Origins and patterning of avian outflow tract endocardium

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Summary

Outflow tract endocardium links the atrioventricular lining, which develops from cardiogenic plate mesoderm, with aortic arches, whose lining forms collectively from splanchnopleuric endothelial channels, local endothelial vesicles, and invasive angioblasts. At two discrete sites, outflow tract endocardial cells participate in morphogenetic events not within the repertoire of neighboring endocardium: they form mesenchymal precursors of endocardial cushions.

The objectives of this research were to document the history of outflow tract endocardium in the avian embryo immediately prior to development of the heart, and to ascertain which, if any, aspects of this history are necessary to acquire cushion-forming potential. Paraxial and lateral mesodermal tissues from between somite 3 (midbrain level) and somite 5 were grafted from quail into chick embryos at 3-10 somite stages and, after 2-5 days incubation, survivors were fixed and sectioned. Tissues were stained with the Feulgen reaction to visualize the quail nuclear marker or with antibodies (monoclonal QH1 or polyconals) that recognize quail but not chick cells. Many quail endothelial cells lose the characteristic nuclear heterochromatin marker, but they retain the species-specific epitope recognized by these antibodies.

Precursors of outflow tract but not atrioventricular endocardium are present in cephalic paraxial and lateral mesoderm, with their greatest concentration at the level of the otic placode. Furthermore, the ventral movement of individual angiogenic cells is a normal antecedent to outflow tract formation. Cardiac myocytes were never derived from grafted head mesoderm. Thus, unlike the atrioventricular regions of the heart, outflow tract endocardial and myocardial precursors do not share a congruent embryonic history.

The results of heterotopic transplantation, in which trunk paraxial or lateral mesoderm was grafted into the head, were identical, including the formation of cushion mesenchyme. This means that cushion positioning and inductive influences must operate locally within the developing heart tubes.

Key words: angiogenesis, heart development, quail–chick chimeras.

Introduction

The developing vertebrate heart first appears in the ventral midline as two concentric epithelial cylinders, the myocardium and endocardium, separated by a thick basement membrane complex, the cardiac jelly. These tubes form simultaneously in a rostral-to-caudal sequence from bilateral primordia that have moved medially and downward concomitant with folding of the endoderm to close the pharynx.

The endocardium of the rostral part of the heart, which is called the outflow tract and includes truncal and distal bulbar (conal) regions, is initially connected to aortic arches 1 and 2, both of which are transient. By the fifth day of incubation these arches have degenerated and the outflow tract connects to derivatives of the 3rd and 4th aortic arches and the pulmonary arteries via the aortic sac. During this period the heart shifts caudally, leaving few traces of its rostral subcephalic origins.

Within the outflow tract, and also at the atrioventricular junction, many endocardial cells delaminate, forming presumptive cushion mesenchyme cells. First seen at stage 17–18 (3-days) in the chick embryo, these cushion cells proliferate rapidly and seed the entire thickness of the cardiac jelly (reviewed in Markwald et al. 1990). In contrast, cushion mesenchyme in the distal part of the outflow tract is derived from neural crest cells (Kirby et al. 1983; Sumida et al. 1989).

Regarding the origins of cardiac tissues, fate mapping studies have identified precursor populations in lateral splanchnic mesodermal of late gastrula-staged embryos. Within these bilateral regions, called the cardiogenic plates, some cells become committed to form cardiac myocytes by the early neurula stage (DeHaan, 1963b). In amphibian embryos this determination is facilitated by interactions with pharyngeal endoderm (Jacobson, 1960; Sater and Jacobson, 1989; Lemanski et al. 1979).

The tacit assumption based on numerous descriptive studies is that both endocardial and myocardial precursors are colocalized within the cardiogenic plates,
and remain together during heart morphogenesis. Unfortunately, most of these mapping analyses focussed on myocardial origins. The presence of endocardial vesicles within myogenic splanchic mesoderm from the early cardiogenic plates stage through fusion of left and right primordia was taken as proof of the developmental congruence of these two precursor populations.

The elegant fate mapping studies of Glenn Rosenquist (1966, 1970) suggested that some members of these two lineages in fact lack congruence in the chick embryo. He grafted pieces of [3H]thymidine-labeled chick embryonic disc (ectoderm+mesoderm+endoderm) to stage-matched host embryos at gastrula stages, and examined sections of survivors up to two days later. Rosenquist's most critical observation is that beginning at stage 5 (head process stage) precursors of myocardium and some endocardium segregate and exhibit markedly different behaviors. Myocardial cells remain as an intact epithelial sheet that moves ventrally and medially to form the outer heart tube. In contrast, some cardiogenic plate cells break away and move rostrally, becoming interspersed with craniofacial mesodermal cells. While the specific locations of these cells in the head varied with the exact site of transplantation, progeny of some grafted cells were found in pre-otic paraxial mesoderm at stage 9, and later in the outflow tract endocardium, aortic arches, and cranial cardinal venous plexus.

