Repulsive and attractive semaphorins cooperate to direct the navigation of cardiac neural crest cells

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A B S T R A C T

The cardiac neural crest, a subpopulation of the neural crest, contributes to the cardiac outflow tract formation during development. However, it follows the defined long-range migratory pathway remains unclear. We show here that the migrating cardiac neural crest cells (NCCs) express Plexin-A2, Plexin-D1 and Neuropilin. The membrane-bound ligands for Plexin-A2, Semaphorin (Sema)6A and Sema6B, are expressed in the dorsal neural tube and the lateral pharyngeal arch mesenchyme (the NCC "routes"). Sema3C, a ligand for Plexin-D1/neuropilin-1, is expressed in the cardiac outflow tract (the NCC "target"). Sema6A and Sema6B repel neural crest cells, while Sema3C attracts neural crest cells. Sema6A and Sema6B repulsion and Sema3C attraction are diminished either when Plexin-A2 and Neuropilin-1, or when Plexin-D1, respectively, are knocked down in NCCs. When RNAi knockdown diminishes each receptor in NCCs, the NCCs fail to migrate into the cardiac outflow tract in the developing chick embryo. Furthermore, Plexin-A2-deficient mice exhibit defects of cardiac outflow tract formation. We therefore conclude that the coordination of repulsive cues provided by Sema6A/Sema6B through Plexin-A2 paired with the attractive cue by Sema3C through Plexin-D1 is required for the precise migration of migrating cardiac NCCs.

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I N T R O D U C T I O N

The neural crest is a cellular population contained within the leading edge of the neural folds. These neural crest cells (NCCs) migrate to various tissues in a segmented manner in the developing vertebrate embryo when neural tube closes. Depending on their precise destination within the embryo, NCCs generate neurons, glia, pigment cells, chondrocytes and smooth muscle cells (Gammill and Bronner-Fraser, 2003; Le Douarin et al., 2004). After delaminating from the neural tube, NCCs at the level of rhombomeres (r)6 to r8 migrate ventrally and form third, fourth, and sixth pharyngeal arch arteries to the cardiac outflow tract. There, they fuse to form the septum dividing the single great vessel emerging from the heart (the truncus arteriosus; NCCs (Creazzo et al., 1998; Etchevers et al., 2001; Stoller and Epstein, 2005). These columns of cells contribute to the significant cell mass of the endocardial cushions in the outflow tract (Epstein et al., 2000). When cardiac NCCs are ablated in the chick embryo, various malformations including persistent truncus arteriosus and the absence of various combinations of the third, fourth, and sixth pharyngeal arch arteries occur (Kirby et al., 1983; Waldo et al., 1998). Thus, alterations in this complex developmental program lead to a spectrum of pathologies involving the cardiac outflow and the great arteries that are associated with common forms of congenital heart diseases observed in humans (Gruber and Epstein, 2004).

An unresolved issue in neural crest development is how the NCCs establish their precise pathwaying to distinct target organs. Several lines of evidence have shown a role for axon guidance molecules, including ephrin and semaphorin, in NCC migration along the trunk region (Krull et al., 1997; Wang and Anderson, 1997; Eickholt et al., 1999; Santiago and Erickson, 2002). The trunk NCC ventral route is defined by the inhibitory ephrin expression from the dorsal region (Santiago and Erickson, 2002), while NCC segmental organization is defined by the expression of inhibitory class 3 semaphorins, Sema3A
and Sema3F, located in the odd-numbered rhombomeres (r1, r3, and r5) (Osborne et al., 2005; Gammill et al., 2006). In contrast, relatively little is known about the molecular mechanisms that are responsible for the cardiac NCC migration to the cardiac outflow tract. A wide variety of extrinsic and intrinsic programs have been implicated in the cardiac NCC fate decision (Stoller and Epstein, 2005). Mice lacking these factors typically exhibit the interruption of the aortic arch and persistent truncus arteriosus. A similar phenotype has been observed in mice lacking Sema3C (Feiner et al., 2001), Neuropilin-1 (a receptor for class 3 semaphorins) (Kawasaki et al., 1999), or Plexin-D1 (a receptor or co-receptor for class 3 semaphorins) (Gifler et al., 2004; Gu et al., 2005). Zebrafish lacking Sema3D in NCC or Plexin-D1 in vascular endothelial cells also exhibit the impaired cardiac development (Sato et al., 2006; Torres-Vazquez et al., 2004). These results suggested that the class 3 semaphorin-mediated signal is involved in cardiac outflow tract formation. However, it remains unclear whether these defects are caused by abnormalities in migration of NCCs. Furthermore, neither ligands for plexin-A2 nor receptors for Sema3C in the cardiac outflow tract formation have not been determined.

In order to determine the molecular mechanisms underlying how semaphorins regulate the pathfinding of migrating cardiac NCCs, we investigated the semaphorin receptors and their ligands during NCC development. In NCCs, we identified an initial and a continuous expression of Plexin-A2 and the subsequent expression of Neuropilin-1 and Plexin-D1. Concomitant with the spatiotemporal expression of these receptors, the Plexin-A2 ligands Sema6A and Sema6B were expressed as contact repellents along the migratory routes. Sema3C, the ligand for the Neuropilin-1/Plexin-D1 complex, was expressed as a diffusible attractant at the cardiac outflow tract. Perturbation of ligand or receptor expression blocked the proper NCC migration into the cardiac outflow tract. Thus, these results indicate that the cooperation of an attractant at the front and a repellent in the back of the migrating NCCs generates a polarized guidance output along the long-range migratory pathway.

