Development 128, 3071-3080 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV3393

PlexinA2 and semaphorin signaling during cardiac neural crest development

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Accepted 30 March 2001

SUMMARY

Classic studies using avian model systems have demonstrated that cardiac neural crest cells are required for proper development of the cardiovascular system. Environmental influences that perturb neural crest development cause congenital heart defects in laboratory animals and in man. However, little progress has been made in determining molecular programs specifically regulating cardiac neural crest migration and function. Only recently have complex transgenic tools become available that confirm the presence of cardiac neural crest cells in the mammalian heart. These studies have relied upon the use of transgenic mouse lines and fate-mapping studies using Cre recombinase and neural crest-specific promoters. In this study, we use these techniques to demonstrate that *PlexinA2* is expressed by migrating and postmigratory cardiac neural crest cells in the mouse. Plexins function as co-receptors for semaphorin signaling molecules and mediate axon pathfinding in the central nervous system. We demonstrate that *PlexinA2*-expressing cardiac neural crest cells are patterned abnormally in several mutant mouse lines with congenital heart disease including those lacking the secreted signaling molecule Semaphorin 3C. These data suggest a parallel between the function of semaphorin signaling in the central nervous system and in the patterning of cardiac neural crest in the periphery.

Key words: Neural crest, Heart, Semaphorin, Plexin, Mouse

INTRODUCTION

The importance of neural crest cells during cardiac development has been appreciated for almost two decades (Creazzo et al., 1998; Kirby et al., 1983). Kirby and co-workers, using ablated chick embryos and quail-chick chimeras, demonstrated that neural crest cells migrate to the developing conotruncus and participate in septation of the outflow tract (Kirby et al., 1983). Ablation of premigratory cardiac neural crest cells results in predictable congenital cardiac abnormalities involving the outflow tract and great vessels including persistent truncus arteriosus and double outlet right ventricle (Kirby et al., 1985). Cardiac neural crest cell migration has been extensively documented in avian species. During cardiogenesis, neural crest cells surround aortic arches 3, 4 and 6 and contribute to the spiral septum forming between the ascending aorta and the proximal pulmonary artery (Kirby and Waldo, 1995). Neural crest cells contribute to the endocardial cushions of the outflow tract that will develop into the aortic and pulmonary valves where they may participate in inductive interactions with cushion mesenchyme. This mesenchyme is derived from epithelial-mesenchymal transformation of adjacent endothelial cells.

Cardiac neural crest also is likely to mediate, through unknown mechanisms, the complex pattern of regression and persistence of right and left sided aortic arch segments that allows for remodeling of the great vessels and the production of the adult asymmetric vasculature. Little is known about how cardiac neural crest cells mediate pathfinding and are constrained to appropriate migratory pathways. Surprisingly, few molecular markers identify post-migratory crest cells in the heart providing few candidates for mediating these cellular functions.

In mammals, a critical role for neural crest during cardiac development has been implicated by analogy to avian models (Waldo et al., 1999) and by the description of several spontaneous or engineered mutations in genes expressed by neural crest cells that give rise to congenital heart disorders affecting the outflow tract and great vessels. These genes are generally expressed by premigratory or migrating cardiac neural crest, or in tissues adjacent to migration pathways. Examples include *Pax3* (Epstein, 1996), Endothelin receptor A (*Ednra*; Yanagisawa et al., 1998), Neurofibromatosis type 1 (*Nf1*; Brannan et al., 1994; Jacks et al., 1994), Connexin 43 (*Cx43*; also called *Gja1*; Lo et al., 1999), *Foxc1* and *Foxc2* (formerly *Mf1* and *Mfh1*; Iida et al., 1997; Winnier et al., 1999). However, mutations in several of these genes affect multiple aspects of cardiovascular development and specific expression by postmigratory neural crest cells in the heart has not been clearly documented for any of these genes. The roles that they play in neural crest migration, differentiation and survival remain unclear. In fact, despite the description of these mutant

phenotypes, the presence of neural crest cells in the mammalian heart remained undocumented until very recently (Jiang et al., 2000; Li et al., 2000; Waldo et al., 1999).

The first evidence that neural crest cells populate the mammalian heart, in a pattern similar to that seen in avians, came from observations of β-galactosidase expression in transgenic mice expressing *lacZ* from a 6.5 kb proximal upstream region of the *Cx43* gene (Lo et al., 1997). Expression was noted in the dorsal neural tube and neural crest derivatives including dorsal root ganglia. Labeled cells were identified in the 3rd, 4th and 6th pharyngeal arches and in the outflow tract of the heart. The pattern of expression in the heart was remarkably similar to the distribution of neural crest cells in chick embryos suggesting, by analogy, that this transgene labels mammalian cardiac neural crest cells. Of note, *Cx43* itself is expressed widely throughout the developing heart and is not restricted to neural crest, indicating that important regulatory elements mediating *Cx43* expression lie outside the 6.5 kb proximal upstream region used to create this transgene (Waldo et al., 1999).

More recently, several groups have performed fate-mapping experiments in the mouse using tissue-specific transgenes and Cre-lox technology. Promoter elements from the *P0*, *Wnt1* and *Pax3* (Jiang et al., 2000; Li et al., 2000; Yamauchi et al., 1999) genes that direct expression to pre-migratory neural crest cells were used to direct expression of Cre recombinase. These transgenic mice were crossed to Cre-reporter mice in which expression of β-galactosidase occurs only in cells that express Cre (Soriano, 1999; Tsien et al., 1996). Since the activation of *lacZ* expression involves a somatic cell genomic rearrangement, cells that express the Cre transgene are indelibly labeled for their lifetime, as are all descendants of those cells. These studies have identified P0, Wnt1 and Pax3 derivatives in the developing heart with apparently similar, but not identical, patterns of expression. These patterns are generally similar to neural crest patterning in avian hearts, suggesting that most or all labeled cells are neural crest in origin. However, it should be emphasized that P0, Wnt1 and Pax3 themselves are not expressed by neural crest cells in the heart, and the use of the Cre-lox labeling method to track neural crest cells in various mutant backgrounds is cumbersome and expensive. Nevertheless, these studies confirm that in mammals, as in birds, cardiac neural crest cells populate the outflow tract of the heart suggesting that cell-type-specific expression of genetic programs exist that mediate neural crest function during cardiac morphogenesis.

We demonstrate that PlexinA2 is expressed by migrating and postmigratory cardiac neural crest cells in the mouse heart. PlexinA2 (Kameyama et al., 1996) is a member of a large family of receptors that recognize secreted semaphorin signaling molecules involved in axon guidance and growth cone collapse in the central nervous system (Tamagnone et al., 1999). We confirm the specificity of PlexinA2 as a cardiac neural crest marker by comparison with available transgenic lines, and we compare and contrast the available labeling techniques for cardiac neural crest. In light of recent data that semaphorin 3C (*Sema3C*) is required for normal development of the aortic arches and for septation of the cardiac outflow tract (see Feiner et al., 2001), we have examined the expression of *PlexinA2* in relation to *Sema3C*, and in *Sema3C*-deficient embryos and in other mouse models with cardiac neural crest defects (Feiner et al., 2001). We document that *PlexinA2*-expressing cardiac neural crest is patterned abnormally in these mutant embryos. These data suggest a model in which PlexinA2 plays a functional role in cardiac neural crest migration by acting as a co-receptor for class 3 semaphorin signaling molecules.

