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Conotruncal myocardium arises from a secondary heart field

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SUMMARY

The primary heart tube is an endocardial tube, ensheathed by myocardial cells, that develops from bilateral primary heart fields located in the lateral plate mesoderm. Earlier mapping studies of the heart fields performed in whole embryo cultures indicate that all of the myocardium of the developed heart originates from the primary heart fields. In contrast, marking experiments in ovo suggest that the atrioventricular canal, atria and conotruncus are added secondarily to the straight heart tube during looping. The results we present resolve this issue by showing that the heart tube elongates during looping, concomitant with accretion of new myocardium. The atria are added progressively from the caudal primary heart fields bilaterally, while the myocardium of the conotruncus is elongated from a midline secondary heart field of splanchnic mesoderm beneath the floor of the foregut. Cells in the secondary heart field express Nkx2.5 and Gata-4, as do the cells of the primary heart fields. Induction of myocardium appears to be unnecessary at the inflow pole, while it occurs at the outflow pole of the heart. Accretion of myocardium at the junction of the inflow myocardium with dorsal mesocardium is completed at stage 12 and later (stage 18) from the secondary heart field just caudal to the outflow tract. Induction of myocardium appears to move in a caudal direction as the outflow tract translocates caudally relative to the pharyngeal arches. As the cells in the secondary heart field begin to move into the outflow or inflow myocardium, they express HNK-1 initially and then MF-20, a marker for myosin heavy chain. FGF-8 and BMP-2 are present in the ventral pharynx and secondary heart field/outflow myocardium, respectively, and appear to effect induction of the cells in a manner that mimics induction of the primary myocardium from the primary heart fields. Neither FGF-8 nor BMP-2 is present as inflow myocardium is added from the primary heart fields. The addition of a secondary myocardium to the primary heart tube provides a new framework for understanding several null mutations in mice that cause defective heart development.

Key words: Chick-quail chimeras, Myocardium, FGF-8, BMP-2, Heart development, Secondary heart field, Outflow tract, Conotruncus

INTRODUCTION

The straight heart tube is formed by ventral midline fusion of myocardial and endothelial cells that differentiate from the bilateral primary heart fields located in the lateral plate mesoderm. Cardiac differentiation is initiated in response to an inductive signal from the anterior endoderm (Schultheiss et al., 1995). Expression of smooth muscle α actin and Nkx2.5 in the primary heart fields constitute the first molecular indication of myocardial lineage (Colas et al., 2000; Antin et al., 1994; Lough and Sugi, 2000).

Mapping studies of these primary heart fields have traditionally relied on whole embryo culture of chick embryos where viability of the explanted embryos is limited to early straight heart tube stages. In the earliest mapping studies, it was assumed that the components of the straight heart tube represented all of the endocardial and myocardial progenitors of the adult heart endo/myocardium (Rosenquist and DeHaan, 1966). However, marking experiments performed in ovo at later, looping stages of heart development suggested that the atrioventricular canal, atria and conotruncus are added secondarily to the straight heart tube during looping (Arguello et al, 1975; de la Cruz et al., 1977; de la Cruz et al., 1987; de la Cruz et al., 1989; de la Cruz et al., 1991). According to de la Cruz, the inflow segments are added first, followed by the outflow segments. Recent studies in mice support the idea that the myocardium of the primary heart tube does not represent all of the myocardium of the septated heart (Christoffels et al., 2000).

In support of the data of de la Cruz, we have identified a previously unrecognized ‘secondary’ heart field in the splanchnic mesoderm that underlies the caudal pharynx. The field provides myocardium to the outflow tract during looping. In contrast, the inflow myocardium appears to be accreted from the bilateral primary heart fields. The secondary heart field expresses Nkx2.5 and Gata-4 prior to differentiation as myocardium, and cells are induced to become myocardium in a manner similar to that which occurs in the primary heart fields.

MATERIALS AND METHODS

Embryos

Fertilized Arbor Acre chicken eggs or Japanese quail eggs were incubated at 37ºC and 90% relative humidity until they reached stages 12-22 (Hamburger and Hamilton, 1951). Chick embryos at stage 14 were placed in shell-less culture by transferring the contents of the egg to hexagonal polycarbonate weigh boats that were then placed in 15 cm Petri dishes with 0.5 cm of distilled water in the bottom of the dish.

For injections, Mitotracker (50 μg, Molecular Probes) was dissolved in 1 ml DMSO and diluted 1:10 with phosphate buffered saline (PBS). Pulled capillaries were used to inject approximately 1-2 nl of the diluted dye into the splanchnic mesoderm just caudal to the cardiac outflow tract. The embryos were reincubated for 48 hours or until they reached stage 22.

To produce chimeras, quail embryos at a comparable stage were dissected from the yolk and placed in PBS. The splanchnic mesoderm just behind the cardiac outflow tract was removed and transferred to the same site in an explanted chick embryo or into the lateral wall of the pharynx. The chick embryos were reincubated for 48 hours or until they reached stage 22.

Embryos for immunohistochemistry or in situ hybridization were collected in DEPC-treated phosphate buffered saline (PBS) and immersion fixed in 4% paraformaldehyde in PBS.

Tissue culture

Conditioned medium was made by culturing ventral pharynx, myocardium or secondary outflow heart field from chick embryos at stage 14-15 overnight in a small volume of Dulbecco's minimal eagles medium (DMEM) with 6% fetal bovine serum. Secondary outflow heart field was removed from embryos at stages 14-15 and cultured overnight in 20 μl of conditioned medium. 5-Bromo-2′deoxyuridine (BrdU, 20 μl of a 40 mM solution, Boehringer) was added to the cultured heart fields for 1 hour before fixation in 4% paraformaldehyde in phosphate-buffered saline as reported by Brand-Saberi et al. (Brand-Saberi et al., 1995). Fixation was carried out for 10 minutes followed by double immunohistochemistry as described above.

Immunohistochemistry

The embryos were embedded in paraffin and sectioned at 8 μm. The sections were incubated as described previously (Waldo et al., 1998). HNK-1 (obtained from American Type Culture Collection, Rockville, MD) was visualized with Alexa 488 goat anti-mouse IgG conjugate (Molecular Probes, Eugene, OR) as reported previously (Waldo et al., 1996). MF20 (red) and QH-1 (green) were visualized with Alexa 568 goat anti-mouse IgG conjugate (Molecular Probes). The MF20, developed by Donald A. Fischman, and QH-1 were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. The mouse ABC Elite kit (Vector) was used for the secondary reaction with DAB visualization. Details of the protocol have been described previously (Waldo et al., 1996).