Materials and methods

Plexin-A2 and Plexin-A4 knockout mice

Generation and genotyping of the Plexin-A4−/− alleles or the Plexin-A2−/− alleles have been previously described (Suto et al., 2005, 2007). Mice were backcrossed for eight generations into the C57B6 background. All animal procedures were carried out in accordance with institutional guidelines.

Construction of cDNAs and transfections

Mouse Neuropilin-1 and Plexin-D1 cDNAs have been described previously (Toyofuku et al., 2004b, 2007). Mouse Sema3A and Plexin-A2 cDNAs lacking stop codons were synthesized by PCR, and then were ligated into the pcDNA3.1/V5-HisA (Invitrogen) and pFLAG-CMV-3 (Sigma) expression vectors, resulting in the production of V5-tagged Sema3A and Flag-tagged Plexin-A2, respectively. Sema6A and Sema6B cDNAs lacking N-terminal signal sequences were synthesized by PCR, and then were ligated into pcDNA4/His/Max-A, resulting in the production of Xpress-tagged Sema6A and Sema6B. Resulting plasmids were transfected into HEK293 cells by Eugene-6-mediated transfection (Roche Molecular Biochemicals). Stable cell lines that overexpressed the plasmid encoded proteins were established by selection with 800 µg/ml G418 (Sigma).

Production of soluble recombinant proteins

To produce soluble Fc-fused proteins, the extracellular domains of Sema6A and Sema6B, and the full length Sema3C, cDNAs were ligated into the pEFBos human IgG1 Fc cassette (Suda and Nagata, 1994). These constructs were then transfected into P3U1 plasmacytoma cells by electroporation. Fc-fused proteins were purified from culture supernatants using protein-G sepharose (Pharmacia).

Generation of RNA interference-based knockdown vector and adenovirus

To suppress selected target proteins for loss-of-function analyses, RNA interference (RNAi) was used. For RNAi plasmid construction to direct the synthesis of short hairpin (sh)RNA, DNA templates for the synthesis of shRNAs in which the sense and anti-sense sequences targeting cPlexin-A2, cNeuropilin-1, or cPlexin-D1 were linked with a 9-nucleotide loop (Table S1) were synthesized, and then paired oligonucleotides were annealed and ligated into the pRNAI-H1.1/shuttle (GenScript Co. NJ). For the construction of adenoviral vectors that direct the synthesis of shRNA (RNAi adenovirus), the expression cassette of the pRNAI-H1.1/shuttle encoding respective shRNA (RNAi plasmid) was excised and ligated into BD Adeno-X viral DNA (BD Biosciences Clontech). After transfection of adenoviral vector into HEK293 cells, adenovirus-containing medium was collected and concentrated.

Semaphorin-alkaline phosphatase binding assay

Semaphorin-alkaline phosphatase binding assays were performed as described previously (Tamagnone et al., 1999). The extracellular domains of Sema3C, Sema6A, or Sema6B were expressed as fusion proteins with placental secreted alkaline phosphatase at the N-terminus (AP-Sema3C, AP-Sema6A, AP-Sema6B). To assess binding, HEK293 cells were transiently transfected with expression vectors encoding Plexins and NP1. After incubation with AP-Sema proteins, cells were fixed and incubated at 65 °C for 100 min to inactivate endogenous AP activity. Cells expressing Plexin, which bound to AP-Sema, were detected histologically.

Surface biotinylation-chase experiment

HEK293 cells expressing Sema6A or Sema6B were biotinylated using a Cellular Labeling and Immunoprecipitation kit (Boehringer Mannheim). After biotinylation, the culture supernatants and the cells were harvested at the indicated times, washed and solubilized in 1% NP-40, after which they were centrifuged at 100,000 g for 1 h to remove membrane debris. The contents of the remaining supernatant and cell lysates were immunoprecipitated with streptavidin and subjected to SDS-PAGE. The immunoblots were probed with anti-Xpress-HRP conjugates (Invitrogen) and developed using ECL reagent (Amersham Pharmacia Biotech. Uppsala, Sweden).

Neural crest explant culture and stripe assay

Neural crest explant cultures were performed as described previously (Eickholt et al., 1999). Briefly, neural tube extending from the mid-optic placode to the 3rd somite, from which the robust migration of cardiac NCCs occur, were dissected from chick embryos at stage 9 and incubated for 5 min in Dispase (1 mg/ml in L-15) to remove the menenchyme and the notochord. For the stripe assay (Eickholt et al., 1999; Vielmetter et al., 1990), neural tube explants were placed on coverslips which had been coated with alternating substratum stripes. Briefly, poly-L-lysine-coated coverslips were placed on silicone matrices. A mix of the purified Sema3C-Fc, Sema6A-Fc, Sema6B-Fc, or control IgG (80 µg/ml) plus fibronectin (1 µg/ml) in PBS was injected into canal and incubated for 3 h at 37 °C. After removing unbound proteins by PBS injection, BSA-FITC was injected into the canal and incubated for 1 h at 37 °C. Coverslips were then removed from matrix, washed with PBS, and then incubated with fibronectin (10 µg/ml) for 3 h at 37 °C. Neural tube explants were cultured on striped substrata for 20 h and examined with anti-HNK-1 antibody (Zymed, 1:100) followed by Rhodamine-conjugated anti-mouse IgG. For adenovirus-mediated
RNAi experiments, purified adenovirus, which directs synthesis of siRNA in vivo, was applied to cultured neural tubes for 6 h and then the explants were used for the stripe assay.

