HHS Public Access

Author manuscript

Cytokine Growth Factor Rev. Author manuscript; available in PMC 2016 February 01.

Published in final edited form as:

Cytokine Growth Factor Rev. 2015 February ; 26(1): 1–6. doi:10.1016/j.cytogfr.2014.05.001.

Eph Receptor Tyrosine Kinases in Cancer Stem Cells

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Abstract

Eph receptor tyrosine kinases (RTKs) and their ligands, ephrins, play critical roles in development, tissue homeostasis, and cancer. Because Eph receptors are expressed in most adult stem cell niches and in many types of cancers, it has been long suspected that this family of RTKs may also regulate the function of cancer stem-like cells (CSCs). This review will focus on recent studies to elucidate the contribution of Eph/ephrin molecules in CSC self-renewal and tumorigenicity, as well as describe efforts to target these molecules in cancer. Because CSCs are often resistant to therapeutic intervention and have been shown to depend on Eph RTKs for self-renewal, targeting Eph receptors may hold promise for the treatment of drug-resistant cancers.

Introduction

It has become increasingly clear that cancer is not a homogenous disease at either the histological or genetic level. Intratumoral heterogeneity has been shown to contribute to both cancer progression and resistance to therapy. Although controversy remains about how to best define cancer stem cells (CSCs), a subpopulation of self-renewing CSCs has been recognized in tumors for their role in facilitating tumor heterogeneity, metastasis, and therapeutic resistance (1, 2). Receptor tyrosine kinases (RTKs) play important roles in maintaining CSC phenotypes, including self-renewal capacity, viability, invasiveness, and tumorigenicity. This article highlights the recent studies to elucidate the contribution of Eph RTKs in the maintenance of CSCs and reviews strategies for targeted inhibition of Eph RTKs in cancer.

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Unique features of Eph receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are important regulators of signal transduction pathways that promote cell growth, survival, and motility during malignant progression of solid tumors. Nearly 50% of RTKs are thought to have oncogenic potential. The Eph receptors belong to the largest RTK family which comprises 14 receptors, accounting for nearly a quarter of the 58 RTKs found in the human proteome [reviewed in (3, 4)]. Structurally, the Eph receptors have the typical RTK topology, with a ligand-binding domain, motifs involving receptor clustering in the extracellular region, a single transmembrane domain, and a cytoplasmic region that contains the kinase domain (Figure 1A). However, compared to other RTKs, Eph receptors have many unique features. For example, unlike many other RTKs, Eph receptors lack a “molecular brake” between the two lobes of the kinase domain (5). Furthermore, not all the Eph receptors contain the typical “gatekeeper” residue that controls access to a hydrophobic binding pocket adjacent to the ATP binding site in the hinge region between the lobes of kinase domain (6).

Activation of Eph receptors by their membrane-bound ligands, or ephrins, on adjacent cells induces receptor oligomerization, leading to trans-phosphorylation and activation of the receptor, termed “forward signaling”. Because ephrins are membrane bound, they are also capable of transducing signals in ligand-expressing cells, referred to as “reverse signaling”. In addition to bi-directional signaling between neighboring cells, Eph receptors and ephrins can be co-expressed in the same cell. In the case when both receptor and ligand are highly expressed, unlike the autocrine signaling of other RTKs, a lateral cis-interaction between the ligand and receptor in the same cell can inhibit Eph receptor “forward signaling” (7–10). In contrast, when lower levels of ligand and receptor are expressed in the same cell, Eph receptors and ephrins are often sequestered in separate microdomains, allowing for parallel activation of “forward” and “reverse signaling” in the same cell (11). Furthermore, Eph receptors can signal independent of ephrin ligands through cross-talk with other receptor systems or oncogenic signaling molecules (12–14) (Figure 1B). These features, as well as cellular context and feedback regulation, contribute to the diversity of Eph receptor activity and functionality. Details of Eph receptor signaling pathways can be found in recent reviews (3, 4, 15).