Quail cells in the quail-chick chimeras were visualized with QCPN, a quail-specific antibody, as described previously (Waldo et al., 1998). Chick Slug antibody was used at 1:50 with the ABC Elite kit for the secondary reaction. The Slug antibody, developed by Thomas M. Jessell, was obtained from the Developmental Studies Hybridoma Bank. BrdU was visualized using the Zymed Kit with DAB as the secondary antibody. The preparations were counterstained with hematoxylin (Anatech, Battle Creek, MI).

Isolation and sequence analysis of cDNA

The GATA-4 probe was made from a GATA-4 PCR fragment cloned by Todd Evans (clone 7) and sent via Katherine Yutzey. A 221 bp fragment of chick Nkx2.5 (Accession Number X91838) was made using RT-PCR with primers and protocol as described previously (Schultheiss et al., 1995). A 225 bp fragment of FGF-2 was cloned using the primers as described by Désiré et al. (Désiré et al., 1998) and cDNA from stage 18 whole embryos. A 239 bp fragment of FGF-4 (Accession Number U14654) was made using primers spanning the region of 321-559bp and cDNA from stage 18 whole embryos. A 393 bp fragment of FGF-8 (Accession Number U55189) was made using primers spanning the region of 17-409 bp and cDNA from hearts from stage 14-18 embryos. A 337 bp fragment of BMP-2 (accession number X75914) was made using primers spanning the region of 239-575 bp and cDNA from stage 14 and 18 embryonic hearts. A 283 bp fragment of BMP-4 (accession number X75915) was made using primers spanning the region of 11-293bp and cDNA from stage 18 whole chick embryos. The fragments were cloned into the vector PCRII (Invitrogen) and sequenced to ensure their identity. PCR fragments containing one of the promoter primers and one of the gene specific primers were generated and labeled with digoxigenin.

In situ hybridization and sectioning of stained embryos

The in situ hybridization was carried out with digoxigenin-labeled riboprobes generated from the cDNA fragment cloned into pCRII (Invitrogen) using one promoter primer and one of the gene specific primers. The protocol followed that described by Wilkinson (Wilkinson, 1992). After examination and documentation of whole-mount staining, the embryos were embedded in paraffin, sectioned transversely at 12 μm and mounted.

RESULTS

Expression of Nkx-2.5 and Gata-4 between the inflow and outflow tracts

Because of our previous interest and expertise in outflow tract development, we first sought a source of myocardial cells to be added to the outflow myocardium. As both Nkx-2.5 and Gata-4 are expressed in the primary heart fields, and are believed to be required for normal myocardial development (Schultheiss et al, 1995; Jiang et al., 1998; Reecy et al., 1999), we examined the mesenchyme adjacent to the outflow of the primary heart tube for Nkx-2.5 and Gata-4 expression at stages 12-18, when new myocardial cells should be added to the outflow tract. Nkx-2.5 was expressed in the ventral pharyngeal mesenchyme between the attachments of the cardiac outflow and inflow tracts at stages 12-18 (Fig. 1A-C). Gata-4 was expressed in the ventral pharyngeal endoderm at earlier stages, but by stages 14-16 was expressed in the Nkx-2.5-positive mesenchyme located dorsal and caudal to the outflow tract (Fig. 1D,E). Both Nkx-2.5 and Gata-4 were expressed in the caudal portion of the distal outflow tract. These results, in combination with those of Sater and Jacobson (Sater and Jacobson, 1990) showing that pharyngeal endoderm retains potency for heart induction until after the third pharyngeal arch is formed, suggest that the splanchnic mesoderm, which underlies the floor of the pharynx and is directly continuous with the outflow and inflow myocardium, could be the site of addition of cells needed for accretion of the definitive outflow segments to the primary cardiac tube.

HNK-1 but not SLUG is expressed by cells in the secondary heart field

HNK-1 was expressed in the Nkx2.5/Gata-4-positive splanchnic mesoderm underlying the pharyngeal endoderm. We have previously noted the expression of HNK-1 in this

mesenchyme and determined through quail-chick chimeric embryos that it is not associated with neural crest (K. L. W. and M. L. K., unpublished). Because of its appearance in other tissues and, during development, at the cardiac inflow and outflow tracts, it appears to be expressed by cells undergoing translocation or differentiation (Vincent and Thiery, 1984; Tucker et al., 1984; Canning and Stern, 1988). From stage 14 onwards, the myocardium of the caudal part of the distal outflow tract was particularly HNK-1-positive, as was the splanchnic mesoderm below the floor of the pharynx immediately caudal to the outflow tract (Fig. 2A-D). The timing of HNK-1 expression in these regions correlated with the period when the secondary or definitive outflow segments are added to the heart tube as shown in the marking experiments of de la Cruz and colleagues (Arguello et al., 1975; de la Cruz et al., 1977; de la Cruz et al., 1987; de la Cruz et al., 1989; de la Cruz et al., 1991).

To examine whether these cells were undergoing a classical epithelial-to-mesenchymal transformation, we used an antibody to Slug, which is expressed in cells undergoing epithelial-to-mesenchymal transformation (Nieto et al., 1994; Romano and Runyan, 1999; Carmona et al., 2000). Slug was expressed in the myocardium of the looping heart as previously reported (Carmona et al., 2000), but not at its poles or in the HNK-1-positive splanchnic mesenchyme caudal to the outflow tract (Fig. 2E).

To determine whether the HNK-1-positive cells at the ends of the cardiac tube expressed myocardial contractile proteins we used double immunohistochemistry for HNK-1 and MF20, a standard myocardial cell marker (Han et al., 1992). In both poles of the heart we found that HNK-1 was expressed in the Nkx2.5-positive mesenchyme below the pharyngeal endoderm. The HNK-1-positive cells in the mesenchyme most distant from the myocardium were negative for MF20 (Fig. 3A,C, green). However, some of the HNK-1-positive cells that were closer to the myocardium were also positive for MF20 (yellow cells in 3A,C). Myocardium more centrally located in the tubular heart, that is, more distant from the attachments of the inflow and outflow to the pharynx, was HNK-1-negative, MF20-positive (Fig. 3A,C). Caudal to the outflow tract, the midline splanchnic mesoderm ventral to the pharynx was strongly HNK-1-positive (Fig. 3B, arrow).

