Neural Crest Cells Retain Multipotential Characteristics in the Developing Valves and Label the Cardiac Conduction System

Tomoki Nakamura, Melissa C. Colbert, Jeffrey Robbins

Abstract—Multipotent neural crest cells (NCCs) are a major extracardiac component of cardiovascular development. Although recognized as contributing cells to the arterial valves at early developmental stages, NCC persistence in the valves at later times or in the adult heart is controversial. We analyzed NCC persistence and contributions to both semilunar and atrioventricular (AV) valves in the mature heart. Two NCC-specific promoters driving Cre recombinase, Wnt1-Cre and P0-Cre, were mated with floxed reporter mice, R26R or CAG-CAT-EGFP, to map NCC fate. Hearts were analyzed before aorticopulmonary (AP) septation through adult stages. As previously demonstrated, strong NCC labeling was detected in ventral and dorsal outflow cushions before AP septation. In contrast to previous reports, we found that substantial numbers of labeled cells persisted in the semilunar valves in late fetal, neonatal, and adult hearts. Furthermore, NCCs were also found in the AV valves, almost exclusively in the septal leaflets. NCCs in the AV valves expressed melanocytic and neurogenic markers. However, cells labeled in the proximal cardiac conduction system exhibited neurogenic and gliagenic markers, whereas some NCCs expressed no differentiation specific markers. These results suggest that cardiac NCCs contribute to the mature valves and the cardiac conduction system and retain multipotential characteristics late in development. (Circ Res. 2006;98:1547-1554.)

Key Words: development • heart valves • embryonic development • transgenic mice • genetics

The importance of the neural crest contribution to cardiovascular development has been recognized for more than 20 years. Neural crest cells (NCCs) provide a major extracardiac source of mesenchyme to the outflow tract (OFT) and postganglionic neural progenitors that innervate the heart. Seminal work using NCC extirpation and analysis of quail chick chimeras demonstrated that NCCs contributed to the aorticopulmonary (AP) septum, the tunica media of the great arteries, the OFT septum, and the semilunar valves. However, crest-derived cells were seldom found beyond the aortic and pulmonary valves, as reported in HtPA-Cre transgenic mice. Several different transgenic lines carry neural crest–specific Cre recombinase activity, including Wnt1, P0, Pax3, and HiPA. These model systems have provided new data on neural crest behavior in mice but have also caused some confusion. Jiang et al. established a standard for following neural crest–derived cells during cardiovascular development, demonstrating that β-gal expression in Wnt1-Cre::R26R-positive embryos reproduced or expanded most of the patterns of neural crest–invested tissues and structures described in quail chick chimeras. Subsequently, other investigators used this double transgenic model to follow NCC migration into the heart or analyze cell lineage in cardiac valves. Other models of NCC-restricted recombination using P0, Pax3, or HiPA with lacZ-based reporter mice show consistent major crest contribution to the great vessels, OFT septum and semilunar valves. Most differences among the data occur in the quantitative levels of recombination or perdurance of expression. For example, the number or intensity of labeled cells in the aortic valves range from insubstantial using Wnt1-Cre recombination to significant cell labeling in the aortic and pulmonary valves, as reported in HiPA-Cre recombined mice. However qualitative differences in expression were noted in the same model of recombination. Poelmann et al. briefly described contiguous β-gal expression in the

Original received November 30, 2005; revision received May 4, 2006; accepted May 9, 2006.
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© 2006 American Heart Association, Inc.
Circulation Research is available at http://circres.ahajournals.org DOI: 10.1161/01.RES.0000227505.19472.69

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proximal conduction system and bundle branches in Wnt1-Cre::R26R embryos that was not described by Jiang et al.\textsuperscript{13} Therefore, inconsistencies in the developmental expression patterns of neural crest–derived cells exist among models, even when the same recombination system is used.

