Electrophysiology and anatomy of embryonic rabbit hearts before and after septation

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Rothenberg, F., V. P. Nikolski, M. Watanabe, and I. R. Efimov. Electrophysiology and anatomy of embryonic rabbit hearts before and after septation. Am J Physiol Heart Circ Physiol 288: H344–H351, 2005.—Mechanisms of cardiac pacemaking and conduction system (CPCS) development are difficult to study, in part because of the absence of models that are physiologically similar to humans in which we can label the entire CPCS. Investigations of the adult rabbit heart have provided insight into normal and abnormal cardiac conduction. The adult and the embryonic rabbit have an endogenous marker of the entire cardiac conduction system, neurofilament 160 (NF-160). Previous work suggested that ventricular septation correlates with critical phases in avian CPCS development, in contrast to the mouse CPCS. Combining high-resolution optical mapping with immunohistochemical analysis of the embryonic rabbit heart, we investigated the significance of ventricular septation in patterning the rabbit embryonic conduction system. We hypothesized that 1) completion of ventricular septation does not correlate with changes in the ventricular activation sequence in rabbit embryos and 2) CPCS anatomy determines the activation sequence of the embryonic heart. We found that preseptated (days 11–13, n = 13) and post-septated (day 15, n = 5) hearts had similar “apex-to-base” ventricular excitation. PR intervals were not significantly different in either group. CPCS anatomy revealed continuity of the NF-160-positive tract connecting the presumptive sinoatrial node, atrioventricular (AV) junction, and ventricular conduction system. The presence of collagen in the AV junction coincided with the appearance of an AV interval. We conclude that ventricular conduction system, neurofilament 160 (NF-160). Previous work indicated that these relatively slowly conducting regions correlated to overlapping zones of labeling of two different markers for myocardial structural proteins (11).

In the mouse model, earliest ventricular activation in the preseptation 10.5 days postcoitum embryo occurred in the apex and was thought to reflect the presence of a mature His-Purkinje pathway (32). These data were in contrast to work in the embryonic chick (7). In preseptation chick embryo hearts, ventricular activation occurred in a “base-to-apex” fashion, whereas completion of ventricular septation was coincident with the emergence of an “apex-to-base” pattern of conduction. It was inferred from this apparent switch that the completion of ventricular septation brought with it maturation of the His-Purkinje pathway through which atrial activation could preferentially reach the common bundle via the AV node. Stable histological markers of the entire chick cardiac conduction system, however, do not exist, and the anatomy that produces this pattern of conduction could not be elucidated.

The rabbit embryo is a unique model of cardiac conduction system development for several reasons. It is a mammalian system with physiology similar to human cardiac physiology. The adult rabbit heart has been used for many decades to study correlates to human cardiac health and disease. Furthermore, an immunohistochemical marker of the cardiac conduction system has been identified, neurofilament 160 (NF-160), that labels the entire cardiac conduction system from early on in development all the way through to the adult (18, 39). Labeling with this marker has been correlated to conduction pathways in the adult (14). We have investigated the physiology and anatomy of pre- and post-ventricular septation embryonic rabbit hearts to determine the anatomic substrates for the emerging patterns of cardiac activation of the developing heart. We hypothesized that septation is not a significant event in the patterning of ventricular activation in rabbit embryos and that could not be determined because, at that time, there was no specific histological marker for them. The electrophysiological-anatomic correlations become more difficult as the conduction system becomes more complex with embryonic age. Detailed intracellular records were combined with electron micrographs of the embryonic heart (3, 4, 25); however, it was difficult to conduct multiple cellular recordings simultaneously.

Recordings from multiple sites (11) with plaque electrodes placed on the surface of the embryonic chick heart revealed regions of slow conduction in the atroioventricular (AV) canal as well as the outflow tract. Immunohistochemical staining indicated that these relatively slowly conducting regions correlated to overlapping zones of labeling of two different markers for myocardial structural proteins (11).
developmental changes in cardiac pacemaking and conduction system anatomy determine the activation pattern of the embryonic heart.

