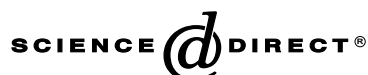


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# Cardiac neural crest contributes to cardiomyogenesis in zebrafish

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## Abstract

In birds and mammals, cardiac neural crest is essential for heart development and contributes to conotruncal cushion formation and outflow tract septation. The zebrafish prototypical heart lacks outflow tract septation, raising the question of whether cardiac neural crest exists in zebrafish. Here, results from three distinct lineage-labeling approaches identify zebrafish cardiac neural crest cells and indicate that these cells have the ability to generate MF20-positive muscle cells in the myocardium of the major chambers during development. Fate-mapping demonstrates that cardiac neural crest cells originate both from neural tube regions analogous to those found in birds, as well as from a novel region rostral to the otic vesicle. In contrast to other vertebrates, cardiac neural crest invades the myocardium in all segments of the heart, including outflow tract, atrium, atrioventricular junction, and ventricle in zebrafish. Three distinct groups of premigratory neural crest along the rostrocaudal axis have different propensities to contribute to different segments in the heart and are correspondingly marked by unique combinations of gene expression patterns. Zebrafish will serve as a model for understanding interactions between cardiac neural crest and cardiovascular development.

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*Keywords:* Zebrafish; Cardiac neural crest; Cardiomyocyte; Lineage analysis

## Introduction

Neural crest cells are derived from dorsal neuroepithelium and migrate to many positions in the developing embryo, where they participate in the formation of a wide variety of structures, including neurons, enteric ganglia, cartilage, connective tissue, thymus, thyroid, and parathyroid. Previously, neural crest contribution to the cardiovascular system has been thought to be important but limited to the endocardial cushions in the outflow tract and aortic arches of the cardiovascular system. In chick, cardiac neural crest originates in the dorsal hindbrain region from the level of the mid-otic vesicle to the caudal boundary of the third somite (rhombomere 6–8) (Kirby and Waldo, 1990; Waldo et al., 1999). Chick cardiac neural crest cells migrate to the third, fourth, and sixth pharyngeal arches, where they initially envelop the endothelium as they migrate ventrally within each pharyngeal arch. From the pharyngeal arch

arteries, they migrate into the developing outflow tract of the heart, where they participate in outflow tract septation and valve formation, which divides the distinct pulmonary and aortic circulatory systems (Waldo et al., 1998). Chick cardiac neural crest cells also contribute to the smooth muscle lining of the pharyngeal arches and cardiac ganglia (Eickholt et al., 1999; Kirby and Waldo, 1990; Waldo et al., 1998). Mammalian cardiac neural crest has been fate-mapped as well by employing the mouse Cre/lox recombination system (Brown et al., 2001; Feiner et al., 2001; Jiang et al., 2000; Li et al., 2000; Yamauchi et al., 1999). Similar to avian cardiac neural crest, murine cardiac neural crest participates in the remodeling of the pharyngeal arch arteries and populates the aorticopulmonary septum and conotruncal cushions prior to and during overt septation of the outflow tract. Cardiac neural crest also contributes to the walls of the proximal coronary arteries (Jiang et al., 2000).

Evolutionarily, heart formation has several conserved steps and several steps that are distinct among different classes of vertebrates. In all vertebrates, the heart forms

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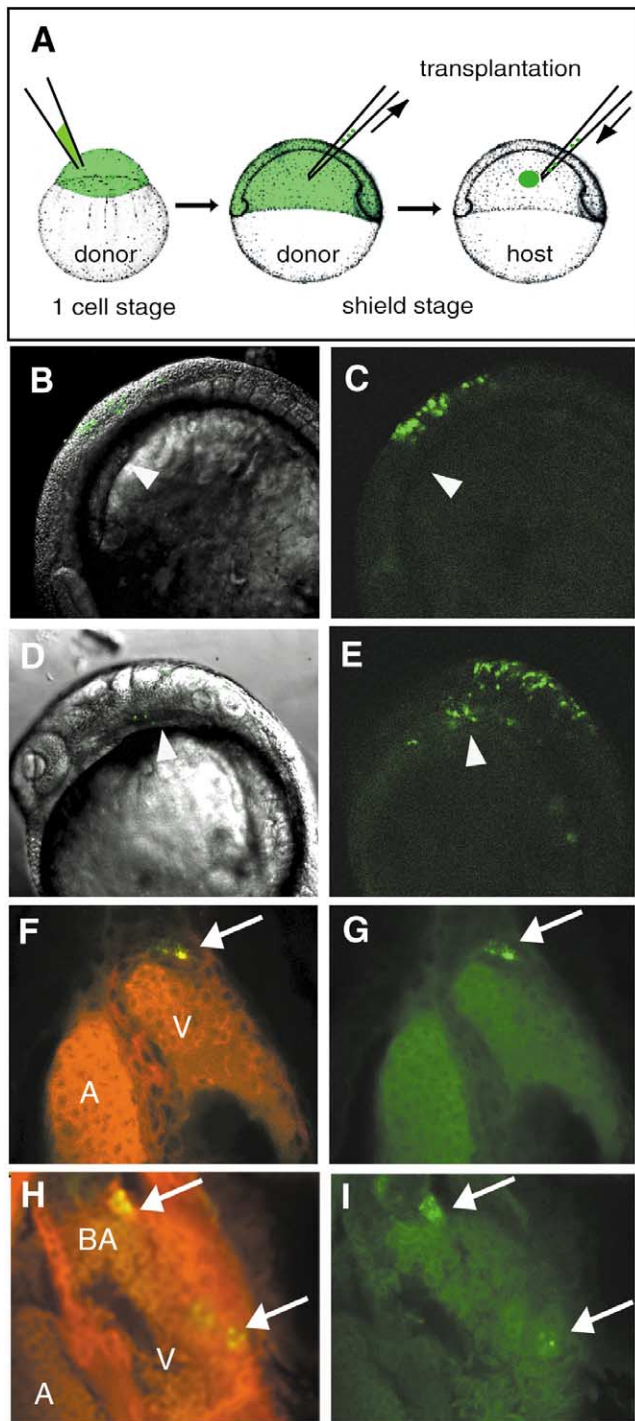


Fig. 1. Transplanted neural crest cells transform into muscle cells in the myocardium. (A) Diagram of cell transplantation procedure. Alexa Fluor 488 was injected into donor embryos at the 1-cell stage. At the shield stage, donor cells were transplanted at the position of neural crest to the homotypic position of an age-matched host. This position does not overlap with the ventral and equatorial mesoderm that will give rise to the heart. (B–E) Lateral view, cranial toward left. DIC and fluorescent images are superimposed in left panels, and fluorescent image in right panels. (B, C) At the 8-somite stage, embryos were selected that had transplanted cells (green label) in the neural crest, not in the lateral plate mesoderm or heart-forming precardiac regions (arrowhead). (D, E) At the 26-somite stage, labeled neural crest cells have migrated toward the heart-forming regions (arrow-

from bilateral primordia that fuse at the ventral midline to form a single linear tube during somitogenesis. The primitive heart tube is divided into two distinct chambers along the rostrocaudal axis, the atrium and ventricle, and loops along the left–right axis. At this point, heart development in birds and mammals diverges from heart development in zebrafish. The zebrafish heart maintains two main chambers, the atrium and ventricle, without structural subdivision of systemic and pulmonary/respiratory circulation, utilizing a single unseptated outflow vessel that consists of the bulbus arteriosus and ventral aorta connected to five pairs of gill arches that retain bilateral symmetry during development (Hu et al., 2000). The zebrafish cardiovascular system can be viewed as a prototypical vertebrate cardiovascular system (Fishman and Chien, 1997) without the subsequent derived modifications to which cardiac neural crest contributes in birds and mammals.

