Neural Crest Cell Contribution to the Developing Circulatory System
Implications for Vascular Morphology?

Maarten Bergwerff, Marlies E. Verberne, Marco C. DeRuiter, Robert E. Poelmann, Adriana C. Gittenberger-de Groot

Abstract—In this study, the distribution patterns of neural crest (NC) cells (NCCs) in the developing vascular system of the chick were thoroughly studied and examined for a correlation with smooth muscle cell differentiation and vascular morphogenesis. For this purpose, we performed long-term lineage tracing using quail-chick chimera techniques and premigratory NCC infection with a replication-incompetent retrovirus containing the LacZ reporter gene in combination with immunohistochemistry. Results indicate that NCC deposition around endothelial tubes is influenced by anteroposterior positional information from the pharyngeal arterial system. NCCs were shown to be among the first cells to differentiate into primary smooth muscle cells of the arch arteries. At later stages, NCCs eventually differentiated into adventitial fibroblasts and smooth muscle cells and nonmuscular cells of the media and intima. NCCs were distributed in the aortic arch and pulmonary arch arteries and in the brachiocephalic and carotid arteries. The coronary and pulmonary arteries and the descending aorta, however, remained devoid of NCCs. A new finding was that the media of part of the anterior cardinal veins was also determined to be NC-derived. NC-derived elastic arteries differed from non-NC elastic vessels in their cellular constitution and elastic fiber organization, and the NC appeared not to be involved in designating a muscular or elastic artery. Boundaries between NC-infested areas and mesodermal vessel structures were mostly very sharp and tended to coincide with marked changes in vascular morphology, with the exception of an intriguing area in the aortic and pulmonary trunks. (Circ Res. 1998;82:221-231.)

Key Words: quail-chick chimera ■ pharyngeal arch artery ■ smooth muscle cell ■ great artery ■ neural crest migration

Neural crest cells have been studied extensively for their migratory behavior and potential to differentiate into a wide variety of cell types. In cardiovascular research, the crest region located between the midotic placode and somite 3, also called the cardiac NC, has received much attention because of its major role in cardiovascular development. Participation in outflow tract septation, media formation of the pharyngeal arch arteries, and contribution to parasympathetic cardiac innervation have been documented using quail-chick chimeras. The importance of the extensive contribution of NCCs to such a wide variety of cardiovascular structures is made clear by the occurrence of congenital malformations involving the NC and presenting more or less severe cardiovascular anomalies. Syndromes associated with 22q11 deletions and vitamin A deficiency are known NC-associated disorders, including cardiovascular malformations. Experimental removal of the cardiac crest in chick embryos, administration of excess retinoids, and deletion of retinoic acid receptor genes also result in severe malformations like ventricular septal defects, common arterial trunk, and aortic arch interruptions. In the present study, the emphasis is not on malformations due to NC disorders but on the actual role of the crest in normal development, focusing on the ectomesodermal lineage that differentiates into vascular SMCs to form the media of the great thoracic arteries.

Vascular SMCs are reported to originate from either the splanchnic mesoderm or the NC. Recently, a third possible origin was ascribed to the endothelial cells, which appear to transdifferentiate into SMCs in the early avian dorsal aorta. Le Lievre and Le Douarin have reported on the distribution of NCCs to the great arteries in the thorax. Other studies have further elaborated on NC seeding of the pharyngeal arches both in birds and in mammals. The tunica media of the great vessels derived from the pharyngeal arch arteries (eg, aortic arch, brachiocephalic arteries, and pulmonary arch arteries) was shown to consist almost exclusively of NCCs, whereas other thoracic vessels (eg, pulmonary arteries, subclavian arteries, and descending aorta) appeared devoid of NCCs and thus entirely mesodermal in origin. This unequal distribution of cells of different embryonic origins may be of influence on differential vascular patterning.