MATERIALS AND METHODS

In situ hybridization

Embryos for whole-mount in situ hybridization were harvested and fixed for 48 hours in 4% paraformaldehyde (PFA) in PBS. Embryos were dehydrated through a graded methanol series and stored in 100% methanol at −20°C. Prior to analysis the chest wall and heads were removed from E12.5 embryos to facilitate probe penetrance and visualization of the heart. Whole-mount in situ hybridization was performed as previously described (Borycki et al., 1999). PlexinA2 probes encompass nucleotides 2121-4330 of the GenBank mouse *PlexinA2* sequence D86949. Bound probe was detected with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular, Indianapolis, IN) and NBT/BCIP substrate. Embryos were postfixed 2 hours in 4% PFA. Some embryos were cleared for better visualization of signal by graded glycerol/PBS series to 80% final glycerol concentration. Sense riboprobes produced no signal. Whole-mount images were digitally captured on an Leica MZ6 stereomicroscope.

For in situ hybridization on paraffin sections, embryos were fixed as above, dehydrated in a graded ethanol series, paraffin embedded and sectioned at 10 μm. Concentrations for digoxigenin riboprobes were determined empirically by serial dilution in control experiments before use. Sections were counterstained with nuclear fast red and mounted in Vecta Mount (Vector laboratories, Burlingame, CA).

For radioactive in situ hybridizations, ^{35}S-labeled sense and antisense riboprobes were synthesized with SP6, T7 or T3 RNA polymerase and ^{35}S-UTP as previously described (Lutz et al., 1994). Hybridization was carried out at 55°C overnight. Successful hybridzation was assessed by overnight exposure of slides to Kodak X-OMAT film. Slides were dipped in Kodak NTB-2 emulsion, exposed for 5-7 days a 4°C, developed and fixed in Kodak Dektol developer and fixer. Cell nuclei were counterstained with Hoechst 33258 (Sigma, St. Louis, MO) and mounted in Canada balsam/methyl salicylate. Sections were digitally photographed on a Zeiss Axioplan 2 microscope. Images were processed with Adobe Photoshop.

Transgenic and mutant mice

Splotch mice were obtained from the Jackson Labs and maintained on a C57/Bl6 inbred background. *Cx43-lacZ* transgenic mice were obtained from Dr Cecilia Lo (Lo et al., 1997). *Cx43-lacZ* mice are a stable transgenic line expressing a nuclear β-galactosidase from a 6.5 kb Cx43 promoter in neural crest cells. *P3pro*-Cre mice were generated by our laboratory (Li et al., 2000). *P3pro*-Cre consists of a 1.6 kb proximal Pax3 promoter upstream of Cre recombinase. *Wnt1*-Cre mice were obtained from Dr Andrew P. McMahon (Danielian et al., 1998). The Rosa reporter mice, R26R, were obtained from Philippe Soriano (Soriano, 1999). *Sema3C*$^{+/-}$ mice were maintained on a CD1 background (Feiner et al., 2001).

X-gal staining

Embryos expressing β-galactosidase transgenes were harvested into cold PBS, and fixed for 2 hours in 2% PFA. To optimize tissue fixation and penetrance of X-gal substrate (Roche Molecular, Indianapolis, IN), the chest wall was opened before fixation and in some cases the heart was removed and incubated in color substrate. Embryos were incubated in X-gal substrate (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% NP-40, 0.1%deoxycholate, 0.1% X-gal in PBS)

at 37°C. For high resolution analysis of β-galactosidase expression patterns, embryos were paraffin embedded, sectioned and counterstained with nuclear Fast Red.

RESULTS

Expression of semaphorin receptor genes during cardiac outflow tract septation

Semaphorin signaling pathways have recently been intensively investigated because of important functions in axon patterning in the central nervous system. Our results, described elsewhere in this issue, have demonstrated additional functions for secreted class 3 semaphorin signals in development of the cardiovascular system (Feiner et al., 2001). The findings of aortic arch interruptions and failure of septation of the truncus arteriosus into aorta and pulmonary artery in *Sema3C*-deficient embryos suggested to us that a functional semaphorin receptor might be expressed by migrating cardiac neural crest cells. This receptor would be capable of mediating Sema3C signals secreted from adjacent tissues and could perhaps mediate cardiac neural crest pathfinding in a manner analogous to axon pathfinding in the CNS.

We investigated the expression of potential Sema3C receptor components during mid gestation when cardiac neural crest migrates to and populates the heart. Class 3 semaphorin receptors are composed of heteromultimers including neuropilin and class A plexin subunits (Tamagnone and Comoglio, 2000). Fig. 1 shows the expression of candidate receptor subunits by in situ hybridization at E12.5 in the neural tube, the aortic arches and the outflow tract of the heart. *PlexinA2* (Fig. 1A-C) is expressed in the dorsal neural tube (arrow, A) and along the aortic arches (B) with a continuous stream of expressing cells extending into the outflow tract of the heart (arrow, C). This pattern suggests expression by cardiac neural crest. *PlexinA1* (Fig. 1D-F) is expressed at low levels or not at all at E12.5. *PlexinA3* (Fig. 1G-I) is expressed in the ventricular zone of the neural tube (G) and diffusely throughout the pharyngeal arch mesenchyme (H) and adjacent to the aortic and pulmonary trunks (I). *Neuropilin 1* (*Nrp1*, Fig. 1J-L) is expressed in the ventral neural tube (J) and in the endothelium and surrounding mesenchyme of the aortic arches (K,L) while *neuropilin 2* (*Nrp2*, Fig. 1M-O) is expressed in the dorsal and ventral neural tube (excluding the ventricular zone, M) and in the mesenchyme surrounding the ascending aorta (N,O). In summary, *PlexinA2* displayed a pattern of expression most consistent with cardiac neural crest, while *PlexinA3*, *Nrp1*, and *Nrp2* had partially overlapping expression patterns consistent with possible functions in cardiac neural crest and/or in adjacent tissues.

