Three-Dimensional Anatomy of the Conduction System of the Early Embryonic Rabbit Heart

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ABSTRACT

The complete embryonic cardiac conduction system is difficult to view in three dimensions, primarily because there has not been a marker of all segments of the normal system throughout all stages of development. Imaging of the conduction system components within the atria has been particularly controversial because different markers reveal different pathways that may or may not represent conduction system components. The conduction system of the adult and embryonic rabbit, however, can be labeled in its entirety with the neurofilament marker, NF-160. The conduction system of rabbit embryos at several stages of development spanning cardiac septation was therefore investigated. Optical mapping of the electrical signature of the conduction system previously revealed a close correlation between the cardiac activation patterns and the anatomy as shown by serial sections. The 3D relationship between the components of the conduction system could only be inferred from the 2D sections. The sections were consequently reconstructed using a commercial software program (AutoQuant). This is the first demonstration of the three-dimensional complete normal rabbit embryonic cardiac conduction system at several stages of development.

Key words: embryo; conduction system; rabbit; three dimensional reconstruction; neurofilament

The embryonic origin of normal and abnormal cardiac conduction has been a focus of recent research with the advent of several new tools, including optical mapping with voltage-sensitive dyes (Rentschler et al., 2001; Reckova et al., 2003; Chuck et al., 2004; Hall et al., 2004; Rothenberg et al., 2005) and transgenic mice that permit labeling of regions that correlate with the anatomical location of the cardiac conduction system (Kupershmidt et al., 1999; Davis et al., 2001; Rentschler et al., 2001; Kondo et al., 2003; Jay et al., 2004). There is some controversy, however, as to whether transgenic markers label true cardiac conduction system cells (Anderson et al., 2004). Large tracts of putative conduction system cells within the atria can be elucidated using the transgenic mouse model that expresses CCS/lacZ in the heart (Jongbloed et al., 2004). These atrial tracts have not been confirmed as being part of the conduction system electrophysiologically or with other histologic methods. In contrast, the neurofilament marker, NF-160, labels all of the components of the cardiac conduction system in the adult rabbit heart (Verheijck et al., 1998; 2003, 2005) and the embryonic rabbit heart (Gorza and Vitadello, 1989; Vitadello et al., 1996). Antibodies against NF-160 and NF-200 have been localized to the atrioventricular node (AVN) and bundle of His in the monkey (Mueller et al., 2003). Antibodies against the neurofilament NAPA-73 have been localized to regions consistent with early conduction system in the ten somite embryonic chick heart (Ciment, 1990). Furthermore, the relationship to impulse conducting cells of the conduction system has been directly confirmed with electrophysiologic recording in the adult rabbit (Dobrzynski et

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al., 2003, 2005). The rabbit embryo, then, is an ideal model for the visualization of the atrial and ventricular components of the normal mammalian cardiac conduction system.

Previous work showed that the electrophysiology of periseptation embryonic hearts was clearly correlated with the location of conduction tissue as determined by 2D sections of NF-160-labeled tissue (Rothenberg et al., 2005). While the normal embryonic conduction system appears continuous from these serial sections, its continuity cannot fully be confirmed without detailed 3D reconstructions. Furthermore, 3D reconstructions of the developing cardiac conduction system can be used to analyze its relationship to surrounding structures for the purpose of understanding the contributions of other tissues and developmental signals that may pass between them. Therefore, a three-dimensional understanding of the anatomy of the conduction system with relation to surrounding structures is critical for understanding the signals that may be used to initiate conduction system formation and remodeling during development, as well as the complex interactions that must occur for impulse generation and conduction throughout embryogenesis. We show here for the first time the entire conduction system at three stages of development, reconstructed from serial sections that mark the expression of the neurofilament protein, NF-160, through the entire embryonic rabbit heart. These reconstructions show that the conduction system truly is a continuous structure from sinoatrial node to Purkinje fibers, even in the looped tubular heart prior to cardiac septation.

