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Review

EPH receptors in cancer

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Summary. EPH receptors and their ephrin ligands constitute the largest sub-family of receptor tyrosine kinases (RTKs) and are components of cell signaling pathways involved in animal development. The ability of the EPH/ephrin guidance system to position cells and modulate cell morphology underlies their various roles in development. In addition, EPH signaling plays an important role in oncogenic processes observed in several organs. These receptors are involved in a wide range of processes directly related with tumorigenesis and metastasis, including cell attachment and shape, migration, and angiogenesis. Accordingly, deregulation of EPH expression and signaling activity could be crucial for the tumorigenic process. This review focuses on EPH receptors' roles in oncogenic transformation and tumor progression.

Key words: EPH receptors, Ephrins, Tumorigenesis, Cancer

Introduction

EPH receptors are the largest family of receptor tyrosine kinases (RTKs), proteins and play a crucial role in many biological processes, such as embryonic development, cell proliferation and differentiation. The first member of the EPH family was identified and cloned in 1987 by Hirai et al. from an Erytropoietin Producing Hepatocellular carcinoma cell line (EPH). To date, 16 receptors (14 found in mammals) and 9 ligands (8 in mammals) have been described.

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EPH receptors and ephrins (ligands) are implicated in a great variety of processes, such as regulation of cell proliferation, migration, cell attachment and shape, axon guidance and synaptic plasticity. EPH receptors play important roles in tumorigenesis and metastasis, and high levels of EPH have been related to angiogenesis in many tumor types, including breast and lung. This review focuses on EPH functions in tumorigenesis, especially in colorectal cancer.

Structure and mechanisms of action

Like other RTKs, EPH receptors are type-I transmembrane proteins with two differenciated domains: an extracellular domain implicated in ligand interaction and a cytosolic domain with tyrosine kinase activity (Labrador et al., 1997; Lackmann et al., 1998).

The extracellular domain of EPH receptors is unique to this RTK family and is composed of a ligand-binding globular domain, a cysteine-rich region and two fibronectin type III repeats, which may be involved in receptor dimerization (Lackmann et al., 1998). The cytoplasmic region of EPH receptors can be divided into different subdomains: a juxtamembrane region (highly conserved, with two autophosphorylation sites), a classic kinase domain, a PDZ-binding motif and a SAM domain that regulates EPH receptor dimerization (Fig. 1) (Pasquale, 2005).

EPH receptors are grouped into two subclasses depending on their ligand specificity. Type A receptors (EPHA) bind preferentially ephrins A, and type B receptors (EPHB) bind ephrins B. Ephrins are membrane-bound proteins divided into two subgroups that differ in their anchorage. Ephrins A have a GDI anchor, while ephrins B have a single transmembrane domain. The cytoplasmatic region of type B ephrins is highly conserved and has a PDZ domain (Fig. 1) (Kalo and Pasquale, 1999).

Ephrins are found in lipid rafts, cell membrane microdomains enriched in cholesterol and sphingolipids, which have recently received considerable attention because they could be involved in many cellular functions, such as signal transduction of extracellular stimuli. The association to the membrane and their distribution in microdomain rafts are necessary for ephrin clustering and for the activation of EPH receptors (Davis et al., 1994). However, while ephrins A are clustered constitutively (Davy et al., 1999), clustering of ephrins B only happens when they are induced (Bruckner et al., 1999).

There are ten different EPHA RTKs (EPHA1-EPHA10) which bind promiscuously to six ephrins A (ephrinA1-ephrinA6). The six EPHB receptors (EPHB1-EPHB6) and EPHA4 interact with three different ephrins B (ephrinB1-ephrinB3).

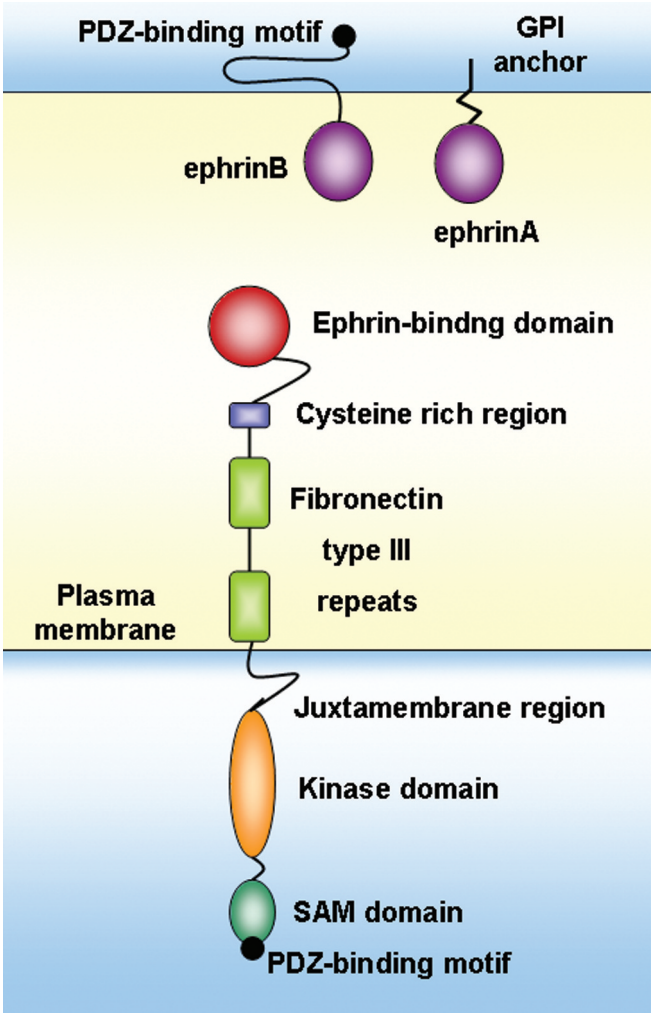

Fig. 1. EPH receptors and ephrin ligands structure. The figure represents the schematic structure of EPH receptors and their ligands (ephrins A and ephrins B).