In light of these results, we focused our attention on *PlexinA2* during cardiovascular development. Fig. 2A shows a whole-mount in situ hybridization of an E11.5 embryo revealing two prongs of *PlexinA2*-expressing cells within the

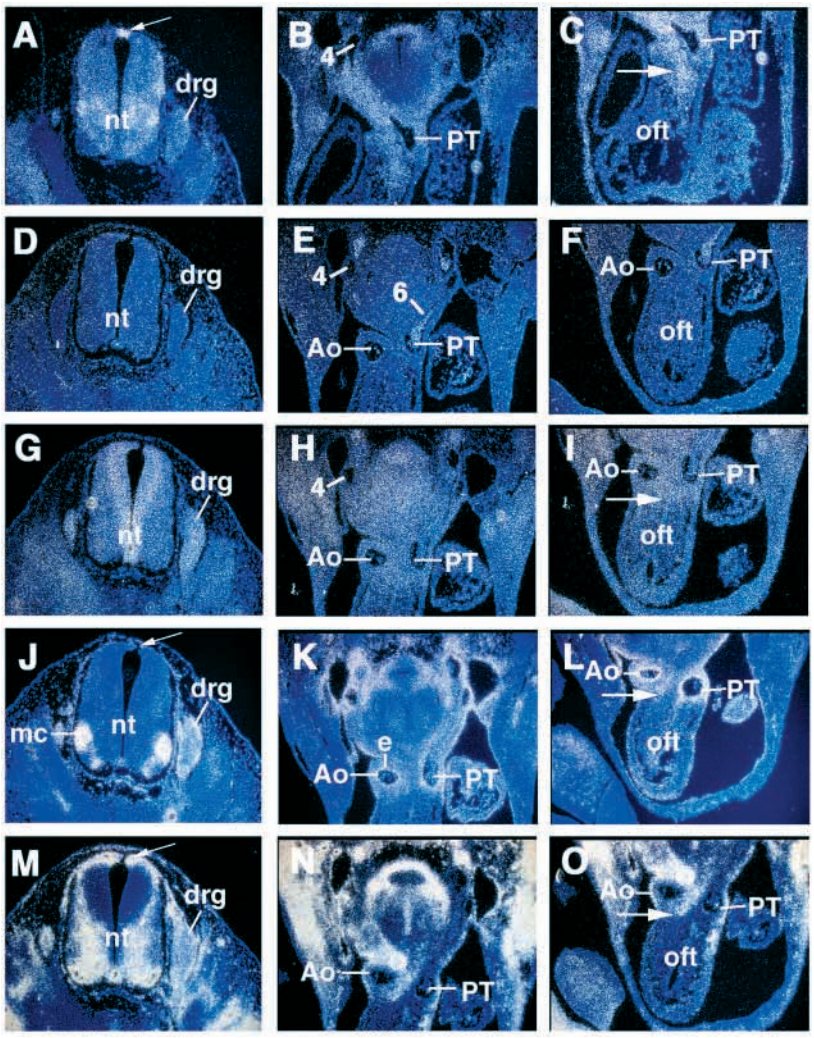

Fig. 1. Expression of potential Sema3C receptor components in mouse embryos at E12.5. Radioactive in situ hybridization analysis of transverse section through the neural tube (nt, panels A,D,G,J,M), aortic arches (B,E,H,K,N) and cardiac outflow tract (oft, panels C,F,I,L,O) is shown. The aortic arch arteries are numbered 4 and 6. (A-C) *PlexinA2* is expressed at high levels in the roof plate of the neural tube (arrow, A) and throughout the ventral neural tube excluding the ventral horns. Expression is also observed in the dorsal root ganglia (drg, A) and adjacent to the pulmonary trunk (PT) and aortic arch arteries (B). (C) A column of expressing cells is seen extending into the outflow tract (oft) endocardial cushions consistent with expression by migrating cardiac neural crest (arrow). (D-F) *PlexinA1* is expressed diffusely at levels barely above background. (G-I) *PlexinA*3 is expressed in the medial region of the neural tube and dorsal root ganglia (G) and broadly throughout the pharyngeal mesenchyme (H) and the mesenchyme surrounding the aorta (Ao) and pulmonary trunk (I). (J-L) *Nrp1* is expressed at high levels in the motor columns (mc) of the neural tube and in the dorsal root ganglia (drgs) (J). *Nrp1* expression overlaps *PlexinA2* expression in the aortic arch mesenchyme (K) and adjacent to the aorta and pulmonary artery (L), but is also seen in the endothelial cells (e) of developing vessels (K). (M-O) *Nrp2* expression is observed in the roof plate (arrow, M) and in the neural tube and drgs. *Nrp2* is expressed by the mesenchyme surrounding the aorta (N), and extending into the outflow tract (arrow, O).

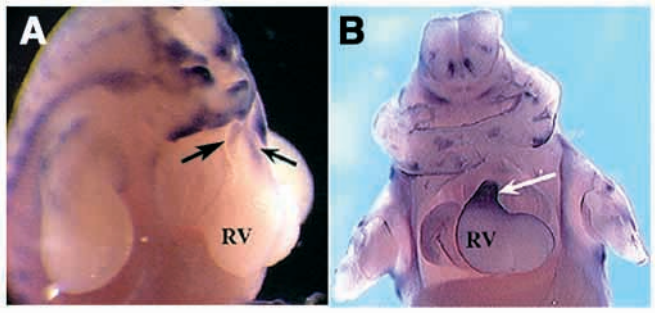

Fig. 2. *PlexinA2* (E11.5) and *Sema3C* (E12) expression in the cardiac outflow tract. Whole-mount in situ hybridization was performed to examine *PlexinA2* (A) and *Sema3C* (B) expression. *PlexinA2* can be seen in two prongs of cells extending into the septating outflow tract (A, black arrows) rostral to the right ventricle (RV). *Sema3C* is expressed in the myocardium of the outflow tract (white arrow, B).

outflow tract of the heart (arrows, Fig. 2A). In avians, two prongs of neural crest cells invade the truncus arteriosus in a similar pattern (Waldo et al., 1998). By whole-mount in situ hybridization, this pattern of expression is distinct from that seen with *Sema3C* (Fig. 2B) which is expressed by the myocardium of the outflow tract adjacent to the outflow endocardial cushions (see below). Since *Sema3C* encodes a secreted ligand, and *Sema3C* mutant embryos have cardiovascular abnormalities suggestive of neural crest-related defects, this complementary pattern of expression further suggested that PlexinA2 might serve as a receptor component on neural crest cells.

PlexinA2 is expressed by cardiac neural crest cells

The pattern of cardiac gene expression shown in Fig. 2A has, to our knowledge, not been previously documented in mammals. While the pattern is reminiscent of neural crest population of the heart in birds, no other specific molecular markers for postmigratory cardiac neural crest have been available in mice. *Pax3*, *CRABP* and others have been suggested previously to label these cells (Conway et al., 1997b), but our studies have found these markers to be extinguished prior to neural crest invasion of the cardiac region, or to be expressed by other cells types (Epstein et al.,