Ephrins and Eph RTKs were originally identified as axon guidance regulators during neural development, and subsequently have been recognized as modulators of physiologic and pathologic processes during embryonic development, normal tissue homeostasis, and disease. Despite the fact that Eph receptors and other RTKs share many common downstream signaling molecules such as Rho and Ras family GTPases and the Akt/mTORC1 pathway, the biological outcomes of ligand-induced Eph receptor signaling are often distinct. For example, while activation of many RTK families leads to cell proliferation, survival, and motility, ephrin-induced Eph receptor signaling can result in growth inhibition and induce cell repulsion (3, 4, 15). In the absence of ligand engagement, however, Eph receptors can also interact with other cell-surface receptors, such as EGFR and ERBB2, resulting in growth promotion and enhanced cell motility (12–14).

Role of Eph receptors in stem cells

Eph receptors have long been implicated in stem cell biology, both during embryonic development and in the adult stem cell niche. EphA2 is highly expressed in embryonic stem (ES) cells (16, 17) and its expression is regulated by E-cadherin (17). Other Ephs and ephrins are also frequently expressed in stem/progenitor cells (18). However, the majority of the functional studies in stem cells have been centered on the nervous system and the intestine.

In the adult brain, engagement of ephrins with their cognate Eph receptors inhibits neural progenitor proliferation in the subventricular zone (19, 20). EphA7 expressed on ependymal cells and astrocytes signals to ephrin-A2 on progenitors and neuroblasts to negatively regulate proliferation and the addition of new neurons to the olfactory bulb (20). Likewise, B class ephrins and Eph RTKs are expressed in the subventricular zone and blocking interactions between ephrin-Bs and Eph receptors resulted in increased progenitor cell proliferation (19). In the hippocampus, EphB receptor forward signaling regulates neurogenesis and migration of progenitor cells (21). In addition, EphB2 controls lineage plasticity of the adult neural stem cell niche (22). Studies of targeted deletion of ephrin-A5 in mice indicated a similar role of A class Eph/ephrin in the hippocampus (23).

In the intestine, stem cells reside at the base of the crypts with cell differentiation proceeding upward toward the top of the intestinal lumen (24). EphB2 and EphB3 are highly expressed in stem cells in the intestinal crypt with their expression gradually decreasing as they migrate towards the top of the crypt. In contrast, ephrin-Bs are expressed complementarily in a gradient with highest expression in the differentiated cells (25). Expression of EphB receptors and ephrin-Bs in the intestine has been shown to be regulated by Wnt signaling. Functionally, EphB/ephrin-Bs are involved in regulation of both cell proliferation and migration, although with distinct signaling mechanisms. EphB2 regulates proliferation in a kinase-dependent manner through Abl kinase and Cyclin D1. In contrast, regulation of cell movement along the intestinal crypt is mediated by EphB2 through a kinase-independent PI3K signaling mechanism (18).

In addition to the brain and intestine, stem/progenitor cells of the skin, bone, and heart are also influenced by Eph-ephrin signaling. In the skin, multiple ephrins and Eph receptors are expressed in two epithelial stem cell populations, the hair follicle bulge stem cells and the basal layer stem cells (26). Disruption of Eph-ephrin interactions results in increased cell proliferation, suggesting ephrins as negative regulators of stem cell self-renewal in the skin (26). In the bone, the expression of Eph receptors and ephrins are regulated dynamically during osteoclast and osteoblast differentiation from monocytes and mesenchymal stem cell, respectively (27–29). Engagement of ephrins to their respective Eph receptors plays a critical role in bidirectional osteoclast-osteoblast communication. Ephrin-B2 is expressed in differentiating osteoclasts, and reverse signaling through ephrin-B2 inhibits osteoclast differentiation via suppression of transcription factors Fos and NFATc1. Forward signaling through EphB4 in the osteoblasts inhibits RhoA activity and osteoblast differentiation (29). In contrast, ephrin-A2 is expressed in early differentiating osteoclasts, and ephrin-A2 reverse signaling appears to stimulate osteoclast differentiation, possibly through enhanced

PLCγ2 signaling. Conversely, forward signaling through the EphA2 receptor inhibits osteoblast differentiation (28). In the heart, cardiomyocytes preferentially express ephrin-A1 ligand, whereas human cardiac stem cells express the EphA2 receptor (30). Recent studies provide evidence that ephrin-A1 promotes the motility of EphA2-positive cardiac stem cells, resulting in enhanced regeneration and cardiac function after myocardial infarction (30).