Recently, we reported on the differentiation of the thoracic arteries during chick embryonic development and suggested...
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Visualized using clear whole-mount distribution patterns of NCCs can be major interference with normal development. In addition, much less invasive than chimera techniques, circumvent lineage systems.30–32 Retroviral introduction of the LacZ gene studied the avian NC lineage27–29 as well as several other cell lineage systems.30–32 Retroviral introduction of the LacZ gene is, in contrast to fluorescent dye injections, beneficial in detecting cell lineages over long time spans and is, at the same time, much less invasive than chimera techniques, circumventing major interference with normal development. In addition, clear whole-mount distribution patterns of NCCs can be visualized using β-gal conversion. Using the retrovirus in combination with immunohistochemistry, we have been able to map distribution patterns of NCCs at any desired developmental stage and to superimpose vascular differentiation patterns in an attempt to relate NC contribution and vascular morphogenesis. For comparison, quail-chick chimeras were also included in the present study. Chimeras have the advantage of labeling virtually all cells emanating from the cardiac crest.

Materials and Methods

Virus

The replication-incompetent virus, designated CXL, that was used in the present study was kindly provided by Dr T. Mikawa (Cornell University Medical College, New York, NY).26 The design of this vector was based on spleen necrosis virus. It lacks the structural viral proteins necessary for replication and carries the LacZ reporter gene.26,27,30,31 The virus was resuspended in a small volume of medium containing 100 μg/mL polybrene (Sigma Chemical Co.) and indigo carmine blue. The harvested virus batch was used for both infection of embryos and titration on cells. Viral titers were determined in duplicate by infection of R2 rat fibroblasts with dilutions of the virus batch on the day of the experiment. Titters varied between 2 and 4×10^3 transducing units/mL.

Infection of Embryos

Fertilized specified pathogen-free white leghorn eggs (ID-DLO) were incubated for 36 to 42 hours at 37°C, windowed, and staged according to Hamburger and Hamilton.22 Embryos between stages 8 and 10 were used for infection with freshly prepared CXL solution containing polybrene. Using glass micropipettes and carefully exerted pressure by a Hamilton syringe connected to an oil-filled system, the neural groove/tube was filled with viral suspension from the somite–4 to –5 region in an anterior direction. Spilling of virus over the neural folds or out of the anterior neuropore was minimized as much as possible. After injection, the eggs were tightly sealed with Scotch tape and returned to the incubator for further development.

Tissue Preparation and β-Gal Staining

Infected embryos were allowed to develop until stages 19 to 40, collected, and further processed. The youngest embryos (stages 19 to 33) were fixed in toto by immersion in 4% paraformaldehyde in PBS (4 hours, 4°C), whereas bigger embryos were first perfused with the fixative via the right atrium, followed by immersion-fixation. After extensive rinsing in PBS, the thoracic viscera were carefully removed from fixed older embryos. Next, embryos and heart-lung specimens were stained by immersion in X-gal solution (PBS containing 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L MgCl2, and 0.1% [wt/vol] X-gal [C14H15BrClNO6, Boehringer]) while being shaken at 37°C for 2 to 4 hours. Again, the Cardiovascular system of larger specimens was perfused with X-gal solution before immersion-incubation. Stained embryos were thoroughly rinsed in PBS, evaluated macroscopically, and processed for immunohistochemistry. The results are based on a total number of 56 successfully labeled embryos.

Immunohistochemistry

β-Gal–stained embryos and heart-lung specimens were swiftly dehydrated in graded ethanol, followed by Paraclear (Earth Safe Industries Inc) and subsequent embedding in paraffin. Xylene-free Paraclear was used to prevent the blue precipitate from dissolving. Sections of 5 μm were cut and distributed over several series of glass slides, allowing for various staining procedures of each embryo. Standard resorcin/fuchsin staining was used to visualize elastic fibers, and monoclonal antibodies against muscle–specific actin (HHF35,34 DAKO A/S) and HNK-135 enabled discernment of differential pathways of NC derivatives. Before antibody incubation, endogenous peroxidase activity was quenched by treatment with 0.3% H2O2 in PBS. Routine immunohistochemical staining was performed using overnight incubations with the primary antibodies diluted in PBS with 0.05% Tween 20 and 1% chicken egg albumin (HHF35, 1:1000; HNK–1 medium, 1:10). Sections were then thoroughly rinsed, and bound primary antibodies were visualized with horseradish peroxidase–conjugated rabbit antimouse antibodies (1:300, DAKO A/S) and treatment with 0.04% diaminobenzidine tetrahydrochloride/0.06% H2O2 in 0.05 mol/L TRIS-malate (pH 7.6) for 10 minutes at room temperature. Then the sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol and Paraclear, and mounted in Entellan (Merck).
dures. In addition to antibodies revealing differentiation antigens (HHF35, HNK-1, and smooth muscle α-actin [1A4, DAKO A/S]), the antibody QCPN (a quail nuclear marker, Developmental Studies Hybridoma Bank) was used to visualize distribution of quail NC-derived cells. Twenty-two successfully transplanted chimeras were used in the present study.

