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Emerging strategies for EphA2 receptor targeting for cancer therapeutics

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Abstract

Importance of the field—High mortality rates with cancers warrant further development of earlier diagnostics and better treatment strategies. Membrane-bound hepatocellular receptor tyrosine kinase class A2 (EphA2) is overexpressed in breast, prostate, urinary bladder, skin, lung, ovary and brain cancers.

Areas covered in this review—This review describes EphA2 overexpression in cancers, its signaling mechanisms and strategies to target its deregulation.

What will the reader will gain—High EphA2 expression in cancer cells is correlated to a poor prognosis associated with recurrence due to enhanced metastasis. Interaction of the EphA2 receptor with its ligand (e.g., EphrinA1) triggers events that are deregulated and implicated in carcinogenesis. Both EphrinA1-independent oncogenic activity and EphrinA1-dependent tumor suppressor roles for EphA2 are described. Molecular interactions of EphA2 with signaling proteins are associated with the modulation of cytoskeleton dynamics, cell adhesion, proliferation, differentiation and metastasis. The deregulated signaling by EphA2 and its involvement in oncogenesis provide multiple avenues for the rational design of intervention approaches.

Take home message—EphA2 has been tested as a drug target using multiple approaches such as agonist antibodies, RNA interference, immunotherapy, virus vectors-mediated gene transfer, small molecule inhibitors and nanoparticles. With over a decade of research, encouraging results with successful targeting of EphA2 expression in various pre-clinical cancer models necessitate further studies.

Keywords

Cancer; EphA2; EphrinA1; Receptor tyrosine kinase; Cell signaling; EphA2 overexpression; Drug targets

1. Introduction

Interconnected and complex signaling pathways orchestrate the key physiological processes such as proliferation, migration, differentiation and apoptosis to maintain homeostasis in the normal cell cycle. Multi-step alterations and deregulations in cellular processes overcome the tight physiological regulation of normal cells and give rise to the pathological states

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implicated in the development of cancer [1]. Receptor tyrosine kinases functioning as proto-oncogenes often display increased activity and play an essential role in the cell signaling pathways in carcinogenesis [2–4]. While several tyrosine kinase receptors have been documented and targeted for their critical role/s in tumorigenesis [5], the cell membrane bound EphA2 receptor, a member of Eph tyrosine kinases receptor family, has generated great interest in the last few years.

The Eph family, the largest group among tyrosine kinase receptor families, is comprised of the EphA (EphA1–10) or EphB (EphB1–6) subclasses of receptors classified as per their sequence homologies and their binding affinity for their ligands, Ephrins (Eph receptor interacting protein) [6–8]. The first member of Eph family was cloned from an erythropoietin producing hepatocellular cancer cell line in 1987 and was named as EphA1 [9]. Subsequently, EphA2 was identified in 1990 by the screening of the human epithelial (Hela cells) cDNA library using degenerate probes designed to hybridize to a highly conserved regions of protein tyrosine kinases [10]. EphA2 was initially referred to as eck (epithelial cell kinase) for its expression in the majority of epithelial cells. The human EphA2 gene is located on chromosome 1, encodes a receptor tyrosine kinase of 976 amino acids with an apparent molecular weight of 130 kDa and has a 90% amino acid sequence homology to the mouse EphA2 [11].

The Eph family contains an extracellular conserved N-terminal ligand-binding domain followed by a cysteine-rich domain with an epidermal growth factor-like motif and two fibronectin type-III repeats (Fig. 1). The extracellular motif is followed by a membrane spanning region and a cytoplasmic region that encompasses a juxtamembrane region, a tyrosine kinase domain, a sterile alpha motif (SAM), and a post synaptic domain (disc large and zona occludens protein (PDZ) domain-binding motif) [12–15]. EphA2 shows a 25–35% sequence homologies with other Eph receptors, and the tyrosine residues are conserved within the juxtamembrane and kinase domain [12,16].

The ligands for Eph receptors, Ephrins, are divided into two subclasses EphrinA (EphrinA1–6) and EphrinB (EphrinB1–3) [13,14]. EphrinA members are anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) linkage while EphrinB members have a transmembrane and a cytoplasmic domain. EphrinA1 (previously known as B61) (Fig. 1) was identified as a cytokine-inducible gene product in human umbilical vein endothelial cells (HUVECs) [17]. Its expression was found to be inducible by stimulation with tumor necrosis factor. Later, it was found to be a ligand for EphA2 receptor based on its ability to bind the extracellular domain of EphA2 receptor tagged to an affinity column [18]. The crystal structures of the extracellular domain of EphA2 receptor alone or bound to the EphrinA1 or EphrinA5 ligand have been recently determined [19,20]. This review focuses on EphrinA1 because this Ephrin is the most extensively studied ligand for EphA2 in cancer, however, EphA2 is likely to also be activated by other EphrinA ligands in cancer cells and tumor vasculature. EphrinA1 is a GPI-anchored protein with an apparent molecular weight of 28 kDa, shows 30–40% amino acid similarities with other Ephrins and has also been characterized from the rat and mouse [12,21,22].

Eph-Ephrin signaling functions like the classical tyrosine kinase receptor-mediated cell signaling wherein a cell bearing an Eph receptor upon binding to EphrinA ligand transmits signals downstream known as forward signaling. In addition, intracellular signals in backward direction are also generated in Ephrin-bearing cells known as reverse signaling (Fig. 1). Accordingly, a contact between two communicating cells and a clustering of membrane-bound Ephrin ligands is required for the Eph-Ephrin bidirectional signaling [8,13,14,23,24]. EphrinA1organizes into micro-clusters upon association of a EphA2 receptor and actin cytoskeleton on the adjacent cell [25]. The EphA2 clustering could be

blocked when the cells were treated with EphA2 antibodies that inhibit ligand binding. The crystal structures of EphA2 extracellular domain also suggest that EphA2 localization at the cell-cell contacts depends on the clustering of EphA2, and a high concentration of EphA2 clustering independent of the Ephrin ligand could potentially impart a phenotype typical of a cancerous cell [20]. The role of Eph-Ephrin signaling has been studied in great detail in the development of the nervous system and spans a wide range of functions such as the development of neuronal networks, axon guidance, formation and remodeling of synaptic connections, and nervous system repair [24]. The interaction of Eph receptors with Ephrins is known to mediate cell-cell repulsion, regulate axon outgrowth, restrict cell migration and maintain well defined boundaries between different anatomical components of the developing brain [13,26]. The Eph-Ephrin interaction also regulates remodeling of vascular network formation during embryonic development [27,28]. A detailed review on Eph receptors and Ephrin ligands in embryonic development and carcinogenesis has been reported elsewhere in a number of review articles [7,15,24,29–31]. The understanding of EphA2 and EphrinA1 expression, signaling, and deregulation is important for developing strategies for cancer therapeutics.

2. Significance of EphA2 expression in cancers

EphA2 mRNA expression is observed in the skin, bone marrow, thymus, uterus, testis, prostate, urinary bladder, kidney, small intestine, colon, spleen, liver, lung and brain [32]. EphA2 expression in the colon, skin, kidney and lung was over ten-fold relative to the bone marrow. EphA2 is also expressed during gastrulation in the ectodermal cells and early embryogenesis in the developing hind brain [11]. In the skin, EphA2 is present in keratinocytes of epidermis and hair follicles but not in dermal cells (fibroblasts, vascular cells and inflammatory cells) [32]. EphA2 is also expressed in proliferating mammary glands in female mice at puberty and differentially expressed during the estrous cycle [33,34].

Besides its expression in embryo and in normal adult tissues, EphA2 is overexpressed in several cancers. In particular, a high level of EphA2 is detected in malignant cancer-derived cell lines and advanced forms of cancer (Table 1). In light of the EphA2 overexpression in pre-clinical models and clinical specimens of many different types of cancer, the increased level of EphA2 expression could be informative in both the prediction of cancer outcomes and in the clinical management of cancer. The differential expression of EphA2 in normal cells compared to cancer cells also signifies its importance as a therapeutic target.

2.1. Breast cancer

In aggressive breast cancer-derived cell lines, Hs578T, MDA-435, MDA-231 and BT549, increased levels of EphA2 protein are observed, while in non-transformed but immortalized human mammary epithelium-derived cell lines, MCF-10A, MCF-12A and MCF-10-2, the normal levels of EphA2 are expressed [35]. In clinical specimens of benign mammary epithelia, 75% (9/12) of the specimens were negative, while 25% (3/12) were weak positive; the average staining intensity was reported as 0.1 when quantified between 0 (no staining) and 3 (maximum staining) range. In breast carcinoma specimens, 92% (11/12) of the cases showed moderate to high staining with average staining intensity of 2.9. A stable overexpression of EphA2 in EphA2-transformed MCF-10A cells resulted in unstable cell-cell contact and fibroblast-like morphology as opposed to the epithelial morphology of the non-transformed cells. While the non-transformed MCF-10A cells did not form tumors in mice, the EphA2-transformed cells readily formed tumors indicating that EphA2 overexpression alone is sufficient to confer a malignant phenotype to normal cells [35]. In addition, a variant of Ras-transformed MCF-10A cells displayed increased EphA2

expression, loss of E-cadherin expression, anchorage-independent growth and a mesenchymal phenotype [36].