Eph receptors in cancer stem cells

Despite controversy on how best to define cancer stem cells (CSCs), recent data from human and mouse models support the presence of self-renewing CSCs (also termed tumor-initiating cells, TICs) that facilitate tumor heterogeneity, metastasis, and therapeutic resistance in a subpopulation of the tumor (1, 2). Recent genome analyses have correlated expression of many Eph RTKs in the tumor epithelium and microenvironment with disease stage, metastasis, recurrence, and patient survival (31). For example, EphA2 expression is elevated in breast, ovarian, and lung cancer, as well as in glioma and melanoma, and high levels of EphA2 are correlated with poor patient survival (32–40). Likewise, levels of several EphB RTKs are also elevated in different stages of colon, lung, and breast cancer (31). Aside from changes in receptor level, somatic mutations have been found in nearly all Eph receptors, some of which were identified as oncogenic mutations (35), whereas others have been found as mutations that inactivate tumor suppressor functions (41, 42). Because Eph receptors are expressed in most adult stem cell niches and in many types of cancers, it has been long suspected that this family of RTKs may also be essential in regulating cancer stem-like cell (CSC) function. However, functional evidence of the role of Eph receptors in cancer stem cells has only emerged recently.

One of the first pieces of evidence for the role of Eph RTKs in CSCs came from studies in malignant glioma where the role of CSCs has been firmly established by recent lineage-tracing studies (43). The EphA2 receptor is overexpressed in human glioblastoma CSCs, and EphA2 expression positively correlated with the size and tumor-initiating ability of the CSCs in this tumor type. Ephrin-A1-induced down regulation of EphA2 inhibited expression of neural stem cell markers and induced astroglial differentiation. Furthermore, EphA2 depletion by either ephrin-A1-induced EphA2 receptor degradation or by RNAi-mediated gene silencing, inhibited CSC self-renewal in vitro and tumorigenicity in xenografts. Together, these studies provided functional evidence of the critical role of EphA2 in cancer stem cells. In addition to EphA2, EphA3 has been shown to be expressed in glioma and also co-expressed with markers of undifferentiated cells in glioblastoma. Knockdown of EphA3 prevented tumorsphere formation and induced neural and glial cell differentiation. An EphA3 activating monoclonal antibody or RNAi-mediated EphA3 knockdown reduced tumorigenicity in tumor xenografts (44). Loss of either EphA2 or EphA3 was shown to be accompanied by an increase in ERK activity. As sustained MAPK signaling was reported to drive differentiation of neural progenitors (45), it is possible that EphA2- or EphA3 maintains CSCs in an undifferentiated state by inhibition of ERK activity (Figure 2).

In addition to regulating the self-renewal of CSCs, EphA2 also appears to modulate the motility and invasiveness of melanoma and glioblastoma stem cells (46) (Figure 2). Overexpression of EphA2 in melanoma cells promotes mesenchymal to amoeboid transition,

expression of stem cell markers, self-renewal of melanospheres, and tumor growth in vivo (47). In glioblastoma, expression of EphA2 in CSCs enhanced intracranial invasion. Using an efna1$^{-/-}$; efna3$^{-/-}$; efna4$^{-/-}$ triple knockout mouse model, Miao et al showed that ephrin-As provide major repulsive guidance cues in the brain microenvironment for infiltrating glioblastoma stem cells. EphA2-dependent glioma stem cell invasion appears to be mediated through cross-talk with Akt. Expression of an EphA2 mutant (S897A) incapable of being phosphorylated by Akt inhibited glioma stem cell invasion in vivo. Interestingly, EphA2 cross-talk with Akt, but not EphA2 kinase activity, was shown to be required for CSC invasion and self-renewal. However, unlike the EphA2 knockdown, the S897A mutation did not significantly affect tumorigenesis in vivo, suggesting an additional mechanism for the regulation of glioblastoma stem cell properties by EphA2.

