is the most informative. At the earliest stages studied (HH 16 to 17), we observed deceleration of activation in the AV canal and outflow tract regions and acceleration of activation in the ventricle consistent with the previous report of de Jong and coworkers (Figure 1). Specifically, within the ventricle, the rapid propagation of impulse appeared to distribute along trabecular ridges organized radially from the AV junction to the outer curvature of the looped, tubular heart. Mapping of the endocardial surface in open hearts suggested that radially arranged trabeculae (Figure 1) continued to account for base-to-apex sequences of activation through to HH 34, when the last examples of hearts demonstrating the immature pattern were observed. Nonetheless, a change from the immature base-to-apex sequence to more mature patterns was first observed starting at HH 29 when a small proportion of mapped hearts demonstrated ventricular activation from two foci, one at the left ventricular base and the other at the apex of the right ventricle (Figure 2a). From HH 31, the proportion of hearts demonstrating this intermediate phenotype increased; however, a growing number of hearts also demonstrated initiation of activation from a single apical focus. This single focus was observed near the right ventricular apex, slightly above the interventricular groove (Figure 2b), a localization confirmed by imaging the right ventricle from the side (data not shown). The proportion of hearts demonstrating such mature apex-first patterns continued to increase from HH 31, encompassing 100% of hearts by HH 36 (Figure 2d). Interestingly, as development proceeded, even the mature pattern demonstrated addition of further complexity, with a second apical focus becoming apparent at the presumptive termination of the left bundle branch from HH 35 in an increasing number of hearts (Figure 2c and online movies, available in the data supplement). Specifically, 2 of 6 hearts and 9 of 16 hearts demonstrating the mature apex-first pattern had breakthrough at both right and left apical foci at HH 35 and 36, respectively.
Timing of the Emergence of Apex-First Activation in the Embryonic Chick Ventricle Is Accelerated by Pressure Overload

The results described in the preceding section confirmed the initial description of conversion of the ventricular activation sequence in the embryo. Most significantly, optical mapping provided a direct and sensitive assay for this discrete marker of the development of HPS function. Previously, we had proposed that hemodynamics and/or mechanical conditioning may be key epigenetic factors in development of the HPS. We thus hypothesized that increased hemodynamic load may accelerate the timing of the conversion, marking maturation of the conduction system. CTB at HH 21 constricts the outflow tract of the embryonic heart, inducing a sustained increase in blood pressure within the ventricle. As conversion normally begins from around HH 29, we focused on stages HH 27 to 28. In line with our hypothesis, CTB resulted in precocious emergence of the mature apex-first

Figure 1. Activation sequence of chick embryonic heart. a, Confocal projection of phalloidin-stained heart at HH 17. Note emerging trabeculae (*) in the ventricular apex. Scale bar=100 μm. b, Labeled image of HH 17 heart from the high-speed camera (dorsal view) with isochrones in 2-ms intervals showing ventricular surface activation. A indicates atrium, AV, atrioventricular canal, V, ventricle, and Ct, conotruncus. c, Time-lapse recording of action potential (first derivative of the filtered data) spread through this looped tubular heart. Bright pixels indicate passing of the activation wavefront. The frame interval is 2 ms. Note slow conduction through the AV canal, fast through the ventricle, and slow again through the conotruncus (individual segments indicated by arrowheads). d, Examples of raw optical recordings of action potential from different heart compartments (“virtual electrode” positions are indicated by color-coded squares in b). Note the contraction artifact in the atrial trace and slower upstroke velocity in the AV and Ct regions. e, Mapping of endocardial activation reveals radial spread of action potential through the trabecular network. Heart at HH 31 was dissected in the frontal plane and imaged from the inside. Dorsal half shows the disposition of the ventricular trabeculae (phalloidin staining, confocal microscopy). Scale bar=100 μm. Star indicates the first activated ventricular region (crest of forming ventricular septum), arrows the direction of spread of activation along the radially aligned trabeculae. RA indicates right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle. f, Activation sequence showing right-to-left atrial activation (top row) followed by ventricular activation (bottom row). Frame spacing is 2 ms.
Specifically, at HH 27 to 28, 9 of 20 banded hearts demonstrated breakthrough at the apex, either as a single apical focus (2 of 20) or in the context of the intermediate phenotype, where two ventricular breakthroughs are observed (Figure 3 and online movies). By contrast, all 27 control hearts consistently showed the immature base-to-apex activation. This evidence of significantly (P < 0.05) accelerated maturation of ventricular conduction system was also present at HH 29 to 30 (Figure 3).

