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Eph receptors and ephrins as targets for cancer therapy

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Abstract

Eph receptor tyrosine kinases and their ephrin ligands are involved in various signalling pathways and mediate critical steps of a wide variety of physiological and pathological processes. Increasing experimental evidence demonstrates that both Eph receptor and ephrin ligands are overexpressed in a number of human tumours, and are associated with tumour growth, invasiveness and metastasis. In this regard, the Eph/ephrin system provides the foundation for potentially exciting new targets for anticancer therapies for Eph-expressing tumours. The purpose of this review is to outline current advances in the role of Eph receptors and ephrin ligands in cancer, and to discuss novel therapeutic approaches of anticancer therapies.

Keywords: Eph receptor • Ephrin • therapeutic target

Introduction

Eph receptors (Ephs) are the largest subfamily of receptor tyrosine kinases (RTKs) [1, 2], with 16 members cloned [3]. They are divided into two groups, EphA and EphB, depending on the types of ligands (ephrins) that they bind [4]. Since the first *Eph* gene was cloned in 1987 [5], the first ephrin ligand was also identified from cancer cells a few years later [6, 7]. Interactions between Ephs and the appropriate ephrin ligand activate bidirectional signalling and transducer signalling cascades. Eph receptors and ephrin ligands play critical roles in various biological functions, such as embryonic patterning, development of the nervous system and angiogenesis. However, deregulated activation of Eph/ephrin signalling in humans is thought to lead to tumorigenesis [8]. A number of studies have demonstrated overexpression of Ephs and ephrins in a variety of human tumours including melanoma [9–11], neuroblastoma [12], malignant glioma [13, 14] and carcinoma of the pancreas [15], breast [16–18], colon [19, 20], prostate [21, 22], lung [23], gastrointestinal tract [24, 25], ovaries [26, 27], oesophagus [28], liver [29, 30] and thyroid [31]. The up-regulation of Ephs and ephrins in human cancer is associated with poor prognosis and high vascularity in cancer, suggesting a detrimental role for the Eph/ephrin system in tumour progression [32]. In addition, it has been suggested that up-regulated Eph expression levels could

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be used as molecular markers for the diagnosis of invasive and metastatic tumours [17]. However, not only up-regulation but also down-regulation of Ephs and ephrins have been associated with tumour progression, and both Eph receptors and ephrin ligands can promote or suppress tumour growth. Eph receptors and ephrin ligands that are preferentially expressed in extremely invasive and metastatic tumours have provided the foundation for potentially exciting new targets for anticancer therapies for these tumours. To date, numerous strategies targeting the Eph/ephrin family have been developed for cancer treatment. This review describes the structure of Eph receptors and ephrin ligands and their signalling pathway, and summarizes the roles of Ephs/ephrins in cancer and anticancer therapies.

Structure of Eph receptors and ephrin ligands

Ephs are divided into two subclasses, EphA (EphA1-10) and EphB kinases (EphB1-6), on the basis of the sequence homology and the means by which they interact with membrane-anchored ephrin ligands. Both EphA and EphB receptors contain a single transmembrane-spanning domain. The extracellular region of Eph receptors is glycosylated, and contains a ligand-binding domain, a cysteine-rich domain and two fibronectin type III repeats. The intracellular region contains a juxtamembrane region with several conserved tyrosine residues, a tyrosine kinase domain, a sterile α motif (SAM) domain and a PDZ-binding motif within the non-catalytic region of the COOH-terminus [33, 34]. On the basis of their structural features and binding specificity to EphA and EphB receptors, ephrins are also divided into two subclasses, ephrinA and ephrinB. EphrinA (A1-A6) ligands are tethered to the extracellular cell membrane *via* a glycosylphosphatidylinositol (GPI) anchor, whereas ephrinB (B1-B3) ligands are transmembrane proteins that possess a short cytoplasmic region with a PDZ-binding motif. EphA receptors typically bind to ephrinA ligands, and EphB receptors bind to ephrinB ligands. However, this does not preclude cross-binding, as has been shown for EphA4, which can bind to ephrinA and ephrinB ligands, and EphB2 which can bind to ephrinA5 [35–37] (Fig. 1A).

Interaction between Eph receptors and their ephrin ligands

Specificity of the binding of ephrins to their Ephs is mediated by the N-terminal glycosylated ligand-binding domain of Ephs [38]. Eph receptors and ephrins expressed in opposing cells interact *in trans* form and activate bidirectional signalling. (Fig. 1B). Eph receptors and ephrins coexpressed in the same cell interact in *cis* form [39], *Cis* interaction has been shown to inhibit *trans* interaction and/or signalling [40, 41]. Upon binding, the Eph/ephrin molecules form heterotetramers to initiate the signal. As a rule, on ephrin binding, Eph clustering leads to activation of the tyrosine kinase domain, resulting in autophosphorylation of certain intracellular tyrosine residues [42].

