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# Terminal diversification of the myocyte lineage generates Purkinje fibers of the cardiac conduction system

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## SUMMARY

**The rhythmic contraction of the vertebrate heart is dependent on organized propagation of electrical excitation through the cardiac conduction system. Because both muscle- and neuron-specific genes are co-expressed in cells forming myocardial conduction tissues, two origins, myogenic and neural, have been suggested for this specialized tissue. Using replication-defective retroviruses, encoding recombinant β-galactosidase (β-gal), we have analyzed cell lineage for Purkinje fibers (i.e., the peripheral elements of the conduction system) in the chick heart. Functioning myocyte progenitors were virally tagged at embryonic day 3 of incubation (E3). Clonal β-gal$^+$ populations of cells, derived from myocytes infected at E3 were examined at 14 (E14) and 18 (E18) days of embryonic incubation. Here, we report that a subset of clonally related myocytes differentiates into conductile Purkinje fibers, invariably in close spatial association with forming coronary arterial blood vessels. These β-gal$^+$ myogenic clones, containing both working myocytes and Purkinje fibers, did not incorporate cells contributing to tissues of the central conduction system (e.g. atrioventricular ring and bundles). In quantitative analyses, we found that whereas the number of β-gal$^+$ myocyte nuclei per clone more than doubled between E14 and E18, the number of β-gal$^+$ Purkinje fiber nuclei remained constant. These data provide evidence that: (1) Purkinje fibers and working myocytes share a common myogenic precursor in the embryonic tubular heart; (2) differentiation of Purkinje fibers may involve earlier commitment to non-proliferation than that of working myocytes and; (3) the peripheral (i.e., the intramural Purkinje fiber network) and central components of the cardiac conduction system are derived from independent parental cells, and that these two components are linked together to establish the sequentially integrated conduction system of the adult heart. We discuss potential mechanisms for induction of Purkinje fibers from embryonic myocytes in association with coronary vasculogenesis.**

Key words: heart, development, Purkinje fiber, connexin, retrovirus, cell lineage, chicken

## INTRODUCTION

Heart contractility is coordinated by conduction of electrical excitation through the specialized tissues of the cardiac conduction system (reviewed by Viragh and Challice 1973; Lamers et al., 1991). In avian heart, this rhythmic sequence of activation initiates at the sinoatrial node and is conducted as an action potential across the atrial chambers to the atrioventricular (AV) ring, a cellular bundle circumscribing the right and left AV valves. From the AV ring, electrical excitation is rapidly propagated along the AV bundle and its branched limbs, finally spreading into working ventricular muscle via an intramural network of Purkinje fibers, ramifying in precise association with the coronary arterial bed (Davies, 1930; Kim and Yasuda, 1980; Vassal-Adams, 1982; Szabo et al., 1986; Gourdie et al., 1993a; Ying et al., 1993; reviewed by Lamers et al., 1991). The main function of this peripheral network of conduction cells is to rapidly distribute an impulse throughout ventricular muscle, thereby synchronizing contraction of the ventricular chambers of the heart. To date, mechanisms governing the differentiation of these essential cardiac tissues are poorly understood, due largely to uncertainties concerning the origin of cells forming the conduction system.

Histologically, cells of the conduction system are characterized by reduced numbers of myofibrils and higher accumulations of glycogen than working myocytes (Viragh and Challice, 1973; Kim and Yasuda, 1980; Szabo et al., 1986; Ying et al., 1993). Recent evidence suggests that gap junctional electrical connections between conduction cells in birds and mammals have characteristic molecular properties (van Kempen et al., 1991; Gourdie et al., 1992; 1993a,b; Bastide et al., 1993; Kanter et al., 1993; Gros et al., 1994). Purkinje fibers in avian heart preferentially express high levels of connexin42, a gap junctional protein absent, or present at lower abundance,

in working myocytes (Gourdie et al., 1993a). In addition to distinctive distributions of connexins, conduction cells also express genes more usually associated with neural and skeletal tissues. These genes include neurofilament antigens (Gorza and Vitadello, 1989; Vitadello et al., 1990), the neural crest-associated markers, HNK-1 (Gorza et al., 1988; Ikeda et al., 1990; Nakagawa et al., 1993) and EAP-300 (McCabe et al., 1992), acetylcholinesterase (Lamers et al., 1987; 1990) and a slow skeletal muscle isoform of the myosin heavy chain (Gonzalez-Sanchez and Bader, 1985). Although these markers distinguish conductive cells from working myocytes, the co-expresssion of neural and muscle genes has confounded our understanding of the origin of the cardiac conduction system. Consequently, two possible origins, myogenic (Patten, 1956) and neurogenic (Gorza et al., 1988; Vitadello et al., 1990) have been proposed for conduction tissues.

In embryonic human heart, a ring-like cluster of cells near the AV junction has been mapped as an initiation site for development of the cardiac conduction system (Wessels et al., 1992). Using a riboprobe to a homeobox gene *Msx-2*, Chan-Thomas et al., (1993) demonstrated the expression of this gene in parts of the 'primary ring' of the chick heart at embryonic day 2 (E2 – HH stage 15+; Hamburger and Hamilton, 1951). *Msx-2* expression later coincided with morphologically distinguishable elements of the central conduction system, including the putative AV ring, but expression was not associated with the peripheral Purkinje fiber network (Chan-Thomas et al., 1993). Overt initiation in differentiation of Purkinje fibers becomes apparent at E10 (Gourdie, 1993a). Based on this central to peripheral sequence of expression, the 'primary ring' has been suggested as containing the precursor cells for the central and peripheral conduction system (Lamers et al., 1991; Wessels, 1992; Chan-Thomas et al., 1993).

