

Development of the cardiac conduction system involves recruitment within a multipotent cardiomyogenic lineage

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SUMMARY

The cardiac pacemaking and conduction system sets and maintains the rhythmic pumping action of the heart. Previously, we have shown that peripheral cells of the conduction network in chick (periarterial Purkinje fibers) are selected within a cardiomyogenic lineage and that this recruitment occurs as a result of paracrine cues from coronary arteries. At present, the cellular derivation of other elements of this specialized system (e.g. the nodes and bundles of the central conduction system) are controversial, with some proposing that the evidence supports a neurogenic and others a myogenic origin for these tissues. While such ontological questions remain, it is unlikely that progress can be made on the molecular mechanisms governing patterning and induction of the central conduction system. Here, we have undertaken lineage-tracing strategies based on the distinct properties of replication-incompetent adenoviral and retroviral *lacZ*-expressing constructs. Using these complementary approaches, it is shown that cells constituting both peripheral and central conduction tissues originate from cardiomyogenic progenitors present in the looped, tubular

heart with no detectable contribution by migratory neuroectoderm-derived populations. Moreover, clonal analyses of retrovirally infected cells incorporated within any part of the conduction system suggest that such cells share closer lineage relationships with nearby contractive myocytes than with other, more distal elements of the conduction system. Differentiation birthdating by label dilution using [³H]thymidine also demonstrates the occurrence of ongoing myocyte transcription to conductive specialization and provides a time course for this active and localized selection process in different parts of the system. Together, these data suggest that the cardiac conduction system does not develop by outgrowth from a prespecified pool of 'primary' myogenic progenitors. Rather, its assembly and elaboration occur via processes that include progressive and localized recruitment of multipotent cardiomyogenic cells to the developing network of specialized cardiac tissues.

Key words: Heart, Conduction system, Retrovirus, Adenovirus, Chick

INTRODUCTION

The heterogeneous tissues of the cardiac pacemaking and conduction system are responsible for setting, maintaining and coordinating the rhythmic pumping of the heart (reviewed Thompson et al., 1995; Moorman et al., 1998; Gourdie et al., 1999). Anomalous development of this specialized network has been implicated in pediatric arrhythmia and other congenital abnormalities of cardiac activation (Schott et al., 1998). In earlier retroviral lineage-tracing work in chick (Gourdie et al., 1995), it was reported that periarterial Purkinje fibers, the most peripheral cells of the developing conduction system (Davies, 1930), share common cardiomyogenic origins with working myocytes. Owing to the close proximity of these Purkinje fiber cells to coronary arteries, it was furthermore suggested that this selection of a specialized phenotype within myocardial clones

occurred as a result of paracrine signals from vascular tissues (Gourdie et al., 1995; reviewed Gourdie et al., 1999). Consistent with this, it was subsequently reported that endothelin-1 (ET-1), a shear stress-induced cytokine prominently expressed by the coronary arterial bed (Yanagisawa et al., 1988; Masaki et al., 1991), prompts embryonic myocytes to express Purkinje-fiber like characteristics in vitro (Gourdie et al., 1998).

While there has been some progress on understanding the mechanisms governing development of peripheral Purkinje fibers, the cellular origins of other components of the conduction system (e.g. the nodes and bundles of the central conduction system), and the molecular signaling processes giving rise to such tissues remain almost entirely uncharacterized. Indeed, this area is distinguished by a number of long-standing controversies (reviewed Gorza et al., 1988; Moorman et al., 1998; Gourdie et al., 1999). First, debate continues as to whether specialized

cardiac tissues have a neuroectodermal derivation (neurogenic cells) or differentiate from populations present in the cardiogenic mesoderm (cardiomyogenic cells). The main evidence supporting a neurogenic origin comes from the prominent expression of neural-associated gene products by certain populations of conductive cells (Vitadello et al., 1998; reviewed Gorza et al., 1988). To date, this question remains unresolved, though lineage-tracing studies of neural crest migration have thus far failed to detect a direct contribution of neural crest to the cardiac conduction system (Gourdie et al., 1995; Poelmann and Gittenberger de Groot, 1999). A second area of discussion has emerged among those who accept a cardiomyogenic derivation for specialized cardiac cells (reviewed Moorman et al., 1998; Gourdie et al., 1999). Based on domain-specific patterns of gene expression in the cardiac primordia, it has been proposed that conduction tissues derive from segment-like domains of fast- and slow-conducting tissue already present in the tubular heart (Moorman et al., 1998). Fundamental to this paradigm is that the electrical architecture of the mature conduction system is remodeled from such segments and that cell fates becomes fixed early within these differentiating modules of gene expression. This model is difficult to reconcile, however, with the low proliferative activity of early central conduction tissues (Thompson et al., 1990, 1995) and retroviral clonal analyses, which reveal that periarterial Purkinje fibers share lineages more in common with nearby working myocytes than with cells in other parts of the conduction system (Gourdie et al., 1995; reviewed Gourdie et al., 1999). Such patterns of relationship between different myocardial cell types would not be expected if all specialized cardiac tissues were generated in toto from the proliferation and differentiation of fate-restricted progenitor cells present in the tubular heart.

To directly address these as yet unresolved questions on the cellular derivation and assembly of the fascicles constituting the central conduction system, we have undertaken lineage-tracing and cell birthdating analyses in the developing chick embryo. The approaches used include complementary strategies based on replication-incompetent adenoviruses (Fisher and Watanabe, 1996) and retroviruses (reviewed Mikawa et al., 1996) to target neural and cardiomyogenic populations putatively contributing to the conduction system. Our study reveals that cells comprising the right AV ring bundle, retroaortic ring bundle, His bundle and bundle branches originate from cardiomyogenic progenitors present in the tubular heart, with no detectable contribution by neural crest. Furthermore, detailed inspection of retroviral clones and cell birthdating indicate the occurrence of continuing and localized patterns of cellular conscription to these large conductive fascicles. This recruitment motif resembles that previously described for periarterial Purkinje fibers and suggests that these bundles also develop by processes that include inductive accretion of nearby populations of non-terminally differentiated cells.