In this report, we revisit neural crest lineage tracing, using 2 well-characterized neural crest–specific promoters for recombination, Wnt1-Cre\textsuperscript{8} and P0-Cre,\textsuperscript{9} and 2 distinct reporter mice, R26R for \(\beta\)-gal expression\textsuperscript{12} and a floxed enhanced green fluorescent protein (EGFP) reporter, to examine crest in situ. This approach facilitated direct comparison between these models at similarly staged developmental times. Furthermore, we bred both recombinase and reporter mice onto a homogeneous C57BL/6J background to eliminate any genetic variation in Cre activity, the behavior of NCCs or reporter expression. As the data are consistent among most models at early developmental stages through midgestation, we concentrated our analyses on the distribution of neural crest–derived cells during later gestation–neonatal stages and in adult hearts. In contrast to prior reports, we see NCCs in AV valves and in late stage expression in the maturing CCS. Although some neural crest–derived cells exhibit melanocytic, neurogenic, and glial characteristics, not all are stained with specific markers, suggesting some may contribute to the newly described neural crest stem cell population in the adult heart.\textsuperscript{17}

Materials and Methods

Transgenic Animals

Animal use was in accordance with Institutional Guidelines with all experiments reviewed and approved by the Institutional Animal Care Committee. Mice were obtained either from our preexisting colonies or, for the P0-Cre animals, From Erik Meyers, Duke University. Fate mapping used a Cre-loxP recombination strategy from embryonic day 11.5 (E11.5) through 8 weeks postnatally. Wnt1-Cre\textsuperscript{8} and Rosa26 (R26R)\textsuperscript{14} mice were bred onto the C57BL/6J background for the majority of experiments, including the majority of experiments in this report. Rosa26 mice were mated to either R26R or CAG-CAT-EGFP reporter mice. The CAG-CAT-EGFP reporter mouse was constructed by inserting a 3.2kb \(\text{Sall–BamHI} \) fragment containing the CMV-\(\beta\) actin promoter and loxP flanked CAT gene\textsuperscript{18} upstream of the enhanced green fluorescent protein (EGFP) cassette. Timed pregnancies were established with the morning of a vaginal mucous plug defined as E0.5.

\(\beta\)-Gal Expression

The Wnt1-Cre::R26R and P0-Cre::R26R mice were prepared and processed for \(\beta\)-gal expression as described\textsuperscript{19} with the following modifications. The thoracic viscera were removed and drop fixed in 0°C 2% paraformaldehyde, 0.2% glutaraldehyde in PBS (pH 7.4). Tissue was washed extensively with PBS and immersed in a PBS/substrate solution containing 2 mmol/L MgCl\(_2\), 5 mmol/L potassium ferricyanide, 5 mmol/L ferrocyanide, and 0.1% X-gal dissolved in dimethyl formamide. For frozen sections, tissues were postfixed with 4% paraformaldehyde in PBS, infiltrated with sucrose, and embedded in OCT. Serial 8-\(\mu\)m sections were cut for embryos younger than E13.5 and 10-\(\mu\)m sections for mice older than E14.5. The samples were counterstained with nuclear fast red.

Immunohistochemistry

Wnt1-Cre::EGFP hearts were fixed overnight in 0°C 4% paraformaldehyde in PBS and prepared for immunohistochemistry as described.\textsuperscript{20} Primary antibodies used were: (1) rat anti-CD31 (1:100 dilution, BD biosciences) to detect endothelial cells; (2) goat anti–procollagen type I (1:100 dilution, Santa Cruz Biotechnology Inc) to identify fibroblasts; (3) goat anti–collagen \(\alpha\) type II (1:100 dilution, Santa Cruz Biotechnology Inc) to identify chondrocytes; (4) rabbit anti–cleaved caspase 3 (1:100 dilution, Cell Signaling Technology) to detect apoptotic cells; (5) rabbit anti–tyrosinase-related protein 1 (TRP1) (1:50 dilution, Santa Cruz Biotechnology Inc) to identify melanocytes and melanocytic precursors; (6) rabbit anti–neuronal class III \(\beta\)-tubulin (TUJ1) (1:2000 dilution, Covance) to identify neurogenic cells; (7) rabbit anti–brain lipid–binding protein (BLBP) (1:1000 dilution), which identifies radial glia.\textsuperscript{21} Alexa Fluor 568 (donkey anti-goat IgG for procollagen type I and collagen \(\alpha\) type II staining and goat anti–rabbit IgG for TUJ1, cleaved caspase 3, TRP1, and BLBP staining, respectively; 1:100 dilution) were used as secondary antibodies. For CD31 staining, either Alexa Fluor 568 (goat anti-rat IgG, Molecular Probe, 1:100 dilution) or Cy5 (goat anti–rat IgG, 1:200 dilution) was used. Images were captured using a Nikon PCM 2000 confocal microscope. For the TUJ1 staining of cardiac NCCs in vitro, neural tubes from the midotic placode to the third somite containing NCCs were excised from E8.5 embryos and explants cultured on fibronectin-coated dishes.\textsuperscript{22} Cells were cultured overnight to obtain early migrating undifferentiated NCC or for 4 days to promote neuronal differentiation. The cultures were fixed and stained using TUJ1 (1:2000) and counterstained with DAPI.\textsuperscript{23}

