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Review

Induction and Patterning of the Cardiac Conduction System

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Introduction

The cardiovascular system is the first organ system to form and function in the developing vertebrate embryo. This comes from the embryo's need for nourishing the entire body while the cardiovascular system is itself developing and maturing. In the case of vertebrates, the heart contracts rhythmically to pump blood unidirectionally, which is contrast to invertebrates, whose hearts stochastically alternate the direction of the contractile wave (see Gourdie *et al.*, 1999). The vertebrate heart achieves this coordinated contraction of the atrial and ventricular chambers due to the precise initiation and transmission of action potentials through a specialized tissue network, the cardiac conduction system (CCS). The components of this specialized cardiac system (Fig. 1) include the sinoatrial (SA) node that generates a pacemaker impulse; the atrioventricular (AV) node that delays an electrical impulse for separating the contraction of the atrial and ventricular chambers of the heart; and His-Purkinje system for the fast and coordinated conduction of impulses to, and throughout, the ventricles. For an overview on the anatomy of the CCS, as well as associated pathology and congenital disease, see Davies *et al.* (1983).

The primordial heart begins its function soon after the bilateral cardiogenic mesoderm fuse to form the heart tube during body folding (Fig. 2; Patten and Kramer, 1933; Manasek, 1968). At early stages of heart function and development, the CCS has not yet developed, let alone integrated the disparate components that make up the CCS. The embryonic heart has developed strategies to enable proper conduction and, therefore, coordinated contraction without a mature CCS. The tubular heart periodically and spontaneously evokes action potentials before the myocardial cells can contract (Hirota *et al.*, 1979; Kamino *et al.*, 1981; Hirota *et al.*, 1985). In the embryonic chick heart at the 7-8-somite stage, these spontaneous action potentials initiate in the primordial sinoatrial region and propagate throughout the myocardium where all cardiomyocytes are electrically coupled *via* gap junctions. As the heart tube undergoes a right-sided looping, cells of the myocardium become contractile (Manasek, 1968). The direction of the electrical excitatory wave along the myocardium – posterior to anterior – produces a contractile wave from caudal to rostral in the primordial heart. This unidirectional propagation of action potentials and contractile wave remains during heart looping until interventricular septation completes (Fig. 3).

Upon separation of the ventricle into the left and right chambers, the wave of electrical impulses that emanates from the atria is then transmitted to the apex of the heart without directly activating ventricular myocytes (Fig. 3; Chuck *et al.*, 1997; Chuck and Watanabe, 1997). In the mouse which has a small heart with a

Abbreviations used in this paper: ANF, atrial natriuretic factor; AV, atrioventricular; β-gal, β-galactosidase; CCS, cardiac conduction system; d*pc*, days *post coitum*; ECE-1, endothelin converting enzyme; ET-1, endothelin-1; ETR, endothelin receptor; FGF, fibroblast growth factor; MDCK, Madin-Darby canine kidney; PDGF, platelet-derived growth factor; ppET, prepro-endothelin; SA, sinoatrial; VEGF, vascular endothelial growth factor.

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thinner myocardium, however, ventricular activation from the apex can be observed slightly prior to the completion of ventricular septation (Rentschler *et al*., 2001; Fig. 3). This dramatic shift in the impulse-conducting pathway depends on the coordinated differentiation and patterning of the CCS. The morphology, electrophysiology and cellular properties of the CCS have long been the subject of characterization. Importantly, new information has been gathered in the last few years uncovering some of the molecular and cellular mechanisms that regulate the differentiation and patterning of these specialized cardiac tissues during development. This review gives an overview of recent advances in understanding the induction and patterning of the CCS.

Functional Elements

The CCS consists of several components, each of which plays a distinct role in coordinating rhythmic heartbeat (Fig. 1). Molecular and functional markers specific for the CCS, as well as CCS subcomponents, have been reviewed in detail elsewhere (Schiaffino, 1997; Moorman *et al*., 1998; Gourdie *et al*., 1999; 2002; Welikson and Mikawa, 2002).

Pacemaker

Pace making impulses are generated at the sinoatrial node (SA-node), embedded in the right atrium. The SA-node is a heterogeneous tissue that varies in morphology and the degree of embedding in the atrial wall from species to species (Boyett *et al*., 2000). In cardiac muscle, which beats rhythmically without external stimulus, the cells with the most rapid inherent rhythm set the rate of beating of the rest of the myocardium. The cells of the SA node display the most rapid rhythm and thus represent the «pacemaker» of the heart. The SA-node is also richly innervated by both the sympathetic and parasympathetic divisions of the autonomic nervous system. The pace making action potential is produced by a slow, diastolic depolarization that involves a number of different ion channels, including T- and L-type calcium channels (Bohn *et al*., 2000).

At present, there are two models for the heterogeneity of the SA node; one, the «gradient model», is one in which there is a gradual change in the properties of node cells from the periphery to the center; secondly, the «mosaic model», is where there is a variable mix of atrial and SA node cells from the periphery to the center. Boyett *et al.* (2000) tentatively conclude that, at least in the rabbit, the «gradient model» best represents the organization of the SA node. This heterogeneity appears to be necessary for numerous reasons, including: protection of the SA node from the hyperpolarizing influence of the surrounding atrial muscle; assistance to the SA node to drive the surrounding atrial muscle and protecting the SA node from action potentials outside the SA node. The complexity of the SA node accounts for the heterogeneity of electrical activity throughout the SA node, the non-radial spread of the action potential from the leading pacemaker site in the SA node, and the block of conduction from the leading pacemaker site towards the atrial septum and pacemaker shift (Boyett *et al*., 2000).