While morphological approaches have provided important insights into the development of specialized myocardial tissues, these techniques are not adequate to address directly questions concerning the cellular progenitors of working and conductive myocardium or indeed the lineage relationships between the central and peripheral components of the conduction system. Here, we address these issues using retroviral cell marking methods established for cell lineage studies during embryogenesis (Sanes et al., 1986; Turner and Cepko, 1987). In the chicken embryo, mesodermal cells lateral to Hensen's node (reviewed by Garcia-Martinez and Schoenwolf, 1993) become committed to cardiac lineage at E1 (HH stage 4; Hamburger and Hamilton, 1951) and complete their differentiation by HH stage 15 (E2; Gonzalez-Sanchez and Bader, 1985). Cardiac neural crest cells initiate migration from the embryonic chick hindbrain at E2-3 and enter the tubular heart at E4, forming the neuronal and glial elements of cardiac ganglia (Kirby and Stewart, 1983; Kirby et al., 1993; reviewed by Kirby, 1988). If, on the basis of this sequence, conduction tissues are of neurogenic origin, then its precursors must be absent from the embryonic heart before E3. If, on the other hand, conduction tissues are of myogenic origin, then its precursor cells must be present in the heart at E3. In our earlier papers (Mikawa et al., 1991, 1992a,b), we have shown that single myocytes can be tagged in situ at E3 using replication-defective viral constructs (Dougherty and Temin, 1986) encoding nuclear- or cytoplasmic-directed β-galactosidase. By analysing the fate of infected cells, we have found that single myocardial progenitor cells, generate cone-shaped colonies of daughter cells traversing the myocardial wall (Mikawa et al., 1992a,b).

In the present study we have analysed clonal populations of cells within discrete β-gal$^+$ colonies in avian heart, using immunohistochemical markers for conduction tissues and working myocytes. Our data show that those β-gal$^+$ clonal populations occurring adjacent to coronary arterial vessels contain both Purkinje fibers and working myocytes. This provides the first direct evidence for differentiation of conductive Purkinje fibers and working contractile myocytes in the same myogenic clonal domain. In no case were Purkinje fibers and central conduction system cells co-localised in the same clonal domain, suggesting different parental lineages for the cells comprising central and peripheral components of the cardiac conduction system. We propose a model for differentiation of the peripheral network of intramural Purkinje fibers that involves: (1) induction of embryonic myocytes to form Purkinje fibers along the ramifications of the coronary arterial bed, and (2) commitment of these specialized conductive myocytes to non-proliferation. The origin of the central conduction system of the heart (i.e., AV ring, AV bundle, and proximal components of the bundle branches) remains to be established.

## MATERIALS AND METHODS

### Retroviral vectors and in ovo infection

The CXL and the SNTZ viral β-galactosidase (β-gal) vectors used in this study are replication-defective constructs derived from a spleen necrosis virus (Dougherty and Temin, 1986). Construction and characterization of the CXL and SNTZ viruses, including proof of helper-virus free stocks and evidence for clonality of discrete β-gal$^+$ patches in developing avian hearts infected with low numbers of virions, has been presented elsewhere (Mikawa et al., 1991, 1992a,b; Mikawa and Fischman, 1992). The two constructs show discrete patterns of localization, with β-gal activity being directed to cell cytoplasm and nuclei for CXL and SNTZ viruses respectively (Mikawa et al., 1992b). Before microinjection, viral stocks were concentrated to $>10^7$ virions/ml by ultracentrifugation of culture supernatants from packaging cells. In ovo pressure microinjection of small volumes of viral suspension (<5 nl), containing polybrene (100 μg/ml), was done onto the right ventricular wall of functional hearts at E3, when myocardial cell migration becomes restricted (Mikawa et al., 1992b). Generally, pure CXL and SNTZ viral suspensions were microinjected separately. However, in a series of control experiments examining clonality of β-gal$^+$ patches, eight hearts were co-injected with suspensions containing a mixture of SNTZ and CXL viruses. Viral targeting of cardiac neural crest, was done by microinjection of CXL virus into the neural tube between somites 1-3 of E2 chick embryos as described by Epstein et al. (1994). All injected eggs were subsequently resealed with Parafilm, returned to a humidified incubator at 37.5°C and were allowed to develop to E14 or E18.

### Histology

Hearts from embryos younger than E18 were routinely fixed with 2% paraformaldehyde (in PBS), reacted with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), inspected as whole mounts and embedded in paraffin as detailed previously (Mikawa et al., 1991). For E18 embryos, the same procedure was followed except that dissected hearts were fixed by immersion in 70% ethanol, rather than paraformaldehyde. This was done to facilitate immunolabeling with ALD58 monoclonal antibodies (see subsequent section). Hearts were oriented for cardiac frontal sectioning across all four chambers, serially sectioned at 10 μm and mounted on poly-l-lysine coated glass

slides. A sequence of eosin-stained reference slides was prepared using every 10th section in the sequence through the heart. The remainder of the sections were used for subsequent immunohistochemical characterization of individual histological features.

