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Role of Semaphorin Signaling During Cardiovascular Development

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Semaphorins are a family of secreted and membrane-bound molecules that play critical functions in diverse biological processes.[1–9] SEMA1A/Fasciclin IV is the founding member of the semaphorin family and was first reported in 1992 as a regulator of axon branching in the growth cone of grasshopper embryos.[10] To date, more than 20 semaphorin members have been discovered in viruses, invertebrates, and vertebrates.[3,5,11] In addition to acting as guidance cues for axon growth in the nervous system, semaphorins have been implicated in many other systems, such as immune responses, tumor angiogenesis, bone development and homeostasis, and cardiovascular development.[1-9]

Semaphorins are categorized into 8 classes. Classes 1 and 2 are found in invertebrates, classes 3 to 7 are present in vertebrates, and class V semaphorins are found only in viruses.[3,12] All semaphorins contain a conserved domain of about 500 amino acids, termed the sema domain, which is located close to the N-terminal end of the molecule[13] (Figure 1). Crystal structures have revealed that the sema domain is composed of a 7-bladed β-propeller fold, arranged radially around a central axis. Each blade comprises a 4-strand antiparallel β-sheet.[14,15] With the exception of certain poxvirus semaphorins, other viral and all animal semaphorins contain a plexin-semaphorin-integrin domain in their extracellular regions, immediately to the C-terminal of the sema domain.[12,16,17] The plexin-semaphorin-integrin domain is a disulfide-rich motif that is found in plexins, semaphorins, and integrins. In addition to the defined sema and plexin-semaphorin-integrin domains, semaphorins can be further distinguished by other specific additional motifs (Figure 1). For instance, classes 2, 3, 4, and 7 contain an immunoglobulin-like domain, class 5 members contain 7 thrombospondin type 1 repeats, and class 3 members contain a basic domain. Some class V members contain an immunoglobulin-like domain, but others do not.[3,12,16] Semaphorins can be membrane anchored or secreted. Classes 1, 4, 5, and 6 are transmembrane proteins, whereas classes 2, 3, and V are secreted proteins. Class 7 proteins are glycosylphosphatidylinositol-linked proteins.[3,11] Some transmembrane semaphorins, such as SEMA6D, also have secreted forms to act both locally and over a long distance.[18,19]

Plexins are the primary receptors for semaphorins[20-22] (Table 1). They are segregated into 4 subfamilies, including PLXNA1-4, PLXNB1-3, PLXNC, and PLXND.[2,6,12,23,24] Similar to semaphorins, all plexins contain a sema domain at their N-terminal ends (Figure 1). The extracellular domains of plexins also contain 3 plexin-semaphorin-integrin motifs and 3 to 6 immunoglobulin-like plexin transcription factors domains. On the intracellular side, plexins are comprised of a rho GTPase-binding domain and a segment GTPase-activating protein domain.[2,6,12,23,24] Recent structural studies have shown that semaphorins form a homodimer through intermolecular disulfide bridges to trigger plexin signaling. Semaphorins and plexins interact with each other through their respective sema domains in a “head-to-head” orientation. Two plexin molecules bind 1 semaphorin homodimer to form a bivalent 2:2 complex to mediate cell-cell signaling. Proteolytic cleavage of the semaphorin dimer results in its dissociation to monomeric semaphorin, which can still bind plexin but fails to trigger signaling.[11,23,25] The same semaphorin molecule can interact with different plexins, and different semaphorins can interact with the same plexins, adding to the complexity of semaphorin-plexin signaling (Table 1). It is noteworthy that the biological activities of the semaphorin-plexin interaction can be further modified by coreceptors. For example, class 3 semaphorins bind to neuropilin (NRP)/plexin complexes, which require NRP1 and/or NRP2 as coreceptors in the complexes.[20-22] In another example, SEMA6D interacts with the PLXNA4/vascular endothelial growth factor (VEGFR2) receptor complex to stimulate endothelial cell migration in the outflow tract (OFT) region of chicken embryonic hearts,

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J Am Heart Assoc. 2018;7:e008853. DOI: 10.1161/JAHA.118.008853.

