Analysis of Cranial Neural Crest Distribution in the Developing Heart Using Quail-Chick Chimeras

Marvin T. Phillips, Margaret L. Kirby, and George Forbes

Previous studies have shown that ablation of cranial neural crest results in heart malformations in chick embryos. Cranial neural crest cells populate all of the pharyngeal arches and provide the mesenchymal walls of the aortic arch arteries. Neural crest cells migrate from the pharyngeal apparatus into the outflow region of the heart. However, it is not known which of the pharyngeal arches contribute ectomesenchyme to the developing heart nor has a pattern of distribution in the outflow region been established. In the present study, premigratory presumptive arch neural crest from quail embryos was grafted homotopically onto early chick embryos. On Day 6 of incubation, the chimeric embryos were fixed and processed for histological evaluation. The neural crest providing mesenchyme to pharyngeal arches 1 and 2 was not associated with the developing heart. Neural crest presumptive for arches 3, 4, and 6 was found distributed to the outflow region of the heart. Neural crest from arch 4 contributed the largest number of cells to the developing aortico-pulmonary and conotruncal septa.

These data suggest that the disruption of neural crest over somites 1 through 3, referred to as cardiac neural crest, results in a high incidence of conotruncal malformations. Ablation of a large part of cardiac neural crest results in a high incidence of persistent truncus arteriosus and ventricular septal defect, while double outlet right ventricle with ventricular septal defect results from small lesions of cardiac neural crest. These data suggest that the disruption of a very small number of neural crest cells can result in a serious defect in normal heart development.

While removal of very small amounts of cardiac neural crest has been shown to cause significant deviations from normal heart development, the exact role and areas of interplay of the specific zones of neural crest cells is not understood. To elucidate the important role that these neural crest cells play in normal conotruncal development, it is imperative that we understand the precise distribution of neural crest to the heart. Therefore, our aims in this study were to map the precise cranio-caudal extent of the cardiac neural crest and to demonstrate the distribution of neural crest cells from each of the pharyngeal arches in relation to normal developing heart.

Materials and Methods

Animal Preparation

Fertilized Arbor Acre chicken eggs (Seaboard Hatchery, Athens, Ga.) and Japanese quail eggs (generously supplied by Dr. Henry Marks, USDA Genetics Lab, Athens, Ga.) were incubated at 38°C and constant humidity in a forced-draft incubator. The eggs were opened at stages 8–9 and the embryos prepared for microsurgery according to the method reported by Narayanan.

Microsurgery

Quail-chick chimeras were prepared by homotopically transplanting premigratory neural crest cells presumptive for each of the pharyngeal arches (Figure 1). Bilateral strips of neural fold containing neural crest presumptive for arches 1, 2, 3, 4, or 6 in the quail were transplanted orthotopically to the chick. In each of the transplantations, the donor and host were at the same stage of development at the time of the transplant, and the chick embryo was the host in each case. The neural fold was excised using a modified Wenger vibrating needle. The neural fold consists of the presumptive dorsal neural tube, neural crest, and some adjacent surface ectoderm. Following microsurgery, all eggs were returned to the preoperative incubator and maintained at 38°C and 97% relative humidity until the circulatory system was well established by visual observation after about 72 hours total incubation. At that time, the heart was stained with 1% crystal violet, prepared for histological evaluation, and examined for any malformations.
time, viable eggs were transferred to a second forced-draft incubator maintained at 37°C and 70% relative humidity until the embryos were immersion-fixed in 10% neutral buffered formalin or Carnoy's fixative at incubation Day 6 (Stage 26) for distribution counts. 

**Tissue Preparation**

Embryos were fixed overnight in neutral-buffered formalin or Carnoy's fixative, dehydrated, and embedded in paraffin. Paraffin-embedded embryos were serially sectioned at 10 μm in the transverse plane and stained according to the method described by Feulgen and Rossenbeck. All sections were examined for the presence of the histologically distinct quail cells. Quail cells can be identified on the basis of a unique centronuclear condensation of heterochromatin.

The quail cells were counted in each section of cardiac outflow from 3 embryos with chimeric arch 3 neural crest and 2 embryos each with chimeric arch 4 or 6 neural crest. A cell was counted only if it had a distinctly quail nucleolus contained in the section.

**Results**

In the chick embryo at 6 days of incubation, the aorta and pulmonary trunk are separated by the aortico-pulmonary septum. However, division of the conotruncal region is not yet complete and active truncal cushions are present. The aorta at 6 days has two major branches: the left and right brachiocephalic arteries whose roots are derived from the third aortic arch arteries. The left fourth arch is in the process of regressing, and the right fourth arch has become the definitive aortic arch.

Neural crest cells presumptive for pharyngeal arches 1 (3 embryos) and 2 (6 embryos) were not found in the thoracic cavity or neck. Cells originating at these two levels of the craniocaudal axis were exclusively associated with structures in the head as described previously. Neural crest cells that migrated into pharyngeal arches 3, 4, and 6 were associated with the developing outflow tract of the heart and with all of the major thoracic arteries. Using light microscopy, it was not possible to distinguish neuronal from mesenchymal derivatives of the neural crest at 6 days of incubation, so it must be assumed that the neural crest cells associated with cardiovascular development represents progenitors of both cell types. Since the cardiac neurons appear in small clusters by about Day 8 of incubation (at the light microscope level), it is probable that the neuronal derivatives represent a rather small fraction of the total population of neural crest-derived cells in the outflow region of the heart and great vessels.