Since some heart structures provided by cardiac neural crest in birds and mammals are absent in zebrafish, it was reasonable to hypothesize that neural crest do not contribute to heart development in zebrafish. Using three distinct lineage analysis techniques, we show in zebrafish that not only do neural crest cells contribute to cardiovascular development but also, surprisingly, cardiac neural crest cells form muscle cells in the myocardium. Our fate-map shows that cardiac neural crest originates from a broader region along the rostrocaudal axis than in chick or mouse. Labeled neural crest cells were observed in all heart segments, including the bulbus arteriosus, ventricle, atrioventricular (AV) junction, and atrium. We also report that distinct regulation of neural crest development along the rostrocaudal axis is correlated with the expression patterns of neural crest marker genes prior to neural crest migration. We show that cardiac neural crest in zebrafish has different fates than previously reported in birds and mammals and propose that understanding cardiac neural crest development in zebrafish will provide insight into the roles of cardiac neural crest in evolution and in cardiovascular disease.

## Materials and methods

### Embryos

Zebrafish, *Danio rerio*, were maintained at 28.5°C on a 14-h/10-h light/dark cycle. Embryos were collected from

head). (F–I) Confocal cross sections of the heart, dorsal view, cranial to the top. Green (lineage-label) and red channel (MF20 immunofluorescence) overlap in left panels. Lineage-labeled cells were shown in right panels. (F, G) At 3 dpf, in the same embryo as shown above, a lineage-labeled transplanted cell (green label and arrow, G) was detected in the ventricular (V) myocardium, and was MF20-positive (yellow label in overlapped images, F). A indicates the atrium. (H, I) In another embryo, transplanted neural crest cells (arrows) were detected in the bulbus arteriosus (BA) and ventricle (V) and were also MF20-positive (yellow labels in H). Myocardial nuclei are not stained by MF20.

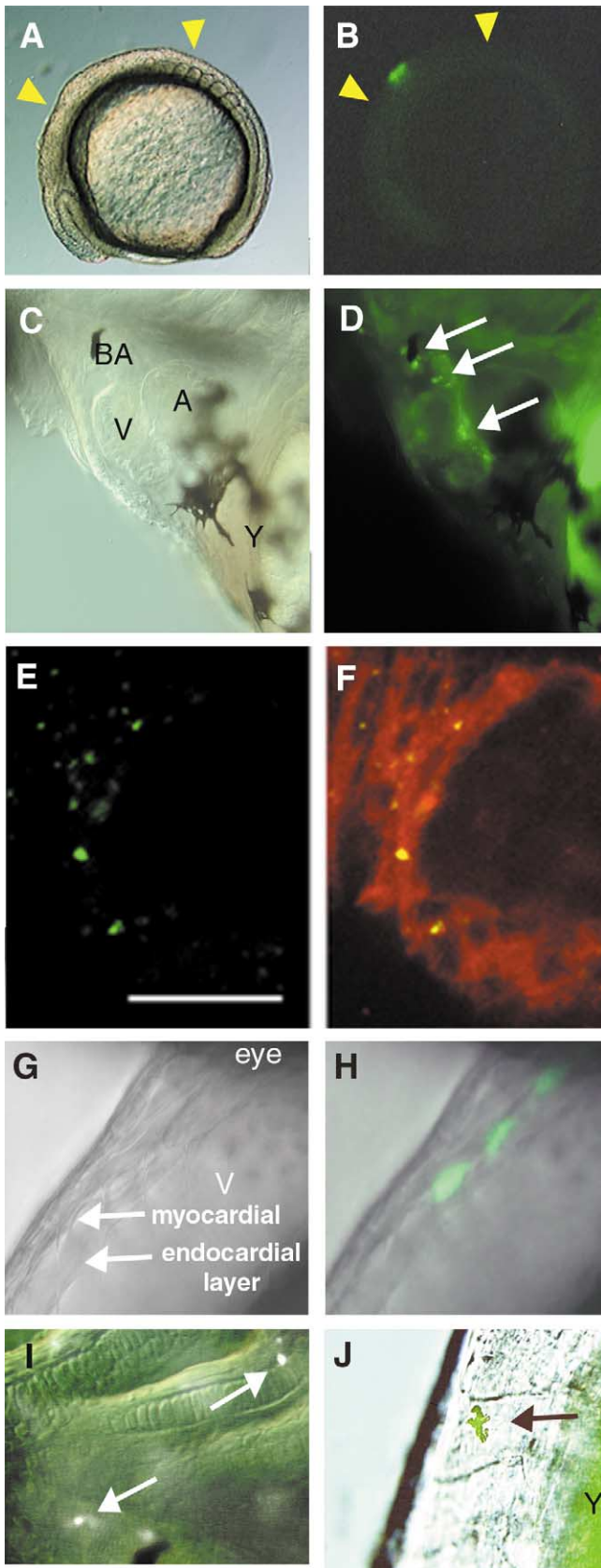


Fig. 2. Two lineage-label approaches mark cardiac neural crest cells that form muscle cells in the myocardium. (A–F, I) Laser uncaging of DMNB-caged fluorescein dextran. Normarski optics (A) and epifluorescence (B)

natural spawning and cultured and staged by developmental time and morphological criteria (Westerfield, 1994). For laser induction experiments, *hsp70-egfp* transgenic zebrafish were obtained as a kind gift from Mary Halloran (Halloran et al., 2000).

#### Cell transplantation

Transplantations were performed essentially as described previously (Ho and Kimmel, 1993). Donor embryos at the one-cell stage were injected by pressure injections of Alexa Fluor 488 dextran (10,000 MW; Molecular Probes), a fixable cell lineage tracer. Donor and host embryos were raised together for the first 3–4 h of development, after which the host embryos were raised at a lower temperature to create the age-matched stage. Both donor and host embryos at the shield stage were dechorionated and positioned into wells made by a plastic mold as described in *The Zebrafish Book* (Westerfield, 1994). Then, 1.2% methylcellulose in embryo medium was spread on the surface of the well to hold the embryo. The appropriate position to transplant was identified with respect to the shield. At the eight-somite stage, embryos with lineage-labeled cells transplanted into the neural crest and neural tube, but not the precardiac mesoderm, were allowed to develop.

#### Laser lineage-labeling

Lineage labeling using DMNB-caged fluorescein dextran (10,000 MW; Molecular Probes) was performed in zebrafish as previously described (Gritsman et al., 2000; Kozłowski et al., 1997; Serbedzija et al., 1998). At the 6- to 8-somite stage, injected embryos were mounted side by side in 1.5% methylcellulose in embryo medium (Warga and

lateral view of an 8-somite-stage embryo in which 15 cells in the neural crest were labeled by laser uncaging (green in B). Yellow arrowheads show cardiac neural crest region in chick. (C, D) Lateral views of the heart in 72-hpf embryo with cranial to the left and dorsal up. Normarski optics (C) and epifluorescence view (D). Arrows indicate labeled cells in the bulbus arteriosus (BA), the ventricle (V), and the atrioventricular junction between the ventricle and atrium (A). Autofluorescence was seen in yolk cells (Y) but not in cells of the embryo. (E, F) Confocal images of the ventricle of a 72-hpf embryo. Green channel (lineage label) shown in left panel, superimposed green and red (MF20) channels shown in right panel. Labeled neural crest cells (green in E) were detected as MF20-positive cells (double staining = yellow in F) embedded in a field of MF20-positive (red) ventricle cardiomyocytes. By 72 hpf, the lineage-labeling begins to appear punctate within the cytoplasm of lineage-labeled cells. Scale bar, 20  $\mu$ m. (G, H, J) Laser activation of *hsp70-gfp* transgene in neural crest lineages resulted in GFP-labeled myocytes at 36 hpf. Lateral views of the heart with cranial to the top and ventral left. Both myocardial layer and endocardial layer in the ventricle were detected in Normarski optics (G). Three fluorescent-labeled cells were detected in the myocardial layer (H). (I, J) Cells labeled by laser uncaging or GFP-activation were also detected in (I) the pharyngeal arches and head cartilage and (J) a pigment cell, indicating that these techniques successfully labeled neural crest cells. Arrows indicate labeled cells.