**Results**

**Stages 19 to 21**

NC-derived cells showing β-gal activity were shown to have migrated into the entire pharyngeal arch region (Fig 1a). The dense mesenchyme of the maxillary buds and branchial arches that surrounds the arch arteries exhibited moderate to extensive blue staining. NCC distribution extended from the most ventrally located arch mesenchyme, alongside the pharynx, and generally up to the paired dorsal aortas and anterior cardinal veins. The NC distribution pattern along the pharyngeal arterial system is depicted in Fig 2a. In contrast to the ectomesenchyme of the pharyngeal arches, the mesenchyme that surrounds the aortic sac region is far less dense, and only a few labeled cells positioned both dorsally and ventrally of the aortic sac and in the endocardial cushion tissue of the outflow tract were shown. Ectomesenchymal contribution to the aortic sac therefore appeared limited at these stages. Endothelial cells in the entire pharyngeal region never showed staining for β-gal activity. By use of a double staining against muscle-specific actins, part of the NCCs in the aortic sac revealed actin expression indicative of primary SMC differentiation (Fig 1b and 1c). The pharyngeal arch arteries, which are also aligned by NC-derived ectomesenchyme, did not yet show signs of early actin expression at this stage in development. The paired dorsal aortas, on the other hand, showed a thin layer of actin-positive cells directly adjacent to the endothelium. These primary SMCs in the dorsal aorta were never shown to be of NC origin in the region posterior to arch 4. However, the anterior dorsal aortas (ie, ductus caroticus and early carotids) showed coexpression of actin and β-gal in a small number of cells, suggesting the onset of SMC differentiation of NC-derived cells.

**Stages 23 and 24**

Whole-mount–stained stage 23/24 embryos clearly demonstrated the disposition of the circumpharyngeal crest area (Fig 1d). Sections revealed massive NC infestation of the arches, whereas further upstream toward the heart, the arch arteries and aortic sac were surrounded by lower numbers of NCCs (Fig 1e and 1f). The lateral sides of the arch arteries clearly
Figure 2. Schematic representation of NCC contribution to the developing arterial system in the chick, as deduced from quail-chick chimeras and retrovirally infected embryos. Density of dots corresponds with relative contribution of NCCs to perienothelial tissue or the media. a, Stage 21. b, Stage 27. c, Stage 31. d, Stage 40. AoAr indicates aortic arch; AoS, aortic sac; AsAo, ascending aorta; BA, brachiocephalic artery; CA, carotid artery; CO, coronary artery; DA, ductus arteriosus; DsAo, dorsal aorta; DsAo, descending aorta; PA, pulmonary artery; PT, pulmonary trunk; SA, subclavian artery; and III, IV, and VI, pharyngeal arch arteries.

showed more seeding with NCCs than the median parts at these stages. Moreover, no indication of a prospective aorticopulmonary septum was found in any of the embryos, as NCC numbers were extremely low at the dorsal side of the aortic sac area. NCCs extended well into the outflow tract cushions, preferably in a dorsally located stretch of cells. In the aortic sac and arch arteries, part of the NCC population was double-stained for LacZ and muscle actin.