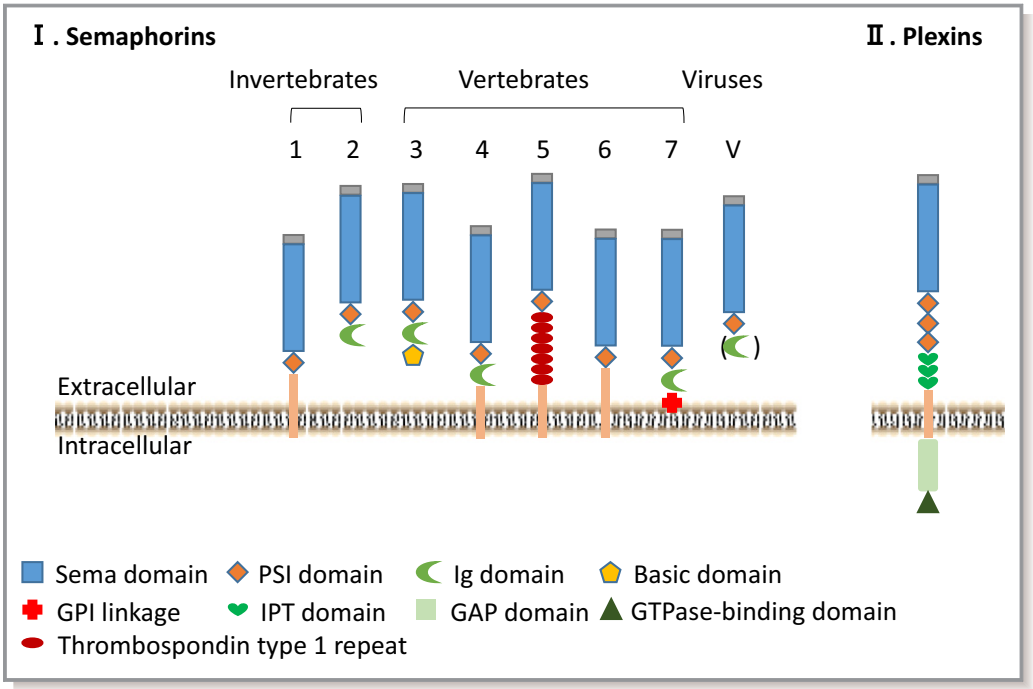

Figure 1. Semaphorins and plexins contain multiple functional domains.

whereas interaction with the PLEXINA4/PTK7 complex inhibits endothelial cell migration in the ventricle region.[18]

Although plexins are the best-studied semaphorin receptors, recent findings suggest that other proteins can also act as receptors by binding to the extracellular domain of semaphorins. For example, TIM2, which belongs to the Tim protein family and is characterized by expression on activated T cells and the presence of conserved immunoglobulin domain and mucin domains, is a receptor for SEMA4A.[26] CD72 is a novel class of SEMA4D receptor on lymphocytes that belongs to the C-type lectin family.[27] Moreover, SEMA7A exerts an essential function by binding and stimulating monocytes through the $\alpha 1\beta 1$ integrin receptor in both the nervous and immune systems.[28]

Brief Introduction of Cardiovascular Development

Cardiovascular development in embryos can be subdivided into heart development and vascular development. The heart is the first functional organ formed in mammals.[9,29-32] Initially, the heart develops from clusters of progenitor cells, which coalesce to form the cardiac crescent and the heart tube.[31-33] Subsequently, the heart tube undergoes elongation, looping, septation, remodeling, and maturation to form the final 4-chambered organ.[31-33] Multiple cell types, including myocardial, endocardial, epicardial, and neural crest cells (NCCs) act coordinately during complicated cardiogenesis in vertebrates. NCCs are a group of pluripotent cells that are generated at the edge of neural tubes. Cardiac NCCs migrate through pharyngeal arches 3, 4, and 6 to enter the distal region of the OFT, where they play a critical role in separation of the aorta root and pulmonary trunk.[34-36] Some cardiac NCCs in the OFT region eventually become interstitial cells in semilunar valves. In addition, NCCs give rise to a group of smooth muscle cells in the pharyngeal arch arteries. In the proximal region of the OFT and atrioventricular (AV) canal region, a subset of endocardial cells respond to signals released from the myocardium and undergo epithelial-mesenchymal transition (EMT) to become cushion mesenchymal cells during midgestation. The cellularized OFT and AV cushions facilitate unidirectional blood flow in embryos and are further remodeled into mature septa and valve structures.[37-41] Most cushion mesenchymal cells become interstitial cells in valves. Vascular development is initiated with vasculogenesis. In this process, mesoderm-derived angioblasts form a primitive vascular plexus,[42] which is further developed into a mature blood vessel system to ensure proper blood supply for the whole body.[43]