The EphA2 receptor has also been implicated in playing a critical role in lung cancer CSCs (48). Expression of EphA2 was correlated with the expression of the cancer stem cell marker ALDH in a human cancer tissue microarray. RNAi-mediated depletion of EphA2 in lung cancer cells significantly reduced ALDH positive populations and the ability to form tumor spheroids in suspension. Knockdown of EphA2 in a cancer stem cell-enriched, sorted population inhibited tumorigenicity in a limiting dilution xenograft model. Although it was not directly tested, JNK signaling may be involved in EphA2-dependent CSCs self-renewal in lung cancer, as EphA2 regulates JNK and mTORC1 signaling in lung cancer cells (35, 48) and JNK signaling has been implicated in stem cells previously (49–51) (Figure 2).

B class Eph RTKs have been shown to be expressed in intestinal stem cells (25). In colon cancer, APC mutations are known to activate the Wnt pathway and upregulate the expression of EphB2, EphB3, and EphB4 receptors (52). The same EphB signaling pathway through Abl and Cyclin D1 in normal cells drives the proliferation of adenoma cells to repopulate the intestinal crypts. However, when EphB-expressing tumor cells reach the surface epithelium, they encounter normal cells expressing ephrin-Bs that restrict tumor cell expansion, resulting in in situ adenoma growth. As tumor development progresses, B class Eph RTK expression is silenced despite the persistence of Wnt signaling, which is concomitant with tumor invasion into surrounding tissues (52). Thus, constitutive Wnt signaling upregulates EphB expression at the early stage of intestinal adenoma formation, but malignant tumor progression at later stages of colon cancer requires EphB silencing. This observation in mice is further supported by clinical data in human cancer in which the loss of EphB expression correlates with the transition from adenoma to adenocarcinoma (52–54).

Targeting Eph receptors in cancer

Because of the tumor-promoting role of Eph receptors in cancer and their known function in regulating the self-renewal and invasion of CSCs, they have emerged as ideal therapeutic targets in cancer. However, some Eph receptors, such as EphB RTKs in colon cancer, exhibit dual roles of tumor promotion and tumor suppression depending on the stage of cancer development and progression (52). Other Eph receptors, such as EphA3, appear to function either as an oncogene or tumor suppressor depending on the tumor cell type (41, 44). Regardless of these caveats, many RTKs serve a tumor promoting role in cancer, and a

myriad of strategies to inhibit these oncogenic receptors have been developed. These include small molecule kinase inhibitors, peptides and small molecules that block ligand-receptor engagement, monoclonal antibodies or soluble ephrin-Fc or Eph-Fc proteins, and monoclonal antibodies or ephrins conjugated with drugs, toxins, or imaging agents.

Kinase inhibitors

Despite dichotomous roles of Eph receptors in cancer, kinase activity of the EphA2 receptor has been consistently linked to tumor promotion in breast cancer and in lung cancer (33, 55–57). Screening efforts to identify ATP-competitive, small molecule inhibitors of Eph RTKs have yielded inhibitors with nanomolar binding affinities to respective Eph RTKs (58). Given the conservative nature of the ATP-binding pocket, these Eph kinase inhibitors also inhibit a variety of other kinase targets. However, several compounds with relative selectivity for the Eph receptors have been discovered using a cell-based screening strategy using a library of type II kinase inhibitors (59). Type II small molecule inhibitors capitalize on the structural conformation of an inactive kinase, targeting the ATP-binding pocket of the kinase domain as well as an allosteric site next to the “FDG” motif in the receptor. Using this approach, a series of compounds targeting EphB2, EphB3, and EphB4, has been identified (60).

