Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 272 (2004) 176–190

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Cardiac neural crest ablation alters Id2 gene expression in the developing heart

Brad J. Martinsen,[a] Allison J. Frasier,[a] Clare V.H. Baker,[b] and Jamie L. Lohr[a,*]

[a] *Department of Pediatrics, Division of Pediatric Cardiology, University of Minnesota School of Medicine, Minneapolis, MN 55455, USA*
[b] *Department of Anatomy, University of Cambridge, Cambridge, England, UK*

Received for publication 22 April 2003, revised 5 April 2004, accepted 12 April 2004
Available online 1 June 2004

Abstract

Id proteins are negative regulators of basic helix–loop–helix gene products and participate in many developmental processes. We have evaluated the expression of Id2 in the developing chick heart and found expression in the cardiac neural crest, secondary heart field, outflow tract, inflow tract, and anterior parasympathetic plexus. Cardiac neural crest ablation in the chick embryo, which causes structural defects of the cardiac outflow tract, results in a significant loss of Id2 expression in the outflow tract. Id2 is also expressed in *Xenopus* neural folds, branchial arches, cardiac outflow tract, inflow tract, and splanchnic mesoderm. Ablation of the premigratory neural crest in *Xenopus* embryos results in abnormal formation of the heart and a loss of Id2 expression in the heart and splanchnic mesoderm. This data suggests that the presence of neural crest is required for normal Id2 expression in both chick and *Xenopus* heart development and provides evidence that neural crest is involved in heart development in *Xenopus* embryos.

Keywords: Heart development; Chick; Xenopus; Neural crest; Secondary heart field; Id2; Cardiac outflow tract; Cardiac neural crest; Helix–loop–helix protein

Introduction

Currently, four members of the Id family have been isolated in chick (Kee and Bronner-Fraser, 2001a,b,c; Martinsen and Bronner-Fraser, 1998) and three members (Id2, Id3, and Id4) in *Xenopus* (Liu and Harland, 2003; Wilson and Mohun, 1995; Zhang et al., 1995). The Id proteins are negative regulators of basic helix–loop–helix (bHLH) gene products and have a highly conserved helix–loop–helix domain, but lack the basic region required for DNA binding (as reviewed in Benezra, 2001; Massari and Murre, 2000). Id genes have been shown to regulate many developmental processes, including myogenesis (Benezra et al., 1990), neurogenesis (Jen et al., 1997; Manova and Bachvarova, 1991), cell growth (Christy et al., 1991), and cell fate specification (Martinsen and Bronner-Fraser, 1998). Id2 expression has been shown in the endocardial cushions, outflow tract (OFT), and valves of the developing mouse heart (Jen et al., 1996); in the cranial neural crest during chick development (Martinsen and Bronner-Fraser, 1998); and in the adult heart in *Xenopus* (Wilson and Mohun, 1995). Despite expression in both the neural crest and heart, no data indicating a clear relationship between Id2 expression and function in the cardiac neural crest (CNC) or during heart development has been presented to date. In this paper, we show that Id2 is expressed in the cardiac neural crest, secondary heart field, and cardiac structures. Ablation of cardiac neural crest cells results in an alteration of Id2 expression and abnormal heart development in both chick and frog model systems. This suggests a conserved role for cardiac neural crest dependent Id2 expression in normal heart development across species.

In chick, the cardiac neural crest (CNC) is an extracardiac population of cells that arise from the neural tube in the region of the first three somites up to the mid-otic placode level, which corresponds to rhombomeres 6, 7, and 8 (Kirby, 2002). These cells migrate into the pharyngeal arches and then on to the heart where they participate in outflow tract (OFT) septation and cardiac innervation (Kirby, 1988, 1989; Kirby and Stewart, 1983; Kirby et al., 1983). Retroviral labeling lineage studies have provided an overall picture of the labeled

* Corresponding author. Fax: +1-612-626-2467.
E-mail address: lohrx003@umn.edu (J.L. Lohr).

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.04.030

CNC cells in their target organs and have revealed two CNC cell entry points into the heart: the arterial pole (Poelmann et al., 1998) and the venous pole (Poelmann and Gittenberger-de Groot, 1999). CNC arterial pole derivatives contain three different subpopulations: outflow tract septum; smooth muscle cells in the great vessel walls; and ganglionic cells in the outer vessel walls and in the subepicardial space of the heart. Additional studies have also revealed that these ganglionic derivatives of CNC form the anterior parasympathetic plexus (APP) of the heart (Kirby and Stewart, 1983; Verberne et al., 1998). CNC cells also migrate through the venous pole of the heart to locations surrounding the prospective conduction system and the atrioventricular (AV) cushions (Poelmann and Gittenberger-de Groot, 1999).

In contrast, very little is known about the contribution of neural crest cells to cardiac development in the frog model system. In *Xenopus*, neural crest cells are induced to form in the lateral and posterior portions of the neural folds, before neural tube closure. During late neurula and early tailbud stages, they migrate from the neural folds through the embryo to generate an array of cell types, both neuronal and nonneuronal, which include the peripheral nervous system, pigment cells, and craniofacial cartilage (Collazo et al., 1993; Mayor and Aybar, 2001; Mayor et al., 1999). *xSnail* (Essex et al., 1993; Mayor et al., 1993), *xSlug* (Mayor et al., 1995), and *xTwist* (Hopwood et al., 1989) are a part of the group of genes activated in the neural fold region that positively control the activation of the genetic cascade required for the development of the neural crest. Expression of these genes begins at the border of the neural plate in *Xenopus laevis* gastrula. Following neurulation, expression of these genes can be detected in migratory neural crest cells. In chick, neural crest cells migrate as closely associated sheets (Bronner-Fraser, 1986) and are restricted to two to three axial segments. In *Xenopus*, neural crest cells are fewer, migrate as sparse individual cells, and are often spread over eight or more myotomal segment lengths (Mayor et al., 1999). Lastly, in chick, a subset of neural crest cells have been found to populate the outflow tract of the developing heart and are labeled "cardiac" neural crest cells (Kirby, 1988, 1989; Kirby et al., 1983); however, a similar population of cells has not yet been identified in amphibia.

The cardiac neural crest is not the only "extracardiac" population of cells required for proper cardiac outflow tract development. It has recently been shown that the outflow tract, instead of being derived from the primary heart field, is added to the heart from a population of cells in the midline splanchnic mesoderm, termed the "secondary" or "anterior" heart field (Kelly et al., 2001; Mjaatvedt et al., 2001; Waldo et al., 2001). Mjaatvedt et al. (2001) discovered that the early formation of the proximal conus (outlet segment of the outflow tract) forms from a novel anterior heart field between Hamburger and Hamilton (1951) (HH) stages 10 to 14. Mjaatvedt et al. (2001) also determined that the remainder of the conus and the distal portion of the outflow tract (the truncus) forms during later stages (stages 16–22) of accretion or recruitment from anterior heart field splanchnic mesoderm. Waldo et al. (2001) also discovered similar accretion between HH stages 14 to 22 from anterior splanchnic mesoderm, which they termed the secondary heart field (SHF). Here, we show for the first time that chick Id2 (chick inhibitor of DNA-binding 2) is highly expressed in the cardiac neural crest (CNC) and is a newly identified marker of the secondary heart field (SHF). In addition, it is expressed in the anterior parasympathetic plexus (APP), outflow tract (OFT), inflow tract (IFT), and atrioventricular (AV) valves of the developing heart. We have examined the expression pattern of *Xenopus* Id2 (*xId2*) in the heart and found that despite significant anatomic differences between the chick and *Xenopus* model systems, *xId2* is also expressed in the venous inflow and outflow tract portions of the developing frog heart. The expression pattern of Id2 in frog is among the first data that suggests the presence of a secondary heart field in this species. Lastly, we present data that shows that the expression of Id2 in the developing heart is altered in the absence of neural crest. Ablation of premigratory cardiac neural crest in the chick alters outflow tract formation and reduces expression of Id2 in the developing heart. Ablation of premigratory neural crest cells in the frog embryo also alters *xId2* expression in the heart and prevents normal cardiac development. In addition to implicating *xId2* in outflow tract development in *Xenopus*, this provides evidence for a neural crest contribution to heart development in the frog embryo.

Methods

Chick cardiac neural crest ablation and in situ hybridization

Protocols for chick cardiac neural crest ablation were followed as previously described, using a modified microcautery unit from Harvard Apparatus (Besson et al., 1986; Kirby et al., 1985). India ink [75 μl in 10 ml albumin or Hank's Balanced Salt Solution (Gibco) plus 1% Penicillin–Streptomycin (Gibco)] was injected under control and experimental embryos for better visualization, and the windowed eggs were subsequently sealed with Parafilm after embryo manipulation. This method of inking and sealing increased the viability of the embryos for long-term survival. The ablations were completed on three to eight somite chick embryos before the onset of cardiac neural crest emigration (CNC) (Boot et al., 2003). Ablated embryos were collected from 4.5 to 9.5 days of incubation and fixed for in situ hybridization.

The protocol used for chick whole-mount in situ hybridization is a combination of protocols developed for chick embryos (Henrique et al., 1995; Wilkinson, 1992; Wilkinson and Nieto, 1993). A linearized plasmid of chick Id2 (either full length or from nucleotide 554 to 848) was used as a template to generate a digoxigenin-labeled antisense probe (Martinsen and Bronner-Fraser, 1998). Chick embry-

os, ranging in age from HH (Hamburger and Hamilton, 1951) stage 10 to day 14.5, were hybridized overnight with the riboprobe and then washed and incubated with a blocking solution containing antidigoxigenin alkaline phosphatase conjugated antibody (Roche). A BM Purple AP substrate (Roche) enzymatic reaction was then completed to visualize the in situ hybridization signal. When the color had developed to the desired extent, the embryos were washed, refixed, and dehydrated with methanol for storage at −20°C. In situ hybridization on sections was completed by following methods previously described (Martinsen et al., 2003) and developed by Etchevers et al. (2001).

Quail-chick cardiac neural crest chimeras

Fertilized quail (*Coturnix coturnix japonica*) and chick (*Gallus gallus domesticus*, White Leghorn) eggs were obtained from commercial sources and incubated at 38°C in a humidified atmosphere to HH stages 9–10 (7–11 somites). A window was cut in the quail shell and a 1:25 mixture of India Ink and Ringer's solution injected into the sub-blastodermal cavity to reveal the embryo. The vitelline membrane in the region of the surgery and the cardiac neural crest (mid-otic placode level to the caudal limit of somite 3, unilateral or bilateral) were removed using a pulled glass needle for dissection. The chick embryo host was similarly prepared, and the cardiac neural crest from the region of the graft site (mid-otic placode level to the caudal limit of somite 3, unilateral or bilateral) was removed immediately before transfer of the quail donor cardiac neural crest. The window was sealed with tape and the host egg was incubated to days 4.5 to 6.5. Embryos were then collected and embedded in paraffin for Id2 in situ hybridization on sections following the protocol described above. Sections through the heart were then analyzed for coexpression of Id2 and QCPN.