Stages 26 to 28
By stages 26 to 28, the third, fourth, and sixth pharyngeal arch arteries are entirely ensheathed by NC-derived cells (Fig 2b). Also, the remnants of the combined first and second arch arteries, still present as small luminized vessels branching from the paired proximal third arches, are surrounded by NCCs. The NCC-containing vessel wall surrounding the third arch artery continues downstream along the paired anterior parts of the dorsal aorta (also called carotid arteries at this stage\(^a\)). In the fourth and sixth arches, however, LacZ labeling stops abruptly at the junction with the dorsal aorta. No NCCs could be found in the posterior part of the dorsal aorta. Occasionally, some LacZ-positive cells were localized in the proximal part of the pulmonary artery (3 of 13 embryos), close to its branching point with the sixth arch artery. No obvious differences in NCC distribution were detected between the left or right part of the pharyngeal arch region. The primary vessel wall of the left fourth arch artery and of both left and right carotid ducts (all of which obliterate during this relatively short time span) was shown to possess a marked NCC contribution.

Double staining for muscle-specific actins suggested that in all aforementioned vessels, NCC located adjacent to the endothelium had started differentiation into vascular SMCs, whereas more peripheral NCC layers were still actin negative (Fig 3).

In chimeras and retrovirally infected embryos, both aortic and pulmonary roots, now separated from each other by the aorticopulmonary septum, showed a remarkably smaller contribution of NCCs than the media of the arch arteries further downstream. Moreover, LacZ-positive and quail cells were preferentially distributed along the medial vessel walls and continued into the condensed mesenchyme and prongs of the aorticopulmonary septum (Fig 4a and 4b). The lateral sides of the bases of both vessels were devoid of NCCs, whereas further downstream, before branching into the arch arteries, NC distribution became entirely circumferential. At these stages of development, the aortic and pulmonary roots and the proximal parts of the arch arteries do not stain for muscle-specific actins.

Stages 30 to 34
Compared with earlier stages (stages 26 to 28), the distribution of NCCs in the vascular system remained basically unaltered. The vascular tree, however, is subject to extensive remodeling during these stages. Its morphology and the distribution of NCCs within the system are depicted schematically in Fig 2c. Most marked differences with earlier stages are those resulting from the degeneration of the left fourth arch artery and of both carotid ducts. In addition, the carotid arteries have lengthened considerably as a result of the caudal positioning of the heart in the thorax with respect to its initially more rostral position near the head and also as a result of the lengthening of the neck itself. Within persisting vessels, LacZ-labeled cells were localized at all possible positions in the NC-derived vessel walls; ie, no preferential labeling could be detected in either peripheral or adluminal cell layers. Blue staining of the condensed mesenchyme of the aorticopulmonary septum was still seen to be continuous with the median side of the pulmonary and aortic outflows, whereas no staining was found at the lateral side of the bases of these vessels.

Between stages 30 and 34, histology of the great arteries is characterized by separation of cell layers of the media due to deposition of matrix components. SMCs in the proximal part of the arterial tree do not express muscle actin at these stages (see also Reference 22). More distally, periendothelial cell layers express muscle actin and are surrounded by a few actin negative cell layers. NCCs were distributed both in proximal actin-negative parts and in more distal parts showing periendothelial actin expression, and they assumed actin-negative as well as actin-positive cellular phenotypes.

Stages 35 to 40
NCC distribution in stages 35 to 40 was confined to the aortic and pulmonary trunks, aortic and pulmonary arch arteries, and brachiocephalic and carotid arteries (Fig 2d). Both media and adventitia were shown to be of NC origin. NC adventitial fibroblasts and NC neural cell types could easily be distinguished by staining the latter with the HNK-1 antibody. The
coronary and pulmonary arteries and the descending aorta were found consistently negative in our study.

In the proximal part of the arterial tree, part of the media cell population starts reexpression of actin from around stage 35–36 onward. This wave of secondary actin expression starts at the interface with the myocardium and results in a markedly layered vessel wall consisting of an actin-negative intima-like layer surrounded by a media that is composed of alternating SMCs and nonmuscular cell layers. This rather thick lamellar wall starts directly after Valsalva’s sinus, thereby narrowing the lumen considerably. At the level of the sinus, both aortic and pulmonary vessel walls were thin and nonlamellar but showed muscle-specific actin positivity in peripheral cell layers up to the connection with the outflow tract myocardium (Fig 4c).