The distinct plasticity in this marker of HPS function led us to search for morphological correlates. Immunohistochemistry of PSA-NCAM, a well-characterized morphological marker of the developing HPS at this stage, delineated both bundle branches and an incipient network of Purkinje fibers in control hearts. In CTB hearts, overall upregulation of PSA-NCAM compared with controls was first apparent at HH 29, and this relative increase persisted through to HH 35 (Figure 4). Birth-dating with tritiated thymidine delineated the His bundle and bundle branches in control and CTB hearts at HH 35. As can be observed in Figures 4e and 4f, and as reported previously, CTB hearts consistently demonstrated a ventricular septal defect (VSD). The His bundle was delineated at the crest of muscular septum posterior to the VSD (Figure 4). The bifurcation of the His bundle and bundle branches were also distinguishable, although not as clearly as in the controls. Importantly, the distribution of cells demonstrating withdrawal from proliferation immediately after CTB (ie, at HH 21) was more extensive in banded hearts compared with nonbanded controls.

**Decreased Loading of the Embryonic Ventricle Delays the Timing of Apex-First Activation**

Because the pressure-overload experiments demonstrated acceleration in the timing of the conversion to mature ventricular activation, we devised experiments to test whether maturation of HPS function could be delayed by decreased loading. For this purpose, we chose a model of decreased left ventricular preload induced by ligation of the developing left atrium. This procedure redirects the blood flow within the developing ventricle, leading to a distinct left ventricular hypoplasia and accompanying decreases in myocyte sarcomeric organization from insufficient mechanical loading. Because complete conversion to mature apex-to-base activation occurs normally at HH 36, we focused our analysis of LAL effects at stages HH 34 and 36. At HH 34, we observed immature base-to-apex ventricular activation in all hearts demonstrating left heart hypoplasia in response to LAL compared with only 36% controls (P < 0.05). At HH 36, two thirds of hearts with left ventricular hypoplasia presented immature base-to-apex activation (P < 0.01), whereas all controls demonstrated the mature phenotype (Figure 5). Fully mature patterns of activation were not seen even in the minority of LAL hearts showing apex-to-base sequences at HH 36. In such cases, no LAL hearts showed left bundle branch breakthrough. Simultaneous breakthrough at the termini of the right and left bundle branches represents the culminating step in the progressive...
maturation of HPS function. Although not present in any LAL hearts, this most mature of patterns was evident in 56% of stage-matched controls. Despite the distinct physiological phenotype resulting from LAL, the morphology and immunohistochemistry of the conduction fascicles in such hearts did not reveal any remarkable differences in HPS patterning (Figure 6), although subtle changes due to the treatment could not excluded.

**Discussion**

This study demonstrates that the normal timing of maturation of the HPS, as revealed by conversion of ventricular activation sequence, depends on hemodynamic loading. Increased pressure loading induced by embryonic outflow tract constriction (CTB) led to a precocious emergence of mature apex-first activation pattern, whereas decreased left ventricular filling caused by LAL led to its significant delay. These findings point to hemodynamics as a key epigenetic factor in development of the cardiac conduction system.

Compared with classic microelectrode recordings, optical mapping offers superior spatial resolution, allowing better appreciation of epicardial activation patterns. The potential shortcoming of inability to see the actual activation wavefront in three dimensions can be overcome by imaging the endocardial surface of a partially dissected heart, as was successfully shown in the present work and previously in a study demonstrating slow impulse propagation through the right bundle branch of connexin40 null mice. To control motion artifacts, we used the actin filament disrupter cytochalasin D. This proved to be effective and significantly improved signal-to-noise ratio. Although no effects of its addition on action potential shape and duration were reported in mouse (reviewed in [27]), we found that in chick it caused slight prolongation of action potential compared with mechanical restraint.28

![Figure 3. Precocious conversion to apex-to-base ventricular activation pattern is observed in CTB hearts. a, In control heart viewed from the back at HH 27, a typical base-to-apex activation pattern is observed. b, Proportion of CTB hearts shows an apex-first activation pattern, normally not seen before HH 31. See online movies for animation. c, Unfiltered optical recordings showing clearly the activation of the ventricular apex (black trace) prior to the base (gray trace) in the CTB heart. Sites of recordings are indicated by black and gray squares in a and b. d, CTB-induced pressure overload accelerates maturation of the HPS, as evidenced by significantly (Pearson’s χ² test) accelerated conversion toward more mature ventricular activation patterns.](http://circres.ahajournals.org/issue)
Detailed evaluation of action potential morphology was not performed, because of the contraction artifacts obscuring the repolarization phase, if no excitation-contraction uncoupler was used, and possible confusion caused by its use that was essential to obtain good activation maps, and the need to change objectives to accommodate the increasing heart size. However, the atrial action potential was shorter than that of the ventricle, with typical morphology already at the earliest stages examined (Figure 1), and little developmental difference was observed in shape and duration of ventricular action potential. Microelec-