One of the compounds discovered to inhibit EphA2 activity, ALW-II-41-27, displayed potent activity in inducing apoptosis in non-small cell lung cancer cells and inhibited tumor growth in a xenograft model in vivo (57). Several lines of evidence suggest that ALW-II-41-27 can serve as a relatively selective EphA2 RTK inhibitor. First, ALW-II-41-27 inhibited EphA2 phosphorylation in cancer cells, whereas NG-25, a structural analog with a similar target spectrum as ALW-II-41-27, had no effect on EphA2 phosphorylation at the same dose and displayed limited effects on cell viability *in vitro* and tumor growth *in vivo*. Second, signaling studies in cells treated with ALW-II-41-27 recapitulated what was observed in EphA2 knockdown cells. Third, depletion of EphA2 by RNAi rendered NSCLC cells much less sensitive to the effects of ALW-II-41-27, relative to the undepleted controls. Finally, *in situ* drug-tumor interaction studies using “KiNativ” mass spectrometry demonstrated the selectivity of ALW-II-41-27 for EphA2 within the Eph receptor family as well as among other kinases. Although the pharmacokinetics, bioavailability, and biological IC_{50} of ALW-II-41-27 need to be further improved, this compound represents a good starting point for further development of EphA2-specific inhibitors with suitable properties for clinical evaluation.

In addition to the identification of inhibitors targeting EphA2, efforts have been devoted to develop selective EphB4 RTK inhibitors (61) due to its prominent role in tumor angiogenesis. Of these, NVP-BHG712 has displayed relative selectivity towards EphB4, inhibiting its kinase activity in the nanomolar range in cell-based assays. In addition, NVP-BHG712 exhibits suitable pharmacokinetic properties after oral administration in vivo, and it has been shown to inhibit EphB4 autophosphorylation in tissue as well as VEGF-induced angiogenesis. Other Eph RTK inhibitors have also been developed, but further studies are needed to investigate their biological effects and target specificities in vivo [reviewed in (58)].

Small molecular antagonists or agonists of Eph receptor

Recent advances in developing small molecule inhibitors capable of disrupting protein-protein interfaces permits the identification of compounds that bind to Eph receptors and inhibit ephrin binding. Combined efforts of high through-put screening and structural analyses have identified the derivatives of salicylates and lithocholic acid as small molecule inhibitors capable of disrupting Eph receptor-ligand interactions. Several salicylates were found to target the binding pocket of EphA4 as well as inhibiting ephrin binding to EphA2 and EphA4 (62, 63). These compounds inhibit a subset of Eph receptor activation and endothelial capillary-like tube formation in cell-based assays.

Another study has found lithocholic acid as a reversible competitive inhibitor of EphA2, which inhibits ephrin binding to several Eph receptors and subsequent receptor tyrosine phosphorylation (64). Although Eph receptor signaling in tumor cells often appears to be ligand-independent, interfering with ligand-receptor interactions may suppress tumor angiogenesis and signaling in the other components of the tumor microenvironment.

Because ligand-induced EphA2 signaling appears to play a role in tumor suppression, small molecular agonists have also been sought. A computer-based screen of existing FDA approved compounds identified doxazosin, an α1-adrenergic receptor blocker, as a selective agonist for EphA2 and EphA4 (65). Similar to ligand stimulation, doxazosin binds to the ligand-binding domain of the EphA2 and EphA4 receptors, inducing receptor phosphorylation and endocytosis. Doxazosin has also been shown to inhibit receptor-dependent ERK and AKT activity, tumor cell migration in vitro, and metastasis in vivo. Doxazosin-induced EphA2 activation is independent of its α1-adrenergic receptor antagonism as demonstrated by unaffected receptor phosphorylation upon co-treatment with phenoxybenzamine, an irreversible α1 adrenergic receptor inhibitor. Since Doxazosin is an FDA approved anti-hypotension drug, it can be, in principle, repurposed for treatment of cancer metastasis. In addition, further efforts directed at improving the potency and separating its activity on Eph receptors from its role in α1-adrenergic receptor antagonism may result in discovery of more efficacious EphA2 agonists.

Biologic agents: Fc-proteins, peptides, and antibodies

In parallel to small molecule inhibition, biological agents have been developed to either activate Eph receptor forward signaling, or block interactions between Eph receptors and ephrin ligands [reviewed in (3, 58). To this end, Ephrin-Fc and ligand mimetic activating antibodies have been successfully used to inhibit tumor progression in mice. The mechanism of how these agents function is not completely clear. One proposed mechanism suggests that these molecules activate Eph receptor forward signaling and/or receptor degradation in tumor cells. Further, Fc-mediated cytotoxicity was noted in some of the agonistic antibodies. Agonistic or antagonistic peptides have also been isolated through a phage display screen. These peptides or antibodies have potential in a wide array of functions, including the identification of circulating tumor cells, suppression of tumor cell proliferation and/or motility, modulation of host response in the tumor microenvironment, or delivery of drugs, toxins, or imaging agents to tumors.