EphA2 overexpression in breast cancer is also linked to estrogen receptor expression with a negative correlation between two molecules [35,37] (Fig. 2). Furthermore, EphA2 overexpressing MCF7 cells show a reduced dependence on estrogen for growth and an enhanced tumorigenic potential in athymic mice [38]. In human epidermal growth factor receptor 2 (Her2) positive breast cancer patients, increased levels of EphA2 mRNA were correlated to a decreased potential for overall and disease-free survival [39]. The constitutive expression of EphA2 in MCF10AHer2 (MCF10A cells overexpressing Her2) cells led to increased proliferation, formation of acinar-like structures and reduced sensitivity towards the Her2 antibody, trastuzumab. A reversal of this effect was observed with the expression of inactive-form of EphA2 in MCF10AHer2 cells.

2.2. Melanoma

EphA2 was found to be increased in melanoma-derived cell lines that metastasize [40]. EphA2 was detected in 67% (12/18 samples) of biopsies from metastatic melanomas, while it was observed in only 22% (2/9 samples) of pre-invasive malignant melanoma lesions [41]. EphA2 was found to be phosphorylated in aggressive melanoma-derived cells and associated with vasculogenic mimicry, i.e., the formation of endothelial cell-like network [42,43]. It is co-localized with the endothelial adhesion molecule VE-cadherin in highly aggressive metastatic cutaneous melanoma (C8161), uveal melanoma (MUM-2B) and aggressive melanoma tumors. Transient suppression of VE-cadherin led to EphA2 de-phosphorylation, and redistribution from the point of cell-cell contacts to random scattering of EphA2 at the cell membrane [44].

EphrinA1 is identified as a melanoma growth factor and is upregulated during the progression of melanoma [41]. In a tissue microarray of melanomas, strong cytoplasmic EphA2 and EphrinA1 staining was present in 16% of the cases that associated with histological thickness of melanomas and tumors proliferation [45]. A correlation of metastatic potential and high EphA2 expression is also observed in human melanoma cell lines derived from patients [46]. Upregulation of EphA2 in F10-M3 cells increased their migration and invasion in two dimensional wound healing and three dimensional matrix invasion Boyden-matrigel assays respectively. EphA2 overexpression changed cellular migration from the mesenchymal- to the amoeboid- type [47].

2.3. Ovarian cancer

EphA2 overexpression is detected in aggressive ovarian cancer-derived cell lines compared to non-transformed cells [48]. Similarly, high EphA2 expression was evident in the clinical specimens of invasive ovarian tumors, while little or no staining was observed in normal ovaries. EphA2 overexpression is significantly and independently associated with poor patient survival [48–50]. The stable overexpression of EphA2 in poorly invasive and low EphA2 expressing A2780 ovarian cancer-derived cells using retroviral vectors resulted in significantly increased anchorage-independent growth [51]. In an orthopic mouse model of ovarian tumorigenesis, exogenous EphA2 overexpression significantly increased tumor growth, angiogenesis and metastasis.

A relationship between EphA2 overexpression and the status of tumor suppressor p53 has also been explored [52]. The incidence of high EphA2 expression in ovarian tumors with p53 null status was associated with poor prognosis. In addition, there seems to be an association of EphA2 overexpression with tumor angiogenesis and invasion [53]. High EphA2 expressing tumors exhibit increased microvessel density when stained for CD31 as a

measure of angiogenesis. In addition, the matrix metalloproteinase (MMP) expression, which degrades the extracellular matrix during cancer progression, has been associated with EphA2 expression.

2.4. Lung cancer

In a retrospective cohort of non-small cell lung cancer (NSCLC), moderate to high immunostaining of EphA2 has been observed in the membrane and cytoplasm in more than 70% of the carcinomas [54]. This increase is comparable in adenocarcinoma, squamous cell carcinoma and large cell carcinomas. The highest level of EphA2 is observed in clinically advanced stages of disease, and the patients with increased EphA2 levels showed a poorer prognosis compared to patients with reduced EphA2 levels. Additionally, patients with brain metastasis of NSCLC displayed high levels of EphA2. EphA2 overexpression was also correlated to poor survival and a history of smoking [55]. A mutation in the fibronectin domain in EphA2 sequences has also been identified in the H2170 lung carcinoma-derived cell line and in squamous cell carcinoma samples (2/28) [56].

2.5. Gliomas

Malignant gliomas show a poor prognosis despite several advances in chemotherapies and radiotherapies [57]. EphA2 overexpression is observed in several glioblastoma multiforme (GBM)-derived cell lines (e.g., A-172MG, DBTRG-05MG, U251MG and G48) compared to normal brain and non-tumorigenic glia-derived cell lines (e.g., H4) [58]. Immunofluorescence studies have determined that the predominant localization of EphA2 is on the membrane in these cell lines. In GBM, EphA2 is predominantly non-phosphorylated which is correlated to decreased levels of EphrinA1. In surgically resected human malignant glioma tissues, a heterogeneous and variable EphA2 staining patterns were observed [59]. Whereas normal brain tissues exhibited minimal staining, anaplastic astrocytoma and glioblastoma multiforme exhibited variable staining patterns. In astrocytic tumors, EphA2 overexpression correlated to a pathological grade and proliferation of tumors [60]. Additionally, EphA2 overexpression negatively correlated to glioblastoma patient survival [61,62].

2.6. Urinary bladder cancer

EphA2 protein expression in invasive bladder cancer-derived cell lines, T24, UMUC-3, and TCCSUP is reported to be increased [63]. Clinical specimens of bladder carcinoma when examined by a semi-quantitative immunostaining showed a differential staining pattern than normal specimens. Of all urinary bladder specimens with Ta grade lesions, 30% showed moderately strong staining, while in the T3 and T4 lesions, 75% and 90% of the samples respectively showed strong staining. In sharp contrast, 85% of normal tissues (n=13) showed weak staining, while the remaining 15% showed moderate staining for EphA2.

2.7. Prostate cancer

In prostate cancer-derived DU-145 and PC-3 cells, intermediate to high levels of EphA2 expression are detected [64]. In the prostate epithelium-derived cells that are transformed either by K-ras (267Ki) or X-irradiation (267X), increased levels of EphA2 expression are observed, while in immortalized human prostate epithelium-derived cells, 267B1, EphA2 is present at a comparatively reduced level. In clinical prostate carcinoma specimens, EphA2 immunoreactivity was increased with an average score of 2.67 (range 1–3) with a positive staining in 60–100% of cells.

2.8 Esophageal, renal, colon and vulvar cancers

EphA2 overexpression is detected in esophageal carcinoma-derived cells and in 50% (40/80) of clinical specimens [65]. In addition, a decrease in the five year survival and prognosis of EphA2-positive cancer was observed compared to EphA2-negative cancers. Metastatic renal cancer-derived cell lines and cancer tissue specimens were found to express increased levels of EphA2 [66]. A correlation of increased EphA2 expression with recurrence and shorter disease-free survival has also been observed. Renal cancers expressing increased EphA2 have been associated with more vascularized higher-grade tumors [67]. Co-expression EphA2 and EphrinA1 was observed in the biopsies from colon cancer and in colorectal adenocarcinoma-derived Caco-2 cells [68]. In another study, increased expression of EphA2 and EphrinA1 was observed in over 59 and 67%, respectively of clinical specimens from colorectal cancer patients [69]. In human colon cancer-derived HCT116 cells, a dose and time-dependent upregulation of EphA2 was noticed after treatment with deoxycholic acid, a component of bile acids and promoter of colon cancer [70]. The upregulation of EphA2 was p53-independent but linked to the activation of MAP kinase pathway. In vulvar cancers, more than 50% of vulvar squamous cell carcinomas express increased levels of EphA2 and EphrinA1 [71].

The aforementioned studies clearly indicate that EphA2 overexpression in cancers could serve as a diagnostic and prognostic marker, in addition to a therapeutic target. The information is significant due to the observed correlation of EphA2 with aggressive features of cancer and reduction in survival. The mechanisms by which overexpressed EphA2 contributes to malignant phenotypes are not entirely clear. Both kinase-dependent and -independent functions of EphA2 have been reported to be involved in aggressive cancer phenotype [72,73]. Additionally, a tumor suppressor function of EphA2 has also been reported in a study where EphA2-knockout mice were more susceptible to a chemical-induced skin carcinogenesis with an enhanced growth rate [74]. It is likely that the EphA2 function in tumorigenesis is dependent on multiple factors such as receptor activation, presence/absence of EphrinA1 and other ligands, and the contribution of a tumor-associated microenvironment towards malignancy.

3. EphA2-EphrinA1 signaling in carcinogenesis

The EphA2-EphrinA1 signaling axis regulates multiple events that are critical for the malignant transformation of normal cells (Fig. 2). The key downstream molecules of this signaling are the phosphatidyl inositol 3’ kinases (PI3K), Src family kinases, Rho and Rac1 GTPases, mitogen activated protein kinases (MAPK) and integrins along with the cross-talks of other oncogenic receptors (e.g., epidermal growth factor receptor, EGFR) that regulate cell adhesion, modulation of cytoskeleton architecture, cell proliferation, migration and development of vascular network.

