Guidance molecules in lung cancer

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Abbreviations: Abl, abelson; ADD, addiction dependence domain; DAPK, death associated protein kinase; DCC, deleted in colon cancer; ECD, extracellular domain; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GPI, glycosylphosphatidylinositol; Ig, immunoglobulin; LOH, loss of heterozygosity; NICD, notch intracellular domain; NRP, neuropilin; NSCLC, non-small cell lung cancer; PDZ, PSD95 discs large zonula occludens; PI3K, phosphoinositide 3-kinase; PSI, plexin semaphorin integrin; RTK, receptor tyrosine kinase; SAM, sterile alpha motif; SCLC, small cell lung cancer; TSG, tumor suppressor gene; UNC5, uncoordinated family member 5; VEGF, vascular endothelial growth factor

Guidance molecules were first described in the nervous system to control axon outgrowth direction. They are also widely expressed outside the nervous system where they control cell migration, tissue development and establishment of the vascular network. In addition, they are involved in cancer development, tumor angiogenesis and metastasis. This review is primarily focused on their functions in lung cancer and their involvement in lung development is also presented. Five guidance molecule families and their corresponding receptors are described, including the semaphorins/neuropilins/plexins, ephrins and Eph receptors, netrin/DCC/UNC5, Slt/Robo and Notch/Delta. In addition, the possibility to target these molecules as a therapeutic approach in cancer is discussed.

Introduction

During normal development, timely and spatially-coordinated signals control cell migration, proliferation, fate and specification, thereby governing tissue arrangement and remodeling. Some of these signals are provided by membrane and extracellular cues that carry information to receptive cells in the environment. Such cues are guidance molecules, which administrate appropriate positioning of cell entities. This process is superbly exemplified by axon pathfinding in the nervous system where protrusions from a neuronal body navigate extreme distances to connect with specific neurologic targets.1 Similar guidance processes orchestrate the development of the vasculature network and the patterning of many if not all human tissues.2,3 Thus, it is not surprising if guidance molecules contribute to tumor growth, activation of the microenvironment and metastasis.4,5 We review here the main families of guidance molecules (Table 1) and focus on their participation in lung development and tumorigenesis.

Lung cancer is one of the most frequent malignant diseases. It ranks first in cancer-related deaths in Europe and the United States and is expected to rapidly increase in Asia. Tobacco exposure accounts for 90% of cases, although other factors such as asbestos, benzene and urban pollution are also associated with lung cancer incidence. In addition, lung cancer is an aggressive disease with a survival of less than 15% at 5 years.

Non-small cell lung cancer (NSCLC) is more common than small-cell lung cancer (SCLC), accounting for about 80% of all lung cancers. It is mainly epithelial in origin and divided into three predominant types. Adenocarcinoma, the most frequent, has glandular differentiation or mucin production and is usually found in the outer part of the lungs. Squamous cell carcinoma shows keratinization or normal epithelium bridges and is usually found near bronchi. Large cell carcinoma, often with neuroendocrine features, can be found anywhere in the lungs. Frequent molecular alterations of NSCLC include mutations/deletions of FHIT, p53, p16, LKB1 and K-Ras genes, as well as overexpression/mutation of EGFR or HER2 and increased telomerase activity. Allelic losses are frequently found in 3p, 6q, 8p, 9p, 13p, 17p and 19q. NSCLCs are currently treated by surgical resection, combination chemotherapy and radiation, with the more recent inclusion of EGFR inhibitors and VEGF antibodies (Beveracizumab).

SCLC is a fast-growing and highly metastatic form of lung cancer with neuroendocrine origin. SCLCs have been traditionally staged as limited or extensive depending on their ability to be incorporated into a single radiation field. Frequent deletions/mutations are found in the FHIT, p53 and RB genes, while c-MYC and BCL2 genes are overexpressed. Telomerase activity is elevated and allelic 3p loss are found in 100% cases in addition to less frequent loss of 4p, 4q, 8p, 10q, 13q, 17p and 22q. Patients with SCLC do not undergo surgery, since subsequent relapse is almost inevitable, and therefore combination chemotherapy has been the predominant treatment.
Table 1. Canonical ligands and their corresponding major receptors in mammals

| Canonical ligand       | Main receptor                        |
|------------------------|--------------------------------------|
| Class 3 semaphorins: SEMA3(A to G) | Neuropilins (NRP1, NRP2), Plexin-A, Integrins |
| Semaphorins from class 4 to 7: SEMA4 to 7 | Plexin-B/C/D |
| Ephrin-A1 (1 to 5)     | EphA1 (1 to 8), EphA10                |
| Ephrin-B1 (1 to 3)     | EphB1 (1 to 4), EphB6                |
| Netrin-1/2/4           | DCC, neogenin, UNC5A-D or UNC5H1-H4 |
| Netrin-G1              | Unidentified                          |
| Netrin-G2              | Unidentified                          |
| Slit1, Slit2, Slit3    | Robo1 to 4                            |
| Delta-like (DII-1/3/4) | Notch1 to 4                           |
| Serrate-like (JAG1, JAG2) | Notch1 to 4                          |

This simplified list refers to receptors that ligands most commonly bind to; some exceptions exist. For instance, SEMA3E can bind plexin-D1 rather than NRP, or ephrin-B1 can bind EphA1.

Guidance Molecules: Structure, Pathway and Functions

Semaphorins and their receptors. Semaphorins constitute a family of about 30 members. They are divided into eight classes based on structural features: vertebrate semaphorins belong to classes 3–7 and include 21 members. All semaphorins share an extracellular cysteine-rich “sema” domain, which is additionally found in their plexin receptors and the Met/Ron family receptors. Vertebrate semaphorins also possess a plexin-semaphorin-integrin (PSI) domain, and diverge by the presence of an immunoglobulin (Ig)-type domain or thrombospondin repeats. Their main difference however, resides in the C-terminus. Class-3 semaphorins (SEMA3A-G) have a basic region and are secreted proteins, classes 4–6 (SEMA4A-G; SEMA5A-B; SEMA6A-D) are transmembrane proteins with a cytoplasmic region and the single class-7 member (SEMA7A) is glycosylphosphatidylinositol anchored to the plasma membrane.

Semaphorins bind and activate plexin receptors. Plexins are transmembrane proteins with the extracellular “sema” domain, PSI and Ig-type domains, followed by an intracellular RasGAP motif, and sometimes by a PSD95 discs large zonula occludens (PDZ) binding motif. Most often, SEMA4-A-7 bind directly to plexins, while class-3 semaphorins bind to neuropilins (NRP1 or NRP2) which transmit signals to plexins (Table 1). SEMA3E is an exception as this semaphorin binds directly plexin-D1 rather than NRP. In some instances, plexins are associated with receptor tyrosine kinases (RTK) like Met and ErbB2, or other membrane proteins.

Notably, neuropilins bind both class-3 semaphorins and vascular endothelial growth factor (VEGF). In light of this finding, binding competition was proposed to explain some of the anti-angiogenic properties of class-3 semaphorins. However, it is still not clear if class-3 semaphorins simply compete with VEGF for NRP1 and NRP2 binding sites or whether they act independently of VEGF. NRP1 and 2 interact with VEGFR-1 and VEGFR-2, and NRP2 interacts also with VEGFR-3, to promote human endothelial cell survival and migration. NRP2 also binds to VEGF-C, a crucial player in lymphangiogenesis with a resulting enhancement of VEGF-C/VEGFR-3 biological effects. This suggests that semaphorin/NRP2 interactions play a role in lymphangiogenesis. Neurpilins also bind other ligands including placenta growth factor 2 (PIGF-2), fibroblast growth factor (FGF), galectin-1, hepatocyte growth factor (HGF) and transforming growth factor (TGF-β). In addition, NRP2 are part of larger complexes that interact with Met and cell adhesion molecules like L1-CAM (L1 cell adhesion molecule).

Semaphorins are expressed in the nervous, cardiovascular and immune systems, in many organs including lung and kidney, both in the embryo and the adult. They contribute to cell communication processes influencing cell positioning and behavior, and thus tissue morphogenesis.

Secretd semaphorins function in a paracrine manner while membrane-bound semaphorins act through cell-cell interactions. However, some semaphorins from class 4 and 6 can be cleaved by proteases and released in the extracellular compartment. Semaphorins employ alternative signaling pathways with a recurrence for small G proteins (Fig. 1). To simplify, we can distinguish four main signaling axes. Secreted class-3 semaphorins bind to NRP2, which act via class-A plexins in two directions: either to regulate CRMP proteins and block tubulin assembly (Fig. 1A) or to inhibit R-Ras through plexin RasGAP activity resulting in integrin inactivation and decreased cell adhesion (Fig. 1B). In the same manner, membranous semaphorins can also inhibit R-Ras activity. Moreover, membranous semaphorins often regulate actin dynamics and cell spreading through upstream Rho regulators (GEF or GAP) depending on which semaphorin/plexin or co-receptor is present in the complex (Fig. 1B and C). In addition, RHo/Rac pathways also participate in SEMA3 signaling. Finally, it is worth noting that some transmembranous semaphorins can signal to the cytoplasm in a reverse manner through their cytoplasmic domain. Examples of effectors in the later case are Abl and EVL, among others (Fig. 1C).