Kimmel, 1990). The fluorescent dye was uncaged in single cells with a 10-s pulse of 352 or 440 nm light from a nitrogen laser (Laser Science Inc.) focused through a 40× objective. Since laser targeting is less accurate in the *z*-axis and cells above or below the focal plane could be activated, the lateral orientation of the embryos allowed us to confine inadvertent activation to the neural tube and to avoid inadvertently targeting cells in the precardiac mesendoderm. Immediately after uncaging, lineage labeling was confirmed by epifluorescence microscopy and embryos with fluorescein-labeled neural crest cells were allowed to develop. Although neural crest cells had migrated to the cardiac tube by 24 h postfertilization (hpf) (data not shown; see Fig. 2G and H for 36 hpf), we found it most reliable to score neural crest cells in the heart at the beating heart stages when the segments of the heart are readily distinguishable (72 hpf). Embryos were anesthetized with tricaine and examined under epifluorescence to assess whether fluorescein-labeled cells were present in the heart. Both ventral and lateral views were examined. Neural crest contributions to the heart were detected in 119 of 398 embryos.

#### *Laser induction of GFP lineage label*

Laser induction of GFP expression in specific cells was performed as previously described (Halloran et al., 2000). At the eight-somite stage, *hsp70-gfp* transgenic embryos were dechorionated and positioned on their side (i.e., lateral view) in order to visualize individual neural crest cells with DIC optics. Lateral orientation was utilized for the same reason stated as above. The same system as used for laser activating DMNB-caged fluorescein dextran was used for *hsp70-gfp* induction. Bursts of 4-Hz laser beam with 440 nm in excess of 2 min were required to activate GFP expression in single neural crest cells.

#### *Immunohistochemistry and confocal microscopic analysis*

Whole-mount immunohistochemistry was carried out as described previously (Chen et al., 1996). Embryos were fixed in 4% paraformaldehyde in PBS, blocked in 5% normal sheep serum and 0.1% Tween in PBS, incubated overnight at 4°C with the anti-myosin heavy chain antibody MF20 (Hybridoma Bank), rinsed, and incubated overnight with rhodamine-conjugated goat anti-mouse IgG<sup>2b</sup> antibody (Southern Biotechnology Associates). Yolk was removed and embryos were mounted in glycerol and viewed by krypton/argon confocal microscopy (Olympus) with a 60× objective.

#### *Whole-mount in situ hybridization*

Whole-mount in situ hybridization was performed by using digoxigenin-labeled antisense RNA probes as previously described (Chen and Fishman, 1996). Probes used: *snail2* (Thisse et al., 1995); *fdk6* (Kelsh et al., 2000); *lefl*

(Dorsky et al., 1999); *wnt1* and *wnt3a* (Dorsky et al., 1998); and *AP2* (Furthauer et al., 1997).

## Results

### *Cardiac neural crest cells contribute to the cardiomyocytes*

It was not known whether neural crest cells contribute to heart development in zebrafish. In order to assess neural crest cell fate in the zebrafish heart, three distinct experimental approaches, with complementary strengths and weaknesses, were performed. The results indicate that neural crest cells migrate to the developing heart and form myocytes in the functional myocardial layer of the zebrafish heart.

#### *1. Cell transplantation*

Donor embryos were injected with Alexa Fluor 488 at the 1-cell stage in order to lineage-label all of the cells in the donor. At the shield stage, approximately 10–50 lineage-labeled cells in the prospective spinal cord region, as based on fate-maps (Kimmel and Warga, 1987; Kimmel et al., 1990; Lee et al., 1994; Stainier et al., 1993), were withdrawn from a donor embryo and transplanted into the homotypic region of an unlabeled age-matched host embryo (Fig. 1A). At the 8-somite stage, the positions of labeled cells were examined in each host embryo, and embryos were selected for further fate-map analysis by stringent criteria: (1) labeled cells in the region of the hindbrain that correspond to the region that gives rise to cardiac neural crest in chick (Kirby and Waldo, 1990; Waldo et al., 1999); (2) no labeled cells in the anterior lateral plate mesoderm, shown to give rise to the heart by previous fate-mappings in zebrafish (Stainier and Fishman, 1992); and (3) no labeled cells in the rest of lateral plate mesoderm. As controls, embryos that had labeled cells in lateral plate mesoderm were found to have labeled hearts later in development and embryos that were not labeled in any of the 3 regions listed above were found to never have labeled hearts (data not shown).

Embryos that met the above criteria for neural crest labeling were photographed by using confocal microscopy at the 8- and 26-somite stages (Figs. 1B–E). Comparison of the 2 stages indicates that neural crest cells have migrated from their original positions, toward the heart-forming region. At 72 hpf, embryos that displayed labeled cells in the beating heart were fixed and processed for immunohistochemistry. Two major cell layers in the adult zebrafish heart are the myocardium, which is highly organized compact musculature several cell diameters thick, and the endocardium, which is composed of a single layer of cells that lines the inner surface of the chambers (Hu et al., 2000). At 72 hpf, the myocardium in the atrium is a single cell layer and in the ventricle is 2- to 3-cell layers thick. Since the myo-

cardial and endocardial layers are separated by a layer of cardiac jelly in the atrium, the cardiomyocytes and endothelial cells can be distinguished by both DIC and confocal microscopy. In addition, myocytes in the myocardium in 72-hpf embryos can be detected by immunohistochemistry with MF20, an antibody specific to myosin heavy chain expressed in skeletal muscle, some smooth muscle, and cardiomyocytes (Bader et al., 1982; Danos and Yost, 1996; Serbedzija et al., 1998). MF20 does not stain endocardium. Transplanted lineage-labeled neural crest cells (green fluorescence) were detected by confocal microscopy in the myocardial layer of the ventricle and bulbus arteriosus (Fig. 1F–I). Transplanted lineage-labeled cells were also detected in the atrium (data not shown). The muscle cell identity of the neural crest cells was confirmed by labeling with MF20 (red fluorescence), giving these cells a yellow color in the overlapped images (Fig. 1F and H). Transplanted cells were also observed in pharyngeal arches (data not shown), consistent with previously described neural crest contributions in zebrafish (Schilling and Kimmel, 1994).

## 2. Laser lineage-labeling

We performed a lineage-labeling experiment by labeling small patches of cells in the neural crest by laser activation of DMNB-caged fluorescein dextran. Laser uncaging is significantly more precise than lineage-labeling by external application of vital dyes, such as DiI (data not shown). In addition, a laser can be focused on single cells under 40 $\times$  objectives. At the 8-somite stage, fluorescein dextran was uncaged in 15 cells within the neural crest by labeling each cell individually (Fig. 2A and B). Labeled cells in the neural crest gave rise to progeny in the bulbus arteriosus, ventricle, and atrioventricular junction at 72 hpf (arrows in Fig. 2D). Immunohistochemistry confirmed that lineage-labeled neural crest cells expressed the MF20 marker (Fig. 2E and F). Other lineage-labeled cells were found in the pharyngeal arches and cartilage (Fig. 2I), confirming that neural crest was successfully labeled by laser activation.