Figure 4. Morphology of ventricular conduction system in control and CTB hearts at HH 35. In control heart, the left bundle branch (arrow) is sharply outlined by both thymidine prelabeling (a and c) and PSA-NCAM (b and d; pseudocolor display of staining intensity), which labels more the distal part and subendocardial Purkinje network. The banded heart (e through h) presents a VSD (*), the boundaries of bundles are less sharply defined, and there is a marked PSA-NCAM upregulation in the left ventricle (f). Scale bar=1 mm (a, b, e, and f). RA indicates right atrium; LA, left atrium; RV, right ventricle; and LV, left ventricle.

Figure 5. Decreased preload in LAL delays HPS maturation. In a majority of control hearts at HH 36, apex-to-base activation with two breakthrough foci (a) is observed. Abnormal persistence of immature base-to-apex activation pattern is shown in heart subjected to LAL and severe left ventricular (LV) hypoplasia (b). RV indicates right ventricle. c, LAL-induced left ventricular hypoplasia is accompanied by significantly (Pearson’s $\chi^2$ test) delayed maturation of the HPS.
tetrode recordings with proper calibration should be more suitable for resolving any regional or developmental differences in action potential morphology.

There were some indications in the literature that development of the ventricular conduction system might show differences between the chick and the mouse. However, the optical mapping and morphological studies in chick and mouse undertaken here and elsewhere suggest that such differences may merely reflect species variation in the timing of key topological events during cardiac morphogenesis rather than their sequence. When such considerations are taken into account, the sequence of events is remarkably similar. In both species, the initial activation sequence follows blood flow through the peristaltoid tubular heart (mouse, embryonic day [ED] 7.5 to 8.5; chick, HH 10 to 14). Concomitant with chamber differentiation and looping, conduction velocity increases in the ventricular region. However, the initial base-to-apex ventricular activation pathway is present in both species when comparable (dorsal) views of the heart are used (mouse, ED9.5; chick, HH 17 to 29 [Figures 1 and 3a]). The functional deployment of HPS is first evidenced in mouse at ED10.5 by an apical epicardial breakthrough at the termination of the forming right bundle branch. As we report here, a similar pattern of right-sided first breakthrough at the apex occurs in chick between HH 29 to 31 (Figure 2). Similarly, in both species, the evidence of left bundle branch function (appearance of a second apical breakthrough site) occurs later (mouse, ED11.5; chick, HH 35). Although the gestation periods are similar between chick and mouse (20 to 21 days), the main sequence of morphogenetic events is much more compressed in the murine, as is the functional maturation of the HPS. While the mouse takes a mere 2 days (ED8.5 to ED10.5) to go from immature peristaltoid to mature apex-first activation, this period stretches between ED2 to ED7 in the avian (HH 14 to 31). This species difference also underscores the usefulness of the chick embryo as an animal model for the types of experiment undertaken in the present study. Together, with the relative

Figure 6. Morphology of ventricular conduction system in left heart hypoplasia. Despite the extreme shrinkage and dorsal shift of the hypoplastic left ventricle (hematoxylin-eosin staining; a and b), PSA-NCAM staining (c through f) on sister sections shows that both left (LBB) and right (RBB) bundle branches are present in their expected location in the upper interventricular septum. Scale bar=1 mm (a and b). RV indicates right ventricle; LV, left ventricle; RBB, right bundle branch; and LBB, left bundle branch.
surgical inaccessibility of the mouse embryo, the compressed “morphogenetic window” in mouse would probably confound resolution of the effects of altered hemodynamic loading similar to what we report here for chick.