In summary, semaphorins control cell protrusion, spreading and adhesion which, when combined, trigger strong migratory responses, most often in a repulsive manner but also attractive depending on the context. In addition, semaphorins can also regulate MAPK or PI3K signaling, proliferation and survival.

Ephrins and their receptors. Eph receptors (for erythropoietin-producing human hepatocellular carcinoma) are the largest subgroup of receptor tyrosine kinases (RTK). Both Eph receptors and their ligands, Eph receptor interacting proteins (ephrins), are divided in two classes, A and B, based on sequence conservation and mutual interaction. In humans, there are fourteen Eph receptors with nine in class-A and five in class-B (Table 1). Eight members of ephrins belong to either the class-A or class-B.

Structurally, Eph receptors are classical RTKs that possess an extracellular domain (ECD), a transmembrane section and cytoplasmic domain with tyrosine kinase activity and a PDZ-binding motif. Ephrin ligands all contain an extracellular receptor-binding domain but differ in their association
and ligands are often expressed at tissue boundaries by alternative cell types in juxtaposition. Eph/ephrins are well represented in the nervous system, the vasculature and virtually all organs where cell-cell interactions contribute to proper tissue morphogenesis.

An interesting characteristic of the Eph/ephrin pair is the bi-directional signaling. Forward signaling from Eph receptors usually engages tyrosine kinase activity and is initiated by ligands from the membrane. Ephrins from class-A are GPI-anchored, while ephrins from class-B are transmembrane and possess a short cytoplasmic domain with a PDZ-binding motif. Ephrins from class-A most often bind EphA receptors and vice versa, despite some exceptions (Table 1).17,18 Since both Eph and ephrins are membrane proteins, they must function in a proximal manner through the binding of ephrin ligands from one cell to Eph receptors on another (Fig. 2). Therefore, receptors and ligands are often expressed at tissue boundaries by alternative cell types in juxtaposition. Eph/ephrins are well represented in the nervous system, the vasculature and virtually all organs where cell-cell interactions contribute to proper tissue morphogenesis.

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Figure 1. Semaphorin signaling. Secreted and membranous semaphorins signal through redundant and alternative pathways. (A) SEMA3-mediated stimulation of plexin-A leads to GSK3-dependent phosphorylation of CRMP and inhibits microtubule assembly by CRMP. Class-3 semaphorins also inhibit Rho-mediated actin polymerization, ERK activation and R-Ras-mediated integrin activation. (B) Upon SEMA4-7 binding, the RasGAP domain of plexin-B/C/D inactivates R-Ras-mediated integrin signaling. This pathway is similarly involved in SEMA3-plexinA signaling. Also, plexin-B/C/D can activate or inhibit RhoA. RhoA regulation sometimes requires the association of the ErbB2 and Met RTK with plexins, which lead to activation of Rho-GEF or RasGAP, respectively. (C) SEMA4-6 reverse signal through their cytoplasmic domain to Abl and EVL to regulate actin dynamics. Gray/black box: R-RasGAP domain. White box: tyrosine kinase domain. Double arrows: proteolytic cleavage. (X) indicates inhibition of the corresponding pathway by SEMA3.
cascades, alternative adaptors and pathways can be used depending on the specific EphA or EphB.

Reverse signaling occurs from class-A ephrins to Fyn and p120-RasGAP, among others, and it is speculated that an additional membrane-associated protein is necessary because of the absence of cytoplasmic tail on class-A ephrins. In contrast, ephrin-B cytoplasmic domains can be phosphorylated and signaling occurs through Axin, Abl, FAK and others (Fig. 2A).

A recurring feature of Eph/ephrin signaling involves cytoskeleton dynamics, cell morphology and migration, which usually but not exclusively leads to repulsion. Also, Eph/ephrin signals act in concert with many RTKs, adhesion molecules, channels and membrane-associated kinases to regulate cell morphology and adhesion.20

Netrins and their receptors. Netrins belong to a family of evolutionarily conserved 60–80 kDa diffusible proteins that present structural similarity with the laminin family of extracellular matrix proteins. Three netrins are expressed in mammals in addition to two related GPI-linked membrane proteins, netrin-G1 and G2 (Table 1). Netrins regulate axon guidance in many organisms and are implicated in axonal outgrowth and orientation of cell migration in the developing nervous system.1 Netrins are bifunctional as they can mediate attraction or repulsion depending on the receptor they bind.21 They are also widely expressed outside the nervous system where they have key roles in branching, morphogenesis and angiogenesis.

Netrin receptors include two main families of proteins: DCC (Deleted in Colon Cancer) including the paralog neogenin, and UNC5 (Uncoordinated family member 5 orthologues). In addition, the membrane-associated adenosine A2b receptor may be a DCC co-receptor.22,23 Also, several laminin-binding integrins (α6β4 and α3β1) can act as direct receptors for netrin-1 and as co-receptors with DCC.24,25 In C. elegans, this interaction regulates the stabilization or trafficking of UNC-40 (C. elegans homolog of DCC) during cell invasion and modulates integrin signaling.26

DCC and UNC5 are single-pass transmembrane receptors that possess extracellular immunoglobulin domains. The DCC extracellular domain contains six fibronectin type III-like repeats. Its cytoplasmic domain includes an addiction dependence domain (ADD). UNC5 receptors contain two thrombospondin-like repeats in the extracellular region, and a zonula occludens-1 and a death domain in the intracellular region. DCC and UNC5 can be cleaved by a caspase in their intracellular region.22

In 1998, Mehlen et al. provided evidence for the first time that DCC regulates apoptosis as a dependence receptor.27 These receptors induce programmed cell death in the absence of ligands, whereas in the presence of their trophic ligands, programmed cell death is inhibited and cell survival, invasion and metastasis are enhanced (Fig. 3). Apoptosis induced by DCC/UNC5 would not involve the classical mitochondrial intrinsic and death-receptor extrinsic apoptotic pathways (Fig. 3A). Yet, the pro-apoptotic signaling is still largely unknown.22,23

Upon netrin-1 binding, DCC/UNC5 form homodimers or multimers (Fig. 3B). This multimerization was recently shown to be sufficient to inhibit apoptosis.28 DCC can associate with

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**Figure 2.** Ephrin signaling. Ephrins signal through reverse signaling and through Eph receptors. (A) reverse signaling occurs through focal adhesion kinase FAK, Abl, the Src family kinase Fyn, Axin and RasGAP. These converge to regulate cell migration through cytoskeleton rearrangement and adhesion. (B) Forward signaling from Eph receptors also engages Abl and RasGAP as well as Rho/Rac to regulate integrin-mediated migration. In addition, Ras downstream signaling controls MAPK and proliferation. Of note, Abl and Fyn signaling are targets of Dasatinib, which specifically inhibits proliferation of lung cancer cells with high expression of Eph receptors. White box: tyrosine kinase domain.
Several studies have described involvement of Slits and their receptors in angiogenesis. Evidence for a pro-angiogenic function comes from cell treatments with soluble Slit, the use of either a soluble extracellular domain of Robo1 (RoboN) or Robo4Fc to inhibit Slit, and by treatment with a monoclonal Robo1 antibody that blocks Robo-Slit interactions. Among Slit/Robo family members, Slit-2 may have an attractive role on Robo-1 expressing vessels. In addition, Robo4 can activate Cdc42 and Rac in endothelial cells leading to the formation of filopodia and lamellipodia. However, this mechanism does not indicate that DCC could perform dual roles, both as a cell surface receptor and as a transcriptional coactivator.
in vitro through direct interaction between Robo1 with the SDF-1 receptor, CXCR4. Furthermore, Slit triggers Robo-DCC interaction with the subsequent loss of responsiveness of DCC to its ligand, netrin-1. This loss may activate apoptotic pathways through caspases 3 and 9.

**Delta/Notch.** The Notch pathway is an evolutionary conserved signaling system that is mandatory for proper embryonic development and functions to regulate tissue homeostasis and maintenance of stem cells in adults. Interestingly, a number of studies also suggest that Notch and Delta are involved in cell migration and act as guidance cues during nerve development. Indeed, the Notch pathway regulates intersegmental nerve guidance and turning during Drosophila embryonic development and is involved in the regulation of microtubule stability and cell migration in vertebrates.

Based on structural homology to Drosophila ligands, the mammalian canonical DSL (Delta, Serrate, Lag2) ligands are Delta-like and Serrate-like Jagged1 (JAG1) and Jagged2 (JAG2) (Table 1). The canonical ligands are responsible for the majority of Notch signaling, but an increasing number of non-canonical ligands have also been described. We will focus only on the canonical ligands in lung development and cancer.