MATERIALS AND METHODS

Histology

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University, Cleveland, Ohio. Pregnant New Zealand white rabbits (gestation days 12, 13, and 15) were euthanized as previously described (Rothenberg et al., 2005) and the embryos harvested. The hearts were extracted and a subset was fixed immediately in 70% ethanol for 10 min, followed by a series through 100%, 70%, 50% saline, 5% sucrose, overnight in 20% sucrose, and embedded in OCT mounting medium (Tissue-Tek, compound 4583; Sakura Finetek, Torrance, CA) for cryosectioning. The hearts were then either stored at −80°C or immediately cryosectioned into 16 μm frontal sections. The serial sections were labeled overnight with anti-NF-160 (1:1,000 mouse IgG; MAB 5254; Chemicon, Temecula, CA) and antisarcomeric antibody (1:1,000 mouse antirabbit IgM; M0874; DakoCytomation, Carpenteria, CA)). Secondary antibodies (AlexaFluor 488 and 594) were purchased from Molecular Probes (Eugene, OR). Negative controls using only the secondary antibodies were negative for labeling (data not shown).

Image Processing

The sections were digitally imaged using a Nikon Eclipse upright microscope, and the sections reconstructed using AutoQuant software (AutoQuant Imaging, Watervliet, NY). Sections were imported as TIFF images, automatically aligned, and reconstructed using appropriate dimensions. The original color of the stain (green) was changed by selecting a different color map lookup table to improve clarity of the image. Only sections containing NF-160 label were used in these images. The movie was generated with the AutoQuant software. Three-dimensional images for viewing with red-blue glasses (with the red lens on the left) were generated with Photoshop. Left-eye and right-eye images were selected from frames of the 3D movie prepared as described above. The images were prepared with Adobe Photoshop. Instructions were obtained from the Web (www.savel.org/old/mad/stereo.html).

RESULTS

The video clips (day 12, day 13, and day 15 http://interscience.wiley.com/jpages/1552-4884/suppmat/) reveal the dramatic anatomical changes that took place within the cardiac conduction system over a span of 3 days. Neurofilament labeling in the day 12 heart showed a concentration of marker on the right side of the atrioventricular junction (Fig. 2, panel 1, arrows), a region that was previously the inner curvature of the linear heart tube only a day before. Physiologically, hearts at this stage of development have sequential atrial-ventricular activation despite the fact that the conduction system appears to be concentrated in this single location with little differentiation (Rothenberg et al., 2005). The neurofilament labeling appeared to be equally distributed dorsoventrally.

Only a day later, the day 13 cardiac conduction system appeared much more differentiated morphologically (Fig. 1, panel A; Fig. 2, panel 2, and video clips http://interscience.wiley.com/jpages/1552-4884/suppmat/). It remained a single unit, however the labeled conduction system elongated in a caudal-cranial direction. The primitive sinoatrial node (SAN) and atrioventricular node (AVN) are now more dorsal in the embryonic heart with respect to their connecting pathways. In fact, the conduction system took on a spiral appearance when viewed from the side. Bundle branches formed, and Purkinje fibers took root in the ventricular trabeculae.

Further development revealed an elaboration and refinement of all conduction system components. In the day 15 embryonic heart, the SAN and AVN were now deeply dorsally located within the conduction system axis, whereas the connecting pathways were also more fully developed in a dorsal-ventral axis (Fig. 1, panel B; Fig. 2, panel 3, and video clips http://interscience.wiley.com/jpages/1552-4884/suppmat/). The bridge between the SAN and AVN looped dorsoventrally, while the bundle branches have a characteristic saddle-shaped appearance draped over the interventricular septum.

DISCUSSION

It is becoming clear that anatomy is critical in defining the pattern of cardiac conduction not only in the mature heart, but in the embryo as well. The mouse model has been and remains an unparalleled tool in the quest for understanding the genetic basis for normal and abnormal cardiac conduction system development (Kupershmidt et al., 1999; Davis et al., 2001; Rentschler et al., 2001; Kondo et al., 2003; Wessels et al., 2003; Jay et al., 2004). The precise anatomic location of conduction system cells using markers obtained through transgenic modifications, however, is controversial because not all markers label the same structures. As an example, the CCS/lacZ marker labels a broad region between what is believed to be the