With the use of molecular tools such as transgenesis in mice, the last decade has seen a great expansion in knowledge of the genetic regulation of cardiac development. By contrast, the epigenetic determinants of heart development remain relatively understudied. Nonetheless, the morphogenetic effects of factors such as myocardial stress/strain and blood pressure and flow in the embryonic heart are beginning to attract increasing attention.7,31,32 Although the present study indicates that ventricular load has direct effects on HPS maturation, the transcriptional and molecular signaling processes that may be downstream of such biophysical prompts are uncharacterized. Responses of embryonic trabecular myocytes to alterations in strain resulting from CTB or LAL, respectively, may be one important factor. Increased (CTB) or decreased (LAL) mechanical loading may accelerate or retard conversion, directly through conditioning of trabecular muscle destined to become conducting fascicles (reviewed in5–6). Another consideration may be the role of paracrine interactions from other mechanosensitive tissues such as the endothelium in prompting myocardial differentiation (reviewed in7–8). It is now well recognized that cardiac, vascular, and endothelial tissues respond sensitively to variation in shear stress and pressure in terms of altered gene expression and cytokine secretion (reviewed in13). Incipient conduction cells present in the ventricular trabeculae of the looped, tubular heart demonstrate intimate contacts with endocardial cells.34 Similarly, progenitors of the most peripheral element of the conduction system in the chick, the perianterial Purkinje fiber, show tight patterns of association with coronary arteries and never differentiate around lower tension vessels such as capillaries and veins.35–37 Vascular and endocardial tissues in the embryonic heart are thus well positioned and primed to transduce mechanical stimuli to embryonic myocytes via paracrine intermediaries. In previous work, we have reported that a shear stress-sensitive cytokine prominently expressed by endothelial tissues, endothelin-1 (ET-1), induces embryonic chick myocytes to express some markers of Purkinje fiber differentiation.38,39 Interestingly, ET-1 secretion by endothelial cells is particularly increased by a combination of pulsatile shear stress and increased blood pressure,40,41 hemodynamic changes particularly pronounced in CTB hearts. Alterations in ET-1 secretion and/or expression of ET-1 signaling pathway components such as endothelin-converting enzyme-1 (ECE-1) in response to altered hemodynamic load thus represent interesting topics for future study.

Studies in mouse suggest that a second factor secreted by endothelial cells, neuregulin-1 (NG-1), also has key roles in both trabecula formation and differentiation of conduction cells.30,42,43 To date, there is no evidence that NG-1 expression or secretion is directly modulated by mechanical factors such as strain or fluid shear stress and pressure. Thus, it is difficult to envisage how NG-1 could be the primary mediator of the plasticity in activation conversion seen here in response to altered loading of the embryonic ventricle. It may be pertinent that it has been reported that ET-1 treatment increases NG-1 expression in cultured endothelial cells,39 suggesting the potential for crosstalk between these two pathways and a basis for an indirect mechanism upregulation of NG-1 by physical force. This being said, it should be emphasized that the specific signaling and/or transcriptional processes governing functional maturation of the HPS remain to be fully characterized.

The present study indicates that conversion to apex-first activation is a sensitive temporal marker of the initiation of mature HPS function. As such, this marker represents a powerful new tool for future studies of the molecular mechanisms regulating morphogenesis of the ventricular conduction system in vivo.

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“Hemodynamics Is A Key Epigenetic Factor In Development Of The Cardiac Conduction System”

by Reckova et al.

Embryonic surgical procedures
At HH 21 (CTB) or 24 (LAL), the embryos were exposed via a window in the shell and an incision of the inner shell membrane. At HH 21, 10-0 nylon suture was passed around the mid-portion of the conotruncus, and tied in an overhand knot snug against its wall, but without constricting blood flow\(^1\). Sham controls had the suture passed and removed. For LAL, the embryo was turned on its right side at HH 24, and after exposing the heart by splitting the chest wall, 10-0 nylon loop was tied around left atrial appendage to restrict the left atrial cavity. Excess thread was trimmed and embryo turned back to its original position\(^2\). Sham controls were turned and opened but not ligated. The window in the shell was then sealed with electrical tape and the embryos reincubated either to HH 27-30 or HH 34-36 for CTB or LAL, respectively.

Optical mapping
Hearts from chick embryos at the appropriate stage were isolated under a dissecting microscope and stained with voltage-sensitive di-4-ANEPPS (0.002% solution in Tyrodes-Hepes Buffer, pH 7.4) for 5 minutes at room temperature. Some hearts were dissected in either frontal or sagittal plane to obtain endocardial views of the
interventricular septum and trabeculae. To reduce undesirable motion, we used Cytochalasin D\textsuperscript{3,4} diluted in dimethyl sulfoxide (DMSO) in concentration 80 µmol/l during the recordings. Our setup for optical mapping is similar to that described by others\textsuperscript{5}. The hearts were then placed into a silicone-lined custom-made copper dish with oxygenated Tyrodes-Hepes solution onto a temperature-controlled stage (Biostage 600, 35°C) of an upright epifluorescence-microscope (Leica DMLFS) fitted with a 12 bit intensified (Videoscope Inc.) high-speed digital camera (Neurocam, EGG-Wallace/Olympus). With green (546 ± 10 nm) excitation and red (590 nm LP) emission filter set, drop in fluorescence intensity corresponds to passing activation potential (Figure 1). To accommodate hearts of different size, objectives from 10x water to 2.5x dry were used. Images were first recorded in high-resolution mode (80x80 pixels) at 500 frames/second. In order to improve temporal resolution up to 1500 frames/second and signal-to-noise ratio, we used in addition asymmetric binning (1:2 to 1:5). Anterior, posterior, and both lateral views of the heart were recorded. In the chick heart in anterior view, the right ventricle comprises the substantive ventricular part\textsuperscript{6,7}. Thus, when studying left ventricular activation transition, the most informative is optical mapping from dorsal view (used throughout this paper). However, anterior and lateral views, as well as different dissections exposing the endocardium, were valuable in understanding the activation patterns.

