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Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption

Leonard Feiner[1], Andrea L. Webber[1], Christopher B. Brown[2], Min Min Lu[2], Li Jia[1], Paul Feinstein[3], Peter Mombaerts[3], Jonathan A. Epstein[2] and Jonathan A. Raper[1,*]

[1]Department of Neuroscience, University of Pennsylvania School of Medicine, 1115 BRB II/III, 421 Curie Blvd, Philadelphia, PA 19104, USA
[2]Department of Medicine, Cardiovascular Division, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA
[3]The Rockefeller University, New York, New York 10021, USA
*Author for correspondence (e-mail: raperj@mail.med.upen.edu)

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SUMMARY

Semaphorin 3C is a secreted member of the semaphorin gene family. To investigate its function in vivo, we have disrupted the *semaphorin 3C* locus in mice by targeted mutagenesis. *semaphorin 3C* mutant mice die within hours after birth from congenital cardiovascular defects consisting of interruption of the aortic arch and improper septation of the cardiac outflow tract. This phenotype is similar to that reported following ablation of the cardiac neural crest in chick embryos and resembles congenital heart defects seen in humans. Semaphorin 3C is expressed in the cardiac outflow tract as neural crest cells migrate into it. Their entry is disrupted in *semaphorin 3C* mutant mice. These data suggest that semaphorin 3C promotes crest cell migration into the proximal cardiac outflow tract.

Key words: Semaphorin 3C, Mouse, Cardiac neural crest, Interrupted aortic arch, Persistent truncus arteriosus, PlexinA2, Chemoattractant, Sema3C

INTRODUCTION

Classic studies performed almost 20 years ago demonstrated that perturbations of migrating neural crest cells induce cardiovascular defects (Kirby et al., 1983; reviewed by Creazzo et al., 1998). Using chick embryos as a model system, Kirby and coworkers ablated regions of the dorsal neural tube before the emergence of migrating neural crest cells and observed subsequent defects in the septation of the cardiac outflow tract (persistent truncus arteriosus or PTA), double outlet right ventricle and interruption of the aortic arch. Using quail-chick chimeras and other labeling techniques to characterize neural crest cell fate during normal development, crest cells were seen to populate the branchial arches and also to help form the septum that divides the truncus arteriosus into the aorta and pulmonary artery (Waldo et al., 1998).

More recently a number of mutations have been engineered in mice that produce similar cardiovascular defects (see Discussion). By analogy to the avian model, these genes are likely to affect cardiac neural crest development or function. In humans, outflow tract and other crest related anomalies are present in patients with DiGeorge syndrome and are often associated with deletions on chromosome 22q11 (Driscoll, 1994). Significant progress has been made in producing mouse models of DiGeorge syndrome by deleting homologous regions of mouse chromosome 16 (Lindsay, 1999). More recent and ongoing studies are identifying candidate genes within this region and characterizing their functional roles in neural crest development (Epstein and Buck, 2000; Merscher et al., 2001; Lindsay et al., 2001; Jerome and Papaioannou, 2001; Guris et al., 2001).

The identification of essential genes is the first step in explaining how cardiac neural crest cells contribute to cardiovascular development. These genes will in turn define complex genetic and developmental pathways that control crest cell specification, migration, differentiation and survival. They will also identify signaling pathways by which crest cells interact with neighboring tissues within the pharyngeal arches and the outflow tract of the heart.

We present evidence that morphogenesis of the outflow tract and great vessels is dependent upon semaphorin 3C (Sema3C), a signaling molecule previously hypothesized to guide extending axons in the developing nervous system. The semaphorins comprise a large family of phylogenetically conserved secreted and transmembrane signaling proteins, some of which function as axon guidance cues (Raper, 2000; Yu and Kolodkin, 1999). Class 3 semaphorins are secreted glycoproteins that contain an approximately 500 amino acid N-terminal semaphorin domain, a C2 type immunoglobulin domain, and a highly basic C-terminal tail. Four of them, including *Sema3C*, are located on mouse chromosome 5 in a region syntenic to human chromosome 7q21-q31 (Tarantino et al., 2000). The first identified vertebrate semaphorin, semaphorin 3A, is a secreted protein purified on the basis of its ability to repel dorsal root ganglion axons in culture (Luo et al., 1993). *semaphorin 3A* knockout mice display a complex

phenotype in which many different tissues are affected (Behar et al., 1996; Taniguchi et al., 1997). Notably, the peripheral nervous system of *semaphorin 3A* mutants is severely defasciculated although most axons ultimately appear to connect with their appropriate targets (Catalano et al., 1998). In knockout animals, axons fail to avoid territories that would normally express semaphorin 3A (Taniguchi et al., 1997). These mutant animals also experience postnatal hypertrophy of the right ventricle (Behar et al., 1996). The cause of this hypertrophy has not yet been identified.

The functions of a related semaphorin, Sema3C, have been characterized in vitro. Sema3C can act as either a repellent or an attractant for axons growing in culture. For example, the growth cones of sympathetic neurons are repelled by Sema3C (Koppel et al., 1997), whereas growth cones of rat cortical axons are attracted towards a source of Sema3C (Bagnard et al., 1998).

We have generated mice deficient in Sema3C. Mutants are cyanotic and die just after birth. Their death is attributable to aortic arch malformations and septation defects in the outflow tract of the heart. Our results suggest that Sema3C expression in the proximal cardiac outflow tract facilitates the entry of migrating neural crest cells that are essential for normal septation.

MATERIALS AND METHODS

Targeted disruption of the *Sema3C* locus

A bacterial artificial chromosome containing the *Sema3C* locus was purchased from Genome Systems. This BAC was digested with *Eco*RI, *Bam*HI or *Xho*I and subcloned into pBluescript. The genomic fragment containing a critical exon was isolated and mapped with restriction enzymes. The majority of this exon was replaced by a *Pac*I site, which was used to insert a cassette consisting of an internal ribosome entry site (IRES) followed by a *tau-lacZ* gene and a neomycin resistance cassette flanked by loxP sites.

Gene targeting

The targeting vector was linearized with *Xho*I. Electroporation and cell culture of E14 cells (Hooper et al., 1987) were carried out as previously described (Mombaerts et al., 1996). Genomic DNA from G418-resistant ES cell colonies was digested with *Eco*RI and analyzed by Southern blot hybridization with external probes 5′ and 3′ to the targeting vector. Mice were backcrossed 4 generations into the CD1 background.