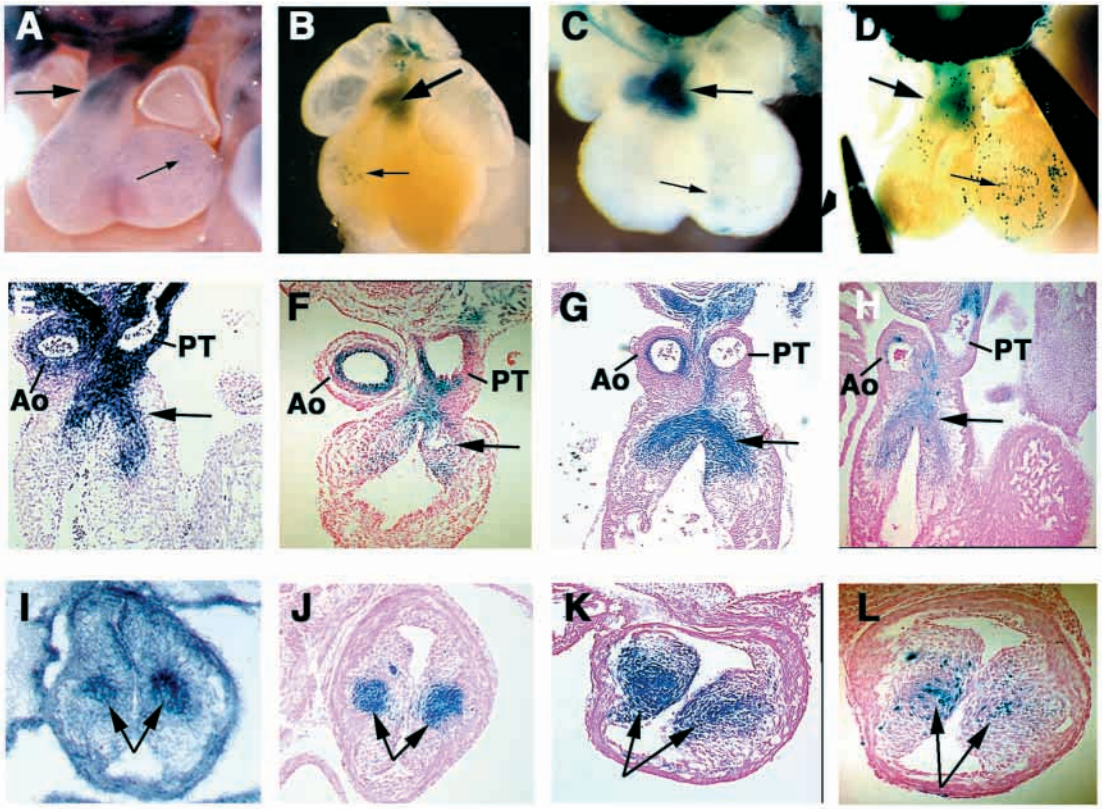

Fig. 3. Comparison of *PlexinA2* expression to fate-mapping and transgenic markers of cardiac neural crest at E12.5. *PlexinA2* expression was analyzed by whole-mount (A) or section in situ hybridization (E,I). For comparison, cardiac neural crest cells were fate mapped using the *Pax3* promoter to drive expression of Cre recombinase and crossing with Cre-reporter mice (B,F,J) or using the *Wnt1* promoter to direct Cre expression (C,G,K). Cardiac neural crest was also labeled with the *Cx43-lacZ* transgene (D,H,L). *PlexinA2* is expressed by two prongs of cells invading the cardiac outflow tract (large arrow, A) in a pattern that resembles that of cardiac neural crest (large arrows, B-D). In frontal sections (E-H), *PlexinA2* and cardiac neural crest are located surrounding the aorta (Ao) and pulmonary trunk (PT) and form a 'saddle' within the outflow endocardial cushions (arrows, E-H). In cross section, *PlexinA2* expression is localized to two clusters of cells within the endocardial cushions (arrows, I). Similar populations of cells are observed within the conotruncal cushions of *Pax3*promoter-Cre (J), *Wnt1*-cre (K), and *Cx43-lacZ* (L) hearts (black arrows). The extent of labeling within the conotruncal mesenchyme is variable in the different mouse lines; *Pax3*promoter-Cre expression (J) is most similar to *PlexinA2* (I). Note that variable numbers of labeled cells are observed within the ventricles of all of these mouse lines (A-D small arrows).

2000). Therefore, we sought to compare the expression of *PlexinA2* in the heart using recently developed transgenic mice that allow for labeling of cardiac neural crest by fate mapping techniques.

Fig. 3 shows a comparison of in situ hybridization for *PlexinA2* at E12.5 (A,E,I) with X-gal stained E12.5 hearts revealing Pax3-expressing neural crest derivatives (B,F,J), Wnt1-expressing derivatives (C,G,K), and cells expressing the 6.5 kb Cx43-promoter-*lacZ* transgene (D,H,L). Fate mapping of Pax3-expressing neural crest precursors relies on the ability of a neural crest-specific element in the Pax3 promoter to direct expression of Cre recombinase in premigratory neural crest cells (Li et al., 2000). Crossing these transgenic mice to Cre-reporter mice results in β-galactosidase expression by neural crest derivatives. A similar approach is used to fate map Wnt1-expressing neural crest percursors (Jiang et al., 2000). The use of *Cx43-lacZ* transgenic mice relies upon the persistent expression of this transgene in neural crest derivatives (Waldo et al., 1999). In all cases, streams of labeled cells can be seen to invade the outflow tract and to reach the level of the outflow endocardial cushions (large arrows, Fig. 3A-D). No labeled cells were seen using any of these techniques, within the atrioventricular endocardial cushions (Fig. 3 and data not shown). However, variable and scattered labeling of cells within the myocardium of either or both ventricles was seen in all cases (arrowheads, Fig. 3A-D). Whether these cells represent neural crest derivatives rather than ectopic expression of the various transgenes remains to be determined, though neural crest derivatives within the myocardium have been noted in other vertebrates (M. Kirby and J. Yost, personal communication).

Examination of *PlexinA2* expression reveals strikingly similar patterns of expression compared to transgenic labeling of neural crest in frontal (Fig. 3E-H) and cross (I-L) sections. In all cases, labeled cells were noted adjacent to the proximal pulmonary artery and aorta and formed a ‘saddle’ within the mesenchymal portion of the outflow endocardial cushions (arrows, E-H). In cross section, the lower extremes of this ‘saddle’ appeared as distinct clusters of labeled cells within the endocardial cushions (I-L). In slightly more rostral sections, these two clusters merged to form the septum between the aorta and pulmonary arteries.

PlexinA2 expression within the endocardial cushions (Fig. 3I) most closely resembled the pattern of *Pax3* derivatives (Fig. 3J), while *Wnt1* derivatives appeared to occupy a larger portion of the cushion mesenchyme (Fig. 3K). This is consistent with earlier reports (Jiang et al., 2000), although these differences were at the time attributed to different sectioning techniques. Whether this difference represents labeling of different populations of cardiac neural crest by these various systems, or whether ectopic transgene expression accounts for the broader *Wnt1* expression domain remains to be determined. In avians, fate mapping studies have yielded results more consistent with the condensed populations of cardiac neural crest revealed by *PlexinA2* expression (Fig. 3I) and seen in the *Pax3* fate-mapping studies (Fig. 3J; Waldo et al., 1998). Together, these studies confirm that *PlexinA2* is expressed by postmigratory cardiac neural crest.