The first step in the activation of the EPH signaling cascade is the juxtacrine interaction between an EPH receptor and an ephrin with 1:1 stoichiometry and nanomolar affinity, at sites of cell-cell contact (Himanen and Nikolov, 2003). EPH-ephrin heterodimerization creates complementary interaction surfaces, which join dimer pairs into tetrameric complexes. This leads to receptor activation and causes its autophosphorylation, which increases their kinase activity (Davis et al., 1994), thereby initiating the receptor-mediated forward signaling.

The majority of cell surface receptor-mediated signals are unidirectional (forward), however EPH-ephrin signaling sends information bidirectionally (Kullander and Klein, 2002). In this way, ephrin ligands (A- and B-type) are able to generate signal transduction and induce cellular effects after interacting with receptors (reverse signaling) (Henkemeyer et al., 1996; Davy and Robbins, 2000).

Forward signaling

Forward signaling triggered by an EPH receptor is an important mechanism regulating cell morphology, adhesion and migration (Murai and Pasquale, 2003). As described above, EPH-ephrin interaction results in receptor activation and its autophosphorylation. Phosphorylated tyrosines may serve as binding sites for SH2 or PTB domains of signaling molecules (Pawson, 2002). These include Src family of non-receptor tyrosine kinases. For example, Fyn binds to tyrosine-602 of EPHA4 (Ellis et al., 1996) and further interacts with EPHB3 through tyrosine-614 (Hock et al., 1998), affecting cell shape, adhesion and migration. Also, Src and Yes, two additional Src family kinases, are able to interact with EPH receptors and regulate the same functions (Zisch et al., 1998).

Rho GTPases cycle between an active, GTP-bound, and an inactive, GDP-bound, conformation. GTPases are involved in cell shape and movement, inducing the generation of stress fibers, filopodia or lamellipodia. RhoGAP is a protein that regulates RhoGTPases activity and also interacts with activated EPH through its SH2 domain (Holland et al., 1997). In this way, forward signaling causes cytoskeleton reorganizations through interacting proteins, which modify Rho GTPases activity. For example, EPHA4 interacts constitutively with efexin, a protein recently identified as a GTPase regulator (Shamah et al., 2001). Efexin is able to activate Rho and inhibit other GTPases, such as Cdc42 and Rac1. Moreover, EPHA is able to inhibit Ras and reduce MAPK signaling (Miao et al., 2001).

There are other signaling proteins that interact with EPH receptors, causing a variety of cellular effects. RasGAP and PI3K interact with EPHB2 or EPHA2, respectively, to control cell adhesion and migration (Pandey et al., 1994; Holland et al., 1997). Grb2, an adapter protein that binds to guanine nucleotide exchange factors, which activate Ras family proteins, is

also able to bind to phosphorylated tyrosine residues of EPHB1 through its SH2 domain, and is involved in signal transduction and cell communication (Stein et al., 1996). Moreover, LMW-PTP, a phosphatase that regulates cell adhesion and mitogenesis, interacts with EPHB1 and EPHB2 (Stein et al., 1998).

Reverse signaling

Reverse signaling is a kinase-independent mechanism induced by ephrin ligands on the surface of the opposite cell. The cytoplasmatic domain of ephrins B is tyrosine phosphorylated upon EPH receptor engagement by Src kinases family or PDGF receptors (Holland et al., 1996, Bruckner et al., 1997). Consequently, cell morphology is modified through reorganization of the actin cytoskeleton by proteins such as Grb4 and FAK. These proteins have SH2 domains, which interact with tyrosine-phosphorylated residues of the cytoplasmatic ephrin domain (Chong et al., 2000). Src kinases can also phosphorylate the PDZ domain of ephrins, allowing the binding of tyrosine phosphatases such as PTP-BL or GRIP to this domain (Palmer et al., 2002). Furthermore, despite their lack of cytoplasmic domain, ephrins A are also able to initiate reverse signaling, modifying cell behavior (Davy et al., 1999), although this pathway is poorly understood.

Additionally, there is another type of signaling, named crosstalk signaling. This mechanism is based on the EPH interaction with other membrane proteins (Murai and Pasquale, 2003). For instance, EPHB2 activated by its ephrin ligand, promotes the clustering and phophorylation of NMDA receptor through Src kinase. This phosphorylation produces calcium entrance induced by glutamate, suggesting an important role in sinaptogenesis (Takasu et al., 2002).

Related to cancer, several EPH-ephrin signal transduction pathways have been implicated in tumorigenesis. For example, the Jak/Stat pathway, involved in cell growth and viability, recently has been described as a new signaling pathway modulated by EPHA4 (Lai et al., 2004). Also, EPHA2 and its ligand ephrinA1 have been identified as transcriptional targets of the p53-family of tumor suppressor proteins (Dohn et

Table 1. Expression of EPHA family members in cancer.