**In ovo RNAi of chick embryo**

Embryos obtained from fertilized chicken eggs (Takeuchi Farm Inc.) were staged according to the Hamburger and Hamilton method. For tracing of NCCs lacking specific target proteins in the developing chick embryos, cPlexin-A2 RNAi plasmid, cNeuropilin-1 RNAi plasmid, or cPlexin-D1 RNAi plasmid were electroporated into chick embryos stage 9 in ovo using an electroporator (CUY-21, BEX Co.). Briefly, tungsten needles, serving as anode and cathode electrodes, were placed on either side of the neuroepithelium. Concurrent with the injection of the solution containing RNAi plasmid (40 pmol/ml) into the neural tube with a sharp glass pipette, three electric pulses were applied (25 V, 50 ms). Eggs were shielded and allowed to develop up to HH stage 20. At 48 h post-electroporation, embryos were removed and fixed. GFP-positive cells in whole-mount embryos were visualized under fluorescence microscopy and sectioned for immunohistochemistry.

**Cell implantation**

Embryos were incubated until stage 9. The eggs were windowed, the vitelline membrane was removed, and an incision was made between neural tube and the surrounding tissue from the mid-optic placode to the 3rd somite (corresponding to r6 to r8). HEK293 cells expressing Sema3C, Sema6A, or Sema6B were pelleted and pushed into position. Eggs were sealed and incubated for up to 24 h.

**Corrosion casting**

Corrosion casting was performed (Polysciences). Briefly, heterozygotes and homozygotes of Plexin-A2+/− mice at P0 were anesthetized with ketamine and perfused with PBS 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 after a small hole was introduced into the right atrium. A 1 ml customized with ketamine and perfused with PBS through the left ventricle between neural tube and the surrounding tissue in the third and fourth through sixth pharyngeal arch arteries and in the mesenchymal tissue at the cardiac outflow tract (Figs. 1C and D). On the other hand, neither Plexin-D1 nor neuropilin-1 were detected in the NCCs at the stage 10. At stage 20, Plexin-D1 expression became prominent in the endothelial cells of the pharyngeal arch arteries and in endocardial and mesenchymal cells of the cardiac outflow tract (Figs. 1E and F). Neurophilin-1 became prominent in the dorsal root ganglion, the endothelial cells of vessels including the pharyngeal arch arteries, and the endocardial and mesenchymal cells of the cardiac outflow tract (Figs. 1G and H). In addition, Plexin-A2, Plexin-D1 and neuropilin-1 expressions (Figs. 1I–N) exhibited a similar distribution of the neural crest cell marker HNK-1 beside neural tube (Figs. 1I’, K’ and M’) and in the mesenchyme of cardiac outflow tract (Figs. 1J’, L’ and N’), suggesting that NCCs express Plexin-A2, Plexin-D1 and neuropilin-1 mRNAs during migration. These expression patterns of Plexin-A2, Plexin-D1 and Neuropilin-1 in chick embryos appeared to be similar to those observed in mouse embryos (Brown et al., 2001; Gitler et al., 2004; Kawasaki et al., 1999).

Thus, the expression pattern of Plexin-A2, Plexin-D1 and neuropilin-1 implicates the role of each receptor in cardiac NCC migration.

**Semaphorin receptor ligand identification and expression during cardiac neural crest development**

Several studies in vertebrates have shown that type A plexins serve as direct receptors for class 6 semaphorins (Toyofuku et al., 2004a; Suto et al., 2005), and in Drosophila, Plexin-A serves as a direct receptor for class 6-related Sema-1a (Winberg et al., 1998). Alkaline Phosphatase (AP)-conjugated Sema6A (AP-Sema6A) specifically binds to cells expressing Plexin-A2 and Neuropilin-1 (Toyofuku et al., 2004b), and in Drosophila, Plexin-D1 and Neuropilin-1 heterodimer, the weak binding to neuropilins-1 alone or neuropilins-1/Plexin-D1 heterodimer, the weak binding to neuropilins-1 alone or neuropilins-1/Plexin-D1 heterodimer, and the weakest binding to Plexin-A1 alone or Plexin-D1 alone (Gitler et al., 2004). Consistent with their results, our binding experiment together with the colorimetric analysis of bound AP demonstrated a direct binding of AP-Sema6A as well as AP-Sema6A to cells expressing Plexin-A2 and the binding of AP-Sema6A to cells expressing both Plexin-D1 and NP1 and the weak binding to neuropilins-1 alone or neuropilins-1/Plexin-A2 heterodimer (Figs. 2A and B).

