Development of the Cardiac Conduction System

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Basic Concepts
In the formed heart, it is convention to distinguish working myocardium (the primary function of which is contraction) from the conduction system (the primary function of which is the generation and conduction of the electrical impulse). The conduction system comprises separate components with distinct functions. The SAN, which contains the leading pacemaker, generates the impulse. The impulse is subsequently conducted, via the atrial myocardium, which in this sense is part of the conduction pathway as well, toward the AVN. With a delay, the impulse is then rapidly transmitted from the AVN via the bundle branches and PPN to ensure a coordinated activation of the ventricular myocardium from apex to base. Classic reports cover the anatomy, pathology, and histology of the adult and developing conduction system.

The myocytes of the conduction system share with those of the ordinary working myocardium four basic elements: (1) contraction, (2) autorhythmicity, (3) intercellular conduction, and (4) electromechanical coupling. In the early embryonic heart tube, an ECG, similar to an adult ECG, can be recorded, indicating the presence of sequentially activated chambers. Given this observation, it is as confusing to accept the presence of a conduction system because it is functionally present as it is to deny its existence because it is not morphologically recognizable. Rather, it is of paramount importance to appreciate that the arrangement of myocyte populations, with distinct contractile, conductive, and pacemaking properties, establishes the coordinated activation of the heart. Departures from these tenets have led to a confusing and fruitless search for so-called “cardiac specialized tissues.”

Early cardiac development starts with the formation of a primary heart tube from the cardiogenic mesoderm (Fig 1); this topic has been reviewed recently. The primary heart tube is a peristaltic pump that moves blood ahead as a result of a unidirectional wave of contractions along the tube. Within this slow-conducting heart tube, fast-conducting and synchronously contracting atrial and ventricular chambers develop; these chambers remain flanked by the slow-conducting primary myocardium of the IFT, AVC, and OFT (Fig 2). The configuration of alternating slow- and fast-conducting segments guarantees that the downstream ventricular segment does not contract before the termination of the contraction of the upstream atrial segment and is responsible for the embryonic ECG. This configuration ensures also that relaxation of the atrial or ventricular segment does not occur before contraction of a downstream flanking segment, by which regurgitation of the blood is prevented. The sphincter-like prolonged peristaltic contraction form of the slow-conducting flanking segments substitutes for the adult type of one-way valves; this phenomenon is essential in a heart in which atrioventricular and semilunar valves have not yet developed. The slow-conducting SAN and AVN will take origin from the slow-conducting myocardium of IFT and AVC, sometimes referred to as “cardiac specialized tissues.”

Development of Polarity
One of the most striking features of the cardiogenic mesoderm and of the subsequently formed cardiac tube is its polarity along the anteroposterior axis. This is not yet the case in our chordate ancestors, the tunicates, in which the position of the leading pacemaker activity is not fixed and thus the heart pumps blood in both directions. The polarity of the vertebrate heart is characterized by the predominance of the atrial phenotype posteriorly (at the inflow/upstream side of the heart) and of the ventricular phenotype anteriorly (at the outflow/downstream side of the heart). Although at both extremities of the heart tube, myocardium is added, dominant pacemaker activity and highest beat frequency are invariably found at the intake of the tube, by which an efficient contraction wave is always ensured. The observation that the first contractions are observed in the middle (ventricular) part of the cardiactube corresponds with the finding that excitation-contraction coupling is first achieved in the ventricular portion. Thus, there is no pacemaker jump from the ventricular portion toward the venous pole of the heart during development. Cells of the future sinus region...
show prepotentials resembling those of the adult pacemaker, and cells of the cardiac tube, the future ventricles, show an electrical behavior similar to that of adult ventricles.15,16

The early development of conduction in the heart has been studied mainly in avian embryos.16,17 When 7 to 10 somites have developed (equivalent age of a human embryo of \( \approx 20 \) days), a single pacemaking area becomes established at the IFT of the heart.18 Pacemaker dominance increases along the anteroposterior axis.3,12 Also, the frequency of the intrinsic beat rate increases along this axis.12–14 In both birds and mammals, the leading pacemaker area is initially found on the left side,14,19–21 but as soon as the sinus venosus has formed (\( \approx 25 \) days in humans), the right side starts to become dominant. The flexibility of the nodal locations may not be surprising, if one takes into account the dimensions of the inflow area at this stage of development and the much bigger adult SAN, in which the leading pacemaker site is not fixed either.22,23 In addition, both right and left IFTs will become incorporated into the right atrium. Thus, in fact, the entire inflow area represents a more or less homogeneous pacemaking area, where the left side predominates. In line with this notion is the observation of node-like cells in the myocardium surrounding the distal portion of the pulmonary veins in adult rat.24 These cells appear to guarantee a unidirectional flow of blood into the left atrium and to prevent regurgitation of the atrial blood into the pulmonary veins.25,26

The molecular signals that impose polarity on the cardiac tube are unknown. Transplantation experiments of cardiomyocytes to another position along the anteroposterior axis of the early embryonic chicken heart have demonstrated that cardiomyocytes initially have the capacity to adapt their phenotype according to the new position.12,27 Retinoic acid induces a posteriorization of the phenotype in the anterior portion of the heart.12,27 These data demonstrate that initially cardiac polarity is not fixed.

In humans and other mammals, the first morphological signs of the SAN are at Carnegie stage 15 (\( \approx 5 \) weeks of...
human development) in the anteromedial wall of the right common cardinal vein, giving rise to the superior caval vein.\textsuperscript{30,31} In chickens and lower vertebrates, it remains a loosely arranged conglomerate of venous sinus myocytes.\textsuperscript{2,3,32-34} How the leading pacemaker area in mammals becomes transformed into a node that is morphologically and molecularly distinct from the surrounding atrial working myocardium and what the nature is of the molecular signals have remained enigmatic so far.

In conclusion, from the beginning onward, polarity is present along the cardiac tube, with the leading pacemaker (SAN) being present at the most posterior part of the developing tube, which guarantees the unidirectional wave of contraction.