CANCER	EPHA FAMILY MEMBERS						
	A1	A2	A3	A4	A7	A8	A10
Breast	(D) Fox and Kandpal, 2004.	(U) Zelinski et al., 2001; Fox and Kandpal, 2004	(U) Fox and Kandpal, 2004	(U) Fox and Kandpal, 2004	(U) Fox and Kandpal, 2004	(D) Fox and Kandpal, 2004	(U) Fox and Kandpal, 2004
Colorectal		(U) Kataoka, 2004; Saito et al., 2004			(D) Wang et al., 2005		
Gastric		(U) Nakamura et al., 2005					
Glioblastoma		(U) Wykosky et al., 2005					
Head and neck	(U) Lin et al., 2004	(U) Miyazaki et al., 2003					
Hematopoietic			(D) Dottori et al., 1999				
Leukemia			(P) Surawska et al., 2004				
Liver	(U) Maru et al., 1998						
Lung	(U) Maru et al., 1998	(U) Kinch et al., 2003	(U) Chiari et al., 2000				
Lymphomas			(P) Smith et al., 2004				
Melanoma	(U) Easty et al., 1999	(U) Easty and Bennett, 2000	(U) Chiari, 2000				
Nonmelanoma skin cancer	(D) Hafner et al., 2006						
Ovary	(U) Herath et al., 2006	(U) Thaker et al., 2004; Herath et al., 2006					
Pancreas		(U) Duxbury et al., 2004		(U) Iiizumi et al., 2006			
Prostate	(D) Fox et al., 2006	(U) Walker-Daniels et al., 1999; Zeng et al., 2003	(D) Fox et al., 2006				
Sarcoma			(U) Chiari et al., 2000				

Abbreviations: U: up-regulated, D: down-regulated, P: present

al., 2001).

EPH receptor family members in cancer

EPHA subclass

EPHA1 was the first EPH receptor identified and was found to be overexpressed in several human carcinomas, suggesting its involvement in tumorigenesis (Hirai et al., 1987). Since then, deregulation of multiple members of the EPH receptor tyrosine kinase family has been frequently observed in a wide range of cancers (summarized in Table 1), suggesting a functional relation between EPH signaling and tumor progression.

EPHA1 overexpression has been reported in head and neck squamous cell carcinoma (HNSCC), lung and ovary cancer (Maru et al., 1988; Hafner et al., 2004; Herath et al., 2006). EPHA1 gene has a well-established potential to act as a classical oncogene; for example, its overexpression in NIH3T3 cells confers the ability to form colonies in soft agar and to create tumors in immunocompromized nude mice (Maru et al., 1990). However, down-regulation of EPHA1 has also been observed in other cancer types, including breast, non-melanoma skin, and prostate cancer (Fox and Kandpal, 2004; Fox et al., 2006; Hafner et al., 2006). In the latter case, Fox et al. reported that the transcript levels of EPHA1 decreased progressively from normal prostate to primary prostate tumor cells and metastatic tumor cells, and this observation was linked to promoter CpG methylation (Fox et al., 2006).

Among the EPHA receptors, EPHA2 has been the most studied, and frequently high levels of this receptor have been correlated with higher tumor grade and poor prognosis, therefore it could be used as an indicator of disease progression. For example, a significant correlation was observed between EPHA2 expression and number of lymph node metastases, poor degree of tumor differentiation and poor survival in esophageal squamous cell carcinoma (Miyazaki et al., 2003). EPHA2 overexpression has also been associated with increased cellular invasiveness in pancreatic adenocarcinoma (Duxbury et al., 2004), and advanced stage of disease, cancer progression and metastasis in gastric and colorectal cancer (Saito et al., 2004; Nakamura et al., 2005). In ovarian carcinoma, overexpression was significantly associated with high tumor-stage and grade, as well as shorter survival (Thaker et al., 2004). In prostate, increased EPHA2 levels were found as prostate epithelial cells progress towards a more aggressive and metastatic phenotype (Walker-Daniels et al., 1999; Zeng et al., 2003). Moreover, EPHA2 overexpression is sufficient to confer malignant transformation and tumorigenic potential on nontransformed mammary epithelial cells (MCF-10A), causing anchorage-independent growth, increasing invasiveness in matrigel, and inducing tumor formation when EPHA2-transformed breast epithelial cells are subcutaneously injected into nude mice (Zelinski et al., 2001). Based on these findings, EPHA2 overexpression could be considered predictive of aggressive cancer behavior and may be an important therapeutic target. Consequently, several strategies have been used to inhibit EPHA2: a) Brantley et al. used soluble EPHA2 receptors to block receptor activation in two tumor models. Soluble EPHA-Fc fusion proteins bind to multiple ephrin A ligands and disrupt binding and signaling through endogenous EPHA receptors, resulting in decreased tumor vascular density, tumor volume, and cell proliferation, as well as increased apoptosis due to inhibition of blood vessel recruitment by the tumor (Brantley et al., 2002; Cheng et al., 2003). Similar results were obtained by Dobrzanski et al. in a pancreatic ductal adenocarcinoma model in mice when EPHA/Fc soluble receptor was administered, inhibiting the growth of primary tumors and the development of metastases (Dobrzanski et al., 2004). b) Another strategy was the specific targeting of EPHA2 with monoclonal antibodies, which were selected for their ability to inhibit features unique to metastatic cells while minimizing damage to non-transformed cells. These antibodies were able to inhibit soft agar colony formation by MDA-MB-231 breast tumor cells, whereas the anchorage-dependent growth of non-transformed epithelial cells remained unchanged (Carles-Kinch et al., 2002). c) Furthermore, siRNA treatment has been used to inhibit EPHA2 receptor in a nude mouse xenograft model resulting in reduction of tumor growth and inhibition of cellular invasiveness and metastasis (Duxbury et al., 2004).