The expression of Sema6A, 6B, and 3C was analyzed in vivo by in situ hybridization at Hamburger–Hamilton (HH) stages 10 and 20 (Figs. 1O–X). At stage 10, Sema6A and Sema6B mRNA expression was first detected in the somite and the dorsal side of neural tube, respectively (Figs. 1O and P). At stage 20, the distribution of Sema6A mRNA was detected in dorsal side of neural tube, myotome and lateral ectoderm (Figs. 1Q and R). Sema6B mRNA expression was detected in dorsal root ganglion, the dorsal side of neural tube and the myotome...
(Figs. 1S and T). On the other hand, Sema3C mRNA expression was not detected at the stage 10 but then was prominent in the anterior horn cells, and the muscular layer of the cardiac outflow tract at the stage 20 (Figs. 1U and V). In this region, HNK-positive cardiac NCCs lie adjacent to the territory of Sema3C mRNA (Figs. 1W and X). Thus, these expression patterns suggest that the interaction between semaphorins and their receptors may play a role in cardiac NCC migration.

NCCs exhibit a repulsive response to both Sema6A and Sema6B but an attractive response to Sema3C

In order to examine the activities of semaphorins expressed along the NCC migratory route, two different assays were employed. First, NCCs isolated from HH stage 10 embryos were placed on alternating stripes consisting of either the semaphorin–Fc chimeric protein (30 nM) plus fibronectin or fibronectin alone to determine the effect of semaphorins on the in vitro migration of NCCs. Although the NCCs did not display any preference in their migration behaviors between stripes containing control IgG plus fibronectin and those containing fibronectin alone (Fig. 3A), a majority of NCCs migrated on the stripes containing fibronectin alone and avoided the stripes containing Sema6A–Fc or Sema6B–Fc (Figs. 3B and C). In contrast, NCCs migrated preferentially on the stripes containing Sema3C–Fc (Fig. 3D). These results also indicate that the ectodomain of semaphorins such as semaphorin–Fc protein is involved in the regulation of NCC migration.

Fig. 1. Expression of semaphorin receptors and semaphorin ligands in chick embryos. (A–N) cPlaxin-A2, cPlexin-D1, cNeuropilin-1 and cRhoB expression were analyzed by either transverse section (A, B, D, F, H, I–N) or whole-mount (C, E, G) in situ hybridization and HNK-1 expression were analyzed by immunostaining (I′–N′). (A, B) In HH stage 10, cPlexin-A2 is expressed in regions lateral to the dorsal side of neural tube (Nt) and in a pattern that resembles that of cRhoB, (C–H) In HH stage 20, cPlexin-A2 expression is detected in the dorsal root ganglion (Drg) and the mesenchyme surrounding the 2nd, 3rd and 4–6th pharyngeal arch arteries (2, 3, 4/6) and extends into the mesenchyme of the outflow tract (Oft) (C, D), cPlexin-D1 expression is detected in the pharyngeal arch arteries (2, 3, 4/6) and the endothelial cells of aortic arteries (Aa, Da), and endocardial cells in the Oft (E, F), cNeuropilin-1 expression overlaps cPlexin-D1 expression in Drg and the endothelial and endocardial cells but is also seen in the tissue surrounding vessels and mesenchyme of the Oft (G, H). cPlexin-A2, cPlexin-D1 and cNeuropilin-1 expressions (arrowheads) are detected in migrating NCCs besides neural tube (I, K, M) and in the mesenchyme of Oft (I′–N′). Migrating NCCs were visualized by immunostaining with anti-HNK-1 antibody in sections adjust to panels I to N, respectively. (O–V) cSema6A, cSema6B and cSema3C expression were analyzed by transverse section (O, P, R, T, V) or whole-mount (Q, S, U) in situ hybridization. (O, P) In HH stage 10, cSema6A expression is detected in the somite (Sm) and the dorsal side of neural tube (Nt), respectively, (Q–V) In HH stage 20, cSema6A expression is also prominent in dorsal side of Nt and myotome (My) and lateral side of ectoderm (Q, R), cSema6B expression is in Drg, My, and the dorsal side of Nt (S, T), and cSema3C expression is in the anterior horn cells (Ah) and myocardial layer of Oft (U, V). (W, X) NCCs were immunostained with an anti-HNK-1 antibody in whole-mount (W) and in transverse sections (X). NCCs migrate in a stream medial to My through the tissue surrounding the aortic arteries (Aa, Da) into the mesenchyme of the Oft. Scale bar, 100 µm. Aa, aortic artery; Cv, cardinal vein; Ct, cardiac tube; Da, dorsal aorta; Drg, dorsal root ganglion; My, myotome; Nt, neural tube; Oft, outflow tract; Tr, trachea.
Furthermore, the NCC responsiveness to each semaphorin was examined by this stripe assay using a neural tube infected with shRNA synthesizing adenovirus against selected target mRNA (RNAi adenovirus). These RNAi adenoviruses efficiently and specifically suppressed target mRNA and protein (Fig. 3E and Fig. S2). When Plexin-A2 expression in NCCs was knocked down by such adenovirus infection, NCCs no longer avoided stripes containing Sema6A–Fc or Sema6B–Fc but demonstrated preference for stripes containing Sema3C–Fc (Figs. 3F–H). In contrast, shRNA-mediated knockdown of either neuropilin-1 or Plexin-D1 suppressed an attractive response of NCCs to Sema3C–Fc without affecting a chemorepulsive response to Sema6A or Sema6B (Figs. 3I–N). Thus, the NCC migratory behavior to each semaphorin was determined by the corresponding receptor.