DSL ligands are type 1 cell-surface proteins containing multiple tandem EGF repeats in their extracellular domains. The DSL domain together with the N-terminal domain and first two EGF repeats are required for DSL ligands to bind Notch. The intracellular regions of DSL ligands lack sequence homology.

In mammals, four Notch family members have been identified (Notch1-4). All are single-pass transmembrane proteins that share structural homology and include a large number of extracellular EGF-like repeats followed by a cysteine-rich or LIN12 (LN) domain, a juxtamembrane region with specific proteinase cleavage sites for 

Notch signaling is intrinsically asymmetric and its activation requires interaction between a ligand-expressing cell and another cell expressing a Notch receptor. Notch signaling is important during pre- and post-natal life to maintain stem cell viability and alteration of Notch function has been associated with hereditary diseases and several cancers.

**Guidance Molecules in Lung Development**

Similar to nerves and vessels, the lung develops by successive branching, generating an architecture of growing complexity until formation of terminal bronchioles. A push-pull model has been proposed, whereby guidance cues act in concert with other critical growth and morphogenetic factors to sculpt the architecture of the respiratory tree.

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**Figure 4.** Slit signaling. Slit ligand binding to Robo receptors triggers several pathways that control actin reorganization and cell migration. The Rho family of GTPases appears to play a central role in Slit/Robo function. Part of the signaling involves the GAP proteins Vilse and srGAP (SLIT-ROBO Rho GTPase Activating Protein) that inhibit Rac and Cdc42 depending on the cellular context. The repulsive output of Robo1 signaling also involves the binding of Ena, an actin bundling protein which can be antagonized by Abl binding and phosphorylation. The protein Mena is involved in Robo4-mediated repulsion. Gray/black box: cytoplasmic conserved motifs (CC0-3).
Semaphorins and their receptors. Semaphorins are expressed and function in the development or maintenance of multiple organs and physiological systems such as the central and peripheral nervous systems, cardiovascular network, lung, kidney, muscle, bone morphogenesis and immune surveillance. Few studies have directly addressed the function of semaphorins in lung morphogenesis. However, in mice, Sema3A is expressed in the mesenchyme and restrains lung budding while Sema3C and Sema3F in the epithelial compartment promote lung branching and support the development of a mature arborization.65,66 Accordingly, we observed expression of SEMA3F at the membrane of type I and II epithelial cells in normal human lungs.67 In murine lungs, Sema3A, 3C and 3F are expressed at specific locations and times during development, and this seems to correlate with concomitant regulation of their receptors NRP1, NRP2 and plexin-A1. Semaphorins also participate in lung vasculogenesis and angiogenesis.

Ephrins and their receptors. Almost all the Eph receptors and ephrin ligands are normally expressed during lung development, but their specific role has not been well characterized. Ephrin-B2, important during lung development, is expressed in branching epithelial buds and distal epithelia in embryonic stages and later localizes to microvascular beds from birth to adulthood.52 Mice with mutant ephrin-B2 lacking the PDZ-binding domain, show changes in extracellular matrix composition which lead to reduced secondary septation and the formation of enlarged alveolae. Ephrin-B2 receptors EphA4, B2 and B4 are simultaneously expressed in the vasculature. In contrast, EphB3 was found in interstitial cells and loss of function induced disorganized matrix. For several other ephrins, not much more than expression data are available. Ephrin-A1, B1, B2 and B3 are expressed in fetal and/or adult lungs.53-59 Eph receptors EphA1, A2, A3 and A4, EphB1 and B4 transcripts are observed at moderate to high levels in lung tissues.53,55 However, EphA8 and EphB1 were not found in this study.

Perhaps most interesting, Eph/ephrins are well-established regulators of vasculature development and angiogenesis. Since the endothelial-epithelial interface is critical to oxygen assimilation in distal airways, Eph-ephrin contribution to lung vasculature also may regulate lung tissue morphogenesis. Indeed, Eph/ephrins are expressed in the blood vessel compartment of the lung and among them, ephrin-A1, B1, B2 and EphB4 are among the most enriched in alveolar capillary endothelial cells in mice.61 However, few specific functions in lung vascularization have been reported. In angiogenesis, ephrin-B2 expression is restricted to arteries or neovascularization sites whereas EphB4 expression is restricted to veins.52,65 In developing vessels of the lung epithelium, there is co-expression of ephrin-B2 and EphB4, which suggests a temporary lack of specification in early lung embryogenesis.64 Later, smooth muscle cells utilize ephrin-B2 to associate with microvessels.65 EphB4 negatively regulates vessel branching and permeability and promotes circumferential growth.66 Conversely, EphA2 increases lung blood vessel permeability.67 More information about the role of these molecules and other guidance molecules in vasculature has been published elsewhere.2,59

Netrins and their receptors. Netrin-1 and its receptors control morphogenesis of endothelial cells and vascular smooth muscle cells. They are implicated in the reorganization of the cytoskeleton, as well as epithelial cell adhesion and migration in lungs, mammary glands and pancreas.7 Netrins are expressed during early stages of murine embryonic development and their localization becomes restricted to the epithelium of large airways at embryonic day 18 (E18).69 They are located at the basement membrane and/or bind locally to epithelial cells at the neck region of elongating endoderm buds to restrict ectopic budding.29 DCC expression peaks early in

Figure 5. Notch signaling. Notch is presented to its ligand as a heterodimer resulting from processing by a furin-like protease during transit to the plasma membrane.165 Ligand binding triggers intra-membrane regulated cleavage of Notch within the juxtamembrane and transmembrane domains, ultimately leading to the release of the Notch intracellular domain (NICD). NICD then translocates to the nucleus where it directly interacts with the CSL (CBF1, Su(H), LAG1) transcription factor. The transcriptional repressor CoR is released. Recruitment of co-activators, including Mastermind, turns on expression of Notch target genes. These targets include a set of transcriptional repressors (Hes1, Hes5, Hey1, Hey2 and HeyL) of the basic helix-loop-helix (bHLH) family that repress expression of other bHLH genes involved in cell differentiation like the ubiquitous E2A factors and Mash1 (mammalian achaete-scute homolog-1), MyoD and others. The ultimate consequence is the regulation of cell fate and differentiation status. Gray box: NICD.

Gene transcription
development with a subsequent switch from localization in the mesenchyme to the epithelium in late development. UNCSH2 is expressed late in development, first localized to both basal and apical surfaces of airway epithelium while later the expression is apical only.69 Netrins modulate the morphogenetic response of lung endoderm to exogenous fibroblast growth factors (FGFs). This effect involves inhibition of localized changes in cell shape and decrease of local ERK1/2 activity. According to this model, the basal lamina plus netrins acts as a kind of “corset,” restricting morphogenesis of the merging bud.

Slit/Robo. The first evidence for involvement of the Slit/Robo family in lung development comes from a Robo1 knockdown mouse model.72 Heterozygous mice born at the expected frequency have no obvious phenotype. However, newborn Robo1−/− mice are usually inactive with labored breathing and 63% die within the first 24 h after birth. Appearance of the lung structures is consistent with a developmental delay. In addition, in an in vitro model using co-culture of endothelial cells and pulmonary epithelial cells on reconstituted basement membrane, Slit3 expression is upregulated during the alignment process of airway epithelium with endothelium.72 Altogether, these results suggest that Slit3 and Robo1 may be key players involved in the maturation of lung tissue during embryonic development. The expression levels and localization of Slit2, Slit3, Robo1 and Robo3 proteins have been studied throughout lung development in embryonic mice and early postnatal life.72-74 However, none of these studies really addressed the exact role of Slit/Robo during lung development.

Delta/Notch. Quantitative expression studies from the developing mouse lung demonstrate a progressive increase of Notch and Notch ligands from E11.5 to adulthood. All Notch molecules are expressed in the lung, Notch 4 being endothelial specific.75-79 Interestingly, by inhibiting γ-secretase, Notch signaling was shown to be unnecessary for lung bud initiation, but rather required to maintain a balance of proximal-distal cell fates in early lung development stages.80

Regarding Notch ligands, JAG1 and JAG2 are expressed in lung mesenchyme while Dll1 expression is restricted to neuroendocrine cells.76,77 Other genes of the Delta/Notch family such as Dll4, Hey1, Jagged2, Notch1, Numb and Siah1b are also expressed in lung capillaries, further supporting a role for Notch signaling in angiogenesis.61 Early lethality of mice with homozygous deletions for Notch1, Notch2, Dll1 and Jagged1 has limited the assessment of these genetic alterations in lung development, forcing researchers to look towards alterations of Notch targets. Among those targets, Hes1 is expressed in Notch1 and Notch3 positive neuroendocrine airway epithelial cells, and Hes1-reactive cells are destined to become Clara cells.76 Furthermore, Hes1 appears to be necessary for mediating the inhibition of neuroendocrine phenotype. Compared to Hes1, Mash1 expression is restricted to neuroepithelial bodies and pulmonary neuroendocrine cells (PNEC), and Mash1 appears to be essential for the expression of DLL1 and the differentiation of PNECs in the developing lung.76,81 Other Notch/Delta targets such as HeyL, Hey1 and Hey2 have been identified in the developing lung but their
function remains unknown. Notch signaling can also affect the immune response in adult and alteration of this pathway has been implicated in some lung diseases such as asthma, “Ondine’s curse” congenital hypoventilation syndrome, chronic obstructive pulmonary disease and inflammation due to microbial infection.