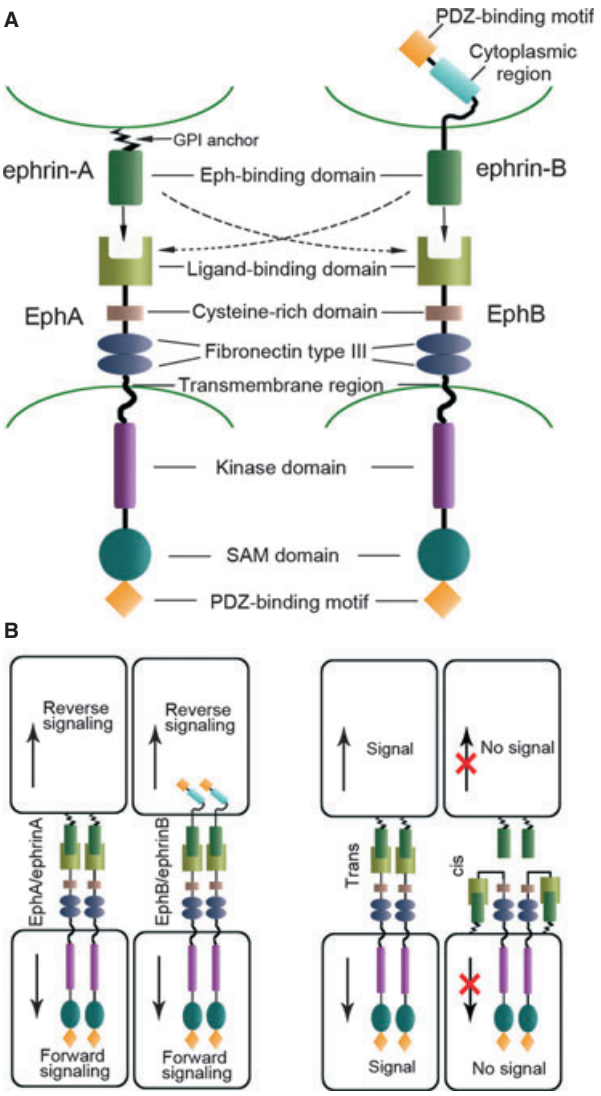

Fig. 1 Eph/ephrin structure and signalling. (**A**) Domain structure of Eph receptors and ephrin ligands. The extracellular region of Eph receptors contains a ligand-binding domain, a cysteine-rich domain and two fibronectin type III repeats. The intracellular region contains a tyrosine kinase domain, a sterile α motif (SAM) domain and a PDZ-binding domain. Both ephrinA (GPI-anchored) ligands and ephrinB (transmembrane) ligands interact with the N-terminal globular domain of Eph receptor. (**B**) Binding Eph/ephrin molecules form heterotetramers to initiate signals. Both classes of Eph receptors and ephrins activate bidirectional signalling: forward signalling and reverse signalling. Eph receptors and ephrin ligands expressed in opposing cells interact in *trans* and lead to bidirectional signal transduction. EphA and ephrinA coexpressed in the same cell interact in *cis*. This impairs receptor activation and inhibits *trans* interaction.

Accordingly, these phosphotyrosine regions bind adaptor proteins, and subsequently trigger downstream signalling pathways that lead to specific biological effects. However, this classical RTK activation does not explain all Eph/ephrin signalling, and ligand-independent

Eph signalling can also occur [43, 44]. For example, EphA8 receptor results in mitogen-activated protein kinase (MAPK) activation in a neural cell line [45], and promotes integrin-mediated cell attachment in a tyrosine kinase activity-independent fashion [46]. A previous study has also indicated that EphB4 can affect cancer cell behaviour in an ephrin-independent manner [47].

Bidirectional signalling

Bidirectional signalling is an important feature of Eph-ephrin signalling and arises dues to activation of signalling pathways in both the receptor-expression and the ligand-expression cells [48]. Forward and reverse signalling are activated by Ephs and ephrins respectively [39] (Fig. 2).

Forward signalling

Eph receptors can regulate biological effects through different kinase-mediated forward signalling molecules and pathways, including small GTPases of Rho members (Rho, Rac and Cdc42), focal adhesion kinase (FAK), the PI3 kinase pathway and Jak/Stat pathway [49, 50]. Small GTPases of the Rho family, which are activated by EphA receptors, control cell shape and movement by promoting the formation of stress fibres (Rho), lamellipodia (Rac) and filopodia (Cdc42) [51, 52]. The EphA receptor activates GTPases through the exchange factor, ephexin. Ephexin, which is preferentially expressed in the nervous system, binds the kinase domain of EphA. EphrinA-induced signals initiate growth cone collapse through the activation of Rho and its downstream effectors [52]. In melanoma and 293T cells, ephrinA-induced recruitment of Crk to EphA3 and a rapid, transient increase in activated Rho causes the retraction of cell processes, cell rounding and membrane blebbing. In addition, SH3 mutant Crk ablates Rho activation and ephrinA-induced cell morphological changes [53]. EphB receptors appear to associate with the exchange factors intersectin [54] and kalirin [55]. Intersectin could activate the Rho-family GTPase Cdc42 and its activity is enhanced by EphB receptor. Activation of the EphB receptor induces translocation of kalirin, an exchange factor for Rac, to synapses and leads to increased local Rac1 activation. The EphB-intersectin-Cdc42 and EphB-kalirin-Rac pathways have been proposed to regulate the EphB-receptor-mediated cytoskeletal reorganization, mesenchymal invasion and migration [54, 55]. Ephs also regulate the activity of small GTPases of the Ras family, including H-Ras and R-Ras [56, 57], H-Ras can in turn activate a MAPK pathway that is very important for transcriptional regulation, cell proliferation, cell migration, neurite outgrowth and axon guidance [58, 59]. Activation of Eph receptors negatively regulates the Ras-MAPK pathway in various cell types [57, 60–62]. For example, activation of EphA2 could down-regulate the Ras-MAPK pathway in fibroblasts, epithelial cells, endothelial cells and tumour cells [57]. EphB2 transiently down-regulates H-Ras activity and MAPK phosphorylation and leads to neurite retraction in the NG108 neuronal cell line [60, 63]. Eph receptor-mediated activation of R-Ras also suppresses the MAPK pathway. EphB activation can reduce integrin-mediated adhesion *via* negative regulation of the R-Ras-MAPK pathway [56]. Focal adhesion kinase, which is a critical component of integrin signalling, may connect Eph receptors with integrins [49]. In PC-3 prostate cancer cells, ligand activation of EphA2 causes dissociation of FAK. EphB2-regulated cell positioning *via* PI3K, independently of kinase activity, because of this, PI3K activity is important for conveying positional information [64]. In contrast, EphB signalling could drive cell proliferation by promoting cell cycle entry. Cyclin D1 is a regulator of cell cycle entry [65]. In Genander's study, EphB2 activity drove cells proliferation through Abl, resulting in post-transcriptional regulation of cyclin D1 protein levels [64]. In breast cancer cells, Abl binds to EphB4 in an activity-dependent manner. EphB4 activates an anti-oncogenic pathway involving Abl family tyrosine kinases and the Crk adaptor protein. This Abl-Crk pathway inhibits breast cancer cell viability and proliferation in addition to motility and invasion [66]. Autophosphorylation of EphA4 could lead to the activation of Jak2, which in turn phosphorylated Stat1 and Stat3, and promoted transcriptional activity and cells proliferation [50]. Taken together, Jak/Stat proteins were considered to be downstream targets of EphA4 signalling.