MATERIALS AND METHODS

Optical mapping. New Zealand White rabbit embryos from days of development near the time of ventricular septation were investigated [day 15 (n = 5), day 13 (n = 5), day 12 (n = 5), and day 11 (n = 6)]. The protocol was approved by the Institutional Animal Care and Use Committee at Case Western Reserve University. The doe was euthanized (described previously, see Ref. 14), and the embryos were immediately harvested. The embryos were left in the uterus and were stored in iced oxygenated Tyrode solution, pH 7.4, for no longer than 1.5 h after harvest. The embryonic heart was excised after cervical dislocation into warm oxygenated Tyrode solution (pH 7.4). The solution was kept at a constant 37°C with a Bioptecs Delta T culture dish (Bioptech, Butler, PA). After the baseline ECG was recorded, the heart was superfused with oxygenated Tyrode solution containing 20 μM of the voltage-sensitive dye 4-(2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium (di-4-ANEPPS; Molecular Probes, Eugene, OR). A lower concentration was required (2 μM) for day 11 embryos. There was a trend toward prolongation of the PR interval in all days of embryonic development (except day 11; there was no visible P wave) after superfusion with di-4-ANEPPS; however, this trend was not statistically significant (PR interval 172 ± 46 ms in saline vs. 206 ± 65 ms in di-4-ANEPPS; n = 10). Motion-reducing agents were not used in this investigation.

Optical records were started after 10 min of staining. Bright-field digital images were taken with an AxioCam MRc5 (Zeiss) mounted on a Nikon E600FN upright microscope. The mapping system is diagrammed in Fig. 1. ECGs were recorded with silver wire electrodes, and the signal was filtered and amplified with CyberAmp 380 (Axon Instruments). A mercury-xenon light source (150 W, OptiQuip 1600) and custom fluorescence filter cube (excitation 510/80 nm, emission 610 nm long pass, 565 dichroic mirror) were used. A 16 × 16-pixel photodiode array (C4675–103, Hamamatsu) was mounted on a second optical port in parallel with the AxioCam and collected signals over 2.0 s. We used ×4 or ×10 objectives. The area recorded by a single pixel of the photodiode array was 87.5 × 87.5 μm at ×10 magnification, and 200 × 200 μm at ×4 magnification. Signals were amplified and filtered by specialized conditioning boards, digitized at 5 kHz, and stored in a computer for later analysis. Custom Labview programs were used for data acquisition and analysis. Movies were constructed with Corel Photopaint and are available as supplemental data for this article at http://ajpheart.physiology.org/cgi/content/full/00770.2004/DC1. Activation maps and waveform plots were prepared with Origin version 7.5 and Microsoft Excel.

Histology and immunohistochemistry. Hearts from day 11, 12, 13, 14, and 15 embryonic rabbits were fixed in a graded series of ethanol. The hearts were embedded in cryosectioning medium (Tissue-Tek, optimum cutting temperature compound 4583; Sakura Finetek, Torrance, CA) after being incubated in a graded series of sucrose and kept at −80°C until sectioning (no longer than 2 mo). Cryosections of 14- to 16-μm thickness were placed on coated slides (catalog no. 12–550–15, Fisher). After being washed with PBS (pH 7.4), the sections were incubated in blocking buffer (PBS, normal goat serum, bovine serum albumin, and Triton X-100) for 30 min and incubated overnight with antibodies against NF-160 (1:1,000 mouse IgG, MAB 5254; Chemicon Temecula, CA), sarcomeric actin (1:1,000 mouse anti-rabbit IgM, DakoCytomation M0874; Carpenteria, CA), collagen type II (1:200 mouse anti-rabbit IgG, MAB 8887; Chemicon), or vimentin (1:500 guinea pig anti-rabbit, GP53; Progen Biotechnik, Heidelberg, Germany). After being washed in PBS, secondary antibodies were placed the next morning for 2 h [Alexa Fluor 594 goat anti-mouse IgM μ-chain and Alexa Fluor 488 goat anti-mouse IgG γ-chain (Molecular Probes) or donkey anti-guinea pig Cy5, AP193S (Chemicon)]. The sections were mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), imaged, and photographed with a Nikon E600 high-resolution digital camera with ACT-1 software.