MATERIALS AND METHODS

Replication-defective retroviruses and infection in ovo

Protocols for construction, propagation and application in cell lineage tracing of the two replication-defective retroviruses, CXL and SNTZ, have been detailed previously (Mikawa et al., 1991; Gourdie et al.,

1995). SNTZ and CXL mediate expression of β -galactosidase (β -gal) in either the cytoplasm (CXL) or at the nuclear envelope (SNTZ) of infected cells. For targeting into tubular heart, fertilized White Leghorn chicken eggs were incubated in a humidified chamber at 38.5°C for 2.5 to 3 days until embryos developed to Hamburger and Hamilton (HH) stages 13 to 17 (Hamburger and Hamilton, 1951) – stages at which the heart contains only myocardial and endocardial cells. A small volume (< 5 nl) of fluid containing a mixture of CXL and SNTZ viruses ($>10^7$ virions/ml), or control solution containing no virus, polybrene (Sigma) at 100 μ g/ml and 0.001% Fast Green (Sigma) was then microinjected into midventricular or atrioventricular segments of tubular hearts. Mixed infections with CXL and SNTZ viruses were done to enable discrimination of monoclonal β -gal+ sectors (Gourdie et al., 1995). Two targeting strategies were devised to infect neural crest cells. In the first, 30–50 nl volumes of concentrated virus plus 100 μ g/ml polybrene were microinjected into the somatopleura of HH stage 10 to 12 embryos directly adjacent the neural tube at the level of the midotic placode to somite 3. The rationale was to precisely target the proliferating pool of recently delaminated neural crest cells occurring in this region. In the second strategy, 30–50 nl volumes of virus were microinjected directly into the neural folds of HH stage 8 to 10 embryos at the level of mid-otic placode to somite 3. Organized cohorts of cells, infected by either of these strategies, demonstrated a pattern of migration consistent with that of cardiac neural crest in chick (reviewed Kirby, 1999). Following targeting of either cardiomyogenic or neurogenic populations, eggs were sealed with parafilm and returned to the incubator until harvesting at E5 to E18.

Replication-defective adenoviruses and infection in ovo

Two *lacZ*-encoding replication-defective adenoviruses were used in this study, expressing β -gal under the control of promoters derived from either human cytomegalovirus (CMV) or avian rous sarcoma virus (RSV). One of these constructs, AdRSVlacZ, was a gift from Drs Michiko Watanabe and Steven Fisher (Case Western Medical School, OH) and is a E1a-deleted construct expressing β -gal with a nuclear-localization signal under the RSV long terminal repeat promoter. The propagation, purification and titer assay of AdRSVlacZ has been described previously (Fisher and Watanabe, 1996; Watanabe et al., 1998). The CMV-lacZ adenovirus (HCMVsp1lacZ) was constructed by Drs Andy Bett and Frank Graham (McMaster University, Canada) and used by permission. HCMVsp1lacZ expresses β -gal in the cytoplasm of infected cells under the control of the Human CMV immediate early promoter. Details of the construction of this vector are given in Bett et al. (1994). HCMVsp1lacZ was grown and purified using methods identical to those used for AdRSVlacZ. To achieve high efficiency infection of tubular chicken hearts, ~500 nl volumes of either recombinant adenovirus (at 10^{10} – 10^{11} plaque-forming units/ml) were injected into the pericardial cavity of E3 White Leghorn chick embryos (Hamburger and Hamilton stages 15–17) according to Fisher and Watanabe (1996). Eggs containing embryos were then resealed with parafilm and placed at 38.5°C in a humidified incubator. Following incubation in ovo to between E4.5 and E19, embryos were harvested and whole embryos or dissected hearts were fixed in 2% paraformaldehyde and immediately X-gal reacted as described below.

Detection of β -galactosidase expression

A two-step X-gal and immunohistochemical protocol was carried out to enhance detection of β -gal-expressing cells in histological analyses (Cheng et al., 1999). Dissected embryos or hearts were perfusion-fixed in paraformaldehyde for 3 hours, washed 3 \times in PBS buffer (1 hour per wash), perfused and immersed with X-gal chromagen and subsequently photographed as whole mounts using a Leica M10 stereomicroscope as described previously (Gourdie et al., 1995). Samples were then dehydrated through a graded ethanol to toluene series, embedded in paraffin, serial sectioned at 10 μ m and slide-mounted according to standard histological practice. Eosin reference slides were prepared

from every tenth section through each sample. To increase sensitivity, selected sections from each heart were subject to staining with antibodies against β -gal as described in Cheng et al. (1999).

Multilabel immunofluorescence microscopy

On other selected sections, double immunolabeling of β -gal was combined with immunodetection of markers of myocardial cell phenotype. The multilabeling protocols carried out were similar to those we have described previously (Gourdie et al., 1993, 1998). In short, primary antibody mixtures of rabbit anti- β -gal and either MF20, a mouse monoclonal marker of muscle lineage (Bader et al., 1982), or a mouse monoclonal marker of the cardiac Purkinje fiber lineage which recognizes EAP300/transitin (McCabe et al., 1995; Gourdie et al., 1995), were applied to tissue sections overnight. Immunolocalized primary antibodies were then secondarily detected using anti-rabbit antibodies conjugated to TRITC (Dako) and anti-mouse antibodies conjugated to either FITC or Cy-5. Following secondary antibody incubation, washing and coverslipping, tissue sections were imaged on a BioRad MRC-1024 laser scanning confocal microscope.

Quantitative analyses of retrovirally infected hearts

Discrete retrovirally infected β -gal+ sectors in the right AV ring and His-bundle were subject to quantitative analyses to compare numbers of conductive and contractive cells at these sites. Only hearts containing small numbers of well-separated β -gal+ sectors were quantitated. For SNTZ-infected sectors, X-gal-labeled nuclei were counted directly. For CXL-infected sectors, X-gal-reacted sections were counterstained with Nuclear Fast Red to delineate nuclei. Right AV ring quantitation was based on four hearts, each containing a single clone incorporating part of the right AV ring. His bundle quantitation was based on three clones, each of which were also located in separate hearts. Counts were based on 5 to 10 serial sections through the extent of each clone. Mean numbers of conductive and contractive cells were compared within the two sites using the Student's *t*-test according to standard statistical procedures.