### Immunofluorescent labeling and microscopy

The eosin reference slides from each heart were searched systematically for discrete perivascular clones. Serial sections, adjacent to the reference sections containing perivascular clones, were processed for immunolabeling by dewaxing, rehydrating, and soaking in blocking solution, as detailed in earlier studies (Gourdie et al., 1991, 1993b). Overnight incubations in the following primary antibodies were subsequently done using; chick ALD58 mouse monoclonal antibodies (Gonzalez-Sanchez and Bader, 1985), chick EAP-300 rat monoclonal antibodies (McCabe et al., 1992), chick connexin42 rabbit polyclonal antibodies (Kanter et al., 1993; Gourdie et al., 1993a), chick atrial and ventricular myosin heavy chain mouse monoclonal antibodies (Sanders et al., 1984; de Groot et al., 1987), and chick vascular smooth muscle caldesmon rabbit polyclonal antibodies (Reckless et al., 1993). All antibodies were used at a 1:10 dilution in PBS, except for those against connexin42, which was diluted at 1:100 in PBS. All incubations were preceded by trypsinization (10 minutes in 0.1% trypsin, 0.1 mM $CaCl_2$, 20 mM Tris buffer), except for those with EAP-300 antibodies. Following incubation in primary antibody, tissue sections were washed and processed for secondary labeling with biotinylated antibody appropriate for the primary antibody used and tertiary streptavidin-fluorescein or streptavidin-Texas-red labeling as described previously (Gourdie et al., 1993b). Double immunofluorescent labeling of connexin42 rabbit antibodies and ALD58 mouse antibodies was carried out using procedures similar to those for single labeling except that the initial antibody incubation was done using mixtures of the two primary antibody probes. Subsequent biotinylated secondary antibody and streptavidin-fluorescein labeling was done in sequence, first immunolocalizing connexin42 with an anti-rabbit-biotin and streptavidin-Texas red complex, then ALD58, with a anti-mouse-biotin and streptavidin-fluorescein complex. Imaging of single and double fluorescent labelings were done using MRC Biorad 500 (Biorad Microscience, Hemel Hempsted, UK) and Leica TCS 4D (Leica UK, Milton Keynes, UK) confocal scanning laser microscopes. Following imaging of immunolabeled specimens, the coverslip was floated free and the tissue was re-stained with eosin and processed for permanent mounting in DPX. All bright-field transmitted light photomicroscopy was done on an EDGE microscope; a specialized instrument that combines high resolution optics with imaging through large specimen depths (EDGE Scientific, Culver City California, USA).

### Quantitative analyses

A quantitative analysis was carried out on seven SNTZ-infected hearts from E14 (four hearts) and E18 (three hearts) embryos. From our earlier work, these two time points fell at significant phases during the differentiation of the terminal Purkinje fiber network in avian heart (Gourdie et al., 1993a). From E14 onwards, histological identification of perivascular Purkinje fibers is straightforward owing to the location and distinctive morphology of these cells. The hearts used were selected to contain low numbers (i.e., <20 per heart) of discrete, well-separated β-gal $^+$ clones within ventricular cardiac muscle tissues. The hearts were serially sectioned, and all sections were then cover-slip mounted. By sequential inspection of the series through each heart using the EDGE microscope, β-gal$^+$ clones were identified and the total number of blue, β-gal$^+$ nuclei was counted in each of these clones. If these clones occurred adjacent to, or were traversed by blood vessels, and if this clone contained perivascular Purkinje fibers, this was recorded and separate counts of the number of β-gal$^+$ nuclei within working myocytes and Purkinje fibers were made. There was no evidence for β-gal$^+$ nuclei in vascular or other non-myocardial tissues in the seven hearts. A total of 50 clones and 21,250 nuclei were counted for the study.

## RESULTS

### Cells of myogenic origin are found within the peripheral network of Purkinje fibers

To examine whether hearts contain precursor populations of Purkinje fibers at E3, we introduced viruses encoding either cytoplasmic- (CXL virus) or nuclear- (SNTZ virus) directed β-gal into the tubular hearts of developing HH stage 17 embryos. The fate of each tagged myocyte was analysed on E14 or E18 by reacting hearts with the chromogenic β-gal substrate, X-

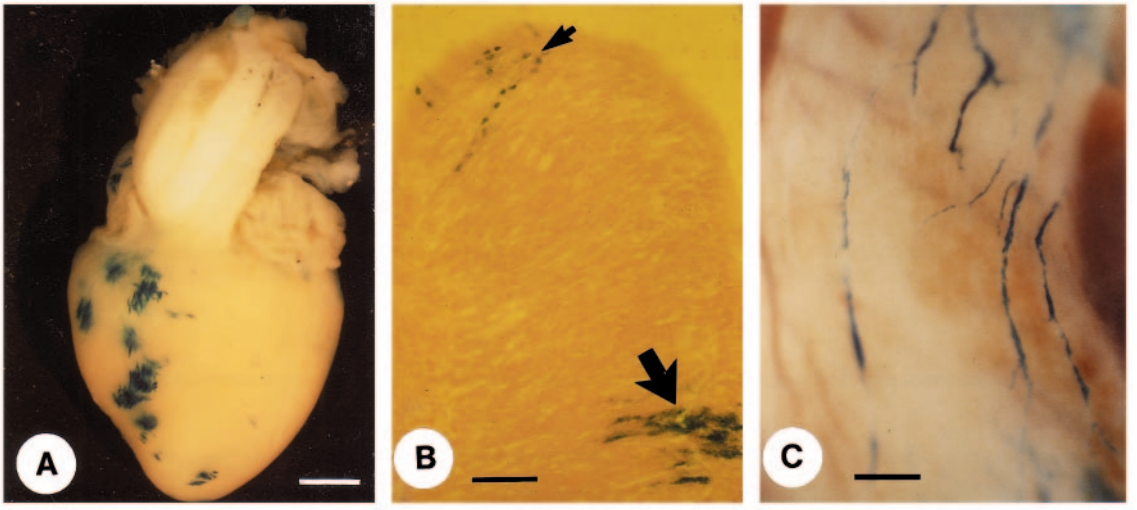


**Fig. 1.** (A) An E14 embryonic heart, reacted with X-gal shows a relatively low number of discrete β-gal$^+$ sectors located mainly in the right ventricle. This heart was taken from an embryo, targeted for CXL viral infection of the tubular heart at E3. (B) A low magnification survey of a SNTZ 'nuclear-tagging' (small arrow) clone and a nearby CXL 'cytoplasmic' clone (large arrow) in avian ventricular myocardium at E8. These two closely adjacent viral colonies, when traced through serial histosections, showed no evidence of intermingling. (C) Detail of the epicardium of an E8.5 embryonic heart showing β-gal$^+$ neuronal cells. This heart was from an embryo, targeted for cardiac neural crest infection by CXL at E2. No evidence for β-gal$^+$ myocardial cells was found in cardiac neural crest-targeted embryos. Scale bars, (A) 500 μm; (B,C), 100 μm.