The inner lining of Valsalva’s sinus was always actin negative and was never shown to harbor NCCs. The outer actin-positive cell layers only showed NC contribution adjacent to the aorticopulmonary septum, which showed clear smooth muscle actin expression at these stages as well (Fig 4d to 4g). Otherwise, the ascending aorta and pulmonary trunk were found to contain an extensive mesodermal contribution in contrast to the arch arteries. Preferential localization of NCCs at the septal side of the bases of these vessel, as was already determined from stage 26 onward, persisted in the older embryos. Further downstream, the NC area expanded within the aortic and pulmonary trunk walls and encircled them entirely before branching into the arch arteries (Fig 4d). Chimeras showed non-NC areas of variable extensions, making it difficult to delineate a true boundary. The coronary arteries were connected with the aorta in this non-NC area and never showed any NCCs in the media or adventitia. At the site of this connection the periendothelial actin-stained muscular coronary arteries enter the lamellar aortic media in an actin negative “window” (Fig 4c and 4d).

Double staining for β-gal and muscle actin revealed that NCCs had differentiated into both SMCs and nonmuscular cells in the media and intima and also into adventitial cells in the great arteries (Fig 5a and 5b). The relative number of β-gal–labeled cells was consistently greater in the media than in intimal cell layers. Chimeric staining also suggested that the media of the arch arteries was composed almost exclusively of NCCs, whereas a number of nonquail cells was often encountered in the innermost part of the lamellar vessel walls. Within the media, no preferential labeling was discerned between SMCs and nonmuscular cells.

During later stages of development, the pulmonary arch arteries can be divided into three distinct segments based on morphological features. The first segment, the proximal...
elastic segment, revealing the aforementioned lamellar structure, harbored an extensive NC contribution, as did the second segment, the muscular DA. The third segment, elastic segment, revealed seeding with NCCs except for its most downstream region. NCC contribution to the distal parts of the aortic and pulmonary arches showed a sudden disappearance, somewhat upstream from where they join to continue as the descending aorta. This sharp boundary was markedly visible in whole-mount \( \beta \)-gal–stained specimens (Fig 5d). Elastic staining revealed differences in the matrix organization between the elastic arch arteries and the elastic descending aorta. NC-derived arch derivatives harbored thin elastic fibers in both longitudinal and circular directions, whereas the non-NC descending aorta contained neatly assembled elastic lamellae, which were circularly arranged in the vessel wall (Fig 6a and 6b). The transition between both elastin organization types was gradual, consisting of thinning of the vascular wall, loss of thin isolated fibers, and concurrent appearance of thick circular elastic lamellae. This transition occurred in the distal parts of the arch arteries, which still consist predominantly of NC-derived cells.

Another sharp boundary of the NC area could be discerned at the junction of the muscular pulmonary artery with the elastic sixth arch artery. No NCCs were ever detected in the pulmonary arteries at stages 35 to 40. In sharp contrast to the arch arteries, the typical muscular pulmonary arteries showed only very little elastin staining and dense periendothelial actin staining instead of alternating SMC and nonmuscular cell layers (Fig 6c and 6d).

The DA was the only part of the NC-derived arch arteries that did not develop into an elastic artery but rather into a typical muscular one. With low elastin levels and periendothelial actin staining, it resembled the pulmonary and coronary arteries.