RESULTS

Electrophysiology of day 13 and 15 rabbit embryo hearts. Activation maps from the dorsal surfaces of representative day 13 (preseptation) and day 15 (postseptation) embryonic rabbit hearts are shown in Fig. 2, A and B, respectively. The supplemental movies show both dorsal and ventral surfaces for the same hearts. Activation across the ventral and dorsal surfaces of the heart was similar in every case and is described below.

The right and left atria of day 13 embryonic hearts activate sequentially, but with no delay between the two. After an AV delay (average PR interval of day 13 hearts was 197 ± 34 ms), the ventricles activated first in the cleft between the two apexes and both right and left ventricles proceeded to activate in parallel in an apex-to-base direction. Activation of the right and left ventricles occurred simultaneously with no apparent difference in conduction velocity as evident by the isochrone map (Fig. 2A).

The day 15 embryonic right and left atria also activated sequentially, but there was a delay in activation between them of −8 ms (Fig. 2B). After a significant AV delay (average PR interval for day 15 hearts was 168 ± 36 ms), the ventricles activated first in the left apex and proceeded rapidly across the left ventricle and then the right ventricle, with an apparently slower rate over the right ventricle.

Anatomy of periseptation hearts is consistent with electrophysiology. NF-160 labeling was performed on complete series of embryonic hearts from days 11, 12, 13, 14, and 15. Critical sections from each period of development are shown in Fig. 3. The most posterior section with NF-160 immunolabeling revealed a small, specifically stained region in the AV junction (green in Fig. 3a). Colabeling with sarcomeric actin (red in Fig. 3a) revealed that these cells are cardiomyocytes. More anterior sections had NF-160 labeling of sarcomeric actin-positive tissue within a bundle connecting the two atria (Fig. 3b). Figure 3c shows a NF-160-positive tract between the atria and the ventricles in the right AV canal. The NF-160-positive cardiomyocytes in the ventricle were draped over the nascent inter-
and more time for complete depolarization than the ventricles. As in the activated and before the LA was activated. In all cases, the left apex showed of the atria—in this representative example, required 12 ms in this example; ventricles were completely delay (110 ms), the ventricles activated at the cleft of the apex with simulta-

each map.

rise to mature conduction system tissues (2).

conduction system (2). The RAR itself may or may not give primary ring that ultimately gives rise to portions of the ring (RAR) in the chick embryo (42). The RAR is a portion of likely represents what has been termed the “retroaortic root (IVS). Labeling was draped over the interventricular septum (IVS). Labeling from the presumptive sinoatrial (SA) node to the ventricular trabeculae at both days of development. a–d: Frontal sections through a day 13 rabbit embryo heart, labeled with sarcomeric actin (red) and NF-160 (green); a is most posterior in the heart; subsequent panels are more anterior to each other. a: Arrow points to the first appearance of NF-160-positive cardiomyocytes in the AV junction between the RA and RV. b: Top arrow indicates NF-160 labeling in what appeared to be the SA node and was directly between both atria. c: NF-160 labeling was draped over the interventricular septum (IVS). Labeling between the atria had a “bundle” appearance. d: Atrial and ventricular bundles meet behind the aortic root [top arrow, retroaortic ring (RAR)]. Diffuse labeling with NF-160 was seen in the trabeculae. A–E: frontal sections through a day 15 rabbit embryo heart, labeled with sarcomeric actin (red) and NF-160 (green). Again, A is the most posterior section with NF-160 labeling in this heart; subsequent sections are more anterior to each other. A: the most posterior expression of NF-160 was at the top of the IVS. B: an interatrial region appeared with more intense expression of NF-160 toward the right side. C: arrowhead on right points to what is likely to be the early atrioventricular node. D: a prominent NF-160-positive bundle extends into the left ventricular apex. E: anterior most sections through IVS have bundles of NF-160 cells on either side of septum. In general, arrows in a–d and A–E indicate NF-160 + cells

ventricular septum and extended to the ventricular trabeculae. Ventricular trabeculae were labeled as well as a bundle that likely represents what has been termed the “retroaortic root ring” (RAR) in the chick embryo (42). The RAR is a portion of the primary ring that ultimately gives rise to portions of the conduction system (2). The RAR itself may or may not give rise to mature conduction system tissues (2).