Guidance Molecules in Lung Cancer

Semaphorins and their receptors. Detailed reviews regarding the involvement of semaphorins and neuropilins in different types of cancer, including lung, have been recently published. Because of space, we refer the readers to these reviews.

The first evidence suggesting semaphorin involvement in cancer was the cloning of SEMA3B and SEMA3F from the chromosome 3p21.3 region, which undergoes frequent loss of heterozygosity (LOH) and less frequent homozygous deletion in lung cancer (Table 2). Since then, most semaphorin studies in lung cancer have focused on these two SEMA3s, which are often downregulated by allelic loss of one allele and hypermethylation of the second. Also, SEMA3B and SEMA3F are regulated by the p53 tumor suppressor, which is frequently mutated or lost in cancer. Furthermore, SEMA3F is a target of ZEB-1, a transcriptional repressor involved in the epithelial to mesenchymal transition (EMT).

In human lung tumors, SEMA3F is lost or delocalized in the cytoplasm, whereas in normal tissues it is found at the membrane of epithelial cells. In addition, loss of SEMA3F correlates with increased membrane VEGF staining and higher tumor grade. In contrast to the loss of SEMA3F, NRP1 and NRP2 levels increase from dysplasia to microinvasive carcinoma and correlate with VEGF expression.

Both SEMA3B and SEMA3F have demonstrated tumor-suppressor activity in vitro and in experimental xenograft models. For instance, SEMA3B transfection inhibited lung cancer cell growth and was associated with apoptosis and PI3K signaling inhibition. This SEMA3B growth inhibitory effect is exerted through induction of insulin-like growth factor-binding protein-6 (IGFBP-6) which is considered to be a TSG. One mechanistic suggestion is that EPHB6 activates MAPK signaling in lung adenocarcinoma. MAPK regulation by EPH/ephrins is common in other cancers, and we can only speculate that other EPH receptors exploit this signaling pathway in lung cancer. Signaling from the PDZ-binding domain of ephrin-B may also be involved, since it is known to play a role in normal lung cells.

One mechanism by which NSCLC tumor cells overexpress EPH/ephrins is chromosomal gain. Increased gene copy numbers of EPHA3, A5 and A8 have been reported in NSCLC and EPHA3 silencing by shRNA reduced the viability of cells displaying gene amplifications (Table 2). Strikingly, ten EPH receptor genes are mutated at moderate-high frequency in lung adenocarcinoma, notably EPHA3 and EPHA5, plus EPHA7, B1 and B6. Mutations arise in the extracellular domain, presumably affecting ligand binding, and in the kinase domain sometimes at a speculated molecular breakpoint. Overall, 16% of adenocarcinomas have mutations or amplifications in the EPH pathway, supporting the notion that EPHs might be proto-oncogenes.

Finally, a few studies have focused on mesothelioma, a particular type of lung cancer associated with asbestos exposure. EPHA2 seems to be more expressed in malignant mesothelioma than in normal pleural mesothelial cells and gene silencing reduces cell growth. Moreover, stimulation by ephrin-A1 decreases EPHA2 levels and mesothelioma growth. About 26% of mesotheliomas have fair to moderate expression of EPHB2, but the significance is unknown.

Another way by which EPH/ephrins may contribute to lung tumorigenesis is their capacity to control tumor angiogenesis. For example, EPHB4 and ephrin-B2 participate in postnatal
and neoplastic angiogenesis, suggesting that normally extinct embryonic EPH/ephrin pathways are reactivated by tumor cells to regulate neangiogenesis. Ephrin-A1 and EPHA2 have been detected in the vasculature of surgically resected human tumors and are essential for maximal effects of VEGF.106

Netrins and their receptors. DCC was identified in 18q21.1, a region of common LOH in colon cancer as well as in many cancers (Table 2).107 It was first suggested that DCC was a tumor suppressor gene. However, this hypothesis was questioned for several reasons, i.e., DCC mutations are rare, DCC mutant mice were not predisposed to cancers and loss of DCC had not been shown to give a selective advantage to tumor cells. Furthermore, SMAD2 and SMAD4, two other tumor suppressor candidates, are also localized in this region affected by LOH and SMAD mutations were frequently identified in pancreatic cancer. However, a number of experiments including forced DCC expression and DCC inhibition have documented roles of DCC as a tumor suppressor gene.22

Over the past decade, evidence has accumulated indicating that netrin-1 and its receptors have an important role in tumor biology. Loss of the apoptotic activity of dependence receptors is advantageous for tumor cell survival and could be achieved by three different mechanisms: loss of the receptors, overexpression of the ligand or inhibition of downstream signaling. The first of these, loss or reduced expression of DCC/UNC5H, was reported in colorectal cancers in addition to various other cancers either through genetic or epigenetic processes.23,107,108 In a panel of 25 lung carcinomas with corresponding adjacent normal tissues, DCC expression was decreased in tumors but netrin-1 expression was increased.109 However, at least two of the UNC5H receptors were always present in tumors with high netrin-1 expression.

Secondly, overexpression of netrin-1 was reported in metastatic breast cancer and colon cancer where it confers a selective advantage for tumor cell survival and invasion.110,111 In addition, netrin-1 overexpression was associated with a worse outcome in poorly differentiated pancreatic adenocarcinoma.112 In lung cancer, 92 NSCLC tumors were examined by immunohistochemistry

| Chromosome | Localization | Gene          |
|------------|--------------|---------------|
| 1          | 1p36, 1p36.1-p35 | EPHA2, EPHB2  |
|            | 1p36.12       | EPHA8         |
|            | 1p34.3        | EPHA10        |
|            | 1p13-p11      | NOTCH2        |
|            | 1q21.2        | SEMA6C        |
|            | 1q21-q22      | Ephrin-A1, -A3, -A4 |
|            | 1q22          | SEMAA4        |
|            | 1q32.2        | PLXNA2        |
| 2          | 2p13.1        | SEMA4F        |
|            | 2q11.2        | SEMA4C        |
|            | 2q33.3        | NRP2          |
|            | 2q36.1        | EPHA4         |
| 3          | 3p21.1        | PLXNB1        |
|            | 3p21.3        | SEMA3F, SEMA3B |
|            | 3p21.1        | SEMA3G        |
|            | 3p12.3        | ROBO2         |
|            | 3p12          | ROBO1         |
|            | 3p11.2        | EPHA3         |
|            | 3q11.2        | EPHA6         |
|            | 3q21.1        | SEMA5B        |
|            | 3q21.3        | PLXNA1        |
|            | 3q22.1        | PLXND1        |
|            | 3q21-23       | EPHB1         |
|            | 3q21-qter     | EPHB3         |
| 4          | 4p15.2        | SLIT2         |
|            | 4q13.1        | EPHA5         |
|            | 4q21-q23      | UNCSC         |
| 5          | 5p15.2        | SEMASA        |
|            | 5q21          | Ephin-A5      |
|            | 5q23.1        | SEMA6A        |
|            | 5q35          | SLT3          |
|            | 5q35.2        | UNC5A         |
| 6          | 6p21.3        | NOTCH4        |
|            | 6q16.1        | EPHA7         |
|            | 6q27          | DLL1          |
| 7          | 7p12.1        | SEMA3A        |
|            | 7q21.11       | SEMA3D, SEMA3E |
|            | 7q21-q31      | SEMA3C        |
|            | 7q22          | EPHB4         |
|            | 7q32.3        | PLXNA4        |
|            | 7q34          | EPHA1         |
|            | 7q33-q35      | EPHB6         |
| 8          | 8p12          | UNCSD         |
| 9          | 9q22.2        | SEMA4D        |
|            | 9q34.3        | NOTCH1        |
| 10         | 10p12         | NRP1          |
|            | 10q22.1       | UNC5B         |
|            | 10q23.3-q24   | SLT1          |
|            | 10q24.31      | SEMA4G        |
| 11         | 11q24.2       | ROBO3, ROBO4  |
| 12         | 12q22-q23     | Netrin-4      |
|            | 12q23.3       | PLXNC1        |
| 13         | 13q33         | Ephin-B2      |
| 14         | 14q32         | JAG2          |

Table 2. Chromosomal localization of human guidance ligands and their major receptors

| Chromosome | Localization | Gene          |
|------------|--------------|---------------|
| 15         | 15q14        | DLL4          |
|            | 15q21.1      | SEMA6D        |
|            | 15q22.3-q23  | SEMA7A        |
|            | 15q25        | SEMA48        |
| 16         | 16p13.3      | Netrin-3      |
| 17         | 17p13-p12    | Netrin-1      |
|            | 17p13.1-p11.2 | Ephin-B3    |
| 18         | 18q21.3      | DCC           |
| 19         | 19q13        | DLL3          |
|            | 19p13.2-p13.1 | NOTCH3      |
|            | 19p13.3      | SEMA68        |
| 20         | 20p12.1-p11.23 | JAG1       |
| 22         | 22q13.33     | PLXNB2        |
| X          | Xq12         | Ephin-B1      |
|            | Xq28         | PLXNA3, PLXNB3 |

Table 2. Chromosomal localization of human guidance ligands and their major receptors
for netrin-1 expression. Netrin-1 was absent or low in normal bronchial and alveolar epithelial cells, whereas both adenocarcinomas and squamous carcinomas expressed high levels of netrin-1, which like in the embryonic pattern was localized in the cytoplasm and in both apical and basal membranes. No statistically significant differences were observed at different stages, but netrin-1 expression was more frequent and more intense in adenocarcinomas than squamous cell carcinomas. In situ hybridization indicated that netrin-1 is not expressed in the stroma cells but is expressed in tumor cells. By either inhibiting netrin-1 expression or its interaction with receptors in two lung cancer cell lines, apoptosis was demonstrated to be dependent on UNC5H1 and UNC5H2 receptors but not DCC. In addition, blocking caspase-9 resulted in inhibition of apoptotic activity by the UNC5H receptors.