The pacemaker activity is the first element to function in the CCS. When the primitive heart tube forms, all epithelioid myocytes are electrically active, but pace making impulses are evoked predominantly by myocytes in the posterior inflow tract, the presumptive sinus venosus and atrium (Kamino *et al*., 1981; Yada *et al*., 1985). Impulses spread to the anterior end of the heart, towards

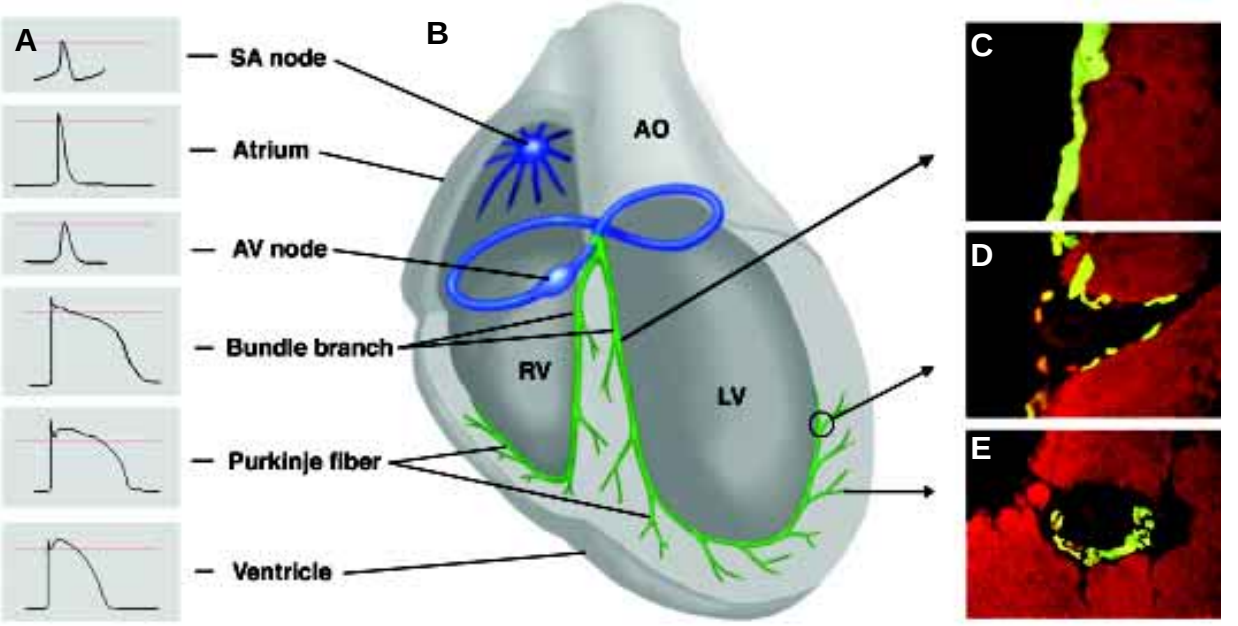

Fig. 1. Components of the cardiac conduction system. (A) *Panels show action potentials for components of the chick heart, in* **(B)**. *The panels on the right demonstrate immunofluorescence to detect slow tonic myosin heavy chain in Purkinje fibers (green) and myosin-binding protein C in ventricular myocytes (red).* **(C)** *Subendocardial Purkinje fibers.* **(D)** *Branch point from subendocardial Purkinje fibers.* **(E)** *Intramural Purkinje fibers. ao, aorta; av, atrioventricular; lv, left ventricle; rv, right ventricle; sa, sinoatrial.*

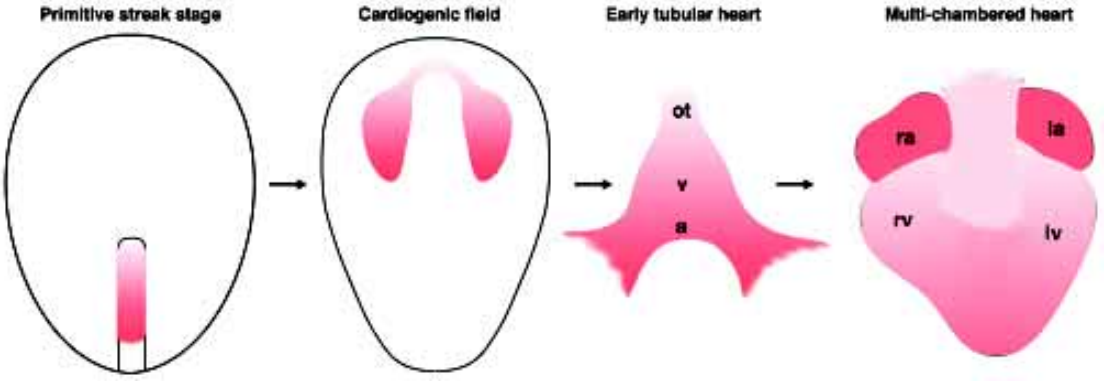

Fig. 2. Diagrammatic representation of the fate of cardiomyocyte precursors. *Developmental progression is represented from left to right. A color gradient represents the location of cardiac cells and their precursors as the heart develops, the darker being the most posterior of cardiac precursors to ingress through the primitive streak. a, atria; la, left atrium, lv, left ventricle; ot, outflow tract; ra, right atrium; rv, right ventricle; v, ventricle.*

the outflow tract, through gap junctions between the epithelioid myocytes (Fig. 3), generating a posterior-to-anterior contractile wave along the heart tube (Patten and Kramer, 1933). Although the presumptive ventricle and atrium are molecularly distinguishable at this stage (Yutzey *et al.*, 1994), action potentials propagate throughout a straight heart tube without any local changes in velocity. In mammals, the SA node becomes morphologically distinct, well after the heart becomes contractile, located at the junction of the right common cardinal vein and the wall of the right atrium (Virágh and Challice, 1980). Little is known about the mechanisms that induce and maintain the pacemaker cells at this predictable site of the heart. Interestingly, in lower vertebrate species, the pacemaker resides in the sinus in a fashion (Virágh and Challice, 1983) analogous to the primitive sinus of embryonic higher vertebrates (Kamino *et al.*, 1981). This spatial and functional relationship prompts one to consider the evolutionary origins of the SA node in higher vertebrates.

AV-delay

The atrioventricular (AV) node is located at the base of the interatrial septum and positioned near the endocardium (Tawara, 1906; Fig. 1). The main function of the AV node is to slow the pacemaking impulse from the atrium to the ventricular myocardium. Like cells of the SA node, cells of the AV node are interspersed with connective tissue and extensive vasculature. In mammals, the AV node is separated from the endocardium by a