Numerous signaling pathways and downstream transcription factors are required for normal cardiogenesis and blood vessel development.[9,31-33,42-51] In the past 2 decades, accumulated studies have indicated that impaired semaphorin signaling results in various cardiovascular disorders during development and in multiple disease states. In this article we summarize recent discoveries regarding the function of semaphorins during mammalian cardiovascular development, with a primary focus on members of the SEMA3, SEMA5, and

Table 1. Semaphorins and Their Corresponding Plexin Receptors[2,5,6,8]

Semaphorins	Organisms	Plexins
SEMA1A	Invertebrate	PLXN A
SEMA1B	Invertebrate	PLXN A
SEMA2B	Invertebrate	PLXN B
SEMA3A	Vertebrate	PLXN A1, A2, A3, A4, D1
SEMA3B	Vertebrate	PLXN A1, A2, A3, A4
SEMA3C	Vertebrate	PLXN A1, A2, B1, D1
SEMA3D	Vertebrate	PLXN D1
SEMA3E	Vertebrate	PLXN B2, D1
SEMA3F	Vertebrate	PLXN A1, A3, A4
SEMA4A	Vertebrate	PLXN B1, B2, B3, D1
SEMA4B	Vertebrate	PLXN B2
SEMA4C	Vertebrate	PLXN B2
SEMA4D	Vertebrate	PLXN B1, B2, C1
SEMA4G	Vertebrate	PLXN B2
SEMA5A	Vertebrate	PLXN A1, A3, B3
SEMA5B	Vertebrate	PLXN A1, A3
SEMA6A	Vertebrate	PLXN A2, A4
SEMA6B	Vertebrate	PLXN A1, A2, A4
SEMA6C	Vertebrate	PLXN A1
SEMA6D	Vertebrate	PLXN A1, D1
SEMA7A	Vertebrate	PLXN C1
Poxvirus A39R	Virus	PLXN C1

SEMA6 families, whose activities during cardiovascular development have been supported with gene inactivation studies in mice.

Versatile Activities of Semaphorins During Cardiovascular Development

Role of Class 3 Semaphorins During Cardiovascular Development

SEMA3A Role

SEMA3A regulates cardiac innervation patterning and is essential for heart rate control.[52] *Sema3A*$^{-/-}$ mice lack a cardiac sympathetic innervation gradient, which leads to sinus bradycardia, abrupt sinus slowing, and stellate ganglia defects.[52] In support of the direct role of SEMA3A in regulating innervation in cardiomyocytes, myocardial-specific overexpression of *Sema3A* resulted in prolonged action potential duration, reduction of sympathetic innervation, and spontaneous ventricular arrhythmia.[52] The role of SEMA3A in regulating sympathetic innervation is not limited to developing hearts. In a rat myocardial infarction model, overexpression of *Sema3A* in left stellate ganglion and myocardium reduces sympathetic reinnervation in the myocardial infarction border zone and the susceptibility to malignant arrhythmia.[53,54] In an independent gene inactivation study, the major cardiac phenotype of *Sema3A* homozygous mutant mice is postnatal right ventricle hypertrophy.[55] The differential phenotypes reported in the literature are likely due to the specific knockout strategies and/or mouse strains used by different groups.