Chamber Formation and the Development of an ECG

A fundamental property of the primary heart tube is the slow conduction of the impulse,\textsuperscript{6,35,36} resulting in a slow and peristaltic contraction,\textsuperscript{37} paraphrased by Patten and Kramer\textsuperscript{38} as “a progress of the contraction wave along the cardiac tube as striking and characteristic as intestinal peristalsis.” We have dubbed this early slow-conducting myocardium the “primary myocardium,” as opposed to (atrial and ventricular) working myocardium.\textsuperscript{5} Among other things, the primary myocardium is characterized by action potentials, which display slow depolarizations reminiscent of pacemaker action potentials and typical of slow voltage-gated calcium ion channels.\textsuperscript{39,40}

With further embryonic development, atrial and ventricular chambers that contract synchronously and sequentially begin to develop (Fig 2).\textsuperscript{37,38} This is accompanied by the development of an adult type of ECG, which merely reflects the sequential activation of the atrial and ventricular chambers rather than the presence of a morphologically recognizable conduction system.\textsuperscript{3,41} The synchronous contractions of the atrial and ventricular working myocardium indicate that these segments are characterized by the development of high-conduction velocities.\textsuperscript{6,35,42,43} Concordantly emerging are action potentials, which have a fast rising phase and high amplitude characteristic of fast voltage-gated sodium ion channels.\textsuperscript{39,40} Paff et al\textsuperscript{34} concluded that in the embryonic chicken heart after 42 hours of incubation (\approx 25 days of human development) “it would appear that a conduction system consisting of a pace-making sinuatrium, atriocentric-junctional tissue, ventricle and conus regions is developing.” Thus, the authors consider the entire embryonic heart as a conducting unit without the presence of a morphologically identifiable “adult conduction system,” which is in line with the view of Patten,\textsuperscript{45} who concluded from experimental studies that “the whole of the primary myocardium constituting the wall of the myocardial tube was acting as a conducting tissue.” It should be noted as well that an atrioventricular delay has developed before the development of a morphologically identifiable AVN.\textsuperscript{36,47} The AVC was recognized as the zone of slow conduction.\textsuperscript{3,6,35,41-43,48} Therefore, it functions as the “AVN equivalent” in a heart in which atrial and ventricular myocardial masses have not yet been insulated by fibrous tissue. In fact, the AVC represents “primary myocardium” remaining between the atrial and ventricular chambers.\textsuperscript{6}

Segments of slow conduction (remaining primary myocardium) also persist at both extremities of the heart.\textsuperscript{43,50} Paff and coworkers\textsuperscript{53} have already concluded that the OFT remains the least differentiated part (compare with “primary myocardium”) of the tubular heart\textsuperscript{50} and that “the prolonged contraction of the OFT produces a sphincter-like closure of the OFT at the end of systole. Thus without valves, little regurgitation of blood occurs.”\textsuperscript{51} Later, Paff and Boucek\textsuperscript{49} reported delayed contractions of the OFT and delayed propagation of the impulse from ventricle to OFT as C waves in the ECG. Finally, de Jong et al\textsuperscript{6} recorded slow conduction in all three flanking segments (IFT, AVC, and OFT), the functional significance of which has been pointed out above (“Basic Concepts”). The sphincter function of the OFT is in part retained in the formed dog heart, where it has been shown that the musculature surrounding the right ventricular OFT maintains the normal tonus during ventricular relaxation and so provides the necessary support for the pulmonary semilunar valves.\textsuperscript{52}

The AVN as a nodal structure becomes only gradually identifiable from about Carnegie stage 15 (\approx 5 weeks of human development) onward.\textsuperscript{53,54} In the chicken, it remains an indistinct entity, and the ativoventricular junction has been supposed to fulfill a role similar to that of the AVN.\textsuperscript{34} It is of great interest that in dog and pig hearts the entire lower rim of the left and right atria just above the fibrous annulus, ie, the former AVC, still has “nodal characteristics,” based on the presence of nodal-like action potentials and low abundance of the gap-junctional protein Cx43 and its encoding mRNA.\textsuperscript{55-57}

In summary, with the process of chamber formation, fast-conducting atrial and ventricular segments are being formed within the slow-conducting primary myocardium of the embryonic heart tube, so that the cardiac tube becomes a composite of alternating slow- and fast-conducting segments. ECGs show that this arrangement of segments provides the embryonic heart with an “electrical architecture” similar to that in the formed heart. The molecular basis underlying the compartmentalization is beginning to emerge through the analysis of the developmentally regulated patterns of transcription factors and of the modular patterns of expression of the lacZ transgene under direction of various cardiac promoter constructs.\textsuperscript{58}

Development of the Nodal Phenotype

In the formed heart, the nodal myocytes are said to display a number of “embryonic characteristics,”\textsuperscript{3,2,59,60} Similar to embryonic cardiomyocytes, nodal myocytes are small compared with the myocytes of the surrounding atrial working myocardium, and they have poorly organized actin and myosin filaments and a poorly developed sarcoplasmic reticulum.\textsuperscript{2} Therefore, in early embryonic hearts they can hardly be distinguished from the surrounding myocardium by unique histological characteristics.\textsuperscript{2,30,57,61} Instead, they initially are indicated by their separate arrangement and topography, which have obviously been the cause of much controversy. The signals that lead to the so-called “aggregation of the nodal area” are unknown. Innervation that occurs in the same
time period\textsuperscript{30} may play a role, but its significance to nodal development has yet to be assessed. When the atrial working myocardium differentiates, the nodes become more easily identifiable because the nodal myocytes remain “primary” in many aspects (see below).

So far, a universal description of the “nodal molecular phenotype” is lacking, indicating that many phenotypical features are not restricted to the nodal myocyte. However, several classes of genes display a pattern of expression that allows, within a species, the distinction of the nodal myocytes from the surrounding atrial working myocardium. Data are summarized in Fig 3. Added to the complexity is the interspecies variability, not only with respect to the type of genes expressed and the level of expression (eg, the interspecies variability of desmin expression\textsuperscript{62–64}) but also with respect to the number and pattern of the nodal cells that express the gene. This has hampered the interpretation of the functional significance of the patterns of gene expression considerably and the general use of these genes as markers to delineate the nodes morphologically.