Xenopus embryos, neural crest ablation, and in situ hybridization

Albino *X. laevis* females were induced to ovulate by injection of 1000 units of human chorionic gonadotropin, pH 7.2 (Sigma), into the dorsal lymph sac. Eggs were expressed from the ovulating females and fertilized with minced testis from pigmented males suspended in a modified Ringer's solution at one-third strength (R/3) plus 50 mg/ml of gentimicin sulfate (Sigma). The fertilized eggs were dejellied in a 2% cysteine, pH 8.0, and incubated in R/3 at either 17°C or 23°C. Embryos were staged for use in experiments according to Nieuwkoop and Faber (1967). Vitelline membranes were manually removed before ablation experiments. *Xenopus* neural crest ablations were done using a modified microcautery unit (Harvard Apparatus or Stoelting) to remove the superficial portion of the neural folds in a bilaterally symmetric pattern that overlaps with the *xslug* expression pattern at stages 15–18 (Figs. 6A,B). Embryos were cultured in modified Ringer's solution until late tailbud stages and then fixed for in situ hybridization (ISH) in a 3.7% formaldehyde solution (MEMFA) (Danos and Yost, 1995; Harland, 1991) for 1–2 h and stored in methanol at −20°C.

RNA probe synthesis and whole-mount in situ hybridization were performed using a standard protocol for *X. laevis* embryos (Harland, 1991; Sive et al., 1995). Using PCR methods, base pairs 1–229 for *xId2* (from a sequence published in Zhang et al., 1995) were cloned and a digoxigenin-labeled antisense RNA probe to *xId2* was synthesized from a cDNA insert in pGEM-T, cut with *Sal*I and transcribed with T7 RNA polymerase. After hybridization and color development, embryos were refixed in a 3.7% formaldehyde solution (MEMFA) and cleared in a 2:1 mixture of benzyl benzoate and benzyl alcohol for analysis of expression pattern and photography or were embedded in gelatin for cryostat sectioning.

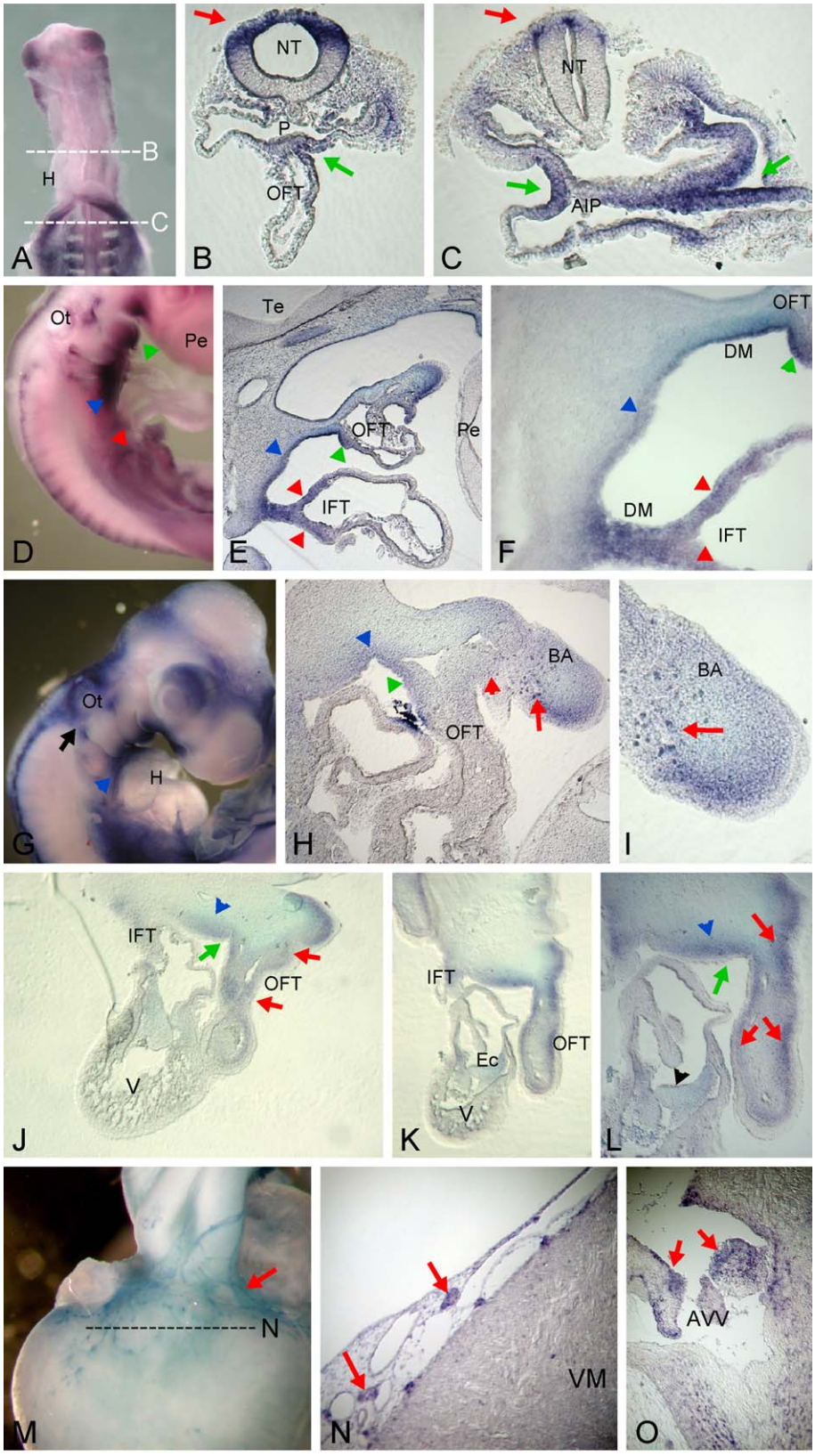
Immunofluorescent staining of Xenopus and quail-chick chimera sections

X. laevis embryos developed to stages 33 and 37 and were fixed and stored in Dent's fixative (80% methanol, 20% DMSO) at −20°C. Embryos were rinsed in 1× PBS, incubated in 5% sucrose, then 15% sucrose until embryos sunk in the solution and then were embedded in 7.5%

Fig. 1. Chick Id2 gene expression during cardiac neural crest and heart development. (A) Ventral view of Hamburger and Hamilton (HH) stages 10–12 chick embryo, showing expression in the anterior intestinal portal (AIP) at the base of the fusing heart tube. (B) Section at the pharynx (P) level shown in panel A. The red arrow points to expression in the neural tube (NT) and neural crest, while the green arrow points to expression in the splanchnic mesoderm (SM) of the secondary heart field (SHF) that is continuous with the outflow tract (OFT) myocardium. (C) Section at the level of the AIP. Red arrow points to expression in the cardiac neural crest (CNC) and NT. Green arrows point to expression in the SM of the AIP. (D) Right lateral view of a HH stage 18 embryo showing expression in the OFT (green arrowhead), SHF (blue arrowhead), and inflow tract (IFT) (red arrowhead). (E) Sagittal section through the developing heart shown in panel D. Expression can be seen in the splanchnic mesoderm (epithelium) (blue arrowhead) of the secondary heart field that is continuous with expression in the caudal wall of the OFT (green arrowhead) and associated dorsal mesocardium (DM), as well as the cranial and caudal walls of the IFT (red arrowheads) and associated dorsal mesocardium (DM). (F) Higher magnification of panel E. (G) Right lateral view of a HH stages 21–22 (day 3.5) embryo that shows expression in migrating cardiac neural crest (black arrow) and secondary heart field (blue arrowhead) next to the heart (H). (H) Sagittal section through the heart and branchial arch (BA) 3 shown in panel G. Expression can be seen in the SHF (blue arrowhead), caudal wall of the OFT (green arrowhead), and early (red arrowhead) and late migrating CNC (red arrow). (I) Higher magnification of the BA 3 shown in panel H. (J–L) Sagittal section through the heart and BA of the HH stages 25–26 (day 4.5) embryo shown in Fig. 2A. Id2 Expression is down-regulated in the SHF (green arrows), and upregulated in the ventral pharyngeal mesenchyme (blue arrowheads). Red arrows show migrating CNC entering the OFT. Black arrowhead and "Ec" denote expression in the atrioventricular (AV) endocardial cushions. (M) Ventral view of a day 13 heart shows Id2 expression in the anterior parasympathetic plexus (APP) (red arrow). (N) Transverse section through the heart shown in panel M. Red arrows show neurons expressing Id2 in the APP. (O) Section through the AV valves (AVV) of a day 14.5 heart shows expression of cId2 (red arrows).

gelatin (15% sucrose in 0.1 M PBS) for cryostat sectioning. Transverse *Xenopus* sections were degelatinized at 37°C in PBT (PBS + 0.2% Triton X-100) for 15 min.

Chick Id2 in situ hybridization on quail-chick cardiac neural crest chimera sections was performed first as described above in the chick in situ hybridization methods



section and then subsequently used for immunohistochemistry of QCPN (quail nuclei marker) as described below.

For immunoflourescent staining, the slides were washed in PBT at room temperature for 15 min with gentle rotation. Sections were blocked in PBT + 2% BSA + 10% goat serum for 1–2 h with gentle rotation. Sections were then incubated with either MF20 (an antibody to myosin heavy chain) for *Xenopus* sections or QCPN (an antibody to quail nuclei) for quail-chick chimera sections diluted 1:1 in the blocking solution for 2.5 h at room temperature. Sections were washed in PBT overnight at 4 °C, then 3 × 15 min at room temperature on day 2. Sections were then incubated in secondary antibody for 3 h at room temperature. Secondary antibody was Alexa Fluor 568 rabbit antimouse IgG (H + L) (Molecular Probes) diluted 1:100 in blocking solution. Sections were rinsed 3 × 15 min in PBT at room temperature and then viewed under a fluorescent scope and photographed. The MF20 monoclonal antibody developed by Donald A. Fischman, MD, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. QCPN supernatant generated from hybridoma cells (DSHB, University of Iowa) was a gift from M. Bronner-Fraser (Caltech).

Results and discussion

Early expression of chick Id2 in the neural crest, secondary heart field, and developing heart

Recent data has shown expression of chick Id2 (Martinsen and Bronner-Fraser, 1998) and chick Id4 (Kee and Bronner-Fraser, 2001a) in migrating cranial neural crest at the post-otic level. Here, we present a detailed analysis of the expression of Id2 in the developing chick cardiovascular system.