Reverse signalling

The interaction between Ephs receptors and their ephrins not only induces forward signalling by the Eph receptor but also leads to reverse signalling by the ephrin ligand [67]. EphrinA ligands can transmit signals despite lacking a cytoplasmic domain. The mechanisms of reverse signalling of ephrinA ligands are thought to be associated with ephrinA clustering and recruitment of regulatory proteins [68]. EphrinA ligands are anchored to the membrane by covalent linkage to GPI, and depend on transmembrane coreceptors for transmitting signals intracellularly [69]. It has been shown that signalling through ephrinA ligands is mediated by the recruitment of adapter proteins. The ephrinA ligands are targeted to lipid rafts, which contain some signalling molecules such as caveolin proteins and G proteins. This indicates that ephrinA ligands may activate a number of signalling pathways [70]. Src-family tyrosine kinases are important regulators of signalling through GPI-anchored proteins [71, 72]. Davy *et al.* [68] demonstrated that activation of Src kinase family members is important for signalling downstream of ephrinA5. Interaction of ephrinA5 with EphA-Fc fusion proteins has been shown to recruit and activate the Src-family kinase, Fyn. Subsequently, Fyn was shown to increase tyrosine phosphorylation of p80, and induce a change in the cellular architecture and adhesion of the ephrinA5-expressing cells. Bonanomi *et al.* [69] showed that Ret, which is also a RTK, is required for motor axon attraction mediated by ephrinA reverse signalling. Because of this, Ret is a transmembrane coreceptor and is dependent on ephrinA ligand for transmitting signals. Recent data demonstrate that activation of ephrinA2 and ephrinA5 by EphA3 leads to a β1-integrin-dependent increase in the adhesion of ephrinA-expressing cells to laminin [73]. This may be because of p120 (120-kDa raft membrane protein), which plays a role in coupling ephrinA activation to integrin activation. Blocking the p120 can abolish the increase in cell adhesion. EphrinA5 engagement activates

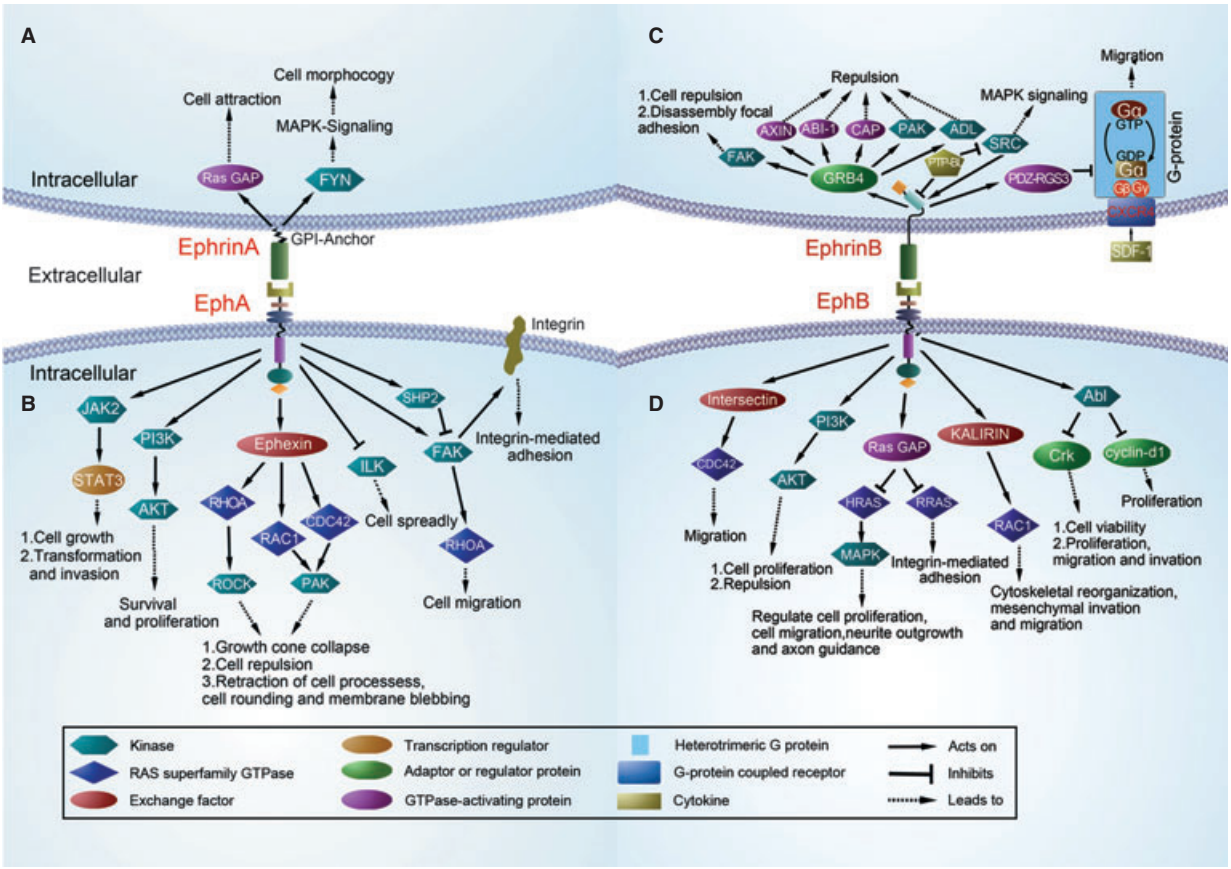

Fig. 2 EphA/ephrinA bidirectional signalling. (**A**) Stimulation of ephrinA5 recruits and activates the Src-family kinase, FYN. Subsequently, FYN induces a change in the cellular architecture and adhesion of ephrinA5-expressing cells [68] and results in mitogen-activated protein kinase (MAPK) activation [74]. (**B**) EphA4 activates signal transducers and STAT3 [50]. EphA receptors directly activate GTPases of the Rho family (RHOA, RAC1 and CDC42) through the exchange factor Ephexin [51, 52]. This pathway involves EphA2 and PI3 kinase in endothelial cells [151]. EphA2 inhibits Akt [190, 191] and inactivates focal adhesion kinase (FAK) through the SHP2 phosphatase [48]. EphA2 activates RHOA through FAK [192, 193]. EphA1 inhibits integrin-linked kinase (ILK)[194]. EphB/ephrinB bidirectional signalling. (**C**) Growth Factor Receptor Bound protein 4 (GRB4) contains a SH2 domain and can link ephrinB ligands to a signalling network that modifies cell morphology [77]. EphrinB1 disrupts focal adhesions through GRB4 [48]. The phosphatase PTP-BL is recruited to the ephrinB carboxy-terminal tail. PTP-BL dephosphorylates ephrinB and inactivates Src [35]. PDZ-RGS3 binds constitutively to ephrinB and catalyses the hydrolysis of GTP to GDP in the G-Alpha subunit of heterotrimeric GPCR. It also inhibits SDF1–mediated cell chemotaxis through the CXCR4 [195]. (**D**) EphB forward signalling activates RAC1 and CDC42 exchange factors [48, 146, 193]. EphB2 activates Ras GAP to inhibit the H-RAS and R-RAS [48, 196]. EphB2 regulates cell positioning *via* PI3K [64]. The EphB4 receptor suppresses breast cancer cell tumorigenicity through an Abl-Crk pathway [66]. EphB2 regulates cell proliferation through an Abl-cyclin D1 pathway [64].