Concluding remarks

Eph receptor tyrosine kinase (RTK) family members are often highly expressed across many types of cancer and elevated expression levels have been linked with poor clinical outcomes. Recent studies also implicate many of the same oncogenic Eph RTKs in cancer stem cell self-renewal, invasion, and tumorigenicity in vivo. As cancer stem cells facilitate tumor cell heterogeneity, metastasis, and therapeutic resistance, targeting Eph receptors may hold promise for treatment of drug-resistant cancer. However, much is still unknown about how Eph receptors regulate cancer stem cells at the molecular level. For example, although mechanisms for EphA2-dependent CSC self-renewal and invasion are known to be distinct, it is not completely understood what signaling pathways downstream of EphA2 regulate stem cell self-renewal and inhibit differentiation. Furthermore, while CSCs appear to express abundant Eph receptors that promote migration and invasion, the expression profile of ephrins in the tumor microenvironment remains to be determined. Ephrin expression on neighboring cells may determine the spread of CSCs locally, while ephrins in the distant organs may affect the dormancy status of Eph receptor-expressing CSCs in micrometastases. Finally, due to the dichotomous roles of Eph receptors in cancer, the effectiveness of a particular Eph-targeting strategy is likely to depend on the tumor type, stage, and microenvironment. Accordingly, the development of effective Eph targeting agents requires a holistic understanding of Eph receptor signaling in different cellular contexts during tumor progression. Despite these challenges, Eph receptors remain attractive therapeutic targets because of their critical roles in tumor growth, angiogenesis, invasion, metastasis, and cancer stem cell function. Therefore, targeting Eph receptors may provide inhibition of multiple processes in tumor progression with a single agent. Because Eph RTK inhibitors may also target Eph expressing cancer stem cells, these inhibitors hold promise to reduce tumor recurrence, metastasis, and therapeutic resistance to conventional chemo- or molecularly targeted therapies.

Acknowledgements

This work was supported by the Department of Veterans Affairs through a VA Merit Award (J. Chen), NIH grants R01 CA95004 (J. Chen) and F-31 CA167878 (K. Amato), and Thoracic Program pilot project funds from the Vanderbilt-Ingram Cancer Center Supporting Grant P30 CA068485 (J. Chen).

Biography

Jin Chen, M.D., Ph.D. is a professor of Medicine at Vanderbilt University School of Medicine. She holds joint appointments as Professor of Cancer Biology and Professor of Cell & Developmental Biology, and is a member of Vanderbilt-Ingram Cancer Center. Her laboratory performed pioneering studies on determining EphA2 receptor function in tumor initiation, metastatic progression and tumor angiogenesis. Dr. Chen’s laboratory is currently funded by grants from National Cancer Institute, Department of Defense, and Department of