## 3. Lineage labeling by laser induction of *hsp70-gfp* in transgenic fish

For a third lineage-labeling approach, the *hsp70-gfp* transgene (Halloran et al., 2000) was used to label neural crest cells. At the eight-somite stage, individual neural crest

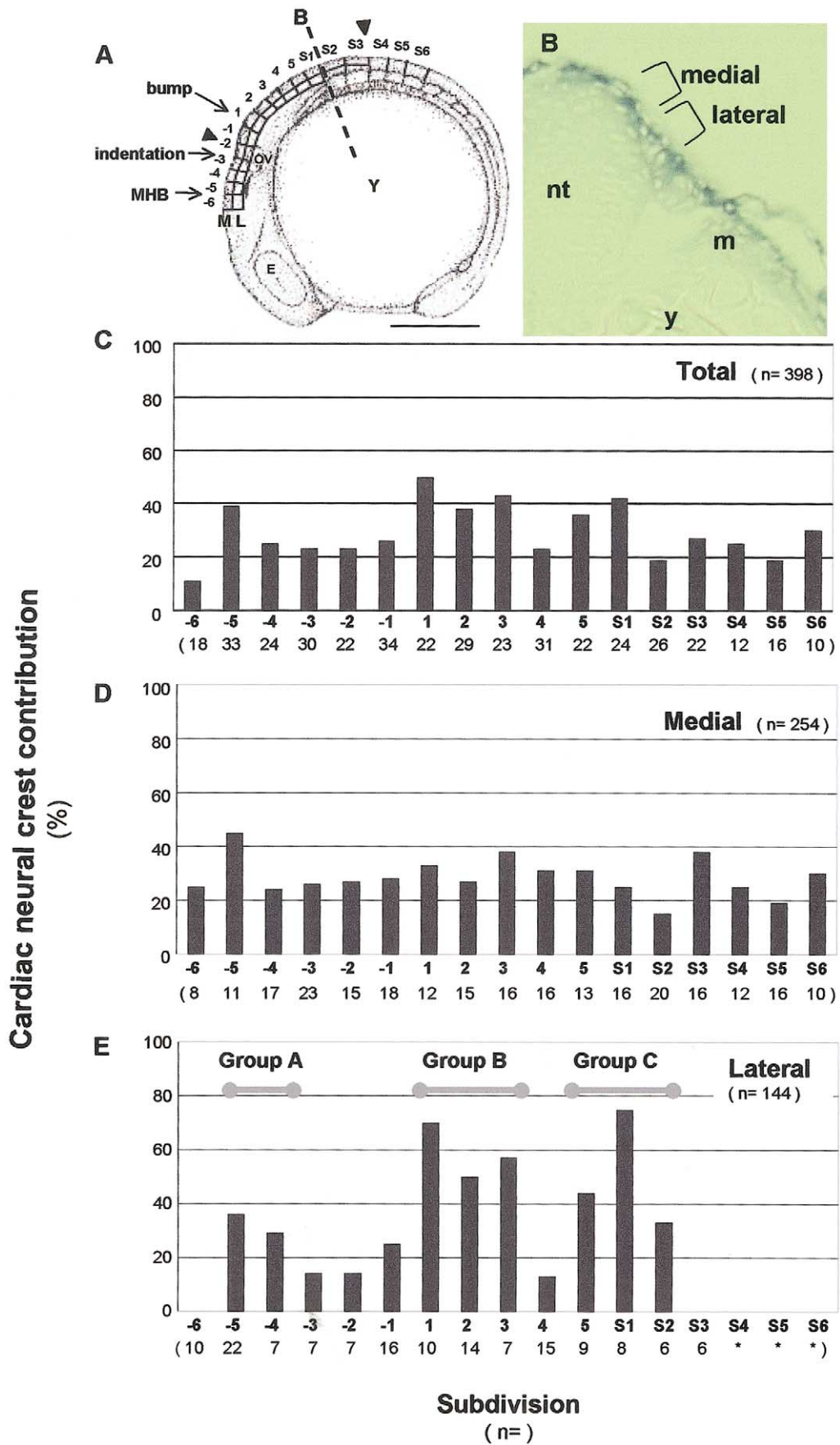
cells were identified and heat shocked by warming with a laser. At 36 hpf, a laser-labeled embryo had three adjacent labeled cells in the myocardial layer of the ventricle. Unlike the uncaged fluorescein dextran (approach 2), green fluorescent protein continued to fill the entire cytoplasm of each lineage-labeled cell, making it easier to confirm that lineage-labeled cells were incorporated into the myocardial layer of the developing heart (Fig. 2G and H). Other GFP-labeled cells were found in the pharyngeal arches and cartilage (data not shown) and pigment cells (Fig. 2J), as expected from neural crest lineages.

Three different approaches showed that zebrafish neural crest contributes to heart development and contributes predominantly to the developing myocardial layer and not the endocardial layer. Immunohistochemistry with MF20 showed that labeled cells were myocytes in the myocardium, suggesting that neural crest cells had transformed into cardiomyocytes.

## Fate-map of zebrafish cardiac neural crest

Having found that labeled neural crest cells migrate to the heart and are incorporated into the myocardial layer, we sought to define the regions along the rostrocaudal and mediolateral axes of premigratory neural crest that contribute to cardiac neural crest. In order to pursue a complete fate map of zebrafish cardiac neural crest, we selected laser uncaging of DMNB-caged fluorescein dextran (approach 2, above) as the most reliable and rapid technique for a large-scale fate-mapping study. There are four distinct advantages of this technique: (1) the laser can be precisely focused on individual cells within well-defined regions of neural crest. In contrast, cell transplants can spread labeled cells to multiple tissues, requiring examination of large numbers of host embryos to find those that have labeled cells only in the correct positions. (2) The lineage label can be detected immediately after laser activation, in order to confirm that the lineage label is exclusively confined to targeted neural crest cells. In contrast, *hsp70-gfp* takes several hours to be strongly expressed, during which time neural crest cells migrate from their initial positions. (3) Hundreds of embryos can be precisely lineage labeled. (4) Two types of controls indicated that the lineage labeling was specifically confined to the cells that were targeted by the laser. First,

Fig. 3. Cardiac neural crest originates from a broad rostrocaudal region in zebrafish. (A) Topological map used for fate mapping. A schematic drawing of an 8-somite embryo shown in a lateral view with cranial to the left and dorsal up (modified from Kimmel et al., 1995). Neural crest were divided rostrocaudally into 17 divisions designated –6 through S6. Arrowheads show cardiac neural crest area in chick. Each division was further subdivided into medial (M) and lateral (L) layers along the orthogonal axis. Line B indicates region of cross section in (B). Y, yolk; E, eye; OV, otic vesicle; MHB, midbrain/hindbrain boundary; M, medial; L, lateral subdivision; scale bar, 250  $\mu$ m. (B) Cross section of the 8-somite embryo, indicating medial and lateral neural crest populations with respect to the neural tube (nt), lateral mesoderm (m), and yolk (y). (C–E) Each subdivision was labeled in separate embryos, and percent of embryos in which labeled cardiac neural crest cells were detected in the heart was scored. Scores within each rostrocaudal division were combined (C) or presented separately for the medial (D) and lateral (E) subdivisions. Bold numbers and letters below each graph correspond with the rostrocaudal topological map position. Lower numbers, in parenthesis, indicate the number of appropriately labeled embryos. Lateral neural crest from 3 regions (Groups A, B, and C) displayed large contributions to the heart. In lateral subdivision positions –6 and S3, labeled neural crest did not contribute to the heart. In divisions S4–S6, it was difficult to define lateral neural crest subdivisions because of the developing somites (\*).



embryos in which nonneural crest cells were labeled, or neural crest cells beyond the bounds of the cardiac neural crest region were labeled, never displayed label in the heart ( $n = 24$ ). Second, embryos in which the DMNB-caged fluorescein dextran was injected but not laser activated, never displayed lineage label in the heart ( $n = 18$ ). These controls also indicate that the other manipulations of the embryos, including brief examination under fluorescent microscopy, did not cause inadvertent uncaging or labeling of the heart.