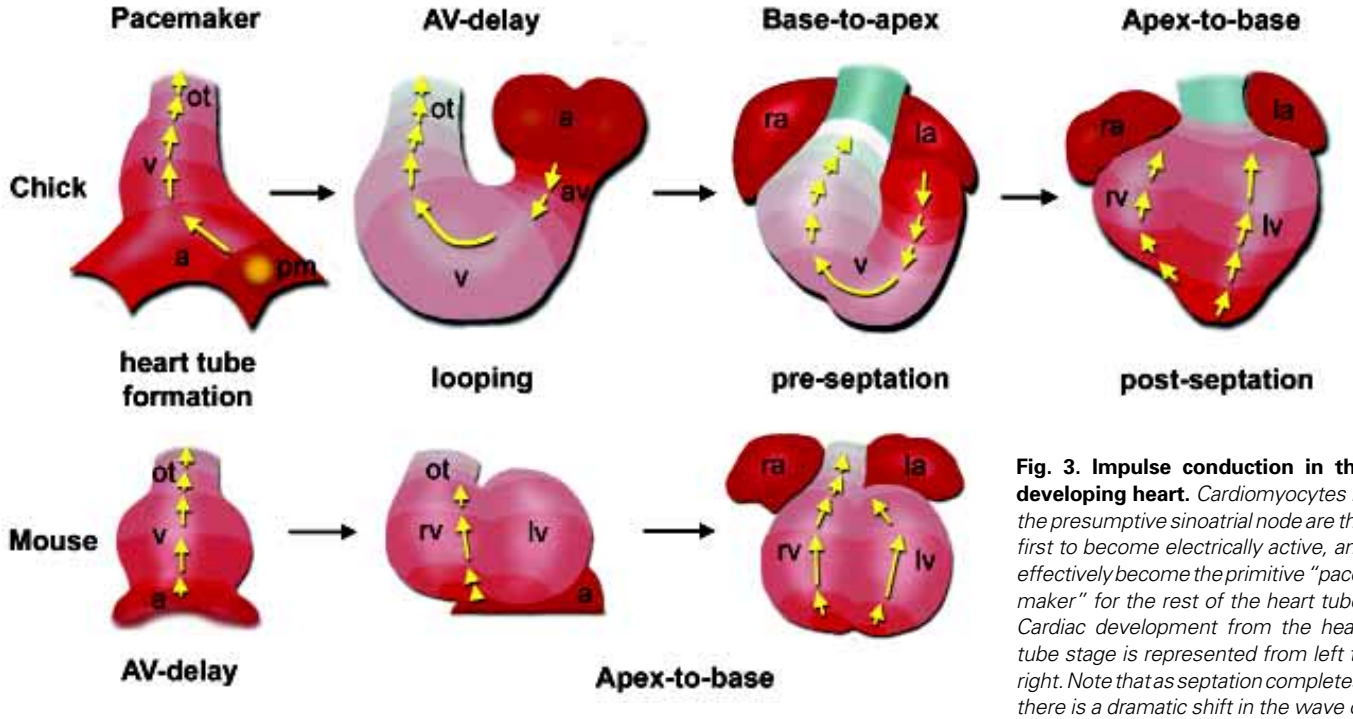

Fig. 3. Impulse conduction in the developing heart. *Cardiomyocytes in the presumptive sinoatrial node are the first to become electrically active, and effectively become the primitive "pacemaker" for the rest of the heart tube. Cardiac development from the heart tube stage is represented from left to right. Note that as septation completes, there is a dramatic shift in the wave of impulse dissemination (yellow arrows). This appears to be in contrast to the situation in the developing mouse heart; recent findings using the MC4 transgenic line to visualize the developing murine cardiac conduction system (Rentschler* et al.*, 2001; Rentschler, Morley and Fishman, unpublished), indicate that there is an apex-to-base change in conduction before septation is complete. The rate of impulse dissemination in different regions of the heart is represented by a color gradient, red being greatest. a, atria; av, atrioventricular canal; la, left atrium; lv, left ventricle; ot, outflow tract; pm, pacemaker; ra, right atrium; rv, right ventricle; v, ventricle.*

thin layer of atrial myocardium. The cells in the periphery of this region are flat and spindle like, while more irregularly shaped fibers are located more deeply (Thaemert, 1973). The superior and right margins are composed of more loosely connected fibers and tend to intermingle with the muscle fibers of the right atrium and interatrial septum. The inferior region of the node becomes more regularly aligned, as it becomes continuous with the atrioventricular bundle. The peripheral cells in the medial portion of the node are globular like and contain hardly any myofibrils.

The AV-delay first becomes detectable when the looping heart undergoes a morphogenic constriction to divide atrial and ventricular chambers (Fig. 3; Lieberman and Paes de Carvalho, 1967; de Jong *et al.*, 1992). This AV delay gives rise to an effective peristaltic wave of myocardial contraction of the looping heart. Myocytes at the AV-junction preferentially express connexin45 (Cx45) (Alcolea *et al.*, 1999), a low conductance gap junction channel that is also expressed in the SA node as well as the AV node of the mature heart (Coppen *et al.*, 1999). It remains to be determined whether myocytes in the AV-junction of looping stage hearts differentiate into cells of the AV-node (Cheng *et al.*, 2000).

Rapid Impulse Conduction

From the AV node, pacemaking impulses are transmitted to the ventricular conduction network that consists of the AV-bundle, bundle branches and Purkinje fibers. The main function of the ventricular conduction network is to rapidly propagate and transmit impulses to the ventricular muscle. Our knowledge of how this is facilitated has been aided by the discovery that the high-conductance gap junction proteins, Cx40 (previously termed Cx42) and Cx43, are expressed in a spatially restricted manner, namely in cells of the more distal elements of the CCS (Gros *et al.*, 1992; Gourdie *et al.*, 1993a; Gourdie *et al.*, 1993b; Gourdie, 1995). His (1893) first described the bundle of cells that forms a connection between the atrial and ventricular chambers in the mammalian heart. The AV bundle, that also bares his name, the His bundle, originates at the posterior right atrial wall near the atrial septum above the atrioventricular groove. The AV bundle passes over the upper margin of the ventricular septal muscle, where their respective fibers intermingle with each other. It then bifurcates near the aorta into a right and a left bundle branch, the latter terminating at the base of the aortic leaflet of the mitral valve. The pioneering work of His advanced the thinking about the conduction of excitation in adult mammalian hearts – that this excitation could be conducted from the atria to the ventricles through continuous, muscular tissue (Tawara, 1906).

The left and right bundle branches eventually lead into the Purkinje fibers. Purkinje fibers are distributed widely throughout the subendocardium of the left and right ventricles (Fig. 1). In some species they penetrate transmurally deep into the myocardium. The tips of Purkinje fibers are electrically coupled to muscle cells *via* gap junctions, thereby initiating an apex to base contraction of the ventricle. Of the components that comprise the CCS, the pattern of the intramural Purkinje fibers shows the most variation between species (Truex and Smythe, 1965). One common theme amongst vertebrate hearts is, however, in the pattern or location of the proximal aspect of the Purkinje fiber network (reviewed by Moorman *et al.*, 1998; Welikson and Mikawa, 2002). These conduction cells are invariably arranged in a sub-endocardial pattern, and are termed the subendocardial Purkinje fibers.