The netrin-1 gain of expression in tumor cells of epithelial origin may have two additive effects in an autocrine manner. Inhibiting dependence receptor-induced cell death confers a selective advantage to tumor cells. In addition, it would favor blood vessel maintenance and development, as netrin-1 was shown to control survival of endothelial cells and promote angiogenesis despite apparently contradictory observations resolved by the dependence receptor hypothesis.

The third mechanism to inhibit apoptotic activity is downregulation of DAPK, the downstream effector of UNC5H2-induced cell death. In the lung, aggressive metastatic mouse carcinoma clones did not express DAPK in contrast to their low-metastatic counterparts. DAPK restoration in highly-metastatic Lewis lung carcinoma cells suppressed their ability to form lung metastases and delayed local tumor growth. The authors proposed a model where loss of DAPK expression provides a unique mechanism that links suppression of apoptosis to metastasis. DAPK can be also inhibited by DNA methylation in lung cancer.

Slit/Robo. Slit/Robo are suspected to have pro-tumoral and pro-angiogenic functions in different cancer models. However, a Robo1-/- mouse model suggested that Slit/Robo signaling functions as tumor suppressor in lung cancer. In this model, two-thirds of the mice that survived to adulthood showed early signs of morbidity. At autopsy, bronchial epithelial hyperplasia and focal dysplasia were observed in most mice. In human lung cancer, Slit/Robo expression is often reduced either because of LOH or inactivation by DNA methylation. Of note, ROBO1, SLIT2 and SLIT3 are located in 3p12.3, 4p15, 2123 and 5q35-q34 respectively and these locations are affected by homozygous deletions or frequent LOH in lung cancer (Table 2). However, no substantial frequency of mutations was found for either ROBO1 or SLIT2. Methylation of the ROBO1 promoter and SLIT3 CpG islands are also rare events in lung cancer, but SLIT2 promoter methylation occurs in almost one-half of lung and breast primary tumors. Nevertheless, no experimental data clearly prove that Slit/Robo are TSGs in lung cancer. The fact that ROBO4 levels, as measured in the serum of patients with advanced NSCLC, correlated with poor survival is unconvincing, since it is not known if the circulating ROBO4 represented a degraded form, and whether its increased levels correlate with impaired angiogenesis in tumors.

Delta/Notch. Notch signaling in lung cancer exhibits properties suggesting both tumor promotion and inhibition, depending on the cell type. Notch1 and Notch2 are frequently expressed in NSCLC; however, the role of Notch1 in lung cancer remains obscure. On one hand, Notch1 inhibits growth of A549 adenocarcinoma cells in vitro and in vivo. On the other, hypoxia upregulates Notch1 and sensitizes cells to small molecule γ-secretase inhibitors. Notch2 is frequently overexpressed in lung cancers as the result of genetic alterations affecting chromosome 1p (Table 2). Notch3 mRNA expression was detected in 7 of 25 NSCLC but not in SCLC lines. In addition, Notch3 is the target of chromosome 19 translocations in NSCLCs. Interestingly, inhibition of Notch3 increases growth factor dependence sensitivity to EGF receptor inhibitors and is associated with a reduced phosphorylation of MAPK. The γ-secretase inhibitor, MKR-003, also inhibits Notch3 activation and induces apoptosis of lung tumor cells in vitro and in vivo. Together, these results suggest that at least Notch2 and Notch3 may have pro-tumor capabilities in NSCLC.

Notch1 is rarely expressed in SCLC, whereas a subset of SCLC exhibits Notch2 expression. In contrast to NSCLC, SCLC appears to be growth inhibited by overexpression of Notch1 and Notch2. Indeed, activated Notch1 and Notch2 cause a marked G1 arrest in SCLC cells, accompanied by upregulation of p21−wat/cip. A prominent function of Notch signaling is to inhibit the transcriptional activities of the widely expressed E2A proteins. This inhibition may occur as a consequence of forming inhibitory complexes of E2A protein with Hes/HERP/HEY proteins, as well as promotion of E2A protein ubiquitylation and degradation by Notch. Indeed, when cultured SCLC cells are induced to overexpress the E2A protein, E12, the ability of Notch1 to induce hASH1 degradation is blunted. Together, these observations indicate that Notch signaling rapidly induces degradation along with inactivation of E-proteins and tissue-specific bHLH proteins such as hASH1. Interestingly, Notch1 was much more potent than Hes1 in causing hASH1 silencing and proteasomal degradation, as well as SCLC G1 cell cycle arrest. These findings are consistent with a model suggesting that Hes–Hey heterodimers may form a more potent Notch effector complex than Hes1 alone. Thus, loss of hASH1 may be critical in mediating the growth inhibitory effect of Notch1 in SCLC. A recent study supporting the idea that Notch1 could have anti-tumor properties on neuroendocrine tumors shows that the induction of Notch1 by valproic acid inhibits carcinoid cell proliferation in vitro and carcinoid tumor growth in vivo.

The role of Notch ligands in lung cancer has been poorly studied, yet a recent report shows that among Notch ligands, JAG1, JAG2, Dll1 and Dll3 are highly expressed in lung cancer cell lines. Moreover, JAG1 expression is dependent upon EGFR activation whereas JAG2 is not. JAG1 depletion (but not JAG2) induces apoptosis, whereas JAG2 depletion induces increased expression of inflammatory related genes. These results suggest that in the same cell type, JAG1 and JAG2 may have distinct functions and that JAG2 can regulate cytokines involved in anti-tumor immunity.
Are Guidance Molecules and their Receptors Targets for Lung Cancer Therapy?

These five families of guidance molecules are involved in tumor development and regulate tumor cell growth, migration, metastasis or angiogenesis. Thus, their manipulation represents new approaches in cancer therapy. However, given the complexity of the various systems in different contexts, it will be necessary to precisely understand the nature of the alteration in order to best take advantage of therapeutic opportunities.

Semaphorins and their receptors. Since semaphorins and their receptors have emerged as regulators of lung tumor development and progression, there is currently strong emphasis on elaborating therapeutic molecules that affect this pathway. To date, significant progress has been achieved although most interest has been centered on neuropilins as downstream VEGF receptors.

Researchers have come up with different strategies to impair the VEGF-neuropilin pathway, such as polysaccharide sulfate-induced neuropilin internalization, neuropilin dimerization inhibiting peptides and VEGF-binding blocking peptides. One of the most promising studies demonstrated that neuropilin antibodies that specifically prevent VEGF binding to NRP1 or NRP2 efficiently reduce tumor growth, angiogenesis and tumor metastasis in animal models. Metastasis could also be inhibited by blocking lymphangiogenesis with an anti-NRP2 treatment that blocks VEGF-C binding to NRP2. This treatment did not affect the primary tumor growth but reduced metastasis to the lung without affecting normal lymphatics. Such a treatment might avoid side effects like lymphedema. Moreover, these antibodies were shown to have additive effects with anti-VEGF antibodies. Thus, there is substantial enthusiasm for targeting neuropilin in cancer.

Semaphorin-based therapies could also prove valuable in clinical practice. For instance, injection of the extracellular domain of SEMA6D reduced tumor growth and angiogenesis in mice. In some situations where semaphorin inhibition is desired, antibodies that block semaphorin-neuropilin binding have been designed and their efficacy as therapeutic agents will likely be evaluated in the future. One option that has not been reported to our knowledge is to target plexins, possibly by screening chemical libraries for plexin agonists or small molecule inhibitors. This might be of interest considering the recent findings that plexins are mutated in some cancers. Given that there are two neuropilins, but nine plexins, it appears reasonable that therapeutic targeting of the latter will be more specific. Alternatively, development of specific inhibitors of semaphorins proved to be a feasible approach, as evidenced by independent reports in the nervous system. Interestingly, a dual VEGF-SEMA3A signaling inhibitor, ZD4190, decreases lung tumor xenograft growth in mice and has additive effects with a Src kinase inhibitor. Therefore, there is hope to target the semaphorin pathway for cancer patient treatment, even if this field is still on the emerging slope.