Proximity to the cardiac outflow tract initiates myocardial differentiation

To demonstrate directly that the outflow tract lengthens by accretion of cells from these secondary centers, mitotracker, a fluorescent dye, was injected into the splanchnic mesoderm behind the outflow tract at stage 14 (Fig. 4A). The hearts were examined using confocal microscopy at stage 22. The myocardium of the most proximal segment, the conus, was fluorescent while that of the distal segment, the truncus arteriosus, did not fluoresce (Fig. 4B), indicating that two new segments were added to the outflow tract between stages 14 and 22. As the truncal myocardium was not fluorescent, it was most probably generated after the time of the injection and arose from an uninjected region of the splanchnic mesoderm. Significantly, after the injection, the splanchnic mesenchyme caudal to the outflow tract at stage 22 was not fluorescent, but a tiny spot of fluorescence, signifying the original site of injection, could be seen cranial to the attachment of the outflow tract to the body wall (Fig. 4B, arrow). An examination of the outflow attachment to the ventral pharynx showed that the attachment point has a dynamic relationship with the pharynx and is located progressively more caudally. Thus, at stage 12 the outflow joins the pharynx ventral to pharyngeal arch/artery 1, and by stage 24 is located ventral to pharyngeal arches/arteries 4-6 (Fig. 5).

Quail-chick chimeras were generated to examine the potential of the ventral pharyngeal mesoderm for differentiating as myocardium. Donor tissue from quail ventral pharyngeal mesenchyme was transplanted into the corresponding region in chick embryos at stage 14 (Fig. 4A). The embryos were collected at stage 22 and double-labeled for an anti-quail antibody (QCPN) and MF-20. The quail implants formed rounded bodies comprised of quail cells, which were encircled by MF-20-positive myocardium derived from the quail graft (Fig. 4C,D). When similar grafts were implanted in the lateral wall of the pharynx, not in proximity to the cardiac outflow tract, they formed vesicles that did not contain MF-20-expressing cells (data not shown).

From these results and double immunohistochemistry with HNK-1 and MF-20, it appeared that proximity to the primary heart tube influenced the conversion of splanchnic mesoderm cells to myocardium.

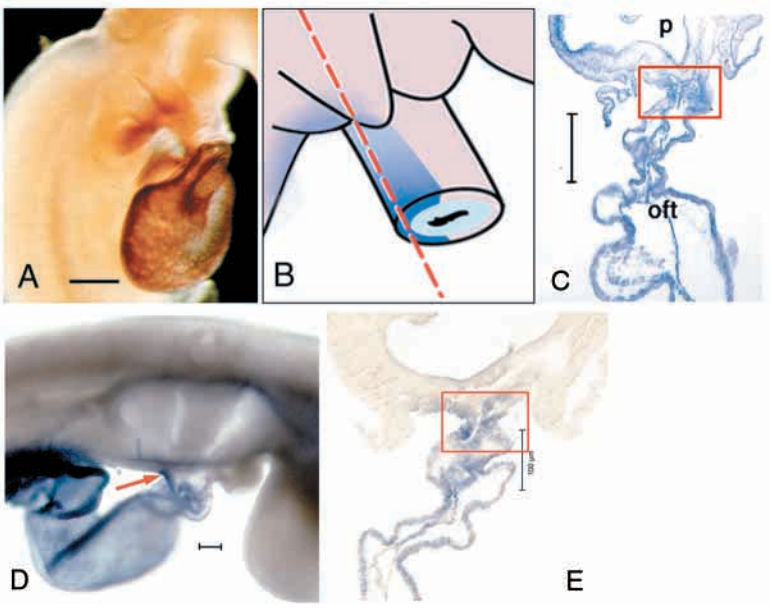

Fig. 1. Nkx2.5 and Gata-4 are expressed in the splanchnic mesoderm of the pharyngeal floor. (A) Whole-mount (stage 14) chick embryos after in situ hybridization for Nkx2.5. (B) Diagram illustrating the distal cardiac outflow tract as it is seen in A. The broken line indicates the plane of section seen in C,E. (C) Nkx2.5 mRNA can be seen in the caudal wall of the distal cardiac outflow tract (oft) and in the mesenchyme dorsal and caudal to the cardiac outflow tract (pharynx, p). (D,E) In situ hybridization showing expression of GATA-4 in the region where Nkx2.5 is expressed (arrow in D). Scale bars: 100 μm in A,C,E; 50 μm in D.

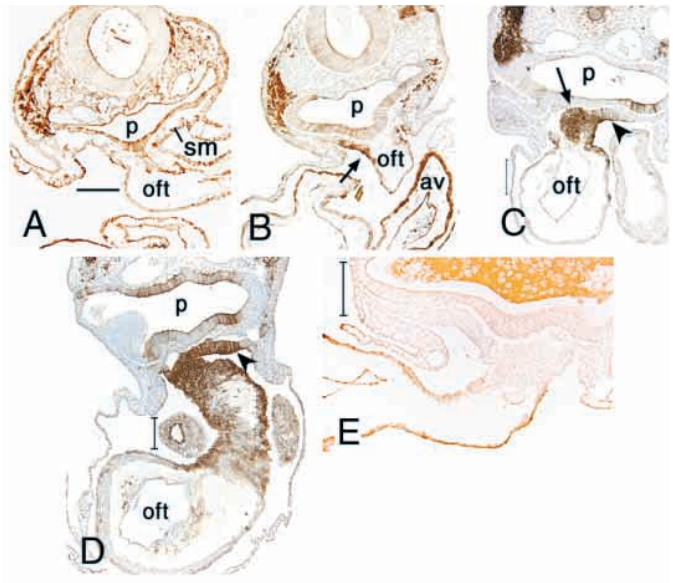

Fig. 2. HNK-1 expression in the splanchnic mesoderm underlying the pharynx at stages 12 (A), 14 (B), 16 (C) and 18 (D). Over time, HNK-1 expression increases in the cranial splanchnic mesoderm and distal outflow tract and decreases in the dorsal mesocardium. (A) Section through the outflow tract (oft) showing its attachment to the ventral pharyngeal wall (p, pharynx). The splanchnic mesoderm (sm) flanking the outflow myocardium is not HNK-1 positive. (B) At stage 14, the cells in the splanchnic mesoderm flanking the primary myocardium of the outflow tract are HNK-1 positive (arrow). (C) HNK-1 expression (arrow) in the distal outflow tract myocardium is continuous with the splanchnic mesoderm caudal to the attachment of the outflow tract to the pharynx (arrowhead). (D) HNK-1 expression in an extended length of caudal outflow myocardium is continuous with that in the splanchnic mesoderm (arrowhead). (E) The myocardium of the outflow tract is positive for Slug antibody but the splanchnic mesoderm is devoid of Slug expression. Scale bars: 100 μm; A and B are the same magnification.