Myocyte Origin of the Conduction System

Much of our knowledge of the development of the vertebrate heart stems from studies conducted on the chick embryo. Historically, there has been much debate as to the origin of cells of the CCS, particularly Purkinje fibers. This, in part, is rooted in the observation that cells of the CCS necessarily display expression of markers similar to neuronal cells as well as those expressed by muscle cells (reviewed by Schiaffino, 1997; Moorman *et al.*, 1998; Welikson and Mikawa, 2002). Given the invasion of the heart by neural crest cells that do express «neuronal» factors, and the expression of common markers by CCS cells and neuronal cells, such as HNK-1 and neurofilament proteins (Gorza *et al.*, 1988; Vitadello *et al.*, 1990), it is not surprising that hypotheses arose about a neural crest origin for the CCS (Gorza *et al.*, 1988).

Fate map studies in the chicken embryo have identified that cells from three distinct embryonic origins, cardiogenic mesoderm, neural crest and the proepicardial organ, constitute the heart (reviewed by Mikawa, 1999). Mesoderm bilateral to Hensen's node becomes committed to myocyte and endocardial cell lineages just after gastrulation begins, and form the double-walled tubular heart (reviewed by Garcia-Martinez and Schoenwolf, 1993). Cardiac neural crest cells migrate towards the heart tube from the neural tube at somite levels 1-3 and form great vessel smooth muscle and cardiac ganglia (reviewed in Kirby, 1988; Poelmann *et al.*, 1994). Prior to the arrival of cardiac neural crest cells to the heart, cells of the proepicardium also migrate from the mesothelium towards the looping, beating heart tube, forming the epicardium (Ho and Shimada, 1978; Virágh and Challice, 1981; Hiruma and Hirakow, 1989; Männer, 1993) and coronary vessels (Mikawa and Fischman, 1992; Poelmann *et al.*, 1993; Mikawa and Gourdie, 1996; Dettman *et al.*, 1998).

Of these three distinct sources of cardiac cells, that elements of the distal CCS, namely the Purkinje fibers, and elements of the proximal conduction system, such as the AV-node, His bundle and bundle branches, are derived from working myocytes was demonstrated beyond doubt in a series of retroviral lineage studies in the chick embryo (reviewed by Mikawa, 1999; Gourdie *et al.*, 2002; Mikawa *et al.*, 2002). In these studies, replication-defective retroviral vectors, encoding recombinant β-galactosidase (β-gal), were used to infect, and thus «tag», single cells of each of three cardiogenic primordia, the mesoderm and heart tube (Mikawa *et al.*, 1992a,b; Gourdie *et al.*, 1995; Cohen-Gould and Mikawa, 1996; Cheng *et al.*, 1999; Wei and Mikawa, 2000), cardiac neural crest (Gourdie *et al.*, 1995; Cheng *et al.*, 1999), and proepicardium (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). β-gal-positive Purkinje fiber cells were exclusively and frequently found in myocyte clones. In contrast, no conduction cells are produced from cardiac neural crest or primordial epicardial cells. Evidence consistent with the non-neurogenic origin of the conduction system has recently been obtained by studies on neural crest derivatives in the mouse embryo (Epstein *et al.*, 2000; Jiang *et al.*, 2000). These studies show that Purkinje fibers are differentiated from a subset of contractile myocytes, not from the neural crest as previously suggested (Fig. 4).

Individual myocyte precursor cells give rise to a series of progeny that migrate more centripetally than circumferentially to form clones that often span the full thickness of the myocardium, i.e., from the epicardial to endocardial surface of the muscle wall

(Fig. 4; Mikawa *et al.*, 1992b; Mikawa, 1995; reviewed in Mikawa and Fischman, 1996; Mikawa, 1999). This clone-based differentiation is a common motif in both proximal conduction fascicles, such as the His bundle (Cheng *et al.*, 1999), and in the distal Purkinje system (Gourdie *et al.*, 1995). Importantly, in no case were Purkinje fibers and cells of the proximal conduction system found in the same β-gal-positive clones. This revealed that conduction cell differentiation occurs within individual myocyte clones that occupy only a segment of the myocardium and, thus, clonally related conduction cells only form a segment of the Purkinje network.

Birth-dating studies indicate that the local recruitment of the proximal conduction system, such as the AV-node, His bundle and bundle branches, ends soon after ventricular septum formation completes (Cheng *et al.*, 1999), while recruitment of cells to Purkinje fiber network continue until hatching (Gourdie *et al.*, 1995; Cheng *et al.*, 1999; Litchenberg *et al.*, 2000). The finding of Purkinje fiber differentiation within individual myocyte clones has provided a new insight into the mechanism by which the Purkinje system expands and is patterned during heart development, lending strong support for the model of recruitment to the CCS from cardiomyocytes rather than a simple "outgrowth" model.

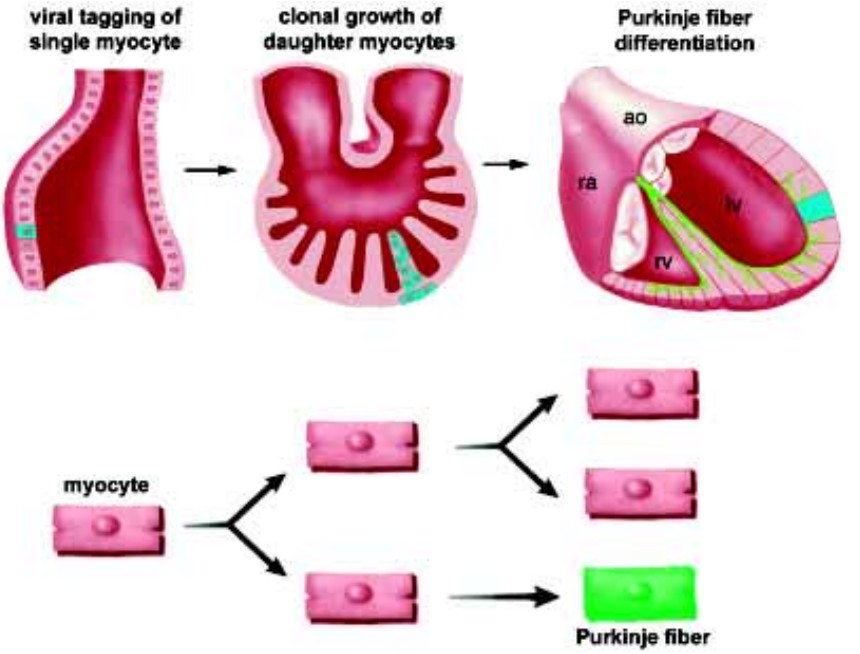

Fig. 4. Identification of the myocyte origin of Purkinje fibers. *Lineage tracing experiments where myocytes were "tagged" using replication-incompetent retroviral vectors demonstrated that Purkinje fibers of the cardiac conduction system are derived from clonally related myocytes. Furthermore, these experiments demonstrated that Purkinje fibers were recruited locally and that the conduction system did not expand simply by outgrowth and branching. Blue represents "tagged" cardiomyocytes and clonally related daughter cells. Green represents Purkinje fibers of the conduction system. ao, aorta; lv, left ventricle; ra, right atrium rv, right ventricle.*