Label dilution of [³H]thymidine

To establish a time course of recruitment to different elements of the conduction system, label dilution (pulse-wait) experiments with [³H]thymidine were undertaken using methods established for CNS birth dating in the rat (Nornes and Das, 1972) and chick (Yurkewicz et al., 1981). Fertilized White Leghorn eggs were opened and low-levels (1-10 μ Ci) of high specific activity [³H]methyl thymidine (80 Ci/mmol) were applied to the air sac membrane at either 2, 4, 6, 8, 10 or 12 days of embryonic incubation. Dose was normalized to changing total embryonic thymidine requirement to be depleted in 24-48 hours and to yield approximately 1 autoradiographic grain/nucleus/day (Thompson et al., 1999). Control embryos labeled at 5 times this dose completed septation with no apparent effect. Following incubation, embryos were killed at either 12 or 20 days of incubation, the hearts were removed, fixed in 2% paraformaldehyde and processed for slide mounting and immunolabeling as described above. Autoradiography was carried out on xylene-cleared, serial 5 μ m sections dipped in 42°C ARG emulsion (Kodak NTB-2), stored at 4°C for 20 days and developed with Kodak D19 at 20°C for 10 minutes. Imaging was carried out on a BioRad MRC1024 microscope in reflectance mode or by using a transmitted light detector on the microscope in dark-field illumination. Image stacks from serial sections were superimposed by NIH-image and Adobe Photoshop 4.0 software.

RESULTS

Replication-incompetent adenovirus identifies a cardiomyogenic origin for central conduction cells

Microinjection of replication-defective adenovirus into the pericardial cavity of embryonic day 3 (E3) chick embryos has

been reported to result in infection of tissues comprising the tubular heart (Fisher and Watanabe, 1996). In agreement with this, we determined that either of two adenoviral constructs expressing β -gal (*lacZ*) under the control of RSV (AdRSV*lacZ*) or CMV (HCMVsp1*lacZ*)-derived promoters mediated high levels of transgene expression throughout the embryonic heart at E4.5 (Fig. 1A,B). In whole mounts of X-gal-reacted E4.5 embryos infected thus, the heart was the only organ showing significant β -gal expression (Fig. 1A). No adenoviral infection was detected in neural tissues in embryos targeted by pericardial microinjection (Fig. 1A). Histology of E4.5 β -gal hearts infected by either the RSV or CMV constructs disclosed that β -gal expression was confined to myocardial cells in the atria, outflow tract and ventricles, with no transgene expression in endocardial or epicardial cells (Fig. 1A,B). While intense and uniform in E4.5 embryos, β -gal expression levels in working myocardial tissues decreased progressively in intact hearts from older embryos infected at E3 with adenovirus. Over a time course that included E10, E15 and E19 sample points, only sparse and isolated β -gal-positive sectors were noted on the external epicardial surfaces of intact hearts from E15 and E19 embryos. However, upon dissection of an E15 heart infected with AdRSV*lacZ*, close inspection of its external and internal surfaces revealed frequent β -gal-positive cells distributed across the endocardium of the ventricles. Based on EAP300-immunolocalization (Gourdie et al., 1995; McCabe et al., 1995), this conspicuous population of cells was identified as subendocardial Purkinje fibers (Fig. 1C). Systematic histological examination of other E10 (11 hearts), E15 (5 hearts) and E19 (3 hearts) samples revealed that relative numbers of β -gal-positive to β -gal-negative cells were also significantly higher in components of the peripheral and central conduction system relative to working myocardium (Fig. 1C,D). Tissues retaining such conspicuous β -gal expression included the right atrioventricular ring, retroaortic ring, His bundle, bundle branches and Purkinje fibers (both subendocardial and periarterial). This spatiotemporal pattern of infection revealed by β -gal expression was consistent in repetitions of the developmental time course and was similar for adenovirus-mediated β -gal expression driven by either RSV or CMV promoters.

The episomal genomes of replication-defective adenoviral constructs are thought to be diluted in infected host tissues by cell division (reviewed Mikawa et al., 1996). Specialized myocardial tissues are characterized by early withdrawal from proliferation, unlike parietal myocardium (Thompson et al., 1995, 1999). We hypothesized that such tissue-specific differences in proliferation kinetics may be an explanation for the time course of adenoviral infection observed (i.e. as disclosed by β -gal). Such kinetics were confirmed by well-established techniques using incorporation of [³H]thymidine into cells undergoing DNA synthesis and subsequent autoradiographic analysis of radioactive label dilution (Nornes and Das, 1962; Yurkewicz et al., 1981). Fig. 1E shows a dark-field autoradiograph of a chick heart exposed to [³H]thymidine on E2 and allowed to develop to E12. The section plane is comparable to that shown in the adenovirus-infected His bundle and left bundle branch in Fig. 1D. Dense accumulations of silver grains are retained in the non- or slowly proliferating tissues of the right atrioventricular bundle, retroaortic ring, His bundle and left bundle branches. At this stage, the working