Connexins

A crucial characteristic of cardiogenesis is the development of alternating slow- and fast-conducting segments. Gap junctions are held to be responsible for the intercellular transfer of the depolarizing action potentials in the myocardium.\textsuperscript{65} Gap junctions are aggregates of membrane channels composed of protein subunits, dubbed connexins, that are encoded by a multigene family.\textsuperscript{66} Five different connexins are expressed in the mammalian heart (Cx37, Cx40, Cx43, Cx45, and Cx46).

In the early myocardium, both the number and size of gap junctions is small but increases during development.\textsuperscript{36,67,68} However, gap junctions remain scarce in the developing SAN and AVN.\textsuperscript{30,48,56} Cx40 and Cx43 protein and mRNA\textsuperscript{57} were found to be undetectable in the flanking segments (OFT, AVC, and IFT) and the developing nodes and to be rare or undetectable in the adult structures of the rat.\textsuperscript{56,57,69,70} guinea

Figure 3. Expression patterns in the fetal and adult conduction system, schematically represented in the cardiac conduction systems of humans and of the most frequently used experimental animals. The expressions of the most well-documented markers for the conduction system are depicted. The explanation of the symbols used appears in the lower part of the figure. The gene expression patterns in the working myocardium are not represented. The numbers in parentheses indicate the following: 1, the expression of Cx40 is scarce in the human and bovine SAN and, hence, not indicated; 2, not all cells display coexpression of \(a\) and \(b\)-myosin in the SAN and AVN; and 3, troponin-I is expressed throughout the whole fetal heart in the rat. LBB and RBB indicate left and right bundle branches, respectively; L & M, neurofilaments of ~70 and 150 kD, respectively.

Figure 4. Molecular phenotype of the nodes. Cx43 mRNA cannot be detected in the myocardium of the rat fetal SAN surrounding the superior caval vein at the entrance of the right atrium (a), where \(\alpha\)-MHC mRNA expression is clearly visible (b); also, the right atrioventricular ring bundle (RAVRB) and AVN/AVB (arrows) do not reveal expression (c). A detailed developmental study has been published elsewhere.\textsuperscript{57} In the cardiomyocytes of the SAN surrounding the nodal area in the adult human heart, visualized by the expression of \(\alpha\)-MHC (d), Cx43 protein cannot be detected either (e).\textsuperscript{74}
pig,\textsuperscript{70,71} cow,\textsuperscript{71–73} and human\textsuperscript{71,74,75} (Fig 4). In human SAN and AVN tissue, the small and scarce gap junctions display faint expression of Cx40 and Cx45.\textsuperscript{72,73} The low abundance of connexin expression in the nodes corresponds with the slow conduction velocities observed in the nodes and the absence of fast sodium currents.\textsuperscript{76} The poor coupling of the nodal cells appears to be a requirement to prevent silencing of the pacing nodal myocytes by the much bigger atrial/ventricular working myocardium.\textsuperscript{77–79}

The low abundance of connexin expression in the nodes has been very useful, in conjunction with the use of node-specific intermediate filament markers, in delineating the interdigitation of nodal and atrial myocardium.\textsuperscript{72,77} Cx43 and the intermediate filaments display an almost mutually exclusive pattern of expression in the atrial and nodal myocardium, respectively; ie, an abrupt rather than a gradual increase in the number of gap junctions is found at the transitions of the nodal tissue to the atrium. Consequently, a gradient in the molecular phenotype of the nodal myocytes may not be the explanation for the proposed gradient in resistivity that is essential for the pacemaker function of the SAN.\textsuperscript{77} Instead, the electrical gradient seems to be the result of a gradual change in the morphology of the nodal cell toward its periphery and a decrease of the number of nodal cells toward the atrial working myocardium rather than a gradient in molecular phenotype.\textsuperscript{80}

**Contractile Proteins**

Whereas the functional significance of specific connexin isoform expression in the nodes can be envisioned, this is more problematic in the case of the so-called persistent expression of genes encoding “embryonic and/or skeletal” contractile protein isoforms, which are often mentioned as characteristic of the conductive tissues.

The expression of the myosin isoforms starts before contraction is being observed, as shown in the chicken, where coexpression of α- and β-MHC has been described.\textsuperscript{81} With the confinement of the expression of α- and β-MHC to the atrial and ventricular working myocardium, respectively, coexpression of the MHCs becomes characteristic for the nodal areas. In the SAN, coexpression of the MHCs is a common feature in a wide variety of species, including chickens,\textsuperscript{33} rats,\textsuperscript{82} cows,\textsuperscript{83} and humans.\textsuperscript{31,84} In chickens, both MHCs are coexpressed throughout the entire node, whereas in mammals, the β-isoform is expressed at the rim of the SAN only. Also, the atioventricular nodal cells coexpress both myosin isoforms in chickens,\textsuperscript{37,85} cows,\textsuperscript{86} and humans.\textsuperscript{83,87} In the developing AVN of rats\textsuperscript{82} and mice (authors’ unpublished data, 1997), however, no expression of β-MHC was found, which may be a characteristic of small animals or merely reflect phylogenetic interspecies variation.

In cows, a nodal myosin isoform immunologically related to the fetal skeletal myosin isoform has been observed in the nodal tissues but not in the Purkinje fibers of the ventricular conduction system.\textsuperscript{83,88} The atrioventricular nodal cells that were positive for the skeletal fetal MHC antibody did not reveal immunoreactivity for β-MHC.\textsuperscript{83} Also, in rats, a MHC isoform related to embryonic skeletal MHC has been localized in the nodal regions from 13.5 days of development onward.\textsuperscript{89} In contrast to the bovine heart, where the expression of the fetal skeletal MHC isoform persists,\textsuperscript{43} the expression of the rat fetal isoform decreases a few days after birth. Unfortunately, the antigen could not be visualized on Western blots of cardiac protein extracts.