Results

Distribution of NCCs in the Aortic Arch System

NCCs contribute to AP septation and the remodeling of arch arteries with the process completed by late embryogenesis. We used 2 neural crest specific transgenic mice, Wnt1-Cre and P0-Cre, crossed with R26R reporter mice to compare NCC distribution in the aortic arches. To exclude the potential for long-lived \(\beta\)-gal expression complicating the analyses and to follow labeled cells directly, a CAG-CAT-EGFP reporter mouse was also generated. The data show a strong concordance of expression resulting from recombination initiated from the 2 neural crest–specific promoters (Figure 1). At late fetal stages, neural crest–derived cells are distributed in the ascending aorta, right brachiocephalic artery, left carotid artery, ductus arteriosus, and pulmonary trunk, although pulmonary trunk distribution is somewhat faint. The descending aorta and both pulmonary arteries are devoid of staining. The overall distribution patterns are identical between Wnt1-Cre::R26R and Wnt1-Cre::EGFP, indicating that \(\beta\)-gal expression at these stages is not attributable to long-lived enzymatic activity (Figure 1A, A’, B, B’).
ison of P0-Cre::R26R with Wnt1-Cre::R26R expression patterns of β-gal–expressing cells in the heart verified NCC-specific distribution (Figure 1C, C').

**Persistence of NCCs in the Valvular Regions During Development and Cardiac Maturation**

NCCs invade both ventral and dorsal cushions in the OFT before initiation of AP septation at around E11.5. However, the ultimate contribution of NCCs to the valves in late development and postnatal stages is controversial. When we analyzed the distribution of cardiac NCCs at late fetal stages and in the adult, we found that significant numbers of NCCs persisted in both the semilunar and AV valves (Figures 2 and 3). Distribution patterns were identical between the Wnt1-Cre::R26R and P0-Cre::R26R mice.

**Figure 2.** NCCs persist in the valvular leaflets of the OFT. A, Wnt1-Cre::R26R valves. Top panels, Pulmonary valve (PV). The 2 leaflets adjacent to the AP septum (right and left posterior cusp, respectively) contain numerous NCCs (arrowheads), whereas in the distal leaflet (anterior cusp, asterisk), there are substantially fewer labeled cells. Bottom panels, Aortic valve (AoV). Staining is similar to pulmonary valve leaflets. The leaflets close to AP septum are strongly labeled (arrowheads) with weak staining in the distal leaflet (asterisk) (×10, counterstained section; ×1.6, 8 weeks postnatally). P1 and P5 denote postnatal days 1 and 5. B, P0-Cre::R26R valves. The staining patterns in the pulmonary and aortic valves of the P0-Cre::R26R are similar to Wnt1Cre::R26R valves, although the number of stained cells is somewhat reduced (×10). Arrowheads and asterisks denote the individual cusps as outlined in A.