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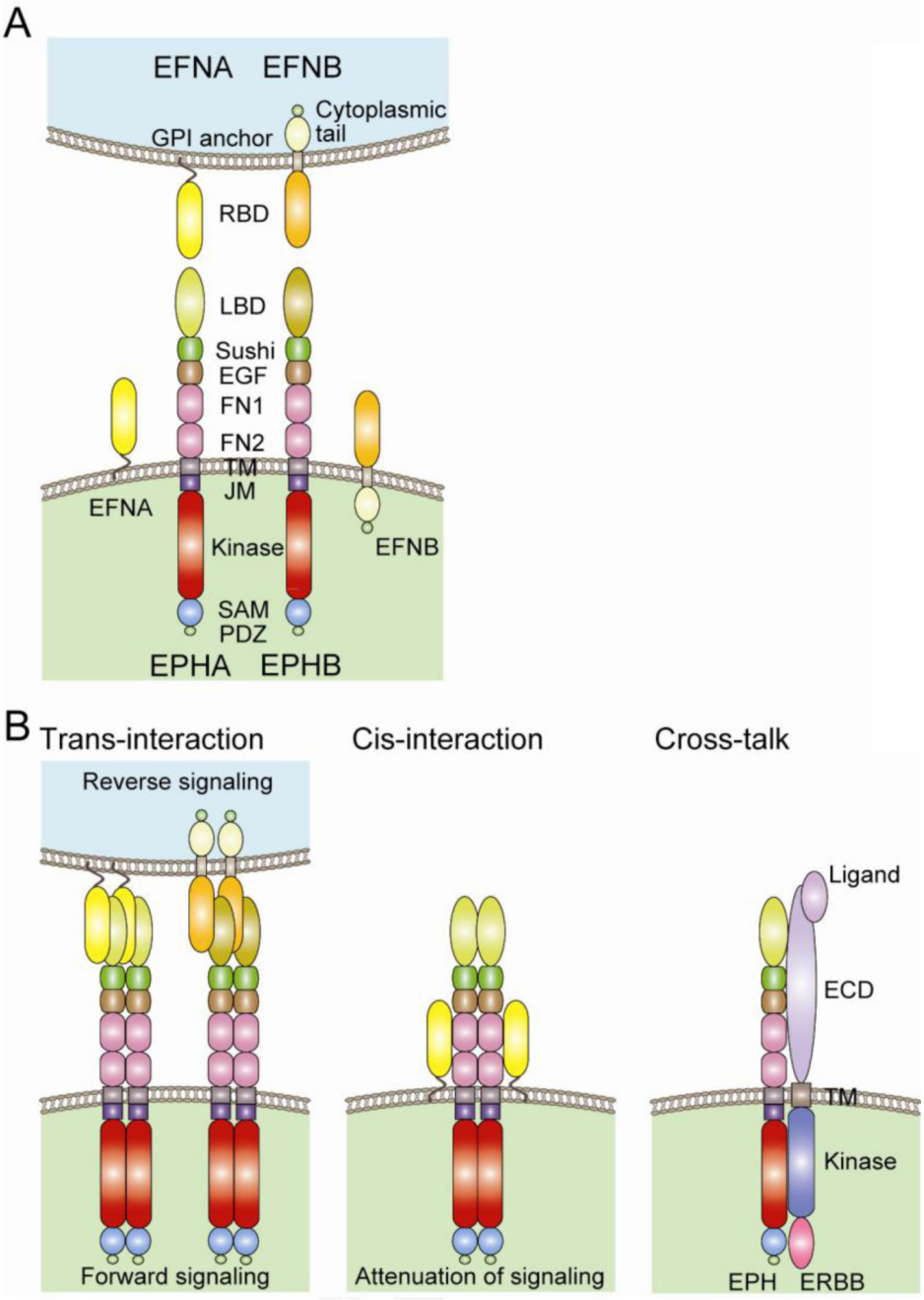

Figure 1. Structure and signaling properties of Eph receptors

(**A**) Eph receptors can be divided in two classes, EphA and EphB, based on sequence similarity and preference for binding either the GPI-anchored ephrin-A ligands (EFNA) or the transmembrane ephrin-B ligands (EFNB). Eph receptors contain a ligand- binding domain (LBD), motifs involving receptor clustering (Sushi, EGF, FN1 and FN2), a transmembrane domain (TM), a juxtamembrane domain (JM), a kinase domain, a SAM domain, and a PDZ-binding motif. (**B**) Binding of ephrins to Eph receptors induces receptor clustering and signaling. Trans-interactions between ephrins and Eph receptors induces bi-

directional signaling (left panel). Cis-interactions between Eph receptors and ephrins within the same cell leads to attenuation of receptor signaling (middle panel). Eph receptors can also cross talk with other growth factor receptors such as those in the ERBB family, resulting in enhanced receptor signaling (right panel).