In rats, expression of the major embryonic form of troponin I persists in the adult AVN.\textsuperscript{90,91} This embryonic troponin I isoform is identical to the isoform expressed in slow skeletal muscles.\textsuperscript{92} The mRNA can be visualized in hearts from 10 days of development onward and decreases after birth. These findings are underscored by the observation that 4200 nucleotides of the upstream sequences of the human slow skeletal troponin I gene are able to confer expression of the reporter gene to the adult mouse AVN.\textsuperscript{93} The onset of expression of cardiac troponin I occurs later in rat heart development (11 days of development) and persists in the entire adult heart. Hence, transiently, the fetal myocardium displays coexpression of both isoforms, similar to the adult AVN.

The functional significance of the expression of the slow β-MHC isoform, of the fetal skeletal myosin isoform, and of the slow skeletal troponin I isoform in nodal cells remains unaccounted for. It may be the obligatory consequence of the nodal (“embryonic”) program of gene expression.

**Cytoskeletal Proteins**

**Desmin**

Desmin, the major component of the intermediate filaments in Purkinje fibers,\textsuperscript{82} is already expressed in early mouse and rat myocardium.\textsuperscript{84,94} It has been shown that specifically phosphorylated desmin isoforms are present in the conduction system (nodes, bundle, and bundle branches) of the adult cow.\textsuperscript{95} As mentioned above, several monoclonal antibodies were raised against desmin-like proteins, reacting specifically with the bovine conduction system and allowing the delineation of the conduction system.\textsuperscript{72,73,96} The high abundance of desmin in the conduction system has led to the suggestion that it could play a role in the reduction of the changing mechanical stress during systole and diastole in the myocytes of the conduction system.\textsuperscript{84,95} In a study in which desmin immunoreactivity was compared in several species, including cows, humans, and rats, Eriksson et al\textsuperscript{99} demonstrated that high levels of desmin were correlated with the morphologically well-differentiated Purkinje fibers of hoofed animals and that low levels of desmin were correlated with the morphologically poorly differentiated ventricular conduction system of the rat. This notion is in line with a study of rat heart development by Ya et al,\textsuperscript{96} who concluded that the expression of smooth muscle proteins and desmin is a temporary parameter for the process of myofibrillar organization in the developing cardiomyocyte. They observed an only slightly higher expression of desmin in the prenatal and postnatal ventricular conduction system.

Finally, it has been reported that 1 kb of the human desmin promoter specifically drives transgene expression in the cardiac conduction system, as judged from in toto X-galactosidase staining of mouse embryos at 8 days and at later stages.\textsuperscript{97} The endogenous gene is expressed in the entire early embryonic heart.\textsuperscript{98} In this early stage, expert transmission electron microscopy\textsuperscript{30,53,61} has not allowed the unambiguous
recognition of the conduction system. Moreover, the presence of expression of the transgene in the conduction system requires analysis of histological sections rather than of intact hearts on in toto staining.

Neurofilament
Three types of neurofilament have been described in mammals with molecular weights of $\approx 70 \text{kD}$, $\approx 150$, and $\approx 200 \text{kD}$, dubbed NF-L, M, and H, respectively. Immunoreactivity for the L and M subunits was found in all parts of the adult rabbit conduction system. Data have been substantiated at the mRNA level. Neurofilament mRNA can be detected from 9.5 days of development onward, and the protein is detectable slightly later at the sinoatrial junction and at both sides of the wall of the AVC of the rabbit heart, where it colocalizes with desmin. It may be of interest to analyze neurofilament expression in conjunction with connexin isoform expression (to delineate the nodal areas) during rabbit cardiac development. This would permit the distinction of the nodal areas from the specialized fast-conducting atrial tracts that could also be positive for neurofilament [see “The Atrial (Internodal) Conduction System” below].

Recapitulation
In an uncomplicated view, the phenotype of the nodal myocytes could be dubbed "embryonic" because of their electrical (embryonic conduction velocities and action potentials) and contractile (embryonic isoforms and poor sarcomeric organization) characteristics. Nevertheless, it seems too simplistic to consider the nodes as mere remnants of the embryonic myocardium, since they have become physiologically highly specialized. Elevated levels of the calcium-release channel/type-1 inositol trisphosphate receptor, $\alpha_\text{a}$, and $\alpha_\text{c}$ isoforms of the sodium pump (Na$^+$/K$^+$-ATPase), G protein $\alpha$-subunit, and the AT$_2$ receptor subtype have been reported in the adult node and/or ventricular conduction system compared with the working myocardium. It is to be expected that analyses of the developmental patterns of expression of this type of proteins will provide insight in the maturation of the nodes in the near future. Such studies may shed new light on a number of intriguing questions regarding the development of the nodes. For example, how do cells of the primary myocardium escape differentiation into working myocardium of atrium and ventricle, and what causes them to differentiate into nodal direction? How do they sort out positionally to form the intricate nodal structures of the formed heart, and what type of interactions with surrounding myocardial and nonmyocardial cells are involved?

Development of the Ventricular Conduction System
In the formed mammalian heart, the ventricular conduction system comprises the AVB, left and right bundle branches, and a PPN, which extends into the periarterial Purkinje fibers in birds only. There exists a great degree of interspecies variability in the type of Purkinje cells, which are clearly distinct from the working myocardium in birds and hoofed animals, less clear in humans and dogs, and almost indistinguishable, or perhaps even absent, from the working myocardium in rodents. Also, there exists within a species considerable diversity that is related to the position of the cells in the conduction system, with those at the periphery (transitional cells) being almost indistinguishable from the working myocardium. In fetal and neonatal human hearts, an additional right atrioventricular ring bundle has been described, whereas in the embryonic human heart a septal branch and a retroaortic root branch have also been described. Finally, in the adult avian heart, the entire system is present. How does this system develop, how does it fit in the model of the segmented heart, and what is its origin?