The subclavian arteries remained devoid of NCCs during the greater part of the developmental range we studied (Fig

**Figure 4.** Interestingly, the most proximal part of the arterial tree, ie, the ascending aorta and pulmonary trunk, remained largely devoid of NCCs throughout development. a and b, Adjacent sections of a stage-28 chimera, stained with QCPN (\( \alpha \)-quail) and HHF35 (\( \alpha \)-actin), respectively. Note in these sections, at the level of the pulmonary outflow, the markedly present condensed mesenchyme (CM) of the aorticopulmonary septum and the area between the dashed lines, which is not seeded by NCCs. IV and VI indicate the fourth and sixth arch artery, respectively; T, trachea. Bar=300 \( \mu \)m. c and d, Schematic representations of the outflow tract area at stage 40. Panel c depicts smooth muscle actin expression patterns, clearly demonstrating a lamellar pattern of alternating SMCs and nonmuscular cells in the elastic media of the ascending aorta (AsAo) and pulmonary trunk (PT), as well as typical periendothelial actin staining in the muscular coronary artery (CO), which connects to the aorta in an actin-negative “window.” M indicates myocardial cuff of the outflow tract. Panel d depicts the presence of NCCs in this region, indicated by dotted areas. Note that in this region, actin expression patterns appear to be independent of cell origin. e, f, and g, Adjacent sections of a stage-40 chimeric embryo stained with QCPN (e), HHF35 (f), and 1A4 (g). Apart from the NC-derived aorticopulmonary septum, the aortic (Ao) and pulmonary roots are for the greater part of mesodermal origin. Bar=250 \( \mu \)m (e, f, and g).
6e). However, in the oldest embryos studied, we found marked β-gal staining in a proximal cuff around the subclavian artery. Intriguingly, this NCC-derived cuff corresponded with a lamellar actin expression pattern. More distally in the subclavian arteries, loss of β-gal staining coincided with a change in actin patterns from the lamellar type to periendothelial expression.

From stage 35, the brachiocephalic, common carotid, and inner/outer carotid arteries showed a marked NC contribution along their entire length, possibly extending into the head region (Fig 6f). In the carotid arteries, lamellar actin expression patterns are replaced by periendothelial actin staining in a cranial direction. The contribution of NCCs to these vessels, however, remained unalteredly high along the entire tract well up into the neck irrespective of changes in vascular morphology.

In addition to the SMC population in the arterial system, NCCs were also found to contribute to the developing vascular walls of the anterior cardinal veins in the neck region (Fig 6g). These venous NCCs had been present around the anterior cardinal veins at earlier stages but had not yet been clearly associated with them in terms of mesenchymal condensations and actin expression. Nascent actin expression in the anterior cardinal veins occurred at around stage 31, and by stage 35, contribution of NCCs to venous SMC layers could easily be established. Smaller veins running alongside the anterior cardinal veins and in the NC-infested thyroid region were associated with NCCs as well, but these veins did not yet develop a distinct primary media within the embryological time span studied. In contrast to arterial NC-derived cells, NCCs in the venous vessel wall did not reach the heart but were present in an area with a posterior boundary at the level of the thyroid.

**Discussion**

Early migratory behavior and mapping of hindbrain NC cells within the pharyngeal arches have previously been studied in both avian and mammalian embryos. However, accurate mapping of the subsequent NC contribution to the vasculatory system that arises within the pharyngeal arch region has been poorly illuminated for later phases of embryological life. Results from the present study serve to fill this hiatus and
to complete detailed mapping throughout in ovo development. In addition, this study provides a direct link to the fate of NC-derived vascular cells in terms of differentiation, both on a cellular and tissue level, based on NCC behavior during normal development.

NC Distribution in the Anterior Vascular System: New Findings and Theories

In addition to the earlier reported NC contribution to the arch arteries, we now show that the paired dorsal aortas harbor NCCs as well, in the region anterior to arch 4 (ie, carotid ducts and early carotid arteries). The anterior dorsal aortas reside in the dorsalmost part of the circumpharyngeal crest area and consequently develop a primary tunica media in which crest cells will have been incorporated.