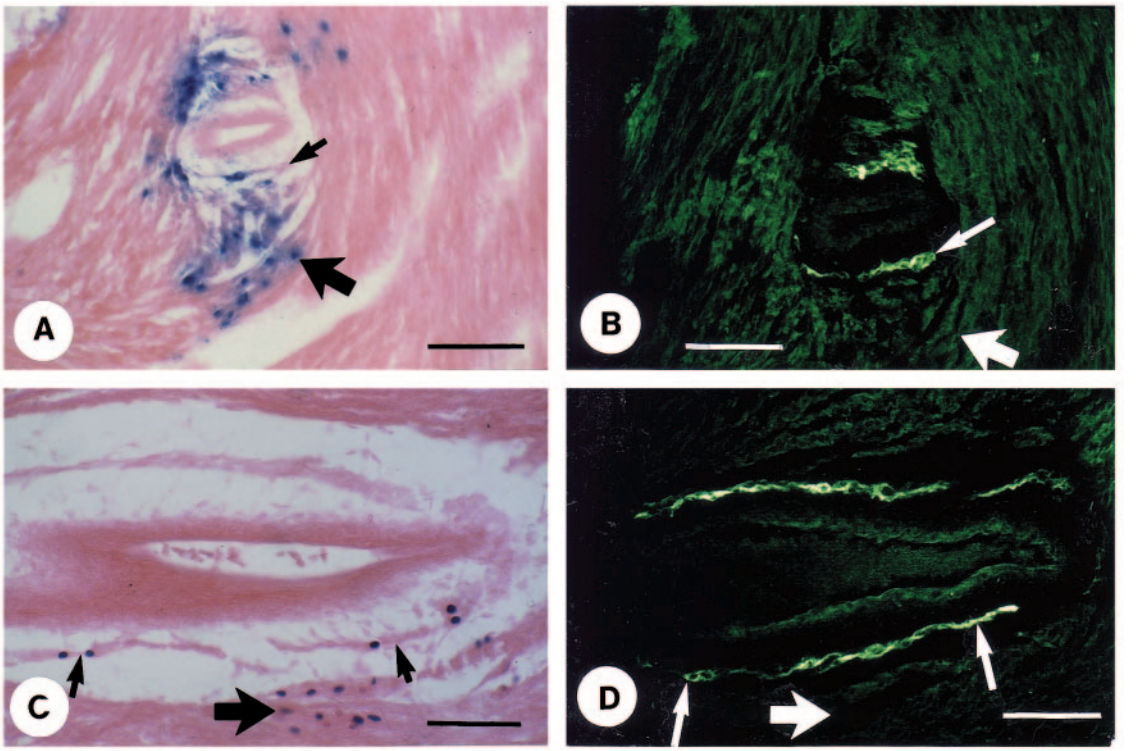


**Fig. 2.** (A) An eosin-stained section, showing a single SNTZ colony (arrowed) next to a blood vessel in the apical ventricular myocardium of an E18 heart. β-gal- abeled nuclei are observed in working myocytes (large arrow) and in sister perivascular Purkinje fibers (small arrow). (B) The same perivascular Purkinje fibers shown in A are immunolabeled with ALD58 (small arrow), an immunological marker of conduction tissues in avian heart. SNTZ-labeled working myocardial cells are unlabeled (location indicated by large arrow). (C) Eosin-stained section through a second clone in the ventricle of another E18 heart, showing distinctive strands of perivascular Purkinje fibres (small arrows), adjacent to a longitudinally sectioned blood vessel. The large arrow indicates the location of sister β-gal$^+$ working myocardial cells. (D) Purkinje fibers at this clone are immunolabeled with ALD58 antibodies (small arrows). Working myocardial cells are unlabeled (large arrow). Scale bars, 100 μm.

Gal. As described in our previous work, daughter cells of individual, infected myocytes formed clustered sectors of β-gal$^+$ cells in myocardial wall (Fig. 1A). Clonality within such β-gal$^+$ domains, derived from singly infected myocytes, has been examined and described previously (Mikawa et al., 1992a,b). In the present study, E3 hearts were also co-infected with CXL and SNTZ viruses at relatively low viral titers such that less than 20 clonal domains would be generated in each infected heart. One heart, bearing 3 cytoplasmically marked and 12 nuclear-tagged clones was completely serially sectioned. Careful inspection of each section in this sequence, revealed no intermingled clones, though on one occasion cytoplasmically marked and nuclear-tagged populations of cells were observed in close proximity to one another (Fig. 1B). As a control, we infected the cardiac neural crest of a series of embryos with CXL-virus and did obtain hearts bearing β-gal$^+$ parasympathetic postganglionic cardiac neurons (Fig. 1C).

Initial identifications of Purkinje fibers within myogenic clones were done in both E14 and E18 hearts. In avian heart, Purkinje fibers form perivascularly, closely adjacent to coronary vessels (Davies, 1930; Kim and Yasuda, 1980; Vassal-Adams 1982; Gourdie et al., 1993a; Ying et al., 1993; reviewed by Lamers et al., 1991). We selected 50 β-gal$^+$ ventricular clones, exhibiting no proximity with other β-gal$^+$ sectors, from seven embryos. We found that between one third and one half of these discrete clones were located adjacent to coronary vessels (see Table 1. Hereafter, such clones will be referred to as perivascular clones). Within perivascular clones, we always found β-gal$^+$ cells in the fibrous region surrounding blood vessels (Figs 2 and 3). These perivascular β-gal$^+$ cells were distinguishable as Purkinje fibers from sister β-gal$^+$ working myocytes within the same clonal domain, owing to their cellular shape and location. A total of 9 discrete clones were observed in the interventricular septa of the seven hearts, including a small perivascular β-gal$^+$ colony (66 β-gal$^+$ nuclei, 13 of which were perivascular cells) occurring closely adjacent to, but not encompassing the AV bundle (Fig. 4, large arrows). Careful inspection of sequential sections through the central conduction systems of the seven hearts revealed that no β-gal$^+$ cells were identifiable within central conduction tissues (e.g. AV ring, bundle and proximal bundle branches). Given the high frequency of β-gal$^+$ labeling in perivascular Purkinje fibers and the absence of this marker from central conduction cells, it seems unlikely that these two tissues share closely related parental lineages. There was no evidence for β-gal$^+$ cells in the Purkinje fiber network or other myocardial tissues of hearts from cardiac neural crest-targeted embryos.