Ephrins and their receptors. As described, a subset of lung cancers exhibit Eph/ephrin mutations or amplifications. Therefore, these molecules might represent therapeutic targets, at least in patients whose tumors contain relevant molecular alterations. For instance, lung cancer cell lines with high copy number of EPHA3, A5 or A8 are more sensitive to the broad-spectrum Bcr/Abl-Src kinase inhibitor, Dasatinib. Interestingly, EPH/ephrins are known to signal through Abl and the Src family kinase FYN (Fig. 2), suggesting that Dasatinib directly inhibits ephrin signaling. Alternatively, overexpression of the EGFR family member, ErbB2, in a mouse cancer model correlates with sensitivity to EphA2 inhibition since EphA2 amplifies ErbB2 signals.

These findings might be relevant to lung cancer where EGFR pathways are commonly affected. Targeting EphA2 appears promising since it would simultaneously block several aspects of tumor progression involving tumor growth, vasculature and microenvironment.

An effort has been placed on engineering potential therapeutic agents that target the ephrin pathway and several strategies have been employed. For example, a small molecule inhibitor competitively blocks ephrin-A5 binding to EphA2 and EphA4 receptors. Conversely, ephrin-mimetic peptides allow specific drug delivery to EphA2 expressing tumor cells. Drug-conjugated human monoclonal antibodies that selectively recognize EphA2 have also been used as "trojan horse." Other efforts, such as an ephrin-A1 cytotoxin and manipulation of EphA2 function have been reported. Finally, EphB2 receptor can be inhibited using azurin, a metalloprotein with structural homology with the EphB2 receptor-binding region. Also, a soluble monomeric EphB4 inhibits the tri-dimensional and in vivo growth of cancer cells.

In summary, there is considerable enthusiasm that agents targeting the Eph/ephrin system will find their way to the clinic for the treatment of lung cancer.

Netrins and their receptors. Targeting netrin-1 might be attractive as a treatment for lung cancer either by inhibiting netrin-1 expression or by preventing its interaction with receptors. For example, DCC-5Fbn, a 100 amino-acid decoy DCC fragment, affects the ability of netrin-1 to trigger receptor multimerization thereby inhibiting its antiapoptotic effect. Inhibition of netrin-1 binding to its receptors might benefit patients whose primary tumors show high levels of netrin-1. Such an approach might not only eradicate tumor epithelial cells but also angiogenic vessels. However, induction of apoptotic death in netrin-1 producing cells by interfering with netrin-1 activity is restricted to human cells and animal models that fail to recapitulate the process of NSCLC in humans. Better understanding of the roles of netrins in cancer will be necessary to induce apoptosis specifically in tumor cells.

Slit/Robo. Some molecules interacting and interfering with Slit/Robo have proved to have potential anti-tumor capabilities in animal models by inhibiting tumor angiogenesis. Despite the fact that lung cancer therapy has not been studied yet, results obtained in melanoma and in chemically induced oral carcinoma, are promising for lung cancer. In these models, both the extracellular domain of Robo1 and a monoclonal antibody directed against the first immunoglobulin domain of Robo1 (R5) inhibited tumor angiogenesis and tumor growth in vivo. Interestingly, interfering with Slit2/Robo1 signaling did not affect...
the expression profile of VEGF. This suggests that inhibiting Slit-Robo signaling could potentially address the clinical problem of drug resistance in patients treated with VEGF antagonists, such as bevacizumab, in addition to metastasis promotion and perturbation of normal angiogenesis.157,158 Soluble Robo4 could have potential for cancer therapy as Robo4 is specifically expressed on endothelial cells.159,160

**Delta/Notch.** Although not reported in lung cancer patients, a few clinical trials involving the γ-secretase inhibitor MK-0752 are ongoing for patients with breast cancers, leukemia and lymphoma. Valproic acid, which induces Notch1 expression in carcinoma cells and subsequent G arrest, is also under investigation in several clinical trials, including one for patients with SCLC, and it is used in combination with other molecules.157 The use of compounds affecting Notch signaling in angiogenesis might have potential for future therapies.47 Dll4 is expressed specifically in tumor vasculature and weaker in adjacent normal vessels, suggesting that therapies directed against Dll4 signaling, as opposed to therapies affecting VEGF signaling, might specifically affect tumor growth without altering other organ functions. Blockade of Dll4-Notch has been tested in various tumor cell line models in mice, with reduction of tumor growth from 50 to 90%. Remarkably, the reduced tumor growth is associated with an increase of non-productive vessels together with a concomitant decrease of ephrin-B2. Furthermore, inhibiting Dll4-Notch interaction using Dll4-Fc or anti-Dll4 antibody affects growth of tumors that were resistant to anti-VEGF treatment.

**Conclusion**

It has become apparent in the last several years that guidance molecules are involved in lung cancer development/progression through interacting with cell survival, migration and tumor angiogenic pathways. The observation that alterations such as LOH or amplification frequently affect the chromosomal locations of several guidance genes in lung cancer also suggests their importance in this disease (Table 2). Moreover, mutations and inactivation by DNA methylation have also been reported for some genes.

In further support of the importance of these molecules in lung cancer, some therapeutic success has been achieved against specific targets. Most of these approaches have been carried out on human cancer cell lines and in animal models. For instance, targeting the neuropilins as VEGF co-receptors appears to be an effective strategy both for angiogenesis as well as lymphangiogenesis. In addition, there is enthusiasm for modulating the semaphorin pathway: while class-3 semaphorins have received the greatest attention because of their interaction with the neuropilins, other components can be targeted. It is also our opinion that the other guidance molecules, including the EPH/ephrin, netrin/DCC/UNC5, Slit/Robo and Delta/JAG/Notch pathways provide equally interesting targets.

On a cautionary note, there is considerable complexity in these pathways. For example, one ligand can bind different receptors and one receptor can bind different ligands with different biologic consequences. Thus, predicting the biologic consequences of any given intervention may be difficult and considerable experimentation will be required. Moreover, there appears to be cross-talk between pathways. Finally, with increasing knowledge come unexpected consequences, such as reports that VEGF blockade is associated with increased invasion and metastases, at least in experimental models.157,161-163 Nevertheless, this represents an exciting area of investigation and promise for the future.

**References**

1. Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. Science 1996; 274:1123-33.
2. Hinck L. The versatile roles of “axon guidance” cues in tissue morphogenesis. Dev Cell 2004; 7:783-93.
3. Suchting S, Bicknell R, Eichmann A. Neuronal clues to vascular guidance. Exp Cell Res 2006; 312:668-80.
4. Chedotal A, Kerjan G, Moreau-Fauvarque C. The brain within the tumor: new roles for axon guidance molecules in cancers. Cell Death Differ 2005; 12:1044-56.
5. Klagenbrun M, Eichmann A. A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. Cytokine Growth Factor Rev 2005; 16:535-48.
6. Potiron VA, Roche J, Drabkin HA. Semaphorins and their receptors in lung cancer. Cancer Lett 2009; 273:1-14.
7. Soker S, Takashima S, Miao HQ, Neufeld G, Klagesbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998; 92:735-45.
8. Miao HQ, Soker S, Feiner L, Alonso JL, Raper JA, Klagesbrun M. Neuropilin-1 mediates collapsin-1/semaphorin III inhibition of endothelial cell motility: functional competition of collapsin-1 and vascular endothelial growth factor-165. J Cell Biol 1999; 146:233-42.
9. Favier B, Alam A, Barron P, Bonnin J, Laboudie P, Fons P, et al. Neuropilin-2 interacts with VEGFR-2 and VEGFR-3 and promotes human endothelial cell survival and migration. Blood 2006; 108:1243-50.
10. Karpapanen T, Heckman CA, Keskitalo S, Jeltsch M, Ollila H, Neufeld G, et al. Functional interaction of VEGF-C and VEGF-D with neuropilin receptors. FEBS J 2006; 20:1462-72.
11. Uniewicz KA, Fernig DG. Neuropilins: a versatile family of receptors and ligands in blood vessel development and tumor angiogenesis. J Cell Physiol 2008; 217:416-29.
12. Kruger RP, Aurandt J, Guan KL. Semaphorins complex: what is their role? Cell Res 2007; 17:897-906.
13. Willecke K. Semaphorins: key players in tumor angiogenesis and metastases. Curr Opin Pharmacol 2007; 7:197-203.
14. Lucchesi BR. Semaphorins: key players in tumor angiogenesis and metastases. Curr Opin Pharmacol 2007; 7:197-203.
15. Klotzle B, Richard G, Behr C. The orthologous human and murine semaphorin 6A-1 proteins (SEMA6A-1/Sema6A-1) bind to the endothelial vasodilator-stimulated phosphoprotein-like protein (EVL) via a novel carboxy-terminal zyxin-like domain. J Biol Chem 2008; 283:39647-53.
16. Toyoshima T, Zhang H, Kumanogoh A, Takegahara N, Yabuki M, Harada K, et al. Guidance of myocardial patterning in cardiac development by Sema6D reverse signaling. Nat Cell Biol 2004; 6:1204-11.
17. Surawaska H, Ma PC, Salgia R. The role of ephrins and Eph receptors in cancer. Cytokine Growth Factor Rev 2004; 15:419-33.
18. Zhang J, Hughes S. Role of the ephrin and Eph receptor tyrosine kinase families in angiogenesis and development of the cardiovascular system. J Pathol 2006; 208:453-61.
19. Pasquale EB. Eph-ephrin bidirectional signaling in physiology and disease. Cell 2008; 133:38-52.
20. Arvanitis D, Davy A. Eph/ephrin signaling: networks. Genes Dev 2008; 22:416-29.
21. Hong K, Hinck L, Nishiyama M, Poo MM, Tessier-Lavigne M, Stein E. A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell 1999; 97:927-41.
40. Stein E, Tessier-Lavigne M. Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science 2001; 291:1928-38.