Chick Id2 is expressed in the developing cardiac neural crest (CNC), secondary heart field (SHF), outflow tract (OFT), and inflow tract (IFT), as well as in the neurons surrounding the developing aorta, pulmonary artery, and base of the heart [anterior parasympathetic plexus (APP)]. At the 5-somite stage (HH stages 8–9), Id2 is expressed in the cranial neural folds, including the neural folds at the level of the CNC, and by the 11-somite stage (HH stages 10–11) it is expressed in migrating cranial and cardiac neural crest cells (Martinsen, 1998; Martinsen and Bronner-Fraser, 1998). At HH stages 10–12 (10–14 somites), during fusion of the bilateral endocardial heart tubes, Id2 is expressed in the anterior intestinal portal (AIP) (Fig. 1A). Cross-sectional analysis at this stage, and at HH12, shows strong expression of Id2 in the dorsal neural tube, neural crest, and AIP (Figs. 1B,C, red arrows). Id2 is also expressed in the secondary heart field, which is comprised of splanchnic mesoderm (SM) underlying the midline endoderm of the pharynx (Fig. 1B, green arrow), and in the SM at the region of the AIP (Fig. 1C, green arrows). Expression of Id2 in the secondary heart field (SHF) can be seen even more clearly at HH stage 18 (Fig. 1D, blue arrowhead) and HH stages 21–22 (Figs. 1G,H, blue arrowheads), when secondary heart field cells are being added to the outflow tract (Figs. 1D–F,H, green arrowheads). At HH stage 18, Id2 is also highly expressed in the inflow tract (Figs. 1D–F, red arrowheads), and splanchnic mesoderm between the cardiac outflow and inflow tracts (Figs. 1E,F, blue arrowheads). This continuous expression of Id2 between the outflow and inflow tract in the splanchnic mesoderm and dorsal mesocardium may provide new clues to a potential link between secondary heart field, inflow, and outflow tract development. It is currently believed that the inflow tract is derived from the primary heart field and not the secondary heart field (Kirby, 2002), but our findings suggest the need for further investigation. Also, note that Id2 is expressed only in the caudal wall of the distal OFT and the dorsal mesocardium (DM) at HH stage 18 (Figs. 1E,F, green arrowheads) and HH stages 21–22 (Fig. 1H, green arrowhead), and not in the cranial wall of the outflow tract, which is consistent with secondary heart field cell markers like Gata-4 and Nkx2.5 (Waldo et al., 2001).

This early expression data shows, for the first time, that chick Id2 is expressed in the secondary heart field during the accretion of SHF cells to the developing outflow tract, and thus provides another molecular marker of the secondary heart field. This also suggests that this family of HLH transcription factors is involved in the development of the SHF and cardiac OFT. It has been shown that BMP-2 is highly expressed in the distal rim of the primary outflow region of the heart and that BMP-2 balances proliferation and differentiation of the SHF myocardium (Yutzey and Kirby, 2002). Because BMP-2 can stimulate the expression of Id2 in vitro (Locklin et al., 2001), it is plausible that it may act through Id2 in vivo to stimulate the proliferation of the secondary heart field and cardiac neural crest cells that reach the region of the distal outflow rim.

Down-regulation of chick Id2 in the secondary heart field

As the chick embryo progresses from day 3.5 (HH 21–23) to 4.5 (HH 25–26) of development, expression of Id2 in the splanchnic mesoderm of the secondary heart field (Figs. 1E,F,H, blue arrowheads) is down-regulated (Figs. 1J,L, green arrows), while expression in the ventral pharyngeal mesenchyme adjacent to the region of the SHF is up-regulated and expanded (Figs. 1J,L, blue arrowheads). The down-regulation of Id2 at HH stages 24–25 correlates with the completion of secondary heart field myocardium accretion to the outflow tract. Expansion of Id2 expression in the ventral pharyngeal mesenchyme adjacent to the SHF region and OFT at HH stages 21–25 correlates with appearance of late migrating cardiac neural crest in the pharyngeal arch 3 mesenchyme (Figs. 1H,I, red arrows) and early migrating

cardiac neural crest in the OFT (Fig. 1H, red arrowhead; Figs. 1J,L, red arrows; Fig. 2A, red arrow; Figs. 3A,C,E,G, red arrowheads). This suggests the possibility that late migrating cardiac neural crest migrating through the region of the secondary heart field may influence the final stages of accretion of secondary myocardium to the OFT (HH 21–23). It may also indicate a difference in the function of early vs. late migrating cardiac neural crest. A recent study has shown that myocardial cells from the SHF fail to be added to outflow myocardium in CNC ablated embryos, but why cells in the SHF behave abnormally after neural crest ablation is not known (Yelbuz et al., 2002). This data suggests that neural crest cells may not only regulate outflow tract development but may play a role in the regulation of the secondary heart field.

Late expression of chick Id2 in the outflow tract, inflow tract, and heart

Day 5.5 (HH stage 28) and day 6.5 (HH stage 30) chick hearts show marked expression of Id2 in the developing outflow tract (Figs. 2C,E, red arrows), atrioventricular (AV) sulcus (Figs. 2C,E, green arrows and Fig. 3E, green arrow), and in the ventral mesenchyme associated with the inflow tract. Expression in the atrioventricular region can be seen in the AV sulcus at day 4.5 (HH stages 25–26) (Fig. 2A, green arrow), and in the AV endocardial cushions at day 3.5 (HH stage 21–23) and day 4.5 (HH stages 25–26) (Figs. 1K,L, black arrowhead). Id2 expression in the AV endocardial cushions is maintained as they are being sculpted into atrioventricular valves at day 14.5 (HH stages 40–41) (Fig. 1O, red arrows).

Outflow tract expression of Id2 is best described by using nomenclature for chick cushion development as in Qayyum et al. (2001). At day 5.5 (HH stage 28), Id2 expression can be seen in the proximal right cushion (PR) of the OFT, which is consistent with a prong of neural crest (Fig. 3A, red arrowhead). Poelmann et al. (1998) showed for the first time that there is an extension of the CNC to the rim of the right ventricular outflow tract (base of the pulmonary outlet in the right ventricle). It has also been suggested that the ultimate

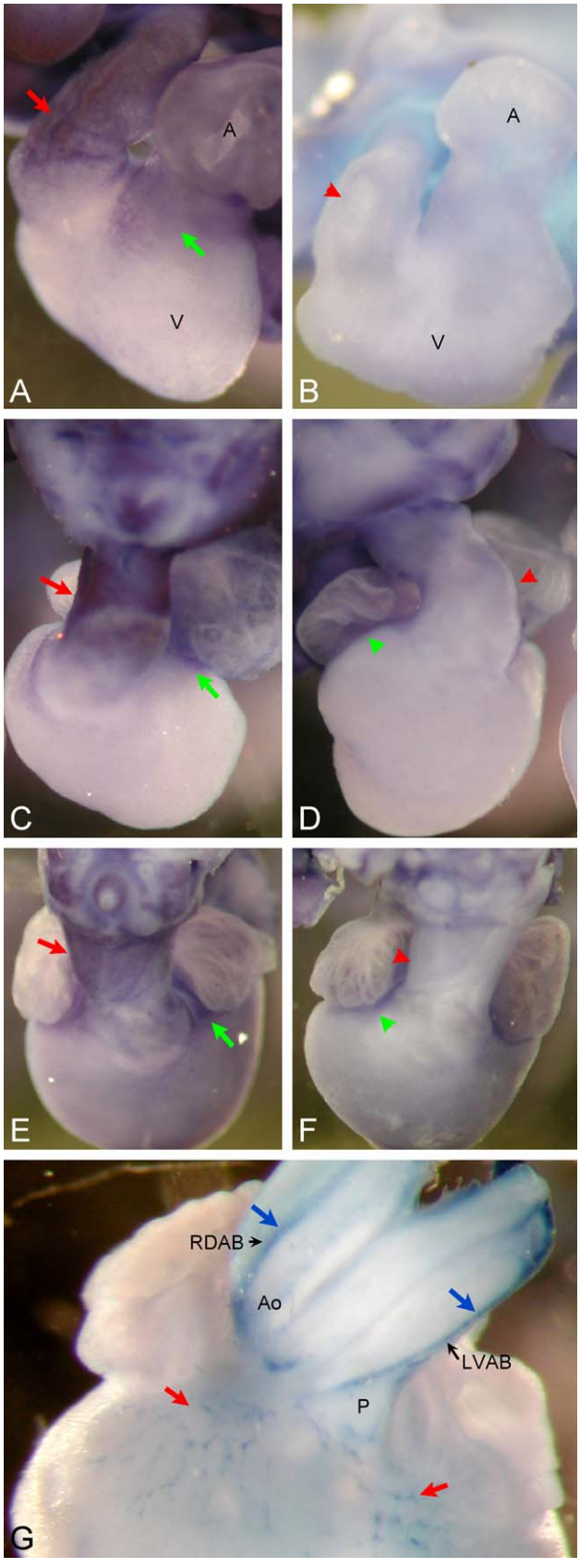


Fig. 2. Chick Id2 expression in the outflow tract and heart requires the presence of cardiac neural crest. (A, C, E) Id2 expression in control (sham operated) day 4.5 (100% have Id2 expression, $n = 15$), day 5.5 (67% have Id2 expression, $n = 7$), and day 6.5 (100% have Id2 expression, $n = 7$) hearts, respectively. Id2 expression can be seen in the outflow tract (red arrows) and atrioventricular sulcus (AVS) (green arrows). (B, D, F) Id2 expression in day 4.5 (only 13% have Id2 expression, $n = 35$), day 5.5 (only 10% have Id2 expression, $n = 10$), and day 6.5 (only 23% have Id2 expression, $n = 9$) hearts following early cardiac neural crest ablation (3–8-somite stage), respectively. Loss of expression in the outflow tract (red arrowheads), but not in the AVS (green arrowheads). (G) Id2 expression in a control day 9.5 heart shows expression in the anterior parasympathetic plexus (APP) (red arrows). Also at this stage, Id2 is expressed in developing cardiac neural crest derived neurons of the Xth (vagal) cranial nerve, including the right dorsal arterial branch (RDAB) that runs along the aorta (Ao), and the left ventral arterial branch (LVAB) that runs along the pulmonary artery (P) (blue arrows).