Morphological Development
A closer look at Fig 5a reveals two component parts in the ventricular conduction system. The first part is a “drape-like” part that is positioned on top and astride the ventricular crest (AVB and bundle branches), extending at the luminal side of the ventricular myocardium. It penetrates into the compact myocardium. This part brings the depolarizing impulse to the ventricles. Its position in the developing tubular heart can easily be envisioned (Fig 5b). The second part of the ventricular conduction system (Fig 5a) surrounds the subaortic outlet of the ventricle and the right atrioventricular junction just above the atrioventricular annulus. It is a bended oval ring, which, depending on the optic angle, will be perceived as a figure-8-shaped ring. This ring of myocardium has been demonstrated in the early fetal human heart and could be traced back to the myocardium surrounding the primary interventricular foramen in the 5-week embryonic heart. To understand the remodeling of the primary interventricular foramen, one should appreciate that the term “interventricular foramen,” used in many textbooks, is confusing, because it is not interventricular only, as will be pointed out below. The ventricular compartments develop from the primary heart tube by the formation of trabecular pouches (Fig 2). The ventricular septum develops by apposition of ventricular myocytes at the outer side, leaving a foramen, dubbed the primary interventricular foramen, between the inner curvature and the top of the ventricular septum. With diastole, both ventricles are filled (the right ventricle via the primary interventricular foramen); with systole, both ventricles are emptied (the left one via the primary interventricular foramen); and so the “crossing” flows of blood are separated in time. Thus, the essence of the position of the primary interventricular foramen is that it demarcates both the inlet of the right ventricle and the outlet of the left ventricle. As will be clear from Fig 5a, this is still the position in the formed heart. With septation, the primary interventricular foramen becomes divided by extension and fusion of the endocardial cushions. By this process, the right atrioventricular junction becomes physically separated from the left ventricular outlet.

The developmental fate of the myocardium surrounding the primary interventricular foramen has been followed in human cardiac development. (Fig 5c to 5e) on the basis of the expression of an epitope, dubbed GIN2 because it reacts with an antibody raised against an extract from the chicken nodose ganglion. Because of the growth of the OFT toward the left, part of the primary interventricular ring...
myocardium that is also part of the proximal OFT expands toward the left and forms the subaortic outlet. Because of the growth of the AVC toward the right, part of the primary interventricular ring myocardium that is also part of the lower rim of the AVC expands to the right and forms the lower rim of the right atrioventricular junction, where the right atrioventricular ring bundle is positioned. The fibrous insulation of the right atrium will take place at the ventricular side of this ring, so that part of the embryonic ventricular segment becomes the lower rim of the right atrium. That is why we have preferred the use of the term “ventricular conduction system” rather than “atrioventricular conduction system.”

Conventional light and electron microscopic studies report the first morphological signs of the primordia of the AVB and bundle branches at 5 to 6 weeks of human development and at 10 embryonic days of mouse development. We have not the faintest notion of the regulatory pathways involved in the complex remodeling of the inner curvature of the heart and the morphogenesis of the conduction system in this part of the heart. A first clue might come from chicken cardiac development, during which the pattern of expression of the Drosophila muscle segment–related homeobox gene, Msx-2, is almost entirely consistent with the GIN2 expression pattern in humans. It is also unknown what the precise contributions are of the AVC and of the primary interventricular ring myocardium to the formation of the AVN and AVB and what kind of mechanisms are involved in preventing the penetrating AVB from interruption by fibrous tissue during the process of atrioventricular fibrous insulation. The origin of the AVB from the embryonic ventricular compartment might be crucial, as the insulating fibrous tissue does not grow into atrial or ventricular myocardium, but in between.

**Segments and Rings**

How does the concept of the development of cardiac segments relate to the ring concept, in which the conduction system, encompassing the nodes and the ventricular conduction system, is reported to take origin from a series of rings of specialized tissue? From a functional point of view, the embryonic heart consists of five segments. Within the atrial and ventricular segments, right and left components develop, bringing the total number of segments to seven.

If one accepts that in the ring concept the cardiac specialized tissues are understood to mean “histologically distinct,” ie, distinct from the surrounding developing working myocardium, then the flanking segments (IFT, AVC, and OFT) are indeed distinct, and IFT and AVC myocardium will give rise to the formation of the nodes. One also has to accept that contrary to the ring concept, the rings are in fact segments, since they need to have a certain length to perform
their sphincter-like function in a heart in which valves have not yet developed.6,50,51 Although the term “specialized cardiac tissue” seems at first glance surprising in view of the current histological, molecular, and electrophysiological data, one should realize that this terminology originates from the beginning of this century and is based on phylogenetic considerations about the adult hearts of lower vertebrates, where these areas have been dubbed “specialized” and have been attributed sphincter-like functions.2,123 The low conduction velocities measured in the flanking segments of the embryonic mammalian heart are amazingly similar to those measured in the junctional areas of the amphibian heart,124 indicating that the basic architecture of the vertebrate embryonic heart follows a phylogenetically old pattern.

It is ironic that the only ring of conduction tissue that has been clearly recognized in fetal and neonatal mammalian hearts has been the right atrioventricular Purkinje ring.122,125,126 In fact, this ring is part of the “primary interventricular ring,” originating from the remodeling of the primary heart tube at the inner curvature, by which it becomes also part of the AVC myocardium where the AVN will develop.

**Molecular Phenotype of the Ventricular Conduction System**

The great intraspecies and interspecies variability in the morphology and histology of the constituent Purkinje cells of the ventricular conduction system2,25,100,111 is also reflected in the diverse patterns of gene expression in the ventricular conduction system, the significance of which has remained largely elusive.60 Nevertheless, the common functional principle in the ventricular conduction system of all species is to achieve a coordinated contraction of the ventricular myocardium from apex to base. This is already realized early in the vertebrate evolution in fish,127 suggesting the presence of preferential pathways of conduction in the single ventricle of the fish heart. In the hearts of higher vertebrates, conduction velocities are higher in the Purkinje network than in the working myocardium to achieve the coordinated ventricular contraction.128 In this context, it is of great interest that the spongy trabecular myocardium of the ventricle of the fish heart is architecturally reminiscent of the trabeculated embryonic ventricles, where the trabeculae display the higher conduction velocities.129 This phylogenetic correlation therefore appears to suggest an origin of the ventricular conduction system from the TVC. Although preferential conduction may be in part the consequence of the larger diameter of the constituent cells, it should be reflected in the molecular phenotype of these cells as well. The data are summarized in Fig 3.