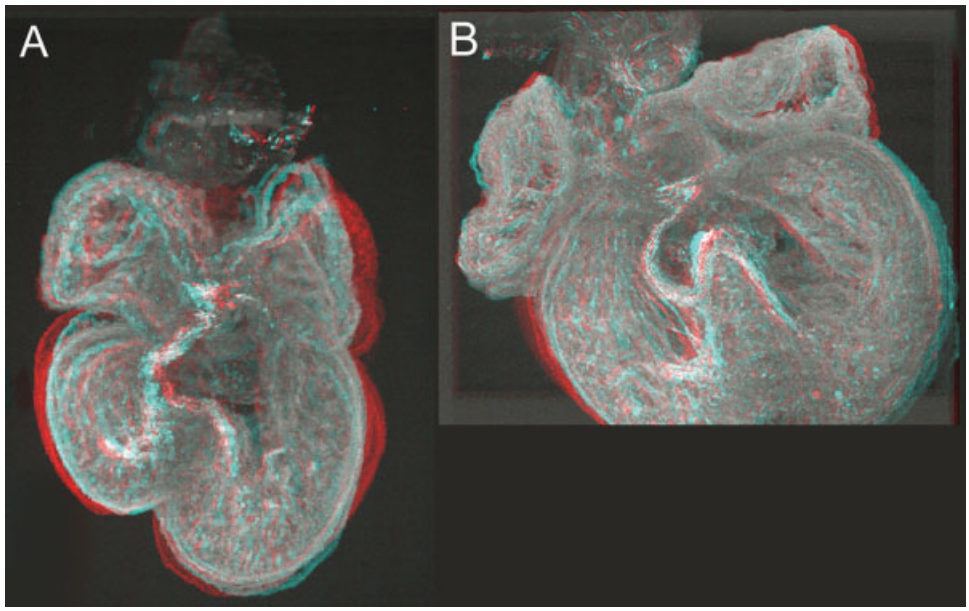

Fig. 1. Three-dimensional images of the cardiac conduction system of a day 13 (**A**) preseptation embryonic rabbit heart and (**B**) day 15 postseptation embryonic heart as seen from the anterior surface. This is the first demonstration of the normal mammalian primitive conduction system in its entirety at several stages of development. These images clearly show that even before cardiac septation is complete, the conduction system in the normal embryonic mammalian heart is present and continuous.

sinoatrial node and the atrioventricular node (Jongbloed et al., 2004), not seen in the cGATA-6 enhancer transgenic mouse (Davis et al., 2001). The advantage to investigating a normal rabbit is clear: the conduction system is not genetically manipulated and is therefore normal. Labeling with NF-160 is present in all elements of the conduction system in the adult rabbit (Verheijck et al., 1998; Dobrzynski et al., 2003, 2005), and these anatomical regions have been confirmed to be conduction system elements electrophysiologically (Dobrzynski et al., 2003, 2005). NF-160 labeling is likewise present in all conduction system cells in the embryonic rabbit, but is also present throughout development, unlike other markers (Gorza et al., 1988; Gorza and Vitadello, 1989; Vitadello et al., 1996). This argues that there is a fundamental difference in conduction system tissues that, despite the fact that all cardiomyocytes can transmit electrical impulses, makes these labeled cells designed for this purpose. Therefore, while accessory pathways and ectopic arrhythmogenic sites might be capable of transmitting impulses, they are not designed for that as suggested by the limited NF-160 labeling and are therefore potentially more arrhythmogenic. This model system then could be critical in elucidating signals that turn cardiomyocytes into NF-160 expressing conduction system cells.

A possible disadvantage may be that this marker appears to label all conduction system elements only in the rabbit for reasons that are still unknown. This suggests that inferences about conduction system development derived from these investigations can only be limited to the rabbit. However, it is unlikely that signals important for conduction system induction would be relevant only to the rabbit since NF-160 works only in this setting. Furthermore, an understanding of the basis for this species difference may provide valuable clues to the signals that initiate conduction system formation.

Whether this species difference would confer functional changes as well is also unknown, but should not diminish the power of this model as an investigative tool for several reasons. First, this investigative baseline in the rabbit model system is entirely normal, whereas the more commonly reported markers of the conduction system in the transgenic mouse require a genetically altered baseline, also of unclear relevance to the human heart. Second, with respect to the mouse model of cardiac conduction, the normal physiology of the rabbit appears to be more similar to the human in that the resting adult heart rate is approximately 150–200 bpm, as opposed to nearly 600 bpm in the adult mouse. With regard to cardiac electrophysiology, the rabbit again is more similar to the human than the mouse. For example, the action potential in the rabbit has a long plateau phase like the human's (Valentin et al., 2004), and the rabbit atrioventricular node can contain fast and slow pathways, as seen in humans also (Dobrzynski et al., 2003). Furthermore, the rabbit heart has been shown to be an effective model for testing of potentially proarrhythmic agents due to the high degree of physiologic similarity (Valentin et al., 2004). The rabbit, then, may be a more relevant model system in cardiac physiology investigations. However, the ability to alter genetic components of relevant developmental pathways is still clearly necessary. The power of the transgenic approach is only in its infancy in the rabbit system (Marian et al., 1999; Bosze et al., 2003; Nagueh et al., 2004). All tools at our disposal, therefore, will be valuable in our attempts to understand the signals relevant to conduction system formation.