Concerning the rest of the members of the EPHA subgroup, EPHA3 is frequently down-regulated in prostate carcinoma cell lines, through promoter CpG methylation (Fox et al., 2006). EPHA4 overexpression has been observed in pancreatic ductal adenocarcinoma (PDAC), and its constitutive exogenous expression in PDAC-derivative cells caused a more rapid growth rate in comparison to cells transfected with mock vector, whereas EPHA4 siRNA transfection drastically attenuated PDAC cell viability, suggesting the involvement of EPHA4 in pancreas carcinogenesis, and pointing at this EPH receptor as a potential therapeutic target in pancreatic tumors (Iiizumi et al., 2006). Also, a significant reduction of EPHA7 expression has been reported in colorectal cancer, and this fact was linked to aberrant CpG methylation (Wang et al., 2005).

EPHB subclass

Deregulation of members of the other subclass of the EPH family, EPHB receptors, has also been reported in many tumor types (summarized in Table 2). Wu et al. have found a positive correlation between high EPHB2 expression and poor survival in both ovarian and breast carcinoma, suggesting its prognostic value and its potential as a predictive marker (Wu et al., 2004, 2006). Furthermore, overexpression of EPHB2 has also been reported in migrating glioblastoma cells. The treatment

of the astrocytoma cell line U87 with EPHB2 antibody inhibited the migration and invasiveness, although conversely these capacities were promoted in U251 cells stably transfected with EPHB2 (Nakada et al., 2004). Although overexpression of EPHB2 is observed in some tumor types, in gastrointestinal cancers, low levels of EPHB2 expression have been reported and found to be significantly associated with advanced disease stage and poor survival (Lugli et al., 2005). In colorectal carcinoma (CRC), a progressive reduction in EPHB2 levels has been reported in the progression from normal epithelial cells to benign adenomas and to low and high stage tumors, as well as lymph node and liver metastases, demonstrating a clear tendency to decreasing EPHB2 levels as CRC progress towards a more aggressive and metastatic phenotype. The loss of EPHB2 expression was also significantly associated with poor tumor differentiation and shorter patient survival. In vitro functional assays showed that EPHB2 does not affect the cell proliferation itself, but rather inhibits cell adhesion and migration (Guo et al., 2006). However, studies with mice intestinal stem cells show that proliferation is reduced in colon crypts lacking EphB2 (Holmberg et al., 2006). The loss of EPHB2 in colorectal cancer has been also reported by Batlle et al., who additionally observed that inactivation of EPHB2 accelerated tumorigenesis initiated by APC mutations in colon and rectum of $Apc^{Min/+}$ mice, demonstrating that loss of EPHB2 expression represents a critical step in colorectal cancer progression (Batlle et al., 2005). Additionally, low EPHB2 levels have been recognized as a molecular marker to identify serrated colorectal carcinomas, a tumour type morphologically different from conventional CRCs (Laiho et al., 2007).

Concerning the mechanisms responsible for this EPHB2 down-regulation in CRC, we have detected aberrant promoter CpG methylation and frameshift mutations. Promoter hypermethylation affected 54% of the 101 CR tumors investigated, and EPHB2 expression was restored after treatment of EPHB2-methylated colon cancer cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, demonstrating the functional relevance of the aberrant methylation of the EPHB2 promoter. On the other hand, frameshift mutations were found in 41% of CRC tumors with microsatellite instability (MSI) affecting an A9 track in exon 17. This frequency was significantly lower in MSI adenomas (21%), in agreement with the progressive loss of EPHB2 during CRC progression, suggesting that EPHB2 inactivation may be important for the transition from adenoma to carcinoma (Alazzouzi et al., 2005). In addition, a high mutation rate was also found in MSI gastric tumors (39%), while significantly fewer mutations were found in MSI endometrial tumors (14%),

Table 2. Expression of EPHB family members in cancer.

CANCER	EPHB FAMILY MEMBERS			
	B2	B3	B4	B6
Bladder			(U) Xia et al., 2006	
Breast	(U) Wu et al., 2004	(D) Fox and Kandpal, 2004	(D) Fox and Kandpal, 2004., (U) Kumar et al., 2006; Wu et al., 2004	(D) Fox and Kandpal, 2004; Fox et al., 2006
Colorectal	(U) Lugli et al., 2005; (D) Alazzouzi et al., 2005; Batlle et al., 2005; Guo et al., 2006		(D) Batlle et al., 2005; Davalos et al., 2006; (U) Stephenson et al., 2001; Liu et al., 2002	
Endometrium			(U) Takai et al., 2001; Berclaz et al., 2003	
Gastric	(U) Lugli et al., 2005			
Head and neck	(U) Lugli et al., 2005		(U) Sinha et al., 2003; Masood et al., 2006; Sinha et al., 2006	
Leukemia			(P) Surawska et al., 2004	(P) Surawska et al., 2004
Lung	(U) Ikegaki et al., 1995			
Melanoma	(U) Yang et al., 2006	(U) Easty and Bennett. 2000; Yang et al., 2006	(U) Yang et al., 2006	(D) Hafner et al., 2003
Mesothelioma			(U) Xia et al., 2005a	
Neuroblastoma	(P) Tang et al., 2001			(U) Tang et al., 1999; 2000a,b
Ovary	(U) Wu et al., 2006		(U) Wu et al., 2006	
Prostate		(U) Chaib et al., 2001; (D) Fox et al., 2006	(U) Xia et al., 2005b	

Abbreviations: U: up-regulated, D: down-regulated, P: present

suggesting a functional role for EPHB2 in gastrointestinal tumor progression and highlighting the existence of differences in the tumorigenic pathways leading to gastrointestinal and endometrial cancer (Davalos et al., 2007).