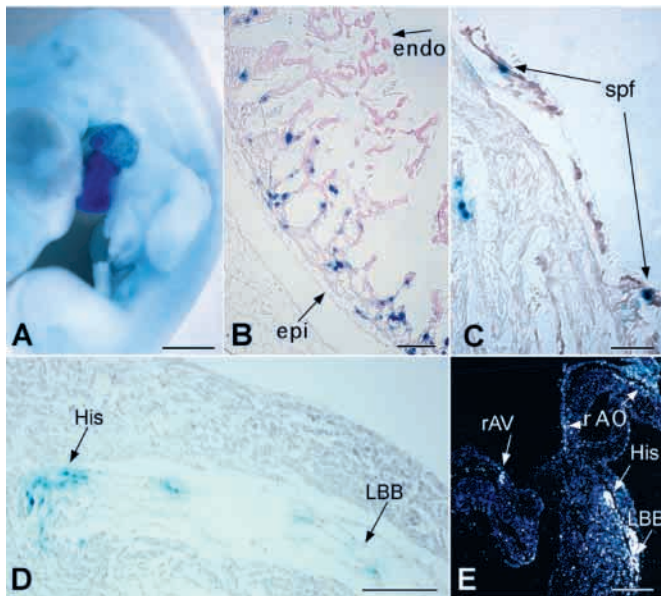


Fig. 1. (A) An X-gal-reacted E4.5 embryo infected with replication-defective adenovirus (AdRSVlacZ) on E3. (B) Histological section showing the right ventricle of an AdRSVlacZ-infected E4.5 embryo following X-gal reaction. The uninfected tissues of the epicardium (epi) and endocardium (endo) are arrowed. (C) Histological section through the ventricle of an E15 heart infected with AdRSVlacZ at E3. Prominent nuclear-localized β -gal signal (blue) is co-localized with EAP300 (brown) in subendocardial Purkinje fibers (spf). (D) Histological section of an E15 heart infected with AdRSVlacZ at E3. Frequent β -gal⁺ cells are restricted to the central conduction tissues of the His bundle (His) and left bundle branch (LBB). (E) Dark-field autoradiograph of a histological section through a heart from a chick embryo labeled at E2 with [³H]thymidine and sacrificed at E12. Labeling is seen in the right AV-ring (rAV), His bundle (His), retroaortic bundle (rAO) and left bundle branch (LBB). Scale bar, 1 mm in A,E; 200 μ m in B; 50 μ m in C,D.

myocardium of the embryonic chick continues rapid hyperplastic growth and the density of autoradiographic grains is accordingly lower in these tissues.

There is no detectable contribution by neurogenic cells to the central conduction system

In the preceding section, it was shown that pericardial targeting with replication-defective adenoviruses at E3 results in infection of cardiomyocytes specifically in the tubular heart and that later, slowly proliferating cohorts derived from these initial infected cells become restricted to the central and peripheral conduction system. This spatiotemporal pattern would be consistent with a significant fraction of all specialized cardiac cells being derived from cardiomyogenic progenitors. However, this does not exclude a co-contribution by neural crest. To determine whether neurogenic populations contribute directly to the conduction system, nanoliter volumes of replication-incompetent retroviruses were precisely microinjected into either the neural folds of HH stage 8-10 embryos (35 tagged embryos analyzed) or somatopleura adjacent the neural tube of HH stage 10-12 embryos (74 tagged embryos analyzed), at the level of the mid-otic hindbrain to somite 3. Retrovirus precisely targeted at either of the two loci

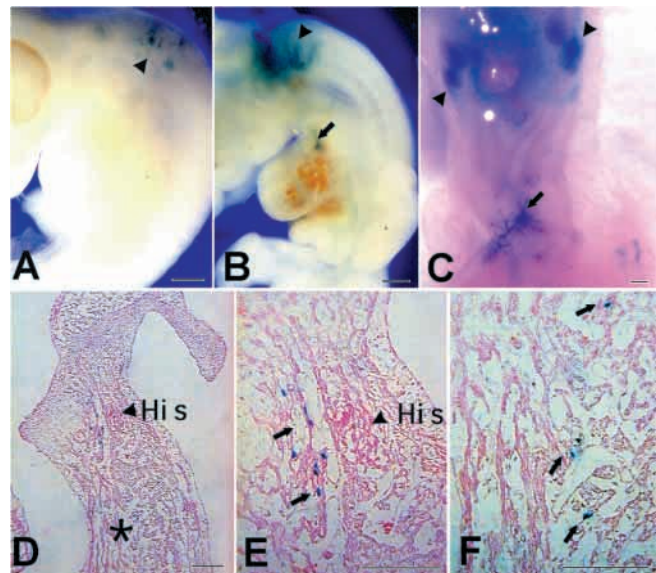


Fig. 2. (A) β -gal⁺ cells (arrowheads) are detected adjacent to the hindbrain of an E4.5 embryo following targeting of neural crest progenitors by co-microinjection of retroviruses at E2. (B) In an embryo targeted similarly, β -gal signal is observed migrating from the hind brain and into the outflow pole (arrowed) of the embryonic heart. (C) Whole mount showing the outflow region of a heart from an E9 embryo targeted by viral microinjection into neural crest progenitors at E2. β -gal⁺ tissues are observed in parts of the great vessels distal to the heart (arrowheads) and in a nerve-like plexus at the base of the outflow region (arrowed). (D) Low power of a histological section through the interventricular septum of a neural crest targeted embryo at E10 (His bundle, arrowhead). (E) Higher magnification of a region shown in D reveals a group of β -gal⁺ cells (arrows) adjacent to the His bundle (arrowhead). (F) β -gal⁺ derivatives of neural crest targeting (arrowed) are seen scattered through myocardium of the interventricular septum. An asterisk in D indicates the location of the high magnification shown in F. Scale bar, 500 μ m in A-C; 250 μ m in D-F.

resulted in tagging of populations of cells with indistinguishable patterns of migration from the hindbrain, through the branchial arches and into the embryonic heart via the developing outflow tract (Fig. 2A,B). Such migratory behaviors conform to those previously characterized for the

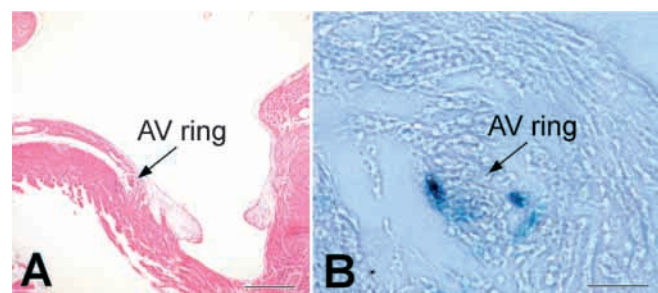


Fig. 3. (A) An eosin-stained section through the atrioventricular junction of an E14 heart that was targeted with retrovirus at E3. The right AV ring bundle is arrowed. (B) A higher magnification of the arrowed region in a sister section reveals β -gal signal in the cytoplasm of CXL-infected cells (blue) within the right AV ring bundle (arrowed). Scale bar, 500 μ m in A, 50 μ m in B.