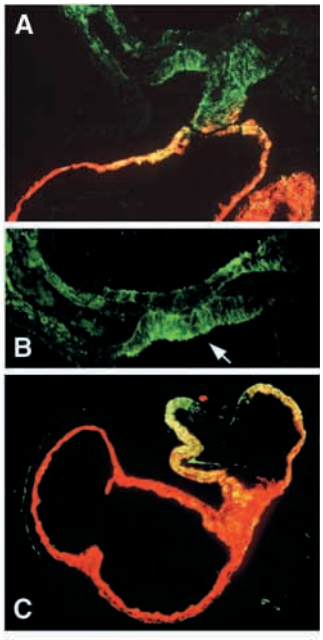

Fig. 3. At stage 16 HNK-1-positive cells express a myocardial phenotype. Double labeling by immunohistochemistry to show the relationship of HNK-1-positive cells (green) to myocardial cells (red) in the cardiac outflow and inflow tracts. The poles of the heart exhibit a gradient of HNK-1 and myosin protein expression during looping, as seen in transverse sections of the outflow and inflow poles of a stage 16 chick embryo double labeled for myosin. (A) The distal outflow tract shows HNK-1-positive/MF20-negative cells in the splanchnic mesoderm and HNK-1-negative/MF20-positive cells in the myocardium, separated by myocardium that is positive for both HNK-1and MF20 (yellow). (B) The splanchnic mesoderm caudal to the attachment of the outflow tract with the pharynx (arrow) is HNK-1 positive/MF20 negative. (C) The atrium displays a gradient of overlapping protein expression, similar to that seen in the outflow myocardium.

Expression of FGF-8 and BMP-2

Because FGF and BMP cytokine family members are essential in induction of myocardial cells from the primary heart fields, we examined the expression of FGF-2, FGF-4 and FGF-8, and BMP-2 and BMP-4 in the pharynx at stages 14 and 18. FGF-2 and FGF-4 and BMP-4 were not expressed in or near the secondary heart field at either stage. At stage 14, FGF-8 was expressed in the lateral pharynx in both the ectoderm and endoderm (Fig. 6A,B). Expression was continuous craniocaudally from arch 1 to the arch 6 region in the ectoderm, but expressed in the endoderm only where it was close or in contact with the ectoderm. Expression in arches 1 and 2 was less than in arches 3-6. By stage 18, the expression of FGF-8 was much reduced so that it was only between the maxillary and mandibular prominences of arch 1 and in the cleft/pouch of arch 2. Expression continued at a reduced level in the lateral ectoderm of arches 3-6 (Fig. 6C,D).

At stage 14, BMP-2 was expressed most strongly in the splanchnic mesoderm just caudal to the outflow myocardium. The myocardium also showed positive expression although less intense than that in the splanchnic mesoderm. The dorsal mesocardium and myocardium of the inflow tract was also positive (Fig. 6E,F,H). It was not expressed in the splanchnic mesenchyme underlying the pharyngeal endoderm between the cardiac outflow and inflow tracts (Fig. 6G). By stage 18, BMP-2 expression was notable in the endocardium but was completely absent from the secondary heart field (Fig. 6I).

Secondary heart field induces myocardial differentiation

To determine whether the lateral endoderm/ectoderm or secondary heart field acted as a signaling center, we explanted various tissues to produce conditioned medium, which was then used in secondary heart field cultures. The secondary heart field was tested for its ability to differentiate as myocardium using MF-20 as a marker. Proliferation was also assessed using BrdU. Conditioned medium was made using explanted lateral pharyngeal endoderm/ectoderm, outflow myocardium or secondary outflow field. Unconditioned medium, or medium conditioned by secondary heart field, distal outflow myocardium or endoderm/ectoderm, induced myocardial differentiation, although MF20 staining was poor or absent in the endoderm/ectoderm conditioned medium (Fig. 7A,B,D). However, in every case exposure to endoderm/ectoderm caused a robust proliferative response in the secondary heart field (Fig. 7C). Proliferation was completely absent in the secondary heart field cultured in secondary heart field-conditioned medium.

Because myocardial differentiation from the primary heart field is dependent on BMP-2 induction, we examined whether BMP-2, which is expressed in both the secondary heart field and outflow myocardium might be necessary for secondary myocardial differentiation. Noggin (15 μg/ml, gift from Dr Richard Harland) was added to a duplicate set of secondary heart field cultures in the various conditioned media. In most cases, myocardial differentiation was less robust in the presence of noggin and proliferation was increased (Fig. 7E-