Second, we also examined the guidance activities of semaphorins on NCC migration in vivo by implanting semaphorin-expressing cells to ectopic positions in the HH stage 10 chick embryo. Surface biotinylation-chase experiment showed the progressive decrease in full-length (150 kD) of Sema6A and Sema6B in the membrane fraction, in parallel with the increase in cleaved extracellular region (100 kD) of these semaphorins in the supernatant (Fig. 4A), indicating that Sema6A and Sema6B expressed in HEK293 cells probably exerts their activities as locally released soluble proteins and as membrane-bound proteins. Embryos receiving cell implants were sectioned and examined by immunohistochemistry using an anti-HNK-1 antibody (HH stage 20) (Figs. 4B–E). To quantitatively analyze the effect of semaphorin expressed in cells on NCC migration in vivo, we implanted semaphorin-expressing cells to ectopic positions in the HH stage 10 chick embryo. Surface biotinylation-chase experiment showed the progressive decrease in full-length (150 kD) of Sema6A and Sema6B in the membrane fraction, in parallel with the increase in cleaved extracellular region (100 kD) of these semaphorins in the supernatant (Fig. 4A), indicating that Sema6A and Sema6B expressed in HEK293 cells probably exerts their activities as locally released soluble proteins and as membrane-bound proteins. Embryos receiving cell implants were sectioned and examined by immunohistochemistry using an anti-HNK-1 antibody (HH stage 20) (Figs. 4B–E).
the NCC migration, we measured the distance between the edge of implant and NCCs around implant (Figs. 4F–J). Compared with control implants (Figs. 4B, F and J), implants of either Sema6A- or Sema6B-expressing cells resulted in skewed NCC migrations. That is, NCCs steered around the Sema6A- or Sema6B-rich regions encircling the implanted cells (Figs. 4C, D, G, H and J). On the other hand, many NCCs were attracted to and accumulated around the Sema3C-expressing implants (Figs. 4E, I and J). These results indicate that NCCs exhibit two different semaphorin type-specific responses: repulsion to Sema6A or Sema6B and attraction to Sema3C.

NCCs lacking semaphorin receptors lost their ability to migrate into the cardiac outflow tract

To investigate whether semaphorin signals are necessary for NCC navigation to the cardiac outflow tract in vivo, we knocked down the expression of cPlexin-A2, cPlexin-D1 or cNeuropilin-1 of neural cells in the neural tube before the robust migration of NCCs occurred in this region. This knock down was achieved by electroporating the pRNAT-H1.1/shuttle plasmid (RNAi plasmid) encoding shRNA and GFP into the right side of the neural tube in the stage 9 chick embryos. shRNAs synthesized by RNAi plasmid successfully suppressed the target mRNA and protein levels, when their RNAi constructs were cloned into adenovirus and transduced in NCCs (Fig. 3A and Fig. S2). At 48 h post-electroporation with RNAi plasmid, electroporated embryos were sectioned and immunostained with an anti-HNK-1 antibody (Figs. 5E–H). A majority of HNK-1-positive NCCs were successfully electroporated with RNAi plasmid. GFP-positive cells that expressed control shRNA could migrate normally into the cardiac outflow tract (Figs. 5A and E). When Plexin-A2 expression was knocked down, most of the GFP-positive cells lingered in the dorsal region.
Fig. 5. Distribution of NCCs lacking cPlexin-A2, cPlexin-D1 or cNeuropilin-1 in chick embryo. After electroporation of RNAi plasmid into the right-side of the neural tube at r2 to 8 of HH stage 10 embryo, the distribution of GFP-positive NCCs (green) in HH stage 19–22 chick embryos were detected by fluorescence microscopy (A–D) or by immunohistochemistry on transverse sections with an anti-GFP antibody (green) and an anti-HNK-1 antibody (red) (E–H and high power shown in panels I–L). Note that a majority of cells migrating from neural tube (Nt) show the overlap of GFP-positive labeling with HNK-1-positive labeling (I–L). (A, E, I) Migrating NCCs electroporated with control RNAi plasmid reside in their characteristic positions. (B, F, J) Migrating NCCs electroporated with cPlexin-A2 RNAi plasmid distribute in regions dorsal–lateral to the neural tube (Oft). (C, G, K) Migrating NCCs electroporated with cPlexin-D1 RNAi plasmid diffusely distribute in the longitudinal and transverse axes without in the cardiac outflow tract (D, H, L) Migrating NCCs electroporated with cNeuropilin-1 RNAi plasmid diffusely distribute in a similar pattern to that of cPlexin-D1. Numbers in panels A–D indicate the number of pharyngeal arches. Ot, otic vesicle. (M). Localization of GFP-positive NCCs into three domains defined therein: dorsal domain (D), middle (M), and ventral (V). N, notochord; F, ventral endoderm of foregut. (N) Quantification of the numbers of GFP-positive NCCs located in distinct domains of chick embryo. In the dorsal domain, GFP-positive cells in neural tube were estimated as neuroepithelial cells instead of NCCs and were omitted. Data in each domain obtained from 10 experiments are summarized as a percentage of the total GFP-positive NCCs. Horizontal bar indicates SEM. Scale bar, 100 µm.
region and failed to egress from the area where Sema6A and Sema6B were expressed (Figs. 5B and F). The GFP-positive cells with suppressed Plexin-D1 expression distributed diffusely in the longitudinal-transverse axes instead of entering the cardiac outflow tract, even though they still migrated out from the dorsal region (Figs. 5C and G). A similar distribution of GFP-positive cells was observed when cNeuropilin-1 expression was suppressed (Figs. 5D and H).

