Patterns during Cardiovascular Development

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We used transgenic mice in which the promoter sequence for connexin 43 linked to a lacZ reporter was expressed in neural crest but not myocardial cells to document the pattern of cardiac neural crest cells in the caudal pharyngeal arches and cardiac outflow tract. Expression of lacZ was strikingly similar to that of cardiac neural crest cells in quail–chick chimeras. By using this transgenic mouse line to compare cardiac neural crest involvement in cardiac outflow septation and aortic arch artery development in mouse and chick, we were able to note differences and similarities in their cardiovascular development. Similar to neural crest cells in the chick, lacZ-positive cells formed a sheath around the persisting aortic arch arteries, comprised the aorticopulmonary septation complex, were located at the site of final fusion of the conal cushions, and populated the cardiac ganglia. In quail–chick chimeras generated for this study, neural crest cells entered the outflow tract by two pathways, submyocardially and subendocardially. In the mouse only the subendocardial population of lacZ-positive cells could be seen as the cells entered the outflow tract. In addition lacZ-positive cells completely surrounded the aortic sac prior to septation, while in the chick, neural crest cells were scattered around the aortic sac with the bulk of cells distributed in the bridging portion of the aorticopulmonary septation complex. In the chick, submyocardial populations of neural crest cells assembled on opposite sides of the aortic sac and entered the conotruncal ridges. Even though the aortic sac in the mouse was initially surrounded by lacZ-positive cells, the two outflow vessels that resulted from its septation showed differential lacZ expression. The ascending aorta was invested by lacZ-positive cells while the pulmonary trunk was devoid of lacZ staining. In the chick, both of these vessels were invested by neural crest cells, but the cells arrived secondarily by displacement from the aortic arch arteries during vessel elongation. This may indicate a difference in derivation of the pulmonary trunk in the mouse or a difference in distribution of cardiac neural crest cells. An independent mouse neural crest marker is needed to confirm whether the differences are indeed due to species differences in cardiovascular and/or neural crest development. Nevertheless, with the differences noted, we believe that this mouse model faithfully represents the location of cardiac neural crest cells. The similarities in location of lacZ-expressing cells in the mouse to that of cardiac neural crest cells in the chick suggest that this mouse is a good model for studying mammalian cardiac neural crest and that the mammalian cardiac neural crest performs functions similar to those shown for chick. © 1999 Academic Press

Key Words: chick; mouse; connexin 43; quail–chick chimera; heart development; cardiac neural crest.

INTRODUCTION

The importance of the neural crest in cardiovascular development has been shown by migration and ablation studies in chick embryos. The migration studies have largely been done using quail–chick chimeras, which take advantage of the fact that quail cell nuclear morphology is distinctly different from that of chick. The cardiac neural crest, a subpopulation of the cranial neural crest, sustains aortic arch artery development and directly participates in septation and remodeling of the cardiac outflow tract (Kirby *et al.,* 1983; Kirby and Waldo, 1995; Waldo *et al.,* 1996). Mapping studies carried out using quail–chick chimeras have shown that the tunica media of the arch arteries, the condensed mesenchyme of the outflow septum, most of the walls of the aorta and pulmonary trunk, the cardiac ganglia, the ultimobranchial gland, and the connective tissues of all

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the pharyngeal glands are derived from the cardiac neural crest (Le Lièvre and Le Douarin, 1975; Kirby *et al.,* 1983; Kirby and Stewart, 1983; Kuratani and Kirby, 1991, 1992; Kirby, 1993; Miyagawa-Tomita *et al.,* 1991; Waldo *et al.,* 1998). After cardiac neural crest ablation in the chick embryos, the outflow tract fails to divide into separate systemic and pulmonary channels (called persistent truncus arteriosus or PTA). PTA in this model is accompanied by aortic arch artery anomalies and hypoplastic pharyngeal glandular derivatives (Kirby *et al.,* 1983, 1985; Nishibatake *et al.,* 1987; Bockman *et al.,* 1987).

The process of outflow tract septation and the configuration of the formed aorticopulmonary septum are similar in chicken, rat, mouse, and human embryos (Thompson *et al.,* 1983, 1984; Thompson and Fitzharris, 1985; Ya *et al.,* 1998b; Sumida *et al.,* 1989; Bartelings and Gittenberger-de Groot, 1989; Fananapazir and Kaufman, 1988), and it has been shown using a fluorescent marker that early neural crest migration in the rat is similar but not identical to that in the chick (Tan and Morriss-Kay, 1985; Fukiishi and Morriss-Kay, 1992). In addition, a large number of genetic studies, including gene knockouts and mutations, provide indirect evidence for neural crest participation in cardiovascular and pharyngeal development in mouse (Franz, 1989; Srivastava *et al.,* 1997; Gruber *et al.,* 1996; Sucov *et al.,* 1995; Srivastava and Olson, 1996). However, progress in understanding cardiac neural crest in mammals has been hindered by the lack of a marker specific for these cells.

In the mouse embryo, connexin 43 (Cx43) is highly expressed by neural crest cells and their derivatives (Lo *et al.,* 1997). Onset of expression in the cranial neural crest is coincidental with emigration of the cells from the dorsal neural tube and continues as the cells migrate into the pharynx and cardiac outflow tract (Lo *et al.,* 1997). Cx43 is a member of the family of proteins that make up gap junctions (Lo *et al.,* 1997). Gap junctions are specialized cell surface membrane channels that mediate intercellular passage of small molecules such as calcium ions, inositol triphosphate, and cAMP (Kumar and Gilula, 1996). Since gap junctions are widely distributed in embryos, they are thought to mediate signaling during many basic developmental processes such as patterning, differentiation, tissue induction, cell migration and proliferation, tissue growth, epithelial–mesenchymal interactions, attachment and fusion of tissues, and tissue condensation (Yancey *et al.,* 1992; Ruangvoravat and Lo, 1992; Sullivan *et al.,* 1993; Rosendaal *et al.,* 1994; Green *et al.,* 1994; Dealy *et al.,* 1994; Minkoff *et al.,* 1994, 1997; Dahl *et al.,* 1995; Perez Valazquez *et al.,* 1996; Miragall *et al.,* 1997; Blackburn *et al.,* 1997; Lo *et al.,* 1997; Ewart *et al.,* 1997).

Lo *et al.* (1997) constructed a transgenic mouse line with lacZ expression driven by Cx43 promoter sequence in neural crest cells and many of their derivatives. LacZ was not expressed in myocardial cells or other tissues which also normally express Cx43 because the regulatory sequence required for Cx43 expression in these other tissues was not present in the 6.5-kb promoter fragment used to make the lacZ construct. Although they found lacZ-positive cells in the cardiac outflow tract of whole-mount embryos at 10.5 dpc and later stages, it was unclear where in the outflow tract these cells were located, if they participated in outflow septation, and how they got there. Similarly, in the work of Fukiishi and Morriss-Kay (1992), who worked with DiI-labeled cardiac crest cells in rat embryos, it was unclear where neural crest cells were located in the heart because they investigated early migration prior to the time when septation would occur. An article by Fananapazir and Kaufman published in 1988 describes formation of the aorticopulmonary septum in the mouse; however, a marker for neural crest cells was not available, making it difficult to identify early and late migration of the crest cells into the aortic sac and proximal outflow, respectively. If the lacZ-positive cells in the transgenic Cx43-lacZ mouse embryo generated by Lo *et al.* (1997) faithfully represent the cardiac neural crest population, this transgenic model could be used to study heart and great artery development in mammals as has been the case in quail–chick chimeras (Waldo *et al.,* 1998). Furthermore, since very little is known about the genes expressed by the cardiac neural crest, or their function in development, it is of value to know if cardiac neural crest cells express Cx43 in the heart during outflow septation and what role gap junctions might play in this context. In fact, the possibility of a role for Cx43 in remodeling of the cardiac outflow tract is indicated by the occurrence of right ventricular outflow tract malformations in Cx43 null mutant mice or mice overexpressing Cx43 in subpopulations of neural crest cells (Reaume *et al.,* 1995; Ewart *et al.,* 1997).

One way of determining whether lacZ expression driven by the Cx43 promoter (referred to as Cx43-lacZ) faithfully represents cardiac neural crest cell development is to correlate its expression with the migration pattern of cardiac neural crest cells in quail–chick chimeras. A precise map of the pattern of cardiac neural crest cell distribution during cardiac outflow septation has recently been completed (Waldo *et al.,* 1998). This study showed that the neural crest cells surrounding the aortic arch arteries are continuous with the condensed mesenchyme of the outflow septation complex and with a seam of neural crest cells between the fusing conal cushions. They also participated in closure of the ventricular septum. Furthermore, a subpopulation of cardiac crest cells migrates into the muscular walls of the pulmonary infundibulum (but not aortic vestibule). Significantly, it is this same region of the right ventricular outflow that is affected in the Cx43 null mutant mouse (Reaume *et al.,* 1995).

Using Cx43-lacZ transgenic mouse embryos ranging from 9.5 to 14.5 days postcoitum (dpc) and stage 18–24 quail–chick chimeras newly generated for this study, as well as stage 25–34 quail–chick chimeras previously generated for an earlier study (Waldo *et al.,* 1998), we show that, with some caveats, mesenchymal cells expressing lacZ in the mouse embryo mirror cardiac neural crest cells in the pharyngeal region and the dividing outflow tract of quail–

chick chimeras. In addition we have been able to note some important differences in septation of the outflow tract as it occurs in mouse and chick. Furthermore, by studying the lacZ-positive cell populations in the developing pharynx and heart, we show the most direct evidence to date that cardiac neural crest plays a similar role in outflow septation and aortic arch artery repatterning in mammalian embryos. The Cx43-lacZ transgene provides the best marker currently available for mammalian cardiac neural crest cells and can be used for studying outflow septation and aortic arch artery repatterning in the mouse. Finally, the timing and location of Cx43 expression suggests that gap junctional communication of cardiac neural crest cells is important in the septation process and in the development of the great arteries.