In support of the clinical relevance of SEMA3A function in rodent cardiomyocytes, addition of SEMA3A to cardiomyocytes derived from human-induced pluripotent stem cells inhibited the $K_v4.3$ (I_{to}) channel, as observed in heterologous human embryonic kidney cells.[56] Furthermore, several missense mutations in *SEMA3A* (R552C, R734W, and I334V) were shown to be associated with Brugada syndrome and unexplained cardiac arrest.[56,57] These mutations impaired the ability of SEMA3A to inhibit the $K_v4.3$ (I_{to}) channel.[56]

SEMA3A is also important for normal development of blood vessels. Knocking out *Sema3A* in mice led to abnormal patterning of anterior cardinal veins in the head and intersomitic vessels in the trunk region.[58] The cranial blood vessels in mutants remain at the primitive capillary plexus stage and fail to remodel. The mouse vascular defects can be observed in the CD-1 background but not in 129/Sv background, suggesting the involvement of other genetic factors in determining vascular phenotypes in *Sema3A*-null mice. Severe defects in dorsal aorta development were also observed in zebrafish with *sema3a* either knocked down or overexpressed[59]; however, a similar phenotype was not reported in *Sema3A* mutant mice. A further mechanistic study showed that SEMA3A acts as a selective inhibitor of VEGF-mediated angiogenesis via disruption of focal adhesion kinase/Src signaling and as a potent inducer of microvascular permeability via activation of NRP1.[60]

SEMA3C Roles

SEMA3C/PLXNA2 signaling and SEMA3C/NRP1 signaling are required for NCC development, which is essential for proper septation of the cardiac OFT.[61-63] Using NCCs isolated from Hamburger Hamilton 10 chicken embryos, Toyofuku et al found that SEMA3C promoted NCC migration through PLXND1 and NRP1.[64] *Sema3C* complete knockout mice are cyanotic and die shortly after birth from interruption of the aortic arch, persistent truncus arteriosus, and septation defects in the OFT.[61] These morphological defects are likely caused by failure of NCCs to migrate into the proximal OFT.[61] A recent study using a conditional gene inactivation approach indicated that SEMA3C expressed in NCCs activates NRP1 in endocardial cells of the OFT to promote EMT in OFT

cushions,[63] which are essential for proper OFT septation and semilunar valve formation. A recent study systematically examined the cis-regulatory elements that control the proper expression of *Sema3C* in the OFT and pharyngeal arch regions.[65] This group of researchers found that transcription factors FOXC1 and FOXC2 can directly bind the FOX binding sites in the enhancer region of *Sema3C* to promote its transcription in the OFT myocardium. In the pharyngeal arch region, expression of *Sema3C* is repressed by TBX1-FGF8. This study strongly supports the idea that proper spatiotemporal expression of SEMA3C is essential for normal septation of the OFT.[65]

SEMA3C can also regulate blood vessel formation. It inhibits VEGF-induced endothelial cell adhesion and migration through PLXND1 and NRP1 receptors in both in vitro and in vivo assays.[66] Moreover, the local administration of SEMA3C into the vitreous body of a retinopathy of prematurity model prevents the formation of pathological retinal angiogenesis.[66]

SEMA3D Roles

Functions of SEMA3D during cardiovascular development have been found in multiple vertebrates. Knocking down expression of *sema3D* in zebrafish led to dysmorphic hearts with smaller ventricles, smaller atrium, and thickened myocardial wall.[67] Endocardium was present in *sema3D*-knockdown fish; however, AV valves and trabeculation were absent.

The function of SEMA3D in mammals appears to be different from that in zebrafish. Rather, mammalian SEMA3D provides repulsive cues to direct normal endothelial cell development. In vitro analyses using primary human umbilical vascular endothelial cells showed that exogenous SEMA3D inhibits endothelial cell migration and tube formation and that this activity requires PLXND1/NRP1[68,69] and activation of the PI3K/AKT pathway.[68] Inactivation of *Sema3D* in mice led to total anomalous pulmonary venous connection in which pulmonary veins abnormally enter the coronary sinus.[70] These results suggest that signals provided by SEMA3D are particularly important for endothelial cells of pulmonary veins in vivo. *Sema3D*$^{-/-}$ mice can survive to adulthood but show severe cardiomegaly due to dilation of right atria and ventricles accompanied by left-to-right shunt, which is likely secondary to the total anomalous pulmonary venous connection defect.[70] Furthermore, a point mutation (F602L) in SEMA3D was identified in a human patient with partial anomalous pulmonary venous connection.[70]

In addition to the loss-of-function allele, a gain-of-function allele was also identified in a human patient who carried a duplication of the 5′ half of *SEMA3D*.[71] This patient displayed transposition of the great arteries, ventricular septal defect, and coarctation of the aorta. The authors speculated that migration of cardiac NCCs into the OFT is impaired in patients. However, a role of SEMA3D in NCCs has not been demonstrated using animal models.