Cre recombination

The neo-selectable marker was removed from the targeted mutation by crossing *Sema3C* heterozygotes (*neo*^{in}) to CD1 mice from a *cre* pedigree (bcre-23) that consisted of 6 kb of the 5′ flanking region of the *Brn4*/*Pou3f4* promoter driving the expression of the Cre recombinase gene (gift from K. Ahn and E. B. Crenshaw III).

PCR genotyping

Three to five mm of mouse tail was placed into 100 μl of buffer containing 0.5 mg/ml proteinase K, 50 mM KCl, 10 mM Tris/HCl pH 8.3, 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20. After 2-10 hours digestion at 55°C, samples were heat inactivated for 10 minutes at 95°C. 1 μl of this sample was added to a standard 15 μl PCR reaction with the following primer sets to amplify the wild-type and mutant alleles respectively in *Sema3C*(*neo*^{in}) mice; 5′-ttcccagtggggtagaacctagagc-3′, 5′-gctatcaggacatagcgttggctac-3′, and 5′-gagtctgtttctacagaaatgcatgggtt-3′, 5′-ttcccagtggggtagaacctagagc-3′. In *Sema3C*(*neo*^{out}) mice the following primer sets were used to amplify the wild-type and mutant alleles respectively 5′-ttcccagtggggtagaacctagagc-3′, 5′-gcgattaccgttgatgttgagtggc-3′, and 5′-gagtctgtttctacagaaatgcatgggtt-3′, 5′-ttcccagtggggtagaacctagagc-3′. The conditions for the thermocycler were: 94°C for 1 minute, 29 seconds followed by 94°C for 31 seconds/60.9°C for 1 minute 10 seconds/72°C for 1 minute 30 seconds (35 times), 72°C for 5 minutes.

RT-PCR

RNA was isolated from E12.5 wild-type and *Sema3C* mutant embryos by $CsCl_2$ gradient centrifugation (Chirgwin et al., 1979). The Superscript Preamplification System Kit (GibcoBRL) was used to generate first strand cDNA using 2 μg of total RNA from each mouse sample. PCR was performed using one of 3 primer sets. Primer set #1 5′-aaatggctggcaaaggatcct-3′, 5′-cggtccacagcaatctttgt-3′, primer set #3 5′-cggggcaccgtgcaaaggtc-3′, 5′-cagtggggtagaacctagagc-3′, primer set #2 5′-ctgactgcgagctgatgattt-3′, 5′-attggggctagtatagaca-3′. 30 cycles of PCR (same conditions as above) were performed and samples were run on a 1.5% agarose gel.

Corrosion casting

To examine the structure of the vascular system, corrosion casting (Polysciences # 07349) was performed. Briefly, P0 wild-type and *Sema3C* mutant mice were anesthetized with ketamine and perfused with phosphate-buffered saline through the left ventricle after a small hole was introduced into the right atrium. A 1 ml tuberculin syringe was used to inject polymer into the left ventricle under gentle pressure. After perfusion with the polymer, the resin was left overnight to harden. After the mass had fully cured, the tissue surrounding the cast was corroded away by using Maceration Solution (Polysciences) at 50°C.

In situ hybridization

Digoxigenin-labeled antisense and sense riboprobes were synthesized by in vitro run-off transcription of linearized plasmids, using SP6, T7 or T3 RNA polymerase. Sense control probes gave no signal. Whole-mount in situ hybridization was performed as described by Borycki et al. (Borycki et al., 1999). Radioactive in situ hybridization was performed as described previously (Wawersik and Epstein, 2000).

Histology

Embryos and newborn mice were fixed in 4% paraformaldehyde in PBS for 24-48 hours. The skin was removed from newborn mice before fixation. After dehydration and embedding in paraffin wax, 8 μm sections were placed on glass slides. After dewaxing in xylenes and rehydration, sections were either stained with Hematoxylin and Eosin, or processed for in situ hybridization.

RESULTS

Generation of mutant mice

To elucidate the role of Sema3C in mouse development, the *Sema3C* locus was disrupted by homologous recombination in ES cells. Work in our laboratory has demonstrated that class 3 semaphorins have small and widely scattered exons (unpublished observations of L. F. and M. J. R.). It was important, therefore, to identify and replace an exon expected to be crucial for *Sema3C* function. Structure-function studies have demonstrated the importance of the semaphorin domain for the functions of class 3 semaphorins (Koppel et al., 1997). A highly conserved, cysteine rich region of the semaphorin domain including the residues CCLARDPYCAWD is present in every class 3 semaphorin (Behar et al., 1999). When these amino acids are deleted from a semaphorin 3A expression

plasmid, no functional protein is secreted from transfected 293T cells. Cell extracts from transfected cells are devoid of collapsing activity. Thus, deletion of these residues destroys the growth cone collapsing activity of semaphorin 3A (data not shown). Furthermore, class 3 semaphorins must dimerize to have collapsing activity (Koppel and Raper, 1998; Klostermann et al., 1998). Removal of the CCLARDPYCAWD-encoding exon alters the reading frame and thereby causes premature termination of the protein before the cysteine residue that is known to promote dimerization of Sema3A. Deletion of the CCLARDPYCAWD-containing exon and the concomitant premature termination of Sema3C is therefore likely to produce a null allele.

A diagram of the targeting vector we used to mutate *Sema3C* is shown in Fig. 1A. The majority of the CCLARDPYCAWD-containing exon is replaced by a cassette that includes an IRES followed by a Tau-*lacZ* fusion protein and a neomycin resistance cassette flanked by *LoxP* sites (Mombaerts et al., 1996). This targeting vector was electroporated into E14 ES cells (Hooper et al., 1987). Ten of 96 clones that developed resistance to G418 had undergone homologous recombination. Three of these ES cell lines were expanded and injected into blastocysts to derive chimeric mice. Chimeras from all 3 lines were capable of germline transmission of the mutation. The targeting of ES cells and germline transmission were confirmed by Southern blot hybridization (Fig. 1B). The neomycin resistance cassette was removed by crossing mice carrying the mutant *Sema3C* locus to mice that express Cre recombinase in the germ cell lineage (gift from K. Ahn and E. B. Crenshaw III). Proper recombination was verified by PCR with a primer set flanking the excised cassette (data not shown).