MAPK, through both integrin-dependent and integrin-independent pathways, which in turn regulate cell architecture and morphology [74]. These studies indicate that ephrinA can influence cellular biological behaviour by transducing signals. EphrinB ligands are similar to Ephs in that they contain a single transmembrane domain, a cytoplasmic region and a PDZ-binding motif. EphrinB reverse signalling also involves Src kinases family that are responsible for ephrinB phosphorylation following Eph receptor binding [48, 67, 75, 76]. Phosphorylated ephrinB can initiate reverse signalling through SH2 or PDZ domain-containing proteins [77, 78]. The adaptor protein, Grb4, contains an SH2 domain and can link ephrinB ligands to a signalling network that modifies cell morphology [77]. The GTPase-activating protein PDZ-RGS3 binds to the cytoplasmic C terminus of ephrinB through its PDZ domain. PDZ-RGS3 can mediate signalling that is induced by ephrinB1 [78]. EphrinB also plays significant roles in boundary formation. In Zebrafish embryo studies, bidirectional signalling between ephrinB2 and EphB2 at rhombomere boundaries restricts cell intermingling [79]. Loss of the ephrinB2 cytoplasmic domain in mice results in defects in vasculogenesis and angiogenesis, which are very similar to those observed in ephrinB2-knockout mice [80]. EphrinB signalling was also shown to play critical roles in vascular development by a corneal micropocket assay [81]. Therefore, this suggests that reverse signalling is required for vascular development of vasculature.

Ephs and ephrins in developing and adult tissues

The Eph/ephrin system is associated with various signalling pathways, and participates in diverse biological processes such as cell proliferation and viability, cytoskeletal organization, cell migration and embryonic development. It plays key roles in the development of the nervous system, for example, in axon guidance [82], axon fasciculation [83] and neural crest cells migration [84]. EphB receptors and ephrinB ligands regulate synaptogenesis, including the establishment and modification of the post-synaptic specialization [85, 86]. Eph/ephrin signalling is also essential for formation of villi and crypts in the intestinal epithelium [87].

Most Eph receptors and ephrin ligands are not only expressed during development but are also expressed in adult tissues [88]. Hafner *et al.* [19] investigated the expression of 12 Eph receptors (EphA1–A8 and EphB1–B6) and 8 ephrin ligands (ephrinA1–A5 and ephrinB1–B3) in 13 different type of healthy human tissue, including brain, lung, liver, spleen, colon, small intestine, kidney, bladder, prostate, testis, uterus, thymus and bone marrow. They reported that except for EphA8 and ephrinA2, all members of the family were expressed in all investigated normal tissues. However, Eph and ephrin proteins are expressed at much lower levels in adult compared with embryonic tissue [89]. Some articles demonstrate that low-level expression of Eph and ephrin in the adult gut [87], vasculature [90] and kidney [91], and could continue play a role in tissue architecture. In contrast, the high-level expression of Eph and ephrin proteins has been studied in very few normal tissues. For example, the expression of Eph and ephrin is relatively strong in the brain, where Eph may participate in the processes of synaptic plasticity, learning and memory [92].

Ephs and ephrins in cancer

It is generally recognized that Eph receptors and ephrin ligands play considerable roles in carcinogenesis, cancer progression and neovascularization of various human malignancies. The expression of Eph receptors and ephrin ligands has been identified in multiple types of human tumours, including melanoma [9–11], neuroblastoma [12], malignant glioma [13, 14] and carcinoma of the pancreas [15], breast [16–18, 74], colon [19, 20], prostate [21, 22], lung [23], gastrointestinal tract [24, 25], ovaries [26, 27], oesophagus [28], liver [29, 30] and thyroid [31]. The expression of Ephs and ephrins is often reported to be up-regulated in human tumours [93]. Thus, these up-regulated Ephs and ephrins may provide molecular markers for the diagnosis of invasive and metastatic tumours [17]. There are also reports describing the down-regulation of the Eph/ephrin family in cancer [18]. For instance, EphA6 was down-regulated in colorectal and renal cell carcinoma, and EphB2 staining was reduced in hepatic cell cancer compared with the surrounding benign liver tissue [19]. Data from recent studies demonstrate that both Eph receptors and ephrins have roles in tumour promotion and tumour suppression. The role of Ephs and ephrins in certain cancer is discussed in further detail below (Table 1).

Breast cancer

There is a significant relationship between Eph expression levels and invasiveness and aggressiveness in breast cancer [94]. EphA2 overexpression has been found to cause oncogenic transformation and to promote tumourigenesis and metastasis in murine models of breast cancer [16, 95, 96]. Elevated expression of EphA2 may promote tumour cell malignancy by interacting with both ErbB2 and epidermal growth factor receptor (EGFR) [97, 98]. Hypermethylated in cancer 1 (*HIC1*), a tumour suppressor gene, encodes a transcriptional repressor that is silenced in many human tumours. Recent research on breast cancer identified EphA2 as a direct target gene of HIC1. Infection of breast cancer cell lines with a retrovirus expressing *HIC1* was shown to reduce EphA2 mRNA and protein [99]. Thus, deregulation of the EphA2 pathway by silencing *HIC1* might play an important role in the progression of breast cancer.

EphB4 provides a survival advantage in breast cancer by attenuating the inherent cell death pathways and up-regulating antiapoptotic proteins. EphB4 knockdown has been found to inhibit breast cancer cell viability, migration and invasion *in vitro* and tumour growth *in vivo* [100]. Furthermore, EphB4 receptor signalling is also able to suppress breast tumour cell growth and motility [66]. Recently, a retrospective study demonstrated that EphA2, EphA4, EphA7, EphB4 and EphB6 were significantly correlated with poor prognosis of breast cancer patients [101]. This suggests that these Eph family members may become useful targets for therapeutic intervention and potential indicators for clinical assessment of tumour prognosis.