Since EphA2 and EphrinA1 are both membrane anchored, a cell-cell contact is required for triggering a bidirectional signaling [7,8,12,75]. In addition, soluble and monomeric EphrinA1 has also been reported as a physiological ligand that could evoke EphA2 signaling independent of cell-cell contact [76]. Upon the binding of EphrinA1 in a ‘lock and key’ mechanism [12], the EphA2 receptor becomes tyrosine phosphorylated by transphosphorylation and interacts with several proteins to elicit downstream signaling [77]. An interaction of EphA2 with PI3K is identified using a yeast two hybrid strategy [78]. A src-like adapter protein (SLAP) containing Src homology-2 (SH2) and SH3 domains, but lacking tyrosine kinase activity, is also identified [79].

In prostate cancer-derived PC3 cells, the activation of EphA2 occurs within minutes upon treatment with a dimerized EphrinA1 fused to human IgG. Activation of EphA2 is

associated with the recruitment of a SH2 domain containing tyrosine phosphatase (SHP2) to the activated EphA2 complex and dephosphorylation of focal adhesion kinase (FAK) and its downstream target paxillin [80]. The biological consequences of EphA2 activation in prostate cancer PC3 cells were cellular rounding and a reduction in integrin-mediated adhesion. However, when the cell culture plasticware coated with EphrinA1 was seeded with the serum-starved mouse immortalized fibroblastic cells (NIH-3T3), the cells adhered and spread [81]. These experiments further revealed that besides EphA2, FAK, $p130^{CAS}$ and paxillin were also tyrosine phosphorylated with the localization of $p130^{CAS}$ and FAK to the punctuate structures in adhered cells. The $FAK^{-/-}$ and $p130^{CAS-/-}$ cells also adhered to the EphrinA1 coated surfaces but failed to spread, a defect that could be rescued by re-expressing FAK or $p130^{CAS}$. These results thus demonstrate that EphA2- EphrinA1 signaling promotes cell adhesion and spreading in a FAK- and $p130^{CAS}$ -dependent manner. The seemingly contradictory results of EphA2-EphrinA1 signaling not only indicate the complexity of the signaling mechanisms but also the importance of the genomic context of the cell lines. Evidently, PC3 cells that overexpress EphA2 show metastatic behavior and harbor aberrant signal transduction pathways compared to NIH-3T3 cells in the genomic context. The complexity of EphA2-EphrinA1 signaling is further elucidated by its effects on the MAPK signaling pathway where both activating and suppressing functions with a common outcome of diminished cell growth and tumorigenicity are reported. EphA2 overexpressing breast (MDA-231) and prostate (PC3) cancer cells when treated with EphrinA1-Fc (recombinant EphrinA1 fused to the Fc portion of immunoglobulin) or an EphA2 antibody activated the MAPK pathway within minutes. This is coupled with the binding of activated EphA2 to the adaptor protein Shc (via SH2 and PTB domains) and GRB2, and the induction of a negative regulation of MDA-231 cells to extracellular matrix [82]. In a study in PC3, pRNS-1-1 prostate cancer, primary bovine aortic endothelial and mouse embryonic fibroblast cells revealed an inhibition of the MAPK signaling [83]. In pRNS-1-1 cells, EphrinA1-Fc stimulation suppressed cell proliferation, clonogenicity and BrdU (5-bromo-2-deoxyuridine) incorporation indicating a slower growth rate.

When stimulated with a hormone (testosterone), NIH-3T3 fibroblast cells expressing a fusion protein comprising of oncogenic human Raf-1 coupled to the hormone-binding domain of androgen receptor which led to the activation of the MAPK pathway and upregulation of EphA2 [84]. The hormone treatment also upregulated EphA2 expression in mammary MCF-10A cells expressing the oncogenic Raf-1. In addition, activation of EphA2 by EphrinA1 ligand reduced EGF-induced extracellular signal-regulated kinases (ERK) phosphorylation in BT159, MDA-157 and HBL100 cells but not in MDA-231 cells. This suggests a cell-dependent cross-talk between EphA2 and the EGFR signaling resulting in a negative regulation of EGFR via the EphA2 signaling [84]. An increase in EphA2 expression levels also correlated to the inhibition of Ras and associated protein kinase B (Akt) activity in NIH-3T3 fibroblasts [85].

The EphA2-EphrinA1 signaling regulates tumor-associated angiogenesis that is required for the survival and maintenance of tumor growth [86]. EphA2-deficient mice displayed decreased microvascular density when implanted with 4T1 mammary adenocarcinoma-derived cells, indicating a role of EphA2 in promoting angiogenesis in tumor microenvironment [87]. The mechanism for the regulation of angiogenesis by EphA2 is demonstrated to be via the PI3K signaling. Upon binding to EphrinA1, EphA2 phosphorylation followed by PI3K recruitment occur resulting in activation of the Vav family of Guanine Nucleotide Exchange Factors (GEF) and Rac1-GTP overexpression [88]. EphA2 overexpression is also associated with angiogenesis and microvessel density in mammary tumorigenesis [89]. EphrinA1 has also been suggested to serve as a pro-angiogenic factor since siRNA-mediated knockdown of EphrinA1 reduces endothelial cell migration *in vitro* and tumor microvasculature density *in vivo*.

4. Altered regulation of EphA2 in cancers

The diverse biological effects of Eph-Ephrin signaling in several vital developmental biological processes are tightly regulated [7]. In light of the understanding of the role of EphA2 and EphrinA1 axis in cancers, it is particularly intriguing to learn how the regulation of Eph-Ephrin is diverted towards carcinogenesis. Expression of EphA2 and EphrinA1 is found to be inversely correlated in a panel of breast cancer cell lines [84]. EphA2 expressing cells display a mesenchymal-like phenotype (E-cadherin-negative and vimentin-positive), while EphrinA1 expressing cells display epithelial characteristics (E-cadherin-positive and vimentin-negative). E-cadherin is known to regulate development processes, and its downregulation is required during epithelial to mesenchymal transition [90]. Furthermore, E-cadherin and EphA2 are co-localized at the sites of cell-cell contact in the non-neoplastic MCF-10A epithelial cells. A functional link between EphA2 phosphorylation and E-cadherin expression has been established [73]. Expression of E-cadherin in neoplastic MDA-231 cells restores EphA2 phosphorylation and localization at the sites of cell-cell contact. E-cadherin also regulates EphA2 expression [91]. Expression of E-cadherin cDNA in embryonic stem (ES) cells lacking E-cadherin restores EphA2 expression levels to that of wild type-ES cells. Additionally, in non-epithelial NIH-3T3 cells stable expression of E-cadherin induces EphA2 expression.

A correlation of EphA2 expression with cell adhesion has been recently described in adherent or suspension cell lines [92]. High EphA2 expression is detected in adherent cells compared to cells grown in suspension. Moreover, suspension cells when adapted and selected to grow as adherent cells express increased levels of EphA2 compared to parental cells in suspension. Adhesion-induced expression of EphA2 is dependent on EGFR and on the activation of MEK and Src signaling. Additionally, EphA2 is reported to be a direct transcriptional target of Raf-MEK and MAPK signaling pathways [84].

EphA2 and EphrinA1 are also reported to be regulated by the p53 family of proteins [93]. A putative p53 response element has been identified by screening bacterial artificial clones and is located in the promoter region of EphA2. It can be activated by wild-type but not mutated p53. EphA2 is induced by other p53 family proteins and by treatment with DNA damaging agents. Ultraviolet radiation also induces EphA2 with a MAPK signaling-dependent mechanism in melanoma cell lines having mutated p53 [94].

Ligand-mediated EphA2 mRNA upregulation is dependent upon the MAPK signaling as a MEK1 inhibitor, PD98059, decreases EphA2 mRNA upregulation [95]. The ligand binding also concomitantly stimulates EphA2 protein degradation and turn-over indicating that an elevated EphA2 expression in cancers could be due to enhanced gene expression and protein stability.

Regulation of EphA2 stability may also play an important role in malignancy. For example, breast cancer-derived cells with diminished cell-cell contacts may have a paucity of EphrinA1 binding to EphA2, resulting in lower EphA2 phosphorylation and an altered intracellular localization. Activation of EphA2 by **E**-cadherin expression restored its normal phosphorylation and localization [73]. Treatment with a subset of biologically active EphA2 monoclonal antibodies that recognized extracellular epitopes increased EphA2 phosphorylation and degradation [31,96]. Similarly, treatment with EphrinA1 led to internalization and degradation of EphA2 indicating that ligand binding is required to maintain normal protein levels. In MDA-231 cells, the mechanism of EphA2 degradation was due to its association with the ubiquitin ligase c-Cbl [97,98].