The quail–chick chimera system has been used to trace morphogenetic movements and identify fates of many craniofacial tissues, including neural crest cells (LeLièvre and Le Douarin, 1978; Noden, 1978, 1983a), placodal cells (D'Amico-Martell and Noden, 1983), neural epithelium (Couly and Le Douarin, 1987), surface ectoderm (Couly and Le Douarin, 1990) and mesoderm (Noden, 1983b, 1986). These analyses have been possible because of the prominent nucleolar heterochromatin condensation unique to quail cells. The production of antibodies that detect endothelial and hemoblastic cells in quail but not chick embryos and adults (monoclonals: Peault et al. 1983; Pardanaud et al. 1987; polyclonals: Lance-Jones and Langenauer, 1987; Noden, 1988, 1989) has permitted direct analyses of angioblast origins and endothelial tissue assembly. Studies using these antibodies have shown that endothelial cell precursors are found throughout most intra-embryonic mesodermal tissues in the 5-somite embryo (Noden, 1989, 1990a; Coffin and Poole, 1988; Poole and Coffin, 1988, but see Pardanaud et al. 1989).

Transplantation of quail mesoderm into chick hosts has revealed the widespread presence of highly invasive angioblasts. These are single cells that are committed to the endothelial lineage and move extensively through mesenchymal tissues before forming endothelial tissues (Noden, 1989, 1990b; Christ et al. 1990). Invasive angioblasts are present prior to the onset of blood vessel morphogenesis in all head mesoderm except the notochord and prechordal plate and in somites and their precursors. They are also endogenous to prospective limb bud mesoderm (Noden and Li, 1990; Feinberg and Noden, 1991; Rosenquist and Noden, unpublished observations). Blood vessel development resulting from invasive angioblast movements augments both angiogenesis, which is growth by sprouting and branching of existing vessel walls, and in situ formation of endothelial vesicles that anastomose with each other and with angiogenic cords (Sabin, 1917; reviewed by McClure, 1921; Wagner, 1980; Noden, 1989, 1990a,b).

The initial objective of the experiments presented herein is to define whether head paraxial and adjacent lateral mesoderm contains angioblasts that contribute to the avian endocardium, as suggested by Rosenquist, and subsequently to cushion mesenchyme. This has been accomplished by transplanting head mesoderm at different axial locations from quail into chick embryos, and examining for labeled (i.e. quail) cells in the outflow tract two to four days later.

The developing heart exhibits many asymmetries and regional differences. As a result of cell shape changes and uneven rates of myocardial proliferation, the bulboventricular loop bends initially to the right side (Manasek et al. 1972). Prior to the site-specific formation of cushions, local differences in trabeculation and pacemaker depolarization rates are evident; the latter are programmed in subsets of myocardial precursors in the cardiogenic plates, suggesting that initiation of both organ- and regional-specific myocardial characters is initiated during gastrulation (Barry, 1942; DeHaan, 1963b). However, myocardial tissue retains regulative capacity with respect to its rostrocaudal axis until the onset of somitogenesis (Orts Llorca and Collado, 1967). This may be related to the ability of endoderm to affect the behavior of individual mesenchymal cells (DeHaan, 1963a; Linask and Lash, 1986; Reiss and Noden, 1989; Davis et al. 1989).

How these early spatial programming events occur is unknown. Tissue recombination experiments (Mjaatvedt et al. 1987) indicate that the sites of cushion mesenchyme formation are characterized by unique factors localized in the cardiac basement membrane complex. These are produced largely, perhaps exclusively, by myocardial cells surrounding outflow tract and atrio-ventricular junction regions (Krug et al. 1985, 1987; Kitten et al. 1987). It is not known whether putative cushion mesenchyme-forming cells represent a unique endocardial subpopulation based on their prior history, or are induced solely in response to localized changes in basement matrix composition.

The second objective of this study is to determine whether the ability of angioblasts to form outflow tract endocardium and cushions is obligatorily linked to their embryonic history. This issue has been resolved by grafting a variety of normally non-cardiogenic angioblasts into the head region and following their movements and patterns of cytodifferentiation.