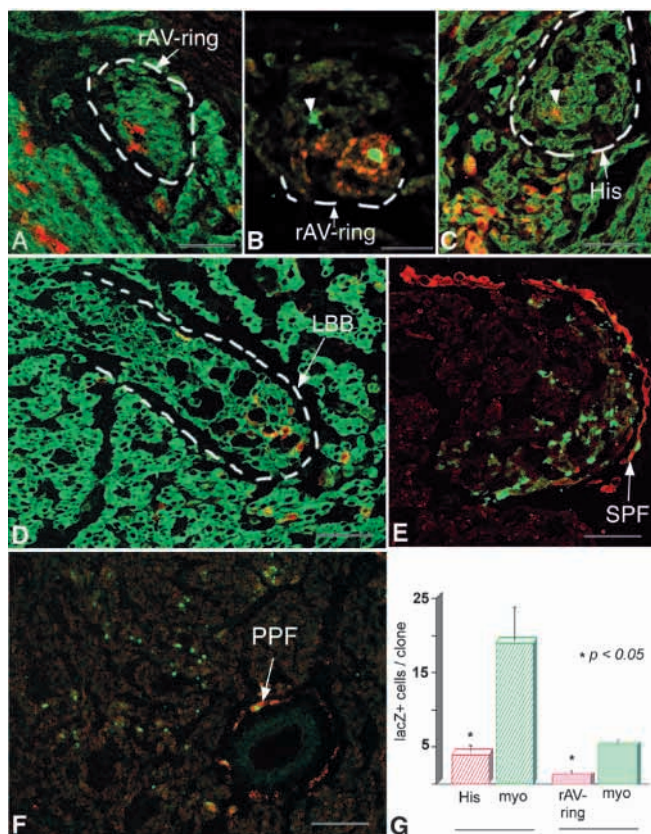


Fig. 4. (A) Double immunostaining for β -gal (red) and a muscle-specific marker (MF20-green) in a sister section to that shown in Fig. 3B (of the right AV ring). β -gal and MF20 signals are observed in cells incorporated within the right AV ring (dashed lines) and also in adjacent working myocytes. (B) Double immunostaining for β -gal (red) and a conduction-specific marker (EAP300, green; McCabe et al., 1995) in the right AV ring. β -gal-labeled cells are observed within the EAP300-positive conduction fascicle (dashed lines) and also in an adjacent EAP300-negative working myocyte (arrowhead). (C,D) A clonal motif similar to those seen in A,B is also observed in separate β -gal sectors incorporating the His bundle (dashed lines in C) and the left bundle branch (dashed lines in D). (E,F) Double immunolabeling for β -gal and EAP300 in sections from E18 hearts infected at E3. EAP300-positive (red) subendocardial (SPF) and periarterial (PPF) Purkinje fiber cells and unlabeled ventricular myocytes occur within the same discrete β -gal+ (green) sectors. (G) Quantitative analysis of conductive cell and myocyte numbers within retroviral clones at the His Bundle and right AV ring. On average, myocytes significantly ($P < 0.05$) outnumber central conductive cells within clones. Scale bar, 30 μ m in A-E; 100 μ m in F.

cardiac neural crest in chick (reviewed Kirby, 1999). Similarly, in intact hearts from older embryos (E10-E14), retroviral targeting of prospective neural crest by either protocol resulted in β -gal-positive sectors in the walls of the aorta and pulmonary arteries proximal to the heart and in superficial nerve-like plexuses at the base of the outflow tract (Fig. 2C). This pattern of retroviral infection in cardiac neural crest derivatives is consistent with that reported previously (Gourdie et al., 1995; Poelmann et al., 1998). Histology of neural-crest-targeted hearts revealed virally tagged cells in cushion and myocardial tissues (Fig. 2D-F). Focal concentrations of β -gal-

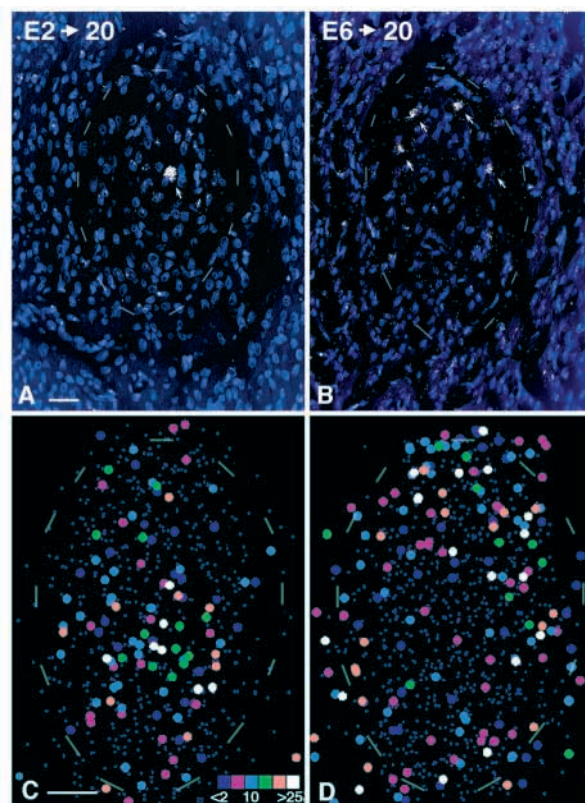


Fig. 5. (A,B) Dark-field images of single sections incorporating the His bundle (dashed lines) of E20 hearts labeled with tritiated thymidine at E2 and E6. (A) A single labeled nucleus is seen near the center of the fascicle. (B) Multiple labeled nuclei are seen at the edge of the fascicle. (C,D) Compilation of labeled nuclei in 10-15 sections through the His bundles (dashed lines) shown in A and B. The color scale inset shows autoradiographic grains/nucleus. Grain numbers per nucleus of less than 2 (i.e. < 2 dark purple on the inset scale) correspond to background levels. Counts of 5 (light purple on scale) or more grains per nucleus were considered positive [3 H]thymidine labeling. The higher the grain count, the more likely that the cell did not divide after treatment of [3 H]thymidine (highest counts of greater than 25 grains are white on scale). Scale bars are 20 μ m.

expressing neural crest cells were occasionally found near, but never within, central conduction bundles (Fig. 2D,E). Conspicuous populations of dispersed β -gal-positive cells were also found throughout the working myocardial tissues of the interventricular septum with no apparent association with known components of the conduction system (Fig. 2F).