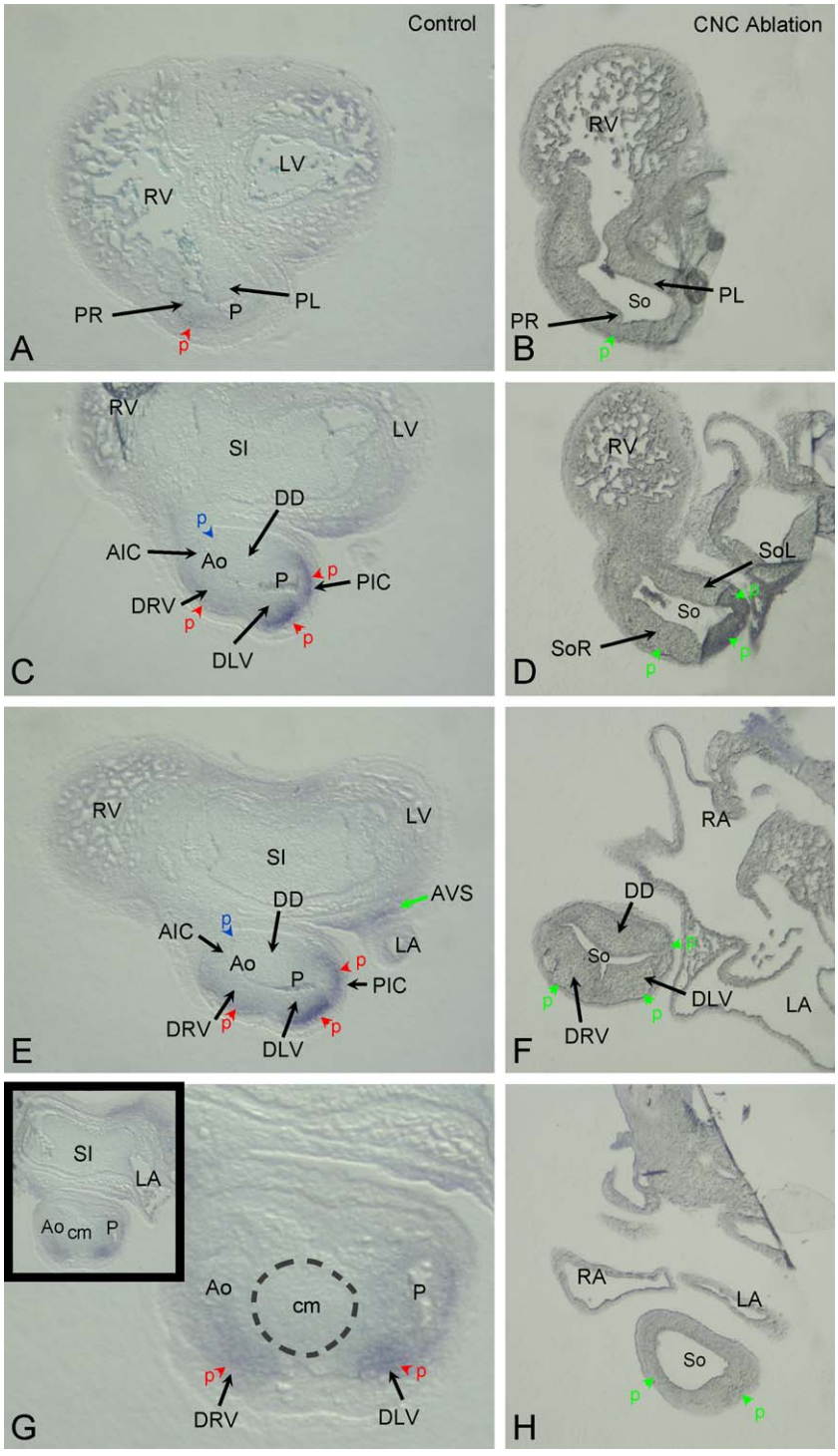


Fig. 3. Loss of chick Id2 expression in a day 5.5 heart following early cardiac neural crest ablation (3–8-somite stage). Selected sections showing chick Id2 expression in a control day 5.5 heart and a day 5.5 heart after early cardiac neural crest ablation. (A, C, E, G) Expression of Id2 from proximal to distal within the developing outflow tract and heart of a control day 5.5 heart. (B, D, F, H) Loss of Id2 expression from proximal to distal within a day 5.5 outflow tract and heart following early cardiac neural crest ablation. (H) Early cardiac neural crest ablation resulted in a single outflow vessel (So) with no septation or cardiac neural crest derived condensed mesenchyme (cm). Red p and arrowhead, prong of cardiac neural crest (CNC) with Id2 expression. Blue p and arrowhead, prong of CNC with no Id2 expression. Green p and arrowhead, loss of CNC prong and Id2 expression after early CNC ablation. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; Ao, aorta; P, pulmonary trunk; PR, proximal right endocardial cushion; PL, proximal left endocardial cushion; SI, septum intermedium; DD, distal dorsal endocardial cushion; AIC, aorta intercalated endocardial cushion; PIC, pulmonary intercalated endocardial cushion; DRV, distal right ventral endocardial cushion; DLV, distal left ventral endocardial cushion; AVS, atrioventricular sulcus; So, single outflow vessel; SoR, single outflow vessel right endocardial cushion; SoL, single outflow vessel left endocardial cushion. OFT labeling based on that of Qayyum et al. (2001).

fate of these deep CNC cells is apoptosis, which would explain the absence of any neural crest derived septal structures below semilunar valve level (Poelmann et al., 1998; Qayyum et al., 2001). These deep CNC cells are also hypothesized to release or mobilize growth factors as they die in morphogenetically active areas of the heart (Poelmann et al., 1998). Thus, Id2 not only marks the secondary heart field and ganglia of the anterior parasympathetic plexus (APP), but it may also mark a population of deep migrating cardiac neural crest cells whose ultimate fate is cell death.

Id2 expression is also seen in a pattern consistent with expression in prongs of crest in the distal left ventral cushion (DLV) and pulmonary intercalated cushion (PIC) of the OFT (Fig. 3C, red arrowheads). Even more distally, at the level of the septum intermedium (SI), Id2 is expressed in prongs of crest directly under the myocardium in both the left and right distal ventral cushions of the OFT, and in the pulmonary vessel wall at the level of the atria (Figs. 3E,G, red arrowheads). OFT expression of Id2 begins to decrease between days 7.5 and 8.5 (HH stages 32–34), which coincides with the completion of OFT septation. The expression pattern of Id2 before day 8.5 of chick development is consistent with a role in secondary heart field, cardiac neural crest, and outflow tract development.

After day 9.5 (HH stages 35–36) of chick development, the expression of Id2 is most prominent in cardiac neural crest derived structures. Id2 expression is seen in developing CNC-derived neurons of the Xth (vagal) cranial nerve, including the right dorsal arterial branch (RDAB), which runs along the aorta, and the left ventral arterial branch (LVAB), which runs along the pulmonary artery (Fig. 2G, blue arrows). Id2 is also highly expressed in the neurons of the anterior parasympathetic plexus (APP) from day 9 to 13 (Fig. 1M,N, red arrows and Fig. 2G, red arrows) a pattern that is consistent with a role for Id2 in cardiac neural crest associated innervation of the heart. Previous cardiac neural crest lineage tracing experiments, using retroviral infection of the reporter gene LacZ, show a similar expression pattern in the cardiac ganglia of the APP (Bergwerff et al., 1998; Poelmann et al., 1998), as do quail-chick chimera studies on the distribution of the CNC in the cardiac nervous system (Verberne et al., 2000). This expression pattern is also similar to Sox 10 expression in the developing APP (Montero et al., 2002). Thus, the Id2 expression data presented here shows for the first time that Id2 specifically marks the anterior parasympathetic plexus (APP) of the heart.

Cardiac neural crest derived cells in the septating outflow tract express chick Id2

Chick Id2 is coexpressed with QCPN in cardiac neural crest (CNC) cells of the septating outflow tract of quail-chick (CNC) chimera embryos, verifying that cardiac neural crest derived cells in the septating outflow tract express Id2 (Fig. 4). Fig. 4 shows Id2 gene expression and QCPN (quail nuclei marker) immunofluorescent staining at three different sagittal levels of a day 6.5 quail-chick unilateral CNC chimeric heart. Id2 is expressed in the aortic and pulmonary (P) vessel walls and the CNC cells of the forming aorticopulmonary septum (APS) of the outflow tract (Oft) (Figs. 4A,D,H,I,N, red arrows). QCPN marks quail CNC grafted cells in the aortic and pulmonary (P) vessel walls and the aorticopulmonary septum (APS) of the outflow tract (Oft) (Figs. 4B,F,J,L, white arrows). Merged images of Id2 gene expression and QCPN immunofluorescent staining of the same section show overlapping expression (Figs. 4C,E,G,K,M,O, white arrows). A higher power view of the APS shows individual quail cardiac neural crest cells expressing Id2 (Fig. 4E, white arrow heads), verifying that Id2-expressing cells in the outflow tract are cardiac neural crest derived. Interestingly, cells expressing Id2 in the atrioventricular sulcus (AVS) (Figs. 4N,P, red arrows) are not positive for QCPN (Fig. 4Q, white arrow heads), suggesting that these cells are not cardiac neural crest derived. These cells are most likely derived from the endocardial cushions of the AVS and this is consistent with the expression of Id2 in the forming atrioventricular valves at later stages.

Chick Id2 expression in the outflow tract and heart requires the presence of cardiac neural crest

The chick system is particularly conducive to experimental manipulation of the developing cardiovascular system, and recent studies have lent support to the idea that the role of cardiac neural crest in mammalian cardiovascular development is similar to that in the chick (Lo et al., 1997; Waldo et al., 1999). Ablation of the premigratory cardiac crest in the chick results in persistent truncus arteriosus, or complete lack of septation of the outflow tract, and a variety of other structural conotruncal defects (Kirby and Farrell, 1999), as well as abnormalities of the myocardium. Heart defects are present in about 90% of embryos surviving to days 8–11 after neural crest ablation (Nishibatake et al., 1987) and abnormal heart development can be seen as early as only 1–2 days of incubation (Kirby and Farrell, 1999). In this study we scored, via whole-mount in situ hybridization, the expression of Id2 in day 4.5 to 9.5 control chick hearts with chick hearts after early cardiac neural crest ablation (Fig. 2).