**Materials and methods**

**Transplantations**

Orthotopic grafts of head mesoderm, including paraxial
Fig. 1. These illustrate the discrepancy in quail endothelial cell identification based on staining using either the Feulgen method (A,C) to reveal the nucleolar heterochromatin marker or monoclonal (B) or polyclonal (D) antibodies. A and B are the same section from the distal outflow tract of a normal stage 21 quail embryo. The quail nuclear marker is clearly visible in myocardial and cardiac mesenchymal cells (most of which are probably neural-crest-derived) but many endocardial nuclei lack this characteristic heterochromatin condensation (arrows). In B all endocardial cells are labeled with the QH-1 antibody. C and D are sections 16 μm apart from a quail-chick chimera (orthotopic graft of lateral mesoderm, at the level of somitomere 5-6) fixed at stage 19. A few cells displaying the quail nuclear marker are visible (arrows). However, this method fails to identify the actual number of quail endothelial cells which are revealed in D by the anti-quail antibody. The overall distribution of quail endothelial cells in the arteries of this embryo is shown in Fig. 4 (bottom). A,B, 420x; C,D, 280x.
mesoderm from approximately somitomere 3 (beside the midbrain) through somite 5 and lateral mesoderm from the middle third of this region, were performed at Hamburger-Hamilton (1951) stages 8.5 through 9.5 (3 to 7 somite stages). Unlike somites, somitomeres are domains in a continuous mesenchyme and the shallow, superficial demarcations between them are not grossly visible. Therefore, the identity of a somitomere is an approximation based on its relation to more easily recognized neural tube landmarks, as identified by Meier (1981, 1982). Similarly, there are no visible features delineating paraxial from lateral mesoderm in the head; this is an arbitrary distinction using the lateral border of the pharyngeal roof as a boundary.

Host embryos for heterotopic grafts were of a similar stage, but quail donor tissues included mesoderm located caudal to somite 5, often within the segmental plate, from embryos at stages 7 through 10. Heterotopic grafts from these different donor sites and stages produced identical results, and the data have been pooled.

The methods that I use to transplant tissues between quail and chick embryos have been detailed elsewhere (Noden, 1978, 1983a,b, 1986). Fertilized eggs from White Leghorn chickens (Gallus gallus domesticus) and Japanese quail (Coturnix coturnix japonica) are incubated at 37°C (±1°C) at 70% humidity for 28-40 h then windowed to expose the embryos, which are stained with 0.2% neutral red. In this study, grafted tissues typically were 100-150 μm in length, which is slightly longer that one somite. Many contained a patch of overlying surface ectoderm to help maintain their integrity and orientation. The presence or absence of the latter does not affect subsequent development of angioblasts.

A total of 94 chimeric embryos containing quail angioblasts and endothelial cells have been examined. Of these, 38 contained labeled endocardial cells and are described in this study; the rest contained quail angioblasts that did not contribute to the heart and have been described previously (Noden, 1989). Most embryos were fixed in Carnoy's or Zenker's between 12 h and 9 days after surgery, with the greatest number fixed at stages 18-25 (3-5 days). Paraffin sections cut at 8 μm were cleared and hydrated. Representative sections were Feulgen stained to identify quail cells based on the presence of the nucleolar marker; the remaining serial sections were treated with antibodies to quail endothelium. Reconstructions of endothelial channels were done by tracing every 3rd section using the Eutectics VDP-3 system; these outputs were scanned, then displayed using Adobe Illustrator on a Macintosh IIX.

**Immunocytochemistry**

Two primary antibodies have been used: QH1, a mouse monoclonal antibody produced by Pardanaud et al. (1987) and available through the Developmental Biology Hybridoma Bank, and polyclonal chick anti-quail antibodies. The latter were produced using the method described by Lance-Jones and Lagenauer (1987); both serum- and yolk-derived antibodies have been used. Following quenching of endogenous peroxidase with 3% H₂O₂, blocking of nonspecific binding with 1:20 normal goat serum, and application of primary antibody (1:200-1:800) for 30 min at room temperature, secondary antibody was added for 30 min. This was either biotinylated goat anti-chick immunoglobulin (KPL, 1:300) or biotinylated horse anti-mouse IgG (Vector, 1:200). Antibody was visualized using the streptavidin-biotin-peroxidase kits from Amersham, with nickel-cobalt intensification. Control slides lacking primary antibody were routinely included. Some sections were lightly counterstained with thionin or cresyl violet.