Colorectal cancer

Several studies have identified a role for Ephs and ephrins in colorectal cancer. A recent study demonstrated significant up-regulation of EphA1 in over 50% of colorectal cancer cases ($P = 0.005$), whereas many of the remaining patients showed down-regulation of EphA1 [102]. In addition, EphA1 overexpression was more in stage II compared to stage III colorectal cancer. Low EphA1 expression has been significantly correlated with poor survival. Similar to EphA1, overexpression of EphA2 and ephrinA1 was more common in the early stage than in the late stage of cancer. On the other hand, reduced expression of ephrinA1 inhibits growth of HT29 colorectal cancer cells [103]. Recently, we reported that EphA3 expression positively correlated with tumour size, histological grade, depth of invasion, lymph node metastasis, distant metastasis and pTNM stage. In addition, patients with high expression of EphA3 had the lowest survival rate ($P = 0.001$) [104]. Therefore, EphA3 may play an important role in the progression of tumours, and appeared as one of the specific molecular markers for assessment of tumour biological behaviour and prognosis. Loss of EphB expression was correlated with colorectal cancer progression, suggesting that reduction of EphB activity could promote tumorigenesis [20]. Supporting this suggestion is the converse finding that highly elevated expression of EphB2 is associated with a longer survival time in colorectal cancer [20, 105, 106]. Reduced expression of the *EphB2* gene, as well as high expression of *EphA4* gene, has also been suggested to promote liver metastasis

Table 1 Expression of Eph receptors and ephrin ligands in cancers compared with normal tissues.

Cancer type	Expression	Ephs/ephrins	References
Breast cancer	Up	EphA2, EphB4	[16, 141, 142, 197–199]
	Down	–	–
Colorectal cancer	Up	EphA1, EphA2, EphA3, EphA8, EphB4, ephrinA1, ephrinB2	[5, 28, 104, 109, 110, 200, 201]
	Down	EphA6, EphA7, EphB1, EphB2	[20]
Prostate cancer	Up	EphA2, EphA3, EphA5, EphA6, EphA7, EphA8, EphA10, EphB3, ephrinA2	[202]
	Down	–	–
Brain tumour, GBM	Up	EphA2, EphA3, EphA4, EphA7, EphB2, EphB4, ephrinB3	[19, 116, 117, 120, 189]
	Down	–	–
Melanoma	Up	EphA2, EphA3, EphB3, ephrinA1	[10, 121, 125, 189, 203]
	Down	EphA4	[204]
Lung cancer	Up	EphA2, EphB3	[23, 127]
	Down	–	–
Hepatocellular cancer	Up	EphA3, ephrinA1	[30, 129]
	Down	–	–
Gastric cancer	Up	EphA1, EphA2, EphA3, EphB2	[24, 130, 131, 133, 135]
	Down	ephrinB1	[25]

in colorectal cancer [107]. Overexpression of the EphA4 gene and reduced expression of the EphB2 gene may thus be a useful predictor of liver metastasis in patients with colorectal cancer. In addition, the overexpression of EphB3 enhanced cell-cell contacts and suppressed tumour growth in HT29 colorectal cancer [108]. Furthermore, EphB4 and ephrinB2 were highly expressed in colorectal cancer compared to the normal mucosa [109, 110]. This suggests that EphB4 and ephrinB2 may play a role in the progression of colorectal cancer.

Prostate cancer

A number of Eph receptors and ephrin ligands have been detected in prostate cancer. Walker-Daniels *et al.* [22] reported that EphA2 is overexpressed in human prostate cancer compared with benign prostate tissues, and overexpression of EphA2 has been linked with metastasis. EphA2 has also been shown to induce an inactive conformation of integrins and inhibit cell spreading, migration and integrin-mediated adhesion through rapid recruitment of the protein tyrosine phosphatase SHP2, and subsequent dephosphorylation and inactivation of FAK [49]. A recent experiment in prostate cancer tissue and cell lines showed that the frequency of EphA7 methylation was higher in cancer with higher Gleason scores [21]. In addition, ectopic expression of EphA7 in DU145 cells was able to inhibit cell colony formation, but not cell growth. As far as metastatic cancer is concerned, for example, Astin and colleagues [111] analysed the dynamics of prostate cancer cell lines co-cultured with fibroblasts, and demonstrated that the unimpeded migration of metastatic PC-3 cells towards fibroblasts was dependent on activation of EphB3 and EphB4 by ephrinB2.

Brain tumours

Eph receptors and ephrin ligands are involved in the development of the central nervous system [82–84, 112–114]. EphA2 overexpression in glioblastoma multiforme (GBM) has been indicated to be a critical mediator of invasiveness and thus also represents an attractive molecular target for the development of therapeutics against GBM [115]. Overexpression of EphA4 enhances cell proliferation and migration through promoting the FGFR1 signalling pathway [116]. EphA7 protein is also overexpressed in GBM, and is correlated with poor survival of GBM patients [117]. This may because of the fact that EphA7 could promote tumour neovascularization. Therefore, the local release of EphA7 inhibitors in to GBM could restrain tumour angiogenesis and improve patient outcome. Recent work demonstrated that EphA7 is an important mediator of neural progenitor apoptosis during brain development [118]. EphB2 expression is higher in glioblastomas, especially in invasive ones, than in low-grade astrocytomas or normal brain tissue [119]. EphB2 tyrosine phosphorylation can promote glioma migration and invasion, whereas blocking

EphB2 could inhibit these aspects of tumorigenesis. Together, these data suggest that EphB2 has potential value for therapeutic intervention. Expression of ephrinB family members was determined in invading glioblastoma cells and glioma cell lines including U87, T98G, U251 and SNB19. *EphrinB3* mRNA was up-regulated in all of these cells and promoted RAC1-dependent invasion of glioma cell lines [120]. Furthermore, ephrinB3 expression and phosphorylation were correlated with increasing human glioma grade.

Melanoma

Initial studies reported that some members of the Eph receptors family are abnormally expressed in melanoma cells compared with melanocytes. In addition, EphA2 expression was significantly higher in metastatic cell than that in primary melanoma cells [121, 122]. EphA2 forward signalling in malignant melanoma can promote vasculogenic mimicry [123]. Moreover, Udayakumar *et al.* verified that EphA2 is an important oncogene in melanoma by analysing EphA2 levels in a panel of melanoma cell lines [124]. EphrinA1, a ligand of the EphA2 receptor, is not only a growth factor for melanoma cells [125] but is also angiogenic and a chemoattractant for endothelial cells. In addition, ephrinA1 was found to be expressed in 67% of metastatic melanomas, and 43% of advanced primary melanomas, but only in the occasional lesions [10]. Together, these studies suggest that ephrinA1 may play a role in promoting melanocytic cell growth and inducing vascularization in advanced melanomas.