The SH2 domain containing 5’ inositol phosphatase 2, SHIP2, is implicated in the regulation of EphA2 endocytosis. In a heterotypic SAM-SAM domain interaction, SHIP2 was

demonstrated to interact with EphA2 upon ligand binding. Further, RNAi-mediated suppression of SHIP2 increased EphA2 degradation [99]. The molecular chaperone heat shock protein 90 (HSP 90) is also overexpressed in cancers and is implicated in enhanced stability of EphA2 in tumor cells [100]. An inhibitor of HSP-90, 17-DMAG (17-dimethylaminoethylamino-17-dimethoxygeldanamycin) induces EphA2 degradation in renal cell carcinoma-derived SLR20 cells.

5. Targeting EphA2 overexpression in cancers

The ultimate goal of cancer therapy is to achieve selective tumor killing while sparing normal cells. With the identification of novel molecular markers for cancers, strategies based on targeting these proteins have become a key component of drug discovery. For example, the trastuzumab, a monoclonal antibody against receptor tyrosine kinase Her2, when used alone or combination with chemotherapy, has helped tumor regression [5,101]. Due to the function of EphA2-EphrinA1 signaling and the consequences of EphA2 overexpression in several cancers, targeting EphA2 overexpression in cancers seems to be an attractive candidate for cancer therapeutics (Fig. 3).

5.1. Antibodies

Monoclonal antibodies are designed against the extracellular domain of EphA2 by immunizing mice with plasmids encoding for the fusion of human EphA2 extracellular domain to immunoglobulin heavy chain [96]. Anti-EphA2 specificity and functionality was assessed in *in vitro* matrigel and soft agar assays where the EphA2 antibodies inhibited formation of tubular network and colonization of breast cancer-derived MDA-231 cells. EphA2 agonist monoclonal antibodies also reduced EphA2 levels in ovarian cancer-derived HeyA8 cells *in vitro* and in intraperitoneal tumors implanted into nude mice [102]. Prolonged treatment of the tumor-bearing mice with EphA2 agonist monoclonal antibody or in combination with a mitosis inhibitor, paclitaxel, significantly reduced tumor growth. The growth inhibitory function of EphA2 antibodies is attributed to the induction of EphA2 phosphorylation, internalization and subsequent degradation, and inhibition of Ras/MAP kinase pathway and Akt activation. Thus, EphA2-specific monoclonal antibodies could function similarly to EphrinA1 and diminish the oncogenic potential of breast cancer cells.

Conversely, EphA2 overexpression enhances the resistance of breast cancer cells towards trastuzumab [39]. The inhibition of EphA2 by an anti-EphA2 antibody restored the sensitivity in trastuzumab-resistant cells in *in vitro* and in orthotopic xenograft models. In order to target EphA2 overexpression on cancer cells and direct T cell cytotoxicity, bispecific antibodies were generated. These bispecific antibodies consisted of single chain antibody fragments from EphA2 and CD3/T-cell receptors [103]. The EphA2 epitope on the bi-specific antibody was selected based on its accessibility and specificity on transformed cells [104], while a deimmunized human CD3ε-specific mouse mAbL2k provided the second component [105]. In human colon carcinoma-derived SW480 cells, the EphA2/CD3 bispecific antibody demonstrated potent cytotoxicity in the presence of unstimulated CD3+ cells in an *in vitro* and in a human xenograft nude mice model.

5.2. Immunoconjugates

Immunoconjugates utilize a monoclonal antibody to deliver a chemotherapeutic agent to induce cytotoxicity in cancer cells. EphA2 targeted monoclonal antibodies are excellent candidates for the development of immunoconjugates since EphA2 is selectively overexpressed in several cancers while expressed at comparatively reduced levels in normal cells. It is thus expected that EphA2-targeted immunoconjugates will induce cytotoxicity selectively in cancer cells while sparing normal cells. An EphA2 monoclonal antibody, 1C1,

coupled to monomethyl auristin phenylalanine (MMAF), a chemotherapeutic agent, via a non-cleavable linker maleimidocaproyl (mc), (1C1-mcMMAF), was evaluated for its efficacy in EphA2-positive ovarian cancer-derived HeyA8 cells [106]. Treatment of HeyA8 cells with 1C1-mcMMAF reduced EphA2 expression levels in a time-dependent manner. The phenotypic changes associated with the treatment were reduced cell proliferation and increased apoptosis. In orthotopic ovarian cancer models, 1C1-mcMMAF treatment caused tumor regression and enhanced survival. In another study, 1C1conjugated molecules reduced EphA2 expression in endometrial cancer-derived Hec-1A and Ishikawa cells [108] due to decreased cell viability and increased apoptosis. The 1C1 antibody labeled with copper molecules (^{64}Cu) showed encouraging results for the development of an EphA2-specific non-invasive imaging technology [107].

5.3. EphrinA1

In glioblastoma multiforme-derived U-251MG cells that overexpress EphA2, soluble EphrinA1 seems to serve as a functional ligand for EphA2 [76]. These cells were transfected with EphrinA1 cDNA and selected for stable clones expressing EphrinA1, which showed a substantial decrease in EphA2 levels and significantly reduced cell proliferation and migration. Furthermore, treatment of parental cells with the conditioned medium from EphrinA1 expressing cells also resulted in downregulation of EphA2 expression accompanied by cellular rounding suggesting that EphrinA1 is released into the medium and is functional in eliciting an anti-tumor cellular response.

EphrinA1 has also been used in molecularly targeted therapies by conjugating with a bacterial endotoxin. Bacterial toxins such as *pseudomonas endotoxin A* have been shown to be potent and lethal to eukaryotic cells. These toxins can be modified to replace their natural eukaryotic cell receptor-binding domain with a target-specific ligand. A derivative of pseudomonas endotoxin A, PE38QQR was fused to EphrinA1 to generate EphrinA1-PE38QQR conjugated cytotoxin [109]. EphrinA1-PE38QQR was cytotoxic to glioblastoma, breast cancer and prostate cancer-derived cell lines. EphA2 protein levels declined within four hours of treatment with EphrinA1-PE38QQR in U-251MG cells and induced caspase-dependent apoptosis.

5.4. Small molecule inhibitors

Small molecule kinase inhibitors represent a class of cancer-therapeutics that target specific protein-protein interactions known to have important roles in tumor growth and progression. These inhibitors can be cost-effective when compared to natural ligands, but their specificity and off-target effects need to be carefully considered. Following the initial success of imatinib, a small molecule kinase inhibitor to treat chronic myeloid leukemia (CML) [110,111], several second-generation kinase inhibitors have been developed to treat many hematopoietic malignancies. Development of small-molecule antagonists that can selectively inhibit the function of the overexpressed EphA2 receptor or block the receptor and ligand interactions, are important for generating targeted therapies and studying the function of EphA2 in *in vitro* models. However, to date, only a few small molecule inhibitors of EphA2 have been identified. In a high throughput screen, two derivatives of 2–5-dimethylpyrrolyl benzoic acid were identified that selectively inhibited EphA2 binding to its ligand [112]. These compounds were not toxic to the cells and selectively inhibited ligand-driven phosphorylation of EphA2. In addition, these compounds were found to selectively inhibit EphA2-mediated retraction of cell periphery in PC3 prostate cancer-derived cells.

Another small molecule tyrosine kinase inhibitor, dasatinib, has been reported to possess potent inhibitory activity against EphA2 [113]. Dasatinib is an orally active inhibitor

developed to target multiple kinases including the Bcr-abl and Src family kinases [114]. Potent anti-tumor activities in both *in vitro* and *in vivo* tumor xenograft models [115–117] were demonstrated, and this drug was approved by the Food and Drug Administration (FDA) in 2006 for the treatment of imatinib-resistant chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia. Treating breast cancer cells *in vitro* with dasatinib has reduced EphA2 expression, phosphorylation, and associated kinase activity [118]. *In vitro* phosphorylation assays indicated a dose-dependent affect of dasatinib on the kinase activity of EphA2 [113]. In human pancreatic cancer-derived cells, BxPC-3, PANC-1, and MIAPaCa-2, treatment with dasatinib decreased EphA2 phosphorylation in a dose-dependent manner and suppressed growth. In addition, dasatinib inhibited EphrinA1-induced interaction of EphA2 and Cbl and the subsequent internalization and degradation of EphA2. , The treatment transiently inhibited EphA2 receptor tyrosine kinase signaling in the BxPC-3 xenograft mouse model [113]. These results indicate that dasatinib could function as an inhibitor of pancreatic cancer. Sensitivity of EphA2 and several other members of the Eph family of receptor tyrosine kinases to dasatinib has also been confirmed by proteomic and gene profiling approaches [118–121]. Dasatinib is currently being evaluated in clinical trials (NCT00563290 and NCT00895960) for squamous cell carcinoma and in combination with radiotherapy for glioblastoma (http://www.clinicaltrials.gov).

5.5. Virus vectors

Adenovirus (Ad) vectors have been extensively utilized as gene delivery vehicles [122–124]. A repertoire of Ad vectors have been developed and tested for their ability to specifically target and induce gene expression in cancer cells. Many of these approaches are in the pipeline for human cancer gene therapy applications. Ad vectors have also been utilized to target the deregulated EphA2 and EphrinA1 signaling in cancers.