Fig. 2. Sagittal section showing the outflow tract-ventricle junction and branchial arches 1 (mandibular) and 2 (hyoid) of a stage 22 chimeric embryo (heterotopic graft of segmental plate mesoderm in place of somitomere 5). Discrete patches of quail endocardial cells (arrows) are interspersed among unlabeled chick endocardium. 108x.

Fig. 3. Section showing the graft (encircled) in a stage 26 chimera. Quail mesenchymal cells are faintly immunopositive, but are easily distinguished from endothelial cells of quail (dark) or chick (unstained) origin. Note that some host angioblasts have entered the graft. 180x.
Results

Validation of the antibodies

Application of anti-quail endothelial antibodies to sections from chimeric embryos results in the staining of many endothelial cells that do not exhibit the characteristic quail nuclear marker following Feulgen staining (Fig. 1). There are three possible explanations for this consistent observation: (1) the antibodies are not species specific, (2) the carbohydrate epitope recognized by the antibody can transfer from quail to adjacent chick tissues, or (3) the quail heterochromatin marker is not retained in all endothelial cells. Control slides in which primary antibody was deleted excluded nonspecific staining by secondary antibodies as a basis.

Alternative no. 1 can be eliminated; labeled cells in chimeric embryos are found only in tissues surrounding the implant, and are never seen in normal chick embryos. The second explanation is negated for several reasons. Fig. 1D shows immunopositive cells as a continuous endocardial sheet with abrupt boundaries. The position of this labeled sheet corresponds to the original right distal cardiac anlage. Moreover, there is no range of labeling intensity, which would be expected if the cell surface carbohydrate was spreading nonspecifically to neighboring endothelial cells. This dichotomy between labeled and unlabeled cells is most apparent in chimeric vessels that show multiple interspersed patches (clones?) of quail and chick cells (Fig. 2).

Finally, the quail nuclear marker is often not detectable in endothelial cells of normal quail embryos (Fig. 1A,B). Thus, presence of the nucleolar heterochromatin marker is not a reliable marker for quail endothelial cells.

Angioblast dispersal

Most grafted mesodermal cells are not angiogenic. These remain as an intact mass, often irregular in shape, usually located dorsolateral to the roof of the pharynx (Fig. 3) or beneath lateral surface ectoderm. Laterally placed grafts occasionally shifted ventrally during lateral body folding, and were subsequently found near the root of the tongue. Previous studies have documented the subsequent myogenic and chondrogenic differentiation of grafted cephalic mesodermal cells (Noden, 1983b, 1986).

From the implant, invasive angioblasts disperse in all directions, reaching dorsally to thealar region of the mid- and hindbrain, rostrally to the optic vesicle, and ventrally to the heart. Fig. 4 illustrates the contributions of implant-derived angioblasts to the major arterial channels in the head in two cases. The widespread contributions of quail angioblasts to veins and smaller blood vessels, especially the meningeal plexus, is illustrated in Fig. 5.

Origin of outflow tract endocardial cells (n=13)

Orthotopic grafts of craniofacial paraxial mesoderm reveal that outflow tract endocardial precursors are located at the level of somitomeres 4 through 7 in the...
5-somite embryo. Their numbers are most abundant in the somitomere 5–6 region, which flanks the rhombencephalon immediately rostral to and underlying the otic placode primordium. The more rostral precursors contribute preferentially but not exclusively to aortic sac and distal outflow tract endocardium. A greater number of labeled outflow tract endocardial cells were present when grafts were placed more laterally, but medial paraxial mesoderm does contain endocardial precursors.

Both the number and the distribution of quail cells in the outflow tract endocardium varied greatly (Figs 6 and 7). In some cases only one or a few single cells or isolated patches were found, while in others large sheets of labeled cells constituted up to half the perimeter of the endocardium (Fig. 1D). Two animals contained single patches of quail endocardial cells in the right ventricle (Fig. 8), and five had labeling in the sinus venosus. The latter were continuous with heavy quail angioblast contributions to the common and cranial cardinal veins and the lateral thoracic plexus. In no cases were quail cells found in the myocardium.

In four of these orthotopic chimeras immunopositive mesenchymal cells were evident beneath labeled endocardial cells (Fig. 9). A few of these cushion cells also expressed the quail nucleolar marker, which mimics the situation observed in normal quail embryos. Quail mesenchymal cells were found only in proximity to patches of quail endocardium and were juxtaposed

Fig. 7. In these cases large areas of outflow tract endocardium were formed by grafted quail angioblasts. (A) Stage 16.5 chimera, also shows labeled cells in the 3rd aortic arch (3) and dorsal aorta (d.a.). B is a section illustrating proximal outflow tract endocardial cells in a stage 26 embryo. (A) 132×; B, 320×.