41. Forcer C, Ye X, Granger L, Corset V, Shin V, Bredesen DE, et al. The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. Proc Natl Acad Sci USA 2001; 98:3416-21.

42. Giniger E, Jan LY, Jan YN. Specifying the path of the intersegmental nerve of the Drosophila embryo: a role for Delta and Notch. Development 1993; 117:431-40.

43. De Bellard ME, Ching W, Gossler A, Bronner-Fraser M. Disrupting segments of neural crest migration and ephrin expression in delta-1 null mice. Dev Biol 2002; 249:120-31.

44. Ferrari-Tonielli G, Bonini SA, Bertinoli P, Uberti D, Momo M. Microtubule stabilizing effect of notch activation in primary cortical neurones. Neuroscience 2008; 154:946-52.

45. Fuss B, Josten F, Feix M, Hoch M. Cell movements controlled by the Notch signalling cascade during foregut development in Drosophila. Development 2004; 131:1387-95.

46. Hashimoto-Torii K, Torii M, Sarkisian MR, Barlet CM, Shen J, Radtke F, et al. Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral neuron. Neuron 2008; 60:273-84.

47. Thurston G, Noguera-Triese I, Vancopoulus GD. The Delta paradoxB: DLL4 blockade leads to more tumour vessels but less tumour growth. Nat Rev Cancer 2007; 7:327-31.

48. Bray S. Notch signalling in Drosophila: three ways to use a pathway. Semin Cell Dev Biol 1998; 9:191-200.

49. Ito T, Kagoshima M, Sasaki Y, Li C, Udaka N, Kutsukawa T, et al. Repulsive axon guidance molecule Sema3A inhibits branching morphogenesis of fetal mouse lung. Mech Dev 2008; 97:35-45.

50. Kagoshima M, Ito T. Diverse gene expression and function of semaphorins in developing lung: positive and negative regulatory roles of semaphorins in lung branching morphogenesis. Genes Cells 2001; 6:559-71.

51. Brannibulla E, Constantin B, Dabrak, H, Rach: J. Semaphorin SEMAP localization in malignant human lung and cell lines: A suggested role in cell adhesion and cell-migration. Am J Pathol 2000; 156:959-50.

52. Wilkinson GA, Scirrty JC, Reinhardt DJ Klein R. Role for ephrinB2 in postnatal lung alveolar development and elastic matrix integrity. Dev Dyn 2008; 237:2220-34.

53. Wohlfahrt JG, Karagiannidis C, Kunzmann S, Epstein CM, Shen J, Radtke F, et al. Interaction between Reelin and Notch signaling regulates neuronal migration and elastic matrix integrity. Dev Dyn 2008; 237:2220-34.

54. Kitsukawa T, et al. Repulsive axon guidance molecule ephrin-A2. Biochem Biophys Res Commun 1998; 252:378-82.

55. Fox GM, Holst PL, Chure HT, Lindberg RA, Jansson AM, Bus R, et al. CDNA cloning and tissue distribution of five human EPH-like receptor protein-tyrosine kinases. Oncogene 1995; 10:897-905.

56. Beckmann MP, Ceretti DR Baum P, Vanden Bos T, James L, Fazlal, T, et al. Molecular characterization of a family of ligands for eph-related tyrosine kinase recep- tors. EMBO J 1994; 13:3757-62.

57. Bohme B, Holtrich U, Wolf G, Luzius H, Graschik KH, Szeberndt K, et al. PCR mediated detection of a new human receptor-tyrosine-kinase, HEK 2. Oncogene 1993; 8:2857-62.

58. Ceretti DP, Vanden Bos T, Nelson N, Koulousky C, Reddy P, Marsakovsky E, et al. Isolation of LERK-5, a ligand of the eph-related receptor tyrosine kinases. Mol Immunol 1995; 32:1197-205.

59. Fletcher FA, Carpenter MK, Shilling H, Baum P, Zegler SF, Gimpel M, et al. LERK-2, a binding protein for the receptor-tyrosine kinase ELK, is evolutionarily conserved and expressed in a developmentally regulated pattern. Oncogene 1994; 9:5241-7.

60. Hafner C, Schmitz G, Meyer S, Bataille F, Hau P, et al. p53RDL1 regulates p53-dependent apoptosis in colorectal cancer (DCC). J Biol Chem 2003; 278:30425-7.

61. Favre CJ, Mancuso M, Maas K, McLean JW, Baluk P, McDonald DM. Expression of genes involved in vascular development and angiogenesis in endothelial cells of adult lung. Am J Physiol Heart Circ Physiol 2003; 285:1917-38.

62. Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiaia TM, et al. Ephrin-B2 selectively marks arteri- ous vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. Dev Biol 2001; 230:151-60.

63. Wang HU, Chen ZF, Anderson DJ. Molecular distinction and angiogenic features between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 1998; 93:741-53.

64. Schwartz MA, Caldwell L, Caffasso D, Zheng H. Emerging pulmonary vascularization lacks fate specifi- cation. Am J Physiol Lung Cell Mol Physiol 2009; 296:71-81.

65. Foo SS, Turner CJ, Adams S, Compagno A, Aubyn D, Kogata N, et al. Eph-B2 cells control motility and adhesion during blood- vessel-wall assembly. Cell 2006; 124:161-73.

66. Eberer R, Eichelbacher U, powjabo V, Koen T, Djourov V, Lin N, et al. EphB4 controls blood vascular morphogen- esis during postnatal angiogenesis. EMBO J 2006; 25:5228-41.

67. Larson J, Schmitt S, Schreuder W, Carpenter DC. Endothelial Eph receptor stimulation increases lung vascular permeability. Am J Physiol Lung Cell Mol Physiol 2008; 295:431-9.

68. Eichmann A, Makinen T, Alitalo K. Neural guidance molecules regulate vascular remodeling and vessel navi- gation. Genes Dev 2005; 19:1013-21.

69. Dalvin S, Anselmo MA, Prodhian P, Konatuzski K, Schnittjer JT, Kinane TB. Expression of Notin-1 and its two receptors DCC and UNC5H2 in the developing mouse lung. Gene Expr Patterns 2003; 3:729-83.

70. Liu Y, Stein E, Oliver T, Li Y, Brunken WJ, Koch M, et al. Novel role for Netin in regulating epithelial behavior during lung branching morphogenesis. Curr Biol 2004; 14:897-905.

71. Xian J, Clark KF, Fordham R, Pannel R, Rabbith TH, Rabbith PH. Inadequate lung development and bronchial hyperplasia in mice with a targeted deletion in the Durl1/Robo1 gene. Proc Natl Acad Sci USA 2001; 98:15062-6.

72. Greenberg JM, Thompson FY, Brooks SK, Shannon JM, Akeson AL. Slit and robo expression in the develop- ing mouse lung. Dev Dyn 2004; 230:350-60.

73. Anselmo MA, Dalvin S, Prodhian P, Konatuzski K, Aidlen JT, Schnittjer JT, et al. Slit and robo: expression patterns in lung development. Gene Expr Patterns 2003; 3:139.

74. Clark K, Hammond E, Rabbith P Temporal and spatial expression of two isoforms of the Durt1/Robo1 gene in mouse development. FEBS Lett 2002; 523:12-6.

75. Guseh JS, Bores SA, Stanger BZ, Zhou Q, Anderson JM. Basic helix-loop-helix transcription fac- tors regulate the neuroectoderm differentiation of fetal mouse pulmonary epithelium. Development 2000; 127:931-21.

76. Post LC, Terner M, Hogan BL, Notch/Delta expression in the developing mouse lung. Mech Dev 2000; 98:95-5.

77. Shan L, Arter JC, Sklar J, Sunday ME. Notch-1 regulates pulmonary neuroectoderm cell differentiation in cell lines and in transgenic mice. Am J Physiol Lung Cell Mol Physiol 2007; 292:500-9.