In 6 out of 9 embryos with chimeric arch 3 neural crest, quail cells were found as far caudal as the level of the semilunar valves (Figure 2). In 3 of the 9 embryos, arch 3 crest migrated only to the level of the brachiocephalic arteries. In chimeras with arch 3 cells extending to or caudal to the semilunar valves, the quail cells were concentrated in the dorsal truncal cushion (Figure 3).

In all embryos with chimeric arch 4 neural crest (n = 4), quail cells were found caudal to the semilunar valves in the truncal cushions. Quail cells from arch 4 were seen in both dorsal and ventral cushions with the heaviest concentration in the dorsal cushion (Figure 3).

In all embryos with chimeric arch 6 neural crest (n = 5), quail cells were also found caudal to the semilunar valves in the truncal cushions. Quail cells from arch 6 were seen primarily in the dorsal cushion (Figure 3).

Arches 3 and 6 were found to supply the smallest number of cells to the truncal and aortico-pulmonary regions of the developing outflow septa, while arch 4 supplied the largest number (Figure 2). Cells derived from arch 4 had the greatest craniocaudal distribution in the outflow region (Figure 2). Cells derived from arch 4 neural crest outnumbered cells from either arch 3 or arch 6 by a 4:1 ratio. The largest population of neural crest-derived cells was located in the aortico-pulmonary septum just cranial to the semilunar valves (Figure 2).
Discussion

On the basis of ablation studies, the term cardiac neural crest has previously been applied to the neural crest originating from the neural fold adjacent to somites 1-3. This area of neural crest seeds ectomesenchyme to pharyngeal arches 4 and 6. The present study shows that neural crest seeding ectomesenchyme to arch 3 also participates in septation of the outflow tract of the heart. Therefore, the term "cardiac neural crest" should apply to the region of neural crest originating from the level of the presumptive otic placode to the cranial limit of the fourth somite. This includes all of the neural crest that has previously been shown to seed ectomesenchyme to pharyngeal arches 3, 4, and 6.

Figure 2. A map showing distribution of quail cells from pharyngeal arches 3, 4, and 6 to the outflow tract of the chick heart at 6 days of incubation. The conus arteriosus was caudal to the area where neural crest was found. Section 1 was at the level of the lower limit of the truncus (the conotruncal boundary is not distinct so these are approximate levels). The truncus extends to about the level of section 21. The aorticopulmonary septum extends from section 22 to section 45. The aorticopulmonary septum was completely closed while the truncal septum was still in the process of fusing.

Figure 3. Chimeras of arch 3 (A and B), arch 4 (C and D), and arch 6 (E and F) showing the distribution of quail cells in the truncal cushions (DC = dorsal cushion; VC = ventral cushion). The cranio-caudal level of the outflow tract is similar for all three embryos and is approximately at the semilunar valve cusps. For arch 6 the angle of the section is slightly more oblique and includes some of the presumptive right ventricular wall. Bar for A, C, and E represents 100 μm. Bar for B, D, and F represents 200 μm.
Previous studies have shown that ablation of neural crest seeding arches 3, 4, and 6 results in a wide range of heart malformations including persistent truncus arteriosus, double outlet right ventricle, and ventricular septal defect.\textsuperscript{3-7} The cardiac malformation was shown to be dependent on the amount of neural crest removed.\textsuperscript{7} Ablation of a 2-somite length or more of neural crest resulted in persistent truncus arteriosus while ablation of a 1-somite length of neural crest caused double outlet right ventricle. Since these areas of neural crest seed the aorticopulmonary and conotruncal septa, it is apparent that normal development of these septa depend on the presence of a critical number of the crest cells presumptive for arches 3, 4, and 6. Recently, we have observed that persistent truncus arteriosus results only after ablation of neural crest seeding arches 3, 4, or 6 — the area that this study has shown to be cardiac neural crest. Ablations of neural crest seeding arches 1, 2, 3, 4, or 6 cause double outlet right ventricle and inflow tract anomalies. Even very large ablations of crest seeding both arches 1 and 2 can only result in double outlet right ventricle and inflow tract anomalies but never persistent truncus arteriosus.

Although this paper does not show neural crest migrating directly from the pharyngeal arches into the outflow region of the heart, Stewart et al\textsuperscript{16} have recently shown with scanning electron microscopy that the pharyngeal arch region is flattened following removal of neural crest seeding those arches. Flattening of the arches morphologically was accompanied by disturbances in hemodynamic measurements that included decreased vitelline artery pressure.

Although neural crest seeding of pharyngeal arches 1 and 2 does not contribute directly to outflow tract septation, these cells probably determine the wall characteristics of aortic arches 1 and 2 during their existence and thus may influence heart development by altering hemodynamic parameters downstream rather than by directly influencing the heart. This may be the case when very short lengths of cardiac neural crest are removed, since this also produces double outlet right ventricle.

Since double outlet right ventricle can be produced by ablation of short segments of cardiac neural crest, the primary problem may be altered aortic arch characteristics resulting in hemodynamic changes rather than affecting outflow septation. It will be important in future studies to determine the effects of neural crest ablation on hemodynamic characteristics of the aortic arch arteries and how and why these changes influence positioning of the inflow and outflow tracts of the developing heart.

References


Key Words • neural crest • quail-chick chimeras • heart development • pharyngeal arches
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