METHODS

Cx43-LacZ Mice

The construction and breeding of the Cx43-lacZ mice have been described previously. A 6.5-kb promoter fragment from the mouse Cx43 gene was used to drive lacZ expression in neural crest cells (Lo *et al.,* 1997). This lacZ reporter construct, which included a nuclear localization signal, lacks regulatory sequences for driving expression of lacZ in other tissues that normally express Cx43 such as the myocardium, condensing mesenchyme of the limb, metanephric mesenchyme, keratinocytes of the skin, branching bronchioles of the lung, and gonadal tissue. Since this promoter construct does not drive lacZ expression in all these tissues that normally express Cx43, it has become a useful tool for studying neural crest development in mice. Transgenic males were mated with outbred CD-1 females. The morning on which a vaginal plug was identified was designated 0.5 dpc. Litters were harvested at 12-h intervals from 9 to 14.5 dpc. The embryos were fixed in 2% paraformaldehyde for 30–45 min and washed for 30 min twice in phosphate-buffered saline. X-gal staining was carried out overnight at room temperature and the embryos were postfixed in 3.7% formaldehyde and photographed. The pharyngeal, or neck and thoracic, regions were embedded in paraffin and sectioned at 10 μm, mounted serially, and counterstained with eosin Y. Some of the sections of the outflow tract were stained with antibody to myosin heavy chain to demonstrate myocardial cells during the process of septation.

Quail–Chick Chimeras

Quail-to-chick chimeras were prepared by homotypic transplantation of the neural folds giving rise to rhombomeres 6, 7, and 8, which are the origin of the cardiac neural crest. The method has been described in detail (Waldo *et al.,* 1998). The chick embryo was the host and the quail embryo the donor in each case. Donor embryos were Japanese quail embryos incubated for 25–28 h (comparable to stage 9–10 of Hamburger–Hamilton (1951)). The neural folds were removed from a donor and host using electrolytically sharpened tungsten needles. The neural folds from the chick embryo were discarded while those from the quail donor were transferred to the chick in 2 μl physiological saline and placed into the appropriate site. The windows were sealed with cellophane tape and the eggs returned to the incubator. At stages 18 to 35, the embryos were removed from the shell, dissected free of the embryonic membranes, fixed in 4% paraformaldehyde or Carnoy's fixative, and staged. The thoracic region and caudal neck were removed, embedded in paraffin, sectioned at 10 μm, and mounted serially. The slides chosen for comparison with mouse sections were stained with QCPN antibody, which specifically recognizes quail cells (Selleck and Bronner-Fraser, 1995).

Immunohistochemistry

The slides were treated for 30 min in 3% hydrogen peroxide in methanol and washed for 10 min in tap water, 5 min in 0.1 M Tris, pH 7.4, and 5 min in Tris with 2% fetal bovine serum (FBS). Then they were incubated in undiluted QCPN antibody (Developmental Studies Hybridoma Bank) overnight at 4°C or myosin heavy chain antibody (Accurate Chemical and Scientific Corp.) diluted 1:1000 or undiluted neurofilament 2H3 antibody (Developmental Studies Hybridoma Bank) for 1 h at room temperature. The slides were washed in Tris and Tris/FBS for 5 min each. At this point, the mouse ABC Elite kit (Vector) was used, repeating the washes after each incubation period. After visualization with DAB (Sigma), the slides were washed briefly in water, dehydrated, cleared in xylene, and mounted with Cytoseal (Stephens Scientific). A counterstain was unnecessary.

RESULTS

We have examined aortic arch artery repatterning and outflow tract septation in Cx43-lacZ mouse embryos from 9.5 to 14.5 dpc. Cells resembling cardiac neural crest were lacZ-positive (blue). The development of the great arteries and outflow tract and the distribution of cardiac neural crest cells in the mouse were compared with quail–chick chimeras in which quail cells (QCPN-positive, brown) replaced the endogenous chick cardiac neural crest cell population. For the purpose of comparison, we have included descriptions and unpublished photomicrographs derived from a recent study on outflow septation in avians (Waldo *et al.,* 1998). In addition, as the route by which the neural crest cells initially migrated into the aortic sac and cardiac outflow tract from the pharyngeal arches was not clear from earlier studies using quail–chick chimeras, we have further analyzed new chimeras encompassing stages 19 through 24. We found that cardiac neural crest cells entered the aortic sac from the pharynx around stage 19 to 20 and the conotruncus by stage 22. These cells migrated lateral to the aortic sac bilaterally and divided into two streams, one of which progressed in the submyocardial mesenchyme and the other in the subendocardial mesenchyme. The submyocardial population on each side later condensed to form the prongs of the aorticopulmonary septation complex. We were not able to establish the fate of the subendocardial population.

The remodeling or repatterning of the aortic arch arteries converted these initially bilaterally symmetrical vessels into the adult great arteries, a process that is dependent on the presence of normally functioning neural crest cells (Kirby *et al.,* 1997; Waldo *et al.,* 1996). In the mouse
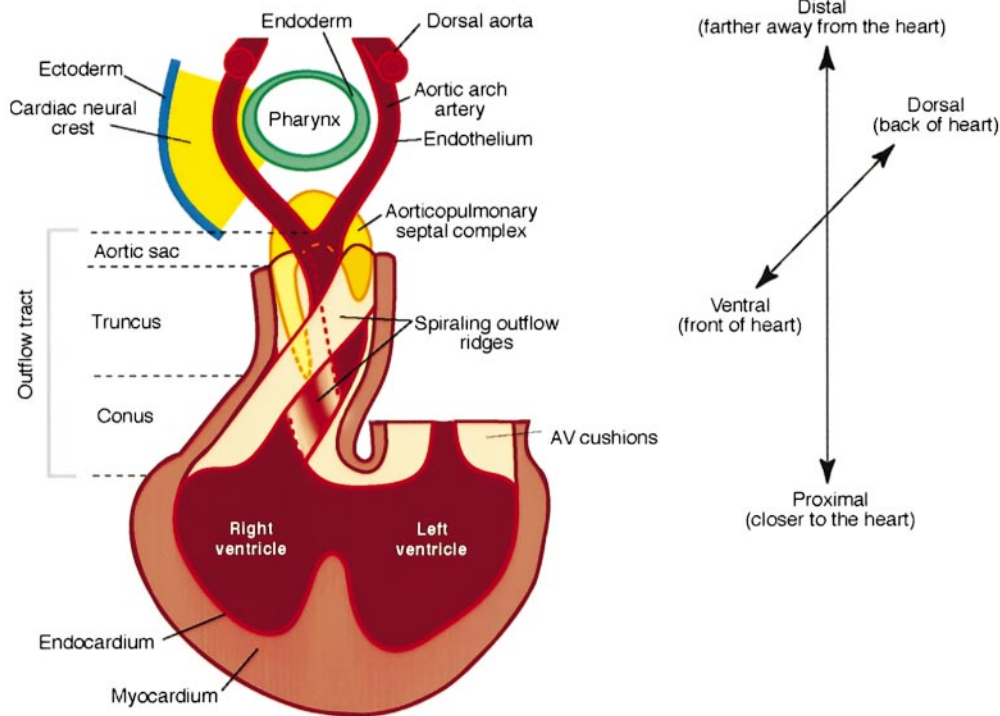
FIG. 1. Diagram of the arch arteries and the outflow tract. The aortic sac connects the distal part of the heart loop, the conotruncus, with the bilaterally symmetrical aortic arch arteries. The conotruncal lumen is lined with endocardium that continues as the endothelium of the aortic sac and arch arteries. Externally, the conotruncus is surrounded by a sleeve of myocardium that reaches but does not cover the aortic sac. Cardiac jelly intervenes between the myocardium and the endocardium and provides an acellular matrix that becomes seeded by endocardially derived mesenchymal cells. The endocardially derived mesenchyme cells undergo rapid proliferation to fill the cardiac jelly and create two long spiraling outflow tract ridges. In preparation for septation, neural crest cells migrate into these ridges to form long columns of cells adjacent to the myocardium, one column in each ridge. As septation is initiated, the columns of crest cells condense into prongs that are united distally by a bridge of condensed neural crest cells spanning the lumen of the aortic sac between aortic arch arteries 4 and 6 and dividing it into the nascent aorta and pulmonary trunk. Together, the two prongs and bridge of condensed crest-derived mesenchyme form the aorticopulmonary septation complex configured roughly into an upside-down U. As outflow septation progresses, the bridging part of the aorticopulmonary septum lengthens, at the expense of the prongs, into the truncus where the lumen is divided into the nascent aortic and pulmonary semilunar valves. Septation of the conal part of the conotruncus occurs by fusion of the proximal ridges along a seam of neural crest cells located beneath the endocardium.

embryo, the period of aortic arch artery remodeling began about 11 dpc. By 14.5 dpc, the adult pattern of great arteries could be seen. Arch artery remodeling occurred at incubation days (ID) 5.5 to 9 in the chick.

In cardiac outflow septation, the lumen was divided and the tissues of the outflow tract underwent major remodeling. The aortic sac and truncus (Fig. 1) were divided by the aorticopulmonary septation complex into the aorta and pulmonary trunk and their semilunar valves. This was the earliest formed and most distal portion of the outflow septum. The conal septum was closed by apposition of the proximal outflow ridges (Fig. 1; Waldo *et al.,* 1998), resulting in the formation of the right and left ventricular outflow tracts (i.e., the pulmonary infundibulum and the aortic vestibule), which underwent continued remodeling. Outflow tract septation began in the aortic sac of mouse and chick, at 11.5 dpc and ID 4.5, respectively, and was com-

plete by 14.5 dpc and ID 9. Since the outflow septum was directly continuous with pharyngeal arch mesenchyme, our observations began with the pharyngeal region.