Early cardiac neural crest (CNC) ablations, completed between 3- and 8-somite stage (HH stages 8–9), resulted in an almost complete loss of Id2 expression in the outflow tract (Figs. 2B,D,F, red arrowheads). Sectional analysis of a heart of an early CNC ablated embryo collected at day 4.5 reveals loss of Id2 expression in the AV cushions, as well as in the region underlying the distal ventral OFT cushion and the distal dorsal and ventral OFT cushions (data not shown). In addition, sectional analysis of a heart of an early CNC ablated embryo collected at day 5.5 shows loss of Id2 expression in the region underlying the proximal right OFT cushion (PR) (Fig. 3B, green arrowhead); underlying the distal right ventricular endocardial cushion (DRV);

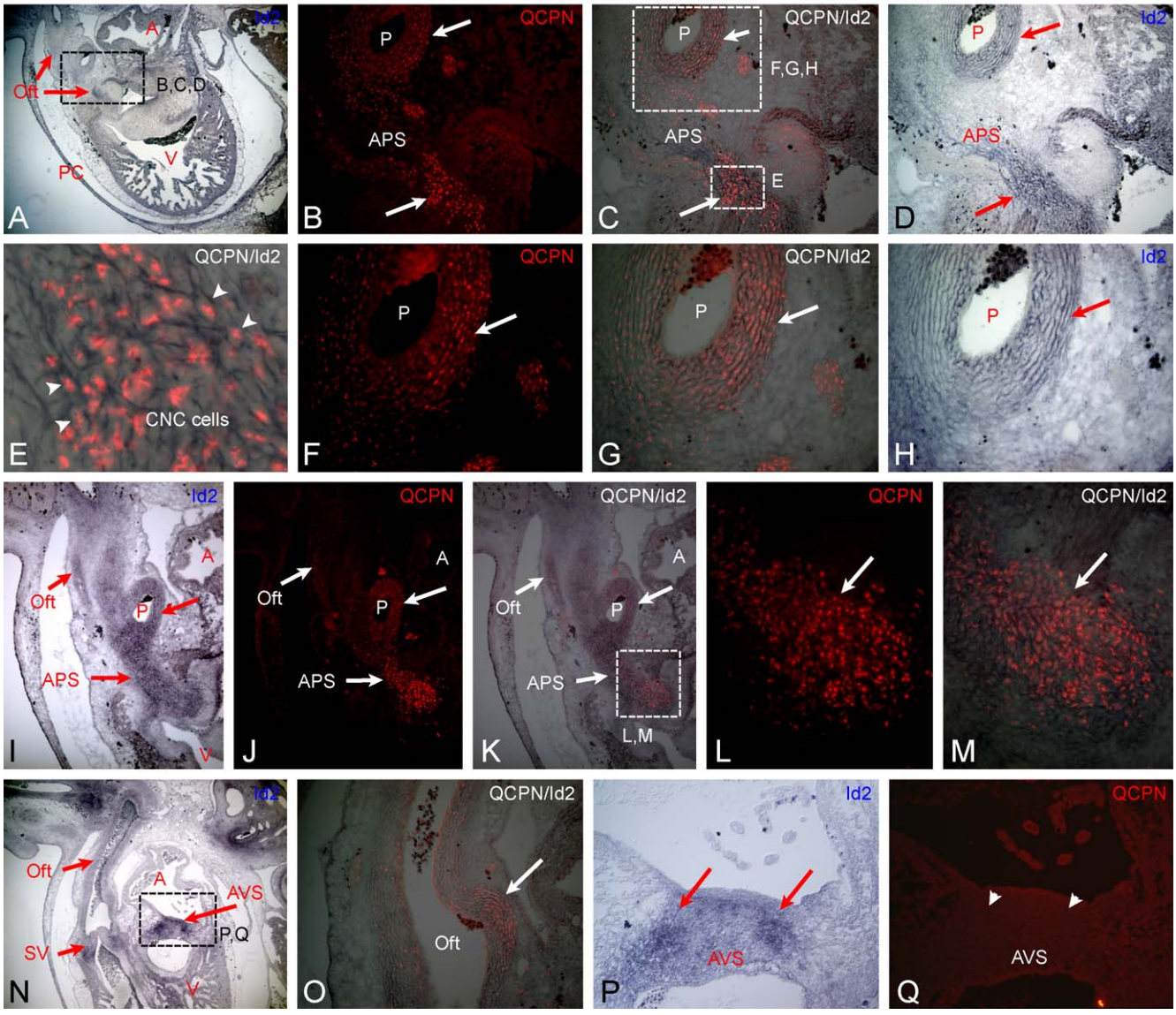


Fig. 4. Cardiac neural crest derived cells in the septating outflow tract express chick Id2. Id2 gene expression and QCPN (quail nuclei marker) immunofluorescent staining are shown at three different sagittal levels of a chick host heart collected at day 6.5 after receiving a unilateral quail CNC graft at the 8-somite stage. (A, D, H, I, N) Id2 (blue) is expressed in the aortic and pulmonary (P) vessel walls and the CNC cells of the forming aorticopulmonary septum (APS) of the outflow tract (Oft) (red arrows). (B, F, J, L) QCPN (red) marks quail CNC grafted cells located in the aortic and pulmonary (P) vessel walls and the CNC cells of the forming aorticopulmonary septum (APS) of the outflow tract (Oft) (white arrows). (C, E, G, K, M, O) Merged images of Id2 gene expression (blue) and QCPN (red) immunostaining of the same section show overlapping expression. (E) A higher power view of the APS (denoted by small white dashed box in panel C) shows individual quail cardiac neural crest (CNC) cells expressing Id2 (white arrow heads), (N, P) cells expressing Id2 (blue) in the atrioventricular sulcus (AVS) (red arrows) are not positive for QCPN as shown in panel Q (white arrow heads). Black dashed box in panel A, region of higher power view shown in panels B, C, and D. Large white dashed box in panel C, region of higher power view shown in panels F, G, and H. Small white dashed box in panel C, region of higher power view show in panel E. White dashed box in panel K, region of higher power view shown in panels L and M; Black dashed box in panel N, region of higher power view shown in panels P and Q; Oft, outflow tract; PC, pericardium; A, atria; V, ventricle; APS, aorticopulmonary septum; P, pulmonary trunk; CNC, cardiac neural crest cells; Id2, Id2 gene expression; QCPN, quail nuclei marker; QCPN/Id2 merged fluorescent and brightfield; SV, semilunar valve; AVS, atrioventricular sulcus.

underlying the distal left ventricular endocardial cushion (DLV); underlying the pulmonary intercalcated endocardial cushion (PIC) (Figs. 3D,F, green arrowheads); and underlying the endocardium of the distal single OFT (Fig. 3H, green arrowheads). The early CNC ablated embryo shown (Figs. 3B,D,F,H, So), collected at day 5.5, has malformed cushions showing no signs of early outflow tract septation, creating an early single OFT. Interestingly, the expression of Id2 in the AV sulcus does not seem to be altered in day 5.5 and day 6.5 hearts after early cardiac neural crest ablation (Figs. 2D,F, green arrowheads). Id2 expression marking the neurons along the aorta and the anterior parasympathetic plexus (APP) is completely lost in a 9.5-day heart after early cardiac neural crest ablation (data not shown). Limited data

in the 9.5-day heart after early CNC ablation is notable for malposition of the great vessels suggestive of transposition or double outlet right ventricle (data not shown).

Xenopus Id2 expression in the neural crest and splanchnic mesoderm

In recent years, a growing number of genes expressed in the neural crest cells of *X. laevis* have been identified, and among these are the Id genes, including Id2. However, a link between neural crest cells and *Xenopus* heart development has yet to be established and it is unclear whether a secondary heart field exists in *Xenopus*. Thus, we report here for the first time that the expression pattern of *xId2* in *Xenopus* is located not only in the neural crest, but also in the splanchnic mesoderm underlying the floor of the pharynx in a region analogous to what has been described in chick as a secondary heart field. The Id2 expression pattern in this region is also very similar to the chick secondary heart field markers, Gata-4 and Nkx2.5 (Waldo et al., 2001). Lastly, the effect of neural crest ablation of premigratory neural crest cells on *xId2* expression and heart formation in *X. laevis* suggests a link between neural crest cells and normal heart development.

The expression pattern of *Xenopus* Id2 (*xId2*) in the developing heart has many similarities to the expression of chick Id2 during chick heart development. During neurula stages (stages 15–20, Nieuwkoop and Faber, 1967), *xId2* is strongly expressed in the neural folds, and at stage 20, with the neural tube closed, *xId2* expression is seen in the neural tube (NT) and in the ventral mesoderm (Figs. 5A–F). By early tailbud stages, *xId2* expression is present in the NT, somites (S), and the branchial arches (BA) (Figs. 5G,H). At stage 37/38 *xId2*, expression is apparent in the heart (H), eye (E), and otic vesicle (O) as well as the somites and branchial arches (Figs. 6E and 8C). A more detailed analysis of cardiac expression in a stage 39 embryo shows expression of *xId2* in the caudal portion of the cardiac outflow tract (OFT), in the cardiac inflow region (IFT), and the splanchnic mesoderm (SM) (Figs. 6C,G). Transverse sections through a stage 39 (tailbud) *Xenopus* embryo after whole-mount in situ hybridization (ISH) with *xId2* reveal expression in the neural tube, eye, and nerve root ganglia (Fig. 6F, green arrows). A slightly more caudal section of the same embryo shows *xId2* expres-

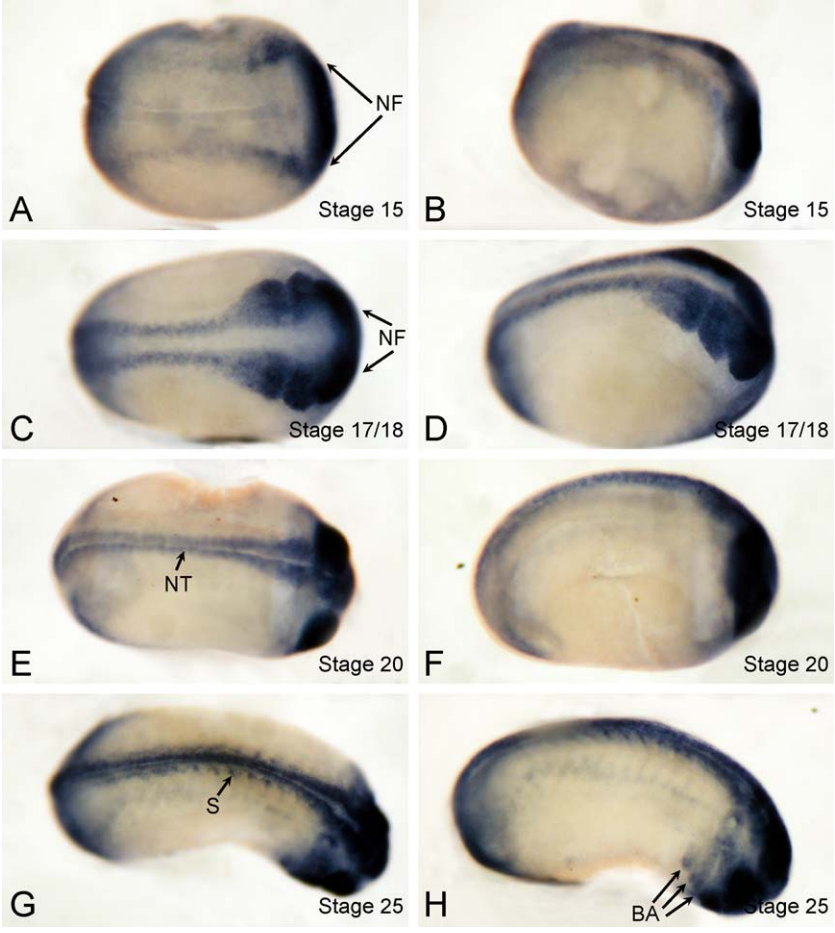


Fig. 5. *xId2* expression in staged *Xenopus* embryos. A, C, E, and G are dorsal views with the anterior of the embryo to the right. B, D, F, and H are right lateral views of the embryos. (A–F) Whole-mount in situ hybridization for *xId2* shows expression in the neural folds (NF) and along the length of the closing neural tube (NT). (G, H) shows *xId2* expression in the somites (S) and branchial arches (BA).