So far, it is unclear why NCCs would populate the larger part of the thoracic arterial system except for certain areas. In contrast to NCCs that differentiate into, for example, the enteric nervous cells, NCCs in the vasculature populate only those areas that during their stage of anlage reside in an anterior region corresponding with the position of the premigratory cardiac crest cells themselves. In both the arterial and venous systems, the NC area has a clear posterior boundary, which, because of remodeling processes, becomes harder to delineate as a clear anterior-posterior axis-related boundary during later development. Hox gene-encoded positional information in NCCs is proven to be involved in patterning of the branchial arches and most likely is involved in determining NC distribution among the arch arteries as well. The fact that the descending aorta and subclavian and pulmonary arteries do not receive NCCs appears to be related to their posterior positions in early development, whereas the coronary arteries have not yet formed their endothelial scaffolding properly at the time of major NCC migration in the pharyngeal arch region, which occurs in a cranial-caudal sequence between stages 15 and 25. Once NCCs have stopped migration and started differentiation to some extent, they are less likely to migrate further toward still unpopulated areas at a later point in development. This might explain why thoracic arteries that arise later in development, like the coronary arteries, recruit their primary SMCs from the local mesenchyme, which is mesodermal in origin.

In the present study, older chimeric embryos generally showed very few chicken cells in the vessel wall, located mainly in the intima-like layer. This suggests that the relative contribution of mesoderm-derived cells at final stages in development is very small in these vessels, except for the intima. These findings suggest a role of mesoderm-derived cells in the genesis of this intima-like layer in conjunction with NCCs during avian embryonic development. These mesodermal cells are virtually absent at earlier stages, suggesting selective proliferation of a small number of progenitor media cells, or they might arise via endothelial-mesenchymal transformation in a process similar to that described in the early avian endocardial cushions and dorsal aorta.

Other intriguing parts of the pharyngeal arch complex concerning the NCC contribution are both the aortic and pulmonary trunks. In retrovirus and chimera experiments,
both vascular roots appeared to have a dual origin in which mesodermal cells clearly outnumbered NCCs. Moreover, NCCs generally turned out to be spatially organized toward the median (septal) side at the very bases, gradually substituting mesodermal cells in a downstream direction; a somewhat similar phenomenon was described by Takamura et al.\(^2\) The chick ascending aorta and pulmonary trunk, or at least their very bases, are generally hypothesized to originate from the septated aortic sac. In contrast to designated derivatives of the arch arteries (aortic arch, brachiocephalic arteries, and pulmonary arch arteries), the aortic sac appears to develop mainly from the mesoderm, incorporating only a minor NC contribution. Actual translation of these chick NCC patterns into the fully remodeled mammalian arterial tree remains difficult. The aortic sac in mammals is considered to develop into the entire ascending aorta, brachiocephalic artery, and pulmonary trunk.\(^9,47\) However, the extension of the proximal cuff, which we found largely devoid of NCCs, is unknown in these major mammalian vessels. Unfortunately, long-term tracing studies, similar to those in avian models, cannot be performed in intrauterine mammalian development, and specific markers for mesenchymal cells and SMCs derived from the NC, which are expressed for a prolonged period of time, have not been identified yet.

Our present finding of the contribution of NCCs to the media of the anterior cardinal veins is the first so far. This finding was accomplished by use of both retrovirus and chimera experiments and strengthens the hypothesis that vascular NC distribution is dictated by the presence of endothelial tubes at the early hindbrain level in both the arterial and venous system. Unlike the outflow tract of the heart and the great arteries, however, the venous system was never described in chicks NCCs have the potential to differentiate into typical SMCs, which express desmin and \(\alpha\)-smooth muscle actin and are surrounded by a laminin and collagen IV basement membrane, and into cells lacking those characteristics (non-muscular cells). Cellular phenotype in the pharyngeal arch derivatives, therefore, appears not to be lineage-related.

On the basis of (immuno)histological examination only, we recently suggested that NC-derived thoracic arteries differ from mesodermal arteries.\(^2\) In the present study, we confirm this hypothesis. In general, it turned out that the typical lamellar vessel wall structure only developed in vessels that have an NC contribution. Interestingly, the boundaries of NC areas tend to coincide with marked changes in vascular morphology. At least three good examples of this phenomenon were seen: (1) the junction between pulmonary arch artery (NC-derived) and pulmonary artery (mesoderm), (2) the proximal subclavian artery (NC)–distal subclavian artery (mesoderm), and (3) the junction of the fourth and sixth arch arteries (NC) with the ascending aorta (mesoderm).