## Perivascular cells of a myogenic origin are Purkinje fibers

The phenotype(s) of perivascular groups of cells, including Purkinje fiber candidates, were examined by immunohisto-

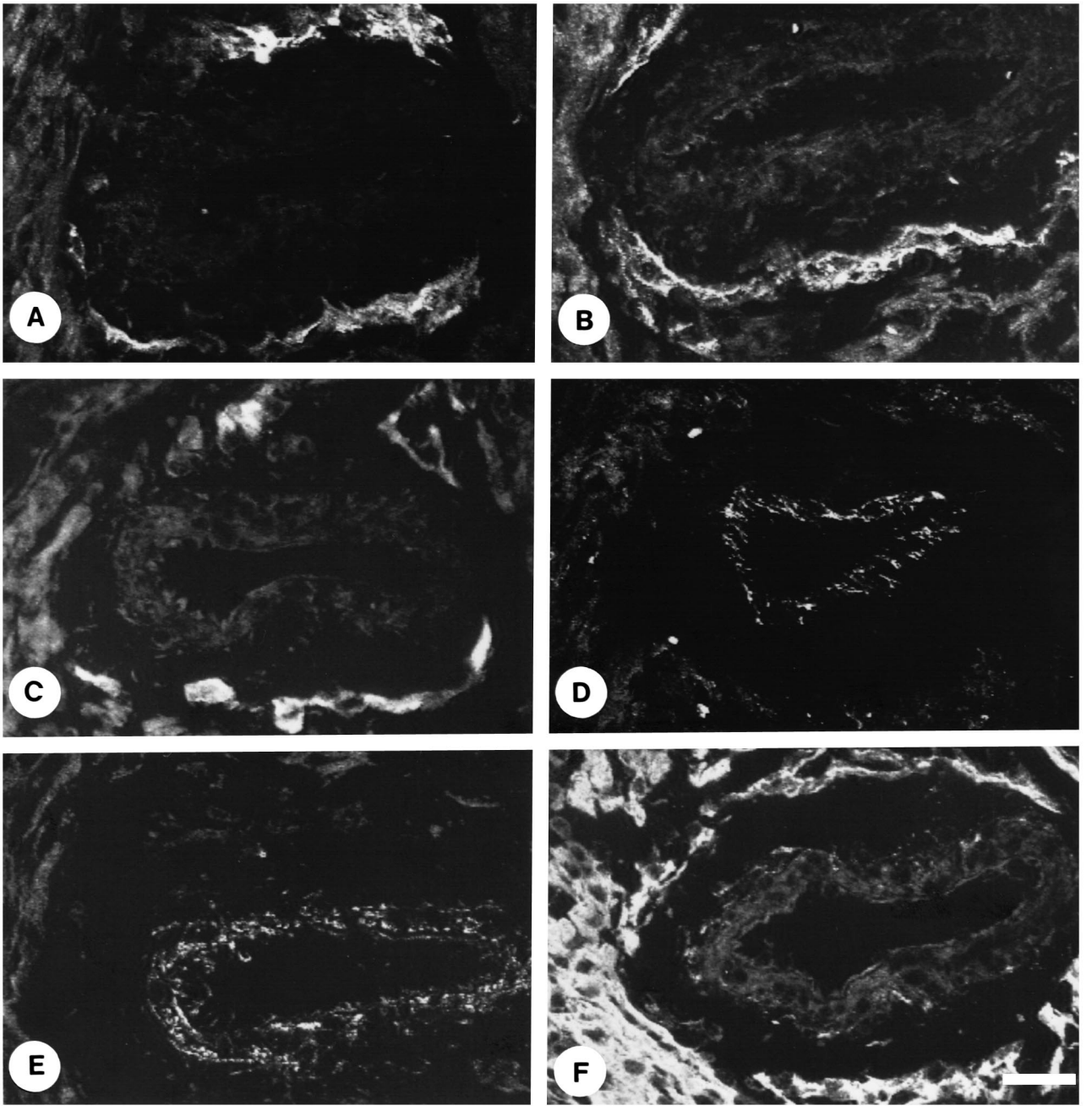


**Fig. 3.** Molecular phenotyping of a perivascular β-gal$^+$ clone. (A) The ALD58 immunolabeled perivascular region shown in Fig. 2B is illustrated at higher magnification. The subsequent five images in the plate represent serial sections through this perivascular region following immunolabeling for different tissue markers. The identity of ALD58$^+$ cells as Purkinje fibers is further confirmed using three other markers of avian conduction tissues. (B) Atrial myosin heavy chain, (C) EAP-300, and (D) connexin42. (E) Caldesmon antibodies are immunolocalized in the vascular smooth muscle of the arteriole. (F) Ventricular myosin heavy chain immunolabeling of myocardial tissues surrounding the arteriole. Magnification is the same for all panels: scale bar, 25 μm.

chemistry. Since cytoplasmic precipitation of blue chromogen following X-gal reaction prevented imaging of the immunofluorescent signal in cells expressing cytoplasmic β-gal, we analysed myogenic clones expressing nuclear-directed β-gal. As demonstrated by Galileo and co-workers (1990), focal precipitation of chromogen near the nucleus enables immunolabeling of structures in the plasma membranes and cytoplasms of virally tagged cells (Figs 2 and 3). Fig. 2A shows a survey of a periarterial SNTZ colony of nuclear-tagged cells in the ventricle of an E18 embryonic chick heart. Light microscopic inspection of serial sections indicated that this group of β-gal$^+$ cells was one of 10 independent ventricular colonies occurring in this heart. The clone illustrated, extended through 14 10-μm sections and contained a total of 536 β-gal$^+$ nuclei. Of this total, 21 nuclei (approx. 5%) could be identified at higher magnifications to be within distinctive perivascular strands (Fig. 2A, small arrow). In Fig. 2B, the same perivascular cells shown in 2A are immunolabeled with ALD58 antibodies, a marker of