Lung cancer

There are relatively few studies of Eph/ephrin family in lung cancer. EphA2 was found to be overexpressed in patients who subsequently developed brain metastases, whereas low expression of EphA2 was related to disease-free survival or contralateral lung metastasis [23]. The above data suggest that high levels of EphA2 could be used to identify the patients that are at risk of lung cancer metastasis to the brain. *EphA2* mutations were demonstrated to be present in lung squamous cell carcinoma and were associated with increases in tumour invasion and survival. Whether or not *EphA2* mutations could serve as a potential therapeutic target for lung squamous cell carcinoma requires further study [126]. In addition, Ji *et al.* [127] found that overexpression of EphB3 in NSCLC cell lines promoted cell growth and migration. Recently, this research group reported that they identified a novel EphB3-binding protein, the receptor for activated C-kinase 1 (RACK1). RACK1 regulates the assembly of signal complexes including protein phosphatase 2A, Akt and itself in response to EphB3 activation, resulting in inhibition of NSCLC metastasis [128].

Hepatocellular cancer

EphA3 was previously found to be expressed at higher level in hepatocellular carcinoma (HCC) than that in corresponding healthy tissue [129]. Remarkably, one novel missense mutation, a GAC to GTC transition (D219V) was found in the extracellular domain of *EphA3*, and two genetic alterations in the intracellular SAM domain of EphA3 appear to be polymorphisms [29]. In addition, the overexpression of ephrinA1 was more frequently detected in poorly differentiated HCC than in well differentiated HCC. This indicates that ephrinA1 may be associated with the malignant phenotype of HCC.

Gastric cancer

Wang *et al.* [130] found that EphA1 protein was significantly associated with depth of invasion and cancer stage. Furthermore, patients with EphA1 up-regulation had a shorter survival time than those with absence or downregulation of EphA1. EphA2 has been associated with malignant transformation and was positively correlated with tumour invasion, lymph node metastasis and TNM stage [24, 131]. Knockdown of EphA2 expression could inhibit gastric cancer cell proliferation and invasion *in vitro* and *in vivo* [132]. This indicates that the specific inhibition of EphA2 may be useful for gastric cancer therapy. More recently, we found that increased EphA3 expression was positively correlated with vascular endothelial growth factor (VEGF), microvessel density (MVD) and patient survival. Thus, EphA3 may play important roles in the angiogenesis and prognosis of gastric cancer. The combined detection of EphA3, VEGF and the determination of MVD, to some extent, can reflect the biological behaviour of gastric cancer and could be used to guide the choice of chemotherapy and molecular targeting therapy [133].

EphB2 mutations have been identified in human gastric tumours [134]. Reduced expression of EphB2 was significantly associated with advanced disease stage, poor histological differentiation and poor survival rate [135]. EphrinB1 are frequently overexpressed in gastric cancer, and the expression of ephrinB1 is especially high in poorly differentiated invasive tumour cells [25]. Accumulating evidence demonstrates that expression of B-type ephrins is closely associated with tumour cell invasion. Reduction of ephrinB1 expression inhibits migration and invasion of scirrhous gastric cancer cells *in vitro* without affecting tumour cell proliferation or apoptosis [136].

Functions in tumour angiogenesis

Angiogenesis, the formation and growth of new blood vessels by sprouting from existing vessels [137, 138], is critical for tumour growth and metastasis by supplying the tumour with nutrients, growth factors and oxygen [139]. Several Eph receptors and ephrins play an important role in tumour angiogenesis by mediating communication of vascular cells with other vascular cells, as well as tumour cells [140]. There is considerable evidence to support the tumour-promoting role of the Eph/ephrin family in angiogenesis [141].

Forward signalling induced by EphA2 is known to promote angiogenesis [142]. *In vitro* and *in vivo* experiments also found that EphA2 forward signalling increases vascular permeability though phosphorylation of claudins [143, 144]. Additional studies revealed the expression of both EphA2 and ephrinA1 in tumour cells of two xenograft

models from human breast cancer and Kaposi sarcoma, as well as in human cancer specimens [141]. A further study indicated that EphA2, which was positively correlated with VEGF expression, was overexpressed in squamous cell carcinoma of the tongue and was also implicated in angiogenesis [145]. EphB4 and its cognitive ligand ephrinB2 not only play an essential role in embryonic vessel development and vascular remodelling but also participate in tumour angiogenesis. It has been suggested that ephrinB2 promotes the vascular formation and remodelling in EphB4-positive tumour tissues [146]. In addition, Martiny-Baron *et al.* demonstrated that EphB4 forward signalling is an important mediator of VEGF-induced angiogenesis, because of that VEGF-induced angiogenesis could be inhibited by the inhibition of EphB4 forward signalling [147]. EphrinB2 reverse signalling is also required for VEGF-induced angiogenesis through regulation of VEGFR2 endocytosis [148].

Eph receptors and ephrin ligands as tumour therapeutics targets

Expression of Eph receptors and ephrin ligands are often up-regulated in various human malignant tumours. Decreases in Eph receptor levels can effectively suppress tumour growth in animal models. Therefore, Eph receptors and ephrin ligands represent probable new targets for anticancer therapies. To date, numerous strategies targeting Eph/ephrin family have been developed for cancer treatment, which we will elaborate below (Table 2).