The Pharyngeal Arches and Aortic Arch Arteries

Prior to cardiac outflow septation in the mouse and the quail–chick chimera, the paired caudal pharyngeal arches (3, 4, and 6) were filled with mesenchyme that was pierced by a centrally placed aortic arch artery. This artery extended ventrodorsally through its pharyngeal arch to connect the ventrally located aortic sac (Fig. 1) with the dorsal aortas (Figs. 2A, 2B, 2E, 2F). In the quail–chick chimera, the mesenchyme that filled each pharyngeal arch was derived from quail cells (cardiac neural crest cells; Figs. 2A, 2B). The pharyngeal ectoderm and endoderm were free of quail cells (Fig. 2B). Each aortic arch artery was invested with neural
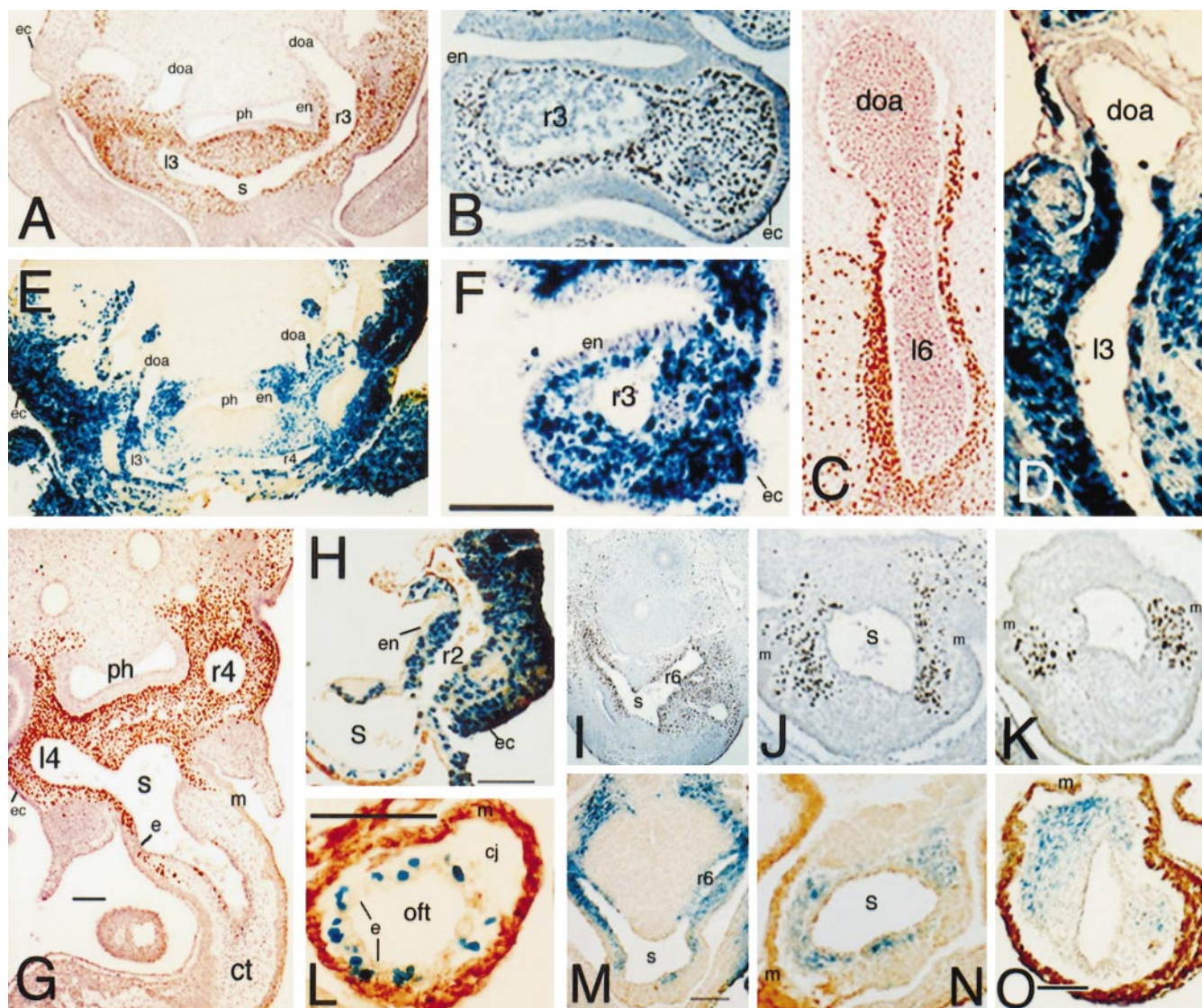
FIG. 2. Preseptation. (A, B, C, G, I, J, K) Quail–chick chimeras stained with QCPN (brown). (E, F, D, H, M, N, O) Cx43-lacZ mouse embryos treated for β-galactosidase histochemistry. The myocardium is labeled with an antibody to cardiac myosin (brown) in H, L, N, O. (A) Stage 22. Neural crest cells fill the pharyngeal area and surround the third arch arteries (l3, r3), separating them and the aortic sac (s) from the pharyngeal endoderm (en). doa, dorsal aorta; ec, ectoderm; ph, pharynx. (B) The mesenchyme of the right third pharyngeal arch is mostly cardiac neural crest cells. (C) Neural crest cells in the sixth (l6) arch arteries end abruptly where they join the dorsal aorta. (D) In a day 11.5 mouse embryo, the walls of the third arch artery show intense Cx43-lacZ expression that terminates abruptly at the junction with the dorsal aorta. (E) Cx43-lacZ-positive cells fill the pharyngeal area at the level of left third and right fourth (r4) arch arteries. (F) LacZ-positive mesenchyme invests the third arch artery. (G) Neural crest cells fill the fourth pharyngeal arches and migrate lateral to the aortic sac into the conotruncus (ct) in two columns, one beneath the endocardium (e) and the other beneath the myocardium (m). (H) LacZ-positive cells surround the endothelium of the aortic sac. The mesenchyme and ectoderm of the second pharyngeal arch (r2) is lacZ positive while the endoderm of the pharynx is lacZ-negative. (I, J, K) Stage 24 chimera; distal-to-proximal transverse sections through the outflow tract at the level of: (I) the sixth arch arteries and aortic sac, (J) the distal conotruncus at the junction of aortic sac with truncus, and (K) the distal truncus. Two columns of cells continuous with the mesenchyme surrounding the arch arteries have penetrated the conotruncal cushions and migrated beneath the myocardium toward the heart. (L) Transverse section at midoutflow level (oft) where lacZ-positive cells surround the lumen. cj, cardiac jelly; e, endothelium; m, myocardium. (M, N, O) LacZ expression in transverse sections of the outflow tract beginning distally (M) and moving proximally toward the heart (O). In (M), the sixth arch arteries surrounded by lacZ-positive cells branch from the aortic sac. (N) The distal conotruncus at its junction with the aortic sac. LacZ-positive mesenchyme nearly surrounds the aortic sac. (O) At the conotruncal bend, and distal part of the conus, lacZ-positive mesenchyme adjacent to the myocardium has begun to condense into prong-like structures on either side of the outflow tract. (F, G, H, L, M, O) Bar, 100 μm.

crest cells that, at later stages, began to condense and formed the smooth muscle of the tunica media of the persisting arch arteries (Figs. 2A, 2B, 2C). This coat of quail cells ended abruptly at the junction of the arch arteries with the dorsal aorta whose walls consisted of non-neural crest-derived cells (Fig. 2C). Neural crest-derived endothelial cells have never been observed in quail–chick chimeras. In mouse embryos, this pattern of neural crest cell distribution was mirrored by lacZ expression in the mesenchyme of the pharyngeal arches and the walls of the arch arteries with lack of expression in the walls of the dorsal aortas (Figs. 2D, 2E, 2F). However, large numbers of lacZ-positive cells were located in the ectoderm covering the pharyngeal arches (Fig. 2E). The mouse endoderm and endothelium were uniformly negative for lacZ at all stages examined (Fig. 2F).

Initial Migration of Neural Crest Cells into the Outflow Tract and Formation of the Prongs of the Aorticopulmonary Septum

Cardiac neural crest cells migrated from the base of each aortic arch artery in the quail–chick chimeras into the loose mesenchyme surrounding the endothelium of the aortic sac at stage 19 and into the truncus by stage 22 (Figs. 1 and 2G). Some of these cells continued into the conus. A second population of crest cells migrated bilaterally around the aortic sac and into the conotruncal ridges subjacent to the myocardium to form the two prongs of the aorticopulmonary septation complex (Table 1 and Fig. 2G). By stage 24 these ectomesenchymal cells had increased in number sufficient to form recognizable prongs (Figs. 2I, 2J, 2K).

In the 9.5-dpc mouse heart, scattered lacZ-positive cells surrounded the lumen of the aortic sac and distal outflow tract subjacent to the endothelium (Table 1, Figs. 2H, 2L). Although no lacZ-positive cells underlying the myocardium were present, within the next 2 days two columns of lacZ-positive cells appeared in the mouse outflow cushions subjacent to the myocardium, to form the prongs of the septation complex (Figs. 2M, 2N, 2O and 3A, 3D). From quail–chick chimera studies we know that the condensed mesenchyme of the prongs is composed of neural crest cells. However, in the mouse, lacZ expression was not uniform in the cells comprising the condensed mesenchyme of the prongs, and some cells located in the uncondensed cushion mesenchyme were also positive (Figs. 2O and 3D).