Analysis of the recordings

Recordings were digitally processed using custom software (Universal Mapping, written by and available from Dr. Martin Biermann, University of Muenster, Germany) as
described previously\textsuperscript{8}. First, the raw optical recordings (in sequence of TIF images) were converted to format compatible with Universal Mapping. Within this program, the profiles of individual pixel intensity values over time were digitally filtered (Butterworth low-pass filter, cutoff at 100 Hz for activation map construction or 60 Hz for movies). No signal averaging was used; however, we determined empirically that the individual beats in the recorded sequence were identical. First derivative was computed for each channel (supplemental Figure 1), and its peak (the maximum upstroke velocity defined as dP/dt max) was used for construction of isochronal activation maps. Thus, activation time for each pixel was calculated with 2 ms precision (full resolution mode) based on the peak of the first derivative of its grey level value over time. The activation maps were then manually superimposed on bright field high-resolution images of the hearts recorded immediately after mapping by a different camera (Figures 2, 5). For display, the traces of individual channels were normalized (re-scaled, supplemental Figure 1), and movies (examples shown in online data supplement) of action potential propagation were constructed. For graphic visualization of activation sequence, the image of the moving activation wavefront (the first derivative) was more informative, as demonstrated previously by others\textsuperscript{9}.

**Cell birth-dating study with tritiated thymidine**

Central parts of chick conduction system (His bundle and its branches) can be visualized by label-dilution principle\textsuperscript{10} based on their slow proliferation, and thus retention of radiolabel. Fertilized White Leghorn eggs were opened and 4 μCi of [3H]-methyl thymidine (specific activity 90.0 Ci/mmol) in 200 μl of Tyrodes-Hepes solution was
applied to the air sac membrane at HH 17 or 21, 24 hours before CTB or LAL, respectively. The eggs were reincubated until HH 35 (CTB) or HH 36 (LAL). The hearts were perfused with cardioplegic solution and Dent’s fixative, processed into paraffin and 8 μm sections were cut and mounted. After rehydration, sections were stained with Propidium Iodide (1:10,000) and dipped at 42°C in 50% ARG emulsion (Kodak NTB-2), stored at 4°C for 21 days, and developed with 50% Kodak D19 at 20°C. Combined dark field and epiflorescence images (Figure 4) were collected on a Leica DMLB microscope with Hamamatsu C5810 3CCD camera. Images were then processed using Adobe Photoshop.

Additional morphological examination

For three-dimensional visualization of trabecular patterns, we referred to our previous studies using scanning electron microscopy or confocal microscopy.

Supplemental References


Supplemental Figure 1. a: Main window showing normalized values of pixel intensity over time from a 2-cycle recording of a chick embryonic heart at HH 24 with mechanical motion inhibition. Zoom window (b) shows ventricular action potentials after inversion of the data. Raw data is in red, filtered data in white, and the first derivative (also filtered with 100 Hz cutoff) in yellow. Activation marks (also in yellow) are generated automatically at the peak of the first derivative.
Legend to online movie supplements

Supplemental movie 1.
Activation sequence of chick embryonic heart. Following the activation of the atria and atrioventricular delay, ventricles at later embryonic stages are activated from the ventricular apex. A single focus corresponding to right bundle branch breakthrough is typical at HH stage 34, while from stage 35, presence of two foci reveals functionality of both right and left bundle branches.

Supplemental movie 2.
Pressure overload induced by conotruncal banding (CTB) accelerates maturation of ventricular activation sequence by HH stage 27. In contrast to control hearts, which were without exception activated from base to apex (left), some CTB hearts presented with mature-like "apex-first" pattern, normally not observed until 2 days later.