**Figure 3.** NCCs are prominent and persist in the AV valves. Atria have been removed (dotted areas shown in C). A, Wnt1-Cre::R26R hearts in a posterior view during development (left column) and postnatal maturation at 8 weeks (right column). β-Gal–expressing cells are first observed in the AV valves at E12.5 (arrows) and are prominent at E14.5 (whole hearts left most columns with enlargements of the boxed areas shown to the right). These stained cells persist in the valves during late-fetal (E17.5) and postnatal stages (P1) and into adulthood (8 weeks). B, Posterior view of AV valves of P0-Cre::R26R hearts, which are also labeled both prenatally (E16.5) and postnatally (P5). Whole hearts left most column; enlargements of the boxed areas are shown to the right. C, Wnt1-Cre::EGFP heart (E17.5, posterior view). EGFP-positive cells are prominent in the AV valve leaflets.
contain numerous NCCs, as demonstrated by robust expression (Figure 2A and 2B), whereas proportionately fewer cells of neural crest origin.

The cardiac cycle, further demonstrating that the cells in the opening/closing of EGFP-positive valve leaflets throughout the cardiac cycle. Sections of P0Cre::R26R at E16.5 (top) and P5 (bottom) are on the right. β-Gal–expressing cells are confined exclusively to the septal leaflets in both the mitral and tricuspid valves. Sections are counterstained with nuclear fast red (×16).

Following AP septation, OFT cushions undergo remodeling and form the semilunar valvular leaflets. Similar to previous reports, the 2 leaflets adjacent to the AP septum contain numerous NCCs, as demonstrated by robust β-gal expression (Figure 2A and 2B), whereas proportionately fewer β-gal cells appear in the distal leaflet. Staining is apparent in both the pulmonary and aortic valves from as early as E13.5 (Figure 2B). Strikingly, it remains prominent at late fetal stages (E16.5, E17.5) and persists through the neonatal and into the early adult at 8 weeks (Figure 2). To visualize the presence of neural crest–derived cells in the valvular leaflets, video microscopy of the Wnt1-Cre::EGFP-positive embryo hearts (E17.5) was used to record the valvular motion (video file in the online data supplement available at http://circres.ahajournals.org). We observed the opening/closing of EGFP-positive valve leaflets throughout the cardiac cycle, further demonstrating that the cells in the valve in late development contain a significant proportion of cells of neural crest origin.

At E12.5, a small number of β-gal–expressing cells in Wnt1-Cre::R26R embryos are first detected migrating into the AV canal and AV valves, and, by E13.5, these become more numerous (Figure 3A). The labeled cells visible in the whole-mounted material in all models were found exclusively in the septal leaflet, whereas the mural leaflet was almost devoid of staining (Figure 3A and 3B, left panels, and 3C). This finding was confirmed by examining histological sections (Figure 4). Again, the pattern and distribution of the labeled cells was identical in both the Wnt1-Cre::R26R and P0-Cre::R26R hearts with the cells persisting into adulthood. It is well known that NCCs migrate into the heart via the truncus arteriosus (arterial pole). Avian studies further demonstrated a second entry point for migrating NCCs, which contribute to the innervation of posterior part of the cardiac nervous system (venous pole).

We asked whether NCCs migrate into the AV valves from either 1 or both entry points. Consistent with previous studies, some NCCs enter the heart from the venous pole at E12.5, penetrate the heart and migrate into the AV valves (Figure 5A through 5C). Histological sections at E12.5 revealed that NCCs were seen exclusively in the dorsal part of AV valves (venous pole), whereas the cells from the arterial pole also begin to migrate toward the anterior aspect of the AV valves (Figure 5D and 5E). The location of the group of NCC cells is consistent with the hypothesis that cells seen in the AV valves by E17.5 could have originated from the arterial pole (Figure 5F).

The Wnt promoter driving Cre recombinase is expressed specifically in the dorsal neural tube in the early embryonic stage. Wnt1 expression is extinguished as the crest cell lineage migrates away from the neural tube and the promoter remains silent thereafter. We wished to confirm that Cre expression was not activated at later stages. Cre immunostaining at E9.5 showed localized expression in the dorsal neural tube and overlapped with NCCs. We were unable to detect Cre expression in either migrating NCCs at any later times or in NCCs in the AV valves (supplemental Figure IA). Additionally, using a sensitive RT-PCR–based assay for Wnt1 transcripts, we found no evidence of wnt1 activity at later developmental stages (supplemental Figure IB). The results verified that NCC labeling in the AV valves was not caused by illicit Cre expression or reactivation of wnt1.