Sema3C mRNA is reduced and appropriately altered in *Sema3C* mutant mice

Owing to the absence of specific antibodies for the mouse semaphorins, we analyzed *Sema3C* mRNA in *Sema3C* mutant mice and their wild-type littermates to determine the effectiveness of the *Sema3C* mutation. Three primer sets were used in RT-PCR reactions to detect different regions of the *Sema3C* mRNA (Fig. 1D). Primer set 1 (blue) amplifies a region of the mRNA 5′ of the targeted region, primer set 2 (red) a region 3′ of the targeted region, and primer set 3 (green) a region internal to the targeted region. All three primer sets amplify appropriately sized PCR products in wild-type animals (Fig. 1E). In *Sema3C* mutant mice, only the primer sets to either side of the deleted exon are capable of detecting *Sema3C* mRNA. Thus, while *Sema3C* mRNA is produced in *Sema3C* mutant animals, this message does not contain the exon modified by the targeting construct (Fig. 1E, green lanes).

Sema3C mRNA is barely detectable by in situ hybridization in E12.5 mutant embryos (data not shown). Thus, the mutant form of *Sema3C* mRNA lacks a critical functional domain and is only expressed at a fraction of the wild-type level. The targeted *Sema3C* allele contains an IRES followed by a sequence encoding a Tau-*lacZ* fusion protein. The purpose of this addition is to harness the endogenous *Sema3C* promoter to drive the expression of *tau-lacZ* in cells that normally express *Sema3C*. Perhaps because of the low levels of *Sema3C* mRNA in mutant mice, no β-galactosidase is detectable by X-gal histochemistry or immunocytochemistry in tissues that normally express *Sema3C* mRNA (data not shown).

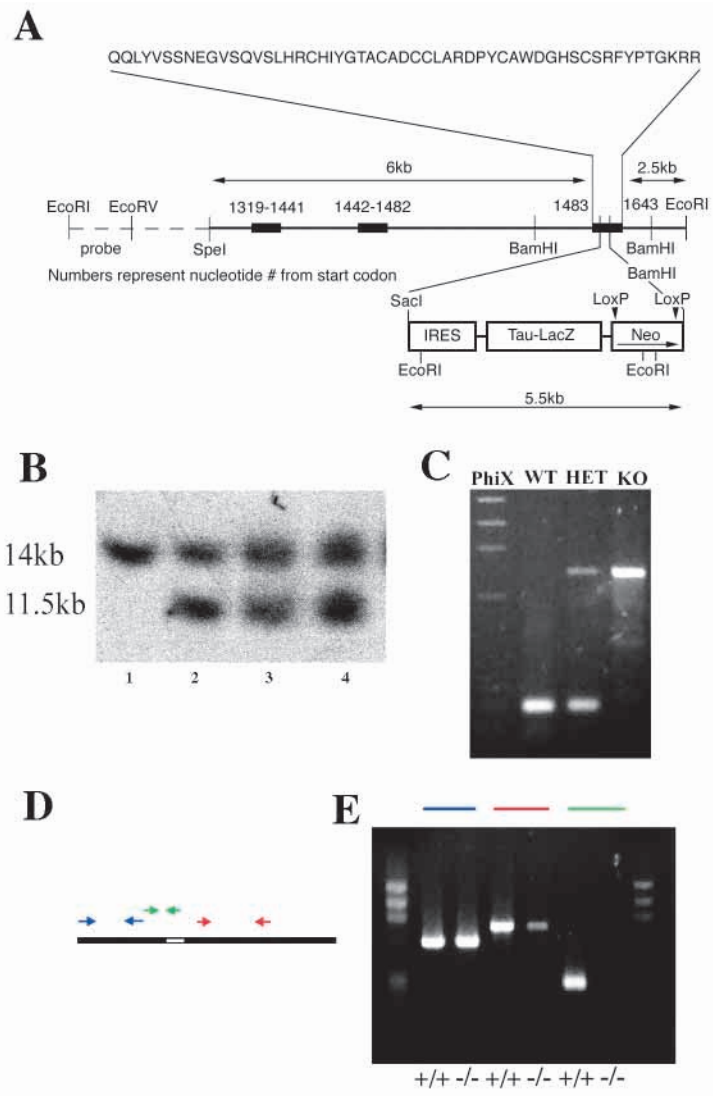

Fig. 1. Generation of *Sema3C* mutant mice and mRNA analysis. (A) A diagram of the targeting vector used to disrupt *Sema3C* is shown. A majority of the targeted exon was replaced by a cassette that included an internal ribosomal entry site followed by a *tau-lacZ* gene and a neomycin resistance cassette flanked by loxP sites (see text). (B) Southern blot analysis shows wild-type (lane 1) and three targeted ES cell lines (lanes 2, 3 and 4). (C) PCR genotyping of wild-type (WT), heterozygous (HET) and *Sema3C* mutant (KO) mice. PhiX, ϕX174 DNA-HaeIII digest (D) Schematic of *Sema3C* transcript showing the locations of primers used for RT-PCR analysis. White inset represents the coding region replaced by the targeting vector. Primer sets 1, 2 and 3 (see text) used in RT-PCR reactions are color coded in blue, red, and green, respectively. (E) Analysis of RT-PCR from mRNA extracted from whole E12.5 wild-type and knockout embryos reveals that *Sema3C* mRNA is produced in mutants but that the mutant message does not contain the exon replaced by the targeting construct (primer set #3; ‘green’ product).

Sema3C mutant mice die from persistent truncus arteriosus and interruption of the aortic arch

Mice heterozygous for the *Sema3C* mutation are grossly indistinguishable from their wild-type littermates. Some progeny of heterozygous mice die within the first 24 hours after birth. These sick neonates are cyanotic and when genotyped are found to be homozygous for the *Sema3C* mutation. Interestingly, the penetrance of this phenotype is highly dependent on the strain of mouse. Postnatal mortality of *Sema3C* mutant mice is lower than 50% on both the 129 and C57BL/6 backgrounds. As the mutation was bred onto the CD1 background, however, the penetrance increased with each successive backcross. Crossing heterozygotes from the fourth backcross generation yields progeny in which the mortality rate for *Sema3C* homozygote mutants is 96% (22 of the 23 mutants in the first 8 litters examined). The powerful effect of strain upon the penetrance of this phenotype may be useful for identifying modifier genes in the future.

Analysis of 8 CD1 litters at birth demonstrates that wild-type, heterozygous and homozygous animals are present in the appropriate Mendelian ratios expected from a heterozygous cross (Table 1). There are occasional *Sema3C* mutant mice that do not die shortly after birth (e.g. 1 of 23 mutants from these 8 CD1 litters). These animals are viable, grossly indistinguishable from wild-type or heterozygous littermates, and fertile. The peripheral nervous system of mutant animals was examined for abnormalities. The cranial nerves of E10.5 and E11.5 *Sema3C* mutant mice were visualized with antibodies that stain neurofilaments and the sympathetic nervous system was examined with antibodies to tyrosine hydroxylase. No misprojections were detected, suggesting that Sema3C by itself is not required for grossly normal peripheral axon pathfinding. A detailed examination of the CNS of *Sema3C* mutant mice is now in progress.