Whether all elements of the conduction system have been labeled is presently under investigation. Reports have shown that regions not associated with the conduction system such as the outflow tract were positively stained using other markers for the conduction system

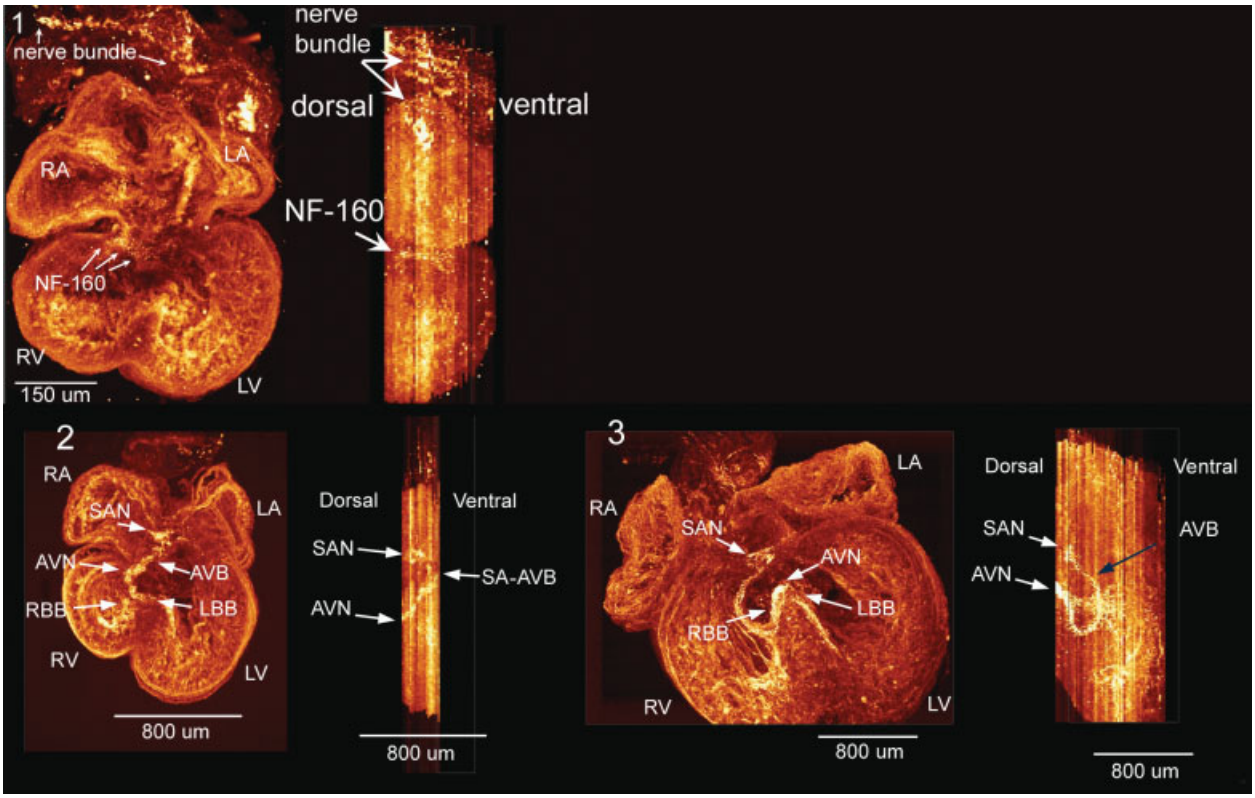

Fig. 2. Legends for movies: (**1**) day 12 rabbit embryo heart, (**2**) day 13, and (**3**) day 15. Panels 1–3 are anterior and lateral views of the day 12, 13, and 15 hearts, respectively. The labels indicate landmarks to aid interpretation of the online movies. SAN, sinoatrial node; AVN, atrioventricular node; SA-AVB, sinoatrial-atrioventricular bundle; LBB, left bundle branch; RBB, right bundle branch. Movies showing complete 360° views of these hearts are shown online (http://interscience.wiley.com/jpages/1552-4884/suppmat/).