Similar to EPHB2, EPHB4 expression in cancer is up- or down-regulated depending on the tumor type. A drastic increase of EPHB4 protein has been observed in endometrial hyperplasias and carcinomas, suggesting EPHB4 as an early indicator of malignant development (Berclaz et al., 2003). Moreover, Takai et al. associated the EPHB4 overexpression with high histological grade and certain clinical stages in endometrial cancer (Takai et al., 2001). An increase in breast carcinoma has also been reported and high levels of EPHB4 correlated with histological grade and stage. In addition, strategies to block EPHB4 expression, both using siRNA and antisense led to a dose-dependent reduction in cell survival and increased apoptosis in breast tumor cells (Wu et al., 2004; Kumar et al., 2006). Wu et al. have also reported high EPHB4 expression in ovarian carcinomas, and found an association with poor survival and poor response to chemotherapy, suggesting EPHB4 as an independent predictor of chemotherapy response (Wu et al., 2006). In prostate, normal tissue has little or no expression of EPHB4, while the protein was present in the majority of tumors. siRNA and antisense experiments have shown a significant inhibition of cell growth and viability, migration, and invasion. Moreover, treatment with EPHB4 antisense has shown antitumor activity in a tumor xenograft model (Xia et al., 2005b). In bladder cancer and mesothelioma, EPHB4 expression was also found to be up-regulated. As in prostate cancer, substancial growth was observed after EPHB4 knockdown by antisense and siRNA in cell lines and murine tumor xenograts (Xia et al., 2005a, 2006). In primary squamous cell carcinoma of head and neck (HNSCC), EPHB4 expression has been observed, whereas normal tissue counterparts did not show detectable EPHB4 expression; and EPHB4 levels correlated directly with higher stage and lymph node metastasis. Furthermore, EPHB4 siRNA and antisense assays inhibited tumor cell viability and induced apoptosis, suggesting EPHB4 as a novel target also in the treatment of HNSCC (Sinha et al., 2003, 2006; Masood et al., 2006). In melanoma cells, a correlation has been found between high EPHB4 expression, malignance and enhanced cell migration, and disruption of EPHB4 activity resulted in an inhibition of cell migration and affected the actin cytoskeleton organization (Yang et al., 2006). Stephenson et al. reported EPHB4 up-regulation in colorectal tumors (Stephenson et al., 2001), however an exhaustive analysis by immunohistochemistry revealed a specific gradient of EPHB4 expression from the lower crypt to the colonic flat mucosa, and a substantial variability of EPHB4 expression in colorectal tumors from complete lack of immunoreactivity to very high levels of expression ((Davalos et al., 2006); Fig.2). Furthermore, our group has shown that low EPHB4 tumor levels identify a subset of colorectal cancer patients with poor prognosis and high risk of recurrence, and demonstrated that promoter hypermethylation was a common mechanism associated with the loss of EPHB4

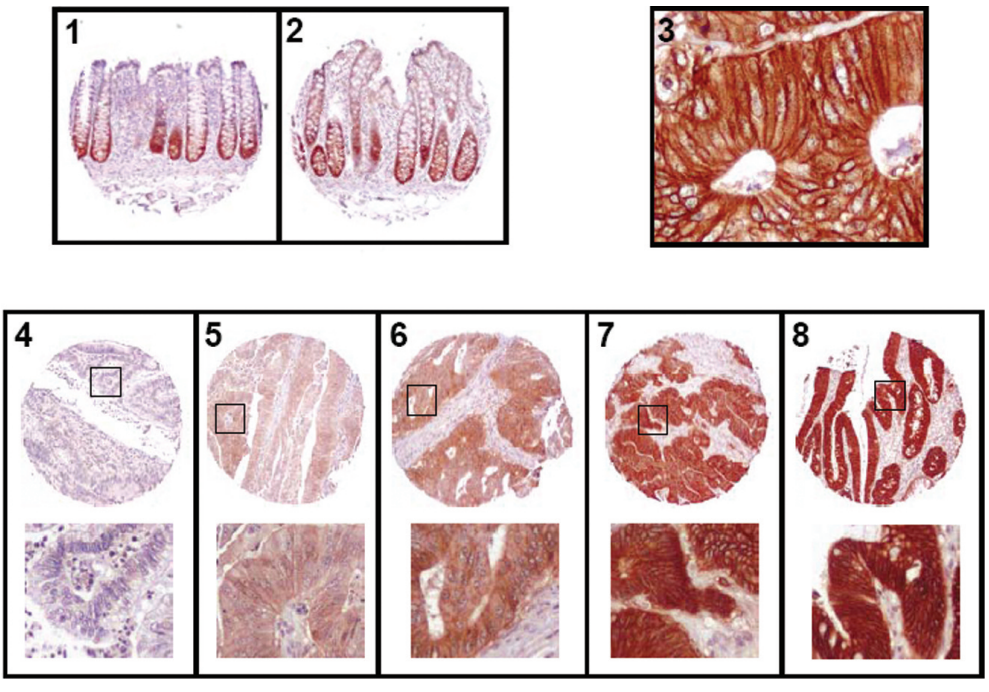

Fig. 2. EPHB4 as a prognostic marker in colorectal cancer. **1 and 2**, immunohistochemical staining revealed a gradient of EPHB4 expression in the normal colonic epithelium, with maximal levels in the lower crypt; **3**, EPHB4 expression was localized to the cellular membrane in the majority of the colorectal tumors investigated; **4 to 8**, there was great variability in EPHB4 expression in colorectal tumors.