Human Ad serotype 5 (HAd5) vector was engineered to express a secretory-form of EphrinA1 (HAd-EphrinA1-Fc) [125]. HAd-EphrinA1-Fc consisted of an extracellular domain of EphrinA1 (without GPI) and is conjugated to the Fc portion of IgG_1. Infection of cultured cells with HAd-EphrinA1-Fc resulted in efficient expression and secretion of EphrinA1 from targeted cells. Infection of MDA-231 cells with HAd-EphrinA1-Fc led to the phosphorylation and enhanced degradation of EphA2. Additionally, infected-MDA-231 cells were not only less viable *in vitro* but were also significantly impaired in tumorigenicity in immune-deficient nude mice xenografts. Secondly, to mimic an immunocompetent host, EphA2 overexpression was investigated in MT1A2 cells that were generated from polyoma virus middle T antigen harboring transgenic mice [126,127]. Infection of MT1A2 cells with HAd-EphrinA1-Fc inhibited cell viability and colonization in soft agar. Implantation of HAd-EphrinA1-Fc infected MT1A2 cells *in vivo* or intratumoral inoculation with HAd-EphrinA1-Fc following tumor development in an FVB/n mice led to significantly impaired tumor growth [127]. These results suggest that Ad vector-mediated EphrinA1 expression can be utilized successfully in human gene therapy applications.

Ad vector-mediated overexpression of EphA2 in non-transformed or Her2 overexpressing MCF-10A cells has been reported to enhance proliferation and invasiveness of MCF-10A cells overexpressing Her2 thus indicating the pro-oncogenic role of EphA2 [128]. In pancreatic cancer, a fiber-modified Ad vector was designed to improve Ad transduction in cancer cells by averting the Coxsackie and Ad receptor (CAR) and specifically targeting the EphA2 receptor expressing cancer [129]. While the CAR receptor is the major route for Ad transduction in cells, its expression is reported to be low in cancer cells resulting in reduced transduction efficiency [124]. Incorporation of a peptide, Tyr-Ser-Ala (YSA), targeting the EphA2 receptor has been integrated into the H1 loop of Ad vector and resulted in enhanced transduction of Ad vector to pancreatic cancer cells [129]. These results indicate that

modified Ad vectors in combination with other therapies will be a useful resource for anti-cancer therapies.

5.6. Immunotherapy

Since cancer is a heterogeneous disease with the presence of distance metastasis as a leading cause of cancer-related deaths, the potential of the immune system to recognize and reject cancer cells offers a novel approach towards cancer therapeutics. EphA2 overexpression could also be a candidate for cancer immunotherapy since epitopes on EphA2 are reported to be differentially displayed in normal versus cancer cells [104]. A subset of EphA2 antibodies reacts strongly to breast (MDA-231) and lung (A549) cancer cells but does not to normal immortalized breast (MCF-10A) cells. This strong reactivity is linked to the differential cell-cell contacts between normal and malignant cells and is not due to any mutations in EphA2 sequences or differences in the binding affinity of antibodies, indicating that EphA2 epitopes have the potential for therapeutic targeting.

Although EphA2 is an endogenous protein that is overexpressed with deregulated function in several cancers, it could also be an immunological target. To determine the role of EphA2 in cancer immunotherapy, CD4+ and CD8+ T cell epitopes have been derived from EphA2 protein sequences and tested for the development of anti-tumor immunity. EphA2 protein sequences displayed several peptide sequences that could bind to human leukocyte antigens (HLA-A0201 and HLA-DRB*401) and also revealed proteosomal cleavage sites that could be predicted by the use of neural network and PAProC algorithms [66,130]. The CD4+ and CD8+ T cells isolated from renal cell carcinoma (RCC) patients showed detectable reactivity towards EphA2 epitopes [66]. In addition, the EphA2-derived peptides increased the number of IFNγ secreting CD8+ T cells isolated from RCC patients with disease-free status. EphA2 peptides were also demonstrated to be naturally processed and presented in several cancers and capable of inducing tumor-specific CD8+ T cell response [131]. In addition to their role in immunotherapy, EphA2-derived peptides could also predict cancer progression and prognosis [66,131].

Dendritic cells (DC)-pulsed with EphA2-derived peptides were used in immunizing mice and it resulted in significantly inhibiting EphA2 growth in EphA2-positive colorectal cancer-derived MC38 cells but not in EphA2-negative BL6 cell-induced tumor growth. In addition, splenocytes from mice treated with DC-pulsed with EphA2 peptides showed anti-MC38 immuno-reactivity and development of antitumor immunity, indicating a promising role for EphA2-derived DC vaccines in colorectal cancers [132]. Recently, HSP-90 inhibitor 17-DMAG not only promoted EphA2 degradation but also enhanced EphA2-specific CD8+ T cell immunoreactivity. This effect could be augmented by combining anti-EphA2 monoclonal antibodies to 17-DMAG [100]. These studies clearly suggest that endogenous EphA2 overexpression in cancers could be utilized as a target for immunotherapy and makes it a promising approach to target metastatic cancers.

5.7. RNA-interference

To suppress EphA2 overexpression, sequence-specific silencing of EphA2 gene expression has been tested with many RNA interference (RNAi) approaches. Initially EphA2 antisense oligonucleotides were tested in MDA-231 cells by transfection. This approach reduced EphA2 expression level and significantly decreased colonization of MDA-231 cells in soft agar suggesting that reducing EphA2 levels by anti-sense nucleotides could reduce tumorigenicity [96]. In addition, suppression of elevated EphA2 levels in low molecular weight protein phosphatase (LMW-PTP)-transformed MCF-10A cells by antisense nucleotides led to reduction in soft-agar colonization [133].

In pancreatic adenocarcinoma-derived MIA PaCa2 cells, EphA2 expression was suppressed by RNAi and it also inhibited tumor growth in a nude mice xenograft model [134]. Biweekly treatment with EphA2-specific siRNA retarded tumor growth by 70% in six weeks. The retardation in tumor growth was accompanied by an increase in caspase 3 activity and the presence of apoptotic cells.

In an indirect approach, an EphA2 partner and endocytosis regulator, SHIP2, was targeted via retrovirus vector-mediated RNAi in MDA-231 breast cancer cells. SHIP2 silencing enhanced EphrinA1-induced EphA2 internalization and degradation [99]. SHIP2 expression levels have also been documented to be increased in breast cancer-derived cell lines and clinical specimens. The suppression of SHIP2 reduces cell proliferation and clonogenicity, thereby suggesting that targeting of SHIP2 and EphA2 is a potentially attractive therapeutic strategy for malignant breast cancer [135,136].

In human glioma-derived U-251 cells, suppression of EphA2 by RNAi reduced cell proliferation and increased caspase 3 activity and apoptosis [137]. Since EphA2 is also regulated by EGFR signaling in the EGFR overexpressing human head and neck carcinoma-derived cell line, HN5, and the human epidermoid-derived cell line, A-431, EphA2 levels are increased when EGFR is activated by EGF. In HN5 cells, suppression of EphA2 expression by RNAi significantly decreased EGF-induced migration *in vitro* wound healing assays. These results indicate that targeting EphA2 reduces EGFR-mediated cancer cell migration and suggest a cross-talk between EphA2 and EGFR signaling [138,139].

In malignant mesothelioma (MMC)-derived cells, overexpression of EphA2 increased cell proliferation and haptotaxis in boyden chamber assays, while suppression of EphA2 by RNAi significantly decreased cell proliferation and down-regulated *in vitro* migration [140]. Efficacy of *in vivo* delivery of siRNA targeting EphA2 has been demonstrated in an orthotopic mouse model of ovarian cancer using the neutral lipid liposomes, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC). In a combinatorial approach in ovarian cancer models wherein EphA2 protein was suppressed along with a focal adhesion kinase (FAK) using DOPC-mediated RNAi delivery, enhanced suppression of tumor growth was observed [141,142]. Additionally, in another approach, a cell line derived from an MMTV- Neu (Her2) tumor was subjected to EphA2 suppression and resulted in slowed growth rate and reduced ERK signaling [128]. These studies demonstrate RNAi-mediated EphA2 suppression as a viable approach targeting EphA2 overexpressing cancers. This strategy along with other oncogenic signaling could be used to improve the anti-tumor response of EphA2 targeted approaches.

5.8. Nanoparticles

For cancer diagnosis and treatment, much like molecular therapies, nanoparticle-based therapy offers the possibility of targeting predominately cancer cells [143]. The feasibility of gold-coated silica nanoshells to target human breast cancer-derived SKBR3 cells was demonstrated by illuminating the nanoshell-treated cells with a diode laser and thus inducing thermal destruction of the targeted cells [144]. Polyethylene glycol (PEG)-coated nanoshells have been used in a murine immunocompetent colon carcinoma model [145]. These nanoshells absorb the light near infrared spectrum (NIR) in addition to being biocompatible and non-toxic [146]. In order to target EphA2, the nanoshells were conjugated to EphrinA1-Fc with a PEG derivative [147]. These EphrinA1 nanoshells showed binding to EphA2 overexpressing PC-3 cells but not to EphA2-deficient human dermal fibroblast (HDF) cells. Upon treatment with a NIR laser, EphrinA1-nanoshell bound PC-3 cells are killed, indicating the specificity of an EphrinA1-nanoshell approach towards the targeted killing of cancer cells.