The Inductive Role of the Cardiac Vasculature

In avian hearts, both subendocardial and intermyocardial components of the Purkinje fiber network persist (Moorman *et al.*, 1998). Intramyocardial Purkinje fibers penetrate from the subendocardium into the myocardium following coronary artery branches, but not venous or capillary networks (Davies, 1930; Vassal-Adams, 1982; Gourdie *et al.*, 1995; Takebayashi-Suzuki *et al.*, 2000). The unique location of Purkinje fiber recruitment within individual myocyte clones of the chick heart has provided an important insight into the cellular and molecular factors involved in the inductive events that pattern the Purkinje fiber system in the embryonic heart. This section discusses the possibility that paracrine interactions between embryonic myocytes and cardiac endothelial cells play a key role in the local recruitment of conduction cells from beating myocytes.

As with the ontogeny of the CCS, the origins of the coronary vasculature have been a controversial subject. There have been two major schools of thought on the subject of the formation of the blood vessels of the heart. One supports the notion of sprouting out from the base of the aorta, that is to say, an entirely angiogenic process. The other supports the idea that an existing vascular bed contacts the base of the aorta to allow circulation to the closed coronary vascular system. Fate mapping studies in the chick have demonstrated that epicardium-derived cells contribute to the coronary vasculature, perivascular fibroblasts and intermyocardial fibroblasts (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Dettman *et al.*, 1998; Pérez-Pomares *et al.*, 1998). The coronary vasculature in the embryonic chick becomes functional at around E14 (Rychter and Ostádal, 1971a; Rychter and Ostádal, 1971b; Vrancken Peeters *et al.*, 1997) when the emerging vascular network grows into the aorta (Bogers *et al.*, 1989) and then circulating blood drains into this network (Virágh and Challice, 1981).

Definitive proof of the discontinuous nature of the formation of the coronary vasculature was provided by cell-tagging and lineage tracing using replication-defective retroviral vectors, thus demonstrating a vasculogenic process (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). That the coronary vessels form by a vasculogenic process precludes the theory of the coronary vasculature arising from the base of the aorta. For further applications on the use of replication defective retroviral vectors, see the reviews by Mikawa *et al.* (1996), Fischman and Mikawa (1997), and Hyer and Mikawa (1997). The model that paracrine interactions of myocytes with developing coronary arteries play an inductive role in conduction cell differentiation (Gourdie *et al.*, 1995; Mikawa and Fischman, 1996; Mikawa, 1999) has been tested experimentally by two complementary approaches: inhibiting as well as promoting coronary arterial branching (Hyer *et al.*, 1999). Inhibition of coronary vessel development resulted in a significant loss of intramural Purkinje fiber differentiation, indicating the necessity of coronary arterial beds for intramural conduction cell differentiation. Furthermore, ectopic Purkinje fibers developed along arteries that were ectopically induced in the myocardium (Hyer *et al.*, 1999). Thus, coronary arterial beds are not only necessary but also sufficient for recruiting adjacent myocytes to differentiate into conduction cells.

Periarterial Purkinje fibers are, however, not commonly seen in mammalian hearts (Moorman *et al.*, 1998). For example, the

mouse heart develops subendocardial, but not intramyocardial, Purkinje fibers (reviewed in Moorman *et al.*, 1998; Welikson and Mikawa, 2002). Our studies in the mouse suggest that the endocardial endothelial cells may play a role in this process (Fig. 5). The mouse CCS in the embryonic and neonatal hearts have recently been visualized by way of a *LacZ* reporter gene (Rentschler *et al.*, 2001) in the MC4 (*Engrailed-LacZ* transgenic) mouse line (Logan *et al.*, 1993). Expression of the *LacZ* reporter gene in the CCS in this case resulted from the rather serendipitous insertion of the reporter into a particular region of the genome, rather than the control of the *engrailed* promoter. It thus appears the reporter construct came under the control of a CCS-specific promoter. The transgene is expressed from 8.5 days *post coitum* (d*pc*) through to the neonatal period and its expression in the ventricle is exclusive to myocytes adjacent to the endocardium.

To investigate the potential role of endocardial cells in the expression of the *LacZ* reporter of the MC4 embryonic heart, we conducted experiments co-culturing myocytes of the transgenic mouse line with several cell types including endocardial endothelial cells (Fig. 5). The transgene expression remained in cultured heart tubes or ventricular segments dissected from embryos at 8.5 and 9.5 d*pc*. In contrast, the *LacZ* expression was significantly diminished when myocytes were isolated and cultured as a monolayer. The strong expression of the transgene was restored when myocytes were co-cultured with endocardial cells. Myocytes from 13.5 d*pc* and neonatal hearts, however, exhibited low but detectable levels of *LacZ* expression without endocardial cells, suggesting that endocardial cells are not required to maintain *LacZ* expression once initiated. Interestingly, a higher level of *LacZ* expression was induced by co-culturing myocytes with endocardial cells, although there was no change in the number of cells positive for the transgene. Neither MDCK cells nor embryonic fibroblasts stimulated *LacZ* expression amongst MC4 cardiomyocytes. These cell culture experiments demonstrate that endocardial cells have the ability to maintain higher levels of *LacZ* expression in cardiomyocytes. The data also suggest an ongoing need for these cell-cell interactions, at least for some period of time, for the maintenance or differentiation of conduction cells of the heart.

Since endothelial cells are the only cell type common to the endocardium and arteries, along which adjacent myocytes differentiate into Purkinje fibers, it was suspected that endothelial cell derived signal(s) might play a role in recruiting and/or maintaining conduction cells. Recent studies in chick and mouse embryos have provided a new basis for elucidating the molecular mechanism(s) involved in this inductive event in the myocyte lineage.