**Leu-7, HNK-1, GIN2, and NCAM**

Antibodies to Leu-7, HNK-1, and GIN2 have been used to delineate the developing conduction system in rats,130–136 rabbits,137 and humans.113,130 In humans and rats, Leu-7, HNK-1, and GIN2 share almost the same spatiotemporal distribution in the heart113,131 and, hence, possibly the same epitope. No reactivity has been observed in mice. The GIN2 antibody is raised against a protein extract from chicken ganglion nodosum and was originally used to identify migrating neural crest cells.118 The HNK-1 antibody recognizes a complex sulfate-3-glucuronyl carbohydrate moiety,138 which is present on a series of molecules involved in cell adhesion139 and extracellular matrix interactions.140 The antibody is accepted as a marker for the chicken neural crest but is not absolutely specific.141 Immunoelectron microscopic studies in developing rat heart have demonstrated that the epitope is predominantly present on cell surfaces and extracellular matrices of nodal and AVB myocytes. Facing mesenchymal cells display the epitope as well; it is prominently present in wide intracellular spaces and rarely observed in cell-cell contact areas and on the surfaces of the myocytes of the working myocardium.134

Interestingly, whereas NCAM is expressed throughout the ventricular myocardium, it is polysialylated in the ventricular trabeculae and astride the ventricular septum in a pattern resembling the developing ventricular conduction system in chickens, although polysialylated NCAM is also present in some other parts of the heart, such as the OFT.138 This may indicate that the developing ventricular conduction system is in a process of insulation from the surrounding working myocardium, since the polysialic acid moiety would attenuate cell-cell interaction in general. In line with this conclusion is the recent observation that expression of the polysialic acid epitope is at the periphery of the expression of the HNK-1 epitope.142 The carbohydrate epitope recognized by the HNK-1 antibody is absent from cardiac NCAM in chickens, although it is present in neural NCAM.143 In the developing human heart, NCAM predominates in the nodal areas.144 Until detailed molecular analyses of the proteins involved and their encoding genes have become available, one can only speculate about the functional significance of this expression and of the HNK-1 epitope, regarding its role in cell-cell interactions, cell adhesion, and differentiation during cardiac development. As yet, their principal significance is their use as molecular markers.

The rat studies of Nakagawa et al131 demonstrate HNK-1 expression in the early ventricular myocardium. As soon as the first signs of the development of the compact myocardium arise, the epitope becomes confined to the interiorly localized trabecular cells and becomes predominant on top and astride the developing septum.132,135 From this stage onward, human embryonic material is also available.113 Strong expression can be observed along the ventricular crest anteriorly and posteriorly up to the inner curvature and thus in the myocardium surrounding the primary foramen, tapering off toward the trabeculae (Fig 5b). Expression is never observed in the CVC, including the free walls and ventricular septum. Taken together, this may imply that the ventricular conduction system in its entirety (AVB, bundle branches, and PPN) takes origin from the TVC, including the primary interventricular ring myocardium. With development, the proximal part of the ventricular conduction system (AVB and bundle branches) becomes insulated from the ventricular working myocardium by fibrous tissue, similar to the insulation of the atrial and ventricular working myocardium. In this respect, it is of interest that this implies that the atria, CVC, and TVC have become, to a large extent, distinct myocardial compartments of cellular communication. In many respects, the TVC
coordinates. The most comprehensive data on connexin expression in the developing and formed heart are from mice and rats. In both rat and mouse embryonic hearts, Cx43 expression is considerably higher in the TVC than in the CVC (septum and ventricular free wall).56,68,69,146,147 Surprisingly, at the mRNA level the pattern is the other way around, indicating substantial posttranscriptional control.57,148 In adult rat hearts, the AVN, AVB, and the proximal parts of the bundle branches do express Cx40 rather than Cx43.56,69 The developmental activation of expression of Cx40 follows a posteroanterior gradient, being highest in the atrial segment, followed by the TVC.149,150 In contrast to Cx43, expression of Cx40 becomes predominant in all components of the ventricular conduction system.70,71,149,151,152 Cx40 is also the predominant isoform in the conduction system of cows, pigs, dogs, and humans.71,75,153 The Cx40 homologue in chicken Cx42 is preferentially expressed in the ventricular conduction system as well.154 The protein appears remarkably late in development (9 days of development), when the ventricular conduction system is already well developed. Since the impulse displays a preferential pathway much earlier via the trabecular component,8,129 other gap junctional proteins should be involved. Accordingly, Cx40 knockout mice display almost no phenotype.155

**Contractile Proteins**

Sartore et al156 first demonstrated that cells of the adult chicken ventricular conduction system had a distinct expression pattern of myosin isoforms, characterized by the coexpression of the atrial and ventricular MHC isoforms (α- and β-MHC). Subsequently, this was also noticed in mammalian species, including rats,157 rabbits,158 cows,86,158 and humans.87 Initially, both isoforms are expressed in opposite gradients and only gradually become confined to either the atrium or ventricle.61,47,81,159 Coexpression persists a relatively long time in the slow-conducting flanking segments (IFT, AVC, and OFT) but also in the fast-conducting TVC.6,129 Similar observations were made in the rat.155 These observations indicate that with regard to the contractile properties, the “embryonic” phenotype persists in the TVC, whereas at the same time the TVC becomes more differentiated with respect to the conducting properties.