In order to target metastasis in EphA2 overexpressing cells, magnetic nanoparticles have been utilized to capture metastatic cells [148]. In this study magnetic $CoFe_2O_4$ nanoparticles were conjugated to the Ephrin-mimetic peptide, YSA, to specifically target EphA2 in ovarian cancer cells. These magnetic nanoparticles were demonstrated to bind to EphA2 on HeyA8 cells through YSA peptide. Further in a dialysis-like treatment approach, the cells were removed from the mouse peritoneal cavity by the application of a magnetic field. This demonstrated that magnetic nanoparticles conjugated to a cancer cell-specific peptide could selectively remove cancer cells from the abdominal cavity. In another targeted approach, the YSA peptide conjugated to core/shell hydrogel nanoparticles (nanogels) was utilized to target EGFR expression via EphA2 receptor in Hey cells [149]. These nanogels were synthesized from *N*-iso-propylmethacrylamide crosslinked with *N*, *N*'-methylenebis(acrylamide) and encapsulated EGFR targeting siRNA. Treatment of EphA2 expressing Hey cells with EGFR-targeted nanogels resulted in a time and dose-dependent knockdown of EGFR expression and enhanced the sensitivity towards the chemotherapeutic drug docetaxel. This effect was not observed in EphA2 lacking SKOV3 cells, thus indicating the specificity of EGFR siRNA delivery via nanogels, and illustrating the potential of EphA2 as a target for therapeutic drug delivery.

Biodegradable nanoparticles comprising of L-phenylalanine (Phe)-conjugated poly (γ-glutamic acid) [γ-pga-Phe] were evaluated for EphA2-derived peptide vaccines [132,150]. Vaccination with EphA2-derived peptides immobilized by nonoparticles elicited anti-tumor effects against EphA2 overexpressing mouse MC38 hepatic tumors and was not associated with any toxic effects. The data indicate that nanoparticles can be utilized to reduce toxic effects of adjuvants while inducing acquired immunity against tumor antigens.

5.9. Combination therapies

Preclinical studies have tested the combination of EphA2-directed therapies with other anticancer therapies, such as chemotherapeutic drugs or molecular therapies. EphA2 siRNA when used in combination with chemotherapeutic drug paclitaxel was found to be more effective in inhibiting growth of HeyA8 or SKOV3 orthotopic ovarian tumors in mice than treatment with the control siRNA and paclitaxel [151]. This combination treatment reduced growth of HeyA8 and SKOV3 tumors by 67 and 82%, respectively, and resulted in a more than 74% reduction in microvessel density. Similarly, treatment with an EphA2-agonistic monoclonal antibody, EA5, in combination with paclitaxel resulted in substantially increased inhibition in tumor growth compared to treatment with the control IgG and paclitaxel [102]. In mice bearing HeyA8 or SKOV3ip1 tumors, combination treatment with the anti-EphA2 antibody (EA5) and paclitaxel resulted in 88 to 92% reduction in tumor weight compared to the IgG control. A 77 to 80% reduction compared to paclitaxel was achieved along with prolonged survival of the tumor-bearing mice.

EphA2 siRNA has also been tested in combination with siRNA targeting FAK or Src tyrosine kinases [141]. A combination of EphA2 and FAK siRNA resulted in a significant decrease in ovarian tumors and a reduction in tumor microvessel density compared to monotherapy. Since EphA2 overexpression is downregulated by treatment with either EphrinA1 or agonist antibodies in a proteasomal-dependent manner, EphA2-directed therapies could be augmented by treatment with agents that promote proteasomal degradation [97]. As previously described, HSP-90 inhibitor, 17-DMAG, enhances tumor recognition by CD8+ T cells in EphA2 overexpressing renal cancer cell lines [100]. Treatment with EphrinA1 or EphA2 monoclonal antibodies also enhanced CD8+ T cell recognition of EphA2-positive renal cancer cell lines [152].

The cross-talk between EphA2 and EGFR signaling suggests that simultaneously inhibiting both EphA2 and EGFR overexpression could provide better anti-tumor response. The

inhibitory effects of EphrinA1 on MAPK signaling could be reversed by treating cells with EGF [92,138]. Furthermore, EphA2 expression influences the sensitivity of estrogen receptor-directed chemotherapies. Stable EphA2 overexpression in estrogen receptor-positive and estrogen-dependent breast cancer-derived MCF7cells has enhanced tumorigenicity of these cells *in vitro* and *in vivo* [38] and showed reduced sensitivity towards tamoxifen. The sensitivity to tamoxifen could be restored by treatment with EphA2 antibodies. Additionally, EphA2 overexpression was identified as a contributing factor towards the development of resistance to Her2-targeted trastutzumab monoclonal antibody therapy [39]. The functional cross-talk of EphA2 with other oncogenic alterations along with the encouraging results from pre-clinical studies clearly indicates the importance of combination therapies in targeting EphA2 overexpression in cancers.

6. Conclusions

Understanding of the function of EphA2 and its ligands in cancers has improved considerably over the last few years with the accumulated evidences clearly indicating a pro-oncogenic role of EphA2 in cancer progression and metastasis. The differential expression of EphA2 with substantially high levels in cancer cells compared with normal cells is documented in cell lines, and clinical specimens derived from cancer patients with several types of cancers indicate a generalized importance of EphA2 in carcinogenesis. This information is significant in the light of correlation of EphA2 overexpression to recurrence and shorter disease-free survival, and is also valuable for an early diagnosis. The mechanisms by which EphA2 overexpression occurs and then contributes towards cancer phenotype are not entirely clear and depend on the genomic context and cell type-specific function in different cancers. Nonetheless, the studies point towards the role of oncogenic signaling transduction pathways coupled with the contributions of cross-talk with other receptor tyrosine kinases. In addition, the phosphorylation of EphA2 resulting in its degradation and turnover has important functional consequences in tumor biology. EphA2 overexpression, functional alterations and gain of oncogenic function provide an opportunity to target EphA2 for therapeutic intervention. Targeting EphA2 overexpression by EphA2 antibodies, RNAi, adenoviral vectors, immunotherapy, small molecule inhibitors and by other approaches have shown promising results in both *in vitro* and *in vivo* cancer models. Since EphA2 expression also regulates tumor-associated angiogenesis, there is a potential to inhibit cancer survival by modulating the tumor microenvironment and generating a bystander affect by targeting EphA2. Overall, the mounting evidences clearly support that the aberrant EphA2 expression contributes towards oncogenesis, angiogenesis and tumor metastasis.

7. Expert Opinion

Cancer is a major global public health care burden and a leading cause of death. The recent statistics showing encouraging trends towards a reduction in cancer incidences and mortality highlight the need to find better diagnostic, treatment and prevention strategies [153]. In cancer therapeutics, the goal is to specifically regress and eliminate the cancerous cells while sparing the normal cells and to prevent recurrences of the disease. The fact that cancers are heterogeneous with diverse etiologies and prognosis makes the task of developing a basic understanding about carcinogenesis and therapeutics more challenging. As knowledge grows, the targeted and individualized cancer treatment approaches are revolutionizing cancer therapeutics. Each technology comes with its own limitations as cancer cells develop escape mechanisms, mutations and resistance towards new therapeutics.

Studies on the role of oncogenic receptor tyrosine kinases have generated many options for targeted anti-cancer therapeutic approaches. One family of receptor tyrosine kinases, the Eph receptors, and their ligands are not only regulators of embryonic development but also implicated in carcinogenesis. The similarities and differences between normal development and cancer development may hold many clues about understanding oncogenesis. Since the discovery of the EphA2 receptor in 1990, the role for this receptor and its dependent or independent on its ligands/s has been ascribed as the key aspects of cell transformation and development of malignancies. Therefore, EphA2 serves as a suitable candidate for cancer therapeutics (Fig. 4). Importantly, EphA2 could be a biomarker and indicator of cancer prognosis since it is overexpressed in many cancers. The different methodologies to target EphA2 have the potential for promising treatment options for cancer patients.

One of the strategies of developing better EphA2-directed therapeutic options for cancers is the use of adenoviral (Ad) vectors as a gene delivery system. Ad vectors expressing a secretory-form of EphrinA1 showed promising anti-cancer effects *in vitro* and *in vivo* in EphA2 overexpressing breast cancer models. One of the main concerns towards the development of Ad vectors for therapeutics is the prevalence of neutralizing antibodies against Ad in the general population. The potential solutions are the development of non-human Ad, rare human Ad or modified human Ad vectors recapitulating the merit of human Ad vectors but circumventing the preexisting Ad vector immunity, and devising multi-modality therapeutic approaches. The development of a potent innate immune response by Ad vectors could also be an inherent advantage in cancer gene therapy. Additionally, the combination of Ad-mediated cancer cell-directed cytotoxicity with immunostimulatory molecules could overcome the development of immuno-tolerance against cancer antigens.

The usefulness of the differential EphA2 expression levels in directing an adaptive immune response or apoptosis against cancers is very encouraging for cancer immunotherapy. Such approaches can be advantageous not only in determining cancer prognosis but also in preventing its recurrence. The differential display of EphA2 epitopes in normal vs. cancer cells is an advantage for the development of immunotherapy. However, caution should be exercised with EphA2-targeted immunotherapy approaches, since EphA2 is an endogenous protein, and the possibility of mounting an auto-immune response should be carefully considered.