Myocyte clones that incorporate central or peripheral conduction cells always include nearby working myocytes

Site-specific targeting by retroviruses provides no evidence of detectable contribution by neural crest to the central conduction system. Based on this result, and our adenoviral data pointing to cardiomyogenic progenitors in the tubular heart, questions on the process of conductive network formation in chick can now be more precisely framed. Utmost of these questions is whether development of the conduction system occurs via outgrowth from a fixed pool of early committing progenitors or whether cardiomyogenic fate is less

fixed and its elaboration occurs via ongoing recruitment from a pool of multipotent cells. In a previous study (Gourdie et al., 1995), we reported that, while retroviral infection of peripheral conduction cells was common, infection of central conduction tissues could not be detected – at least in the 7 hearts examined. Here, we have undertaken the same analysis in a much larger sample of E14 and E18 hearts from embryos microinjected at E3 with retrovirus. Dual infections with CXL (cytoplasmic) and SNTZ (nuclear) were carried out as a control such that monoclonal sectors of viral infection could be discriminated from polyclonal sectors (Gourdie et al., 1995). Of the 854 tubular-heart stage embryos targeted, 204 hearts were subsequently identified as containing well-separated domains of CXL- or SNTZ-mediated β -gal signal suitable for histological analysis of fate selection within virally defined clones.

A total of 9 hearts (i.e. 4.5% of the 204 β -gal+ hearts) were identified as containing sectors of retrovirally infected cells incorporated in 'atrial' or 'ventricular' components of the central conduction system (Figs 3, 4). A single sector of either nuclear or cytoplasmic virus was found in the central conduction system of each of these hearts. Figs 3A,B and 4A illustrate sections through one of these sectors, in which CXL-infected cells are observed in the right atrioventricular ring (rAV ring). Fig. 4B shows retrovirally infected β -gal-expressing cells (green) incorporated within, and in working atrial myocytes adjacent to, the EAP300-labeled (red) right AV ring bundle. Other components in which monoclonal domains of retrovirus were located included the retroaortic ring bundle, His bundle (Fig. 4C) and bundle branches (Fig. 4D). Five of the sectors identified were compact and located exclusively along free wall segments of the right AV ring. Two other hearts demonstrated relatively discrete, retrovirally infected domains incorporating the His bundle and left and right bundle branches. The two remaining β -gal-expressing zones were initially identified in the right AV ring, but could be traced in continuous distributions that extended into the retroaortic ring and His bundle tissues. None of the 9 virally infected domains incorporating central conduction tissues ever extended into subendocardial (Fig. 4E) or periarterial elements (Fig. 4F) of the peripheral conduction system. The virally tagged cells in the central conduction system were of myocardial (i.e., MF20+, Fig. 4A,C,D) and conductive phenotype (i.e., EAP300+, Fig. 4B) and always occurred as part of a sector that incorporated nearby β -gal+ working myocytes. Indeed, Fig. 4 indicates that the co-existence of conductive cells and working myocytes within discrete, virally defined sectors is a found in all parts of conduction system in which retroviral infection was detected. A quantitative analyses of β -gal-positive cells in four clones incorporating either right AV ring or His bundle indicated that contractile cells ($P < 0.05$) outnumbered conductive cells by an average of 5 to 1 (Fig. 4G). This quantitative data is consistent with the [3 H]thymidine birth dating and adenoviral studies (e.g. Fig. 1) which indicate that commitment to specialization in large conductive bundles is accompanied by slowed proliferation. A final point on the β -gal-positive sectors incorporating the right AV ring is that these clones contained both atrial and ventricular working myocytes (see Fig. 4A,B). Morphologically, the right AV ring has been viewed an atrial structure, demarcating a relatively strict segmental boundary between the atria and ventricles. The

heterogeneous myocardial phenotypes emerging in right AV sectors suggest that this concept may require revision.

The conduction system develops during incubation in ovo by ongoing accretional recruitment of cardiomyogenic cells

The preceding results indicate that central conductive cells share common parental cells with nearby sister working myocytes, but not with peripheral conduction cells (e.g. Fig. 1E). This provides further evidence of the cardiomyogenic origin of most specialized cardiac tissues. However, three further implications arise from this data. First, they conclusively demonstrate that cardiomyogenic fate is not necessarily fixed in the E3 tubular heart. Second, the data provide further confirmation of the separate genesis of the central and the peripheral conduction system. Finally, they suggest a process of localized and inductive recruitment within virally defined clones occurring throughout the central and peripheral conduction system. In this final section of the results, we use cell birth dating protocols to confirm the occurrence of this recruitment process and define its spatiotemporal time course.

In a previous study, we showed that the proportion of clones containing periarterial Purkinje fibers increased significantly between E14 (21 clones counted) and E18 (29 clones counted), thereby demonstrating that recruitment to the most peripheral parts of the conduction system is ongoing until just prior to hatching. Owing to the small number of clonal sectors identified in the large conductive bundles, we were unable to use a similar approach for the central conduction system. In Fig. 1E, radioactive label retention in slowly dividing cells can be seen to sharply delineate all elements of the central conduction system including the right AV ring, His bundle, bundle branches and retroaortic ring. Fig. 5A,C show a quantitative analysis of label density in the His bundle of an embryo labeled with [3 H]thymidine at E2 and allowed to develop to E20. Superimposition of 10-15 sections (e.g. Fig. 5C) through the His bundle reveals a core of more heavily labeled cells in the center of the fascicle. If exposure to radiolabel is carried out on E6 and the same quantitative analysis is undertaken at E20, a different distribution of autoradiographic grains is revealed (Fig. 5B,D). Here, heavily labeled cells are found more often at the periphery of the His bundle. This pattern was duplicated in a repeat of this experiment and indicates a lamellar recruitment of cells to slowed proliferation and differentiation around the early core of His bundle cells, which continues through the first 8 to 10 days of incubation. This is further illustrated in Fig. 6A, which shows co-localization of EAP300 and [3 H]thymidine labeling in the His bundle (arrowhead) of an E20 embryo pulse-labeled on E8. An EAP300/[3 H]thymidine-positive Purkinje fiber is also seen at the periphery of an artery in this image. If exposure to [3 H]thymidine is undertaken on E12, such peripheral elements of the conduction system (i.e. periarterial and subendocardial Purkinje fibers) are the most prominent population of cells showing retention of the radiolabel (Fig. 6B,C). In these hearts, recruitment of cells to the central conduction system appears to be largely complete, as no prominent concentrations of silver grains are found to delineate central conduction bundles (negative data not shown). Together with the viral lineage-tracing data, the label dilution analysis