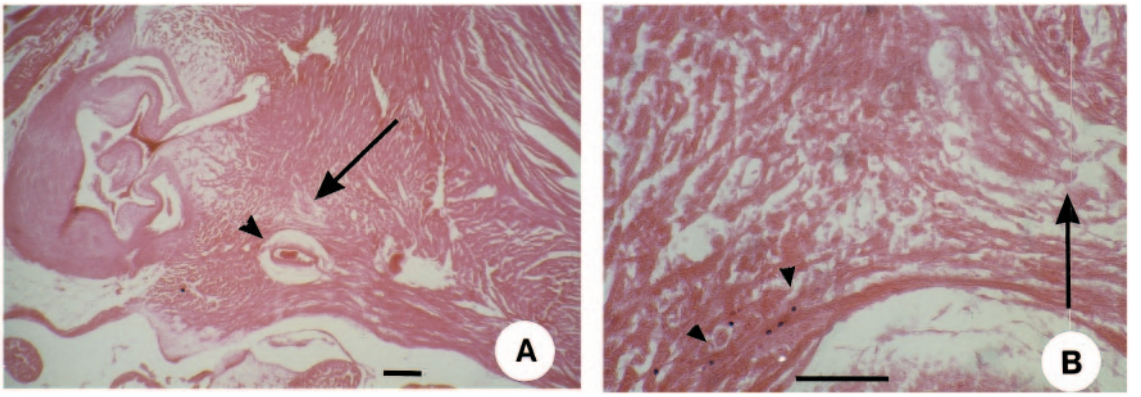


**Fig. 4.** (A) A low power survey of a section through the interventricular septum of an E18 chick heart in the plane of the AV bundle (large arrow). The small arrowhead indicates the location of a small perivascular β-gal$^+$ sector. (B) A higher magnification showing perivascular β-gal$^+$ nuclei (small arrowheads) and nearby AV bundle tissue (large arrow). No labeled nuclei are located in the central conduction cells of the AV bundle. Scale bars, (A) 150 μm; (B) 50 μm.

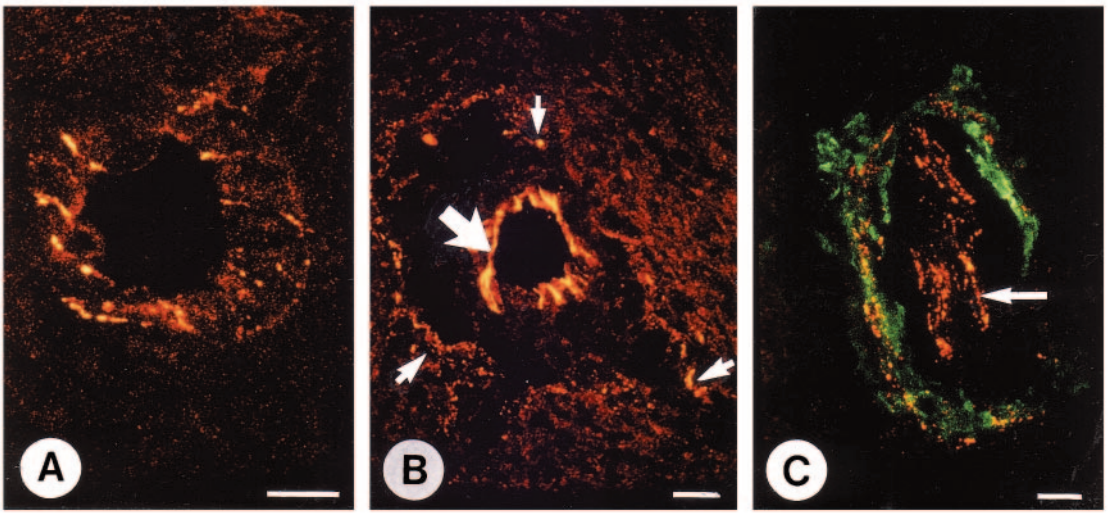


**Fig. 5.** Connexin42 immunolocalization in vascular endothelium and embryonic myocardium during the differentiation of perivascular Purkinje fibers. (A) Connexin42 immunolabeling is first observed at the lumen of early coronary vascular structures in the ventricle of E9 chick embryos. (B) A day later, at E10, strong connexin42 signal is still evident in vascular endothelium (large arrow), but prominent immunolabeling of putative conductive cells, surrounding early blood vessels, now becomes evident (small arrows). Faint and substantially less abundant connexin42 signal is seen associated with the working myocardium. (C) Punctate connexin42 immunolabeling (red) is co-localized with ALD58 immunolabeling (green) in perivascular ventricular myocardium in an E20 embryo. This double labeling confirms the identity of the highly connexin42$^+$ cells adjacent to the blood vessel as Purkinje fibers. The vascular endothelium is still strongly immunopositive for connexin42 (arrowed). Scale bar, (A) 10 μm; (B,C) 5 μm.

avian conduction tissues, confirming the identity of the β-gal$^+$ strand as Purkinje fibers. Adjacent β-gal$^+$ working myocardial cells (large arrow) were unlabeled for ALD58. A smaller perivascular β-gal$^+$ clone (130 working myocardial and 20 Purkinje fiber nuclei), in another E18 chick heart, is shown in Fig. 2C. This colony was one of 12 independent ventricular clones occurring in this heart. The β-gal$^+$ perivascular strands included within the colony were confirmed in sister sections as Purkinje fibers by immunolabeling with EAP-300 and ALD58 antibodies (Fig. 2D). Fig. 3A is a higher magnification detail of the ALD58-immunolabeled region arrowed in Fig. 2A. In subsequent sections through this region, the conductive phenotype of ALD58$^+$ cells was further corroborated using three other markers of avian ventricular conduction tissues: atrial myosin heavy chain (Fig. 3B), EAP-300 (Fig. 3C), and connexin42 (Fig. 3D). To confirm the vascular nature of the luminal structure illustrated, Fig. 3E shows immunolabeling of vascular smooth muscle with antibodies against caldesmon. The tissue surrounding the artery was strongly labeled with antibodies against ventricular myosin heavy chain, a well-characterized marker of ventricular myocardial cells (Fig. 3F).