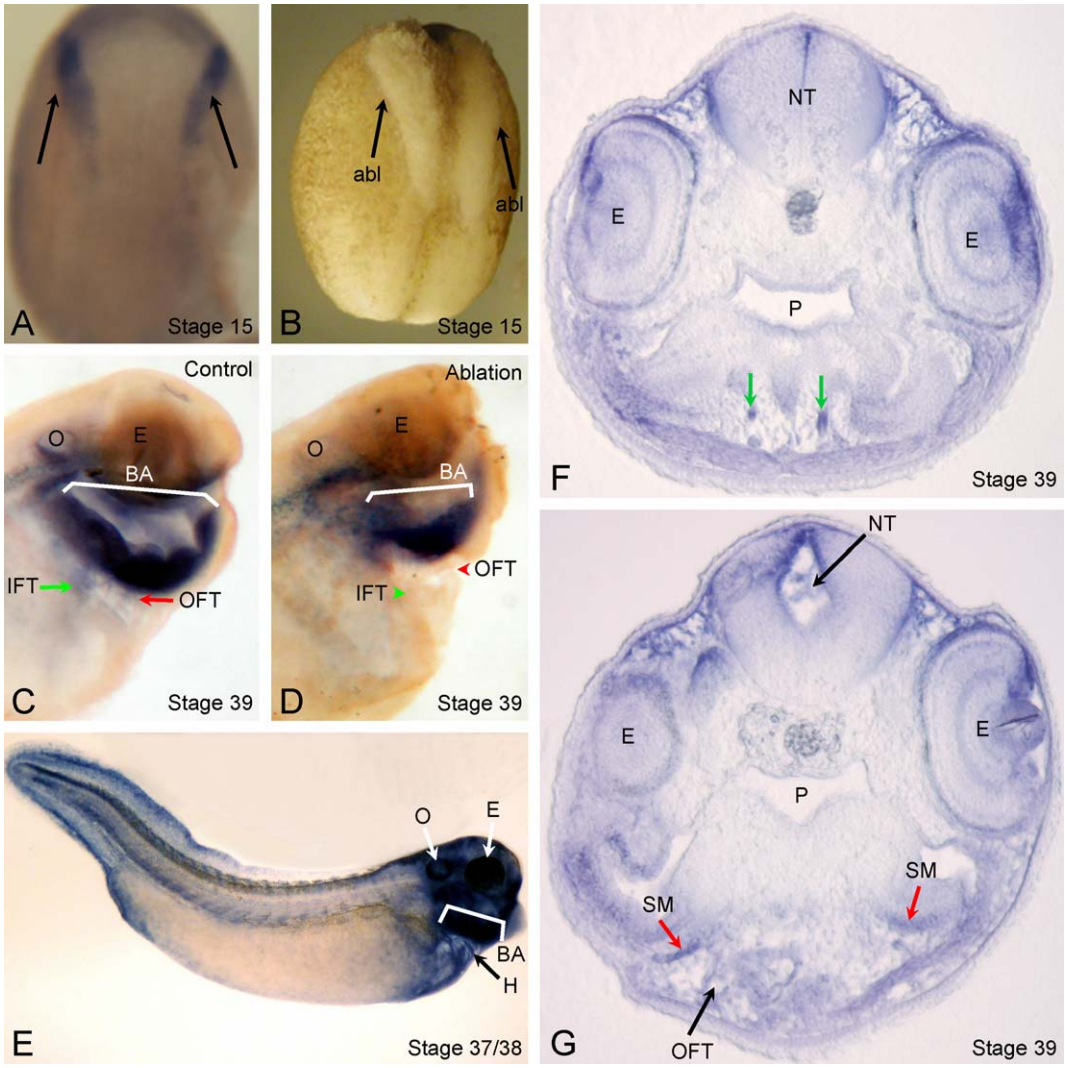


Fig. 6. Results of *Xenopus* neural crest ablation and *xId2* expression patterns. (A, B) *xSlug* expression in the neural crest was used as a guide (A) for stage 15 neural crest ablations (B, abl). (C, D) Whole-mount *xId2* in situ hybridization (ISH) was carried out on stage 39 control and neural crest ablated embryos. (C) There is *xId2* expression in both the inflow tract (IFT) (green arrow) and outflow tract (OFT) (red arrow) of the developing heart, as well as the otic vesicle (O), eye (E), and branchial arches (BA) in the control embryo. (D) In the ablated stage 39 embryo, there is lack of expression in the IFT (green arrowhead), OFT (red arrowhead), and reduced expression in the BA. (E–G) Control embryos underwent whole-mount in situ hybridization (ISH) for *xId2* and were fixed at stage 37/38 for whole-mount analysis and at stage 39 for sectional analysis. (E) *xId2* expression is located in the branchial arches (BA), eye (E), otic vesicle (O), and heart (H). (F, G) *xId2* expression is located in the neural tube (NT), eye (E), outflow tract (OFT), pharynx (P), nerve ganglion (green arrows), and splanchnic mesoderm (SM, red arrows).

sion in the cardiac outflow tract (OFT) and splanchnic mesoderm (Fig. 6G, red arrows, SM). *XId2* gene expression can be seen in the splanchnic mesoderm underlying the floor of the pharynx in stages 33 and 37 control *Xenopus* embryos (Figs. 7C,D and 8D, red arrows), which is MF20 negative (Figs. 7A,B and 8A,B, white arrows). The expression of *xId2* in the splanchnic mesoderm (Figs. 7C,D and 8D, red arrows) is continuous with *xId2* expression in the distal primary outflow myocardium (m) (Figs. 7C and 8D, green arrows), which is MF20 positive (Figs. 7A,B and 8A,B, bright red immunofluoresence). This expression pattern is similar to Gata-4, Nkx2.5, and Id2 in chick, which mark the secondary heart field. Thus, *Xenopus* may also have a secondary heart field, which expresses *xId2* and contributes to cardiac outflow tract formation, analogous to the secondary heart field (SHF) in the chick.

Xenopus neural crest required for proper heart development and xId2 expression

The contribution of cardiac neural crest to heart development is well described in the chick embryo, and conotruncal defects similar to those seen in neural crest ablated chick embryos account for a significant percentage of human congenital heart disease. In contrast, the role of neural crest in heart formation and function has only recently been tested in zebrafish (Li et al., 2003; Sato and Yost, 2003), and we believe that the work presented here is

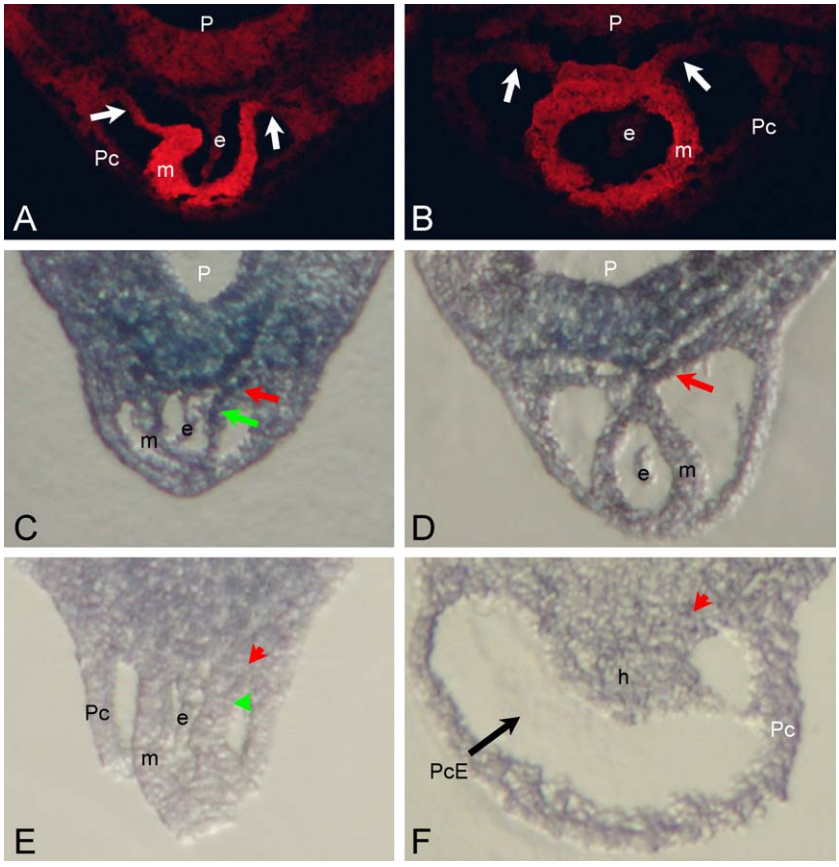


Fig. 7. Analysis of the heart morphology and *xId2* expression patterns of stage 33 *Xenopus* embryos, following neural crest ablation and in situ hybridization (ISH) for *xId2*. (A, B) Transverse sections of a stage 33 control embryonic heart immunostained with the myocardial marker MF20. White arrows indicate (MF20 negative) undifferentiated splanchnic mesoderm cells continuous with the newly formed cardiac myocardial cells expressing myosin heavy chain (MF20 positive). (C, D) Sections through a stage 33 control embryo following ISH for *xId2*. In the control embryos, there is a visible myocardium (m), endocardium (e), and pharynx (P). [See Mohun et al. (2000) for reference to *Xenopus* heart morphology.] There is *xId2* expression in the splachnic mesoderm (red arrows) as well as the outflow track myocardium (green arrow). (E, F) Sections through a stage 33 embryo following stage 15 neural crest ablation and ISH for *xId2*. There is a lack of *xId2* expression in the splachnic mesoderm (red arrowheads) and the outflow tract myocardium (green arrowhead). There are also morphological differences in heart development following neural crest ablation. Note the enlarged myocardium (m), the thickened pericardial layer (Pc), and the pericardial edema (PcE). Morphology is variable among neural crest ablated embryos; (E) sometimes there is visible formation of a myocardium (m) and endocardium (e), (F) whereas in other embryos there is only an undistinguishable mass of heart cells (h).