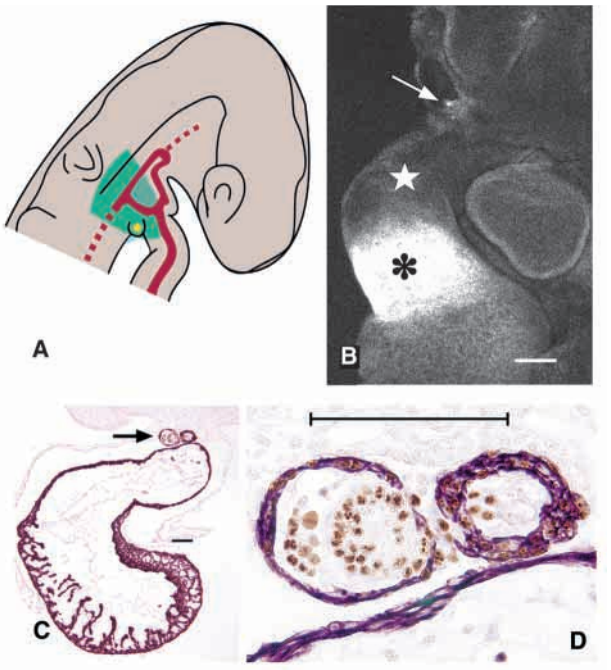

Fig. 4. New outflow tract myocardium is derived from splanchnic mesoderm caudal and adjacent to the outflow. (A) Diagram of a stage 14 chick embryo showing the location of the secondary cardiac outflow field (yellow asterisk), which is located at this stage adjacent to the second arch (green). (B) Confocal image of a stage 22 chick embryo injected into the secondary heart field just caudal to the outflow tract with mitotracker at stage 14. Two new segments, a proximal fluorescent segment (asterisk) and a more distal non-fluorescent segment (star), were added to the primary tube between the time of injection at stage 14 and acquisition of this image at stage 22. The fluorescent spot (white arrow) cranial to the outflow tract shows the needle entry site for the mitotracker injection, which was originally posterior to the outflow tract. (C) The secondary heart forming region was removed from a stage 14 quail embryo and placed in the comparable site of a chick embryo at a similar stage. A transverse section at the level of the distal outflow myocardium shows two small vesicles with quail cells (QCPN antibody, brown) adjacent to the outflow tract (arrow). (D) Higher magnification of the vesicles reveals an inner lumen lined with quail cell epithelium, surrounded by MF20-positive (purple) myocardial cells. Scale bars: 100 μm.

H). However, myocardial differentiation appeared unaffected in the secondary heart field cultured in secondary heart field-conditioned medium, which could perhaps be explained by the combined presence of abundant BMP-2 from the conditioned medium and the cultured secondary heart field itself overriding the noggin inhibition. This also might explain the paucity of proliferation in these cultures with or without noggin. Proliferation in the endoderm/ectoderm-conditioned medium with noggin was remarkable for its abundance although it would be difficult to make the case that noggin altered proliferation in these cultures.

Accretion of myocardium by the cardiac inflow tract is from the primary heart fields

The inflow does not lengthen much after stage 12, which means that it is finished by the time secondary myocardium is being added to the outflow tract at stage 14. We examined

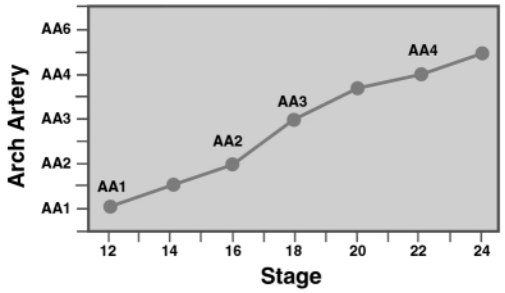

Fig. 5. Position of the outflow tract over time, relative to the aortic arch arteries.The outflow tract has a dynamic relationship with the pharynx during the looping stages. The outflow tract originally attaches ventral to arch 1 and is progressively displaced caudally to lie below pharyngeal arches 4 and 6. AA1-4, aortic arch arteries 1-4.

the possibility that accretion of myocardium to the inflow tract is also from a secondary heart field. At stage 11, the inflow end of the heart and its attached dorsal mesocardium was HNK-1 positive, while the remaining heart tube was negative (Fig. 8A). Subsequently, the mesenchyme adjacent to the inflow lost its HNK-1 reactivity while the mesocardium just behind the outflow myocardium became positive (Fig. 2C). NKX2.5 and GATA-4 were expressed by the inflow myocardium, dorsal mesocardium and extended slightly into the splanchnic mesoderm underlying the pharynx (Fig. 8B,D), while myocardial differentiation occurred slightly into the dorsal mesocardium but not into the splanchnic mesoderm (Fig. 8C). Neither BMP-2 nor FGF-8 was expressed in a pattern that suggested induction in the same manner as that observed for the cardiac outflow tract (Fig. 8E,F). Because of the continuity of the cardiac inflow tract with the sinus venosus and systemic veins, all of which are invested in myocardium (Franco et al., 2000), it appeared that accretion of myocardium that occurs close to the time that the single heart tube is fusing could be a continuation of this process of fusion. In this case the myocardium would be added from the primary heart fields rather than from a special secondary heart field, as appears to happen in building the definitive outflow tract.

DISCUSSION

These results show that (1) new segments are added to the straight heart tube during looping; (2) the definitive outflow myocardium is generated by splanchnic mesoderm caudal to the primary outflow tract, while the definitive inflow myocardium appears to be generated from a caudal continuation of the primary heart fields bilaterally; and (3) the secondary heart field generates outflow myocardium via similar signaling pathways and transcriptional regulation as cells in the primary heart fields although the sources of inductive signals are different because (4) proximity to the primary myocardium is necessary for differentiation of the secondary myocardium. Because these cells originate from splanchnic mesoderm distinct from the primary heart fields, it seems appropriate to refer to it as a secondary heart field. The secondary heart field generates ‘secondary’ or definitive outflow myocardium, which is distinct from myocardium of