SEMA3E Role

SEMA3E is a potent repulsive guidance cue for endothelial cells. *Sema3E* mRNA is robustly expressed in the caudal region of each somite in E11.5 mouse embryos from in situ hybridization analysis.[72] Knocking out *Sema3E* led to disorganized intersomitic vessels, suggesting the essential role of SEMA3E in guiding intersomitic vessel formation and patterning. Further detailed examination of the *Sema3E*-null mice revealed more vascular defects, including the paired dorsal aortas and fusion of a large plexus of blood vessels.[73,74] To further support SEMA3E as a repulsive cue for endothelial cells, electroporation of a SEMA3E expression plasmid into E3 chicken embryos reduced vessel formation in the area where SEMA3E was ectopically expressed.[72]

SEMA3E appears to act primarily through PLXND1 in blood vessels. Alkaline phosphatase-tagged SEMA3E (AP-SEMA3E) bound to COS cells expressing PLXND1, but not NRP1 or NRP2.[72] AP-SEMA3E bound to the blood vessels in sections from wild type but not *PlnxD1* null embryos. Functional analysis showed that addition of SEMA3E caused collapse of PLXND1-expressing COS cells. Unlike SEMA3D, SEMA3E-mediated cytoskeletal reorganization does not require NRP1.[68] Inactivation of *PlxnD1* results in similar organizational defects in the somatic vasculature as observed in *Sema3E*-null embryos.[72]

The Role of SEMA5A During Cardiovascular Development

SEMA5A is the only known member of the SEMA5 family for which there is clear genetic evidence to support its role during cardiovascular development. SEMA5A is a proangiogenic molecule that potently induces endothelial cell proliferation and migration and inhibits apoptosis.[75,76] Treatment of immortalized human dermal microvascular endothelial cells with SEMA5A significantly increased their migration. Furthermore, subcutaneous injection of SEMA5A-containing matrigel enhanced blood vessel sprouting.[75] Knocking out *Sema5A* in mice led to embryonic lethality between E11.5 and E12.5.[77] A thorough examination of the cardiovascular system in mutants revealed that the number of secondary and tertiary branches of blood vessels in the cranial region was reduced, although the capillary network was not affected.[77] No other cardiovascular defect was reported in mutant embryos. Therefore, the proangiogenic activity of SEMA5A is essential only in the cranial region in vivo. The major cause for the death of *Sema5A*-null embryos remains unidentified, as the vascular defect in the cranial region is unlikely lethal to the embryo.

The Role of Class 6 Semaphorins During Cardiovascular Development

SEMA6A Role

In vitro analysis using human umbilical vascular endothelial cells showed that SEMA6A promotes endothelial cell survival and growth by modulating VEGFR2 signaling.[78] The vascular defects in *Sema6A*-null animals are limited to the eye. At the P4 stage, hyaloid vessels displayed a significantly reduced network complexity in mutant animals. Also at the same stage, the extension of the vascular network from the optic nerve to the periphery was also reduced in mutants. The vascular defects in eyes disappeared at the P8 stage.[78] One likely reason is that other members of the semaphorin family are able to compensate for the loss of SEMA6A in mutant eyes at later stages. *Sema3A* is expressed in the vasculature of eyes during both embryonic and postnatal eye development[79] and thus is a likely candidate.

SEMA6A inhibits migration of NCCs isolated from Hamburger Hamilton 10 chicken embryos, in contrast to SEMA3C, which stimulates NCC migration.[64] However, no defect was observed in the OFT and pharyngeal arch arteries in *Sema6A*-null mice. It is thus possible that SEMA6A exerts different activities in chicken and mammals.