NF-160 labeling in the day 15 embryonic rabbit heart revealed some similarities and some differences in conduction system anatomy with the day 13 heart that correlated to changes observed in the activation sequence between the two stages. Similarities included NF-160 labeling in the AV junction in the most posterior sections (Fig. 3A), a positive-staining region between the atria and over the interventricular septum more anteriorly (Fig. 3B), connection of the atrial NF-160-positive region and the ventricular NF-160-positive region through a right-sided muscular band spanning the AV canal (Fig. 3C), and, finally, continuity of the system from the region between the atria all the way to the ventricular trabeculae as described by NF-160 labeling.

Differences observed in NF-160 labeling between the pre- and the postseptation heart were as follows. 1) The NF-160-positive region between the atria was wider, the bulk of this labeling located primarily on the right side of the muscular band separating the atria (Fig. 3B). 2) A long NF-160-positive branch extended into the left ventricular trabeculae within the apex (Fig. 3D).

NF-160 and sarcomeric actin colocalize. Previous investigations showing colocalization of NF-160 labeling with markers of cardiomyocytes used light microscopy (18, 39). Confocal microscopy was performed to support this finding. Figure 4 shows a colabeled region from the trabeculae of a day 14 embryonic rabbit heart. Figure 4C is a stack through a 12-μm-thick section, and Fig. 4D is a single 0.3-μm-thick confocal section. Regions that are NF-160 positive only were identified (red in Fig. 4B) as well as regions positive for sarcomeric actin. Significant colabeling was observed as indicated by the yellow regions (Fig. 4, C and D).

Collagen II identifies more specific subset of fibroblasts in rabbit embryo. The role of fibroblasts in the formation and function of the cardiac conduction system is actively being investigated (15, 33). It was of interest, therefore, to determine the pattern of fibroblasts with respect to the emerging cardiac conduction system. Initial studies of fibroblast location relative to the cardiac conduction system in the embryonic rabbit revealed that anti-vimentin markers stained a large population of cells, consistent with reports that have shown vimentin-positive labeling of cells undergoing epithelial-mesenchymal
bundle branches, and subendocardial Purkinje system—appear to be present and in a continuous pathway before cardiac septation in the day 13 rabbit embryo. Previous investigations with NF-160 labeling in the rabbit embryo heart did not demonstrate the SA node as such (18). Those authors showed a region between the sinus venosus and the right atrium that might represent the SA node as depicted here (Fig. 4 in Ref. 18). Differences in labeling seen in this study may reflect subtle differences in age of development, different breeds of rabbit, or differences in antibody production. Although all relevant conduction system components appear to be present at these early stages, it will be necessary to follow the NF-160-positive structures into adulthood. For example, if the SA node is indeed the posterior (dorsal) structure identified in this investigation, then the SA node must traverse anteriorly and laterally to its final position in the mature heart at the base of the superior vena cava.

The timing of atrial activation obtained with optical mapping techniques is consistent with the immunohistochemical anatomy observed. In the day 13 embryo, the nearly simultaneous activation of the atria is consistent with the SA node location at the base of the right atrium between the two atria. After 2 days of development, the right atrium clearly activates before the left. Consistent with this, the NF-160-positive region becomes an elongated band between the atria. The delay in interatrial timing may be a result of the widening of the NF-160-positive band, infiltration with collagen-positive cells, changes in fiber orientation, or introduction of other extracellular elements that slow conduction through cardiac tissues.

We have shown a continuous NF-160-positive tract connecting the atrium to the ventricles in both pre- and postseptated embryonic rabbit hearts. The presence of a similar right-sided bundle has been shown in the rabbit (18, 41) and other species

DISCUSSION

**Preferential conduction pathways are present in tubular embryonic heart.** The presence of NF-160 labeling has been correlated to electrophysiologic activity in the adult rabbit (14) and to anatomic sites consistent with the conduction system in the embryonic and adult heart (17, 18, 39). In this investigation, we have shown that changes in the pattern of electrical activation over several stages of embryonic rabbit heart development correlate with changes in the morphology of the conduction system as marked by the expression of NF-160.