In order to estimate the distribution of the migratory population, serial sections of some of the electroporated embryos were analyzed by measuring the area occupied by GFP-positive cells. Transverse sections of embryo were visually divided into three domains (dorsal, middle, and ventral), and the number of GFP-positive cells in each domain was counted (Fig. 5I). Compared to the experiments using a control RNAI plasmid (D:M:V = 14: 9: 77), the experiments using a cPlexin-A2 RNAI plasmid or cNeuropilin-1 RNAI plasmid exhibited the diffuse distribution in the pharyngeal arches (D:M:V = 18: 62: 20 for cPlexin-D1 RNAI and 15: 64: 21 for cNeuropilin-1 RNAI) (Fig. 5J). Thus, the distinct semaphorin signaling pathways is required for the correct pathfinding of NCCs. The departure of NCCs from the dorsal side of the trunk is dependent on the Plexin-A2-mediated repulsion, and the destination of NCCs is dependent on the Plexin-D1/Neuropilin-1-mediated attraction.

**Cardiovascular malformations of Plexin-A2−/− mice**

Suto et al. have recently generated a Plexin-A2−/− allele and demonstrated that Plexin-A2, in cooperation with Plexin-A4, and their putative ligand SemalA6, all regulate the lamina-restricted projection of mossy fibers in the hippocampus (Suto et al., 2007). We analyzed the binding of AP–Sema6A, AP–Sema6B, and AP–Sema3C to NCCs derived from the neural tube of Plexin-A2 mutant mice (Fig. 6A). AP–Sema6A, AP–Sema6B and AP–Sema3C bound to NCCs in Plexin-A2−/− mice, but AP–Sema6A and AP–Sema6B did not bind to NCCs in Plexin-A2−/− mice. This finding also confirmed that mouse embryo NCCs respond to Sema6A and Sema6B through Plexin-A2. We next analyzed offspring from intercrosses of heterozygous mice. In 8 out of 13 Plexin-A2−/− mice at E12.5, transverse embryo sections exhibited structural defects in the cardiac outflow of the heart. Aortic and pulmonary channel septation (Fig. 6B), where Plexin-A2 is prominently expressed, was lacking (Fig. 6C, asterisk) (Brown et al., 2001). These results indicate that Plexin-A2 is involved in the cardiac outflow tract in mice, but AP–Sema6A and AP–Sema6B did not bind to NCCs in Plexin-A2−/− mice. These findings also conformed to the findings that mice embryo NCCs respond to Sema6A and Sema6B through Plexin-A2. The plasma and attractive interpretation of these results is that Sema6A and Sema6B work as contact repellents, pushing the underlying NCCs toward the cardiac outflow tract. This proposed function of Sema6A and Sema6B would be consistent with the chemorepellent activity of Sema6A observed in cerebellar granule cell migration through Plexin-A2 (Leighton et al., 2001; Kerjan et al., 2005; Renaud et al., 2008), in spinal motor neuron migration through Plexin-A2 (Bron et al., 2007; Mauti et al., 2007), and in hypothalamic mossy fiber extension through Plexin-A2 and Plexin-A4 (Suto et al., 2007), and that of Sema6B in peripheral sensory and sympathetic ganglion axons through Plexin-A4 (Suto et al., 2005).

The molecular mechanism of semaphorin-mediated growth cone collapse has been extensively investigated. However, how semaphorin–plexin signaling controls cell migration is still unclear. It is likely that Sema6A/Sema6B–Plexin-A2 signaling moderates cell migration through controlling the cell–cell and/or cell–matrix adhesion. Secreted class 3 semaphorins, by binding to neuropilin and to the type A plexin complex, exert their repulsive activity via the suppression of integrin-mediated cell adhesion (Serini et al., 2003; Mauti et al., 2007; Danovaro and Mauti, 2007). Drosophila Sema1a, an invertebrate orthologue of class 6 semaphorins, facilitates motor axon defasciculation at the neuromuscular junction by countering the homophilic adhesion of fasciculin II protein (Yu et al., 2000) and ensures class-specific olfactory axon segregation by countering the adhesion of N-cadherin (Lattemann et al., 2007). In fact, cranial NCC motility is regulated by the integrin-mediated cell adhesion on surrounding tissues (Strachan and Condic, 2004). Alternatively, recent study has demonstrated that Sema6A–Plexin-A2 signaling controls centrosome positioning in migrating cerebellar granule cells (Renaud et al., 2008). In migrating neurons, microtubules linked to the centrosome pull the nucleus during migration, thereby positioning nucleus toward the leading process. Thus, actin and microtubule dynamics involved in the semaphorin-mediated growth cone collapse may regulate cell migration of NCCs through controlling centrosome positioning. Furthermore, our study demonstrated that the extra-cellular region of Sema6A and Sema6B can be cleaved and function as locally diffused repellents. If it is the case in vivo, cells responsive to these semaphorins such as NCCs and cerebellar granule cells could migrate along the concentration gradient of released repellents. Altogether, class 6 semaphorins such as Sema6A and Sema6B could function as NCC releasing molecules, thereby facilitating their detachment from the neural tube and their migration from the dorsal region.