SEMA6D Roles

The initial evidence indicating a role for SEMA6D during heart development came from chicken studies. It was found that SEMA6D could promote endocardial cell migration in the OFT region through the PLXNA1-VEGFR2 receptor complex, whereas it inhibited endocardial cell migration in the ventricle region through the PLXNA1-PTK7 receptor complex.[18] In addition to acting on endocardial cells, SEMA6D can also regulate myocardial wall morphogenesis in chicken embryos through its cytoplasmic domain-dependent reverse signaling.[19] The chicken studies described above applied comprehensive loss-of-function and gain-of-function approaches; however, these activities of SEMA6D in chicken were not replicated in subsequent mouse studies.

We recently developed a novel conditional immortal AV cushion mesenchymal cell line, tsA58-AVM, and used this line to identify *Sema6D* as the regulatory target of bone morphogenetic protein signaling in AV cushions.[80] Conditional inactivation of *Sema6D* in endocardial cells of mouse embryos using the *Nfatc1-Cre* driver led to hypocellular AV cushions at E9.25 and E9.5 due to reduced EMT in the AV canal region. Functional tests revealed that SEMA6D activates Rho through PLXNA1-FARP1 to promote cushion mesenchymal cell formation in the AV canal[80] (Figure 2). Thus, EMT by endocardial cells in the OFT and AV canal both rely on semaphorin signaling, with the OFT region needing SEMA3C and the AV canal region requiring SEMA6D. The AV cushion defect in

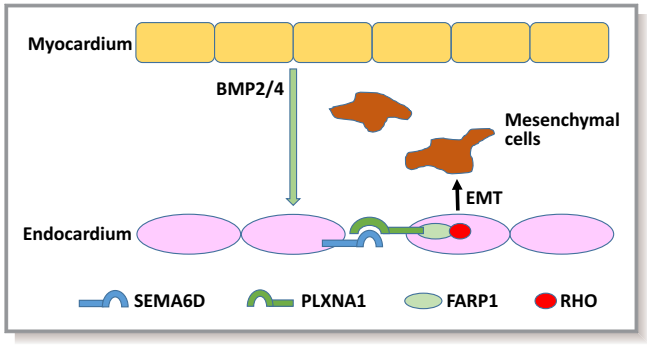

Figure 2. SEMA6D promotes EMT at AV cushions. In responding to BMP ligands released from the overlying myocardium (such as BMP2 and BMP4), expression of SEMA6D in endocardial cells is upregulated in the AV canal region of mouse embryos at E9.0–9.5. SEMA6D acts on adjacent endocardial cells through the PLXNA1/FARP1/RHO axis to promote cushion mesenchymal cell formation and migration in AV cushions. AV indicates atrioventricular; BMP, bone morphogenic protein; EMT, epithelial-mesenchymal transition.

Nfatc1-Cre/Sema6D$^{loxp/loxp}$ embryos was resolved at a later stage (E10.5), likely due to the compensatory effect from increased expression of SEMA6C.[80] Studies of double-knockout mice of *Sema6D* and *Sema6C* are required to test this hypothesis. No defect in the OFT cushions or in the endocardial cells of ventricles was observed in *Nfatc1-Cre/Sema6D*$^{loxp/loxp}$ embryos.[80] No myocardial wall defect was reported in *Sema6D* complete knockout mice.[81] The apparent difference in the functions of SEMA6D between chicken studies and mouse studies suggests that this cytokine has differential roles in the cardiovascular system in birds and mammals.

Summary

Multiple semaphorin molecules play an essential role in regulating cardiovascular development. This conclusion is supported by convincing mouse genetic evidence complemented with tests on other model systems including cell culture, zebrafish, and chicken studies (summarized in Table 2). The activities of semaphorin signaling are highly versatile and include regulation of NCC migration, endocardial cell EMT in the OFT and AV canal regions, cardiac innervation, myocardial wall morphogenesis, endothelial cell migration during blood vessel formation, and patterning of vessel networks.