Division of the Aortic Sac into Aortic and Pulmonary Channels

Cardiac outflow septation was initiated in both the mouse and the chick embryo as the aortic sac was divided into aortic and pulmonary channels by the aorticopulmonary (AP) septum. In the chimeras, the aorticopulmonary septation complex was composed of quail cardiac neural crest cells resembling an upside-down U with each prong of the U embedded in one spiraling outflow cushion and its

arch bridging the lumen of the aortic sac (Figs. 1 and 3C, 3E). As the sac was divided, the short aortic and pulmonary channels shared a common wall (the bridging part of the AP septum) of cardiac neural crest cells, but their free walls were not composed of neural crest cells (Table 1, Fig. 3C).

In the mouse embryo, the aortic sac was divided by the aorticopulmonary septum around 11.5 dpc (Fig. 3B). Here, as in the chimeras, the nascent outflow vessels shared a common wall wherein lacZ expression mimicked the pattern of neural crest distribution in the AP septum (Table 1, Fig. 3B). Furthermore, lacZ was expressed by cells that surrounded the aortic sac and was especially prominent on the aortic side. Comparable neural crest cells were not seen in chimeras (Fig. 3B). Unlike the chimeras, where the prongs extended into the outflow tract only as far as the truncus, the prongs in the mouse outflow tract extended into the distal conus (Figs. 3A, 3D).

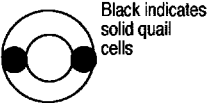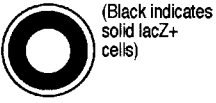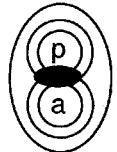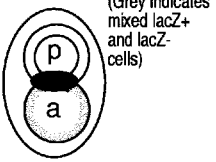### Division of the Truncus into Nascent Aortic and Pulmonary Semilunar Valves
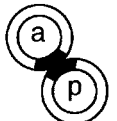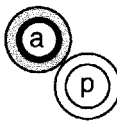
As septation continued from the aortic sac into the truncus by the leading edge of the aorticopulmonary septum, nascent semilunar valves were formed in mouse and the chimeric avian hearts (Figs. 3F, 3I). In the chimeras, neural crest cells from the truncal septum migrated beneath the endocardium into the truncal and intercalated cushions that gave rise to the three leaflets of each semilunar valve (Figs. 3F and 4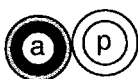A, 4B). Shortening of the originally long prongs (Fig. 3G) occurred coincidentally with the lengthening of the truncal septum as it divided the lumen (Fig. 3F). Closer to the heart, scattered neural crest cells populated the conal ridges mostly subjacent to the endocardium (Fig. 3H). In 12- to 12.5-dpc mouse embryos, lacZ-positive cells delineated the truncal septum (Figs. 3I, 3J), but by 13–14.5 dpc, lacZ expression decreased significantly in the truncal septum, although the remaining expression was consistent with the location of neural crest cells in quail–chick chimeras of comparable ages (Figs. 4E, 4F). While lacZ-positive cells appeared in the valve leaflets, they were not found primarily beneath the endocardium as were the crest cells in quail–chick chimeras (compare Figs. 4A and 4E). In the conus, lacZ-positive cells were present beneath the endocardium in a pattern resembling that of the neural crest cells in the proximal outflow ridges of quail–chick chimeras (compare Figs. 3H and 3K).

Septation of the Conus

Septation of the conus resulted in separation of the pulmonary infundibulum from the aortic vestibule (Fig. 4D). In the chimeras, this occurred by fusion of the conal cushions leaving a seam of cardiac neural crest cells at the fusion line (arrows in Fig. 4D). Myocardialization of the conal cushions started before fusion began and was nearly finished by the completion of septation. A subpopulation of the crest cells migrated far enough into the right ventricle

TABLE 1

Major Differences between Expression of LacZ in the Pharynx and the Outflow Tract
of Mouse Embryos and the Location of Cardiac Neural Crest Cells
in Quail–Chick Chimeras

	Quail Cardiac Neural Crest Cells in Quail-Chick Chimeras	**LacZ Expression in Mice**
Pharyngeal ectoderm	Negative	Positive
Aortic sac (pre-septation)	Black indicates solid quail cells	(Black indicates solid lacZ+ cells)
Aortic sac at septation		(Grey indicates mixed lacZ+ and lacZ– cells)
Aorta & pulmonary trunk above the valve (remodeled aortic sac after septation)		
Mid ascending aorta & pulmonary trunk		
Upper ascending aorta & pulmonary trunk		
Arch of aorta adjacent to ductus		

to reach the trabeculated wall of the right ventricle (Fig. 4B) and take part in closure of the ventricular septum (not shown). As the myocardium surrounding the distal outflow tract shortened, the aortic and pulmonary valves settled closer to the ventricles and the truncal septum became continuous with the seam of crest cells closing the conal septum (Figs. 4A, 4B).

In mouse embryos, fusion of the conal cushions occurred earlier than in chicks and at a time when the outflow tract was still oriented to the right side of the heart (Fig. 4C). Myocardialization of the conus did not occur until well after conal septation was finished at 13 dpc and was not completed until late 14.5 dpc. As myocardialization progressed in the mouse, the pulmonary infundibulum was
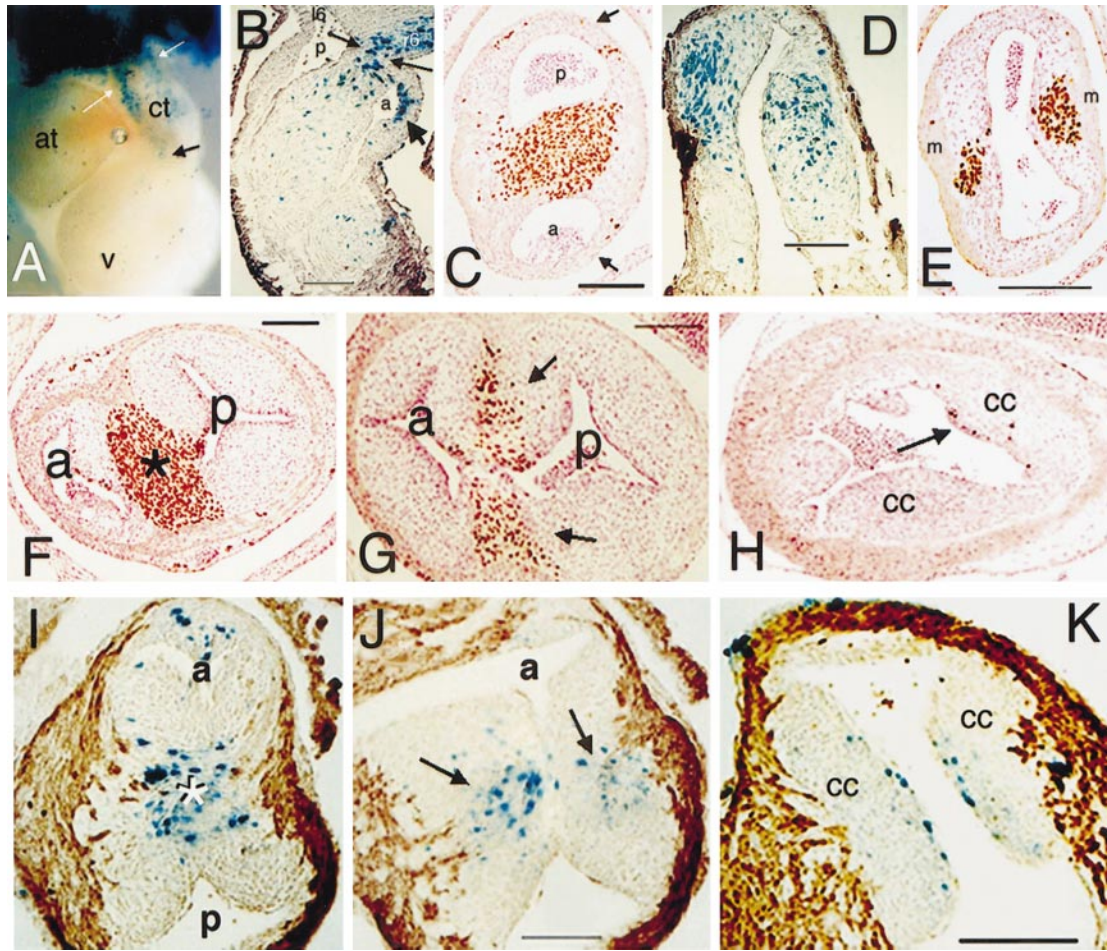
FIG. 3. Septation of the aortic sac and truncus. (A, B, D, I, J, K) Cx43-lacZ mouse embryos treated for β-galactosidase histochemistry. (C, E, F, G, H) Quail–chick chimeras stained with QCPN (brown). The myocardium is labeled with an antibody to cardiac myosin (brown) in B, D, I, J, K. (A) Embryonic day (ED) 11.5 whole-mount mouse heart viewed from the right side. Two columns of lacZ-positive cells (white arrows) extend from the pharynx through the distal conotruncus and into the distal conus (black arrow). at, atrium; ct, conotruncus; v, ventricle. (B) Oblique section through the aortic sac and distal conotruncus. The aorticopulmonary septum (between the two thin arrows) containing numerous Cx43-lacZ-positive cells divides the aortic sac into aortic (a) and pulmonary (p) channels. LacZ-positive cells in the aorticopulmonary septum are continuous with positive cells that surround the right and left sixth arch arteries. More lacZ-positive cells are subjacent to the aortic endothelium (fat arrow). (C) Transverse section of the aortic sac of a quail–chick chimera showing the neural crest-derived aorticopulmonary septum separating the aortic and pulmonary channels. (D) Oblique section through the conotruncus where two large populations of Cx43-lacZ-positive cells can be seen in the outflow cushions. (E) Transverse section showing the two prongs of the aorticopulmonary septation complex filled with neural crest cells. (F, G, H) Stage 28. Distal-to-proximal transverse sections through the conotruncus. In (F), the neural crest-positive truncal septum (star) divides the truncal lumen into the nascent aortic and pulmonary semilunar valves. More proximally (G), the two septal prongs, filled with neural crest cells, lie in the truncal cushions. (H) Neural crest cells (arrow) lie under the endocardium of the conal cushions (cc) near the base of the heart. (I, J, K) Distal-to-proximal sections through the conotruncus at the level of the semilunar valves (I), the conotruncal transition (J), and the proximal conus (K). LacZ-positive cells delineate the condensed mesenchyme of the aorticopulmonary septation complex in I (star) and J (arrow) and lie under the endocardium of the proximal conal cushions in K. (B–K) Bar, 100 μm.