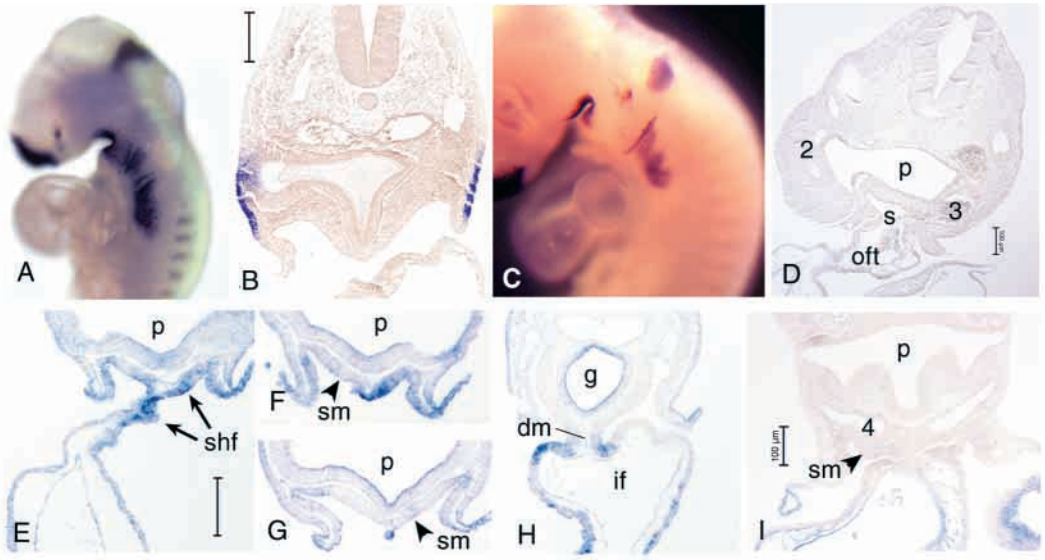

Fig. 6. FGF-8 (A-D) and BMP-2 (E-I) expression at stages 14 and 18. (A) FGF-8 expression in the lateral pharynx. Moderate expression in arches 1 and 2 with pronounced expression in arches 3-6. FGF-8 expression persists in the pharyngeal pouch/grooves after expression in the arches has diminished. (B) Sectioned whole-mount embryo showing that FGF-8 expression in arch 3 is in the ectoderm covering the pharyngeal arches and in the endoderm where it meets the ectoderm in the pharyngeal pouches. (C) FGF-8 expression at stage 18 is diminished in the caudal pharyngeal arches and completely absent in arches 1 and 2. Expression remains in the interval between the maxillary and mandibular prominences and the second pouch/groove. (D) Sectioned stage 18 whole-mount showing the greatly diminished FGF-8 expression in arches 2 and 3. (E) BMP-2 expression in the caudal outflow tract and splanchnic mesoderm that is continuous with the outflow tract at stage 14. (F) BMP-2 expression at stage 14 stops abruptly caudal to the outflow tract in an oblique section. (G) The splanchnic mesoderm between the inflow and outflow at stage 14 is devoid of BMP-2 expression. (H) BMP-2 expression can be seen in the inflow myocardium and dorsal mesocardium at stage 14. (I) By stage 18, BMP-2 is no longer expressed by the splanchnic mesoderm or myocardium but can now be seen in the endocardium. All scale bars are 100 μm.

the primary heart tube because of its different origin and time of addition to the heart tube.

The outflow myocardium has been recognized for a long time as being molecularly distinct from ventricular myocardium (Ruzicka and Schwartz, 1988). The primary myocardium begins to regionalize and proceeds to maturation through a set program of contractile isoform expression, while the outflow myocardium retains characteristics of myocardium in the primary heart tube (Christoffels et al., 2000). For example, expression of α smooth muscle actin, the first myocardial contractile gene to be expressed in myocardial cells derived from the primary heart fields (Colas et al., 2000), continues long after the ventricular myocardium has stopped expressing the smooth muscle isoform in favor of cardiac α actin (Ruzicka and Schwartz, 1988). During septation, the outflow myocardium is incorporated into the right ventricular outflow tract, although some of the myocardial cells undergo apoptosis during this process (Watanabe et al., 1998). The fact that the definitive outflow myocardium is incorporated from a separate heart field well after the primary heart tube has formed helps to explain why it is different from the primary myocardium.

The transformation of cells from the secondary heart field into myocardium appears to require many of the same steps undertaken by cells in the primary heart fields (Fig. 9). The primary heart fields are separated, paired heart fields in the

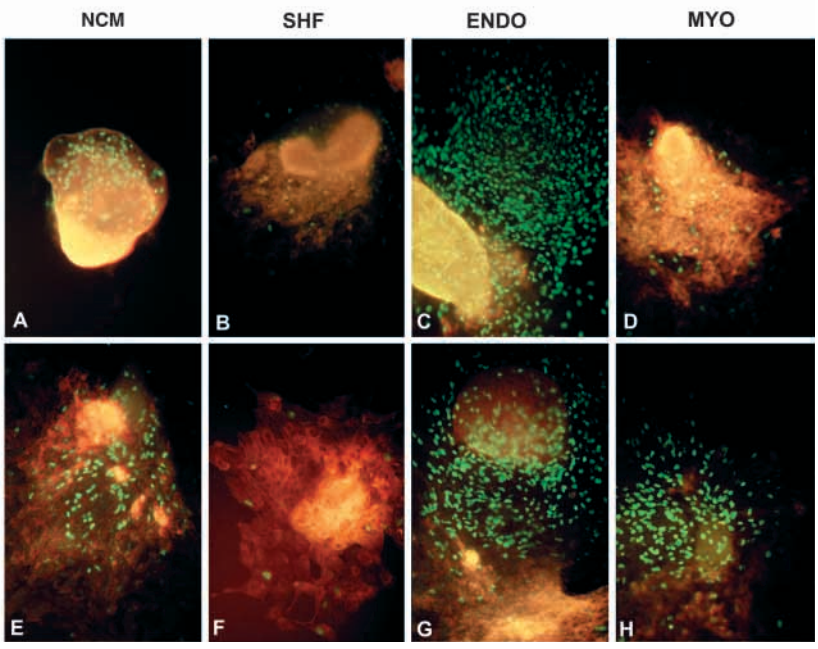

Fig. 7. Myocardial differentiation in cultured secondary heart field is inhibited by noggin. All the panels show secondary heart fields cultured for 24 hours, incubated with BrdU for 1 hour and then doubly stained to show fluorescein-labeled antiBrdU (green) and rhodamine-labeled MF-20 (red). Medium was conditioned as indicated above the panels: A,E, unconditioned medium; B,F secondary heart field-conditioned medium; C,G, pharyngeal endoderm/ectoderm-conditioned medium; and D,H, myocardium-conditioned medium. The heart fields shown in the lower panels were cultured in the conditioned medium indicated with 15 μg/ml noggin protein added. Noggin appears to have decreased myocardial differentiation and increased proliferation, except in the secondary heart field cultured in secondary heart field conditioned medium (B,F). The endoderm/ectoderm conditioned medium caused increased proliferation in both the absence (C) and presence (G) of noggin.