Pathological evaluation of homozygous mice that die shortly after birth reveals cardiac outflow tract and aortic arch abnormalities. The aortic arch is interrupted in 100% of mice that die postnatally. The interruptions occur either between the left common carotid and left subclavian arteries (type B interruption; Fig. 2B) or between the brachiocephalic and left common carotid arteries (type C interruption; Fig. 2C). Type B interruption of the aortic arch could result from inappropriate regression of the left fourth branchial arch artery. Duplication of the left common carotid is infrequently observed (Fig. 2D).

Sema3C mutant mice frequently exhibit defects in septation of the conotruncus known as persistent truncus arteriosus (PTA). Injection of methyl methacrylate resin into the left ventricle is a sensitive method for detecting communication between the aorta and the pulmonary artery (Fig. 3A,C). Seventy-five percent of P0 *Sema3C* mutant mice evaluated by this technique have incomplete septation of the conotruncus (Table 1). In animals with PTA, the common valve of the outflow tract has four cusps (Fig. 3C inset). A ventriculoseptal defect is present in these animals just below the common valve. Histological analysis of the heart of a *Sema3C* mutant mouse with PTA (Fig. 3D) identifies a defect in the membranous portion of the ventricular septum with an intact muscular septum (Fig. 3E). The histology of the ventricular

Table 1. Genotype distribution and frequency of cardiac defects

Genotype of P0 mice (*n*=98)	% Distribution	Cardiac defects			
		Total aA* interruption	Type B	Type C	PTA‡
+/+	27	ND	–	–	ND
+/–	50	0% (*n*=5)	–	–	0% (*n*=6)
–/–	23	96% (*n*=23)	74% (*n*=23)	22% (*n*=23)	75% (*n*=8)

*aA, aortic arch.
‡PTA, persistent truncus arteriosus.

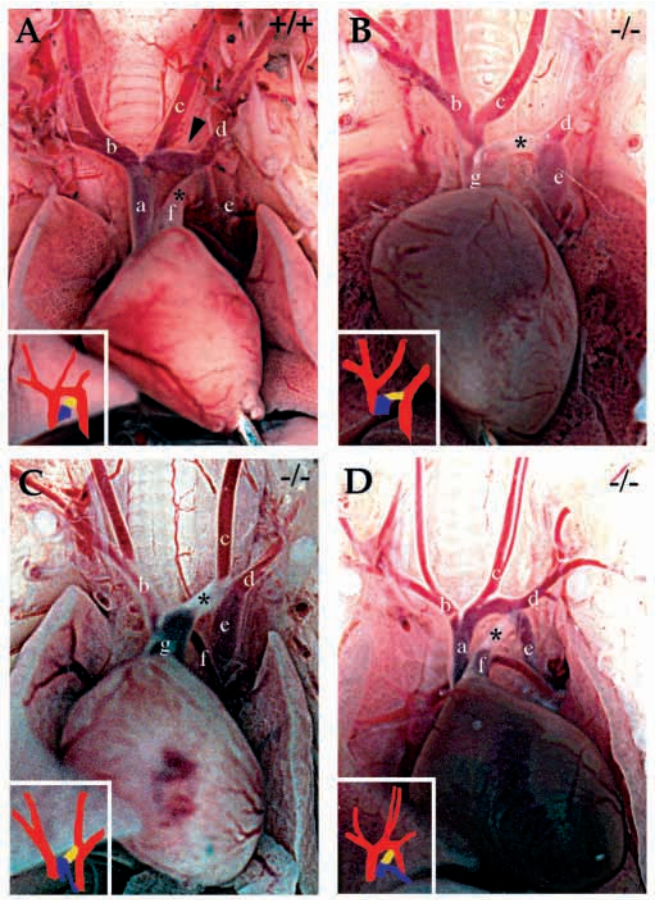

Fig. 2. Cardiovascular defects in *Sema3C* mutant mice. Gross dissections of P0 wild-type (A) and *Sema3C* mutant (B-D) mice with atria removed facilitates visualization of the great arteries. The arch of the aorta is indicated by an arrowhead. The ductus arteriosus is labeled with an asterisk in each panel. The great vessels are labeled as follows: a, ascending aorta; b, brachiocephalic artery; c, left common carotid artery; d, left subclavian artery; e, descending aorta; f, pulmonary artery and g, persistent truncus arteriosus (PTA). (B) Fusion of aortic and pulmonary trunks (PTA) and type B interruption of the aortic arch in a *Sema3C* mutant mouse. The left common carotid artery originates from the PTA. (C) PTA and type C interruption of the aortic arch in a *Sema3C* mutant mouse. The left common carotid artery originates from the descending aorta. (D) Duplication of the left common carotid artery in a *Sema3C* mutant mouse. Insets in each frame present a schematic of the aortic arch with the aorta and tributaries in red, the ductus arteriosus in yellow, and the pulmonary artery in blue.