All of the elements of the primitive embryonic conduction system—sinoatrial (SA) node, AV system, common bundle,
with several markers (10, 13, 23, 32, 42). This bundle has been described as being a part of the primary ring that gives rise to the AV node (41, 42). The most important function of the adult AV node is to slow cardiac conduction from the atria to the ventricles, allowing adequate filling of the ventricles before ejection of blood. Despite the clear presence of conducting cells connecting the atria to the ventricles in the preseptation embryo, there is still a significant slowing of conduction that takes place across the AV junction. This may reflect an infiltration of collagen-containing tissues that occurs before septation (see below), differences in expression of connexins and ion channels in this region, or both. Previous investigations showed that the orientation of cardiomyocyte fibers within the embryonic heart may also play a significant role in the slowing of conduction through the AV junction as observed from the epicardial surface (37).

This continuity of the cardiac conduction pathway helps explain the presence of an apex-to-base pattern of conduction even in the presence of incomplete septation. In the chick it had been proposed that the AV node and His-Purkinje pathway developed independently, requiring subsequent linkage (32). It was thought that the alteration in ventricular activation observed at the time of ventricular septation might be a result of this phenomenon. This work and investigations of the CCS-lacZ mouse (32) support the idea that the conduction system develops from a continuous primordial system that is modified as the embryonic heart undergoes morphological changes necessary to support the organism it serves. The strength of NF-160 as a marker of the conduction system in the embryonic rabbit heart is demonstrated in this investigation by the subtle shift detected in earliest ventricular activation from the midline to the left apex consistent with the appearance of a prominent NF-160-positive band of cardiomyocytes in this region.

Differences between rabbit and chick: not just fur and feathers. Why does ventricular septation confer such a dramatic change in activation of the chick ventricle and not the rabbit ventricle? The answer may be entirely morphological, residing in differences in trabecular structure and growth between the two species. Activation of the embryonic ventricle in the chick has been shown to occur in an endocardial-to-epicardial fashion, much like mature cardiac tissue (11, 31). Assuming this is true for the rabbit and the mouse, the difference in trabecular structure compared with the chick may have considerable significance.

First, recent work using high-resolution optical mapping suggests that there are direct connections from the left lateral atrium to the left base in the preseptated chick heart (F. Rothenberg, unpublished observations). These AV connections may permit early activation of the left base at this stage of development, with subsequent ventricular activation occurring through trabecular subendocardial conduits.

Second, conduction through embryonic ventricular myocardium in the chick appears to be linked to trabecular anatomy (37). Trabeculations in the preseptated chick heart are plate-like sheets, in contact with the full length of the ventricular wall from the inner to the outer curvature (5, 35). Activation of the
ventricles may proceed in a trabecular plate-to-plate fashion, producing the base-to-apex, left-to-right pattern previously observed (7). As the ventricles mature the trabecular orientation changes (5, 35), possibly leading to the alterations in ventricular activation seen at the epicardial surface. After septation the direct AV connections are severed and the trabeculae become rodlike and appear to contact the inner ventricular wall only where they insert at the apex and base (5, 35). The apex-to-base activation pattern in the postseptated hearts could be a reflection of this internal morphology. Changes in epicardial ventricular activation patterns that are seen after septation in the chick may be entirely due to changes in trabecular anatomy rather than dramatic changes that might occur in the AV node-His-Purkinje axis.

In the mouse, where electrical activation of the ventricles is similar to the rabbit embryo of comparable developmental age, the trabeculations in either ventricle are more similar in appearance (35, 40) and do not appear to undergo dramatic changes with morphogenesis. This can also be observed in the day 13 and 15 embryos in the present investigation (Fig. 3). The orientation of the trabeculae with respect to the compact myocardium is similar in both ventricles, and although the interventricular septum in the chick arises from fusion of the platelike trabecular sheets, the mouse interventricular septum has a compact appearance from its inception. In the mouse, interventricular growth toward the AV cushions is thought to be due to expansion of the ventricles on both sides and a consequent movement toward the cushion rostrally (1, 24, 27, 27, 31).