For fate-mapping, multiple morphological landmarks in the 8-somite stage embryo were used to align a topological map (Fig. 3A). The topological map was divided into 17 rostrocaudal divisions, approximately 5 cell diameters each. In order to compare the zebrafish map with the cardiac neural crest map in chick (Kirby et al., 1983; Kirby and Waldo, 1990), the regions analogous to chick neural crest (arrowheads in Figs. 2A and B, and 3A) were designated position  $-1$  through S3. As will be seen below, the zebrafish cardiac neural crest extends both rostrally and caudally beyond this region, so additional topological landmarks were utilized. The rostral end of the topological map was aligned to the developing midbrain/hindbrain boundary (MHB), defined here as the boundary between division  $-6$  and division  $-5$ . The caudal end of the topological map was aligned with the position of emerging somite 6 (S6). Between these readily evident rostrocaudal extremes, four additional landmarks were distinguished in lateral view with DIC microscopy: an indentation on the dorsal side (defined as position  $-2$ ), a bump on the dorsal side which corresponds with rhombomere 6 (defined as position 1), the underlying otic vesicle (defined as position  $-2$  and  $-3$ ), and each of the first six underlying somites (defined as positions S1 through S6).

During lineage labeling, cells were counted from each relevant landmark to define the boundaries of adjacent divisions. These rostrocaudal divisions were further subdivided along the orthogonal axis, into medial (M) neural crest cells adjacent to the neural keel and lateral (L) neural crest cells farther from the neural keel (Fig. 3A and B). Each medial and lateral subdivision was approximately 3 cell diameters wide along the mediolateral orthogonal axis. We labeled groups of 15 cells that occupy each division at the 8-somite stages. Labeled positions were confirmed by epifluorescence microscopy within 5 min after laser activation (Fig. 2A and B). When chamber demarcation was evident (72 hpf), embryos were anesthetized with tricaine and examined under epifluorescence to determine whether any fluorescein-labeled cells were present in the beating heart (Fig. 2C and D). Neural crest cells originating in these subdivisions also contributed to other structures previously shown to receive neural crest contributions, such as pharyngeal arches and cartilage (Fig. 2I; and data not shown).

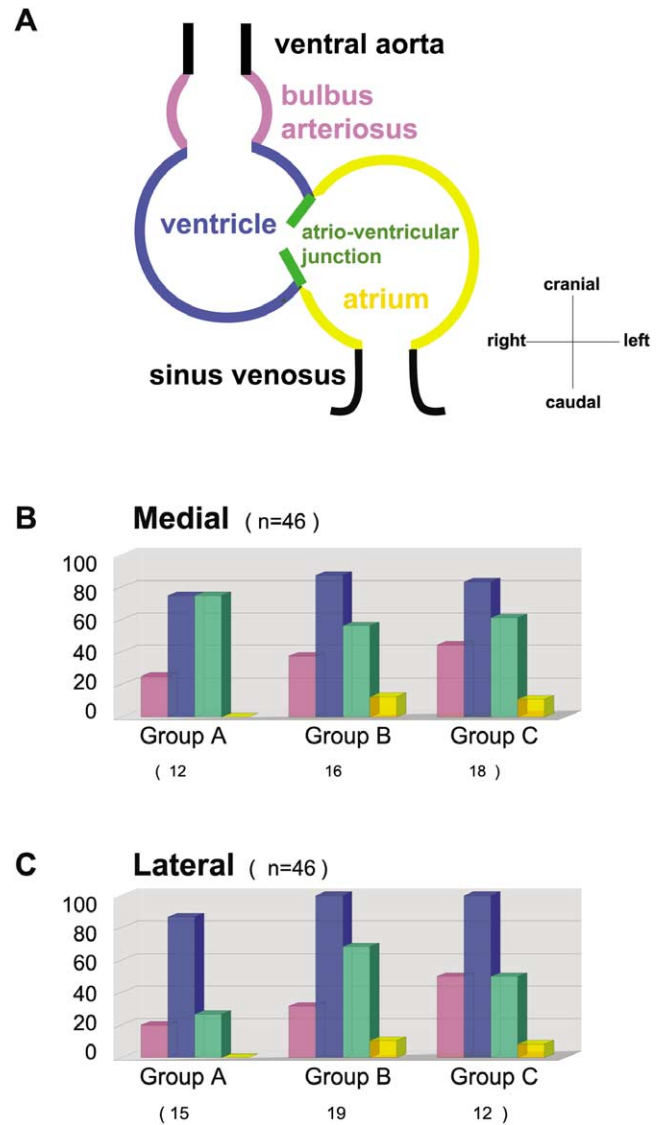


Fig. 4. Cardiac neural crest groups make distinct contributions to heart segments. Groups of cardiac neural crest (Group A, B, and C, defined by fate-maps in Fig. 3D) were labeled and scored for their contributions to four specific heart segments shown in the diagram. (A) Diagram of the zebrafish heart at 72 hpf, ventral view: bulbus arteriosus (red), ventricle (blue), atrioventricular junction (apposed to the site of cushion and valve formation, green), and atrium (yellow). (B) Structural contributions of medial cardiac neural crest cells. (C) Structural contributions to the heart by lateral cardiac neural crest cells. Lower numbers, in parenthesis, indicate the number of appropriately labeled embryos. In Group A, medial neural crest cells contributed to both the ventricle and AV junction (B); however, lateral neural crest cells contributed predominantly to the ventricle (C). There was no neural crest contribution to the atrium from Group A. The neural crest contribution to the bulbus arteriosus was statistically greater in Group C than in Group A.

#### *Broad range of neural crest along the rostrocaudal axis contribute to the heart*

Individual subdivisions in the topological map were labeled, and in each division the percent of embryos that have lineage-labeled cells in the heart was scored (Fig. 3C).

Surprisingly, each of the rostrocaudal divisions along the topological map contained neural crest cells that migrated to the heart, ranging from 11 to 50% ( $n = 10$  to 34 embryos per each division). Labeled cells in position 1, overlying rhombomere 6, contributed to the heart in 50% of embryos. With the exception of position 4, the region (position 1 through S1) analogous to chick cardiac neural crest contributed to the heart in more than 40% of embryos. Interestingly, both cranial neural crest (position -6 to S1) and trunk neural crest (S1 through S6) also contained cells that contributed to the heart. In contrast to the more limited domains of cardiac neural crest reported for avian embryos, neural crest from much more extensive rostrocaudal regions contributed to cardiac development in zebrafish.

#### *Distinctions between medial and lateral neural crest*

Within a given rostrocaudal position, crest cells that originate in lateral positions migrate before crest cells in medial positions adjacent to the neural tube. In trunk neural crest, the mediolateral position before emigration predisposes cells to distinct migratory pathways (Raible and Eisen, 1994). In order to assess whether neural crest cells that originate in medial or lateral positions (shown in Fig. 3B) are distinct in their ability to contribute to heart development, the relative contributions of medial and lateral cells in each rostrocaudal division were further analyzed.