suggests that assembly of the conduction system from E2 occurs by a process of accretional recruitment that continues (albeit not at uniform rates in different parts of the system), almost up until hatching.

DISCUSSION

Here, we have used adenoviral and retroviral lineage-tracing and cell birth dating techniques to show that tissues constituting the conductive fascicles of the central conduction system are derived from cardiomyogenic cells present in the looped, tubular heart. While the possibility of direct neural crest contribution to the conductive bundles of the heart cannot be ruled out, we detected no neurogenic contribution to these structures by the methods used in this study. Interestingly, retroviral clonal and cell birthdating analyses provide independent confirmation of the occurrence of ongoing and localized recruitment of cells to both central and peripheral components of the conduction system. This pattern of conscription of cardiomyogenic cells into specialized cardiac structures is difficult to reconcile with models (reviewed Moorman et al., 1998) that envisage the cardiac conduction system developing in its entirety from the outgrowth of fate-restricted subsets of progenitors present in the tubular stage heart. In addition to giving insight into the ontogeny of cardiac cells, the tissue-specific patterns of adenovirus infection revealed provide a novel strategy for gene targeting the developing conduction system.

While a cardiomyogenic origin for peripheral conduction cells is now widely accepted, there have been recent suggestions that a neural crest contribution to the tissues of the central conduction system remains possible (Vitadello et al., 1998). By using the complementary properties of replication-defective adenoviral or retroviral lineage-tracing constructs, improving the sensitivity of β -gal detection and increasing the numbers of embryos screened, we have now been able to trace virally tagged cells into the right AV ring, retroaortic ring, His bundle and bundle branches following targeting of the looping, tubular heart prior to known neural crest immigration. The results suggest that a significant fraction of cells constituting these parts of the central conduction system are derived from cardiomyogenic progenitors. Conversely microinjection of replication-defective retrovirus into the cardiac neural crest before or during its emigration from the neural tube failed to result in detectable incorporation of β -gal-positive daughter cells in conduction tissues. Interestingly, the data do indicate the migration of some neural crest derivatives to locations nearby developing conductive fascicles in patterns similar to those reported by Poelmann and Gittenberger de Groot (1999). Hence, it cannot be precluded, as was suggested by these workers, that neural crest does not have an indirect role in the maturation of specialized cardiac tissues. However, we also detected large numbers of β -gal+ neural crest cells interspersed within working myocardial tissues in no apparent association with identifiable parts of the conduction system. The reason for this difference between the studies is uncertain. The targeting methods used by the other group involved delivery of large volumes of virus (40-100 nl) and were based largely on a protocol in which the neural tube of E2 chick embryos was flushed with virus-containing medium. Here, we confined

targeting by microinjecting small volumes directly into the specific tissue loci from which cardiac neural crest cells emigrate. Any explanation of the function of neural cells in the heart is likely to be somewhat more complex than solely impacting the differentiation of conduction tissues. The distribution found in the present study would not be inconsistent with neural crest cells also having a role in cushion myocardialization (Van den Hoff et al., 1999). Furthermore, the nodes and bundles of the adult heart are well-innervated structures (Crick et al., 1996). Neural tissues are found throughout the working myocardia of the atria and ventricles. The migratory behavior of neurogenic progenitors revealed by the targeting of the cardiac neural crest is therefore also consistent with patterns of innervation in the mature vertebrate heart and implicate organized central conduction bundles in the oriented migration of such later neural elements.

One model proposes that specific segmental domains of the looped tubular heart contain precursor cells from which the entire conduction system proliferates and differentiates (reviewed Moorman et al., 1998). A long-standing problem with this paradigm has been that non-DNA-synthesizing cells in the tubular heart map to early conduction system (Thompson et al., 1990, 1995). The results of the present study are similarly difficult to reconcile with this model. While all 204 hearts with detectable viral infection examined contained frequent β -gal-expressing subendocardial or periarterial Purkinje fibers, only a fraction of these hearts (9) also had retroviral tracer in central conduction bundles. Put another way, 196 (~95%) of the hearts studied showed no evidence of subendocardial and periarterial Purkinje fibers sharing common progenitors with cells constituting central conduction bundles (including the His bundle and bundle branches). Furthermore, retroviral clones reveal the existence of multipotent precursors in the tubular heart capable of giving rise to both cells incorporated in central conduction bundles and working myocytes adjacent to bundles. Finally, cell birth dating analysis reveal the accretion of newly quiescent myocytes to central conductive bundles continuing at least up until the end of septation (i.e., E8), and possibly longer (i.e., <E12). Together, these data are best explained by an ongoing process of lamellar recruitment of non-terminally differentiated myocytes to forming central conductive fascicles. These results do not imply that all myocytes in the tubular heart have an equal potential for differentiation or that conductive bundles are generated *de novo* from such cells. A number of studies provide undeniable support for the occurrence of patterning events directing the formation of specialized domains of myocardial cells early in tubulogenesis (Thompson et al., 1990, 1995; Wessels et al., 1992; Chan-Thomas et al., 1993; Moorman et al., 1998). However, it appears that the cells subject to these early patterning events do not go on to form the progenitor pools from which all specialized cardiac tissues subsequently differentiate. In light of the present results, these segment-like domains are perhaps best viewed as an initial or primary framework upon which subsequent assembly of the cardiac conduction system proceeds. Such processes, extending well past the end of cardiac septation, would also allow for plasticity in completing terminal arborizations of the His-Purkinje network.