Finally, the trabecular morphology between the left and right ventricles in the periseptation chick embryo is dramatically different (5, 35). Trabeculations within the left ventricle appear to undergo the majority of alterations in the course of septation, the trabecular orientation reflecting the epicardial pattern of activation observed. This is consistent with the observation that the primary change in activation after septation occurs over the left ventricle (Ref. 7 and unpublished observations).

Fig. 7. Confocal images of a frontal section from day 14 rabbit embryo ventricular apex (A), labeled for sarcomeric actin (red; B), connexin43 (Cx43, green; C), and vimentin (blue; D). E: 12-μm-thick stacked confocal series. F: Cx43 is found between sarcomeric actin-positive cells, the cardiomyocytes, and do not appear to be in direct contact with vimentin-positive cells.
In summary, embryonic ventricular activation as observed with optical mapping may be a reflection of trabecular anatomy in the chick and the rabbit rather than events within the conduction system axis.

**Immunohistochemical markers of conduction system axis in rabbit embryo.** Patterns of fibroblast and connexin distribution were investigated. As noted in RESULTS, vimentin is expressed by many migrating mesenchymal cells in the developing embryo (29). For this reason, many more cells than fibroblasts were labeled when antibodies against vimentin-positive cells were used. Antibodies to collagen II appeared to be much more specific for collagen-producing fibroblasts in this setting, always labeling a subset of vimentin-positive cells. Collagen II appears to be a more suitable marker to localize fibroblast activity in the embryo. The implications of collagen appearance and location in the embryonic heart are discussed below.

The distribution of Cx43 and the relationship to fibroblasts and cardiomyocytes were also investigated. The primarily myocardial distribution of Cx43 in the day 14 rabbit embryo is consistent with findings in the rat (38) and mouse (8, 9). Confocal microscopy in the present investigation revealed little to no interaction between Cx43-positive regions and fibroblasts, Cx43 primarily localizing to cardiomyocytes. Antibodies were used in this portion of the study, which may overestimate any interaction between fibroblasts and Cx43 in the embryo because some of these cells may be migrating mesenchymal cells (29). This result is not in agreement with studies of neonatal rat cardiomyocytes (16), in which colocalization of Cx43 and fibroblasts was found by immunohistochemistry and Western blots. It has been suggested (see below) that fibroblasts serve as modulators of cardiac conduction (15). Several investigators have demonstrated gap junctions between cardiomyocytes and fibroblasts, and even between fibroblasts themselves (15, 16). Cardiomyocyte-fibroblast and fibroblast-fibroblast communication may not be instituted until later in development, and whether these connections increase or decrease conduction velocity has yet to be convincingly demonstrated in embryonic hearts.

**Fibroblasts as modulators of cardiac conduction.** Two-thirds of the cells that make up the normal mature heart are noncardiomyocytes, and the majority of these are fibroblasts (26, 28). There is mounting evidence that intracellular communication occurs between fibroblasts and cardiomyocytes, and even between cardiac fibroblasts themselves (6, 15, 16, 33). In vitro optical mapping of cultured cardiomyocyte-fibroblast strips suggests that fibroblasts might modulate cardiomyocyte conduction (15). This has important ramifications for cardiac remodeling in pathological states such as myocardial infarction and ventricular hypertrophy with fibrosis.

The data shown here suggest that fibroblast-cardiomyocyte interactions are also important for the developing heart, in particular in electrical modeling in the AV junction. Separate atrial and ventricular activation occurs only after collagen II (deposited by fibroblasts) overlaps NF-160-positive cells in the AV junction between days 11 and 12 of rabbit embryonic development. This suggests that fibroblast-cardiomyocyte interactions may be necessary in formation of AV nodal slowing of conduction.

In conclusion, high-resolution optical mapping with voltage-sensitive dye combined with immunohistochemical labeling of the conduction system demonstrated the emergence of a complete and continuous preferential pathway in the early rabbit embryo from the SA node to the ventricular trabeculae. This supports the hypothesis that preferential pathways for electrical activation are established very early in the developing heart. The rabbit embryo is an important developmental model for the investigation of the tissue, cellular, and molecular interactions that occur in the emergence of a normal conduction system.