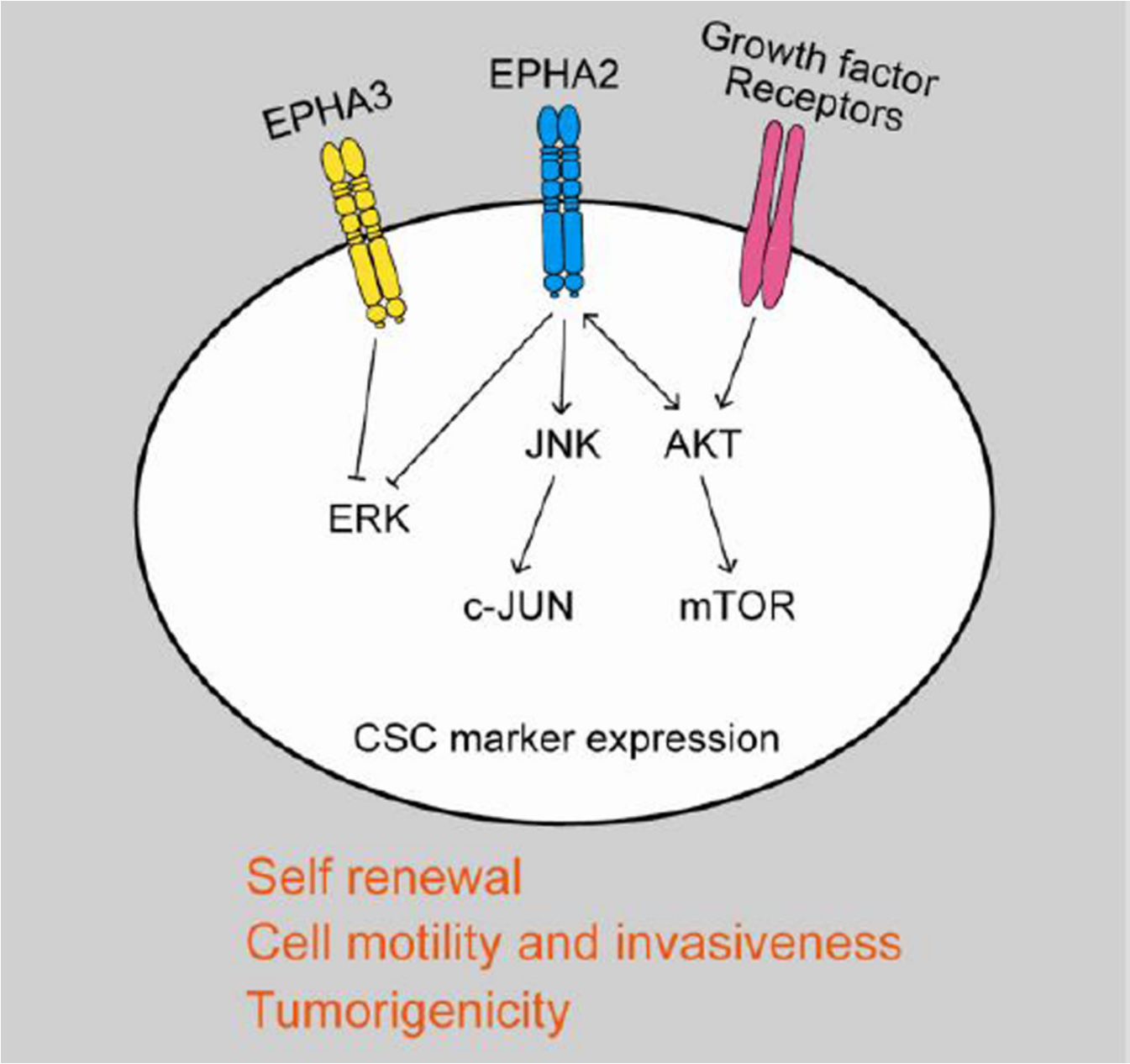

Figure 2. A model of EphA2 and EphA3 receptor signaling in cancer stem cells

EphA2 and EphA3 promote cancer stem cell self-renewal, possibly through inhibition of Erk signaling in glioblastoma stem cells. EphA2-dependent motility and invasiveness in glioma stem cells, however, appears to be mediated through cross-talk with Akt. The JNK and c-JUN signaling pathway was shown to be important in EphA2-dependent tumor cell proliferation and motility, and is likely to regulate stemness in non-small cell lung cancer.