Preventing receptor-ligand interactions

The activation of Eph receptors by ephrin ligands relies on direct contact between cells that express Ephs and ephrins to induce signalling. Preventing receptor-ligand interactions may be useful to inhibit Eph/ephrin function. A large number of molecules can be used for this purpose. As function-blocking antagonists, soluble Eph and ephrin exdomains that bind their corresponding partner can inhibit Eph function during tumour progression and neovascularization [142, 149, 150]. Soluble EphA2-Fc can inhibit EphA forward signalling, but promote reverse signalling, whereas EphB4-Fc can inhibit both forward and reverse signalling. Both EphA2-Fc and EphB4-Fc can suppress tumour growth in mouse models by inhibiting tumour angiogenesis [150–154]. Furthermore, it was reported that soluble monomeric EphB4 significantly suppressed tumour growth in a mouse model [155, 156]. Scehnet *et al.* used the extracellular domain of EphB4 fused with human serum albumin to block ephrinB2 in Kaposi sarcoma cells *in vitro*. This block of ephrinB2 resulted in the inhibition of migration and invasion of Kaposi sarcoma cells in response to various growth factors [154]. Antagonistic antibodies (MAb 2H9) [157] and peptides (TNYL-RAW, SNEW, KYL, *etc.*) [158, 159] that compete with ephrins for binding to Eph receptors are also useful for blocking these interactions. Recently, Lamberto *et al.* [160] have reported that KYL, APY and VTM antagonistic peptides could selectively target EphA4 to inhibit ephrin binding to EphA4. Two isomeric small molecules that selectively inhibit ephrin binding to EphA2 and EphA4 have been identified [161]. The peptides and small molecules could be used to develop pharmaceuticals that selectively targeting Eph receptors with high affinity. Agonistic antibodies have also been used to suppress tumour growth in mouse models. These agonists are activators of Eph-ephrin signalling that stimulate Eph forward signalling and could be used to negatively regulate tumour cell growth and to induce the degradation of Eph receptors in cancer cells [66, 162–164]. By stimulating its ephrinB2 ligand, EphB4 activates an anti-oncogenic pathway (Abl-Crk pathway) that can inhibit breast cancer cell survival, proliferation, motility and invasion [66]. Coffman *et al.* targeted EphA2 on cancer cells using agonistic antibodies that simulate the effect of ligand binding. They showed that agonistic EphA2 antibodies can decrease tumour growth *in vivo* through protein degradation [162]. Therefore, we suggest that Eph agonistic antibodies could be useful in cancer treatment in combination with chemotherapy. There are a great many approaches to identify inhibitors to the Eph kinase domain [147, 165–169]. For example, several 2,5-dimethylpyrroly benzoic acid derivatives are inhibitors of EphA4 receptors and 2,4-bis-anilinopyrimidines are inhibitors of EphB4 receptors. In addition, ALW-II-49–7 was reported to inhibit EphB2 tyrosine kinase activity. Furthermore, NVP-BHG712, which was originally described as an inhibitor of EphB4 kinase, can inhibit VEGF-driven angiogenesis *in vivo* [147]. However, NVP-BHG712 can inhibit many kinases and is also non-selective for different Eph receptor kinases. Dasatinib is a small molecular inhibitor of multiple tyrosine kinases containing Src, BCR-ABL and c-Kit, multiple Eph kinases and platelet-derived growth factor-beta receptor kinases [170]. It is used to suppress proliferation of haematological malignancies [170, 171]. In addition, dasatinib can inhibit growth, migration and invasion of breast cancer cells [172]. Dasatinib can also inhibit invasion, and induce cell apoptosis of ovarian cancer, which was highly sensitive to dasatinib [173]. It was also reported that the potency of dasatinib may be because of an ability to decrease EphA2 phosphorylation [15]. Small interfering RNA (siRNA) that specifically induces destruction of specific mRNA is a powerful tool in the analysis of protein function and targeted therapeutics. Duxbury *et al.* demonstrated that EphA2 siRNA suppresses EphA2 expression, cellular invasiveness, anoikis resistance and FAK phosphorylation *in vitro*, and inhibited tumour growth and metastasis in a pancreatic cancer xenograft model [174]. In addition, knockdown of EphA2 inhibited endothelial expression of EphA2, suppressed ephrinA1- and VEGF-induced cell migration, inhibited cell proliferation and induced cell apoptosis in human glioma cells [175, 176]. It was demonstrated that targeted knockdown of EphB4 expression by siRNA (and antisense oligodeoxynucleotides (ODNs)) led to poor survival of breast cancer cells, and increased apoptosis [100]. Furthermore, antisense ODN-mediated EphB4 knockdown resulted in the suppression of tumour growth in a murine tumour xenograft model. Previous studies have indicated that siRNA targeting the oncoprotein EphA2 was incorporated into the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) for efficient *in vivo* siRNA delivery [177]. Treatment with EphA2-targeting siRNA-DOPC resulted in significantly decreased tumour cell proliferation and tumour growth in an orthotopic mouse model of ovarian cancer [178].

Table 2 Strategies targeting Eph receptors and ephrin ligands for cancer therapy.

Treatment	Target	Tumour	References
Inhibitors of Eph/ephrin interaction			
EphA2-FC, EphA3-FC	ephrinA	Breast cancer Pancreatic cancer	[142, 149, 150]
EphB4-FC	EphB4	Melanoma	[156]
Mab2H9 antagonistic antibody	EphB2	Colorectal cancer	[157]
TNYL-RAW peptide	EphB4	Breast cancer	[158]
SNEW peptide	EphB2	Breast cancer	[158]
KYL, APY, VTM peptide	EphA4	Angiogenesis	[159, 161]
2,5-dimethylpyrroly benzoic acid derivatives	EphA4	Angiogenesis	[161]
Disalicylic Acid-furanyl derivative	EphA2	Prostate cancer	[205]
Activators of Eph forward signalling			
EA2,B233,3F2-WT antibody	EphA2	Breast cancer	[162]
EA5 antibody	EphA2	Ovarian cancer	[163]
mAB208	EphA2	Renal cell cancer	[164]
Dimerized IIIA4 antibody	EphA3	Malignant Melanoma	[183]
YSA, SWL peptides	EphA2	Breast cancer	[206]
Kinase inhibitor			
Dasatinib	EphA2	Prostate cancer Ovarian cancer Pancreatic caner	[15, 173, 207]
Benzenesulphonamide derivative	EphB4	Angiogenesis	[165]
Xanthine derivatives	EphB4	Hepatocellular cancer	[168]
Inhibitor of Eph expression			
EphA2 siRNA	EphA2	Pancreatic cancer Ovarian cancer	[174] [176, 177]
EphB4 siRNA	EphB4	Breast cancer	[100]
Oligonucleotides	EphB4	Breast cancer Bladder cancer	[100, 208]
Imaging agent			
^{64}CU –DOTA-1C1 antibody	EphA2	Colorectal cancer, Prostate cancer, Ovarian cancer, Glioblastoma, Malignant Melanoma	[182]
^{111}Indium-labelled IIIA4 antibody	EphA3	Malignant Melanoma	[183]