From these results we conclude that the perivascular groups of β-gal$^+$ cells are Purkinje fibers, which share common myogenic parental cells with adjacent working myocytes.

## Quantitative analysis of ventricular myocardial clones

In order to characterize the events associated with the commitment of embryonic myocytes to the conductive or working

**Table 1. Quantitation of perivascular SNTZ clones in ventricular myocardium at E14 and E18**

| Embryonic day of incubation | E14 | E18 |
|---|---|---|
| β-gal$^+$ clones | 21 | 29 |
| % Periarterial | 32 (2.4) | 48 (2.3) |
| Working myocyte nuclei per clone | 340 (128) | 798 (132) |
| Purkinje fiber nuclei per clone | 12 (4.2) | 13 (2.6) |

The quantitation was based on a sample of seven hearts, four at E14 and three at E18. A total of 50 β-gal$^+$ clones were surveyed and 21,250 β-gal$^+$ nuclei counted in this study. Standard errors (s.e.m.) are given in brackets.

myocardial phenotype, a quantitative study was carried out on β-gal$^+$ clones in E14 and E18 hearts. These two time points were chosen as our earlier work had indicated that the period between E10 and E20 was the key phase in the initial differentiation of the peripheral conduction system of the chick heart (Gourdie et al., 1993a and Fig. 5). The quantitative data is summarized in Table 1. Perivascular clones always contained Purkinje fibers, in addition to nuclear-tagged working myocardial cells. Intramural Purkinje fibers were never found in association with coronary veins or vascular sinusoids. Between E14 and E18, increases in the complexity of vascularization was matched by significant ($P$<0.001) increases in the proportion of perivascular clones containing conductive cells, suggesting a relationship between Purkinje fiber differentiation and formation of the coronary vascular bed. The average number of β-gal$^+$ nuclei within perivascular clones more than doubled between E14 and E18 consistent with cell division underpinning growth in the myocardium over this period. However, the numbers of β-gal$^+$ nuclei associated with Purkinje fibers in these colonies remained constant ($P$>0.05) between E14 and E18. This result (Fig. 6) suggests conversion to the perivascular conductive phenotype involves a commitment to non-proliferation and mitotic quiescence near or preceding day 14 of embryonic incubation.

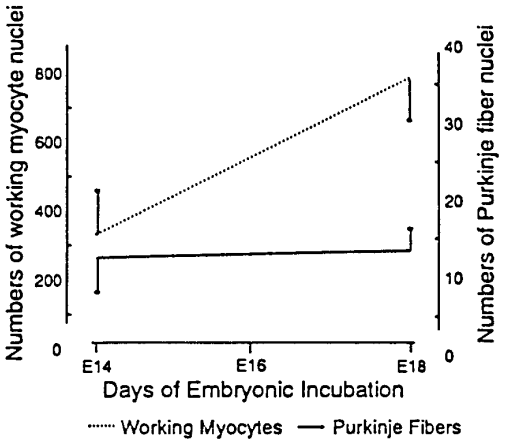


**Fig. 6.** Changes in the number of β-gal$^+$ labeled working myocyte and Purkinje fiber nuclei between 14 (E14) and 18 (E18) embryonic days of incubation in perivascular SNTZ clones. Whilst the numbers of working myocyte β-gal$^+$ nuclei per clone more than double between E14 and E18 (dotted line and left hand y-axis), the numbers of Purkinje fiber β-gal$^+$ nuclei per clone remained constant (solid line and right hand y-axis). Error bars indicate the standard error of the mean (s.e.m) for each time point.

## DISCUSSION

In this study, we present evidence that Purkinje fibers differentiate from contractile myocardial cells present in the embryonic tubular heart. As such, this provides the first direct demonstration that cells of the cardiac conduction system share common myogenic progenitors with working cardiac myocytes. We find no evidence for central conduction tissues providing cellular progenitors for the peripheral Purkinje fiber network. Our data therefore, does not support the concept of a 'primary ring' of early conduction cells giving rise to the entire cardiac conduction system.

The diversification of myocyte phenotype within clonally derived populations provides an important opportunity to characterize the mechanisms involved in differentiation of cardiac conduction tissues. Purkinje fibers in birds have been identified as a network juxtaposing coronary vessels (Davies, 1930; Kim and Yasuda, 1980; Vassal-Adams, 1982; Szabo et al., 1986; Gourdie et al., 1993a; Ying et al., 1993; reviewed by Lamers et al., 1991). The present study demonstrates that parental myocytes, already contractile when virally tagged, generate conductive Purkinje fibers as well as working myocytes within their daughter populations. In earlier work, we have shown low migratory activities of myocytes within myocardial wall during heart formation (Mikawa et al., 1992b). Taken together, we would speculate that contractile myocytes can be recruited to form conductive myocytes when exposed to forming coronary vessels. In support of this, it has been demonstrated that overt coronary vasculogenesis in the avian heart begins at E6 (Rychter and Ostradal, 1971; Mikawa and Fischman, 1992). With ensuing development, a closed vascular system is functional at around E14, and this closed system continues to branch and grow during subsequent maturation of the myocardium into adulthood. Our quantitative study indicates that between E14 and E18, the proportion of Purkinje fiber-containing clones adjacent to arterial vessels increases, suggesting that the peripheral conduction network is laid down in close temporal and spatial association with development of the coronary vascular bed.