**Lineage Analysis of Cardiac NCCs in the Valves**

Previous in vitro analyses demonstrated that, at the onset of migration, NCCs give rise to at least 6 differentiated phenotypes: smooth muscle cells, fibroblasts, chondrocytes, sensory neurons, autonomic neurons, and pigment cells. The significance of the presence of NCCs and their persistence in the valves at late developmental stages was evaluated by immunohistochemistry using lineage-specific antibodies. Initial experiments showed that NCCs in the valvular regions did not colocalize with fibroblasts (collagen I) or chondrocytes (collagen II) (not shown). A recent report described the presence and distribution of melanocytes in the C57BL/6J mouse heart. Using a TRP1 antibody, which marks melanocytic precursors, we explored whether the EGFP-positive cells were wholly or in part neural crest–derived melanocytes. Although no TRP1-positive cells were found in OFT valves in E17.5 hearts, many but not all cells in the septal leaflets of the tricuspid (Figure 6) and mitral (not shown) valves were strongly stained. The heterogeneity of coexpression, apparent at high magnification (Figure 6, panels d’-f’), indicated that not all NCCs were committed to the melanocyte lineage. Compared with late embryonic and early postnatal stages, a large number of mature melanocytes were found in the septal leaflets of AV valves in the adult (supplemental Figure II). These data confirmed that postnatal melanocytes found in the valves were derived from NCCs and that differentiation occurred postnatally.
NCCs in the Heart Express Neurogenic Markers

Positive staining with TUJ1, a pan-immature neural marker, was found in the septal leaflets of both OFT and AV valves as well as in the AP septum (Figure 7A) and colocalized with EGFP-positive NCCs. NCC neurogenic potential was demonstrated in vitro using NCCs isolated from the dorsal neural tube of E8.5 embryos. Neural crest explants were incubated in culture for 24 hours and the emerging, undifferentiated cells stained positive for TUJ1 (Figure 7B). After 4 days in culture, when NCCs have begun to differentiate, positive TUJ1 staining along linear axonal formation was apparent, suggesting that TUJ1 may mark a subpopulation of migrating NCCs. Furthermore, sections of the AV valves taken at 2 weeks postbirth from Wnt1-Cre::EGFP-positive animals no longer stained with TUJ1 (data not shown), indicating that the cells resident in the valves are not neural derivatives.

NCCs Contribute to the Proximal Cardiac Conduction System

NCC in the mouse heart are reported to be contiguous with the CCS. However, their contribution to the conduction system is unclear. Although most NCCs are eliminated via apoptosis in the chick, mouse NCCs are proposed to play a role in CCS maturation. We therefore asked whether areas of the CCS also contain NCCs. When Wnt1-Cre::EGFP mice were examined at E17.5, the proximal CCS, including the posterior internodal tract, His bundle and bundle branches (Figure 8A) all contained EGFP-positive cells. In the rabbit heart, neurofilament 160 (NF160) is recognized as a unique marker for the conduction system in early development into the adult. Immunostaining with NF160 and TUJ1 colocalized with EGFP-positive cells in the conduction system (supplemental Figure III). Sections stained with antibodies against activated caspase 3 showed few if any positive cells present at E12.5, E14.5, and E17.5. The few cells that were positive were not labeled with EGFP (data not shown). To further examine the nature of these cells, a marker for...
whereas a fully differentiated glial marker, GFAP, did not with EGFP-positive cells (supplemental Figure IIIB), a second immature glial marker, GLAST, also colocalized positive cells and was most prominent in the CCS at the in vivo, complementing in vitro analyses of NCC potential to differentiate into multiple cell types. However, our results also differ in that the NCCs are not of ventral origin. It is possible that discrepancies reflect a species difference and/or enhanced sensitivity of NCC to retroviral infection. In mice, recombination occurs early and exclusively in the dorsal neural tube (supplemental Figure I), and we find no evidence of wnt1 activity or insertional reactivation of recombinase at later developmental stages, which might label a second population of migratory cells.