such as HNK-1 and PSA-NCAM in the chick (Chuck and Watanabe, 1997) and rat (Ikeda et al., 1990). Preliminary investigations using these markers in the embryonic rabbit suggest similar staining patterns to the chick and rat (data not shown). It is unlikely that the outflow tract is part of the conduction system. However, the reason for its positive labeling is presently unclear. In addition, evidence from other species suggests the AVN forms from the fusion of two structures in the pre-septation heart (Marino et al., 1983—ferret; James, 1970—human, Arguello et al., 1988—chick) located in the base of the atrioventricular septum and the AV canal. It is possible that the SAN is either not present (has been removed in the dissection) or does not label with NF-160, as it does in the adult. These hypotheses are currently under investigation.

What should be clear is that the combined investigative approach of structure and function in the normal rabbit embryo is an important tool in understanding the normal and abnormal anatomy and physiology of the cardiac conduction system and will provide critical information regarding the relationship between embryonic tissues that must take place for the normal formation of the cardiac conduction system.

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LITERATURE CITED

Anderson RH, Christoffels VM, Moorman AFM. 2004. Controversies concerning the anatomical definition of the conduction tissues. Anat Rec 280B:8–14.

Arguello C, Alanis J, Valenzuela B. 1988. The early development of the atrioventricular node and bundle of His in the embryonic chick heart. An electrophysiological and morphological study. Development 102:623–637.

Bosze Z, Hiripi L, Carnwath JW, Niemann H. 2003. The transgenic rabbit as model for human diseases and as a source of biologically active recombinant proteins. Transgen Res 12:541–553.

Chuck ET, Watanabe M. 1997. Differential expression of PSA-NCAM and HNK-1 epitopes in the developing cardiac conduction system of the chick. Dev Dynam 209:182–195.

Chuck ET, Meyers K, France D, Creazzo TL, Morley GE. 2004. Transitions in ventricular activation revealed by two-dimensional optical mapping. Anat Re 280A:990–1000.

Ciment G. 1990. Precocious expression of NAPA-73, an intermediate filament-associated protein, during nervous system and heart development in the chicken embryo. Ann NY Acad Sci 588:225–235.

Davis DL, Edwards AV, Juraszek AL, Phelps A, Wessels A, Burch JB. 2001. A GATA-6 gene heart-region-specific enhancer provides a novel means to mark and probe a discrete component of the mouse cardiac conduction system. Mech Dev 108:105.

Dobrzynski H, Nikolski VP, Sambelashvili AT, Greener ID, Yamamoto M, Boyett MR, Efimov IR. 2003. Site of origin and molecular substrate of atrioventricular junctional rhythm in the rabbit heart. Circ Res 93:1102–1110.

Dobrzynski H, Li J, Tellez J, Greener ID, Nikolski VP, Wright SE, Parson SH, Jones SA, Lancaster MK, Yamamoto M, Honjo H, Takagishi Y, Kodama I, Efimov IR, Billeter R, Boyett MR. 2005. Computer three-dimensional reconstruction of the sinoatrial node. Circulation 111:846–854.

Gorza L, Schiaffino S, Vitadello M. 1988. Heart conduction system: a neural crest derivative? Brain Res 457:360–366.

Gorza L, Vitadello M. 1989. Distribution of conduction system fibers in the developing and adult rabbit heart revealed by an antineurofilament antibody. Circ Res 65:360–369.

Hall C, Hurtado R, Hewett K, Shulimovich M, Poma C, Reckova M, Justus C, Pennisi D, Tobita K, Sedmera D, Gourdie R, Mikawa T. 2004. Hemodynamic-dependent patterning of endothelin converting enzyme 1 expression and differentiation of impulse-conducting purkinje fibers in the embryonic heart. Development 131:581–592.

Ikeda T, Iwasaki K, Shimokawa I, Sakai H, Ito H, Matsuo T. 1990. Leu-7 immunoreactivity in human and rat embryonic hearts, with special reference to the development of the conduction tissue. Anat Embryol 182:327–334.