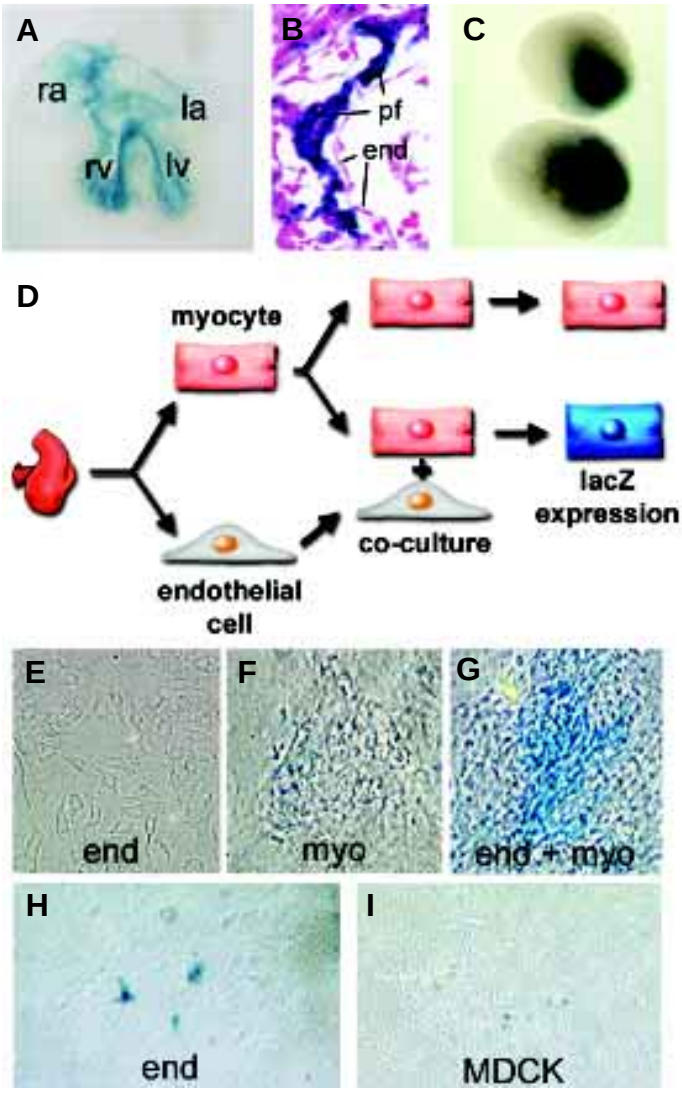

Fig. 5. Endocardial cells promote induction and/or maintenance of a conduction phenotype amongst cardiomyocytes in the murine conduction system. (A) *X-gal staining (blue) of an E13.5 mouse heart from the MC4 transgenic line shows the extent of the developing cardiac conduction system.* **(B)** *A cross section through the ventricular region of an MC4 E13.5 heart after X-gal staining (counter stained with eosin). Note the Purkinje fibers (stained blue) adjacent to endothelial cells.* **(C)** *Ventricular segments of E9.5 hearts cultured* in vitro *for 3 days. Note that LacZ expression persisted in the ventricular segments, whereas in isolated myocyte cultures* **(E)**, *LacZ expression was greatly diminished.* **(D)** *Shows a model for the endothelial induced up-regulation and maintenance of LacZ expression (therefore a propensity for conversion to a conduction cell phenotype) amongst embryonic cardiomyocytes. This model was tested under co-culture conditions; WT (CD1) E9.5 hearts were dissociated and endocardial cells were preferentially isolated and cultured for 2 days to form a monolayer. Cardiomyocytes were harvested from the MC4 transgenic line and added to endocardial cells and cultured for a further 5 days. No LacZ-positive cells were found amongst endocardial cells cultured alone (E), while amongst cardiomyocytes cultured alone, some cells showed low levels of LacZ expression* **(F)**. *When endocardial cells and cardiomyocytes were co-cultured, however, a marked increase in the proportion and levels of LacZ expression amongst myocytes was observed* **(G)**. *To test whether this effect was specific for endocardial cells, the co-culture experiment was used to compare endocardial cells and MDCK cells for the ability to exert an effect on LacZ expression in cardiomyocytes from the MC4 transgenic line. Both co-cultures showed a similar number of X-gal-positive cells, though in the endocardial co-cultures, the myocytes that were X-gal-positive stained more intensely for X-gal* **(H)** *than X-gal-positive myocytes cultured with MDCK cells* **(I)**. *The higher level of myocyte LacZ expression in endocardial co-cultures indicates that another epithelial-like cell line can not recapitulate the effect conferred by endocardial cells. We conclude that a factor secreted by endocardial cells, or direct contact with endocardial cells, encourages the higher levels of X-gal expression in cardiomyocytes. end, endocardial cells; la, left atrium, lv, left ventricle; myo, myocytes; pf, Purkinje fibers; ra, right atrium; rv, right ventricle.*

Endothelial Cell-Derived Factors in Purkinje Fiber Differentiation

Studies in the chick heart have shown that Purkinje fibers differentiate subendocardially and periarterially, but not adjacent to cardiac veins or capillaries (Gourdie *et al.*, 1995; Takebayashi-Suzuki *et al.*, 2000). It has also been suggested that vascular bed-specific phenotypes, including heterogeneity in the endothelial cell population (Gerritsen, 1987; Page *et al.*, 1992; Cines *et al.*, 1998; Rajotte *et al.*, 1998) are dynamically regulated by environmental cues (Aird *et al.*, 1995; Aird *et al.*, 1997; Guillot *et al.*, 1999). Shear stress is one mechanism that regulates the expression and/or secretion of vascular cytokines (McCormick *et al.*, 2001) and is evidently higher in endocardial and arterial endothelial cells than those of veins and capillaries. Recent studies have demonstrated that embryonic myocytes can be induced both *in vivo* (Takebayashi-Suzuki *et al.*, 2000) and *in vitro* (Gourdie *et al.*, 1998) to differentiate into Purkinje fibers by a shear stress-induced cytokine, endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988; Yoshizumi *et al.*, 1989). A conversion from embryonic myocytes to Purkinje fibers was not observed, however, following exposure to other vascular cytokines, such as FGF, PDGF and VEGF.