**Discussion**

NCCs are generated from subsets of neuroepithelial cells through a sequential intrinsic program and obtain mesenchymal properties, most evidently, the capability to migrate to a specific target organ (the epithelial–mesenchymal transformation) (Gammill and Bronner-Fraser, 2003; Le Douarin et al., 2004). The polarized guidance response generated to a very shallow gradient of chemotactic molecules can be established by the asymmetric localization of intracellular signaling components (Charest and Firtel, 2006; Ridley et al., 2003). However, how long-range migrating cells such as NCCs achieve the precise pathfinding to a target organ remained unclear. Here, we show evidence that the polarized NCC guidance response is established by the coordination of contact repellents such as transmembrane class 6 semaphorins expressed along the migratory routes as well as by diffusible attractants such as class 3 semaphorins at the target tissue.

Sema6A and Sema6B initiate NCC migration from dorsal neural tube through Plexin-A2

Cardiac NCC pathfinding requires segmented and dorso-ventral directional cues. The migratory pathway of cardiac NCCs appears to be demarcated by the territory provided by Sema6A and Sema6B. Both in vitro and in vivo analyses reveal that NCCs exhibit a repulsive response to Sema6A and Sema6B through Plexin-A2. The plausible and attractive interpretation of these results is that Sema6A and Sema6B work as contact repellents, pushing the underlying NCCs toward the cardiac outflow tract. This proposed function of Sema6A and Sema6B would be consistent with the chemorepellent activity of Sema6A observed in cerebellar granule cell migration through Plexin-A2 (Leighton et al., 2001; Kerjan et al., 2005; Renaud et al., 2008), in spinal motor neuron migration through Plexin-A2 (Bron et al., 2007; Mauti et al., 2007) and in hypothalamic mossy fiber extension through Plexin-A2 and Plexin-A4 (Suto et al., 2007), and that of Sema6B in peripheral sensory and sympathetic ganglion axons through Plexin-A4 (Suto et al., 2005).
Fig. 6. Cardiovascular phenotype of Plexin-A2 mutant mice. (A) Micrographs of binding assays that test the binding of AP-Sema6A, AP-Sema6B, or AP-Sema3C on NCCs derived from Plexin-A2 mutant mice at E12.5. By using alkaline phosphatase (AP) substrates, significant staining was observed between AP-Sema6A, AP-Sema6B, or AP-Sema3C and NCCs derived from Plexin-A2+/− mice, and between AP-Sema3C and NCCs from Plexin-A2−/− mice. Scale bar, 50 µm. (B) Histology of sequential transverse sections through the aortic sac (Top to bottom) in Plexin-A2+/− mice (left panels) and Plexin-A2−/− mice (right panels) at E11.5. Arrows indicate the sites where the aortopulmonary septum normally forms and which have a high percentage of neural crest-derived cells. Note a septation of the aortic and pulmonary channels of Plexin-A2+/− mice but a lack of septation in Plexin-A2−/− mice. Ao, aortic trunk; E, esophagus; PT, pulmonary trunk; T, trachea; TA, truncus arteriosus. (C) Transverse section in situ hybridization reveals Plexin-A2 expression restricted to the cardiac outflow tract (asterisk) in Plexin-A2+/− mice but not in Plexin-A2−/− mice. (D) The cardiovascular phenotype is characterized by corrosion casting. Methyl methacrylate was injected into the left ventricle of anesthetized P0 Plexin-A2 mutant mice. The cast of Plexin-A2+/− mice shows normal vascular architecture (left panel). Cast of Plexin-A2−/− mice shows the incomplete interruption of the aortic arch (Type B interruption, arrow) and with normal septation of the aortic and pulmonary trunks (middle panel). Another cast of Plexin-A2−/− mice shows persistent truncus arteriosus and interruption of the aortic arch (Type C interruption). The descending aorta has filled because the ductus arteriosus (arrowhead) has not closed completely. Ao, ascending aorta; Ba, branchiocephalic artery; Da, descending artery; E, esophagus; Lca, left common carotid artery; Lsa, left subclavian artery; Pa, pulmonary artery; PTA, persistent truncus arteriosus; Rca, Right common carotid artery; Rsa, Right subclavian artery; T, trachea. (E) Translucent (upper panels) and immunostained images (lower panels) on transverse sections through the septation of aortic arch and pulmonary trunk (arrows) in Plexin-A2 mutant mice. HNK-1-positive NCCs are distributed in the endocardial cushion of the cardiac outflow tract, and they are delineated from MHC-positive myocardial cells. Note the fewer but various amounts of HNK-positive NCCs in Plexin-A2−/− mice when compared with those in Plexin-A2+/− mice. Scale bar, 100 µm for panels A, B, C, and E, 250 µm for panel D. (F) Summary of HNK-1 positive cells in cardiac outflow tract shown in panel E. Data are displayed as mean pixel intensity (arbitrary units) after subtraction of background (mean±SEM, n=5). ⁎ p<0.01 from Plexin-A2+/−.
Sema3C attracts NCCs to the cardiac outflow tract through the Plexin-D1/Neuropilin-1 complex

After migrating through the dorso-lateral territory, cardiac NCCs reach their final target destinations and subsequently differentiate into endocardial and smooth muscle cells. Therefore, in addition to the necessity of Sema3A and Sema6A as an NCC egress from the dorso-lateral region, another guidance cue must be required for attracting to and accumulating NCCs at the target tissue. Our present study demonstrated that Sema3C functions as this attraction cue. The timing of Sema3C expression at the outflow tract region appears to coincide with that of NCC accumulation at the same region. In fact, migrating NCCs accumulate in the cardiac cushion of the cardiac outflow tract and are surrounded by the Sema3A-expressing myocardial layer. Furthermore, Sema3C attracts NCC migration in vitro as well as in vivo.