Figure 7. Migrating NCCs exhibit neurogenic potential. A, Sections from E17.5 Wnt1-Cre::EGFP mice were stained for tubulin (TUJ1). Cardiac NCCs in the OFT and AV valves (enlargement at right) stain positively and colocalize with EGFP-labeled NCCs. Cells clustering in the AP septum and NCCs in the valvular leaflets express neural precursor markers (arrow). AoV indicates aortic valve; TV, tricuspid valve (∼20). The endothelial cells are stained blue using a CD31 antibody. B, Both undifferentiated and differentiated NCCs cultured in vitro express neurogenic markers. Cardiac NCCs isolated from the dorsal neural tube of E8.5 embryo are positive for TUJ1 staining immediately after migrating from explanted tissue (left, 24 hours of incubation, arrowheads). Inset shows that isolated TUJ1-positive cells are also found. Following 4 days in culture (right), axons stain positively for TUJ1, indicating committed neuronal differentiation (∼20).

immature neural crest–derived neuroglia, BLBP, was used. BLBP staining colocalized with some but not all EGFP-positive cells and was most prominent in the CCS at the bundle branch bifurcation (Figure 8B). Immunostaining with a second immature glial marker, GLAST, also colocalized with EGFP-positive cells (supplemental Figure IIIB), whereas a fully differentiated glial marker, GFAP, did not label any of the CCS. Although we have not resolved whether specific NCCs express these different markers, our results suggest that NCCs contributing to the maturating proximal CCS express a unique mixed-cell potential.

Discussion
This study represents the first report that directly compares fate mapping of NCCs between 2 well-characterized NCC-restricted promoters using Cre-loxP recombination strategies on a homogenous genetic background. Previous recombination studies yielded valuable data on fate mapping of NCCs in vivo, complementing in vitro analyses of NCC potential to differentiate into multiple cell types. However, the contribution of NCCs in later developmental stages to valve formation and the CCS has received less attention, and NCC persistence in the adult heart, other than their contribution to the great vessels, has not been determined. Our data show that NCCs persist in the valvular leaflets in the adult heart, are intimately associated with areas of the proximal CCS, and may retain elements of multipotency in late development.

A controversial aspect of these data is that cardiac NCCs penetrate well beyond the arterial pole of the heart. In support of this conclusion, 2 independent bitransgenic labeling systems, P0-Cre and Wnt1-Cre, clearly demonstrate NCCs in both semilunar and AV valves from midgestation onward. Crest cells enter the heart from both arterial and venous poles of the heart and proceed to the AV canal and valve primordia. This is entirely consistent with retroviral labeling studies in the chick and corroborates Wnt1-Cre mapping in mice. However, our results also differ in that the NCCs are not of ventral origin. It is possible that discrepancies reflect a species difference and/or enhanced sensitivity of NCC to retroviral infection. In mice, recombination occurs early and exclusively in the dorsal neural tube (supplemental Figure I), and we find no evidence of wnt1 activity or insertional reactivation of recombinase at later developmental stages, which might label a second population of migratory cells.

It is somewhat surprising that few of the other recombination studies, which have used a variety of neural crest–specific promoters including Wnt1-Cre, have described labeled cells in the AV valves. There are a number of possibilities to explain this discordance in the data. Several authors reported that staining of NCCs mediated by Wnt1-Cre recombination diminished over time and disappeared by E17.5. However, very few investigators performed these studies on a pure background, and a decrease in recombination efficiency may be associated with a mixed genetic background of either the reporter or recombinase mice. For example, background differences and genetic modifiers may be critical to Pax3-Cre–mediated recombination in a knock-in model, with the mixed strain 129/SV::C57BL/6J revealing unexpected derivatives and alterations in null mouse survival. The genetic background of all transgenics used in this study is identical (C57BL/6J), eliminating these potentially confounding variables. It is also possible that, based on early reports of loss of expression, later stages were not a focus and exhaustively evaluated. We have recently learned that labeled NCCs that are HtpA-Cre recombinated are abundantly apparent in the AV valves from E12.5 onward, in agreement with our observations in this report (personal communication, S. Dufour and D. Henderson, 2006). Thus depending on genetic background, labeled NCC can contribute the AV valves using three of four distinct systems for recombination mediated labeling of NCC.