The production and secretion of biologically active ET ligands requires two steps of post-translation processing from its precursor, preproendothelin (preproET) (Xu *et al.*, 1994). The precursor peptide is first digested into bigET by furin proteases. bigET is then cleaved into mature ET by the membrane-bound, ET-specific metalloprotease, endothelin converting enzyme (ECE-1) (Xu *et al.*, 1994; Emoto and Yanagisawa, 1995). Binding of mature ET to the G protein-coupled receptors, ET_A, ET_B and ET_{B2} trigger ET-signaling (Arai *et al.*, 1990; Sakurai *et al.*, 1990). Recent studies in mouse and chick embryonic hearts have revealed that ET_A and ET_B are expressed by myocytes, while ET_{B2} is expressed in the developing valve leaflets (Clouthier *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000; Kanzawa *et al.*, 2002). In contrast, ECE-1 is predominantly expressed in endocardial and coronary arterial endothelium, and absent from myocytes and endothelial cells of cardiac veins and capillaries (Takebayashi-Suzuki *et al.*, 2000). The distinct expression patterns of ET-receptors and ECE-1 in the heart suggest that localized production of ET-ligand to endocardial and arterial endothelia may specifically induce adjacent myocytes to differentiate into Purkinje fibers. Experimental evidence for this idea has been provided by viral-mediated co-expression of ECE-1 and preproET-1 in the chick embryonic heart (Takebayashi-Suzuki *et al.*, 2000). Exogenous co-expression of ECE-1 and preproET-1 in the embryonic ventricular myocardium resulted in the ectopic and precocious differentiation of Purkinje fibers. These results suggest that induction of conduction cells is localized in the ventricular myocardium by the site-specific cleavage of bigET 1 by ECE-1 (Fig. 6; Takebayashi-Suzuki *et al.*, 2000). The implication of hemodynamic-responsive factors, such as endothelin, in the patterning and development of components of the CCS raises the possibility that environmental factors, like shear stress or pressure, may influence this developmental patterning (Fig. 7).

It has been shown that expression of many Purkinje fiber marker genes can be ectopically induced by activation of ET-signaling both *in vitro* and *in vivo* (Gourdie *et al.*, 1998; Takebayashi-Suzuki *et al.*, 2000; Takebayashi-Suzuki *et al.*, 2001). A recent study, however, has found that a subset of genes which are expressed in *bona fide* Purkinje fibers are not induced by ET, suggesting that an ET-independent pathway may also play a role in regulation of Purkinje fiber specific genes (Takebayashi-Suzuki *et al.*, 2001). A potential involvement of other paracrine interactions such as neuregulin and its receptor tyrosine kinases, erbB2 and erbB4, between the endocardium and the myocardium has also been suggested (Moorman *et al.*, 1998; Rentschler *et al.,* 2000). In the embry-

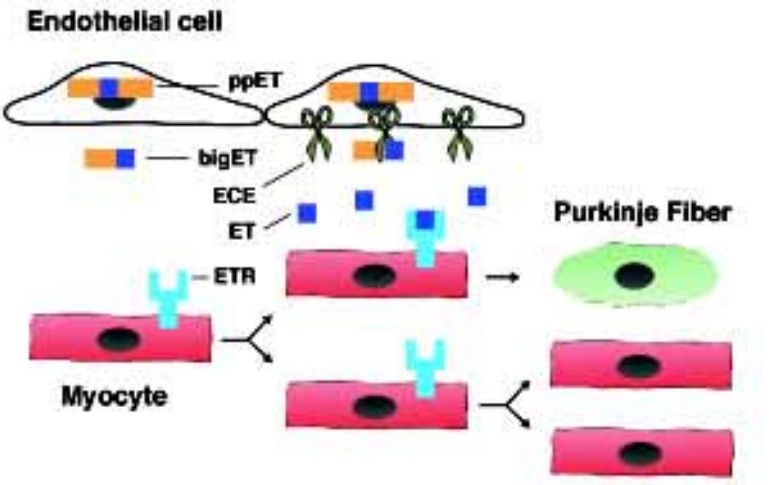

Fig. 6. Model for the conversion of myocytes to Purkinje fibers. *Differential ECE expression allows a spatial regulation of endothelin signaling as only the fully proteolyticaly processed product, endothelin, activates endothelin receptors. In the embryonic chick heart, myocytes expressing endothelin receptors adjacent to ECE-expressing endothelial cells are responsive to this inductive signal and are thus recruited to the cardiac conduction system in a spatially restricted manner. bigET, big endothelin; ppET, preproendothelin; ECE, endothelin converting enzyme; ET, endothelin; ETR, endothelin receptor.*

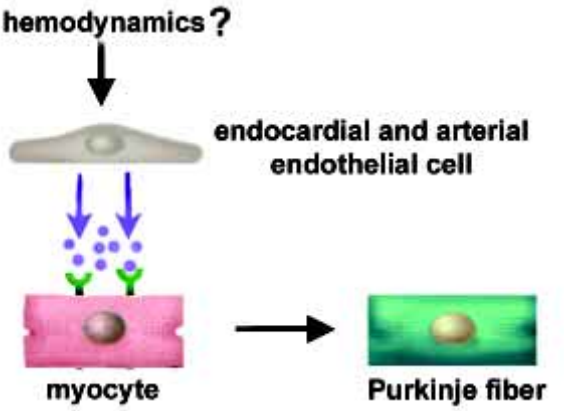

Fig. 7. The role of hemodynamics in conduction system development. *Recruitment of myocytes to the conduction system occurs only in locations adjacent to certain endothelial populations, namely certain endocardial and coronary arterial endothelial cells. What are the cues or factors responsible for this spatial restriction? There is mounting evidence that hemodynamic-responsive factors, such as endothelin that is expressed at higher levels in coronary arterial and a subpopulation of endocardial endothelial cells, play a role in the induction and patterning of aspects of the cardiac conduction system.*

onic mouse heart, neuregulin is expressed by the endocardium, while erbB2 and erbB4 are expressed in the myocardium. A loss-of-function mutation in the *neuregulin* or *erbB* gene in the mouse results in severe cardiac defects, including the loss of trabeculae and cardiac cushion formation (Gassmann *et al.*, 1995; Lee *et al.*, 1995; Meyer and Birchmeier, 1995). These mutant embryos exhibit irregular heartbeats and die. Neuregulin-signaling appears to play multiple roles in the heart, promoting myocyte survival and growth (Zhao *et al.*, 1998) and regulating cardiac cushion formation (Ford *et al.*, 1999). It is also suggested that conduction disturbances in neuregulin signaling-deficient mutants may be associated with insufficient contractile capacity (Moorman *et al.*, 1998). Like ET, neuregulin can induce myocytes to up-regulate some conduction cell markers, such as atrial natriuretic factor (ANF) and skeletal muscle protein (Zhao *et al.*, 1998). Interestingly, neuregulin expression is known to be up-regulated by ET (Zhao *et al.*, 1998). Further studies on the interactions between these signaling cascades will provide an important insight into molecular mechanisms underlying induction, differentiation and maturation of conduction cells.