Fig. 6. Hearts from chimeric embryos fixed at stages 19 (A) and 25 (B). Grafts had been implanted in the left somitomere no. 5 (A) and right somitomere no. 4 (B). A, 108×; B, 134×.

Fig. 8. This stage 24 chimera is one of only two embryos in which labeled endocardial cells were present in ventricular endocardium (arrow). Note the labeled cushion mesenchyme beneath a patch of quail outflow tract endocardium. 130×.
to, and sometimes interspersed with, chick host cushion cells.

**Specification of outflow tract endocardium (n=25)**

Grafts of trunk somitic or presumptive somitic (segmental plate) mesoderm produced results indistinguishable from the orthotopic series described above.

Fig. 9. Three cases, all fixed at stages 24–25, in which immunopositive cushion mesenchyme cells are present beneath patches of quail endocardium. Note in C that the quail nuclear marker is visible in some immunopositive cushion cells (arrows). (A) 161×; B, 134×; C, 210×.

Fig. 10. Reconstructions of the cephalic arterial system following heterotopic grafting of quail trunk mesoderm. The patterns of distribution of graft-derived angioblasts are similar to those seen in control embryos (Fig. 4).

Reconstructions reveal a similar pattern of labeling to major craniofacial vessels (Fig. 10). Grafted trunk angioblasts invade the region of outflow tract endocardial assembly (Fig. 11) and, in marked contrast to any behavior they would have displayed in their normal, i.e. non-cardiac, terminal locations, these cells participated in the formation of endocardial cushions (Fig. 12).

**Discussion**

These transplantation experiments prove that at early somite stages presumptive outflow tract angioblasts reside within cephalic paraxial and lateral mesoderm, preferentially but not exclusively in a region underlying and slightly rostral and lateral to the otic placode. The patterns of quail cell distribution within the outflow tract endocardium suggest that many of these angioblasts become interspersed with host endocardial precursors prior to or during the initial stages of cardiogenesis. Neither somitomeres 4–6 nor adjacent paraxial and lateral mesodermal populations contribute to atrial or ventricular endothelium, suggesting that the precursors of these more caudal regions of the embryonic heart either do not participate in the widespread movements of angioblasts that has recently been described (Noden, 1989) or do so at some distance from outflow tract precursors. As expected, myocardial precursors are not found within head paraxial mesoderm or lateral mesoderm located above or beside the pharynx.

Given that angioblasts within grafted head mesoderm are highly invasive, it is possible that their antecedents may have immigrated into head somitomeres from
Fig. 11. Contributions of heterotopically grafted trunk segmental plate mesoderm to outflow tract endocardium and, evident in B, cushion mesenchyme. (A) stage 23; (B) stage 25; both 108×.

Fig. 12. Low magnification (62×) and enlarged (177×) micrographs showing the contributions to outflow tract tissues of somitic mesoderm (somites 4 and 5 from a stage 8.5 donor) grafted in place of somitomeres 4 and 5. Several small patches and a large area of endocardium were formed by graft-derived angioblasts. Two quail ventricular endocardial cells are evident (arrows). Note the large population of immunopositive cushion mesenchyme beneath the quail endocardium.
lateral splanchnic mesoderm of the cardiogenic plate, as suggested by Rosenquist (1966, 1970). However, initial studies using retrovirus labeling have not yet identified cardiogenic plate cells seeding any part of the embryo other than the myocardium (Mikawa et al. 1990 and personal communication).

The sites at which angioblasts are initially formed and may reside prior to entering the developing outflow tract is not an important factor in the subsequent assembly and remodeling of this endocardial tissue. The same has been shown previously for the aortic arches and other craniofacial vessels (Noden, 1989) as well as for appendicular vessels (Pardanaud et al. 1989; Feinberg and Noden, 1990). The occasional double identification of cushion mesenchymal cells by the presence of immunostaining and of the nucleolar marker, and the restriction of immunolabeling to areas of the outflow tract also containing labeled endocardium, confirm their origin from local endocardial cells.

These results indicate that the delineation of cushion-forming endocardium is a local, intracardiac event. Previous studies have shown that matrix components produced by myocardial cells of the outflow tract and atrioventricular junction regions are necessary prerequisites for the formation and radial movement of the cushion cells (Mjaatvedt et al. 1987; Markwald et al. 1990). The present data augment these findings, indicating that endocardial cells, at least those of the outflow tract, are not preconditioned to undergo an endothelial–mesenchymal transformation due to a unique prior history.

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