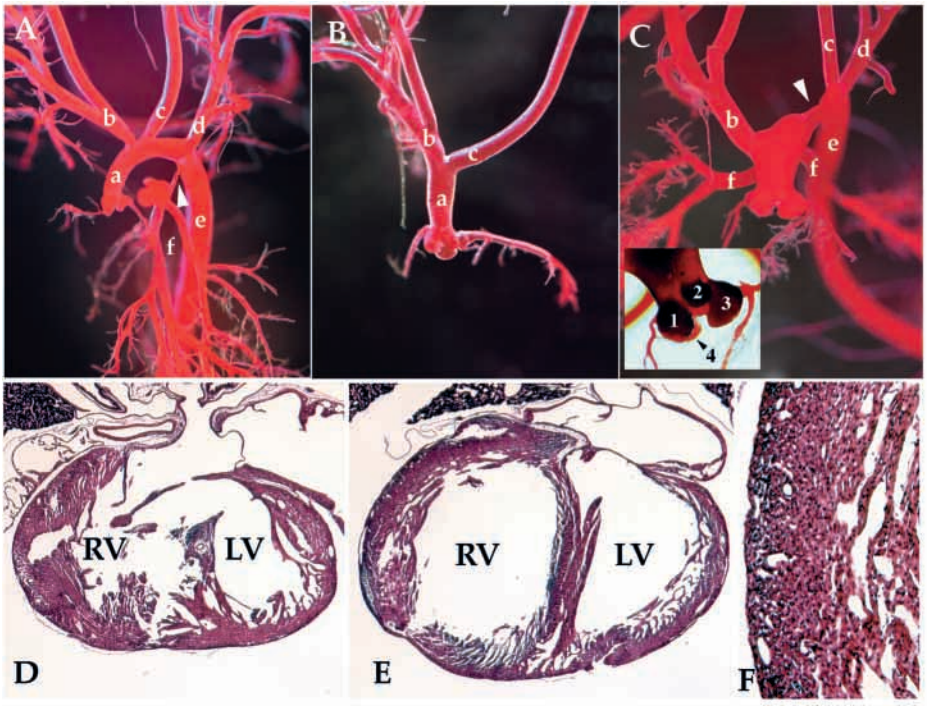

Fig. 3. Cardiovascular phenotype characterized by corrosion casting. Methyl methacrylate was injected into the left ventricle of anesthetized P0 wild-type and mutant mice. (A) Cast of wild-type showing normal vascular architecture and a nearly closed ductus arteriosus (arrowhead). (B) Cast of a *Sema3C* mutant mouse reveals interruption of the aortic arch with normal septation of aorta and pulmonary trunks. The pulmonary vasculature is not filled as the ductus arteriosus has closed. Note the absence of filling of the descending aorta. (C) Another cast of a *Sema3C* mutant P0 mouse shows persistent truncus arteriosus, interruption of the aortic arch, and a single four-leaflet valve (inset-cusps are labeled 1-4). The descending aorta has filled because the ductus arteriosus (arrowhead) has not yet closed completely. (D) Hematoxylin and Eosin-stained paraffin wax section through the heart of a P0 *Sema3C* mutant heart demonstrates a ventricular septal defect in the membranous portion of the ventricular septum. (E) A more posterior section reveals an intact muscular ventricular septum. (F) Higher magnification of the ventricular wall demonstrates normal myocardium and trabeculations in *Sema3C* mutant mice. RV, right ventricle; LV, left ventricle; a, ascending aorta; b, brachiocephalic artery; c, left common carotid artery; d, left subclavian artery; e, descending aorta; f, pulmonary artery.

wall (Fig. 3F) and the structure of the mitral and tricuspid valves appear normal in *Sema3C* mutants (data not shown).

In utero, blood is oxygenated in the placenta rather than the lungs and is diverted from the pulmonary circulation into the descending aorta through a shunt called the ductus arteriosus. The pulmonary and systemic circulations are separated when this shunt closes just after birth. The systemic circulation is maintained in a *Sema3C* mutant embryo before birth because blood can bypass the interruption of the aortic arch by flowing from the heart into the descending aorta through the ductus arteriosis. After birth, however, blood cannot enter the descending aorta once the ductus arteriosus closes. *Sema3C* mutant mice therefore die from cardiovascular defects that are incompatible with postnatal life.

Sema3C is expressed in the developing outflow tract

To better understand the etiology of this phenotype, we studied the expression of *Sema3C* in the cardiac outflow tract during development. *Sema3C* mRNA is expressed in the conotruncus as early as E10.5 and persists through E12.5 (Fig. 4A-C). Thus, expression of *Sema3C* is contemporaneous with the migration of cardiac neural crest cells into the outflow tract (Waldo et al.,

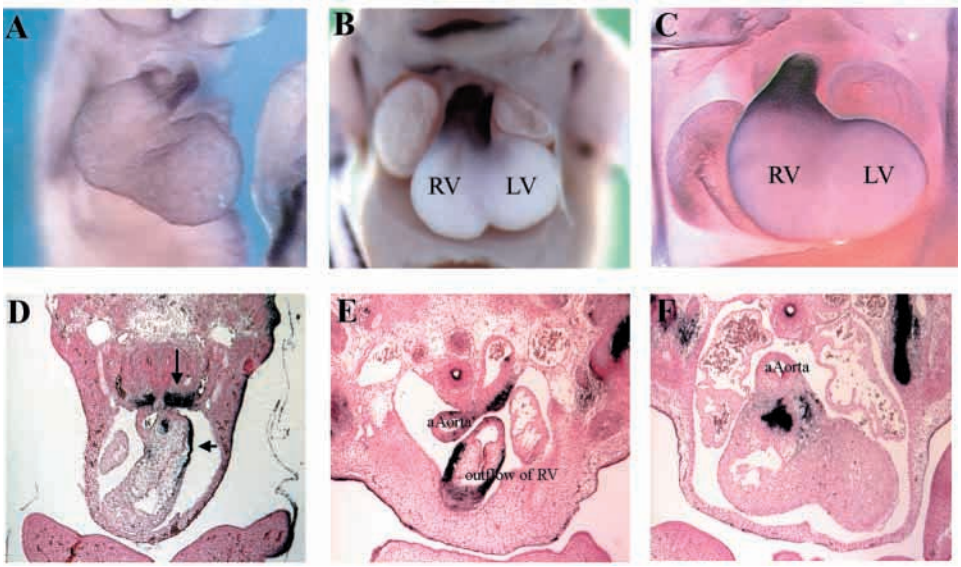

Fig. 4. *Sema3C* expression in the cardiac outflow tract. In situ hybridization using digoxigenin-labeled antisense *Sema3C* riboprobe at (A,D) E10.5, (B) E11.5 and (C,E,F) E12.5. *Sema3C* is expressed in the conotruncus throughout the period of outflow tract septation and remodeling of branchial arch arteries. (A-C) Whole-mount images reveal strong *Sema3C* expression restricted to the outflow tract within the heart. (D) *Sema3C* is expressed by cells surrounding the branchial arch arteries (long arrow) and in the left lateral wall of the conus (short arrow). (E) At E12.5, *Sema3C* is expressed by mesenchymal tissue surrounding the great vessels and myocardium in the right ventricular outflow tract. (F) In a more caudal section closer to the left ventricular outflow tract, *Sema3C* mRNA is expressed in the condensed mesenchyme of the aorticopulmonary septation complex. aAorta, ascending aorta.

1998). More detailed analysis of the expression pattern in sections at E10.5 demonstrates that *Sema3C* is expressed in the mesenchyme surrounding the branchial arch arteries (Fig. 4D). At this stage, *Sema3C* is restricted to the left side of the conotruncus and appears to be expressed in the myocardial cuff.