Table 2. Continued

Treatment	Target	Tumour	References
Antibody-Drug/toxin conjugation			
ephrinA1-PE38QQR	EphA2	Glioblastoma	[13]
1C1-maleimidocaproyl-MMAF conjugate	EphA2	Prostate cancer	[179]
2H9 antibody-vc-MMAE conjugate	EphB2	Colorectal cancer	[157]
Immunotherapy			
bscEphA2 × CD3 bispecific single-chain antibody	EphA2/CD3	Breast cancer Colorectal cancer	[184]
EphA2-DCs	EphA2	Colon cancer	[187]
$EphA2_{833–891}$ peptide	EphA2	Malignant gliomas	[14]
EphA3- and EphB6-derived peptides	EphA3/EphB6	Glioma	[188, 189]

Drug/toxin-conjugated antibody targeting of Eph-positive cancers

Monoclonal antibodies that selectively bind tumour cells provide a vehicle for targeted delivery of cytotoxins. A number of recent studies have provided insight into drug/toxin-conjugated Eph antibodies capable of killing tumour cells that express high levels of Eph receptors [157, 179, 180]. Organic compounds that are suitable for conjugation and delivery by antibodies have been identified. It has been described that auristatins, derivatives of the tubulin polymerization inhibitor, were used as potent cytotoxic agents delivered by conjugated antibodies [181]. Pseudomonas aeruginosa exotoxin A, which is a novel cytotoxin composed of ephrinA1 ligand conjugated to a bacterial toxin, was also used to kill GBM cells overexpressing EphA2 [13]. Jackson *et al.* demonstrated that the anti-EphA2 antibody-drug conjugate (1C1–maleimidocaproyl-MMAF (mcMMAF)) induces degradation of the EphA2 receptor and inhibits tumour growth *in vivo* [179]. In addition, an EphB2 antibody conjugated to monomethylauristatin E specifically killed EphB2-expressing colorectal cancer cells *in vitro* and *in vivo* [157]. However, EphB2 is also expressed in normal tissues, and the potential for this method to destroy normal cells needs to be further investigated. Therefore, advanced technology, drug potency and conjugation methods are urgently needed to develop safe and effective antibody-drug/toxin conjugates for the treatment of cancer.

Antibodies conjugated to imaging agents could be used for PET imaging. EphA2 labelled with ^{64}Cu using the chelating agent 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA) was used for quantitative radioimmunoPET imaging of EphA2-expression tumour-bearing mice. The tumour uptake value of ^{64}Cu-DOTA-1C1 obtained from PET imaging correlated very well with the tumour expression level of EphA2 *in vivo* [182]. In addition, both IIIA4 monoclonal antibodies and ephrinA5 that were labelled with $^{111}Indium$ were successfully used for γ-camera imaging in solid tumour-bearing xenografts [183].

Targets for cancer immunotherapy

Dendritic cell-based tumour vaccines can induce protective antitumour immunity in tumour models, by inducing both the tumour-specific cytotoxic T-lymphocyte and helper-T cell response. There is some evidence that Eph receptors may be useful targets for cancer immunotherapy [14, 184–188]. A bispecific single-chain antibody (bscAb) that simultaneously targets EphA2 on tumour cells and the T cell receptor/CD3 complex on T cells can lyse EphA2-expressing tumour cells *in vitro* and *in vivo* [184]. In an experimental approach, EphA2-derived peptides that induce specific, tumour-reactive $CD8^+$ or $CD4^+$ T cell responses might be able to serve as agents for immunotherapy of renal cell carcinoma [185]. Yamaguchi *et al.* investigated the effectiveness of vaccination dendritic cells (DCs) loaded with EphA2-derived peptides (Eph-DCs) in a murine colon cancer model. They demonstrated that immunization with Eph-DCs suppressed MC38 tumour (with EphA2 overexpression) growth compared with the control group, and in contrast, Eph-DC vaccination had no influence on BL6 tumour (without EphA2 expression) growth [187]. Furthermore, a previous study showed that the synthetic $EphA2_{883–891}$ peptide induces an antigen-specific cytotoxic T-lymphocyte response in human leucocyte antigen $A2^+$ patient-derived peripheral blood mononuclear cells from EphA2-expression malignant gliomas [186]. EphA3- and EphB6-derived peptides were also suggested to be recognized by cancer-specific cytotoxic T cells [188, 189].

Conclusion

The Eph receptors that comprise the largest subgroup of tyrosine kinase, and their ephrin ligands, form a cell-cell system that is associated with various important biological processes, including nervous system development, angiogenesis and tumorigenesis. They regulate

cell-to-cell adhesion, cell proliferation and viability, cytoskeletal organization and cell migration. Increasing experimental evidence indicates deregulated activation of Eph/ephrin signalling in cancer. Our understanding of the Eph/ephrin pathway has improved tremendously. Ephs/ephrins can influence tumour behaviour by bidirectional signalling as well as other signalling modalities. Every tumour cell has to integrate and translate the signals that it receives into corresponding cellular responses, to achieve its overall biological function. However, varying surface densities of Eph receptors and ephrin ligands on tumour cells may influence Eph receptor signalling and the cellular response. The binding characteristic formation of multimers, bidirectional signalling and cross-talk with other molecules and signalling pathways further contribute to the complexity of the Eph/ephrin system. Therefore, a detailed understanding of Eph/ephrin signalling is important to regulate Eph-mediated tumour cell responses, to exploit the tumour-specific expression of Eph receptors and ephrin ligands, and to provide potentially novel targets for anticancer therapies for Eph-expressing tumours.

To date, a number of strategies targeting the Eph/ephrin system have been developed for cancer treatment, such as the prevention of receptor-ligand interactions, targeted delivery of drugs and immunotherapy. However, the complexity of their signalling and biological functions complicates the development of effective therapeutic agents. Strategies targeting the Eph/ephrin system might be useful in tumours in which Eph receptors promote tumorigenesis, and ineffective or even detrimental in tumours in which Eph receptors suppress tumorigenesis. In addition, the side effects of Eph/ephrin-targeting agents on normal tissues expression these family members are not well documented. Further examination of changes in Eph/ephrin expression in tumours and cancer-related Eph/ephrin gene mutations, as well as the underlying molecular mechanisms of bidirectional signalling of the Eph/ephrin system are needed to develop successful, safe and effective therapeutic strategies.

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Disclosures

The authors confirm that there are no conflicts of interest.

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