separated not only from the aortic vestibule, but also from the tricuspid valve. Although lacZ expression was greatly reduced by late septation in mouse embryos, enough cells remained positive to suggest a pattern of septation of the proximal outflow in mouse embryos similar to that in the chimeras (compare Fig. 4A with 4E; Fig. 4B with 4F, Fig. 4C with 4B and 4D, and Fig. 4D with 4F and 4G). However, no lacZ-positive cells were at the site of ventricular septal closure or in the wall of the pulmonary infundibulum. By 14.5 dpc, most of the conus had become myocardialized with the exception of the remnant of the outflow septum, which still had numerous lacZ-positive cells (Figs. 4H, 4I).
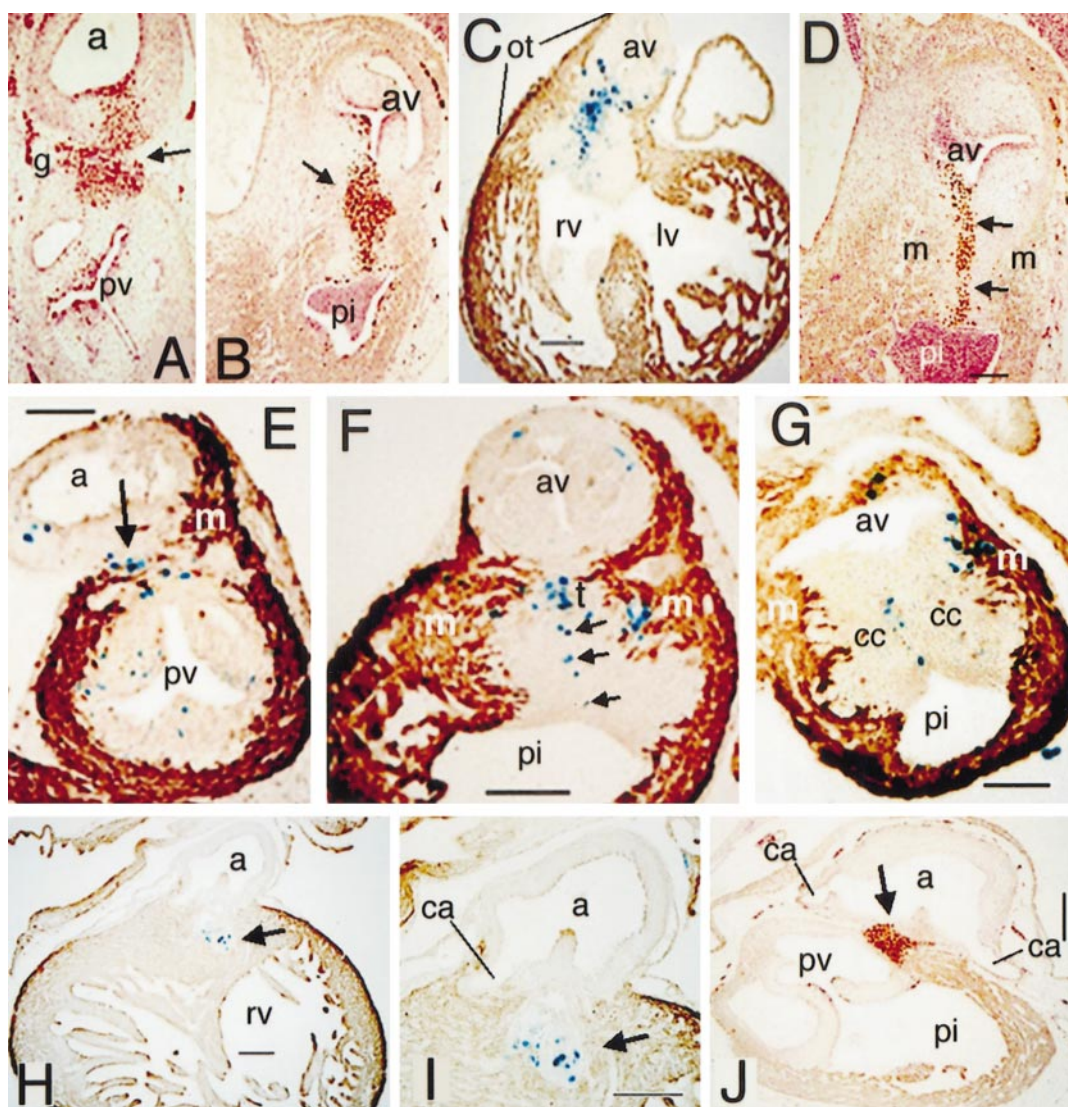
FIG. 4. Septation of the proximal outflow conotruncus. (A, B, D, J) Quail–chick chimeras. The quail cardiac neural crest cells are labeled with QCPN antibody (brown). (C, E, F, G, H, I) Cx43-lacZ mouse embryos in which the myocardium is stained with an antibody to cardiac myosin (brown). (A) The outflow septation complex (arrow) separates the base of the aorta (a) from the pulmonary valve (pv). Neural crest cells lie under the endothelium of the pulmonary valve cushions and are scattered in the cushion mesenchyme. g, cardiac ganglion. (B) Transverse section through the heart at the level of the aortic valve (av) and the pulmonary infundibulum (pi). Neural crest cells fill the proximal part of the truncal septum (arrow) and are interspersed with the myocardium surrounding pulmonary infundibulum. (C) Frontal section through the outflow tract (ot) showing lacZ-positive cells along the fusion line of the conal cushions. rv and lv, right and left ventricles. (D) A seam of neural crest cells marks the fusion line of the conal cushions (arrows). Most of the cushion tissue has been invaded by myocardium (m). (E) Transverse section through the pulmonary valve (pv) and base of the aorta. LacZ-positive cells are scattered in the truncal septum (arrow). The pulmonary valve cushions contain scattered lacZ-positive cells. (F) Transverse section through the pulmonary infundibulum and the aortic valve. Myocardium with intermixed lacZ-positive cells is beginning to penetrate the fused conal cushions. Scattered lacZ-positive cells are in the truncal septum (t) and the seam marking the fused conal cushions (arrows). (G) Transverse section through the heart at the level of the conus. Only a few lacZ-positive cells mark the fusion line of the conal cushions. (H) Transverse section through the heart at the level of the aorta and the base of the right ventricle. The remnant of the outflow septation complex (arrow) contains scattered lacZ-positive cells. (I) A higher magnification of the truncal septum in (H). ca, coronary artery. (J) ED 10 chimera. Transverse section through the pulmonary valve at its junction with the pulmonary infundibulum and the aortic sinuses (a). The remnant of the truncal septum (arrow) shows the abundant presence of cells derived from cardiac neural crest. All bars, 100 μm.

This remnant of the outflow septum in the mouse greatly resembled that of the chick (Fig. 4J).

Elongation, Remodeling, and Final Configuration of the Aorta, Pulmonary Trunk, and Great Arteries

During septation in the quail–chick chimeras, elongation of the great arteries occurred as neural crest cells migrated from the arch arteries into the walls of the nascent aorta and pulmonary trunk (Waldo *et al.,* 1998). By the end of the septation period, the distal two-thirds of the walls of the aorta and pulmonary trunk consisted of neural crest-derived cells while the proximal parts, except for the facing walls, were non-crest-derived cells (Table 1, Figs. 5A, 5B, 5C). The aorta and pulmonary trunk of the mouse embryos also elongated; however, the expression of lacZ during this process did not reflect the neural crest distribution seen in chimeras (Table 1). Instead, the pulmonary trunk was largely lacZ-negative while the aorta had many lacZ-positive cells surrounding the lumen adjacent to the endothelium proximally and throughout the wall more distally (Figs. 5D, 5E, 5F). In contrast, the ductus arteriosus, derived from the left sixth aortic arch artery, showed abundant lacZ expression. In both the aortic arch and the ductus arteriosus, lacZ expression stopped abruptly at the junction with the descending aorta (compare Figs. 5C and 5E), mimicking the abrupt termination of neural crest cells at the same site in chimeric embryos.