James TN. 1970. Cardiac Conduction System: Fetal and Postnatal Development. Am J Cardiol 25:213–226.

Jay PY, Harris BS, Maguire CT, Buerger A, Wakimoto H, Tanaka M, Kupershmidt S, Roden DM, Schultheiss TM, O'Brien TX, Gourdie RG, Berul CI, Izumo S. 2004. Nkx2–5 mutation causes anatomic hypoplasia of the cardiac conduction system. J Clin Invest 113: 1130–1137.

Jongbloed M, Schalij M, Poelmann R, Blom N, Wang Z, Fishman G, Gittenberger-De Groot A. 2004. Embryonic conduction tissue: a spatial correlation with adult arrhythmogenic areas. J Cardiovasc Electrophysiol 15:349–355.

Kondo R, Anderson RH, Kuperschmidt S, Roden DM, Evans SM. 2003. Development of the cardiac conduction system as delineated by minK-lacZ. J Cardiovasc Electrophysiol 14:383–391.

Kupershmidt S, Yang T, Anderson ME, Wessels A, Niswender KD, Magnuson MA, Roden DM. 1999. Replacement by homologous recombination of the mink gene with lacz reveals restriction of mink expression to the mouse cardiac conduction system. Circ Res 84: 146–152.

Marian AJ, Wu Y, Lim D-S, McCluggage M, Youker K, Yu Q-T, Brugada R, DeMayo F, Quinones M, Roberts R. 1999. A transgenic rabbit model for human hypertrophic cardiomyopathy. J Clin Invest 104:1683–1692.

Marino TA, Severdia J. 1983. The Early Development of the AV Node and Bundle in the Ferret Heart. Am J Anat 167:299–312.

Mueller RW, Gill SS, Pulido OM. 2003. The monkey (Macada fascicularis) heart neural structures and conducting system: an immunochemical study of selected neural biomarkers and glutamate receptors. Toxicol Pathol 31:227–234.

Nagueh SF, Chen S, Patel R, Tsybouleva N, Lutucuta S, Kopelen HA, Zoghbi WA, Quinones MA, Roberts R, Marian AJ. 2004. Evolution of expression of cardiac phenotypes over a 4-year period in the β-myosin heavy chain-Q403 transgenic rabbit model of human hypertrophic cardiomyopathy. J Mol Cell Cardiol 36:663–673.

Reckova M, Rosengarten C, deAlmeida A, Stanley CF, Wessels A, Gourdie RG, Thompson RP, Sedmera D. 2003. Hemodynamics is a key epigenetic factor in development of the cardiac conduction system. Circ Res 93:77–85.

Rentschler S, Vaidya D, Tamaddon HS, Degenhardt K, Sassoon D, Morley GE, Jalife J, Fishman, GI. 2001. Visualization and functional characterization of the developing murine cardiac conduction system. Development 128:1785–1792.

Rothenberg F, Nikolski V, Watanabe M, Efimov I. 2005. Electrophysiology and anatomy of embryonic rabbit hearts before and after septation. Am J Physiol Heart Circ Physiol 288:H344–H351.

Valentin J-P, Hoffmann P, De Clerck F, Hammond TG, Hondeghem L. 2004. Review of the predictive value of the Langendorff heart model (Screenit system) in assessing the proarrhythmic potential of drugs. J Pharmacol Toxicol Methods 49:171–181.

Verheijck EE, Wessels A, van Ginneken ACG, Bourier J, Markman MWM, Vermeulen JLM, de Bakker JMT, Lamers WH, Opthof T, Bouman LN. 1998. Distribution of atrial and nodal cells within the rabbit sinoatrial node: models of sinoatrial transition. Circulation 97:1623–1631.

Vitadello M, Vettore S, Lamar E, Chien KR, Gorza L. 1996. Neurofilament M mRNA is expressed in conduction system myocytes of the developing and adult rabbit heart. J Mol Cell Cardiol 28:1833–1844.

Wessels A, Phelps A, Trusk TT, Davis DL, Edwards AV, Burch JBE, Juraszek AL. 2003. Mouse models for cardiac conduction system development. In: Chadwick DG, editor. Development of the cardiac conduction system. New York: John Wiley and Sons. p 44–59.