Although these coinciding spatial boundaries are very suggestive of lineage-related morphogenesis, other findings partly contradict this hypothesis. In both aortic and pulmonary roots, for instance, we demonstrated a spatially organized dual origin of the vessel wall. However, the areas in these vessels that were shown to be of mesodermal origin did not differ morphologically from the NC areas nearby. An NC boundary, the exact position of which could not be established by us, is certainly not reflected in the histology of this part of the arterial tree, suggesting the major influence of other factors, such as hemodynamics. This area nonetheless remains very interesting because of matrix-related pathologies, which predominantly affect this region. Fibrillin-1–associated Marfan’s syndrome\(^53,54\) often presents dilatation or dissection of the ascending aorta, whereas the elastin-related Williams syndrome involves supravalvular aortic stenosis.\(^55\)

In addition, the sixth arch derivative, the DA, was shown to receive an extensive NC contribution but did not join the other arch derivatives in their elastogenic differentiation. NC-derived arteries therefore can differentiate into both elastic and muscular (DA) vessels. Although the DA shares the same origin and presumably comparable hemodynamic and environmental factors with the other arch derivatives, its differentiation deviates rather early in development. Since NCCs carry \(\text{Hox}\) gene–encoded positional information into the pharyngeal arches\(^40,41\), one could speculate on a further refinement of lineage–dependent morphogenesis, as cardiac crest cells differ in \(\text{Hox}\) gene expression according to their original position on the anteroposterior axis. However, cardiac NCCs originating from different rhombomeric origins were shown to mix rather extensively within the circumpharyngeal crest before finishing migration into the forming pharyngeal arches.\(^4,39\) In this way, the sixth arch receives NCCs emanating from the somite 1 to somite 3 level and therefore largely shares its cellular composition with that of arch 4. Yet regardless of these similarities,
only the sixth arch was shown to retain HOX 2.1 (HOXB5) expression, thereby emphasizing its unique identity.

To date, an increasing number of in vitro experiments favor the hypothesis of lineage-related SMC differentiation. SMCs isolated from normally developed NC vessels were shown to differ in vitro from “surrogate” SMCs that infest the arteries after experimental NC ablation. SMCs (of undetermined origin) that replaced the ablated NCCs showed much higher levels of α-actin, tropoelastin, and expression of c-jun during in vitro assays. In addition, in vitro experiments also elaborated on differential responses to TGF-β1 by either thoracic or abdominal aortic SMCs. TGF-β was shown to be growth inhibitory when administered to abdominal (mesodermal) SMCs, whereas it increased DNA synthesis in SMCs isolated from the thoracic (NC) aorta.

Topouzis and Majesky suggested that this differential TGF-β responsiveness might result from lineage-dependent differences in glycosylation of the type II TGF-β receptor. However, the implications of their findings for vessel differentiation in vivo remain to be elucidated, since cellular morphology and protein expression levels of cultured cells, oddly enough, were similar in both groups. Actin expression in vivo, however, was shown to be restricted to only a subpopulation of media cells in the NC-derived aortic arch. All other cells were of a nonmuscular actin-negative phenotype. Nonetheless, differences in potency unraveled by in vitro studies will aid evaluation of morphological and physiological heterogeneity among vessels that so far are considered to be similar. If SMC origin and topographical localization indeed are of major importance in defining the characteristics and performance of the cell, then this should be taken into account in design and evaluation of in vitro experiments as well as in surgical vessel grafting.

Last, origin-related SMC features may well be of significance in vascular pathogenesis. Intriguingly, spontaneous aortic plaques in the chicken were only found in the descending part of this vessel. The proximal part, showing the intricate cellular lamellar organization, which we now know is predominantly of NC origin, does not develop such arteriosclerotic pathology in the adult chicken. Because a proper correlation of human and chick patterning is still lacking and because this phenomenon cannot yet be translated to human arteriosclerosis-prone areas, more research into the NC in mammals will be needed to elucidate its role in vascular biology.

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