By E12.5, expression of *Sema3C* is restricted to the proximal outflow tract and the great vessels (Fig. 4E). Strong expression is also seen in the mesenchyme of the aorticopulmonary septation complex where neural crest cells form the developing septum (Fig. 4F). *Sema3C* is expressed in cells adjacent to neural crest migration pathways, suggesting a non cell-autonomous function. It is also expressed within the aorticopulmonary septation complex, possibly even within the neural crest itself. It is not uncommon to observe an axon guidance molecule to be expressed in neurons that are responsive to that cue. One dramatic example is the modulation of ephrin responsiveness by ephrin expression in retinal ganglion cells (Hornberger et al., 1999). It is possible that cardiac neural crest cells may both express and respond to *Sema3C*.

Abnormal migration of cardiac crest cells in *Sema3C* mutant embryos

Sema3C has been shown to influence growth cone motility and guidance in vitro. Cardiac neural crest cells are known to be required for proper formation and septation of the developing cardiac outflow tract. We hypothesized that Sema3C acts as a guidance cue for migrating cardiac crest cells, that its loss prevents crest cells from populating the aortic arch or the proximal outflow tract, and that the resulting absence of crest cells in these structures is sufficient to induce the cardiac abnormalities in *Sema3C* mutant mice.

We compared the migration of cardiac crest cells into the proximal cardiac outflow tract in wild-type and mutant embryos. Cardiac crest cells were visualized with a series of molecular markers including plexinA2. This transmembrane molecule is a member of a family of receptors that is thought to help mediate semaphorin signaling (Takahashi et al., 1999; Tamagnone et al., 1999). In an accompanying paper, transgenic and fate-mapping techniques demonstrate that plexinA2 is expressed by neural crest cells that surround the aortic arches and migrate into the cardiac outflow tract (Brown et al., 2001). The complementary expression patterns of Sema3C and plexinA2 raise the possibility that plexinA2 is a receptor component for Sema3C. PlexinA2 expression (Fig. 5E) is colocalized with other cardiac crest cell markers, including *Foxc1* (Fig. 5A) and endothelin receptor A (*EdnrA*; Fig. 5C), in the developing heart of wild-type embryos. Crest cells in wild-type embryos are seen to encase the branchial arches by E10.5 (not shown) and they populate the endocardial cushions of the outflow tract by E12.5 (Fig. 5A,C,E). Patterning of neural crest cells in the outflow tract of the heart is altered in *Sema3C* mutant littermates. Altered expression of Foxc1 (Fig. 5B), endothelin receptor A (Fig. 5D), and plexinA2 (Fig. 5F) suggests that neural crest cell invasion of the outflow tract is significantly impaired in mutant embryos.

The branchial arch arteries form normally in *Sema3C* mutant embryos

The most dramatic and fully penetrant cardiac phenotype observed in P0 *Sema3C* mutant pups is interruption of the aortic arch. This anomaly could arise either from a failure in formation of the fourth left branchial arch artery, or from inappropriate regression of this artery segment during the complex process that reconfigures the symmetric embryonic vasculature into the asymmetric adult pattern. This remodeling process is known to require the cardiac neural crest, although the mechanisms by which specific right or left sided segments of branchial arch arteries are maintained or lost remain obscure. Branchial arch remodeling takes place in the mouse between embryonic days 10.5 and 13.5. Examination of sections of E10.5 and E11.5 *Sema3C* mutant embryos demonstrates that the branchial arch arteries are present and normal morphologically (Fig. 6A-H).

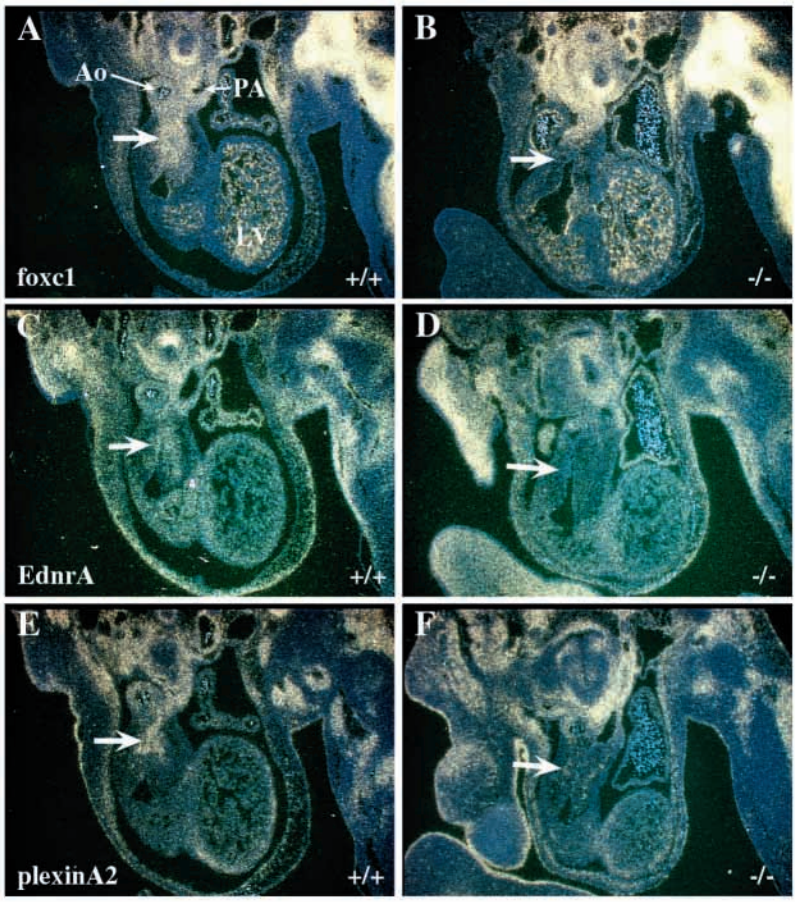

Fig. 5. Altered cardiac neural crest population of outflow tract endocardial cushions in *Sema3C* mutant embryos. E12.5 (A,C,E) wild-type or (B,D,F) *Sema3C* mutant littermates were sectioned and probed for markers of cardiac neural crest cells. mRNAs for (A,B) *Foxc1*, (C,D) endothelin receptor A (*EdnrA*), or (E,F) plexinA2 were visualized with ^{35}S-labeled riboprobes. In wild-type embryos cardiac crest cells are detected populating the endocardial cushions of the cardiac outflow tract (arrows) and surrounding the aortic arch arteries, while in Sema3C mutant embryos few if any cardiac crest cells are detected in comparable locations (arrows). Ao indicates the aorta and PA the pulmonary artery.