The information generated from the crystal structures of Eph-Ephrin complexes and the contact-dependent bidirectional signaling is significant with the potential to mimic and modulate this signaling with Ephrin ligands by designing novel drug candidates. The cancer-specific cytotoxicity generated by EphA2-targeted therapies could lead to the identification of tumor-associated antigens. Additionally, a localized expression of immunostimulatory molecules in solid tumors would allow migration of antigen presenting cells (APC) to the cancer site leading to the presentation of tumor antigens to immune cells and the development of tumor-specific adaptive immune responses. The characterization of cancer cells that escape the immunosurveillance will be helpful in determining the mechanism/s of resistance. RNAi will be useful in uncovering the mechanistic details about the aberrant cell signaling. The application of nanotechnology to the development of novel therapeutic approaches has provided hope for the delivery of better cancer diagnostic and therapeutic tools.

Despite the promise of new approaches, the efficacy of molecular targeted therapies has not been up to the mark. The complex nature of cancer cell survival, growth and metastasis likely precludes a single therapeutic modality to be absolutely effective in each and every situation. Furthermore, the development of resistance and escape mechanisms by cancer cells towards conventional therapies make the task of treating cancer more challenging. A

combination of therapies depending upon tumor characteristics may be more effective in maximizing the benefit and development of individualized therapeutic options. Future studies using combination therapies that simultaneously will target EphA2 along with other oncogenic alterations are needed to evaluate the synergestic effect of this approach. The contribution of EphA2 expression on the development of resistance towards EGFR-targeted therapies has been reported suggesting that cancer-specific alteration in cell metabolism will provide insights into the improvement of cancer targeting approaches.

The role EphA2 in oncogenesis must be explored in conjunction with risk factors such as obesity, smoking, ultraviolet radiation and other environmental factors. Novel methods that induce cancer cell-specific apoptosis, inhibit cell proliferation and cell cycle progression, target oncogenic signaling, block tumor-associated angiogenesis and modulate tumor microenvironment are essential for cancer therapeutics to supplement the existing therapies.

Article Highlights

- One family of receptor tyrosine kinases, the Eph receptors, and their ligands, Ephrins, are implicated into carcinogenesis with evidence of the overexpression of EphA2 in pre-clinical models and clinical specimens of many different types of cancer.
- EphA2 overexpression could be valuable in the prediction of cancer outcomes and its clinical management.
- Eph-Ephrin signaling functions like the classical tyrosine kinase receptor-mediated cell signaling with forward signaling precipitated by contact between two communicating cells culminating in the binding of the receptor and the ligand in a ‘lock and key’ mechanism. In addition, reverse signaling in Ephrin-bearing cells are also generated resulting in a bidirectional signaling.
- The EphA2-EphrinA1 signaling axis regulates multiple events that are critical for the malignant transformation of normal cells including the tumor-associated angiogenesis which is required for the survival and maintenance of tumor growth.
- An elevated EphA2 expression in cancers could be due to enhanced gene expression and/or protein stability, and therefore, targeting EphA2 overexpression offers attractive candidates for cancer therapeutics.
- Various therapeutic strategies targeting EphA2 are being developed including the use of monoclonal antibodies, immunoconjugates, small molecule antagonists, adenovirus vectors, immunotherapy, RNAi-mediated EphA2 suppression, vaccination with EphA2-derived peptides and targeting by nanoparticles.
- The usefulness a combination of EphA2-directed therapies with other anticancer therapies such as chemotherapeutic drugs or molecular therapies has been evaluated in preclinical studies.

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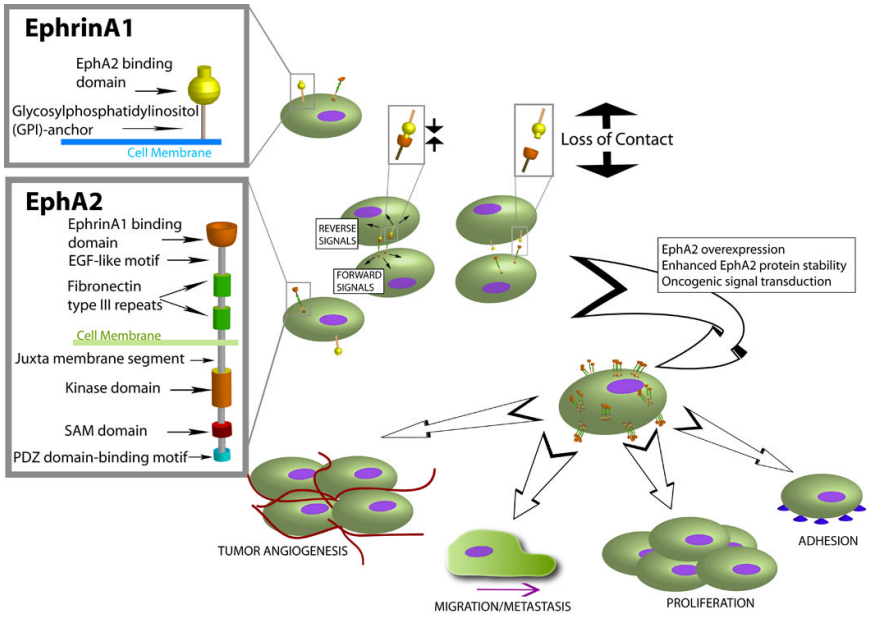

Figure 1. EphA2 and EphrinA1 signaling in cancers

EphA2 receptor tyrosine kinase and its ligand EphrinA1 belong to the Eph family of receptors. The interaction of cell membrane bound EphA2 with EphrinA1 generates forward or reverse signals in the corresponding cells. The cell-cell contact-dependent functioning of Eph receptors and Ephrins is tightly regulated during normal embryonic development and maintenance of homeostasis. However, during oncogenesis due to loss of cell contacts, the normal EphA2-EphrinA1 signaling is disrupted leading to the overexpression of EphA2 and oncogenic signal transduction. This deregulated signaling is implicated in several critical aspects of oncogenesis such as cytoskeleton modulation, cell adhesion, migration, metastasis, proliferation and angiogenesis.

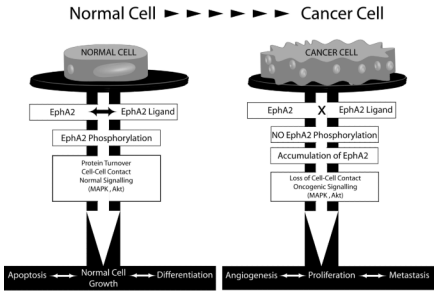

Figure 2. The role of EphA2 in the normal versus the cancer cell

EphA2 is present on the surface of a normal cell and interacts with its ligand (e.g., EphrinA1) which is present on the surface of adjacent cells. Upon interaction, EphA2 becomes phosphorylated and subsequently degraded. The phosphorylation of EphA2 is important for the normal signaling of MAPK and Akt pathways. In cancer cells, EphA2 fails to efficiently interact with its ligand on adjacent cells, leading to the accumulation of the unphosphorylated-form of EphA2 in the cell. This leads to oncogenic signaling; however, the underlining mechanism/s is/are not clear. MAPK, mitogen activated protein kinases; Akt, associated protein kinase B; ↔, interaction; X, no interaction.