Medial neural crest in all rostrocaudal subdivisions contributed to the heart with frequencies ranging from 15 to 45% ( $n = 8$  to 23 embryos per each division) (Fig. 3D). In contrast, three distinct subgroups of lateral neural crest that contributed to the heart with high frequency were identified (Fig. 3E): divisions -5 through -4 (Group A), divisions 1 through 3 (Group B), and divisions 5 through S2 (Group C). Lateral neural crest cells have a strong propensity to contribute to cardiac lineages, with lateral subdivisions in Group B contributing to the heart in 70, 50, and 57% of embryos and cells in lateral subdivision S1 in Group C displaying the largest percentage of embryos with cardiac labeling (75%). Neural crest cells in regions -2 to -3, overlying the prospective otic vesicle and separating Group A and Group B, showed a lower frequency of contribution to the heart. In addition, only a small percentage of embryos displayed a cardiac neural crest contribution from position 4, separating Groups B and C. Groups B and C define a region that is comparable with the chick cardiac neural crest.

Given previous results in chick, it was surprising to find that neural crest rostral to the otic vesicle made significant contributions to cardiac neural crest in zebrafish. More than 29% of embryos labeled in Group A (positions -5 and -4) showed cardiac labeling. The rostral boundary of this group is at the midbrain/hindbrain boundary. This rostral group of cardiac neural crest has not been described in other vertebrates.

#### *Three neural crest groups make distinct contributions to segments in the heart*

The zebrafish heart consists of four morphological segments (Fig. 4A): blood flows sequentially through the sinus venosus (inflow), atrium, ventricle, and bulbus arteriosus (outflow tract). The mature atrioventricular (AV) valves are not present at 72 hpf; however, preavalvular cushion that separates the atrium and ventricle is readily detected and was used here to identify the myocardium at the AV junction.

In order to assess whether distinct groups of neural crest precursor cells along the rostrocaudal axis have different propensities to contribute to different segments in the heart, we labeled neural crest cells in either medial or lateral subdivisions within Group A, B, or C, as defined above. In contrast to labeling of individual subdivisions described above, approximately 30 cells in Group A or 45 cells in Group B or C were labeled, spanning multiple rostrocaudal divisions within a specific group. The contributions to 4 heart segments (bulbus arteriosus, ventricle, AV junction, and atrium) were scored for cells in each of the 6 neural crest groups: medial subdivisions in A, B, and C (Fig. 4B) and lateral subdivisions A, B, and C (Fig. 4C).

The ventricle was labeled by neural crest cells from Groups A, B, and C at high rates (greater than 75% of embryos in each group). In contrast, neural crest contribution to the atrium displayed region-specific restrictions. The atrium was populated by neural crest cells from the medial and lateral subdivisions of Groups B and C, but not by any neural crest cells from Group A ( $n = 27$ ). The exclusion of Group A neural crest cells from the atrium was further confirmed by lineage analysis of specifically labeled subdivisions: labeled cells at position -6 to -3, the level of the anterior hindbrain, never contributed to atrium (data not shown). The myocardium of the AV junction, apposed to the site of prospective AV cushion and valve formation, presents an intermediate case. Neural crest cells in the medial subdivision of all three groups made strong contributions to the AV junction (Group A: 75%, B: 56%, and C: 61% of embryos), as did cells in the lateral subdivisions of Groups B and C (Group B: 68%, C: 50% of embryos). In contrast, lateral cells in Group A appear to be uniquely underrepresented in the AV junction, making only a minor contribution to this structure. Neural crest labeling of the bulbus arteriosus, the outflow structure immediately adjacent to the ventricle, was generally less frequent than labeling of the ventricle and AV junction. Medial and lateral subdivisions within each group made equal contributions. Strikingly, the bulbus arteriosus was labeled twice as frequently by cells from Group C compared with cells from Group A ( $P < 0.01$ ).

The results from fate-map analysis of distinct neural crest contributions to cardiac segments indicate that the rostrocaudal positions of a particular neural crest cell before emigration biases the cardiac segment to which the crest cell



will contribute. These rostrocaudal- and mediolateral-specific constraints in cardiac neural crest cell fates have not been described in other vertebrate embryos.

#### *Cardiac neural crest fate-map corresponds with gene expression boundaries*

In general, neural crest development is composed of a series of events, such as induction, emigration, migration, and differentiation. Though many neural crest markers have been defined, most of them either cease expression once the cells have emigrated or are not expressed until the neural crest cells reach their target sites and differentiate. Similarly, little is known about the genes expressed specifically by the cardiac neural crest during emigration or their function during cardiac neural crest development.

As described above, we found that three groups of cardiac neural crest along the rostrocaudal axis showed distinct contributions to heart segments (Fig. 4). In order to assess whether the regions we have identified can be distinguished by differential gene expression, we analyzed neural crest marker expression patterns relative to the cardiac neural crest fate-map at the eight-somite stage (Fig. 5A). The *snail* zinc finger transcription family member *snail2* is an early neural crest marker (Thisse et al., 1995). Transcription factor *AP2* is expressed in cells bordering the neural plate, including presumptive neural crest cells (Furthauer et al., 1997). One of the fork head domain genes, *fkf6*, is a marker for premigratory neural crest (Kelsh et al., 2000). Members of the *wnt* family, *wnt1* and *wnt3a*, are potential modulators of neural crest cell fate (Dorsky et al., 1998). *lefl* is a transcription factor in the TCF/LEF family and mediates *Wnt* signaling by interacting with  $\beta$ -catenin (Dorsky et al., 1999). All of these genes were expressed in premigratory neural crest cells, but their expression patterns were distinct along the rostrocaudal axis.

By superimposing gene expression patterns on our cardiac neural crest topological map (Fig. 5A), we identified distinct spatial expression patterns that correlate with distinct cardiac neural crest fates (Fig. 5). At the eight-somite stage, *snail2* and *fkf6* were expressed in Groups A, B, and C. *AP2* was not expressed in Group C. *wnt1* was only expressed in Groups B and C. *wnt3a* was expressed in only Group B, and *lefl* was expressed only in Group A. Thus, the fate map groups can be defined by distinct gene expression patterns. Group A expresses *snail2*, *AP2*, *fkf6*, and *lefl* and excludes expression of *wnt1* and *wnt3a*. Group B expresses all genes except *lefl*. Group C expresses only *snail2*, *fkf6*, and *wnt1*. Considering the boundaries between the groups, the boundary between Group A and Group B cells can be defined at the anterior-most extent of *wnt1* expression in the hindbrain and the posterior-most extent of *lefl* expression. In other words, in Groups A and B, the expression of *lefl* and *wnt1/wnt3a* is mutually exclusive, respectively.