expression. Moreover, reintroduction of EPHB4 into EPHB4-deficient tumor cells significantly reduced their long-term clonogenic potential, which taken together contributed to establish EPHB4 as a new putative tumor suppressor gene, and a useful prognostic marker in colorectal cancer (Davalos et al., 2006).

Similarly, EPHB6 expression has been found to be down-regulated in melanoma, and the loss of expression was correlated with progression to metastatic disease (Hafner et al., 2003). Correspondingly, in breast carcinoma EPHB6 expression was found to be reduced in non-invasive carcinoma cells relative to the normal tissue, and transcriptionally silenced by promoter hypermethylation in invasive cases, suggesting the analysis of EPHB6 methylation as a diagnostic and prognostic tool in breast cancer (Fox and Kandpal, 2006). In neuroblastoma, higher levels of EPHB6 were found in low-stage tumors compared to advanced-stage tumors, suggesting high-level expression of EPHB6 as a predictor of favorable disease outcome in this cancer type (Tang et al., 1999, 2000a,b, 2001).

Mechanisms of EPH functions in tumor cells

The EPH-ephrin system plays an important role in a wide range of processes directly involved in tumorigenesis and metastasis, including cell shape, migration, substrate attachment and angiogenesis. For this reason, the deregulation of EPH expression and signaling activity observed in tumors could be crucial for malignant progression.

Cell shape and migration

Although EPH receptors are frequently overexpressed in cancer, most EPH members do not behave like classical oncogenic growth factor receptors. Unlike other receptor tyrosine kinases (RTKs), EPH receptors do not directly affect cell proliferation or differentiation in all tissues. Instead, several studies have related EPH receptor expression to invasiveness and metastatic potential, and this appears to be largely related to the capacity of EPH receptors to modulate integrin activity, small GTPases and to interact with cadherins.

Regulation of integrins

EPH receptors regulate cell migration and attachment to the extracellular matrix by modulating integrin activity. Integrins are a family of transmembrane receptor proteins involved in cell-to-cell and cell-to-extracellular matrix adhesive interactions. Consequently, these proteins are implicated in important mechanisms of tumor growth and metastasis, such as cell migration, invasion and intra- and extra-vasation (Mizejewski, 1999). EPH receptors are able to down- or up-regulate the function of integrins. Miao et al. have shown that activation of endogenous EPHA2 kinase negatively regulates integrin functions through rapid recruitment of the protein tyrosine phosphatase SHP2, and subsequent dephosphorylation and inactivation of focal adhesion kinase (FAK), consequently inhibiting cell spreading and integrin-mediated adhesion (Miao et al., 2000). On the other hand, activation of EPHB receptors may also influence integrin function depending on the receptor:ligand ratio. In this way, EPHB1 is able to detect the cellular density of ephrinB1 and induce different changes in cell-matrix attachment depending on ephrinB1 density (Huynh-Do et al., 1999). EPHB overexpression may result in tumor sensitivity to ligand-induced stimulation and decrease attachment as well as increase mobility (Huynh-Do et al., 1999, Surawska et al., 2004).

Small GTPases

EPH receptors modulate cell shape, migration and attachment through the remodelation of actin cytoskeleton, not only controling integrin activity but also activating small GTPases, such as Rho and Ras.

EPHB receptors are able to activate Rho GTPases (Noren and Pasquale, 2004). These proteins have critical roles in restructuring the cytoskeleton, cell morphology and adhesion, and consequently activation of Rho signaling by EPHB receptors may contribute to the ability of tumor cells to metastasize (Banyard et al., 2000; Croft et al., 2004). In addition to regulating Rho family proteins, EPH receptors also regulate the activity of Ras family proteins. EPH receptors regulate negatively H-Ras, a member of the Ras family, which in its active form triggers signaling pathways such as MAP kinase cascade, involved in cell proliferation, cell adhesion and transformation. Miao et al. have shown that stimulation of endogenous EPHA kinases potently inhibits the Ras/MAPK cascade (Miao et al., 2001). Inhibition of this pathway may explain the inhibition of cell proliferation exerted by EPH receptors. However, under certain circunstances, EPH signaling can also activate rather than inhibit the MAP kinases, affecting the role of MAP kinases in cell adhesion, thus contributing to cell migration and invasiveness (Noren and Pasquale, 2004).