Individual class 3 semaphorins can function as both chemorepellents and chemoaffectants, depending on the particular axon type examined (Fiore and Puschel, 2003; Fujisawa, 2004). Sema3C repels sympathetic axons (Chen et al., 1998; Takahashi et al., 1998) but attracts cortical neurons (Bagnard et al., 1998). There have been several reports that class 3 semaphorins convert attractive and repulsive responses in target cells. Levels of cGMP and cAMP in growth cones may determine the attractive and repulsive responses of rat dorsal root ganglion axons, respectively (Song et al., 1998). Furthermore, the functional difference of class 3 semaphorins may be dependent on their activity on integrin function. The repulsive activity of Sema3A on endothelial cells requires the suppression of integrin function (Serini et al., 2003), while the attractive activity of Sema3C on endothelial cells requires the activation of integrin function (Banu et al., 2006). Thus, the particular interactions of neuropilins and specific plexins may confer the specificity of growth cone responses to each class 3 semaphorin. Sema3C binds to neuropilins-1 with the higher affinity to the Neuropilins-1/Plexin-D1 heterodimer than the Neuropilins-1/Plexin-A2 heterodimer. Although it is not completely ruled out the possibility that Plexin-A2 function as a co-receptor for Sema3C-mediated signal, the stripe assay indicates that Sema3C-induced attraction is mainly mediated through the Neuropilin-1/Plexin-D1 heterodimers.

Defects of the cardiac outflow tract formation in semaphorin–plexin knockout mice

Plexin-A2/+ mice exhibited cardiovascular defects which could be caused by the abnormality of NCC-mediated cardiac outflow tract formation. In fact, the accumulation of NCCs in the cardiac outflow tract seen in Plexin-A2/+ mice appeared to be less than that in Plexin-A2−/− mice (Fig. 6E). Since NCCs from Plexin-A2+/− mice exhibited similar BrdU incorporation and apoptotic cell populations to those from Plexin-A2−/− mice (Fig. S3), the abnormal migratory behavior of NCCs observed in this study might contribute to cardiac outflow tract formation defects in Plexin-A2 mutant mice. It is unknown how some populations of NCCs still migrate into cardiac outflow tract in Plexin-A2−/− mice. Plexin-A4 expression, which is negligible in the cardiac outflow tract (Fig. S1), also binds directly to Sema6A and mediates repulsive activity on the mossy fiber projection (Suto et al., 2005). Since Plexin-A4 expression appeared to be upregulated in the cardiac outflow tract in Plexin-A2−/− mice (Fig. S4), Sema6A signal could be transmitted through Plexin-A4 in Plexin-A2−/− mice. In fact, Plexin-A2+/−;Plexin-A4−/− mice exhibited incomplete septation of the truncus arteriosus at a higher ratio than Plexin-A2−/− mice at E 12.5 (data not shown), suggesting that defects in cardiac outflow tract in Plexin-A2−/− mice is rescued by the Plexin-A4. These results indicate that the low penetrance of the cardiovascular phenotype of Plexin-A2−/− mice is due to the compensatory mechanism through Plexin-A4 in NCCs.

Cardiovascular defects involving the cardiac outflow and the aortic arch have been described in Sema3C, Plexin-D1, and Neuropilin-1 knockout mice (Feiner et al., 2001; Gitler et al., 2004; Kawasaki et al., 1999). Binding experiments revealed that these gene products comprise a receptor-ligand paracrine signal pathway (Gitler et al., 2004 and this study). Reduced NCC accumulation in the cardiac outflow tract is observed in Sema3C−/− and Neuropilin-1−/− mice, but not in Plexin-D1−/− mice. Thus, cardiac defects in Plexin-D1−/− mice may be caused primarily by the dysfunction of other Plexin-D1-expressing endothelial cells, while the NCC migratory behavior could still be compensated by another plexin family member. In the chick embryo, NCCs lacking Plexin-D1 or Neuropilin-1 could egress from the dorsal neural tube and migrate close to the pharyngeal arches but fail to invade the cardiac outflow tract. As time-lapse analyses reveal the failure of NCCs lacking Neuropilin-1 to invade into pharyngeal arches (McLennan and Kulesa, 2007), cardiovascular defects in Sema3C and Neuropilin-1 knockout mice should similarly be caused by the failure of Sema3C-mediated NCC invasion into the cardiac outflow tract.

In conclusion

A key issue in guidance signaling is that migrating cells must achieve a polarized output. A sufficiently robust difference between the front and the back of the cells is required to facilitate migration. One way to achieve this difference is to amplify the initially subtle concentration gradient from one side of the cell to the other (Charest and Firtel, 2006; Ridley et al., 2003). In addition to an intrinsic gradient amplifier, our results demonstrated that long-range migrating cells such as the NCCs use a dynamic balance of attractants and repellents as an effective mechanism to increase guidance polarity. In the developing brain, specific classes of neuroblasts migrate over long ranges, and this neuroblast migration also depends on a dynamic balance of repulsive and attractive molecules (Murase and Horwitz, 2002; Wu et al., 1999; Liu and Rao, 2003). Our results therefore provide a broad implication for our understanding of signaling pathways in long-range cell migration during organogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.028.

References