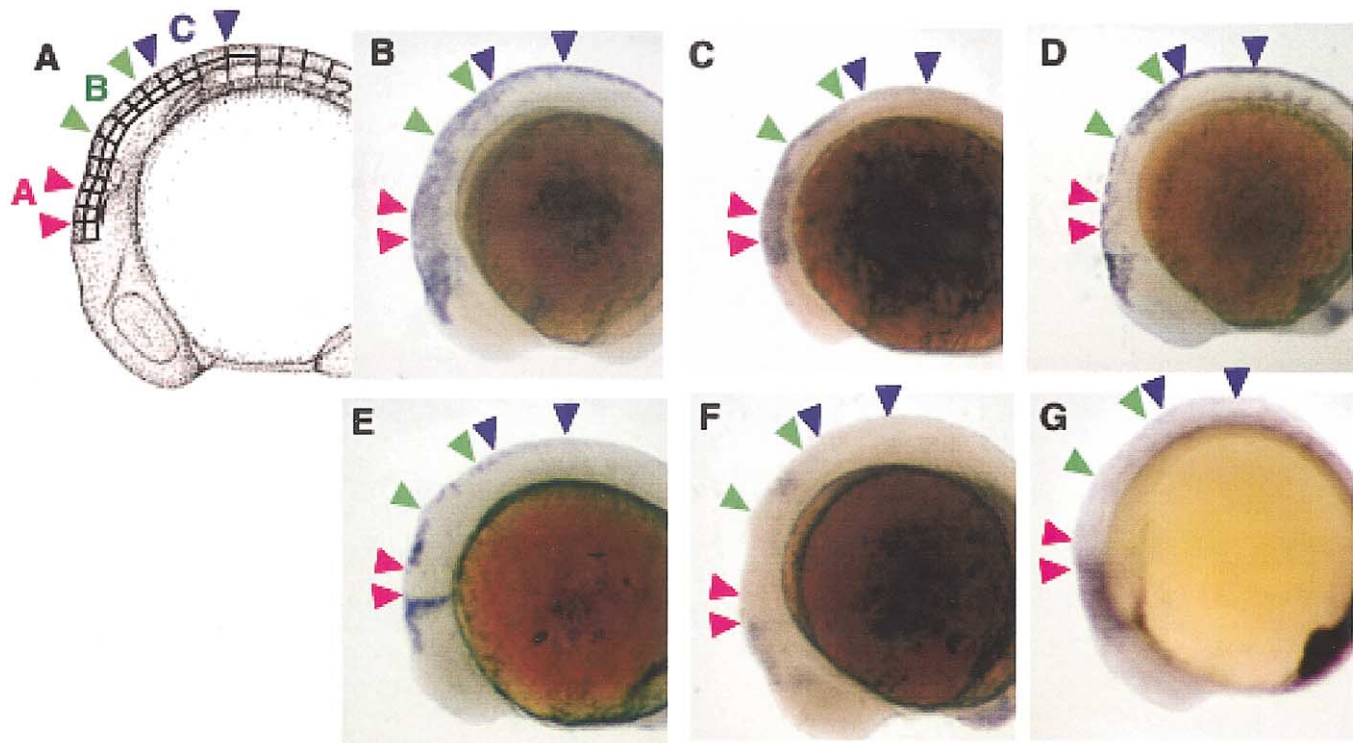
The expression patterns in Group C are dynamic, with *AP2* and *wnt3a* expression being downregulated before the

eight-somite stage (data not shown). This change distinguishes Group C cells from more anterior neural crest cells. Taken together, these data show that the differential expression of *AP2*, *wnt1*, *wnt3a*, and *lefl* corresponds with the segmental identity of the cardiac neural crest cells along the rostrocaudal axis, which in turn corresponds with the differential fates of these groups of cells to contribute to structural segments in the heart.

#### **Discussion**

We present evidence that neural crest cells are a source of MF20-positive muscle cells in the myocardium of developing zebrafish, suggesting that neural crest cells can differentiate into cardiomyocytes. As previously described, neural crest cells are also found in the pharyngeal arches in zebrafish (Schilling and Kimmel, 1994), as in chick and mouse. Since heart structures formed by cardiac neural crest in amniotes are not present in zebrafish, it was surprising that zebrafish have cardiac neural crest cells. Zebrafish cardiac neural crest emigrate from a broad region along the neural plate, ranging from the midline/hindbrain boundary (rostral end of the first rhombomere) to the neural crest above the sixth somite. Three groups along the rostrocaudal axis, including a rostral group not described in other vertebrates, show distinct propensities to contribute to myocardium in a diverse set of heart segments, including the bulbus arteriosus, ventricle, AV junction, and atrium. Each of these fate-map groups correlates with a unique combination of gene expression patterns. Functional alteration of these genes or their expression patterns would test whether they determine neural crest contributions to specific structures in the heart.

In zebrafish, fate-mapping studies revealed that the myocardial progenitors are originally located in 3 tiers of cells at the ventrolateral marginal zone of the blastula (Stainier et al., 1993; Warga and Nusslein-Volhard, 1999). At the onset of somitogenesis, the bilateral primary heart field is elaborately patterned by expression patterns of several genes, such as *nkx2.5*, *gata4*, and *hand2* (Angelo et al., 2000; Chen and Fishman, 1996; Lee et al., 1996; Serbedzija et al., 1998; Yelon et al., 2000). Subsequently, *cardiac myosin light chain 2* and *ventricular myosin heavy chain* mark the subdivision of the heart fields into ventricular and atrial precursors in a mediolateral pattern at the 13-somite stage (Yelon et al., 1999). Recent studies in chick and mice revealed a secondary heart field that contributes cardiomyocytes to the outflow tract later in development (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Here, we describe a developmental source of myocardial muscle cells that arises from outside the primary or the secondary heart fields: cardiac neural crest cells form muscle cells in the zebrafish myocardium. Zebrafish cardiac neural crest contributes to all segments of the heart: the bulbus arteriosus, ventricle, atrioventricular junction, and atrium. This broad contribution to all heart segments suggests that cardiac



H

Group		A		B		C
division	-6	-5-4	-3-2-1	1 2 3	4	5 S1 S2
<i>snail2</i>						
<i>AP2</i>						
<i>fkd6</i>						
<i>wnt1</i>						
<i>wnt3a</i>						
<i>left1</i>						

Fig. 5. Distinct gene expression patterns in three cardiac neural crest groups. (A–G) Lateral views of embryos at the eight-somite stage; cranial to the left and dorsal up. (A) Arrowheads in the diagram show the fate-map boundaries of the three cardiac neural crest groups along rostrocaudal axis: Group A (red), Group B (green), Group C (blue). (B–G) Whole-mount in situ hybridization of neural crest markers indicated a bottom of each panel. (H) Summary of neural crest gene expression patterns. The cardiac neural crest fate-map groups in which these genes are expressed are indicated in gray in the chart. *wnt1* and *wnt3a* are not expressed in group A, and *left1* is weakly expressed. *wnt3a* is only expressed in group B.

neural crest cells participate in structuring the heart. However, further analysis utilizing zebrafish mutants will be required to identify the exact functions of neural crest derived cells in the myocardium. Although neural crest cells are known to transform into many different cell types, such as neuron, glia, cartilage, bone, connective tissue, smooth

muscle, and pigment cells (Knecht and Bronner-Fraser, 2002), the transformation into muscle cells in the major heart chambers has not been reported in other vertebrates.

The region of neural crest that gives rise to cardiac neural crest in chick is restricted from the mid-otic vesicle to the caudal limit of somite 3 (Fig. 6A) (Kuratani et al., 1999).