There remain some outstanding questions regarding the underlying mechanisms by which semaphorin signaling regulates cardiovascular development. We list 3 here. (1) The functions of semaphorins are highly cell-type and/or tissue-type dependent. In most cases it is unclear how such specificity is achieved. Detailed characterization of conditional gene-inactivation mouse models, in which semaphorin/plexin

Table 2. Summary of Functions of Different Semaphorins During Cardiovascular Development

Semaphorins	Cardiovascular Expression	Cardiovascular Defects in Mutants
SEMA3A	Trabecular zone of embryonic hearts, Purkinje fiber, vascular endothelia cells[52,58]	*Zebrafish:* Abnormal dorsal aorta development[59] *Mouse:* sinus bradycardia, abrupt sinus slowing, stellate ganglia defects, right ventricle hypertrophy, abnormal patterning of anterior cardinal veins and intersomitic vessels[52,55,58] *Human:* Brugada syndrome[56,57]
SEMA3C	Neural crest cells, outflow tract myocardial cells[61-63,65]	*Mouse:* interruption of the aortic arch, persistent truncus arteriosus, septation defects in the outflow tract[61-63]
SEMA3D	Mesocardial reflection and proepicardial organ in embryos, neural crest cells[70]	*Zebrafish:* dysmorphic hearts, absence of atrioventricular valves and trabeculation[67] *Mouse:* total anomalous pulmonary venous connection, cardiomegaly[70] *Human:* partial anomalous pulmonary venous connection, transposition of the great arteries, ventricular septal defect, coarctation of the aorta[70,71]
SEMA3E	Notochord, lateral plate mesoderm, caudal region of somites[72,73]	*Mouse:* disorganized intersomitic vessels, paired dorsal aortas, fusion of a large plexus of blood vessels[72-74]
SEMA5A	Atrium septum, endocardial cells, cushion mesenchymal cells, mesoderm surrounding cranial vessels[77]	*Mouse:* reduced number of secondary and tertiary branches of blood vessels in the cranial region[77]
SEMA6A	Hyaloid vessels, retinal vessels[78]	*Mouse:* reduced network complexity in hyaloid and retinal vessels at P4, defects resolved at P8[78]
SEMA6D	Myocardial, endocardial, cushion mesenchymal cells[18,19,80]	*Chicken:* altered endocardial cell migration, reduced myocardial wall trabeculation, small ventricle[18,19] *Mouse:* reduced cushion mesenchymal cell number at E9.5, defect resolved at later stages[80]

genes are specifically inactivated in different cardiovascular cell types, would provide crucial clues to answer this question. (2) Semaphorins can activate many downstream effectors to accomplish their complex biological activities. Unlike many other signaling pathways, such as TGFβ/BMP signaling, there is no canonical pathway that is associated with semaphorin signaling. How the semaphorin/plexin complex on the cell surface selectively activates downstream cytoplasmic effectors in a context-dependent fashion remains largely elusive. (3) Another critical question to be addressed is how semaphorin signaling interacts with other signaling pathways during cardiovascular development. Such interaction may occur at the cell surface through sharing (or competing for) the same coreceptors and/or in the cytoplasm through crosstalk between different cytoplasmic effectors.

Answering the above questions will help us design effective strategies to accurately and specifically modulate semaphorin signaling for therapeutic purposes. Recent studies have shown that tumor angiogenesis can be regulated by different semaphorins.[4,82] A better understanding of the molecular mechanism by which semaphorins regulate blood vessel formation may lead to identification of novel intervention targets for cancer treatment. Another potential area for translational research of semaphorin signaling is regenerative medicine. For example, semaphorin signaling is involved in generation of cushion mesenchymal cells in both the OFT and AV canal regions. These mesenchymal cells are precursors of septa and valves in mature hearts. Our knowledge of semaphorin signaling during OFT and AV cushion development may provide crucial guidance for us to differentiate pluripotent stem cells into valvular/septal cells for tissue repair. This area of research remains a blank in the literature.

In summary, semaphorins are versatile signaling molecules that regulate multiple aspects of cardiovascular development. Studies on semaphorin signaling are highly significant for both basic and translational research.

Acknowledgments

We regret that due to space limitations, the work of all of our colleagues could not be cited here. We thank the members of the Jiao laboratory for their comments and suggestions for the article.

Sources of Funding

Research in the authors' laboratory is supported by NIH R01 (R01HL095783), R03 (R03HD082634), and R21 (R21CA199586) grants awarded to Jiao.

Disclosures

None.

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Key Words: cardiac development • cardiovascular research • heart development