Earlier expression of *PlexinA2* is also consistent with a role for it in cardiac neural crest migration and function. *PlexinA2* expression was detected as early as E8.5 in the neural folds prior to closure of the neural tube (Fig. 4A), although expression was not localized to dorsal neural tube at this timepoint. Shortly thereafter, by E9.5, intense expression was noted in the dorsal neural tube at the level of the first three somites, the region from which cardiac neural crest emerges (arrow, Fig. 4B). Expression in the roof plate persisted through E10.5 (arrow, Fig. 4C) and broad signal was detected in the mesenchyme of the branchial arches surrounding the aortic arches (arrows, Fig. 4D and data not shown). This is the region populated by migrating cardiac neural crest. At E10.5, expression was first noted in the outflow tract of the heart (arrow, Fig. 4E), consistent with our recent identification of cardiac neural crest cells arriving in this location at this stage of development (Epstein et al., 2000). At E11.5, *PlexinA2* expression persists in tissue surrounding the forming aorta and pulmonary trunks and in the outflow tract (arrow, Fig. 4F). Expression is also first noted in the myocardium of the left ventricle. These data suggest that in addition to other expression domains, *PlexinA2* is expressed by pre- and

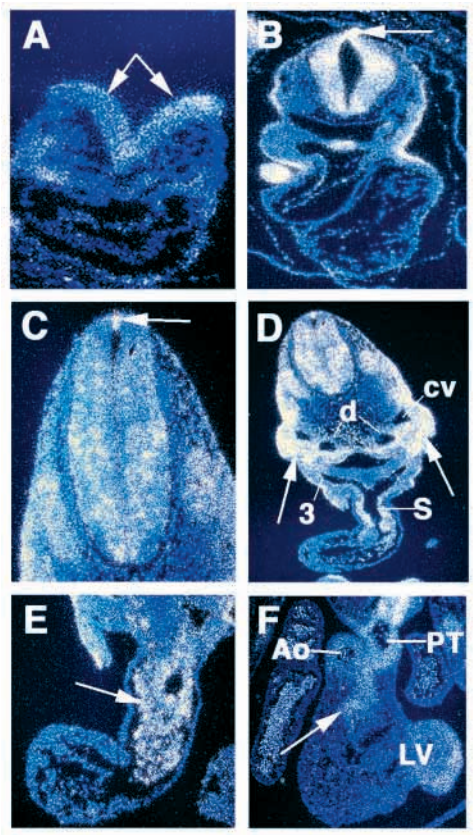

Fig. 4. *PlexinA2* expression during cardiac neural crest migration. *PlexinA2* can be detected by radioactive in situ hybridization in the neural tube as early as day E8.5 (A, white arrows) before neural tube closure. High levels of *PlexinA2* expression are noted in the roof plate of the neural tube at E9.5 (arrow, B) and at E10.5 (arrow, C). Extensive expression is observed throughout the pharyngeal arches (arrows D), and surrounding the aortic arch arteries at E10.5. A large population of *PlexinA2*-expressing cells is seen extending into the conotruncus (arrow, E). At E11.5, *PlexinA2*-expressing cells can be seen surrounding the aorta (Ao) and pulmonary trunk (PT), and extending into the outflow tract mesenchyme (arrow, F). *PlexinA2* expression in the left ventricle (LV) is first seen at E11.5 (F). d, dorsal aorta. S, aortic sac. cv, cardinal vein. 3, aortic arch artery 3.

postmigratory cardiac neural crest cells during mid gestation, when septation of the outflow tract and repatterning of the aortic arches is taking place.

PlexinA2 and *Sema3C* are expressed in distinct and overlapping domains

Since *Sema3C* deficiency leads to cardiovascular abnormalities attributable to neural crest defects (see Feiner et al., 2001), and since plexins can function as components of semaphorin receptors, we examined the relative expression of *Sema3C* and *PlexinA2* during cardiac development. A careful analysis of *PlexinA2* and *Sema3C* expression in adjacent sections of mid gestation embryos reveals a striking complementary pattern in many tissues, while expression overlaps in some important areas. Most notably, both the secreted ligand *Sema3C* and the putative receptor component *PlexinA2* are expressed in the roof plate of the neural tube (large arrows, Fig. 5A,B,G,H). Both genes are also expressed in the condensed mesenchyme of the cardiac outflow tract, a region populated by neural crest (arrows, Fig. 5B,H). However, *Sema3C* is expressed somewhat more broadly in this region (Fig. 5H-J) such that only a subset of the *Sema3C* expression domain overlaps with that of *PlexinA2* in this tissue (Fig. 5I,J). Elsewhere in the neural tube, heart and great vessels the pattern of expression is reciprocal. For instance, *PlexinA2* is expressed in the neural tube excluding the ventral horns (Fig. 5A,B), while *Sema3C* is restricted to the ventral horns (Fig. 5G,H). *PlexinA2* surrounds the ductus arteriosus (Fig. 5A) and dorsal aortae (Fig. 5A,B) while *Sema3C* is in the myocardium of the outflow tract and in the proximal pulmonary trunk (Fig. 5G). This reciprocal pattern of expression is seen in other tissues including the intestine, where *PlexinA2* is present in smooth muscle (Fig. 5C) while *Sema3C* is expressed by intestinal epithelium (Fig. 5D). Similarly, in the lungs, *PlexinA2* is expressed in the bronchial smooth muscle (Fig. 5E) and *Sema3C* is in the underlying endothelium (Fig. 5F). In summary, *PlexinA2* and *Sema3C* are both expressed in the roof plate of the neural tube and in the condensed mesenchyme of the cardiac outflow endocardial cushions. Expression is complementary and in opposing tissues in the conotruncal region and in many other tissues including neural tube, lung and intestine.

PlexinA2 expression in *Sema3C*$^{-/-}$ embryos

Sema3C$^{-/-}$ embryos have conotruncal cardiac defects and interruptions of the aortic arch suggestive of cardiac neural crest defects (Feiner et al., 2001). We determined the expression of *PlexinA2* in wild-type and *Sema3C*$^{-/-}$ littermates