Unique for the chicken ventricular conduction system is the presence of slow tonic myosin, which unfortunately accumulates late in the development in the peripheral Purkinje system only, by which it cannot be used as a developmental marker.156 Recently, the presence of myosin binding protein H, a member of the myosin binding protein gene family, has been reported in late fetal and adult chicken conduction systems.60,61 Because this protein is expressed in adult skeletal muscle but not in adult cardiac muscle, it was suggested that Purkinje fiber differentiation could involve a switch from the cardiac to the skeletal program of gene expression. Another example would be slow skeletal troponin I.90 However, slow skeletal troponin I is also part of the embryonic cardiac program of gene expression, whereas the developmental pattern of myosin binding protein H is not yet known.

**Atrial Natriuretic Factor**

ANF belongs to a family of homologous natriuretic peptides and is implicated in the regulation of body fluid and electrolyte balance. In the fetal and adult human conduction system, ANF has been localized in the AVB and the bundle branches, but not in the nodes.162 Expression has also been observed in the ventricular conduction system of the adult cow,163 pig,164 rabbit,165 and rat.166 Colocalization of the related brain natriuretic factor has been reported as well.167,168 Obviously, the developmental appearance and pattern of ANF expression is of great interest. ANF protein169,170 and mRNA171 have been found during rat development in, apart from the atria, the TVC only from 11 days of development onward, underscoring the notion that the TVC may contribute to the ventricular conduction system (Fig 6).

**Cytoskeletal Proteins**

**Desmin**

Desmin expression has proven to be an unambiguous marker for the adult bovine ventricular conduction system.52,95,96,172 As yet, no developmental data are available. In the mouse, desmin predominates in the TVC.88 In the rat, desmin is only slightly higher expressed in the ventricular conduction system than in the neighboring working ventricular working myocardium, reflecting the lower degree of differentiation of the rat system. In line, α-smooth muscle actin is higher expressed in the TVC than in the CVC and remains higher in the ventricular conduction system in fetal and neonatal stages.64

**Neurofilament**

Gorza and coworkers100–102,177,173 have convincingly demonstrated that in the adult and developing rabbit heart, neurofilament protein and mRNA are excellent markers for the entire conduction system: SAN, AVN, and the ventricular conduction system. So far, this is the only marker that identifies both functionally distinct parts of the conduction system, the nodes and the ventricular conduction system. The ventricular conduction system is first detected as a subendocardial layer in the embryonic ventricular wall.180 Colocalization with desmin has been demonstrated,180 and coexpression with HNK-1 has been noted as well.137 So far, the patterns of expression have not been compared with the expression of functional markers, such as connexins. The rabbit is well suited for electrophysiological studies.174 This fact and the availability of the unique neurofilament marker make the (developing) rabbit heart an exceptionally good model for integrated functional and molecular studies.

**Acetylcholinesterase and Creatine Kinase**

**Acetylcholinesterase**

Acetylcholinesterase activity has been demonstrated in embryonic chicken175 and rat133,176 hearts. High activities were found in the myocardial wall of the OFT, slightly lower activities were found in the ventricular trabeculae, and low
activity was found in the inner layer of the AVC. The localization downstream in the cardiac tube has led to the proposal that acetylcholinesterase may be involved in a calcium-mobilizing muscarinic regulatory system. Such a function would match the gradient in the contraction duration along the cardiac tube, which increases in the downstream direction, and the anteroposterior pattern of expression of the genes encoding sarcoplasmic reticulum calcium ATPase (high at the venous pole of the heart) and its natural inhibitor phospholamban (high at the arterial pole of the heart). However, attempts to identify other components of the cholinergic signal-transduction pathway failed, leaving the function of acetylcholinesterase unknown.

Creatine Kinase

In cardiac muscle, two distinct, but related, cytosolic isoforms of creatine kinase have been described. These isoforms exist as homodimers or heterodimers, dubbed MM, MB, or BB, where M stands for muscle and B for brain. A highly interesting pattern of expression has been observed in the developing human heart. Creatine kinase-M is virtual absent from the flanking segments (IFT, AVC, and OFT) and was much higher in the TVC than the CVC. Higher creatine kinase-M levels are also found to be associated with the developing ventricular conduction system in chicken, rat, and bovine hearts.

The Atrial (Internodal) Conduction System

The question of whether there are internodal tracts between the SAN and AVN has been discussed at length. There is unanimity that “specialized” tracts insulated by fibrous tissue from the surrounding atrial working myocardium do not exist. There is also unanimity that the intricate atrial geometry (the atria are not just perfect globes) in addition to differences in the alignment of the myocardial fibers can lead to preferential conduction pathways. There is no consensus as to the existence of an atrial system, fashioned as tracts or as a kind of network and composed of atrial myocytes with distinct conducting properties, resulting in preferential pathways of conduction. Although there is no proof as yet that such an “atrial conduction system” would exist, the molecular markers are now available to settle this issue. Thus, in the adult rabbit heart, extensive neurofilament staining has been observed in the nodal areas in the atrium, in the sinoatrial ring, and in the ventricular conduction system. However, it is not known whether the extensive immunostaining in the atrium is restricted to the nodes or whether it extends outside the nodes and, if so, whether the extranodal neurofilament-positive cells have distinct conducting properties. Similarly, Leu-7 (HNK-1) displays expression in internodal tracts, branches across the roof of the right atrium, and spreads into the left atrium in the developing human and rat heart.

Again, the central question is whether these cells are different in other aspects as well. The same questions can be raised for the chicken atrium, where Purkinje cells have been described in the atrial trabeculae and a network of loosely arranged Purkinje cells coexpressing α- and β-MHC has been reported; this network connects both atria and has been described in the developing and adult chicken heart.

Origin of the Conduction System

There is debate as to the origin of the conduction system. Owing to the recent observations of so-called “neural proteins,” a neural crest origin has become in vogue. As yet, there is no evidence to support the notion of an extracardiac origin of the conduction system. Current knowledge states that neural crest cells first enter the embryonic heart at 4 days of development in the chicken, which is far beyond the time that adult-like ECGs can be registered.