The molecular mechanisms responsible for directing formation of this initial framework can only be speculated upon at present. RNA encoding the homeodomain transcription

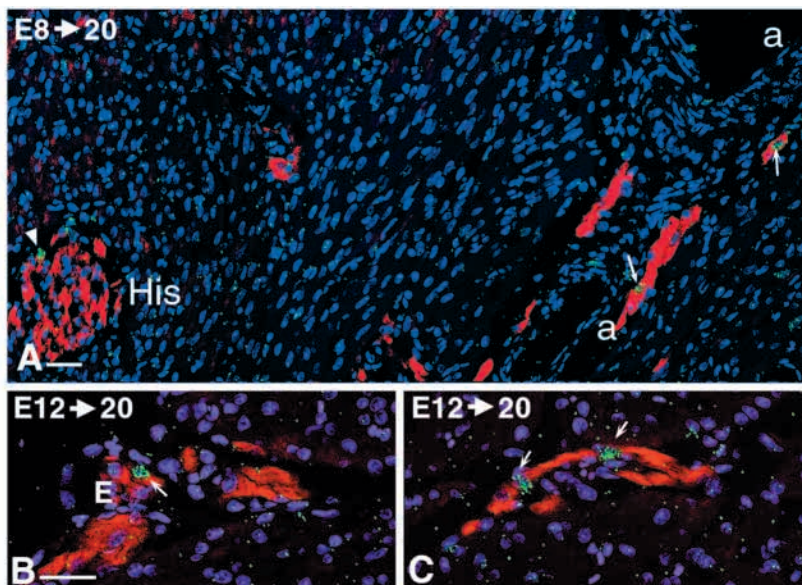


Fig. 6. (A) Heavily labeled nuclei (green spots) within a EAP-300-positive His bundle and periarterial Purkinje fibers in an E20 heart pulsed with [^3H]thymidine on E8. (B,C) Labeled nuclei (green spots) within EAP-300-positive subendocardial (B) and periarterial (C) Purkinje fibers in an E20 heart pulsed with [^3H]thymidine at E12. Scale bars, 20 μm .

factor *Msx-2* is expressed and maintained in central conduction progenitors at early stages of cardiac morphogenesis (Chan-Thomas et al., 1993). Mutation of another transcriptional regulator, *Nkx2.5*, results in aberrant function of central conduction components in humans (Schott et al., 1998). Interestingly, recent studies also suggest that this tissue is also

characterized by the expression of a potassium channel associated protein minK (Kupersmidt et al., 1999) and gap junctional Connexin-45 (Coppens et al., 1999). More pertinent to the present investigation are the mechanisms guiding conscription of cells to the framework once established in the tubular heart. In this regard, perhaps the most interesting question is whether the primary framework is a passive scaffold or whether its constituent cells actively direct the assembly of the conduction system. Recruitment of peripheral conductive cells within the myocyte lineage has been shown to occur in tight spatial and temporal association with forming coronary arterial branches (Gourdie et al., 1993, 1995; reviewed Gourdie et al., 1999). It was consequently suggested that tissues constituting coronary arteries actively induced conversion of myocytes to Purkinje fibers via secretion of a paracrine-like effector. The observation that a blood vessel-associated cytokine, endothelin-1 (Masaki et al., 1991; Yanagisawa et al., 1988), was capable of converting cultured embryonic myocytes into Purkinje fiber-like cells (Gourdie et al., 1998), provided support for this hypothesis. Recent experiments involving global or localized manipulations of coronary branching patterns have provided further direct evidence for an active role of coronary arteriogenesis in the induction and organization of the peripheral conduction network *in vivo* (Hyer et al., 1999). Whether the primary framework is an active organizing focus in development of conduction bundles remains to be shown. In Fig. 7, we propose a model suggesting that inductive processes analogous to those operating in peripheral Purkinje fiber development also act in the formation of cardiac central conduction bundles. Finally, it was previously concluded that the subendocardial and periarterial Purkinje fiber network forms independently of the central conduction system in chick and consequently proposed that a linkage of these two compartments must occur during development (Gourdie et al., 1995). The lineage analyses undertaken in this present study provide complementary data supporting this earlier conclusion. In morphological and electrophysiological studies, Watanabe and co-workers have recently described stage-related changes in the activation of developing chick ventricle that are also

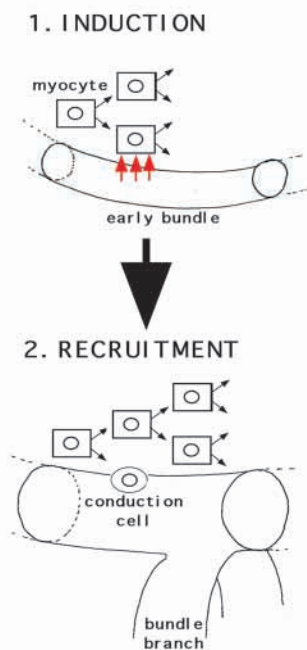


Fig. 7. A model for development of cardiac central conduction fascicles. Following establishment of an initial or primary framework (as represented by a bundle) in the tubular heart, conduction bundles differentiate by inductive recruitment within clones of multipotent cardiomyogenic cells. Sister cells not induced to specialization continue to proliferate and differentiate into working myocytes adjacent to conductive bundles. The specific nature of the inductive signal (i.e. red arrows) is unknown, but it is presumed to be secreted in a paracrine manner similar to that proposed in the induction of periarterial Purkinje fibers (Gourdie et al., 1995).

consistent with discontinuous assembly of the conduction system (Chuck et al., 1995). If we accept that elements of this system form initially as independent parts, then one of the most interesting questions that remains is how these specialized components become functionally joined to form the integrated atrioventricular conduction system of the mature vertebrate heart. Insight into this in situ linkage process, and how it goes wrong in the congenitally malformed heart, may be of direct relevance to understanding the developmental basis of heart block and pediatric arrhythmias.

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