the first evidence of a contribution of neural crest to heart formation in *Xenopus*. To determine the effects of neural crest ablation on *xId2* expression and heart development in *Xenopus*, we ablated the surface cells of the lateral neural folds bilaterally in a stage 15 embryo (Fig. 6B) using *xSlug* expression as a visual guide to the areas of premigratory neural crest formation (Fig. 6A). Thus, neural crest cells were ablated in the entire cranial region to the mid-trunk level of the *Xenopus* embryo (Fig. 6B). We believe that if *Xenopus* has cardiac neural crest cells, they will be contained within this region based on cardiac neural crest mapping in chick and zebrafish (Li et al., 2003). In the ablated embryos, *xId2* expression is reduced in the otic vesicle (O), eye (E), and branchial arches (BA), and is absent in the cardiac outflow tract (OFT) and inflow tract (IFT) (Fig. 6D). In addition, the expression in the splanchnic mesoderm (Figs. 7C,D and 8D, red arrows) and myocardium (Figs. 7C and 8D, green arrows) is lost in the neural crest ablated embryos (Figs. 7E,F and 8E,F, red and green arrow heads). Cardiac formation is also altered in neural crest ablated *Xenopus* embryos. Cardiac findings after ablation of premigratory neural crest in *Xenopus* embryos included an elongated, unlooped heart, and enlarged pericardial cavity (Figs. 6D, 7F, and 8E) and a lack of normal heart tube formation (Figs. 7E and 8F). This *Xenopus* neural crest ablation phenotype is very similar to that seen in recent zebrafish cardiac neural crest ablation experiments (Li et al., 2003) and suggests that the presence of neural crest is required for normal heart development in *Xenopus*.

Conclusions and future directions

The observation that the ablation of the cardiac neural crest results in outflow tract and aortic arch defects occurred 20 years ago, but the delineation of the molecular cues that

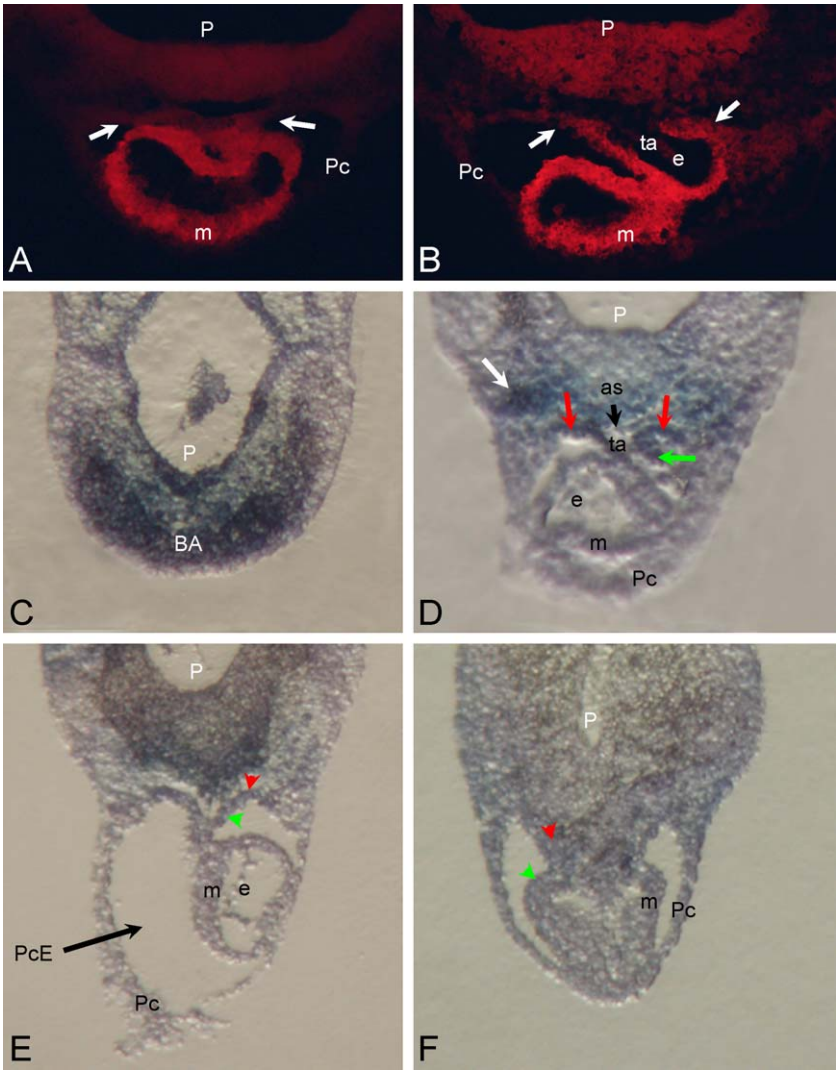


Fig. 8. Analysis of morphology and *xId2* expression patterns of stage 37 *Xenopus* embryos following neural crest ablation and in situ hybridization (ISH) for *xId2*. (A, B) Transverse sections of a stage 37 control embryonic heart immunostained with the myocardial marker MF20. White arrows indicate (MF20 negative) undifferentiated splanchnic mesoderm cells continuous with the newly formed cardiac myocardial cells expressing myosin heavy chain (MF20 positive). (C, D) Sections through a stage 37 control embryo following ISH for *xId2*. (C) There is Id2 expression in the branchial arches (BA), and in a more caudal section (D) there is expression in the splachnic mesoderm (red arrows), myocardium of the outflow tract (green arrow), and the branchial arches (white arrow). Note that the expression in the splachnic mesoderm is continuous with the distal outflow tract myocardium. There is a visible pharynx (P), pericardium (Pc), myocardium (m), endocardium (e), aortic sac (as, black arrow), and truncus arteriosus (ta). [See Mohun et al. (2000) for reference to *Xenopus* heart morphology.] (E, F) Sections through a stage 37 embryo following stage 15 neural crest ablation and ISH for *xId2*. There is a lack of xId2 expression in both the splachnic mesoderm (red arrowheads) and myocardium of the outflow tract (green arrowheads). Morphologically, there is pericardial edema (PcE), an enlarged myocardium (m), and an irregular pericardial layer (pc). (E) The heart that formed in this ablated embryo is an unlooped linear heart tube.

regulate cardiac neural crest and cardiac outflow tract development have been daunting (Baldwin, 1999). In the work presented here, we show that expression of the helix–loop–helix molecule Id2, a known regulator of many developmental processes, occurs in cardiac neural crest, secondary heart field, outflow tract, and innervation of the heart. We show that Id2 is a new marker for the secondary heart field during chick embryonic heart development, and that the expression of Id2 in the developing cardiac outflow tract is altered after neural crest ablation. These findings suggest that Id2 may regulate accretion of the cardiac outflow tract from the secondary heart field, a hypothesis we will test with further experimentation. Although no neural crest contribution to heart development has been shown in *Xenopus*, a role for neural crest in the development of normal structure and function of the zebrafish heart has recently been described (Li et al., 2003; Sato and Yost, 2003). Here we report expression of *Xenopus* Id2 in the splanchnic mesoderm and outflow tract of the embryonic frog heart, suggesting that there is a structure analogous to the chick secondary heart field in *Xenopus*. The true nature of this secondary heart field and its contribution to cardiac formation in *Xenopus* require further study. In addition, our neural fold ablation experiments are the first, to our knowl-

edge, that show a role for neural crest in normal cardiac gene expression and heart formation in *Xenopus*.

Indirect evidence suggests that bHLH proteins may participate in the control of cardiac muscle gene expression. Recent studies have shown that the novel bHLH dHAND and eHAND genes are expressed in early cardiogenic progenitors, as well as the lateral plate (splanchnic mesoderm), heart tube, cardiac neural crest, and branchial arches (Angelo et al., 2000; Olson and Srivastava, 1996; Srivastava, 1999; Srivastava et al., 1995). Inhibition of expression of these factors with antisense oligonucleotides in cultured chick embryos suggest that these factors regulate critical steps in cardiac morphogenesis (Srivastava et al., 1995). The interacting network of HLH containing transcription factors, including Id2, may help regulate the process of cardiac neural crest and heart development. In particular, the proximity of gene expression in the CNC and molecular structure suggests that Id2 and dHAND may interact during this critical period of cell commitment to the cardiac lineage. Understanding the role of Id2 in regulating these interactions and cardiogenesis is the subject of our ongoing work.

Acknowledgments

This work was supported by an American Heart Association Fellowship (#0020507Z) to BJM, an American Heart Association Scientist Development Award (#9930216N) to JLL, and gifts from the Andrew David Sit Foundation and Sit Investment Associates Foundation to JLL. Special thanks to Marianne Bronner-Fraser for her continued support.

References

Angelo, S., Lohr, J., Lee, K.H., Ticho, B.S., Breitbart, R.E., Hill, S., Yost, H.J., Srivastava, D., 2000. Conservation of sequence and expression of *Xenopus* and zebrafish dHAND during cardiac, branchial arch and lateral mesoderm development. Mech. Dev. 95, 231–237.

Baldwin, H.S., 1999. Advances in understanding the molecular regulation of cardiac development. Curr. Opin. Pediatr. 11, 413–418.

Benezra, R., 2001. The Id proteins: targets for inhibiting tumor cells and their blood supply. Biochim. Biophys. Acta 1551, F39–47 (Oct 1).

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., Weintraub, H., 1990. The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. Cell 61, 49–59.

Bergwerff, M., Verberne, M.E., DeRuiter, M.C., Poelmann, R.E., Gittenberger-de Groot, A.C., 1998. Neural crest cell contribution to the developing circulatory system: implications for vascular morphology? Circ. Res. 82, 221–231 (Feb 9).

Besson, W.T., Kirby, M.L., Van Mierop, L.H., Teabeaut, J.R., 1986. Effects of the size of lesions of the cardiac neural crest at various embryonic ages on incidence and type of cardiac defects. Circulation 73, 360–364.

Boot, M.J., Gittenberger-De Groot, A.C., Van Iperen, L., Hierck, B.P., Poelmann, R.E., 2003. Spatiotemporally separated cardiac neural crest subpopulations that target the outflow tract septum and pharyngeal arch arteries. Anat. Rec., Part A 275A, 1009–1018.

Bronner-Fraser, M., 1986. Analysis of the early stages of trunk neural crest migration in avian embryos using monoclonal antibody HNK-1. Dev. Biol. 115, 44–55.

Christy, B.A., Sanders, L.K., Lau, L.F., Copeland, N.G., Jenkins, N.A., Nathans, D., 1991. An Id-related helix–loop–helix protein encoded by a growth factor-inducible gene. Proc. Natl. Acad. Sci. U. S. A. 88, 1815–1819.