Sinoatrial Node

Regarding the origin of the SAN, it is more than probable that it originates from existing myocardium, because its function has been shown from the first heartbeat onward. According to Patten: “The atrial part of the heart is not formed all at once, but by progressive fusion of the paired primordia. This means that at any given phase of development the most recently added part of the atrium is the pacemaker.” A neural
Atrioventricular Node
As the necessary consequence of the development of the (fast-conducting) atrial and ventricular working myocardium at two places in the slowly conducting primary heart tube, a delay of the depolarizing impulse between the two segments becomes manifest, which can be demonstrated in ECG recordings. This way of development does not require the recruitment of additional extracardiac neural crest cells to explain the presence of delayed conduction of the depolarizing impulse from the atria to the ventricles, the central feature of the AVN. The entire AVC functions as the AVN in these stages. It was again Patten who first demonstrated that this is indeed the case. He dissected the AVC in two parts, leaving a small artificial AVB to the atrial and the ventricular compartments. No difference in atrioventricular conduction was observed, irrespective of whether this artificial bundle was left at the position where the AVB normally would develop or at an opposite position.

Ventricular Conduction System
Combined (1) genetic, (2) molecular and functional, and (3) morphological evidence is in favor of the notion that the trabecular component of the ventricle is a separate transcriptional domain that is distinct from the compact myocardium and may give rise to the entire ventricular conduction system.

Genetic Evidence
Mice carrying a loss-of-function mutation in the neuregulin gene do not develop trabeculae and die at 10.5 days of development. Similar phenotypes were obtained in mice carrying a loss-of-function mutation in the neuregulin receptor erbB2 or erbB4. Neuregulin is a peptide growth factor acting via receptor tyrosine kinases. In the heart, expression is confined to the endocardial cells. ErbB2 and erbB4 are expressed in the atrial and ventricular myocardium. The idea is that neuregulin initiates the signal transduction pathway leading to the formation of the trabeculae in a paracrine manner. The atrial myocardium and the compact myocardial zone are apparently normal. These independent loss-of-function mutations in three independent genes indicate that the TVC constitutes a distinct component. The cause of death is unknown. A reasonable explanation is that the heart has insufficient contractile capacity. Another explanation may be the occurrence of conduction disturbances, since the animals display irregular heartbeats.

A second piece of evidence comes from the functional dissection of mice carrying a homozygous loss-of-function mutation in the retinoic X receptor α. The formation of the trabeculations and of the compact myocardial zone is affected in the mutant heart. These mice die at embryonic day 15. Apart from depressed ventricular function, the mice display partial or complete heart block, indicating that the atrioventricular connection has not been appropriately formed.

Molecular and Functional Evidence
With the first morphological indications of the formation of the ventricular trabeculations, Leu-7 (HNK-1) identifies the interiorly localized ventricular myocytes, which will form the TVC. A comparison of the pattern of gene expression in the TVC on the one hand and the CVC on the other hand reveals that the TVC expresses many atrial isoforms (eg, ANF, α-MHC, MLC1A, and MLC2A) (Fig 6) in addition to the (lower expressed) ventricular isoforms, whereas connexins are more abundant in the TVC than in the CVC. Hence, the contractile phenotype of the TVC can be dubbed “embryonic” relative to the CVC, whereas the conducting phenotype is more advanced in the TVC than in the CVC. This notion is underscored by electrophysiological measurements that demonstrate that the depolarizing impulse is preferentially conducted via the trabeculations. Many of the markers remain expressed in the adult ventricular conduction system.

Morphological Evidence
The TVC initially constitutes a large component relative to the compact myocardium, and one might question whether it will give rise exclusively to the conduction system or whether it will also contribute to the compact myocardium. Vassal-Adams concluded that the definitive (ventricular) conduction system takes origin from widespread precursor tissue, which he thought was distributed as a complete dark-staining subendocardial sleeve, which, on trabeculation and septation of the ventricles, becomes dispersed within the trabeculae, forming the intramural and subendocardial Purkinje cells and forming, as it were, a drape over the developing septum. Patten denotes this process as a restriction and reshaping of the “primordial myocardium,” and Robb and Petri indicated that the cells of the outer compact layers are destined to become cardiac working myocardium and that the trabecular layer represented Patten’s “primordial myocardium,” the area that we now would dub the TVC and that has a clearly distinct molecular phenotype. It is remodeled with development and forms a gradient toward the CVC, being most pronounced at the luminal side. The chicken studies are in agreement with previous mammalian studies of Viragh and colleagues, who reported on the developing mouse heart and determined that the AVB and bundle branches are derived from the early trabeculae.

Regarding the extent of the TVC, it is important to appreciate that the ventricular conduction system is a notably larger structure in relation to the compact myocardium in
the embryonic heart than in the adult heart. The width of the embryonic bundle is \( \approx 1/5 \) the width of the septum, and the width of the adult bundle is \( \approx 1/500 \) the width of the septum, which makes the contribution of the “large” embryonic TVC to the “small” adult ventricular conduction system more understandable. With development, most of the new ventricular muscle is formed by proliferation of the compact myocardium to form the septum and the ventricular free walls, where highest DNA-synthetic activities relative to the TVC have been observed. The compact myocardium not only thickens but also balloons out to enlarge the ventricular lumen, forming sponge-like myocardium covered and intermingled with the “true trabecular myocytes” reaching almost to the epicardium. This extensive spongy myocardium is architecturally similar to the condition in the formed heart of lower vertebrates, where a coordinated contraction of the ventricle from apex to base also exists. One might envision that the interiorly localized trabeculations are involved in this referential conduction, forming a primitive ventricular conduction system.

Finally, Gourdie et al. recently addressed the issue of the origin of the peripheral conduction system in chicken heart development. By retroviral tagging of the right ventricular myocardium of chicken ventricles at 3 days of development, “when myocardial cell migration becomes restricted,” they were able to demonstrate a clonal relationship between Purkinje and “working” myocytes. No clones were observed in the AVB and bundle branches. The important conclusion of their study is that it unambiguously demonstrates that the myocardium to form the septum and the ventricular free walls, the interiorly localized trabeculations are involved in this preferential conduction, forming a primitive ventricular conduction system.

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Development of the Cardiac Conduction System


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