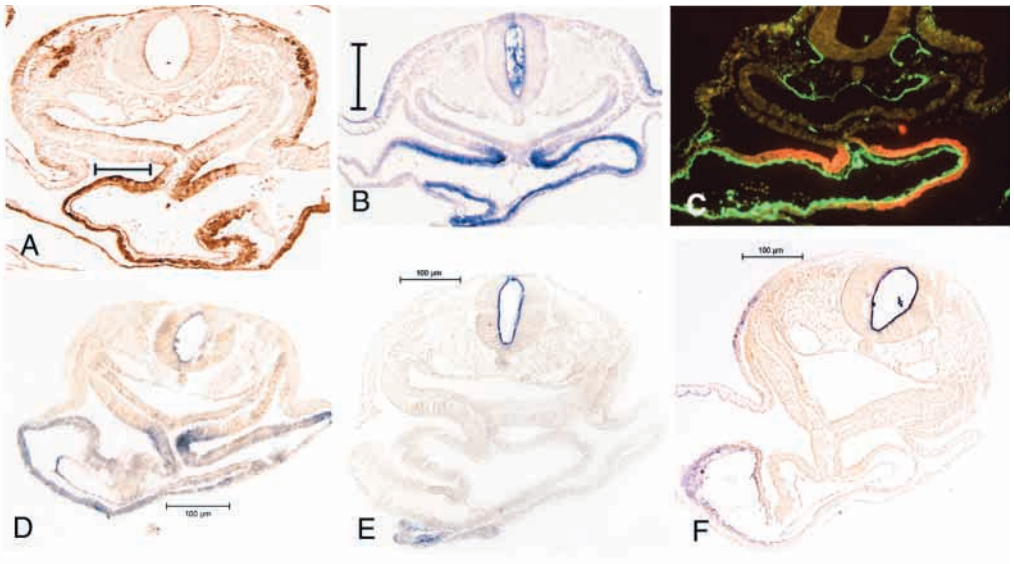

Fig. 8. At stage 11, the inflow tract shows continuity with the primary heart fields. (A) HNK-1 expression in the myocardium and dorsal mesocardium at the cardiac inflow tract. (B) Nkx-2.5 expression overlaps that of HNK-1 but extends slightly farther into the splanchnic mesoderm of the primary heart field. (C) Myocardial differentiation as indicated by MF-20-positive staining (red) in a stage 11 quail embryo double stained for QH-1 to show the endothelium/endocardium (green). (D) Gata-4 expression appears to replicate that of Nkx-2.5. (E) BMP-4 is not expressed in or near the cardiac inflow myocardium. (F) FGF-8 is expressed at very low levels by the ectoderm lateral to the pharynx, but is not seen in or near the cardiac inflow myocardium. All scale bars are 100 μm. Magnification in C is similar to that in B.

lateral plate mesoderm (Rosenquist and DeHaan, 1976; Colas et al., 2000). Differentiation of myocardial phenotype from these fields requires at least three separate inductions. The earliest induction of myocardial potential occurs at gastrulation by activin signaling from the organizer (Antin et al., 1994; Nascone and Mercola, 1995; Yatskievych et al., 1997). Because activin induces both mesoderm and endoderm, and interactions between the two may be responsible for heart induction, the role of activin may be indirect (Logan and Mohun, 1993). In any case, after this initial induction, BMP signaling is necessary to maintain the myocardial lineage potential (Schultheiss et al., 1997; Andree et al., 1998; Yatskievych et al., 1997). Signaling from the anterior endoderm appears to be important in a number of species, including mouse (Arai et al., 1997), chick (Schultheiss et al., 1995), newt (Sater and Jacobson, 1990) and *Xenopus* (Nascone and Mercola, 1995). The endoderm induces Nkx-2.5 and Gata-4 expression, the first molecular indication of myocardial cell commitment, in these fields. Expression of both Nkx-2.5 and Gata-4 appears to be required prior to myocardial differentiation (Schultheiss et al., 1997; Jiang et al., 1998; Reecy et al., 1999; Schlange et al., 2000). Finally myocardial differentiation of the Nkx-2.5/Gata-4-positive cells depends on FGF-2 and BMP-4 signaling from the anterior endoderm (Sugi and Lough, 1994) as treatment with either factor alone does not induce Nkx-2.5 expression in explants of primary heart field mesoderm (Lough et al., 1996; Yatskievych et al., 1997; Barron et al., 2000; Lough and Sugi, 2000). Exposure to FGF-2 for 30 minutes with continuous exposure to BMP-4 elicits myocardial differentiation from mesoderm explanted from the primary heart fields (Barron et al., 2000). Most of these studies have been carried out in chick, but BMP signaling is also required for expression of myocardial markers by the primary myocardium in *Xenopus* embryos (Shi et al., 2000). The single midline primary heart tube forms from the two heart fields during body wall closure. In the chick embryo this occurs at about stages 8-9.

After body wall closure, the secondary heart field lies just underneath the same endoderm that induces Nkx 2.5 and Gata-4 expression in the primary heart fields. Sater and Jacobson (Sater and Jacobson, 1990) showed that the ventral pharyngeal endoderm is still capable of inducing heart development in non-cardiac mesoderm until after the third pharyngeal pouch has formed, which is well into the looping stage of heart development. The ventral pharyngeal endoderm retains the

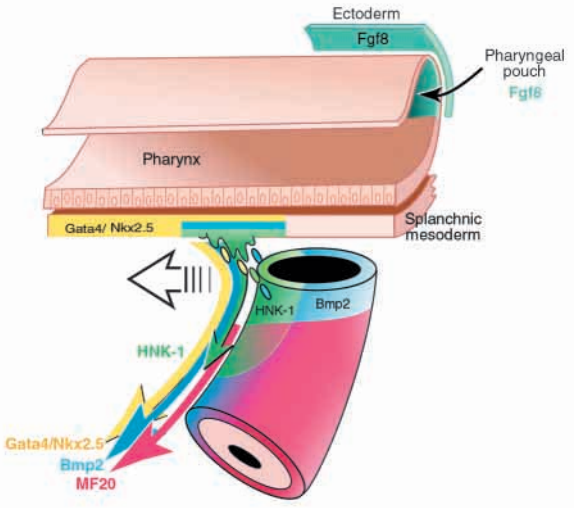

Fig. 9. The dynamic location of events in accretion of myocardium to the outflow tract from the secondary heart field. The splanchnic mesoderm expresses Gata-4 and Nkx2.5. The outflow tract moves craniocaudally across this mesoderm. As the outflow tract approaches, Gata-4/Nkx2.5-positive cells begin to express HNK-1, move into the outflow tract, and begin to express MF20. FGF-8 expressed at the lateral sides of the pharyngeal arches may cause proliferation of the cells in the secondary heart field, while BMP-2 expressed by the distal outflow myocardium and secondary heart field near the outflow tract induce expression of the contractile proteins in the cells being incorporated into the outflow myocardium.

same potency in chick embryos until about stage 16 (M. J. Farrell and M. L. K., unpublished).