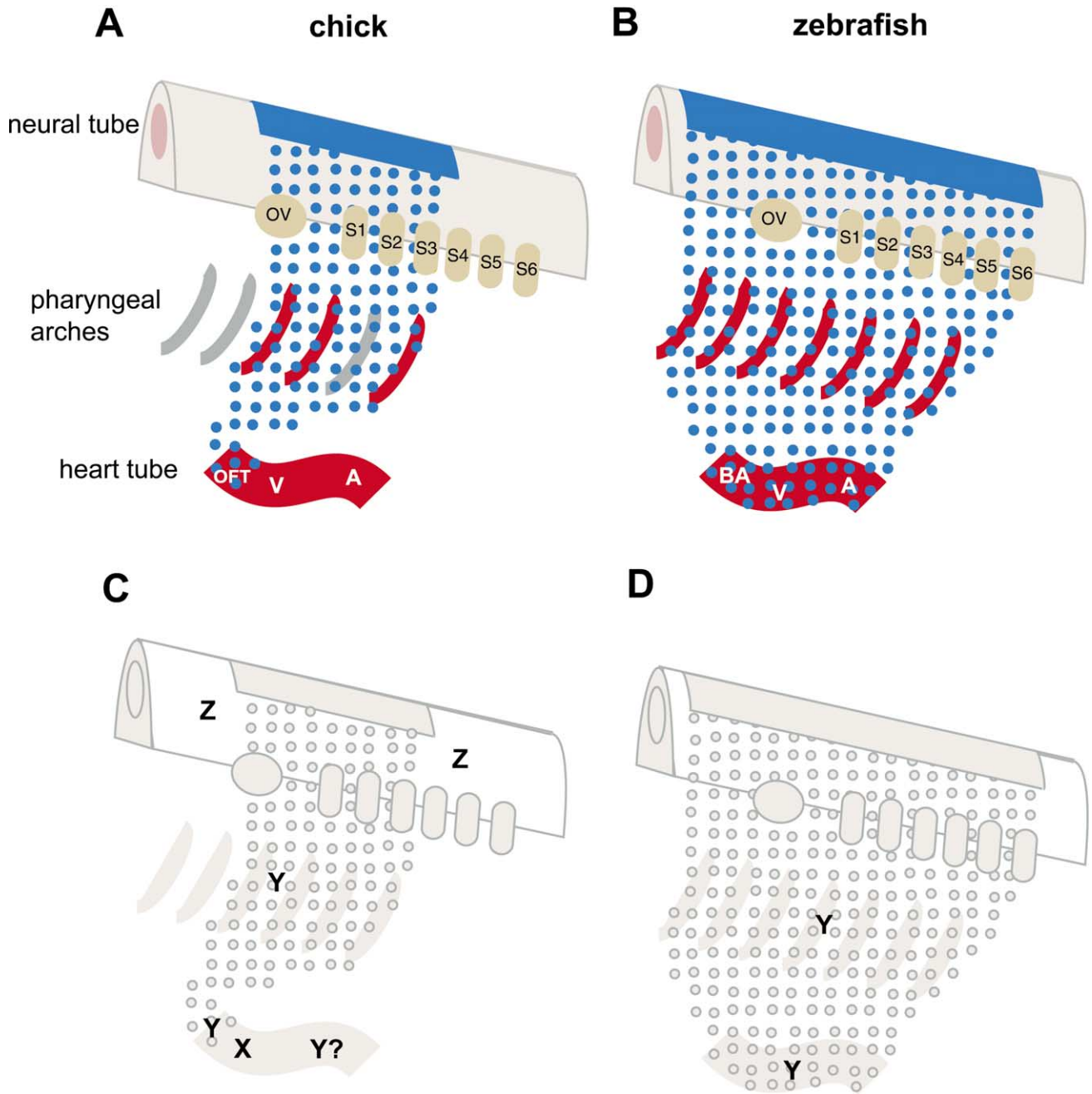


Fig. 6. Models of migrating cardiac neural crest. (A) In chick, cardiac neural crest migrate from the regions from rhombomeres 6–8 (solid blue) to the third somite into pharyngeal arches 3, 4, and 6. Some pharyngeal arches are maintained throughout development (red) and others regress (gray). From the pharyngeal arches, crest cells migrate into the developing outflow tract (OFT) of the heart, where they participate in outflow tract valve formation and septation, which divides the distinct pulmonary and aortic circulatory systems. (B) In zebrafish, cardiac neural crest migrates from an expanded rostrocaudal region (solid blue) ranging from rhombomere 1 to somite 6 into all heart segments (BA, bulbus arteriosus; V, ventricle; A, atrium). (C, D) Models of cardiac neural crest development. “Z” region in chick does not give rise to cardiac neural crest. Crest cells from this region either do not have the appropriate combination of gene expression to specify them to a cardiac neural crest fate or have been usurped for other neural crest developmental functions. Environmental factors “Y” support migration, survival, and differentiation of cardiac neural crest. It is possible that this environment is expanded in zebrafish. Hypothetical stop signals “X” may be present in chick and absent in zebrafish, allowing cardiac neural crest migration into additional heart segments in zebrafish. Loss of these stop signals in other vertebrates, by mutation or environmental insult, may sequentially cause deeper ingressions of crest cells into the heart, erroneous positioning of cardiac neural crest, and subsequent congenital heart disease. OV, otic vesicle.

Cardiac neural crest is thought to represent a transition between cranial and trunk neural crest (Kirby et al., 1985, 1993). In contrast, zebrafish cardiac neural crest originates

broadly from the first rhombomere in the hindbrain region to the caudal boundary of the sixth somite (Fig. 6B). The novel rostral-most cardiac neural crest we have identified in

zebrafish (Group A) has not been reported in other vertebrates, perhaps because the precision afforded by laser-targeted lineage labeling of discrete groups of neural crest cells in large numbers of embryos is not readily feasible in other vertebrates. Alternatively, we hypothesize that the broad rostrocaudal origins of cardiac neural crest in zebrafish represent a “default” fate to contribute to cardiac development, and that in chick some of these crest cells have been converted to noncardiac fates (“Z” in Fig. 6C). Our identification of unique combinations of gene expression patterns in each of three cardiac neural crest groups (Fig. 5H) might provide comparisons to groups of neural crest in other vertebrates. In chick, transplantation of neural crest cells from positions caudal to the cardiac neural crest zone (i.e., caudal to somite 3) into the cardiac neural crest zone demonstrated that transplanted cells are capable of contributing to cardiac neural crest (Verberne et al., 2000). This suggests that caudal neural crest cells are induced to become cardiac neural crest either by local determinants or by determinants along their migratory pathways (“Y” in Fig. 6C). Whether more caudal (beyond somite 6) neural crest cells can contribute to cardiac development when transplanted into the cardiac neural crest region in zebrafish, or whether the zebrafish cardiac neural crest fate map concurs with the “default” commitment of cardiac neural crest along the rostrocaudal axis, awaits cell transplantation experiments.

We find that zebrafish cardiac neural crest migrate into most developing segments of the heart, including the bulbus arteriosus, ventricle, AV junction, and atrium. This is in striking contrast to cardiac neural crest in birds and mammals, which contributes mostly to the outflow tract and does not appear to invade deeply into the developing heart tube. From these observations, we suggest that birds and mammals have acquired critical signals that regulate migration of cardiac neural crest, causing neural crest cells to cease migration shortly after entering the developing cardiac tube. We propose that these “migratory stop” signals are embedded in the migratory tracts of neural crest in birds and mammals and are absent or overridden in the zebrafish cardiac tube (signal “X” in Fig. 6C). In the absence of stop signals, zebrafish cardiac neural crest cells migrate deeper into the developing heart tube and contribute to all heart segments, instead of being retained exclusively in the pharyngeal arch arteries and the outflow tract. Alternatively, the migratory pathways of cardiac neural crest in zebrafish might include entry into the heart tube from both the rostral and caudal ends. Regardless of the migratory pathways, the absence of septation in the zebrafish is clearly not due to an absence of cardiac neural crest.

In general, neural crest cells use stereotypical routes when migrating from the neural tube to their target sites. In the trunk of the chick (Erickson and Perris, 1993) and zebrafish embryo (Raible et al., 1992), for example, cells in lateral positions migrate first and predominantly enter the medial pathway between the neural tube and somite to give

rise to sensory and sympathetic ganglia. Subsequently, cells in medial positions, adjacent to the neural tube, migrate along either the medial pathway or along the lateral pathway between the somite and epidermis to form pigment cells. Here, we find a striking difference between medial and lateral cells in Group A: medial cells contribute strongly to the AV junction and lateral cells do not (Fig. 4).

Zebrafish cardiac neural crest lineages have roles in cardiomyogenesis that have not been observed in birds and mammals. The zebrafish heart represents a prototypical vertebrate heart and provides a powerful model system to determine the genetic mechanisms involved in cardiac development. Analysis of mutations that disrupt the development of cardiac neural crest might reveal mechanisms underlying cardiac morphogenesis. The discovery of zebrafish cardiac neural crest provides a basis for analysis of the diverse contributions of neural crest cells in organogenesis and the mechanisms controlling vertebrate heart development.

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