The arch arteries were remodeled asymmetrically in both mouse and avian embryos to form the adult great arteries. The adult pattern of the great arteries was different in the two species due to basic differences in the way the aortic sac was remodeled. The chimera had a right-sided aorta with two brachiocephalic arteries branching from the left side of its ascending portion, while the mouse had three major arteries branching from the cranial part of a left-sided aortic arch. In mammals, the aortic arch consists of the left fourth arch artery between the left subclavian and common carotid arteries, while the right fourth arch artery becomes the first segment of the right subclavian artery (Congdon, 1922). Since all the persisting arch arteries had a tunica media of neural crest cells in the chimeras (Fig. 5G), we expected the derivatives of these vessels in the mouse to be lacZ positive. This occurred in that lacZ was expressed most intensely in the wall of the aortic arch between the left common carotid and the subclavian artery (Figs. 5I, 5J), in the common carotids (derived from the third arch arteries), and in the proximal part of the right subclavian artery at 13–14.5 dpc (Fig. 5K). The mouse ductus arteriosus (left sixth arch artery in mammals) also expressed lacZ, reflecting the pattern of neural crest distribution in the bilateral ductuses of the chick (Fig. 5H). Significantly, the atrioventricular cushions and pulmonary arteries were free of quail cells in quail–chick chimeras and lacZ expression in mouse embryos (not shown). However, there were two tissues in which cardiac neural crest cells were not apparent in chimeras but showed lacZ expression in the mouse—the

pericardium was very lacZ-positive, and became more so with age, and as mentioned previously, the pharyngeal ectoderm exhibited expression.

Neural Crest Cells Form Cardiac Ganglia

During and after septation, neural crest cells migrated subepicardially into the outflow tract of quail–chick chimeras, external to the myocardium, and formed cardiac ganglia (Figs. 4A and 5A). Although in the mouse embryo no recognizable ganglia had formed in the outflow tract by the end of the septation period, lacZ-positive cells were found in small clusters next to the aorta and pulmonary trunk. Histological sections stained for neurofilament at 14.5 dpc showed very small amounts of fine neurofilament-positive branches near or in these clusters. Additional clusters were also found in the myocardium near the mouth of the coronary arteries (not shown). This distribution is consistent with the expected location of cardiac ganglion cells (Kirby and Stewart, 1983; Kirby *et al.,* 1980).

DISCUSSION

Cx43, a gap junction gene implicated in many developmental processes, is expressed in neural crest and neural crest derivatives in both mouse and chick embryos (Minkoff *et al.,* 1993; Ruangvoravat and Lo, 1992). Cardiac neural crest is essential for normal development of the cardiovascular system in the chick embryo (Kirby and Waldo, 1995). While it is not required for initial formation of the aortic arch arteries, it is essential for normal repatterning of these bilaterally symmetrical endothelial channels into the asymmetric great arteries. In fact, cardiac neural crest cells appear to convey a blueprint to the pharynx for remodeling the embryonic arch arteries into their adult pattern (Kirby *et al.,* 1997). In addition, these cells are essential for the septation and remodeling of the cardiac outflow tract (Waldo *et al.,* 1998). We do not have direct evidence that cardiac neural crest functions in the same way in mammalian embryos; however, the present results show that the timing and location of Cx43-lacZ expression very closely resembles the timing and migration patterns of cardiac neural crest populations in the pharyngeal arches and in the outflow tract just prior to and during outflow septation. Figure 6 summarizes the migration of cardiac neural crest as we perceive it in the mouse. Our data suggest that cardiac neural crest cells in mouse and chick not only have similar migration patterns, but also more than likely serve the same function.

Similarities in Mouse and Chick Cardiac Neural Crest and Heart Development

The most striking similarity was in the neural crest-derived mesenchyme of the caudal pharyngeal arch arteries, which were ensheathed by neural crest cells in the chick

and exhibited lacZ expression in the mouse. Both neural crest cells and lacZ expression ended abruptly at the junction of the arch arteries with the dorsal aorta. In the outflow tract, lacZ expression began at 9.5 dpc in a pattern that exactly reflected the subendocardial population of neural crest cells in chimeras. Streams of lacZ-positive cells appeared in the 10.5-dpc mouse cardiac outflow tract at a time comparable to migration of cardiac neural crest from the pharyngeal region into the outflow tract of the chick embryo. Moreover, the condensed mesenchyme of the outflow septum, which consisted of cardiac neural crest cells, was well represented by lacZ-positive cells during the peak of the septation period, 12–12.5 dpc. Fusion of the conal cushions, demarcated by a seam of cardiac neural crest cells along the closure site, was mimicked by a seam of lacZ-positive cells in the mouse outflow tract at 12.5 dpc. Finally, lacZ expression in the wall of the definitive aorta reflected the distribution pattern of neural crest cells in the wall of the ascending aorta of the chick.

These data carry the implication that Cx43 has a role in repatterning the aortic arch arteries and septation of the outflow tract. Although it has already been shown that Cx43 expressed by the myocardium plays a major part in myocardial development and function (Gros *et al.,* 1978; Fromaget *et al.,* 1990; Gourdie *et al.,* 1992, 1993; Ruangvoravat and Lo, 1992; Chen *et al.,* 1994; van Kempen *et al.,* 1991, 1995, 1996; Dahl *et al.,* 1995; Wiens *et al.,* 1995;

Delorme *et al.,* 1997; Verheule *et al.,* 1997), a role for Cx43 expressed in cardiac neural crest cells in outflow septation and development of the great arteries has not been reported.

Differences in Outflow Septation between Mouse and Chick

The major differences in distribution of quail cells in quail–chick chimeras and lacZ-expressing cells in the Cx43-lacZ mice are summarized in Table 1. While the pattern of lacZ-positive cells correlated very well with cardiac neural crest in the pharyngeal region, differences were observed as neural crest cells began to enter the aortic sac and conotruncus prior to septation. New quail–chick chimera data demonstrated that crest cells migrated into the outflow tract of the quail–chick chimeras by two different routes: a smaller population entered in a single but discontinuous layer just under the endocardium while a second larger population entered opposite sides of the outflow tract just under the myocardium to seed and form the two distinctive prongs of condensed ectomesenchyme of the aorticopulmonary septation complex (stages 19–24). When lacZ-positive cells appeared in the mouse outflow tract for the first time (9.5 dpc), they were just beneath the endocardium in a single layer that became three to four cells thick and completely invested the aortic sac (dark ring around preseptation aortic sac in Table 1). However, there

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FIG. 5. (A, B, C, G) Quail–chick chimeras, stained with QCPN. (D, E, F, H, I, J, K) LacZ-Cx43 mouse embryos; myocardium labeled by cardiac myosin antibody. (A) Only the facing walls of the aorta and pulmonary trunk are composed of neural crest cells, whereas the remainder of the walls consist of non-neural crest-derived cells. (B, C) Transverse section through the proximal (B) and distal (C) aorta at incubation day 7.5–8. The base of the aorta (a) is relatively free of neural crest cells, whereas distally its walls are almost entirely neural crest cells, as is the wall of the ductus (d). Distally, the aortic arch and ductus merge with the descending aorta (dao) and neural crest cells end abruptly at the junction. (D) ED 14.5 heart viewed from the right side; right atrium removed. The walls of the aorta and thymus (th) are lacZ-positive while the pulmonary trunk (p) is lacZ-negative. The lacZ-positive truncal septum (arrow) is located between the valves of the aorta and pulmonary trunk. (E) Frontal section through the ascending aorta, wall of the aortic arch (*), and descending aorta. LacZ-positive cells are found in the wall of the ascending aorta and aortic arch, but end abruptly at the junction of the aortic arch with the descending aorta. (F) Transverse section through the base of the pulmonary trunk, pulmonary valve (pv), and the ascending aorta. Lac Z-positive cells form a layer of cells surrounding the aortic lumen but almost no positive cells appear in the pulmonary trunk. (G) The pulmonary trunk is not present at this level as it has already divided into the left and right ductuses (ld, rd). All of the arch arteries are ensheathed with a thick coat of neural crest cells. rb and lb, right and left brachiocephalic arteries. (H) The ductus connects the pulmonary trunk with the descending aorta. The walls of the pulmonary trunk contain no lacZ-positive cells, but the ductus is very positive for lacZ. (I, J, K) Frontal sections through the arch of the aorta. In I, the wall of the distal end (left side) is lacZ-positive but the remainder of the arch is negative. The area of the aortic wall between left subclavian (ls) and left carotid (lc) arteries has the denser lacZ-positive labeling in J and K. The brachiocephalic artery is also lacZ-positive (b).

FIG. 6. Migration of the cardiac neural crest. (A) Cardiac neural crest cells exit the dorsal neural tube and migrate beneath the ectoderm to the circumpharyngeal area (B) prior to entering the pharynx where they fill pharyngeal arches 3, 4, and 6 (C and D). In the pharyngeal arches they separate each arch artery and the aortic sac from the pharyngeal endoderm (C) and condense against the lumen of each arch artery to form its smooth muscle wall (orange in D). A subpopulation of the cardiac crest cells forms the aorticopulmonary septum (E) which, here, has divided the aortic sac to form the aorta (Ao) and pulmonary trunk (P). In the mouse embryo the Cx43-lacZ marker was not expressed in the wall of the pulmonary trunk (blue in D). The two prongs of the aorticopulmonary (AP) septum extend into the proximal and distal conotruncus in the outflow ridges. Here, the bridge of the aorticopulmonary septal complex has partially divided the distal conotruncus (F, G) to form nascent semilunar valves of the aorta and pulmonary trunk. Another subpopulation of cardiac crest cells aligns itself subjacent to the endocardium along the leading edge of the ridges of truncus (G) and the seam of fusing ridges in the conus (H). In the mouse embryo there are just a few cells that express Cx43 beneath the endocardium of the truncal cushions but in the chick there are many crest cells here. (Oto, otic placode; S1, S2, S3, and S4, somites 1, 2, 3, and 4; DAo, dorsal aorta; AoS, aortic sac; L, lumen of the conotruncus; RV, right ventricle; LV, left ventricle.)