E-cadherin

EPH receptors may also modulate cell attachment through their interaction with E-cadherin. Cadherins are a type of transmembrane proteins that play important roles in cell adhesion. E-cadherin is probably the most widely studied cadherin and it is the main cell adhesion molecule of early embryonic and adult epithelial cells. Down-regulation of E-cadherin causes epithelial-mesenchymal transition during embryonic mesoderm formation and tumor progression in epithelial cells. Zantek et al. have suggested that stabilization of EPHA2-ephrin binding mediated by E-cadherin leads to a block of focal adhesions, causing the loss of cellular

adhesion, which is a hallmark of metastatic cancer (Zantek et al., 1999). Furthermore, E-cadherin is required for accurate EPHA2 location at cell-contact sites; and in the absence of E-cadherin, EPHA2 expression and location are altered in breast cancer cells (Zantek et al., 1999, Orsulic and Kemler, 2000). Reciprocally, EPH may regulate E-cadherin expression and function. However, the mechanisms responsible are not well understood, although Rho GTPases could be implicated since these proteins regulate the endocitic transport of cadherins (Ellis and Mellor, 2000), therefore EPH could modify E-cadherin function through their ability to modulate Rho ans Ras activity. Taken together, deregulation of EPH receptor activity may enhance tumor cell motility, contributing significantly to tumor invasion and metastasis.

Angiogenesis

The growth of solid tumors is heavily dependent upon the generation of new blood vassels and capillaries. This process of vascularization is known as angiogenesis. Unlike classical oncogenes, which often affect only tumor cells, EPH receptors also mediate cell-cell interactions in tumor vasculature and tumor stroma (Brantley-Sieders et al., 2004a). The growth of solid tumors depends directly on the recruitment of new blood vessels, which in turn provide nutrients, oxygen and growth factors essential for tumor survival and malignant progression. Additionally, these vessels constitute an entry point for dissemination of metastatic cells (Folkman, 2002). During angiogenesis, endothelial cells invade the surrounding tissue through matrix degradation, proliferate, and then migrate towards the angiogenic stimulus, where they join in tubular structures that maturate integrating tightly with support cells, such as smooth muscle cells and pericytes (Yancopoulos et al., 2000).

EPH receptors and their ephrin ligands have emerged as a major class of receptor tyrosine kinases that regulate tumor neovascularization (Wang et al., 1998). The first study to assess the role of EPH receptors in tumor angiogenesis were done by Ogawa et al., who screened the expression patterns of EPH proteins in tumor blood vessels of xenografs grown in nude mice from MDA-MB-435 human breast cancer cells or human Kaposi's sarcoma cell line KS1767, as well as tumor specimens, and found that the EPHA2-ephrinA1 pair was constitutively expressed in tumor associated endothelium (Ogawa et al., 2000).

Expression of EPHA2 has been predominantly detected in tumor-associated endothelium, while ephrinA1 expression has been observed predominantly in tumor cells, suggesting that ephrinA1 might act as a pro-angiogenic signal to attract EPHA2-positive endothelial cells. Thus, ephrinA1 may promote angiogenic remodeling through the modulation of endothelial cell migration towards the tumor. Brantley-Sieders et al. have shown that activation of EPHA2 receptors in endothelial cells stimulates PI3K-mediated activation of Rac1 GTPase, promoting endothelial cell migration and vessel assembly. Furthermore, EPHA2-deficient endothelial cells fail to undergo vascular assembly and migration in response to ephrinA1 in vitro, and EPHA2-deficient mice show a diminished angiogenic response to ephrinA1 in vivo (Brantley-Sieders et al., 2004b). Functional data suggesting a role for EPHA-ephrinA molecules in promoting angiogenesis in tumors came from studies using soluble EPHA2 and EPHA3 to block receptor activation in tumor models, where soluble receptors were able to inhibit angiogenesis and, consequently, tumor growth (Brantley et al., 2002; Dobrzanski et al., 2004). In addition, EPH receptors are able to interact with angiogenic factors, such as vascular endothelial cell growth factor (VEGF). For example, VEGF induces endothelial expression of ephrinA1 and phosphorylation of EphA2 receptor (Cheng et al., 2002).

EPH receptors in the intestine

EPHB receptors are key regulators of cell migration and proliferation in the intestinal epithelium, specifically in the intestinal stem cell niche. The intestinal track consists of the small intestine, subdivided into the duodenum, jejunum and ileum; and the large intestine, which can be divided into colon and rectum. The main function of the small intestine is the absorption of nutrients, and for this reason the absorptive surface is increased by numerous protrusions of the luminal epithelial monolayer and the underlying submucosa,

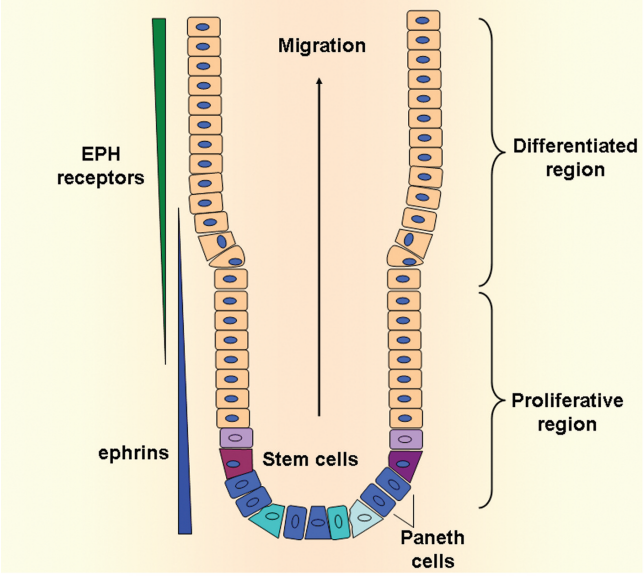

Fig. 3. Diagram of intestinal crypt. Paneth cells and stem cells are located at the bottom of the crypt, where the levels of ß-catenin/TCF4 transcriptional activity are higher. At the top of the crypt, this transcriptional activity decreases and cells mature and differentiate.

termed villi, and invaginations known as crypts of Lieberkühn. Three differentiated cell types can be found in the villi: enterocytes, enteroendocrine cells and goblet cells. Another cell type, the Paneth cells, reside at the bottom of the small intestinal crypts, and immediately above are located the proliferative stem cells, crucial in this self-renewing system. Epithelial cells differentiate as they move towards the top of the crypts, and fully differentiated cells can be found on the villus surface (Reya and Clevers, 2005).