Based on morphological evidence, a number of studies have suggested that the initiation site for conduction system development in vertebrates occurs at a ring-like zone of tissue circumscribing the early interventricular or AV junction (Wessels, et al., 1992; Lamers et al., 1991; Chan-Thomas et al., 1993). While such regions of early specialization probably contain precursors of the central conduction system, it seems unlikely that they also supply the progenitors for an extensive network of peripheral conduction tissues. In the present study, perivascular clones containing Purkinje fibers were common, accounting for between one third and one half of all β-gal$^+$ sectors quantified. In all colonies examined, there was no evidence for co-localisation of peripheral and central conductive cells in the same domain. Indeed, systematic inspection of serial sections through hearts containing perivascular clones never revealed β-gal$^+$ cells in central conduction tissues (e.g., AV ring, AV bundle, and proximal bundle branches). In view of the location and known developmental timing of central conduction system differentiation, these results do not exclude a myogenic origin for central conduction tissues. Indeed we would speculate that as with perivascular Purkinje fibers, central conduction cells

have progenitors in common with working myocytes, but such tissues may already be lineage committed and withdrawn from proliferation by E3, as indicated by DNA-labeling studies (Thompson et al., 1990; 1994). The absence of coronary arteries in the period before E3 and the strong association between vascularization and peripheral Purkinje fiber differentiation do suggest differences in the mechanisms of determination for central and peripheral conduction tissues.

A separate genesis for central and peripheral components of the conduction system poses the question of how they might come to be linked together into the sequentially integrated AV conduction system of the adult heart. It is clear that central conduction functions such as pacemaker activity and delayed propagation at the AV junction are present in the embryonic heart (de Jong et al., 1992). However, the processes that would enable scattered populations of Purkinje fibers to link together and establish continuity with the central conduction system remain to be characterized.

In Fig. 7 we propose a model sequence for the differentiation of perivascular Purkinje fibers. The main elements of this sequence are (i) vasculogenesis, i.e., development of the coronary vascular bed, (ii) conversion of embryonic contractile myocytes adjacent to developing blood vessels into perivascular conductive cells and (iii) withdrawal of perivascular conductive cells from proliferation. The constituent cells of the coronary vasculature do not arise in situ, but migrate into the tubular heart from mesenchymal extracardiac sources (Hiruma and Hirakow, 1989; Mikawa and Fischman, 1992; Poelmann et al., 1993; Waldo et al., 1994). At E3, these migratory coronary precursors can be recognised external to the primitive heart, as grape-like clusters of cells on the dorsal mesocardium. Based on retroviral cell lineage studies, it has been proposed that following the inward migration of vasculogenic cells, the first manifest step in blood vessel development is the formation of discontinuous endothelial channels (Mikawa and Fischman, 1992). Following this, smooth muscle cells migrate to, and ensheath these early luminal structures. We would extend this to suggest that during this process, embryonic myocardium, immediately local to the forming vessel, commits to the conductive lineage. Exactly which cell type(s) is responsible for the potential inductive signal is uncertain. Coronary vascular and perivascular tissues have heterogeneous developmental origins. In addition to mesenchymally derived vascular tissues, neurons making up the cardiac plexus originate from an ectomesenchymal source, the cardiac neural crest (d'Amico-Martel and Noden, 1983; Kirby and Stewart, 1983; Kirby et al., 1993; reviewed by Kirby, 1988). It is likely that developmentally significant interactions occur between neurogenic and vasculogenic tissues in the developing heart. Ablation of the cardiac neural crest leads to disruption of the normal spatial deployment of coronary arteries (Hood and Rosenquist, 1992; Gittenberger de Groot et al., 1993). Localisation of neural crest derivatives adjacent to blood vessels is important in the survival of definitive branches of the coronary arterial system (Waldo et al., 1994). The present study indicates that a direct contribution by migratory neurogenic or vasculogenic cells to the peripheral cardiac conduction system is unlikely, however, an indirect influence by such migratory cell populations in the induction, withdrawl of Purkinje fiber precursors from proliferation and spatial deployment of the peripheral conduction system cannot be excluded.

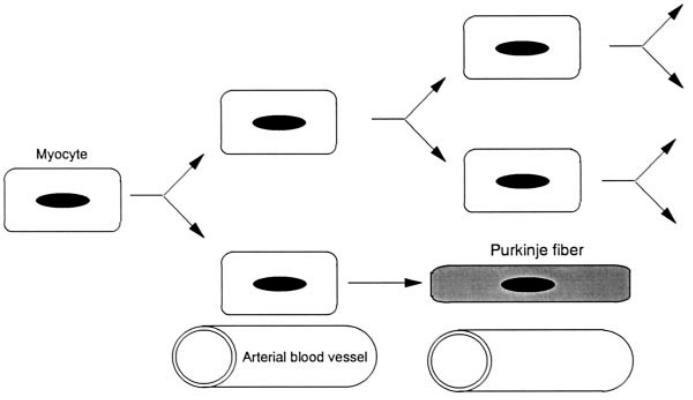


**Fig. 7.** A model for differentiation of perivascular Purkinje fibers. Vasculogenetic formation of coronary blood vessels (see Mikawa and Fischman, 1992) initiates during the post-septation phase (i.e., from E6) of heart development. Angioblasts form luminal structures after migrating into embryonic myocardial tissues (cylinder = arterial blood vessel). Cell populations associated with arterial vessel formation induce contractile myocytes (unshaded rectangular cells) local to the vessel to start differentiation toward the conductive, Purkinje fiber phenotype (shaded cell). Working myocardial cells continue to increase in number by cell division whereas committed perivascular Purkinje fiber sister cells cease proliferation and become mitotically quiescent.

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