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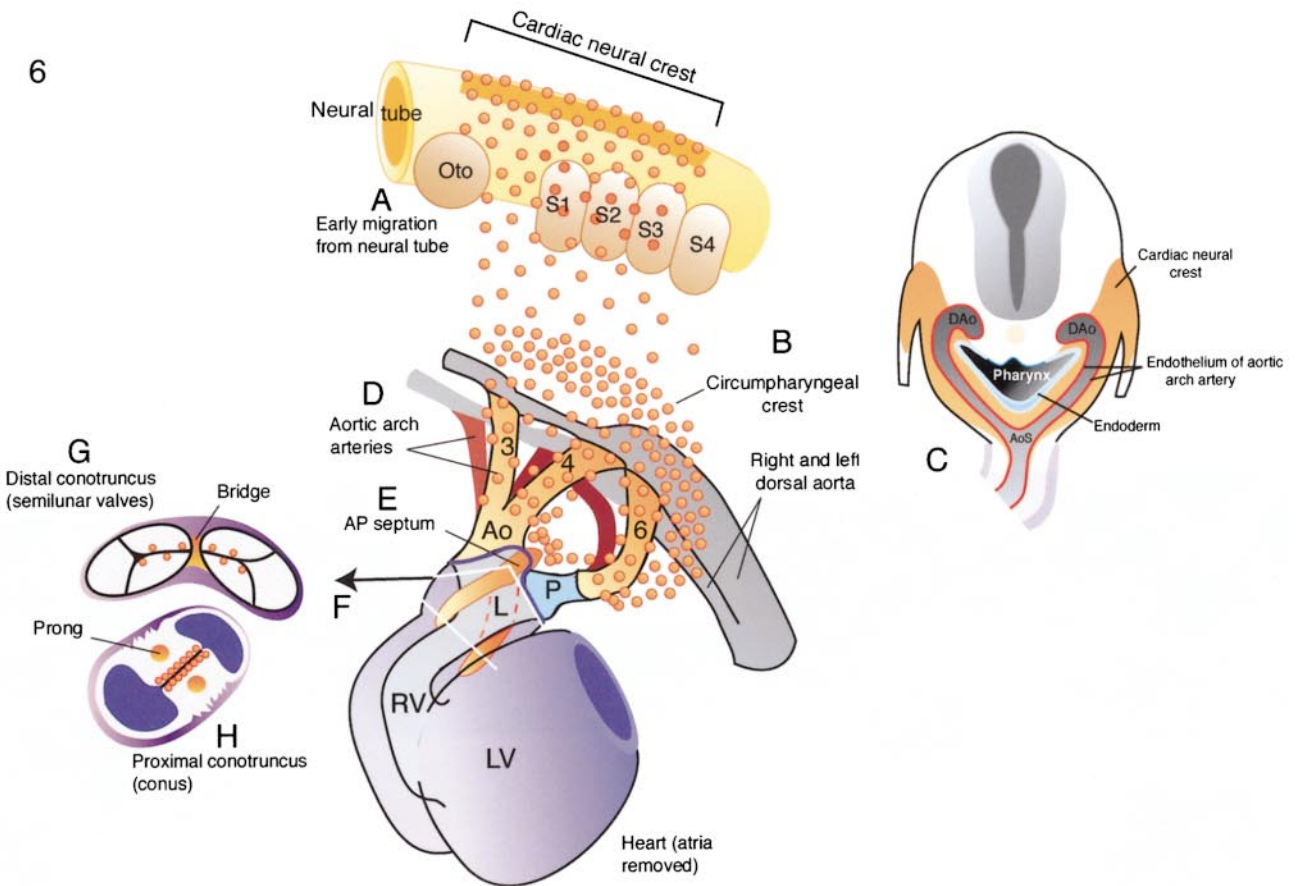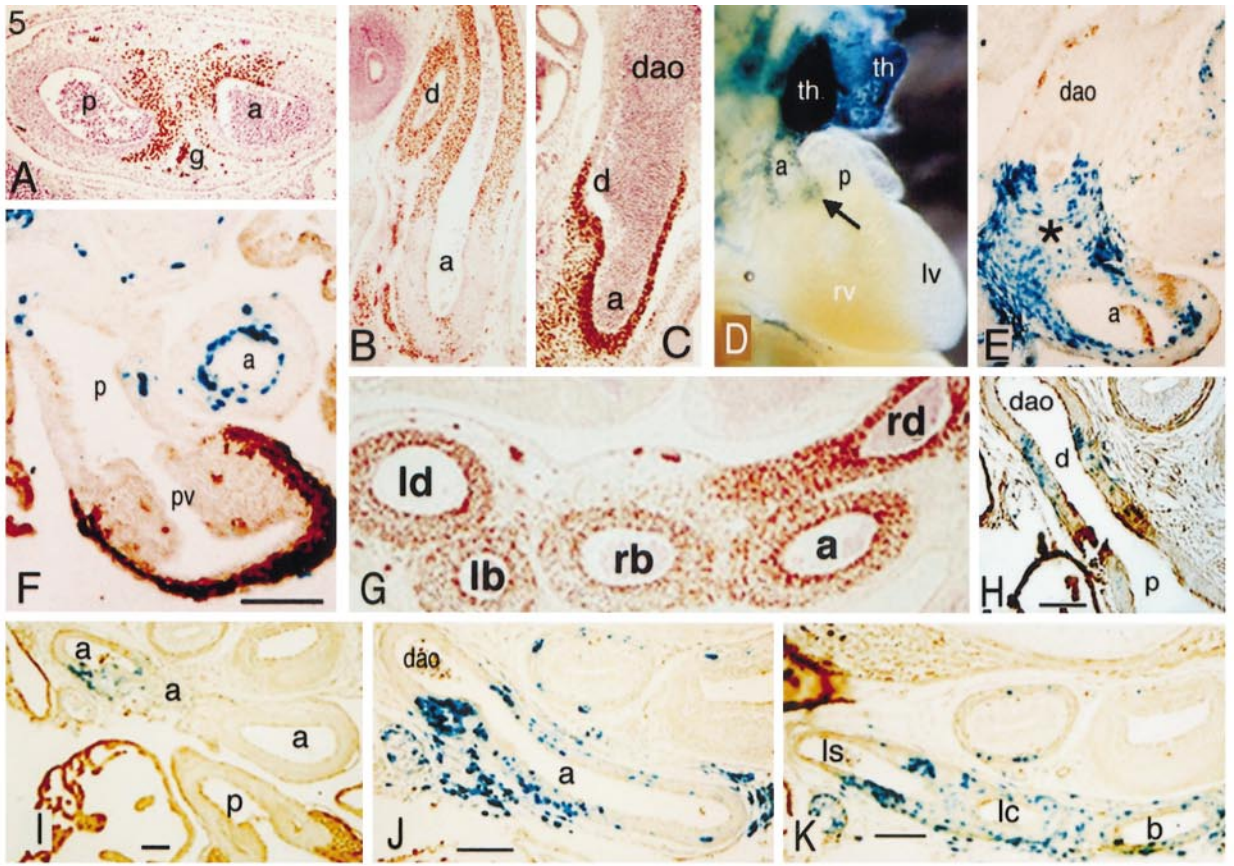
were no lacZ-positive cells near the myocardium. This represents a distinctive difference between the behavior of neural crest cells in the chimeras and in the mouse. In fact, by 10.5–11.5 dpc when two populations of lacZ-positive cells comprising the prongs of the aorticopulmonary septation complex did appear in the truncus, they were continuous distally with the large population of lacZ-positive cells investing the aortic sac.

As the prongs of the aorticopulmonary septation complex began to condense in the mouse outflow tract, lacZ was expressed in many but not all of the condensing cells. Neighboring, scattered cells in the loose mesenchyme underlying the myocardium and in or around the forming prongs also expressed lacZ, and it is unclear whether these cells would eventually take part in forming the prongs or were non-neural crest cushion mesenchyme cells.

There was a distinct difference between the chick and the mouse in the proximal extent of the prongs into the conotruncus. In the chick, the longest prong (septal) did not quite reach the conotruncal transition; however, in the mouse, this prong extended into the distal part of the conus. This pattern is apparently normal for mammals since it has been noted by Bartelings *et al.* (1990) in the outflow tract of human embryos. In chimeras the prongs did not extend into the conus and the crest cells seen in the conus migrated next to the endocardium. In contrast, lacZ-positive cells in the mouse that initially appeared next to the endocardium of the truncus eventually disappeared and only a few were present in the conus at later stages, perhaps because of the more proximal extension of the prongs.

Because the tunica media of the chick pulmonary trunk is composed of neural crest, we were surprised that the mouse pulmonary trunk did not express lacZ. In the chick, the ascending aorta and pulmonary trunk derive originally from the aortic sac and are initially free of neural crest cells in their walls except at the site of the aorticopulmonary septum (Waldo *et al.,* 1998). Subsequent elongation of the aortic arch arteries, which are invested by neural crest cells, is accompanied by displacement toward the heart of the neural crest–non-neural crest interface which marks the junction of the aortic sac with the aortic arch arteries. Thus the walls of the ascending aorta and the pulmonary trunk become invested secondarily by neural crest cells (except in the nonfacing walls just at and slightly above the semilunar valves) (Waldo *et al.,* 1998, 1994). Although lacZ expression in the ascending aorta, aortic arch, and brachiocephalic artery of the mouse embryo mirrors the avian pattern of cardiac neural crest cells, the development of this region in the two species is quite different. In mammals the distal ascending aorta and most of arch, as well as the brachiocephalic artery, are remodeled from the original aortic sac (Van Mierop and Netter, 1969). Because of the presence of lacZ-positive cells surrounding the undivided aortic sac, it is not surprising that these vessels are invested with lacZ-positive cells. However, this presents something of a dilemma. Since the aortic sac in the chick is not surrounded by neural crest cells, a short length of the base of the aorta

and pulmonary trunk is virtually free of neural crest cells except for the remnant of the aorticopulmonary septum between the two vessels. If the lacZ-positive cells surrounding the aortic sac in the mouse are derived from neural crest, this represents a difference in neural crest pattern between mouse and chick. The other possibility is that the cells are not neural crest derived, but express lacZ because of a functional similarity with the neural crest cells surrounding the aortic arch arteries. Paradoxically, the lack of lacZ expression in the pulmonary trunk, which is also derived from the aortic sac in the mouse embryo, fails to reflect the neural crest pattern in chick and is puzzling with regard to the fate of the lacZ-positive cells that initially surrounded the aortic sac. The most likely explanation for this is that the pulmonary trunk probably derives mostly from the distal truncus arteriosus, as suggested by Van Mierop and Netter (1969) for the human embryo. Since the myocardial cuff extends much higher posteriorly than anteriorly, the pulmonary portion of the aortic sac is extremely short when it is divided. In this event, the smooth muscle cells investing the wall of the proximal pulmonary trunk are likely to be derived from transformed myocardial cells as has been observed in rat embryos (Ya *et al.,* 1998b). Because of the shortened posterior aortic sac, the crest cells may have been displaced to the aorticopulmonary septum and/or the aorta. Another possibility is that Cx43 expression is turned off in the wall of the posterior aortic sac as it is divided.