Progenitor cells divide every 12-16 hours, and approximately 200 cells per crypt are generated every day. To maintain epithelial homeostasis, there is an equivalent cell loss at the villus tip and a continuous upward movement of cells (Heath, 1996). In this migration, cells differentiate to enterocytes, enteroendocrine or goblet cells, whereas cells that migrate toward the botton of the crypt differentiate along the Paneth cell lineage (Fig. 3).

Precise regulation of the homeostasis of the system is of key importance, and increased proliferation or reduced cell loss may result in tumor formation. In the epithelium of the normal intestine, there is a gradient of EPHB receptor levels along the crypt-villus axis with maximal expression at the bottom of the crypt. However, the expression of ephrin ligands shows an inverse pattern, with maximal expression in the villus (revised in Clarke, 2006). The transcriptional complex ß-catenin/TCF controls the migration of epithelial cells within the crypt by regulating the expression pattern of EPH members, because EPHB2, EPHB3 and EPHB4 are direct targets of the ß-catenin/TCF pathway (Batlle et al., 2002; Clevers and Batlle, 2006). This EPHB/ephrinB gradient establishes crypt-boundaries between the proliferative and differentiated region and the position of Paneth cells at the bottom of the crypt (Fig. 3).

In colorectal tumors, EPH receptors can behave as tumor suppressor genes. It has recently been shown that the disruption of EPHB2 and EPHB3 genes induced aberrant cell location in the normal epithelium and revealed that EPHB have an important role in cell sorting within the crypts. Although colonic premalignant lesions express high levels of EPH receptors, as mentioned before, its expression is frequently lost in malignant tumors and the loss of EPHB receptors is correlated with high tumor grade. Thus, despite constitutive activation of the Wnt signaling pathway, and the fact that EPHB receptors are direct transcriptional targets of the ß-catenin/TCF complex, down-regulation of EPHB levels in many colorectal cancers seems to significantly contribute to tumor progression. In $Apc^{Min/+}$ mice, an animal model where intestinal tumorigenesis is initiated by APC mutations, dysplastic crypts and small polyps in the large intestine of these animals remain confined to small areas at the surface epithelium surrounded by normal cells, and *EPHB2* seems to exert its function as a tumor suppressor, at least in part, by preventing the expansion and malignant progression of these lesions (Batlle et al., 2005). Furthermore, we have reported the tumor suppressor function of EPHB4 (Davalos et al., 2006), based on the strong correlation found between loss of EPHB4 expression and poor patient survival, and the significant reduction in clonogenic potential after reintroduction of *EPHB4* into cells expressing low levels. Recently, a mechanism for EphB-mediated tumor suppression in CRC has been proposed, where repulsive interactions between EphB and ephrin B restrict the spreading of cancer cells. EphB-expressing tumor cells are excluded from the ephrin-B-positive region in the normal mucosa, thus compartmentalizing incipient adenomas and suppressing tumor progression (Cortina et al., 2007). EphB signaling must then be inactivated before tumor cells can progress beyond this stage. Frameshift mutations and promoter hypermethylation are two such mechanism of inactivation that have recently been proposed (Davalos et al., 2006).

Clinical approaches and perspectives

Differential expression of EPH receptors in tumor and normal cells support their role as early tumor markers and potential therapeutic targets in cancer. For example, the overexpression of EPHA2 in tumor cells and its absence in the normal tissue of origin makes EPHA2 an ideal molecule for antibody targeting, minimizing adverse effects in normal cells (Carles-Kinch et al., 2002, Martiny-Baron et al., 2004). The same strategy has shown inhibition of tumor growth and angiogenesis by soluble EPHB4 in melanoma mouse model (Martiny-Baron et al., 2004). EPHB4 has also been suggested as a novel therapeutic target according to the results obtained using siRNA and antisense transfection to block EPHB4, where a significant inhibition of the viability of tumor cells and induction of apoptosis have been observed in several tumor types, including breast, bladder, mesothelioma, prostate and head and neck cancer (Xia et al., 2005a,b, 2006; Masood et al., 2006). It is necessary, however, an extensive validation of these and other potential therapeutic strategies to develop new chemotherapeutic drugs useful in cancer treatment, given the tumor type specific effects observed. For example, EPHB4, despite being overexpressed in several tumor types, has been reported down-regulated in colorectal tumors. Consequently, therapeutic strategies should be carefully studied and validated taking into account the tumor context.

The clinical importance of EPHB receptors as diagnostic and prognostic markers has been elucidated in the last few years. For instance, colorectal cancer patients with low EPHB2 or EPHB4 tumor levels have significantly shorter survival and higher risk of recurrence than patients with high EPHB4 levels. Therefore, assessment of EPHB4 levels in biopsy or resected tumor samples could be used to identify poor prognosis patients that could benefit from more aggressive treatment options (Davalos et al., 2006). However, there are significant gaps in the basic research

of EPH receptors and more studies should be carried out to elucidate the molecular mechanisms underlying the role of EPH receptors in tumorigenesis.

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