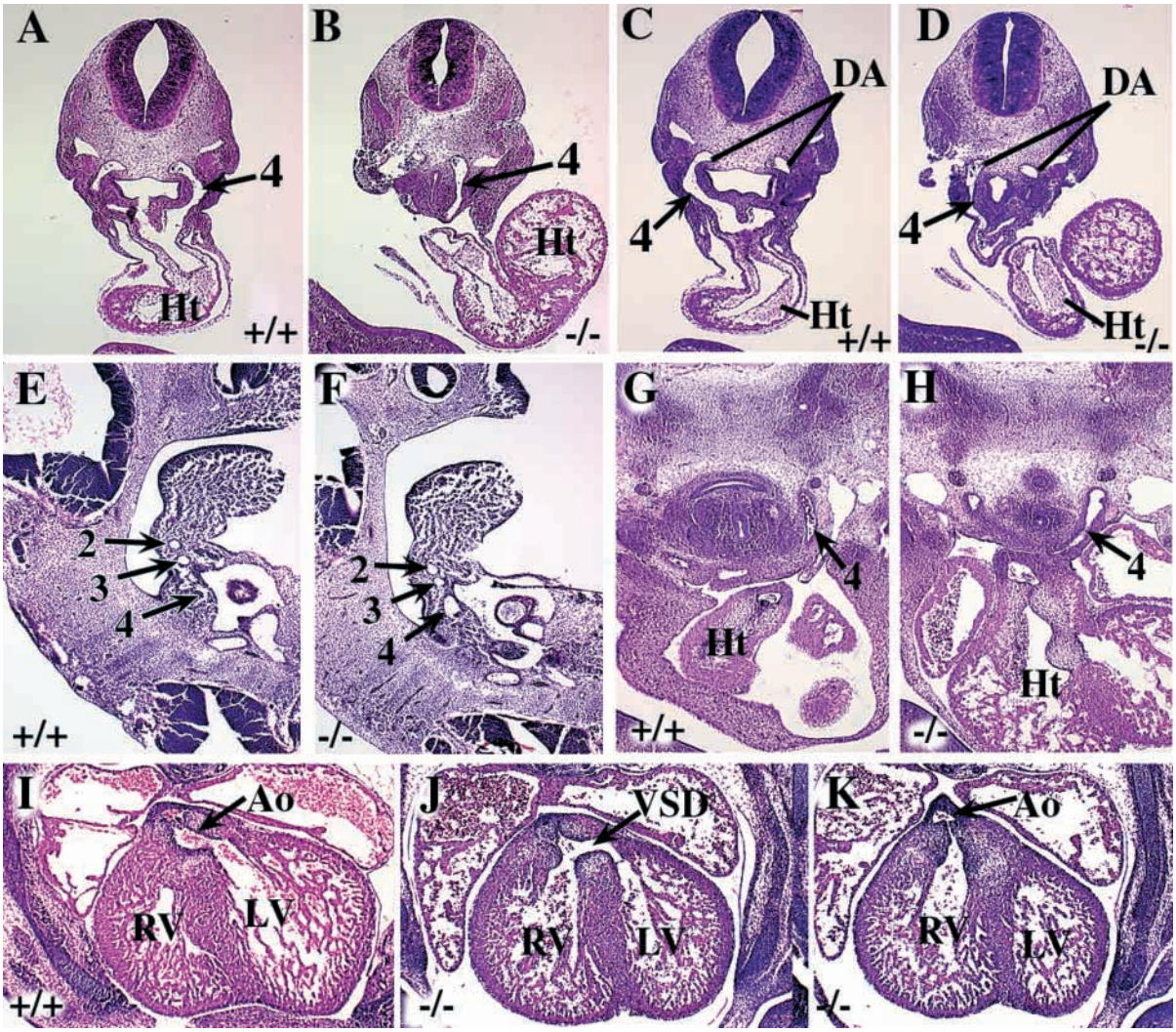

Fig. 6. Normal formation of branchial arch arteries in *Sema3C* mutant embryos. Hematoxylin and Eosin-stained sections of (A,C,E,G,I) wild-type and (B,D,F,H,J,K) *Sema3C* mutant littermate embryos. (A,B) The left fourth branchial arch (arrows, 4) is apparent in both wild-type and mutant E10.5 embryos. (C,D) The right fourth branchial arch (arrows, 4) is present in wild-type and mutant E10.5 embryos. (E,F) Branchial arches 2, 3 and 4 (arrows) appear normal in wild-type and mutant E10.5 embryos cut in sagittal section. (G,H) The left fourth aortic arch is present in wild-type and mutant E11.5 embryos (arrows, 4). (I) An intact ventricular septum and the proximal aorta (Ao) arising from the left ventricle (LV) in a wild-type E13.5 embryo. J and K show a ventricular septal defect (VSD, in J) and the proximal aorta arising from the right ventricle (RV, in K) in a *Sema3C* mutant littermate. This *Sema3C* mutant embryo had a double outlet right ventricle. Ht, heart.

The anomalies seen later must therefore arise from a failure of the arch arteries to remodel correctly.

Consistent with our observation of impaired neural crest migration into the cardiac outflow tract at E12.5, we first detected structural cardiovascular abnormalities at E13.5. Histologic analysis of an E13.5 *Sema3C* mutant embryo revealed a ventricular septal defect (VSD; Fig. 6J) not seen in wild-type litter mates (Fig. 6I). In the mutant embryo, the aorta was seen to arise from the right ventricle (Fig. 6K) resulting in a double outlet right venticle. This malformation is seen after neural crest ablation in chick embryos (Kirby et al., 1983).

DISCUSSION

The deletion of a crucial exon and early truncation of the signaling molecule Sema3C leads to mice with congenital cardiovascular defects. These consist of interruption of the aortic arch and improper septation of the cardiac outflow tract. During cardiac development, the single great vessel emerging from the heart is the truncus arteriosus, from which the symmetric branchial arch arteries originate. A series of complex morphogenic events results in the division of the truncus arteriosus into the aorta and the pulmonary artery, and the realignment of the great vessels so that the aorta emerges from the left ventricle (Olson and Srivastava, 1996). Failure of proper septation of the conotruncus leads to a pathological condition known as persistent truncus arteriosus. Reconfiguration of the heart and outflow tract arise from remodeling of the branchial arch arteries. The branchial arch arteries begin as symmetric sets of vessels that feed the paired branchial arches. Each branchial arch artery contributes to

specific portions of the adult aortic arch or its tributaries (Creazzo et al., 1998). The left fourth arch artery, for example, develops into the portion of the aortic arch between the left common carotid and left subclavian arteries. Thus, the mature configuration of the aortic arch and its tributaries results from the regulated growth and regression of the branchial arch arteries.