Collazo, A., Bronner-Fraser, M., Fraser, S.E., 1993. Vital dye labelling of *Xenopus laevis* trunk neural crest reveals multipotency and novel pathways of migration. Development 118, 363–376.

Danos, M.C., Yost, J., 1995. Linkage of cardiac left–right asymmetry and dorsal–anterior development in *Xenopus*. Development 121, 1467–1474.

Essex, L.J., Mayor, R., Sargent, M.G., 1993. Expression of *Xenopus* snail in mesoderm and prospective neural fold ectoderm. Dev. Dyn. 198, 108–122.

Etchevers, H.C., Vincent, C., Le Douarin, N.M., Couly, G.F., 2001. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128, 1059–1068.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Harland, R.M., 1991. In situ hybridization: an improved whole-mount method for *Xenopus* embryos. Methods Cell Biol. 36, 685–695.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J., Ish-Horowicz, D., 1995. Expression of a delta homologue in prospective neurons in the chick. Nature 375, 787–790.

Hopwood, N.D., Pluck, A., Gurdon, J.B., 1989. A *Xenopus* mRNA related to *Drosophila* twist is expressed in response to induction in the mesoderm and the neural crest. Cell 59, 893–903.

Jen, Y., Manova, K., Benezra, R., 1996. Expression patterns of Id1, Id2, and Id3 are highly related but distinct from that of Id4 during mouse embryogenesis. Dev. Dyn. 207, 235–252.

Jen, Y., Manova, K., Benezra, R., 1997. Each member of the Id gene family exhibits a unique expression pattern in mouse gastrulation and neurogenesis. Dev. Dyn. 208, 92–106.

Kee, Y., Bronner-Fraser, M., 2001a. Id4 expression and its relationship to other Id genes during avian embryonic development. Mech. Dev. 109, 341–345.

Kee, Y., Bronner-Fraser, M., 2001b. Temporally and spatially restricted expression of the helix–loop–helix transcriptional regulator Id1 during avian embryogenesis. Mech. Dev. 109, 331–335.

Kee, Y., Bronner-Fraser, M., 2001c. The transcriptional regulator Id3 is expressed in cranial sensory placodes during early avian embryonic development. Mech. Dev. 109, 337–340.

Kelly, R.G., Brown, N.A., Buckingham, M.E., 2001. The arterial pole of the mouse heart forms from Fgf10-expressing cells in pharyngeal mesoderm. Dev. Cell 1, 435–440.

Kirby, M.L., 1988. Role of extracardiac factors in heart development. Experientia 44, 944–951.

Kirby, M.L., 1989. Plasticity and predetermination of mesencephalic and trunk neural crest transplanted into the region of the cardiac neural crest. Dev. Biol. 134, 402–412.

Kirby, M.L., 2002. Molecular embryogenesis of the heart. Pediatr. Dev. Pathol. 5 (6).

Kirby, M.L., Farrell, M., 1999. The diverse roles of neural crest in cardiovascular development: myocardial function, aortic arch repatterning and outflow septation. Prog. Pediatr. Cardiol. 9, 171–181.

Kirby, M.L., Stewart, D.E., 1983. Neural crest origin of cardiac ganglion cells in the chick embryo: identification and extirpation. Dev. Biol. 97, 433–443.

Kirby, M.L., Gale, T.F., Stewart, D.E., 1983. Neural crest cells contribute to normal aorticopulmonary septation. Science 220, 1059–1061.

Kirby, M.L., Turnage III, K.L., Hays, B.M., 1985. Characterization of conotruncal malformations following ablation of "cardiac" neural crest. Anat. Rec. 213, 87–93.

Li, Y.X., Zdanoqicz, M., Young, L., Kumiski, D., Leatherbury, L., Kirby, M., 2003. Cardiac neural crest in zebrafish embryos contributes to myocardial cell lineage and early heart function. Dev. Dyn. 226, 540–550.

Liu, K.J., Harland, R.M., 2003. Cloning and characterization of *Xenopus* Id4 reveals differing roles for Id genes. Dev. Biol. 264, 339–351.

Lo, C.W., Cohen, M.F., Huang, G.Y., Lazatin, B.O., Patel, N., Sullivan, R., Pauken, C., Park, S.M., 1997. Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. Dev. Genet. 20, 119–132.

Locklin, R., Riggs, B., Hicok, K., Horton, H., Byrne, M., Khosla, S., 2001. Assessment of gene regulation by bone morphogenetic protein 2 in human marrow stromal cells using gene array technology. J. Bone Miner. Res. 16, 2192–2204 (Dec).

Manova, K., Bachvarova, R.F., 1991. Expression of c-kit encoded at the W locus of mice in developing embryonic germ cells and presumptive melanoblasts. Dev. Biol. 146, 312–324.

Martinsen, B.J., 1998. Molecular differences between cranial and trunk neural crest/tube that influence cell fate decisions. Developmental and Cell Biology. University of California, Irvine, pp. 82–114.

Martinsen, B.J., Bronner-Fraser, M., 1998. Neural crest specification regulated by the helix–loop–helix repressor Id2. Science 281, 988–991.

Martinsen, B.J., Groebner, N.J., Frasier, A.J., Lohr, J.L., 2003. Expression of cardiac neural crest and heart genes isolated by modified differential display. Gene Expression Patterns 3, 407–411.

Massari, M., Murre, C., 2000. Helix–loop–helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell Biol. 20, 429–440 (Jan).

Mayor, R., Aybar, M.J., 2001. Induction and development of neural crest in *Xenopus laevis*. Cell Tissue Res. 305, 203–209.

Mayor, R., Essex, L.J., Bennett, M.F., Sargent, M.G., 1993. Distinct elements of the xsna promoter are required for mesodermal and ectodermal expression. Development 119, 661–671.

Mayor, R., Morgan, R., Sargent, M.G., 1995. Induction of the prospective neural crest of *Xenopus*. Development 121, 767–777.

Mayor, R., Young, R., Vargas, A., 1999. Development of Neural Crest in *Xenopus*. Current Topics in Developmental Biology, vol. 43. Academic Press, San Diego, pp. 85–113.

Mjaatvedt, C.H., Nakaoka, T., Moreno-Rodriguez, R., Norris, R.A., Kern, M.J., Eisenberg, C.A., Turner, D., Markwald, R.R., 2001. The outflow tract of the heart is recruited from a novel heart-forming field. Dev. Biol. 238, 97–109.

Mohun, T.J., Leong, L.M., Weninger, W.J., Sparrow, D.B., 2000. The morphology of heart development in *Xenopus laevis*. Dev. Biol. 218, 74–88.

Montero, J.A., Giron, B., Arrechedera, H., Cheng, Y.-C., Scotting, P., Chimal-Monroy, J., Garcia-Perrero, J.A., Hurle, J.M., 2002. Expression of Sox8, Sox9 and Sox10 in the developing valves and autonomic nerves of the embryonic heart. Mech. Dev. 118, 199–202.

Nieuwkoop, P.D., Faber, J., 1967. Normal Table of *Xenopus laevis* (Daudin) North Holland, Amsterdam.

Nishibatake, M., Kirby, M.L., Van Mierop, L.H., 1987. Pathogenesis of persistent truncus arteriosus and dextroposed aorta in the chick embryo after neural crest ablation. Circulation 75, 255–264.

Olson, E.N., Srivastava, D., 1996. Molecular pathways controlling heart development. Science 272, 671–676.

Poelmann, R.E., Gittenberger-de Groot, A.C., 1999. A subpopulation of apoptosis-prone cardiac neural crest cells targets to the venous pole: multiple functions in heart development? Dev. Biol. 207, 271–286.

Poelmann, R.E., Mikawa, T., Gittenberger-de Groot, A.C., 1998. Neural crest cells in outflow tract septation of the embryonic chicken heart: differentiation and apoptosis. Dev. Dyn. 212, 373–384.

Qayyum, S.R., Webb, S., Anderson, R.H., Verbeek, F.J., Brown, N.A., Richardson, M.K., 2001. Septation and valvar formation in the outflow tract of the embryonic chick heart. Anat. Rec. 264, 273–283.

Sato, M., Yost, J.H., 2003. Cardiac neural crest contributes to cardiomyogenesis in zebrafish. Dev. Biol. 257, 127–139.

Sive, H.L., Grainger, R.M., Harland, R.M., 1995. Early Development of *Xenopus laevis*: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Srivastava, D., 1999. HAND proteins: molecular mediators of cardiac development and congenital heart disease. Trends Cardiovasc. Med. 9, 11–18.

Srivastava, D., Cserjesi, P., Olson, E., 1995. A subclass of bHLH proteins required for cardiac morphogenesis. Science 270, 1995–1999.

Verberne, M.E., Gittenberger-de Groot, A.C., Poelmann, R.E., 1998. Lineage and development of the parasympathetic nervous system of the embryonic chick heart. Anat. Embryol. (Berlin) 198, 171–184.

Verberne, M.E., Gittenberger-de Groot, A.C., van Iperen, L., Poelmann, R.E., 2000. Distribution of different regions of cardiac neural crest in the extrinsic and the intrinsic cardiac nervous system. Dev. Dyn. 217, 191–204.

Waldo, K.L., Lo, C.W., Kirby, M.L., 1999. Connexin 43 expression reflects neural crest patterns during cardiovascular development. Dev. Biol. 208, 307–323.

Waldo, K.L., Kumiski, D.H., Wallis, K.T., Stadt, H.A., Hutson, M.R., Platt, D.H., Kirby, M.L., 2001. Conotruncal myocardium arises from a secondary heart field. Development 128, 3179–3188.

Wilkinson, D.G., 1992. Whole mount in situ hybridization of vertebrate embryos. In: Wilkinson, D.G. (Ed.), In Situ Hybridization. IRL Press, Oxford.

Wilkinson, D.G., Nieto, A., 1993. Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol. 225, 361–373.

Wilson, R., Mohun, T., 1995. XIdx, A dominant negative regulator of bHLH function in early *Xenopus* embryos. Mech. Dev. 49, 211–222.

Yelbuz, T.M., Waldo, K.L., Kumiski, D.H., Stadt, H.A., Wolfe, R.R., Leatherbury, L., Kirby, M.L., 2002. Shortened outflow tract leads to altered cardiac looping after neural crest ablation. Circulation 106, 504–510.

Yutzey, K.E., Kirby, M.L., 2002. Wherefore heart thou? Embryonic origins of cardiogenic mesoderm. Dev. Dyn. 223, 307–320.

Zhang, H., Reynaud, S., Kloc, M., Etkin, L.D., Spohr, G., 1995. ID gene activity during *Xenopus* embryogenesis. Mech. Dev. 50, 119–130.