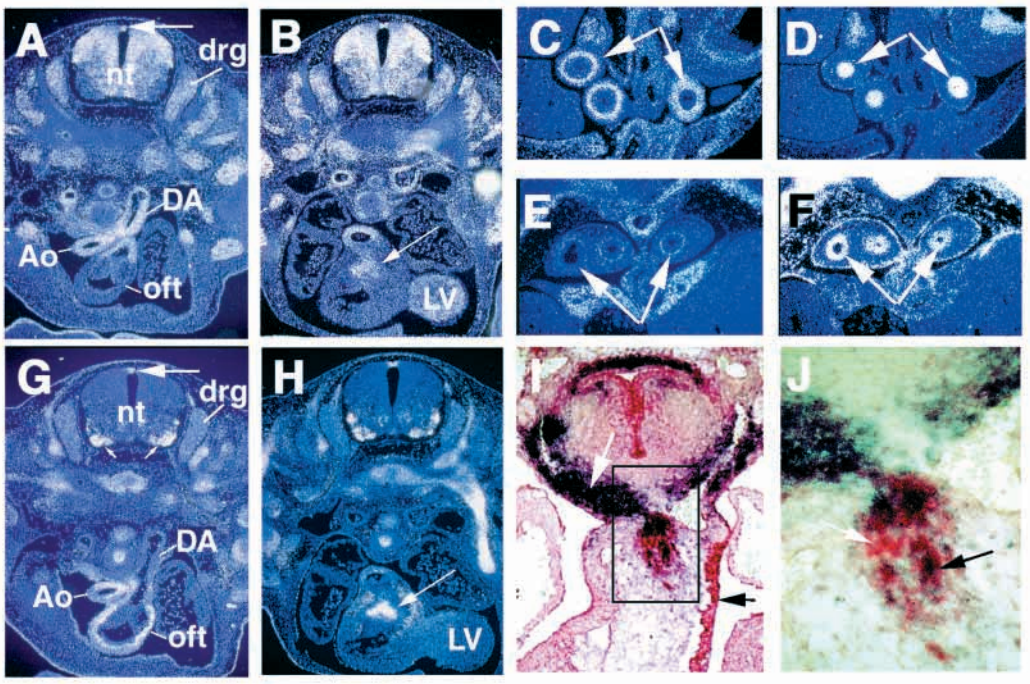

Fig. 5. Complementary and overlapping expression domains of *PlexinA2* and *Sema3C*. *PlexinA2* and *Sema3C* expression patterns were determined by radioactive in situ hybridization (A-H) in E12.5 embryos, and by double label non-radioactive in situ hybridization at E11.5 (I, J). In the neural tube at E12.5, *PlexinA2* (A) and *Sema3C* (G) are both expressed in the roof plate (arrows, A,G) and the dorsal root ganglia (drg, in A,G). In the outflow region of the heart, both genes are expressed around the aorta (Ao, in A,G). Both *PlexinA2* (B) and *Sema3C* (H) are expressed in an overlapping population of cells in the condensed mesenchyme of the outflow tract where neural crest cells reside (B,H white arrows). However, complementary expression is observed elsewhere. *PlexinA2* is expressed in the ductus arteriosus (DA, in A) while *Sema3C* is expressed in the myocardial cuff of the right ventricular outflow tract (oft, in G) and in the proximal pulmonary trunk. In the neural tube (nt), *PlexinA2* expression excludes the ventral horns (A), where *Sema3C* is expressed (small arrows, G). Reciprocal expression domains are observed in the intestines (arrows, C (*PlexinA2*), D (*Sema3C*)) and in the lung (arrows, E (*PlexinA2*), F (*Sema3C*)). Double label in situ hybridization of E11.5 embryos (I,J) reveals overlapping but not identical expression of *Sema3C* (red), and *PlexinA2* (purple) in the aortic arch mesenchyme (white arrow, I) and outflow tract (boxed region, I). *Sema3C* is expressed in the inner curvature of the myocardial wall of the outflow tract (I, black arrow). A higher magnification (J) of the outflow tract (boxed region in I) demonstrates cells that express both *Sema3C* and *PlexinA2* (black arrow), as well as cells that only express *Sema3C* (white arrow). LV, left ventricle.

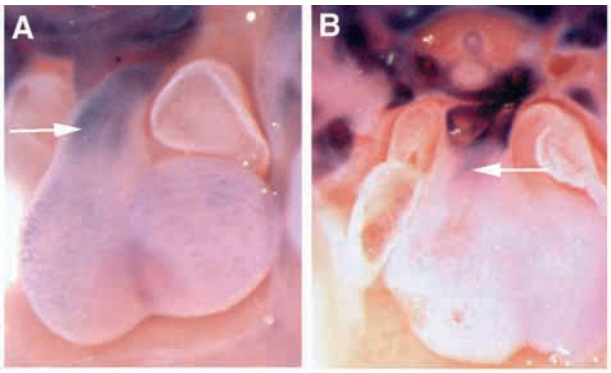

Fig. 6. Altered *PlexinA2* expression in *Sema3C* null embryos. *PlexinA2* expression was compared by whole-mount (A,B) in situ hybridization at E12.5. Prongs of *PlexinA2*-expressing cells observed in wild-type hearts (arrow, A) are reduced in *Sema3C* null embryos (arrow, B). *PlexinA2* expression surrounding the aorta and pulmonary trunk is decreased in *Sema3C* null embryos compared to control.

during the critical time points of conotruncal septation. In mutant embryos at E12.5, *PlexinA2*-expressing cells were seen migrating along the aortic arches and populating the outflow tract of the heart (Fig. 6B). This result indicates that *Sema3C* is not required for neural crest migration to the cardiac region. However, within the conus, *PlexinA2* patterning was different in *Sema3C* mutants compared with controls. *PlexinA2*-expressing cells did not migrate as far distally within the outflow mesenchyme in mutant embryos and did not form the localized clusters of condensed mesenchyme (Fig. 6, see also Fig. 5 in Feiner et al., 2001). Expression within the endocardial cushions was reduced and more diffuse in mutant embryos. It is worth noting that this area of abundant *PlexinA2* expression is directly adjacent to the normal *Sema3C* expression domain in the myocardial cuff of the outflow tract (Fig. 2B), a signal that is missing in *Sema3C*$^{-/-}$ embryos. These results suggest that Sema3C/PlexinA2 signaling may function to modulate final positioning of neural crest cells within the outflow tract while other pathways affect more proximal regions of cardiac neural crest migration.

PlexinA2 expression in *Pax3*-deficient *Splotch* embryos

We also determined the location of *PlexinA2*-expressing neural crest cells in another model with cardiac conotruncal defects. *Pax3* is a transcription factor expressed by pre-migratory neural crest cells in the dorsal neural tube. Mutations in the *Pax3* locus result in the *Splotch* mouse which exhibits failure of outflow tract septation and other neural crest defects (Auerbach, 1954; Franz, 1989). A poorly developed myocardium with a thinned ventricular wall and ventricular septal defects are also present. *Pax3* is also expressed in limb muscle progenitors where it functions to regulate hypaxial myoblast migration at least partially by directly regulating the transcription of the c-*Met* gene encoding a tyrosine kinase receptor that is required for myoblast migration (Bladt et al., 1995; Bober et al., 1994; Daston et al., 1996; Epstein, 1996). *c*-Met is a member of the Plexin family (Artigiani et al., 1999). Interestingly, *PlexinA2* expression was noted in the hypaxial musculature in our studies (Fig. 7C), a conclusion that was supported by the absence of *PlexinA2* expression in these domains that are absent in *Splotch* embryos (Fig. 7F). These regions included limb musculature (Fig. 7C,F) and the diaphragm (data not shown), tissues that are deficient in *Splotch*. We sought to test whether Pax3 might modulate *PlexinA2* expression in neural crest cells in a manner analogous to Pax3 modulation of c-*Met* in myoblasts. However, it is worth emphasizing that Pax3 is not absolutely required for cardiac neural crest migration (Epstein et al., 2000), despite clear evidence that it is required for limb myoblast migration (Daston et al., 1996), indicating important differences in Pax3 function in these tissues.