Regulation of Gene Expression in the Conduction System

The CCS exhibits a complex pattern of gene and protein expression (Schiaffino, 1997; Moorman *et al.*, 1998; Welikson and Mikawa, 2002). Though the exact mechanisms underlying the unique gene expression in cells of the CCS remain unknown, several transcription factors have been detected to be specifically, or preferentially, expressed in conduction cells. The first gene encoding a transcription factor identified in the conduction system was *Msx-2*, a homeobox domain gene homologous to the Drosophila muscle segment homeobox gene (*msh*). This homeobox gene is expressed transiently in progenitors of the proximal conduction system, such as the AV-ring, in developing chick hearts (Chan-Thomas *et al.*, 1993). TBX5, a T-box transcription factor, is also present in the AV-node at higher levels than in the ventricular myocardium of the human embryonic heart (Hatcher *et al.*, 2000). Roles for Msx-2 and TBX5 in formation and patterning of the conduction system remain to be determined.

The gene encoding the cardiac transcription factor, CSX/NKX2.5 (Komuro and Izumo, 1993; Lyons *et al.*, 1995), the mammalian homologue of the *tinman* gene in Drosophila (Bodmer, 1993), is expressed highly in the embryonic and adult conduction system (Takebayashi-Suzuki *et al.*, 2001; Thomas *et al.*, 2001). Mutations in the human *CSX*/*NKX2.5* gene result in an inherited, autosomal dominant disease characterised by atrial septal defects and AV conduction delays (Schott *et al.*, 1998). CSX/NKX2.5 can bind to GATA4, a zinc finger domain protein (Durocher *et al.*, 1997). It has been shown that CSX/NKX2.5 and GATA4 cooperatively activate the expression of ANF (Durocher *et al.*, 1997; Lee *et al.*, 1998), which is expressed at high levels in both the atrium and the ventricular conduction system (Wharton *et al.*, 1988; Hansson and Forsgren, 1993). Indeed, both CSX/NKX2.5 and GATA4 have recently been found to be co-expressed at higher levels in Purkinje fibers of the chick embryo heart (Takebayashi-Suzuki *et al.*, 2001; Thomas *et al.*, 2001). It is still unknown if and how these transcription factors down-regulate ventricular muscle-specific genes in Purkinje fibers or how genes typical of neuronal or skeletal muscle lineages are up-regulated in conduction cells.

While nothing is currently known about mechanisms underlying neuronal cell-type gene expression in conduction cells, the activity of a "muscle-specific" enhancer/promoter of the *desmin* gene in Purkinje fibers has been demonstrated (Li *et al.*, 1993). Desmin, a member of the intermediate filament family (Lazarides, 1982), is expressed in all myogenic cell lineages: skeletal, cardiac and smooth muscle (Hill *et al.*, 1986; Furst *et al.*, 1989; Babai *et al.*, 1990). The proximal 280bp of the 5' regulatory sequence (DES1) of the *desmin* gene has been shown to contain an E-box site for binding of the basic/helix-loop-helix (bHLH) family of muscle determination transcription factors (van de Klundert *et al.*, 1994) and the CArG-box of the serum response element (Treisman *et al.*, 1992). The DES1 fused to a reporter gene, β-gal, has been found to drive expression in skeletal muscle, but not in smooth muscle or the working myocardium of the heart (Li *et al.*, 1993). Importantly, the DES1 enhancer also directs β-gal expression in the Purkinje fibers during embryonic development and after birth, suggesting that a skeletal muscle-specific program could be active in cardiac myocytes that differentiate into specialized conduction cells.

The ability of skeletal muscle-type transcription factors to activate a skeletal muscle program in the embryonic heart has been tested. MyoD ectopically introduced in the developing mouse heart can induce several skeletal muscle proteins in late stage embryonic hearts (Miner *et al.*, 1992). However, ectopic MyoD does not activate Myf-5 or MRF-4 (Miner *et al.*, 1992). The only myogenic counterpart induced in the transgenic mouse by MyoD is myogenin. In the frog heart, MyoD, but not myogenin nor MRF4, has been detected at low levels (Jennings, 1992). Our recent analysis in the embryonic chick heart has also detected *MyoD* expression, but not *Myf5* or *MRF4*, in both *bona fide* and ET-induced Purkinje fibers (Takebayashi-Suzuki *et al.*, 2001). These studies suggest that the transcriptional mechanisms inducing expression of "skeletal muscle" type-genes in conduction cells may be different from those functioning in skeletal muscle. Further investigation will better our understanding of the molecular mechanisms that induce and maintain a unique and complex set of genes that function in the CCS.

Summary

The cardiac conduction system (CCS) is the component of the heart that initiates and maintains a rhythmic heartbeat. As the embryonic heart forms, the CCS must continue to develop and mature in a coordinated manner to ensure that proper pace making potential and distribution of action potential is maintained at all stages. This requires not only the formation of distinct and disparate components of the CCS, but the integration of these components into a functioning whole as the heart matures. Though research in this area of development may have lagged behind other areas of heart development, in recent years there has been much progress in understanding the ontogeny of the CCS and the developmental cues that drive its formation. This is largely due to studies on the avian heart as well as the use of molecular biology approaches. This review gives a perspective on advances in understanding the development of the vertebrate CCS, and reports new data illuminating the mechanism of conduction cell determination and maintenance in the mammalian heart. As much of our knowledge about the development of the CCS has been derived from the chick embryo, one important area facing the field is the relationship and similarities between the structure and development of avian and mammalian conduction systems. Specifically, the morphology of the distal elements of the

mammalian CCS and the manner in which its components are recruited from working cardiomyocytes are areas of research that will, hopefully, receive more attention in the near future. A more general and outstanding question is how the disparate components of all vertebrate conduction systems integrate into a functional entity during embryogenesis. There is mounting evidence linking the patterning and formation of the CCS to instructive cues derived from the cardiac vasculature and, more specifically, to hemodynamic-responsive factors produced by cardiac endothelia. This highlights the need for a greater understanding of the biophysical forces acting on, and created by, the cardiovascular system during embryonic development. A better understanding of these processes will be necessary if therapeutics are to be developed that allow the regeneration of damaged cardiac tissues or the construction of biologically engineered heart tissues.

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