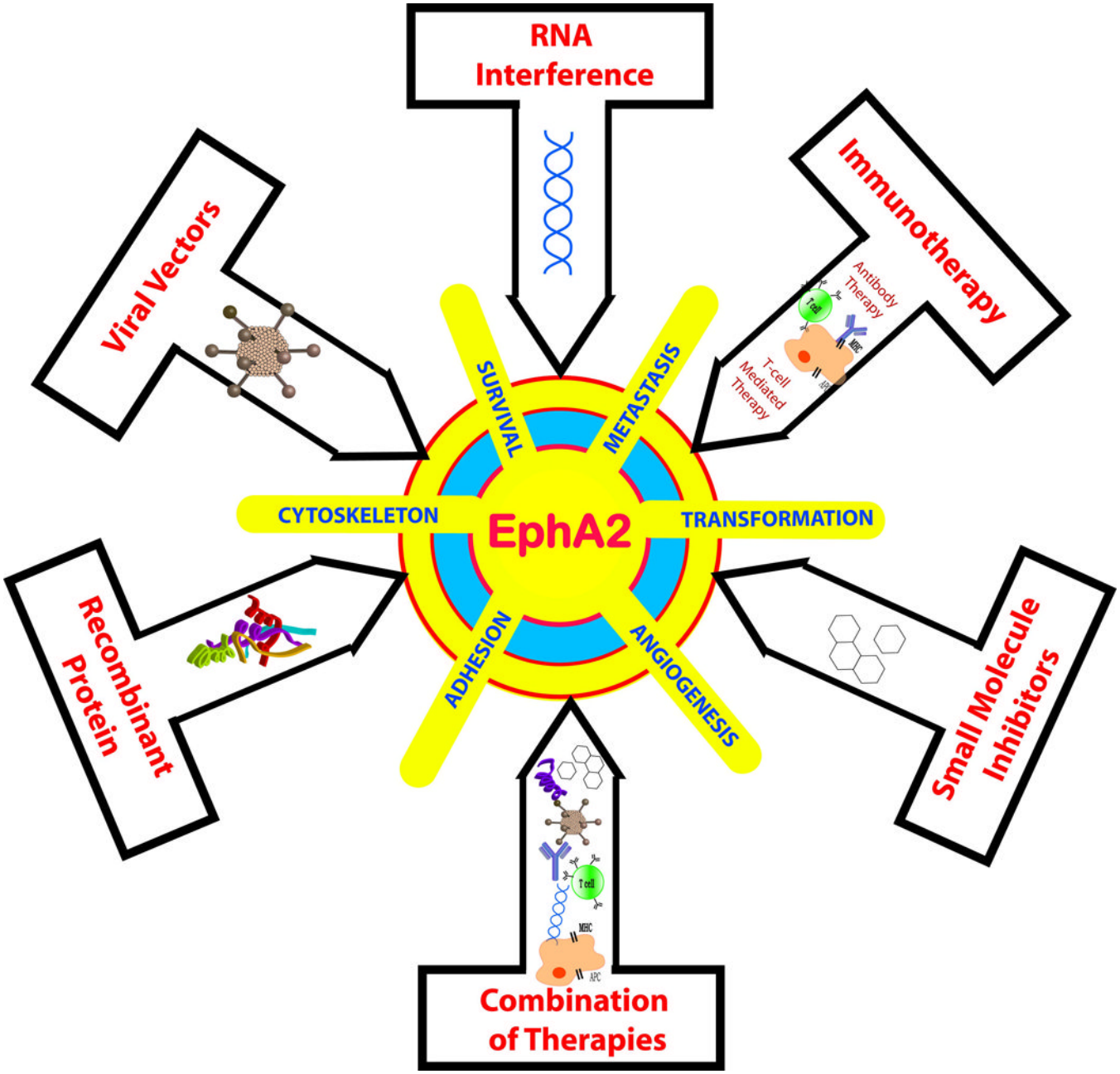

Figure 3. Targeting EphA2 in breast cancer

EphA2 receptor tyrosine kinase is reported to regulate multiple aspects of oncogenesis such as cell survival, cytoskeleton modulation, cell adhesion, tumor-associated angiogenesis and metastasis. The deregulated signaling by EphA2 receptor provides multiple avenues to design rational intervention approaches. Accordingly, EphA2 overexpression has been targeted with several approaches such as agonist antibodies, small molecule inhibitors, viral vectors, RNA-interference, cancer immunotherapy and combination of these therapeutic approaches.

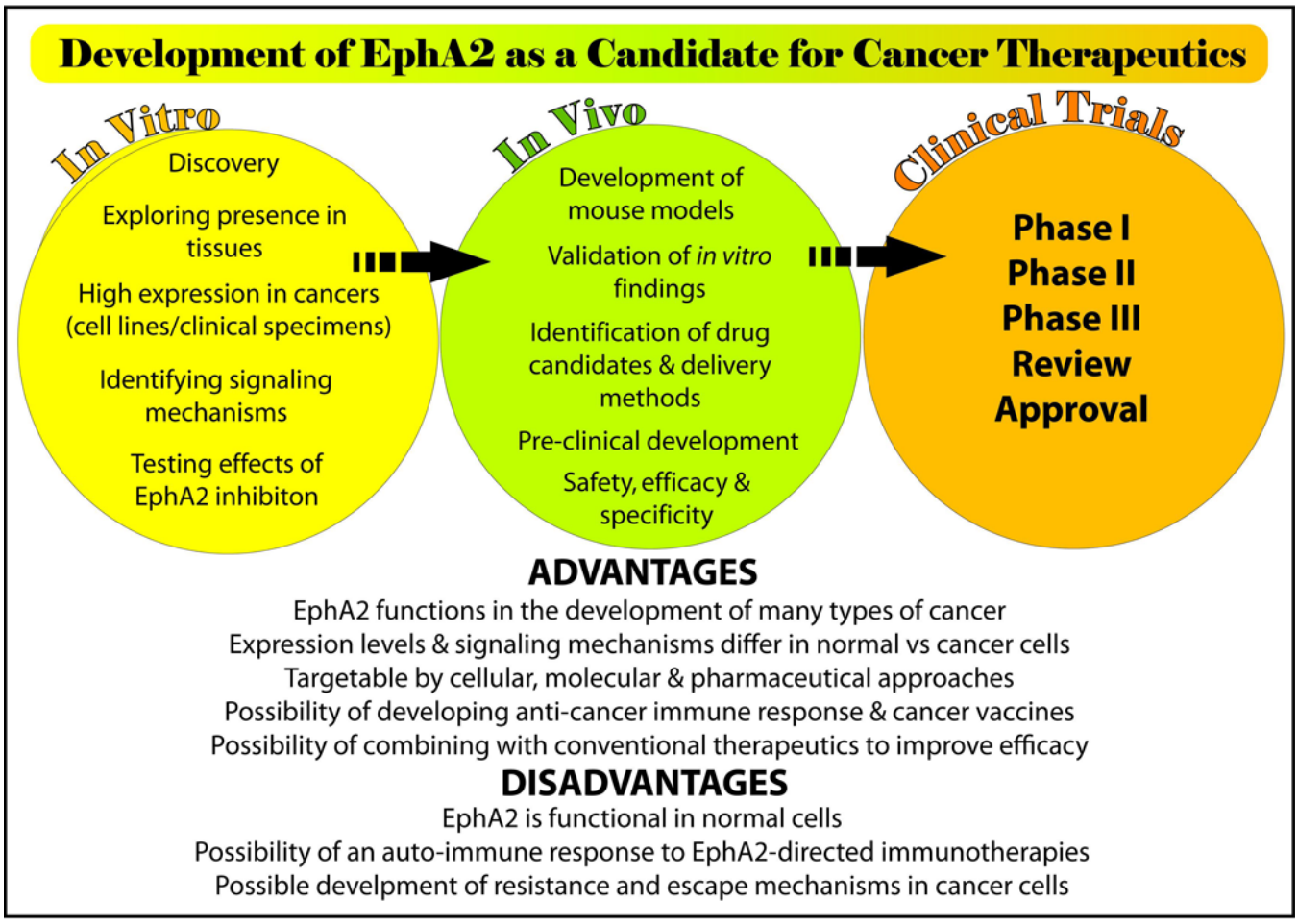

Figure 4. Simplified view of the development of EphA2 as a target for cancer therapeutics

The identification of a differential expression pattern and mechanisms of EphA2 in normal *versus* cancer cells by several *in vitro* techniques (highlighted in yellow) have led to the pre-clinical testing in mouse models of cancers (highlighted in green). After the validation of pre-clinical data and ensuring the safety of various therapeutic strategies, multiple stages of clinical trials and a review process need to be conducted (highlighted in orange) to develop EphA2-targeting anti-cancer therapeutics.

Table 1

Characterization of EphA2 expression levels *in vitro* and *in vivo* study models

ORGAN	EphA2 Expression Levels			REFERENCE
	LOW	MEDIUM	HIGH	
BREAST	MCF-10A, MCF-12A, MCF-10A-2 (non-transformed mammary epithelium)	MDA-436 (transformed mammary epithelium)	Hs5786, MDA-435, MDA-MB-231, BT549 (transformed mammary epithelium), MT1A2 (mouse adenocarcinoma)	[35,127]
BRAIN	Normal brain (frontal lobe), H4 (non-tumorigenic malignant glia cell line)	U-87 MG (Gliobastoma cell line)	A172MG, DBTRG-5MG, U-251MG (human glioblsatoma multiformis / GBM cell lines), G48a (primary GBM explants cell culture)	[58,62,154]
OVARY	HIO-180 (non-transformed ovarian epithelium), A2780 PAR (ovarian cancer)		SKOV3, EG, 222, HeyA8 (ovarian cancer)	[48,52]
URINARY BLADDER	RT4 (low grade papillary tumor cell line)	HT1376 (high grade invasive bladder cancer)	T24, TCCSUP, UMUC-3 (high grade invasive bladder cancer)	[63]
PROSTATE	BC1, TR5P (canine prostate cancer), LNCaP (human prostate cancer)	DU-145 (human prostate cancer)	TR6LM, BF2, CF3, GN4 (lung or bone metastasis-derived canine prostate cancer cells); PC3 (human prostate cancer)	[64,155]
PANCREAS	Capan-2 (pancreatic adenocarcinoma)	BxPC3 (pancreatic adenocarcinoma)	PANC1, MIAPaCa2 (pancreatic adenocarcinoma)	[134]
ESOPHAGUS	TT, CKEk1 (immortalized human esophageal cells)	TE2, TE8, TE13, TE15, TTn (human esophageal cancer)	TE1 (human esophageal cancer)	[65]
LUNG	Pleural mesothelium		MMC-1, MMC-2, MMC-3 (malignant mesothelium)	[54,140]
STOMACH			AGS, SGC-7901 (human gastric adenocarcinoma cells)	[156,157]