During septation of the outflow tract in chick embryos, neural crest cells become a prominent feature of the semilunar valve cushions and leaflets, especially at the margins (Waldo *et al.,* 1994, 1998). Although some scattered lacZ-positive cells were found in the mouse semilunar valve cushions, they were never a prominent feature of the cushions or valve leaflets. Finally, we observed expression of lacZ-positive cells in the pharyngeal ectoderm, epicardium, and pericardium, areas where cardiac neural crest cells have never been seen in the chick embryo at these stages.

While we do not know exactly why there are differences in the locations of cells expressing lacZ in the mouse and cells derived from cardiac neural crest in chick, there are a number of possible explanations. Species differences could affect timing of neural crest migration, cell proliferation, condensation, differentiation, and tissue inductions. Certainly this is the case with the remodeling of the aortic sac and the great vessels where their final configuration is quite different in mammals and birds. Our data suggest that either the great vessels and their branches may form in a different manner in mammals than is currently believed or that some aspects of neural crest migration may differ in mammals and avians. Another possibility is that lacZ expression is lost with the completion of migration and as the neural crest cells differentiate. The loss of lacZ expression has been observed in neural crest outgrowths derived from neural tube explant cultures with the cessation of cell migration and the initiation of cell differentiation (Lo *et al.,*

1997). Similarly, *in vivo* studies of mouse–chick chimeras showed that the expression of lacZ was lost as the migrating mouse crest cells reached the pharynx and began to differentiate (M. L. Kirby, unpublished observations). The expression of lacZ in cells not ordinarily considered to be neural crest derivatives may reflect merely a species difference or use of gap junctions for a function shared with crest cells.

Can Cx43-LacZ Be Used as a Marker for Cardiac Neural Crest?

Because a neural crest-specific marker is not available in the mouse, we hoped to establish whether the Cx43-lacZ mouse is an appropriate model to study cardiac neural crest and its relation to mammalian cardiovascular development. All crest cells express the lacZ marker as they emerge from the neural tube (Lo *et al.,* 1997). This can be seen by the fact that X-gal staining is found in all regions where presumptive crest cells are known to be present, i.e., regions dorsolateral to the neural tube along the entire craniocaudal axis. How long the lacZ marker stays on as crest cells migrate to various distant destinations has not been determined for all crest lineages, although we expect that in most regions they stay on until they reach their target sites and begin to differentiate (based on distribution of lacZ-labeled cells). When they begin to differentiate, we expect that lacZ expression is turned off. This can be seen in the forming dorsal root ganglia, sympathetic ganglia, cranial ganglia, etc. (Lo *et al.,* 1997). Cardiac crest cells are lacZ labeled as they migrate into the pharyngeal arches and outflow tract, but in late stages of fetal heart development, lacZ expression is no longer found in the outflow tract. How long expression is sustained is not known; however, we have shown that many of the crest cells do lose their Cx43 expression by the completion of outflow tract septation. Whether these cells begin differentiation into smooth muscle or connective tissue or undergo apoptosis is not known at this time. Nevertheless, the fact that so many crest cells express lacZ during the early and middle stages of outflow tract septation suggests that Cx43 can serve as a tool for studying the role of cardiac neural crest in heart development. That these cells eventually lose Cx43 expression does not negate the usefulness of this tool as long as the temporal limitations of expression are realized. By comparing cells expressing lacZ in this mouse model with quail cells in quail–chick chimeras, we have shown very close correlation of Cx43 expression in the mouse with the temporospatial patterns of neural crest migration into the outflow tract during septation. Those differences we have noted appear to be related to inherent morphological differences between the hearts of mammals and birds and to differences in the timing of some of the events. For example, the length of the outflow tract, which is short in mammals and quite long in birds, and the presence (birds) or absence (mice) of a temporary groove separating the distal and proximal parts of the conotruncal ridges seem to determine how far toward the heart the tips of the prongs of the aorticopulmonary complex reach. The earlier closure of the mammalian conal septum, prior to its alignment with the ventricular septum, may affect migration of crest cells into the closure site of the ventricular septum. As noted in the previous section, the arterial branches from the left-sided aortic arch in the mouse are organized and located differently from those branching from the right-sided aortic arch in the chick. Thus the mammalian ascending aorta undergoes much more remodeling in mammals than in birds and this may be reflected by the continuous lacZ expression in the mammalian aorta. Finally, in the mammalian heart, remodeling of the conus septum is different from that of birds and gives origin to structures not present in the chick heart (Gessner and Van Mierop, 1970). In the final analysis, the similarities of Cx43 expression to patterns of avian cardiac crest are more striking than the differences and lend support to the idea that the basic role of cardiac neural crest in mammalian cardiovascular development is similar to that reported for the chick, but may also involve additional remodeling due to differences in morphology and timing. With the exceptions noted here, we believe that the Cx43-lacZ mouse model significantly enhances our repertoire of tools available to study cardiac neural crest in a mammalian setting.

Function of Cx43 in Cardiac Neural Crest Cells

Dye-injection studies showing that migrating neural crest cells are functionally linked by gap junctions (Ewart *et al.,* 1997; Lo *et al.,* 1997) suggest that gap junction communication plays a role in migration, perhaps maintaining the integrity of subpopulations of crest cells as they migrate from the neural tube into the pharynx and heart. Cx43 may also be involved in differentiation of some cardiac crest cell lineages. In fact, gap junction communication has been linked with the differentiation of a number of cell types, including both vascular smooth muscle and cardiac myocytes (Rennick *et al.,* 1993; Little *et al.,* 1995; Blackburn *et al.,* 1997; Proulx *et al.,* 1997; Angst *et al.,* 1997; Westfall *et al.,* 1997; Oyamada *et al.,* 1996; van Kempen *et al.,* 1995; Delorme *et al.,* 1995; Fromaget *et al.,* 1992; Gourdie *et al.,* 1990, 1992; Navaratnam *et al.,* 1986; Zhang and Thorgeirsson, 1994; Rosenberg *et al.,* 1996; Fried *et al.,* 1996; Minkoff *et al.,* 1993).

A major role for Cx43 expression may be in tissue remodeling that accompanies and follows cardiac outflow septation. In the chick, cardiac neural crest cells take an active part in septation of the conus, resulting in a separation of the right and left ventricular outflow tracts. A subpopulation of these cells continues its migration into the muscular wall of the right, but not the left, ventricular outflow tract where continued remodeling of tissues occurs. The association of cardiac neural crest cells with sites undergoing active remodeling occurs repeatedly in development of the outflow tract and great arteries. As remodeling occurs, mechanisms such as programmed cell death, cell

proliferation, and tissue mobilization are activated by as yet unidentified factors. It is possible that Cx43 gap junctions in cardiac neural crest cells at sites of remodeling may pass on second messengers associated with tissue remodeling. Interestingly, homozygous null mutations of the Cx43 gene in mouse embryos are associated with normal cardiac outflow septation. Even so, the pups die of right ventricular outflow obstruction caused by abnormal remodeling of the muscular wall of the right ventricular outflow tract (Reaume *et al.,* 1995). It is possible that the loss or reduction of Cx43 in cardiac neural crest cells in the Cx43 null mouse results in an imbalance of the normal processes that occur as part of right ventricular outflow remodeling. Significantly, in the transgenic CMV43 mouse in which Cx43 is overexpressed, the right ventricular outflow tract is functionally obstructed (Ewart *et al.,* 1997), although the phenotype has some differences from the Cx43 null mouse. More recently, it has been demonstrated by Huang *et al.* (1998) that faster or slower migration of neural crest *in vitro* and *in situ* was directly correlated with over- or underexpression of Cx43. A similar correlation was associated with proliferation of the ventricular myocardium. It would appear that the mistiming of cardiac neural crest migration or possibly the malfunction of the crest cells due to altered gap junctional communication may have profound effects on tissue remodeling orchestrated by cardiac neural crest cells. Reaume (1995) also described a normal aortic and pulmonary trunk in the Cx43 null mutant mouse, but others have observed cardiac alignment abnormalities affecting the great vessels (Lo *et al.,* 1997; Huang *et al.,* 1998; Ya *et al.,* 1998a). It is possible that the mistiming of crest migration or its malfunction may also play a role in the development of the aortic and pulmonary trunk. Since one of the functions of the cardiac crest is to initiate elastogenesis in the aorta and pulmonary trunk and their branches (Rosenquist *et al.,* 1988, 1990), it follows that even though the outflow vessels may appear normal, elastogenesis may be perturbed and wall compliance abnormal. The histology and functional status of the aorta and pulmonary trunk have not been investigated, perhaps due to the early death of the pups.

ACKNOWLEDGMENTS

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