The precise role of NCCs in the CCS remains unclear. In the distal cardiac conduction system of the chick, Purkinje fibers differentiate from a subset of cardiomyocytes, although it has been hypothesized that components of the proximal CCS may originate from NCCs. Viral and genetic mapping studies suggest a close association between NCC and the developing conduction system, which our data confirm. In the rabbit heart, NF160 expression marks both the embryonic and adult conduction systems. Vitadello et al described coexpression of NF160 and desmin in conduction myocytes and suggested that they may originate from neuro-
ectoderm. However, Schwann cells, particularly nonmyelinating cells, also express both NF160 and β-tubulin. We see that markers for undifferentiated glia (BLBP and GLAST) appear to be coexpressed with neural markers (NF160 and TUJ1) in NCCs in the His bundle and bundle branches. Although it is possible that NCCs form the neural marker labeled conducting myocardium, it is unlikely, as this labeling pattern occurs only at late developmental stages. More likely, neurofilament labeling represents components of autonomic postganglionic neurons. Until recently, there have been no direct data demonstrating a role for NCC in CSS maturation. Targeted knockout of HF-1b in neural crest results in dysfunctional AV conduction, presumably attributable to defective innervation, whereas NCC ablation in the chick resulted in the His bundles failing to undergo compaction and electrical insulation of the CCS. Our data show that NCCs reside in the proximal but not distal CCS and maintain both neurogenic and gliagenic potential. We think it is likely that NCCs provide neural connections and the gliagenic cells organize compaction of the His bundles and branches.

Recent studies have demonstrated that multipotent NCCs persist in several adult tissues, including PNS ganglia, skin, and the heart. In the latter study, P0-Cre::EGFP-labeled stem cells were directly isolated from the adult heart. The undifferentiated, EGFP-labeled NCCs we see in late fetal/early neonatal hearts may contribute to the described creche of stem cells resident in the adult heart. Our immunohistochemical analysis showed that NCCs in the late fetal heart express early, often overlapping markers for melanocytic or neurogenic and gliagenic potential. On leaving the neural epithelium, NCCs undergo a progressive restriction in developmental potential and cells with various intermediate phenotypes occur along the path toward an ultimate differentiated state. Although we have no direct experimental evidence that supports multipotentiality of the labeled NCCs, we speculate that these cells are not fully differentiated at this stage, as evidenced by the lack of mature markers, and may represent cells somewhere along the developmental pathway.

Sources of Funding
This work was supported by NIH grants HL69779, HL56370, HL074728, HL61638, and HL52318 (to J.R.).

Disclosures
None.

References


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*Circ Res.* 2006;98:1547-1554; originally published online May 18, 2006; doi: 10.1161/01.RES.0000227505.19472.69

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Classification: Basic Science Research 108

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Sources of Support: National Institutes of Health Grants HL69779, HL56370, HL074728, HH61638 and HL52318.

Short title: Neural crest cells
Supplemental Material

Methods

Video Microscopy

Wnt1-cre::EGFP embryos (E17.5) were removed from the mother and the anterior chest wall was quickly dissected in PBS. The embryos were placed in a supine position under a Leica MZ-16FA fluorescent microscope equipped with a GFP3 filter set (excitation 470/40nm and 525/50nm). The video recording system included a Toshiba IK-TU52H three 0.5” CCD analog video camera and valvular motion was captured via using a Pinnacle Systems studio AV/DV capture card.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Tissue was obtained from AV canal, AV valves and interventricular septum of E17.5 C57BL/6J hearts. Samples from E9.5 C57BL/6J neural tube and gut were used as a positive and negative control, respectively. RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was synthesized with 4 µg of total RNA and oligo(dT) using SuperScript III First-Strand synthesis system for RT-PCR (Invitrogen). PCR was performed using GeneChoice Taq DNA polymerase with standard buffer (1.5 mmole/L MgCl₂ final concentration) (PGC Scientific, Frederick, MD). For each reaction, 2 µl of cDNA template was used to PCR amplify Wnt1 transcripts (391 bp). The primer set was as follows; Wnt1 forward primer (5’-TCCCAGGGTTCATAGCGATCCAT-3’), reverse primer (5’-GTGCCCTTTCAACTCGGAAACTCT-3’). PCR conditions included initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation (95°C for 30 seconds), annealing (58°C for 30 seconds) and elongation (72°C for 30 seconds); and 10 minutes of elongation at 72°C. As a loading control, L7 was used and its primer sequence and PCR conditions are as follows;
forward primer (5’-GAAGCTCATCTATGAGAAGGC-3’), reverse primer (5’-AAGACGAAGGAGCTGCAGAAC-3’); initial denaturation at 95°C for 5 minutes; 35 cycles of denaturation (95°C for 30 sec), annealing (55°C for 30 sec) and elongation (72°C for 30 sec); and 10 minutes of elongation at 72°C. The PCR products were loaded on 2% agarose gel and the image was obtained using a Typhoon 9400 scanner (Amersham).