Previous work from other laboratories has demonstrated that both remodeling of the branchial arch arteries and septation of the outflow tract require a subset of neural crest cells known as the cardiac crest. Transplantation of quail neural crest cells into developing chicks has shown that cardiac neural crest cells contribute to the tunica media of the aortic arch and to the septum of the outflow tract (Waldo et al., 1998). When the cardiac neural crest is ablated in the chick, the resulting malformations are variable but commonly include persistent truncus arteriosus and absence of various combinations of branchial arch arteries, including those derived from pharyngeal arches 3 and 4 (interrupted aortic arch) and pharyngeal arch 6 (absent ductus arteriosus; Kirby et al., 1983; Creazzo et al., 1998).

The similarity between the cardiovascular phenotypes in *Sema3C* mutant mice and crest ablated chicks suggests that the mouse phenotype reflects a requirement for Sema3C in some important aspect of cardiac neural crest cell development. Two additional observations are consistent with this hypothesis. First, branchial arch arteries form normally in *Sema3C* mutants. Subsequent interruption of the aortic arch must therefore be attributable to a failure of the branchial arches to reorganize correctly, a process known to require the cardiac neural crest. Second, incomplete septation of the outflow tract is evident very early in *Sema3C* mutant embryos and can be attributed to an impairment in crest cell migration into the proximal portion of the tract.

Several engineered and spontaneous mutant mouse lines have been described with interruption of the aortic arch or improper septation of the conotruncus. The cardiac defects in these lines have been ascribed to abnormalities in cardiac neural crest development. In most of these mutant mice, either septation of the conotruncus or remodeling of the branchial arch arteries is separately disturbed. Pax3, retinoic acid receptors, and connexin 43 are all expressed in cardiac neural crest cells (Franz, 1989; Mendelsohn et al., 1994; Reaume et al., 1995). Disruption of these genes leads to defects in septation of the conotruncus. It has been hypothesized that the cardiac defects arise in these knockout mice because the specification of, or communication between, cardiac neural crest cells is perturbed. Interruption of the aortic arch is not observed in mice with these mutations.

Mutations in the endothelin pathway result in a different set of cardiovascular anomalies. Disruption of this intercellular signaling pathway, which mediates communication between the arterial endothelium and the surrounding mesenchyme, leads to interruption of the aortic arch and abnormal persistence of some vessels that normally regress. This has been demonstrated by targeted disruption of endothelin-1, endothelin converting enzyme-1, or the endothelin receptor A (Clouthier et al., 1998; Kurihara et al., 1995; Yanagisawa et al., 1998). Mice null for *HoxA3* or the winged helix transcription factors, *Foxc1* (*Mf-1*) or *Foxc2* (*Mfh-1*), also die as the result of aortic arch anomalies (Winnier et al., 1999; Chisaka and Cappechi, 1991). Although PTA is observed in 6% of *EdnrA* mutant mice and 10% of endothelin converting enzyme-1 mutant mice, septation of the conotruncus usually occurs normally in these knockout lines.

In contrast to these other mouse mutants, disruption of the *Sema3C* locus causes both aortic arch interruption and persistent truncus arteriosus in the majority of mutant mice (Table 1B). One interpretation of this result is that Sema3C might function upstream of these other genes in a pathway regulating cardiac neural crest development. For example, loss of Sema3C function could affect crest cell specification, block cardiac crest cell migration, or reduce the expression levels of other genes known to be important in crest cell directed remodeling of the cardiac outflow tract. We have not exhaustively assayed the expression levels of genes that are expressed in cardiac crest or those known to affect remodeling of the outflow tract, but our data do show that three of them, *Foxc1*, the endothelin A receptor, and plexinA2 (see Brown et al., 2001) are all expressed in *Sema3C* mutant mice in the regions of the branchial arches normally populated by neural crest cells. These observations argue that cardiac crest cells are properly specified and begin to migrate normally in *Sema3C* mutant mice.

Neural crest cell migration into the proximal cardiac outflow tract, however, appears to be impaired. Since *Sema3C* is normally expressed in the myocardial cuff of the outflow tract, a plausible and attractive interpretation of these results is that Sema3C acts as an attractant for cardiac crest cells and helps to promote their entry into the outflow tract. This proposed function would be analogous to its chemoattractant activity for cultured cortical axons (Bagnard et al., 1998). Alternatively, Sema3C could promote entry indirectly, perhaps by inducing the expression of a permissive or attractive signal in nearby tissues. While our results support the hypothesis that *Sema3C* promotes the migration of cardiac crest cells into the proximal outflow tract, we cannot eliminate the possibilities that Sema3C affects cardiac crest cell proliferation, differentiation or survival within the proximal tract.

Cardiac defects including persistent truncus arteriosus and interruption of the aortic arch have been described in *neuropilin-1* knockout mice (Kawasaki et al., 1999). These mice die between E12.5 and E13.5 and have multiple defects in several organ systems. Neuropilin-1 is capable of acting as a coreceptor for the angiogenic signals vascular endothelial growth factor (VEGF) and placenta growth factor 2 (PlGF) (Migdal et al., 1998; Soker et al., 1998). Cardiovascular defects present in *neuropilin-1*$^{-/-}$ embryos have been interpreted to result from impaired angiogenic signaling. However, neuropilin-1 can also serve as an obligatory coreceptor for a subset of class 3 semaphorins including semaphorin 3A and semaphorin 3C (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Defects in axon pathfinding in *neuropilin-1*$^{-/-}$ embryos can be ascribed to a loss of sensitivity to these guidance cues. The striking similarity between cardiac defects observed in the *neuropilin-1* knockout and in the *Sema3C* mutant mice is consistent with the hypothesis that neuropilin-1 functions as a receptor component for Sema3C in cardiac neural crest. Our results strongly argue that the cardiac defects observed in *neuropilin-1*-deficient embryos are due to the inability of cardiac neural crest cells to respond to Sema3C.

The cardiovascular defects resulting from the disruption of

the *Sema3C* locus closely resemble heart defects seen in human infants with congenital heart disease. Some of these arise as a consequence of microdeletions within chromosome 22q11 (Driscoll, 1994), however, a majority of infants with outflow tract and aortic arch defects do not have this particular chromosomal abnormality (Goldmuntz et al., 1998). Semaphorins, their receptors, and the signaling pathways they influence now constitute a new class of candidate genes for human congenital cardiac disease.

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