Patterning of *PlexinA2*-expressing neural crest cells was abnormal in *Splotch*. While *PlexinA2*-expressing cells were seen diffusely in the outflow endocardial cushions of *Splotch* embryos (arrow, Fig. 7E), a localized collection of expressing cells in the condensed mesenchyme seen in control embryos (arrow, Fig. 7B) was missing. However, it is important to note that *PlexinA2*-expressing cells were clearly present in the outflow tract of *Splotch* embryos (Fig. 7D,E), consistent with our previous studies showing migration of neural crest cells to the hearts of these mutant embryos (Epstein et al., 2000). This

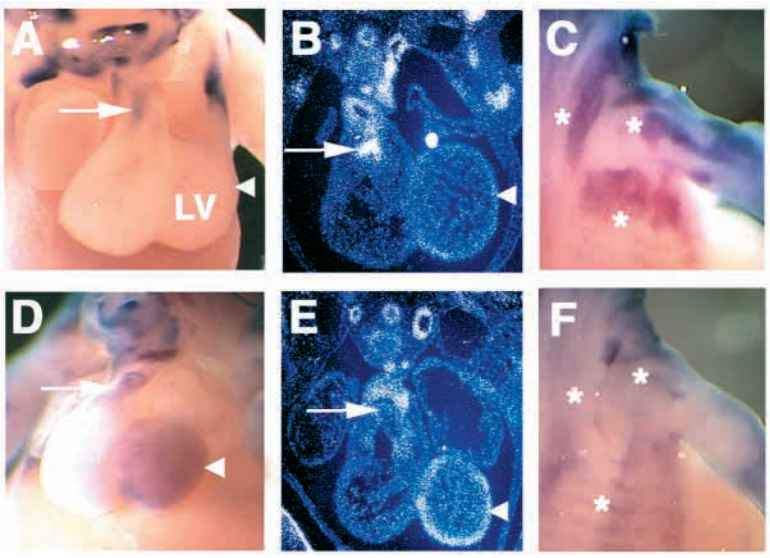

Fig. 7. Expression of *PlexinA2* in *Splotch* embryos. Whole-mount in situ hybridization (A,C,D,F) and radioactive in situ hybridization (B,E) was performed with E12.5 wild-type (A-C) and *Splotch* (D-F) littermates. *PlexinA2* expression is noted in the outflow tract of both wild-type (arrow, A) and *Splotch* (arrow, D) embryos. However, analysis of transverse frontal sections reveals strong expression by a cluster of cells in the condensed mesenchyme of the conotruncal endocardial cushions in wild-type embryos (arrow, B) that is not seen in *Splotch* embryos (arrow, E). *PlexinA2* is normally expressed only weakly in the left ventricular (LV) myocardium at this stage (arrowheads, A,B). In *Splotch* embryo, *PlexinA2* expression is significantly up-regulated in the left ventricle as seen with both whole-mount (arrowhead, D) and radioactive (arrowhead, E) techniques. *PlexinA2* is also expressed in hypaxial muscles of wild-type embryos (C, asterisks), while this domain of expression is absent in *Splotch* embryos (F, asterisks), consistent with the established lack of limb musculature in *Splotch* embryos.

result indicates that Pax3 is not required for *PlexinA2* expression in cardiac neural crest, though we cannot rule out a role for Pax3 in regulation of *PlexinA2* expression not apparent in these studies because of functional redundancy of other genes (e.g. *Pax7*).

Interestingly, we noted dramatic up-regulation of *PlexinA2* in the myocardium of the left ventricle of *Splotch* embryos by both whole-mount (Fig. 7D, arrowhead) and radioactive (Fig. 7E, arrowhead) in situ hybridization. While Pax3 is not expressed in the ventricular myocardium, morphological and functional defects are present in this tissue in *Splotch* embryos (Conway et al., 1997a; Creazzo et al., 1998; Li et al., 1999). Hence, we conclude that Pax3 deficiency results in a secondary elevation in *PlexinA2* expression in the myocardium perhaps functionally related to these morphological and contractile defects.

In summary, analysis of *PlexinA2* expression in *Splotch* embryos is consistent with the existence of secondary alterations in myocardial gene expression in the setting of primary neural crest defects. These data also support the conclusion that neural crest cells can migrate to the heart in *Splotch* embryos, but cannot coalesce to form a functional septation complex.

DISCUSSION

In this report, we present evidence that *PlexinA2* is expressed by cardiac neural crest and that *PlexinA2*-expressing neural crest cells are patterned abnormally in several mouse models of conotruncal congenital heart disease. Identification of *PlexinA2* in migrating and post-migratory cardiac neural crest implicates a new family of signaling molecules, the semaphorins, in cardiac neural crest development.

Plexins are known to function as co-receptors for secreted semaphorin ligands and can be divided into at least 4 classes (for review see Tamagnone et al., 1999; Tamagnone and Comoglio, 2000). Members of the class A Plexins form functional multimeric receptor complexes with neuropilin 1 (Nrp1), neuropilin 2 (Nrp2) or both in order to bind class 3 semaphorin ligands (Chen et al., 1997; Kolodkin et al., 1997; Tamagnone and Comoglio, 2000). The recent observation that mice homozygous null for *Sema3C* exhibit cardiovascular defects (Feiner et al., 2001), combined with the data presented here, suggest that PlexinA2 may form part of a functional receptor for Sema3C on cardiac neural crest cells. Our expression data indicates that both *Nrp1* and *Nrp2* are expressed in appropriate locations during cardiac development to participate in formation of functional receptor complexes with PlexinA2, though this hypothesis will require experimental validation. In vitro binding experiments demonstrate that Sema3C can recognize both PlexinA2/Nrp1 and PlexinA2/Nrp2 complexes (Kolodkin and Ginty, 1997). Interestingly, PlexinA2/Nrp2 complexes do not bind the related ligand Sema3A, and PlexinA1/neuropilin complexes that bind Sema3A bind Sema3C poorly and do not signal (Tamagnone and Comoglio, 2000). Thus, specificity of the cellular response to a particular semaphorin ligand results from the specific Plexin co-receptor present in the complex. Neuropilins appear to subserve less-specific functions in determination of ligand specificity. This is consistent with our expression data suggesting that expression of PlexinA2 by cardiac neural crest may make these cells uniquely responsive to Sema3C.

Sema3C null mice die shortly after birth and display an interrupted aortic arch and persistent truncus arteriosus. These defects suggest abnormalities of cardiac neural crest, though *Sema3C* itself is expressed predominantly in the myocardium surrounding the outflow tract during cardiac development consistent with its role as a secreted ligand affecting cardiac neural crest. Peripartum lethality is likely due to the normal closure of the ductus arteriosus at birth, which results in the loss of blood supply to the descending aorta in the setting of an interrupted aortic arch. Interestingly, other neural crest derivatives, such as the dorsal root ganglia, are normal in *Sema3C*$^{-/-}$ embryos indicating that cardiac neural crest are particularly susceptible to loss of Sema3C signals. This suggests that a specific Sema3C receptor or signaling complex is expressed by cardiac neural crest cells.