**Immunohistochemistry**

Preparation of the heart tissue section was described in the Materials and Methods. The following primary antibodies were used: (1) Rabbit anti-Cre (1:1000 dilution) to detect Cre recombinase expression. (2) Mouse Neurofilament 160 (NF160, 1:200 dilution, Developmental Studies Hybridoma Bank) to detect CCS together with mature neurons. (3): Guinea pig glial glutamate transporter (GLAST, 1:4000 dilution, Chemicon) to identify immature glia. (4): Rabbit glial fibrillar acidic protein (GFAP, 1:1000 dilution, Dako), which identifies mature glia. Alexa Flour 568 (goat anti-rabbit IgG for Cre, 1:100 dilution) was used as secondary antibody. For NF160, streptavidin Alexa Flour 568 (1:100 dilution) was used following M.O.M. immunodetection kit (Vector laboratories). For GLAST and GFAP, biotinylated antibody was used followed by streptavidin-Alexa Flour 568 (1:100 dilution).
**Video file**

Anterior view of the Wnt1-Cre::EGFP mouse heart at E17.5. The opening and closing of the pulmonary valve leaflets can clearly be seen.
Supplemental Figure 1. Cre recombinase is not reactivated in double transgenics. Panel A shows immunohistochemical analysis of E9.5 (a-c) or E17.5 (d-f) embryos using a polyclonal antibody against bacterial cre recombinase. In a-c, Wnt1-Cre is expressed at E9.5 exclusively in the dorsal neural tube. Note migrating NCCs (c, arrowheads) are negative for Cre. In d-f note the absence of anti-Cre staining of AV valves at E17.5. Other sections of embryos at later stages are similarly negative. The bottom panel is a negative control of staining from a non-transgenic mouse at E9.5 (g).

Panel B shows RT-PCR analysis of cardiac tissue for Wnt1 transcripts at E17.5. Lanes 1, 5 are positive control RNA from E9.5 hindbrain and neural tube; lanes 2, 6 are negative control RNA samples from E9.5 gut; lanes 3, 7 contain samples from E17.5 AV canal and interventricular septum; lanes 4, 8 are positive control RNA without reverse transcriptase.
**Supplemental Figure 2.** Adult heart valves (8 wk) contain mature neural crest-derived melanocytes. β-gal expression is detected in: whole mount preparations of P0Cre::R26R valves (top, arrows, X1.6) and Wnt1Cre::R26R (bottom right, arrowheads, X20) hearts, which contain many mature melanocytes that are positive for expression.
Supplemental Figure 3. Some NCCs in the His bundle express immature and mature neural markers. Similar to valvular region, some but not all GFP labeled cells in areas of the conduction system at E17.5 co-localize with TUJ1 staining (upper panels, x20). The His bundle is also stained with mature pan-neuronal marker, NF160 although the numerous cells (arrowheads) other than areas of conduction system remain unlabeled. (lower panels, x20).
Supplemental Figure 4. NCCs in His bundle express immature, but not mature glial markers at late embryonic stage. Both BLBP and GLAST, which mark immature glial precursors, co-localized with areas of conduction system (upper panels, x20). In contrast, no staining with GFAP is seen anywhere in the CCS at E17.5 (lower panels, x20).