78. Tsao PN, Vanconcellos M, Ivolsky KI, Qian J, Lu J, Cardoso WV. Notch signaling controls the balance of ciliated and secretory cell fates in developing airways. Development 2009; 136:2257-307.
98. Koyama N, Zhang J, Hsuin, Miyawara H, Tanaka T, Su X, et al. Identification of IGBP6 as an effector of the tumor suppressor activity of SEMA3B. Oncogene 2008; 27:6581-9.

99. Rolny C, Capparuccia L, Casazza A, Mazzone M, Villadoro A, Cignetti A, et al. The tumor suppressor semaphorin 3B triggers a prometastatic program mediated by inter leukin 8 and the tumor microenvironment. J Exp Med 2008; 205:1155-71.

100. Pan SH, Chao YC, Chen HY, Hung PF, Lin PY, Lin CW, et al. Lumin forms collagen response mediator protein-1 (LRM-1) expression is associated with clinical outcome and lymph node metastasis in non-small cell lung cancer patients. Lung Cancer 2010; 67:93-100.

101. Lugli A, Schipitko H, Maurer R, Mirlicher M, Kiefer J, Huskens P, et al. EphB2 expression across 138 human tumor types in a tissue microarray: high levels of expression in gastrointestinal cancers. Clin Cancer Res 2005; 11:6450-60.

102. Yu J, Bulk E, Ji P, Hascher A, Korschmider S, Berdel WE, et al. The kinase defective EphB6 receptor tyrosine kinase activates MAP kinase signaling in lung adenocarcinoma. Int J Oncol 2009; 35: 759-76.

103. Sos ML, Michel K, Zander T, Weiss J, Frommolt P, et al. Predicting drug susceptibility of non-small cell lung cancers based on genetic lesions. J Clin Oncol In press 2009; 18: 1747-44.

104. Ding L, Gertz G, Wheeler DA, Mardis ER, McEllan MD, Cibulskis K, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature 2008; 458:1060-79.

105. Nauren N, Mohammed KA, Lai Y, Antony VB, Receptor EphA2 activation with ephrinA1 suppresses growth of malignant mesothelioma (MM). Cancer Lett 2007; 258:215-22.

106. Ogawa K, Panzaffini R, Lindberg R, Ata R, Pfeifer AL, Pasquali R. The ephrin-A1 ligand and its receptors, EphA2, are expressed during tumor neovascularization. Oncogene. 2006; 19:6043-52.

107. Fearon ER, Cho KR, Nigro JM, Kern SE, Simons JW, et al. Somatic mutations affect key pathways in lung adenocarcinoma. Nature 2008; 458:1060-79.

108. Shin SK, Nagasaka T, Jung BH, Matsubara N, Kim WH, et al. Human semaphorins A(V) and IV reside in lung cancer and demonstrate distinct expression patterns. Proc Natl Acad Sci USA 2007; 104:12890-5.

109. Dallol A, Forgacs E, Martinez A, Sekido Y, Walker R, Kshibida T, et al. Tumor specific promoter region methylation of the human homologue of the Drosophila Roundabout gene DUTT1 (ROBO1) in human cancers. Oncogene 2002; 21:3200-8.

110. Sundaresan V, Chung G, Heppell-Parton A, Xiong J, Grundy C, Roberts I, et al. Homozygous deletions at 3p12 in breast and lung cancer. Oncogene 1998; 17:1723-9.

111. Georgas K, Burridge L, Smith K, Holmes GR, Chenex-Trench G, Ioannou PA, et al. Assignment of the human slit homologue SLIT2 to human chromosome band 4p13.2. Cyogenet Cell Genet 1999; 86:246-7.

112. Shivaparakash N, Virmani AK, Wontschulji MB, Mackay B, Minna JD, et al. Deletions of chromosome 4 at multiple sites are frequent in malignant mesothelioma and small cell lung carcinoma. Clin Cancer Res 1999; 5:17-23.

113. Girard L, Zochbauer-Muller S, Virmani AK, Gazdar AF, Minna JD. Genome-wide allelotyping of lung cancer identifies new regions of allelic loss, differences between small cell lung cancer and non-small cell lung cancer, and loci clustering. Cancer Res 2000; 60:4896-904.

114. Dallol A, Da Silva NF, Vicacova P, Minna JD, Bieche I, Maher ER, et al. SLIT2, a human homologue of the Drosophila slit gene, has tumor suppressor activity and is frequently inactivated in lung and breast cancers. Cancer Res 2002; 62:5874-83.

115. Gorn M, Anige M, Burkholler I, Muller B, Schefller A, Edler L, et al. Serum levels of Magic Roundabout protein in patients with advanced non-small cell lung cancer (NSCLC). Lung Cancer 2005; 49:71-6.

116. Zheng Q, Qun H, Zhang H, Li J, Hou L, Wang H, et al. Notch signaling inhibits growth of the human lung adenocarcinoma cell line A549. Oncol Rep 2007; 17:847-52.

117. Chen Y, De Marco MA, Graziani I, Gazdar AF, Strock PR, Miele L, et al. Oxygen concentration determines the biological effects of NOTCH-1 signaling in adenocarcinoma of the lung. Cancer Res 2007; 67:7954-9.

118. Garnis C, Campbell J, Davies JJ, Macaulay C, Lam S, Williams R, et al. Involvement of multiple developmental genes on chromosome 1p in lung tumorigenesis. Hum Mol Genet 2005; 14:473-82.

119. Dang TP, Gazdar AF, Virmani AK, Sepetavec T, Hande KR, Minna JD, et al. Chromosome 19 translocation, overexpression of Notch3, and human lung cancer. J Natl Cancer Inst 2000; 92:1355-7.
132. Haruki N, Kawaguchi KS, Eichenberger S, Massion PP, Olson S, Gonzalez A, et al. Dominant-negative Notch3 receptor inhibits mitogen-activated protein kinase pathway and the growth of human lung cancers. Cancer Res 2005; 65:3555-61.

133. Konishi J, Kawaguchi KS, Vo H, Haruki N, Gonzalez A, Carbone DP, et al. Gamma-secretase inhibitor prevents Notch3 activation and reduces proliferation in human lung cancers. Cancer Res 2007; 67:8051-6.

134. Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. Cancer Res 2001; 61:3200-5.

135. Sriuranpong V, Borges MW, Strock CL, Nakakura EK, Watkins DN, Blaumaulder CM, et al. Notch signaling induces rapid degradation of achaete-scute homolog 1. Mol Cell Biol 2002; 22:3129-39.

136. Nie L, Xu M, Vladimirova A, Sun XY. Notch-induced E2A ubiquitination and degradation are controlled by MAP kinase activities. EMBO J 2003; 22:5780-92.

137. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R, Ning L, Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Neri D, Bicknell R. Tumour vascular targeting. Nat Rev Cancer 2005; 5:436-46.

138. Pan Q, Chantry Y, Liang WC, Stawicki S, Mak J, Rathore N, et al. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell 2007; 11:53-67.

139. Balakrishnan A, Penachioni JY, Lamba S, Bleeker FE, Zanot C, Rodolfo M, et al. Molecular profiling of the "plexinome" in melanoma and pancreatic cancer. Hum Mutat 2009; 30:1167-74.

140. Wong OG, Nikumon T, Oninuma I, Zhou C, Blanc V, Brown RS, et al. Plexin-B1 mutations in prostate cancer. Proc Natl Acad Sci USA 2007; 104:19040-5.

141. Naraizaki M, Segarra M, Tosato G. Sulfated polysaccharides identified as inducers of neuropilin-1 internalization and functional inhibition of VEGF-A, and semaphorin3A. Blood 2008; 111:4126-36.

142. Roeth L, Nasarre C, Dhirig, Grosch S, Aunis D, Cremel G, Hubert P, et al. Transmembrane domain interactions control biological functions of neuropilin-1. Mol Biol Cell 2008; 19:646-54.

143. Caunter M, Mak J, Liang WC, Stawicki S, Pan Q, Tong RK, et al. Blocking neuropilin-2 function inhibits tumor cell metastasis. Cancer Cell 2008; 13:331-42.

144. Pan Q, Chantry Y, Liang WC, Stawicki S, Mak J, Rathore N, et al. Blocking neuropilin-1 function has an additive effect with anti-VEGF to inhibit tumor growth. Cancer Cell 2007; 11:53-67.

145. Balakrishnan A, Penachioni JY, Lamba S, Bleeker FE, Zanot C, Rodolfo M, et al. Molecular profiling of the "plexinome" in melanoma and pancreatic cancer. Hum Mutat 2009; 30:1167-74.

146. Wang OG, Nikumon T, Oninuma I, Zhou C, Blanc V, Brown RS, et al. Plexin-B1 mutations in prostate cancer. Proc Natl Acad Sci USA 2007; 104:19040-5.