Further support for a role of semaphorin signaling in neural crest-mediated cardiovascular development comes from the recent description of cardiovascular phenotypes in *Nrp1* (Kawasaki et al., 1999) null mice. *Nrp1*-deficient embryos die around E13.5 with severe cardiovascular defects including an interrupted aortic arch, persistent truncus arteriosus and a poorly developed vasculature. Analysis of the *Nrp1* phenotype is complicated by the fact that Nrp1 is also a co-receptor for vascular endothelial growth factor (VEGF). VEGF is known to be required for vascular development (Ferrara and Henzel, 1989; Keck et al., 1989; Risau, 1997) and the loss of functional VEGF receptors accounts for at least part of the *Nrp1* null phenotype (Kawasaki et al., 1999). However, cardiac outflow tract and aortic arch defects are suggestive of neural crest deficiencies. In light of our data implicating semaphorin signaling in cardiac neural crest development, it seems likely that a portion of the *Nrp1* null phenotype is related to deficient Sema3C signaling.

Defects in *Sema3C* and *Nrp1* null mice are reminiscent of the spectrum of cardiovascular defects including interrupted aortic arch and PTA that are seen in the human DiGeorge and Velocardiofacial syndromes (Goldmuntz and Emanuel, 1997). While many DiGeorge syndrome patients have chromosome 22q11 deletions, many other patients with DiGeorge and related neurocristopathies do not have these deletions. Our analysis of semaphorin signaling molecules during cardiac neural crest development provides additional genes potentially responsible for human congenital cardiovascular disease.

In addition to Sema3C, another class 3 semaphorin ligand, Sema3A, functions during cardiac development (Behar et al., 1996; Taniguchi et al., 1997). Sema3A acts as a repulsive signal affecting neural crest migration in the chick (Eickholt et al., 1999). Sema3A signals via a receptor complex composed of at least PlexinA1 and Nrp1, but it can also bind PlexinA2/Nrp1 complexes (Takahashi et al., 1999). In the chick, cells from neural crest explants from both the hindbrain and the trunk regions of the embryo selectively avoid growth on Sema3A stripes in vitro, and individual cells bind Sema3A and undergo a morphological shape change consistent with a negative response to Sema3A (Eickholt et al., 1999). In mice, two *Sema3A*-deficient lines were generated independently. Taniguchi et al. (Taniguchi et al., 1997) demonstrated a non-lethal phenotype with defects in peripheral nerve projections while Behar et al. (Behar et al., 1996) described perinatal

lethality characterized by hypertrophy of the right ventricle, dilation of the right atrium and bone and cartilage abnormalities. In both cases, the defects seen in *Sema3A* null mice are not suggestive of cardiac neural crest defects. *Nrp2* null mice display defects in selective axon pathfinding during neurogenesis, but display no obvious defects in neural crest or cardiac development (Chen et al., 1997). These results suggest a lack of requirement for *Sema3A* and *Nrp2* during cardiac neural crest development, or the existence of functional redundancy with other genes.

Taken together, the existing data suggest that the most likely receptor complex expressed by cardiac neural crest cells that mediates Sema3C signaling includes PlexinA2 and Nrp1.

By analogy to semaphorin signaling during neurogenesis, semaphorin signaling in the cardiac neural crest may regulate pathfinding and migration. Subtle abnormalities of neural crest positioning may result in congenital heart disease. Consistent with this hypothesis, we have previously observed defects in positioning of cardiac neural crest cell populations in the outflow tract cushions in the *Pax3* mutant *Splotch* (Epstein et al., 2000). In *Splotch* embryos, neural crest cells reach the outflow tract but are positioned in inappropriate lateral domains within the truncus arteriosus (Epstein et al., 2000). This suggested to us that Pax3 might regulate expression of *PlexinA2* in cardiac neural crest, a hypothesis that was also appealing because of the known function of Pax3 to modulate expression of c-*Met* (a plexin-related gene) in myoblasts. However, we found that *PlexinA2* was expressed by cardiac neural crest in the absence of Pax3. Thus, factors other than *PlexinA2* are required for proper neural crest positioning in the heart.

Like *Splotch* embryos, *Sema3C*-deficient embryos have neural crest-related cardiac defects. Abnormalities of outflow tract septation in *Sema3C* nulls present a spectrum of defects ranging from little or no connection between the pulmonary trunk and aorta in mildly affected embryos, to complete failure of septation (persistent truncus arteriosus). We have noted a similar variability in the expression of *PlexinA2* in *Sema3C* mutants. In some *Sema3C* null embryos, there is a dramatic decrease in *PlexinA2* expression in the outflow tract cushions, while expression in other embryos appears close to normal. This phenotype is particularly interesting in that only a subset of the cardiac neural crest, those that populate the 4th aortic arch and a subset that enter the outflow tract, are affected. The ductus arteriosus, a 6th arch derivative develops normally. Thus, signaling between Sema3C and PlexinA2 appears to be critical for only a subset of cardiac neural crest. The expression of *Sema3C* in the myocardial wall of the outflow tract may represent a guidance signal for migrating neural crest cells directing proper positioning in the outflow tract. Additional roles for semaphorin signaling in neural crest differentiation, proliferation or survival remain to be determined.

We have demonstrated that *PlexinA2* and *Sema3C* are expressed in both complementary and overlapping domains in the developing embryo consistent with a ligand-receptor interaction. In the central nervous system, there are important parallels between the ephrin and semaphorin families, both of which mediate axon pathfinding cues. In the case of the ephrin family, examples exist in which a given cell type both expresses and responds to a specific secreted ligand (Hornberger et al., 1999). Thus, it is plausible that cardiac neural crest cells both secrete Sema3C and also respond to Sema3C that is secreted in an autocrine and paracrine fashion.

Expression of *Sema3C* and *PlexinA2* partially overlap at E11.5 in the pharyngeal mesenchyme surrounding the aortic arch arteries. If co-expression of receptor and ligand is required for proper maintenance of the aortic arches, absence of the Sema3C ligand may result in a loss of arch positional identity or survival cues. Subsequent inappropriate arch regression would result in interruptions of the mature arch. Misexpression and transgenic rescue experiments using tissue-specific promoter elements should help to test this hypothesis.

In summary, our data provide evidence suggesting a critical role for semaphorin signaling during cardiac neural crest-mediated heart development. *PlexinA2* is expressed by cardiac neural crest cells and likely functions as a component of a receptor for Sema3C. The importance of semaphorin signaling in both cardiac morphogenesis and neural patterning in the central nervous system suggests parallels between molecular and cellular pathways mediating cardiac neural crest migration and axon guidance. This paradigm suggests new avenues of research relevant to the understanding of neural crest development and the etiology of congenital heart disease.

This work was supported by NIH grants HL62974, HL61475, DK57050, the WW Smith Foundation and AHA GIA 0050052N to J. A. E., NIH AR08584 to C. B. B., and the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG#1530 to A. L. W. We are grateful to C. Lo for providing *Cx43lacZ* mice and to A. McMahon for *Wnt1*-Cre mice.

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