The decision of the Nkx-2.5/Gata-4-positive cells in the secondary heart field to translocate into the outflow tract coincides with the onset of HNK-1 expression at about stage 14. The HNK-1 carbohydrate epitope is part of a cell-surface glycoprotein that mediates cell-cell or cell-substrate interactions. It is widely accepted in avian embryology as a marker of migrating neural crest cells (Bronner-Fraser, 1986), but is it expressed in a number of non-neural crest cell types. Canning and Stern (Canning and Stern, 1988) have noted the onset of HNK-1 expression in epiblast cells as they traversed the primitive streak to become mesodermal.

While it is possible that HNK-1 is expressed by cells in the secondary heart fields in association with their differentiation as myocardium, the time of its appearance is more consistent with the onset of migration or translocation into the outflow tract. We were not able to see any morphological indications of myofibrils in the splanchnic mesoderm using either confocal or transmission electron microscopy (K. L. W and M. L. K., unpublished). It is only as the HNK-1-positive cells from the secondary heart field approach and incorporate into the existing primary myocardium that they commence expression of a myocardial phenotype, as shown here by the onset of myosin heavy chain expression. Once the cells have completed their incorporation into the myocardium, they lose their HNK-1 reactivity.

While FGF-2 and FGF-4 are not expressed in the splanchnic mesoderm or outflow myocardium, FGF-8 is expressed strongly by ectoderm and endoderm of the lateral walls of the pharynx. FGF-8 expression is extinguished in the lateral pharynx by about stage 18, when most of the secondary myocardium has been added to the outflow tract. In the zebrafish, FGF-8 is required for expression of Nkx-2.5 and Gata-4 in cardiac precursors (Reifers et al., 2000).

Because the HNK-1-positive cells begin to express MF-20 only when they are near the outflow myocardium/endocardium, the final signal needed for expression of a myocardial phenotype may emanate from the outflow tract itself. Interestingly, BMP-2 has been shown in previous studies to be expressed by the primary outflow myocardium during the stages that the secondary myocardium is incorporated (Yamada et al., 1999). Smad6, an inhibitory SMAD, is expressed throughout the outflow myocardium at the same time (Yamada et al., 1999), which might protect the newly incorporated myocardium from BMP signaling.

BMP-induced cardiomyocyte differentiation is mediated by TGF-β-activating kinase 1 (TAK1), a novel member of the mitogen-activated protein kinase kinase kinase superfamily (Monzen et al., 1999). TAK1 inhibits cyclin D1 promoter activity and proliferation (Terada et al., 1999). In the present study, secondary heart field cultured with pharyngeal endoderm-conditioned medium showed elevated proliferation. Both secondary heart field- and myocardium-conditioned medium induced cardiomyocyte differentiation and both are sources of BMP-2 during the time that secondary myocardium is incorporated into the looping heart. Thus, signaling from the endoderm/ectoderm may require local signaling by BMP-2 released from the distal myocardium and secondary heart field to initiate myocardial contractile protein expression. If this is the case, proliferation appears to be inhibited concurrent with myocardial differentiation. Because of the robust expression of BMP-2 by the secondary myocardium, it was difficult to completely inhibit myocardial differentiation, even in the presence of the endoderm/ectoderm if this is indeed one of the functions of BMP-2. The addition of noggin, a BMP inhibitor, suppressed myocardial differentiation to some extent but only in a few cases did myocardium not appear in the cultures.

A recent study has shown that FGF-8 in the ventral pharynx caused a decrease in the myocardial transient, concomitant with increased proliferation after neural crest ablation (Farrell et al., 2001). Neural crest cells migrating into the caudal pharynx are believed to block the detrimental effect of the pharyngeal FGF-8.

Failure of the quail cells to be incorporated into the myocardium of the outflow tract in chimeras of the secondary heart field is somewhat puzzling. However, the architecture of the ventral pharynx is established during formation of the foregut pocket by closure of the body wall (Patten, 1971). It is during this process that the bilateral primary heart fields are brought to the ventral midline to form the primary heart tube. Because the cells in the dorsal mesocardium and splanchnic mesoderm are laid down at this time, it is not surprising that grafted cells would not be in an appropriate position to be incorporated into the myocardium of the outflow tract. Grafts in the ventral pharynx are difficult because of the movement of the outflow tract relative to the pharynx and movement of the region because of the heartbeat.

It remains to be determined whether the mammalian myocardium also arises from primary and secondary heart fields; however, recently acquired information suggests that the myocardium of the primary heart tube in mouse embryos does not represent all of the myocardial cells that will build the four-chambered heart (Christoffels et al., 2000). If normal differentiation of the chambers of the heart is dependent on addition of the definitive outflow and inflow, then these chambers may not differentiate normally if the secondary heart fields fail to be induced or the cells do not become incorporated (Christoffels et al., 2000). Embryonic mice with Mef2c or dHAND (Hand2 – Mouse Genome Informatics) null mutations fail to express molecular markers specific to the right ventricle (Srivastava et al., 1997; Lin et al., 1997). If right ventricular chamber specification requires the addition of the secondary myocardium, then the defect in the case of these two mutations may be lack of addition of the secondary or definitive outflow tract to the primary myocardium.

Another mouse with a similar phenotype provides a potential mechanism for movement of cells from a secondary heart field. The hdf mouse mutant, which has a transgenic insertional mutation in the Cspg2 (versican) gene located on chromosome 13, also fails to differentiate right ventricular chamber markers or an outflow tract (Mjaatvedt et al., 1998). Versican expression is highest in the outflow tract during looping (Capehart et al., 1999). The fact that the hdf mouse appears to lack a definitive outflow tract suggests that versican may provide a migratory matrix for incorporation of the HNK-1-positive cells from the secondary heart field. It has recently been found that neural crest cells will not migrate through a three-dimensional collagen-aggrecan gel, but will move through a collagen/versican substrate via engagement of HNK-1 antigen-bearing cell-surface components (Perissinotto et al., 2000).

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