Eph receptors as therapeutic targets in glioblastoma

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The dismal outlook for patients with the most aggressive and common form of adult brain cancer, glioblastoma (GBM), motivates a search for new therapeutic strategies and targets for this aggressive disease. Here we review the findings to date on the role of Eph family receptor tyrosine kinases and their ephrin ligands in brain cancer. Expression of the Eph family of cell surface proteins is generally downregulated to very low levels in normal adult tissues making them particularly attractive for directed therapeutic targeting. Recent Eph targeting studies in pre-clinical models of GBM have been very encouraging and may provide an avenue to treat these highly refractory aggressive tumours.

Eph receptor primary function occurs during early development, where these proteins regulate many cellular processes including adhesive and repulsive mechanisms, mediated by the ability of Eph receptor signalling pathways to regulate key molecules involved in cell motility and adhesion. Although most extensively characterised for their roles in development, Eph receptors and ephrin ligands are re-expressed in a variety of diseases including a number of human malignancies (Pasquale, 2010). Dysregulated cellular de-adhesion and abnormal cytoskeletal functions affecting cell shape and motility are key features of advanced cancers. Eph and ephrins regulate many of these key cellular processes, enabling them to promote tumour invasion and metastasis. The challenge of identifying new targeted therapies with efficacy in GBM is immense. GBMs are the most common malignant primary adult brain cancer and are typically highly aggressive, infiltrative and resistant to conventional therapies. At present treatment involves surgical resection, post-operative radiation and temozolomide chemotherapy (Wen and Kesari, 2008). These treatments are rarely curative, median survival is <15 months with only about 10% of patients surviving for two years without disease recurrence (Stupp et al, 2005).

**EPH AND EPHRIN EXPRESSION AND FUNCTION IN GBM**

**Introduction.** Numerous studies have analysed Eph receptor expression in GBM in seeking to shed light on how this family of receptors might function to promote malignant progression of this disease (Table 1). The accumulated data have led to a better understanding of Eph and ephrin expression levels; despite these efforts very little has been elucidated regarding Eph/ephrin function in GBM. In addition many of these studies were performed prior to recent seminal advances in the neuro-oncology field, defining GBM tumour heterogeneity, molecular subtypes and the identification of putative glioma stem cells (GSCs), which are now thought to be responsible for tumour initiation and recurrence (Vescovi et al, 2006; Verhaak et al, 2010; Brennan et al, 2013). Many of these studies have been performed using long established, serum grown, immortalised cell lines that we now know are no longer representations of the original tumour. In part due to attempts to overcome these limitations, recent larger studies conducted by ourselves and others have started to define Eph function in GBM, with encouraging results.

**EphA/ephrin-A family members.** Eph receptors have been shown to have somewhat perplexing dichotomous roles with both tumour suppressor and tumour promoting functions being described. This phenomenon stems from the fact that Eph–ephrin complexes can initiate both ‘forward’ Eph-mediated and ‘reverse’ ephrin-mediated signalling cascades while receptor-ligand interactions may occur both in cis and in trans with opposing outcomes. Although there are specific examples that run counter to this argument, by and large it appears that EphA receptors, while being highly expressed in many cancers, are relatively kinase-inactive due to either kinase inactivating mutations or low ligand expression (Pasquale, 2010). Indeed, kinase-activated forward signalling tends to inhibit
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Table 1. Summary of Eph & ephrin expression, clinical outcome and function in GBM

| EphA/ephrin-A | Expression | Clinical outcome | Mechanism | Reference |
|---------------|------------|-----------------|-----------|-----------|
| EphA2 | ↑ MES/CLAS subtypes | Poorer outcome | ↑ On GSCs/negatively regulates MAPK PW/ligand dependent and independent roles/effects proliferation, invasion and neovascularization/loss induces differentiation/Akt Phos EphA2 at S897/targeting shows proven anti-tumour responses | Binda et al, 2012; Liu et al, 2006; Wykosky et al, 2005, 2007, 2008a, 2008b; Liu et al, 2007; Hatano et al, 2005; Wang et al, 2008a; Li et al, 2007, 2010; Miao et al, 2009 |
| EphA3 | ↑ MES/CLAS subtype | Poorer outcome | ↑ On GSCs/negatively regulates MAPK PW/proven ligand independent roles/effects proliferation/loss induces differentiation/targeting shows proven anti-tumour responses/functional mutants identified | Day et al, 2013; Wykosky et al, 2007 |
| EphA4 | ↑ | Poorer outcome | ↑ In U251 cells affects proliferation and invasion through EphA4-FGFR1 signalling PW | Fukai et al, 2008, Wykosky et al, 2007 |
| EphA5 | Detectable | Better outcome | No proven effect on proliferation in U118 cells/expressed in dormant GBM/downregulated as tumour advances/may have anti-angiogenic roles | Bruce et al, 1999; Almog et al, 2009; Wykosky et al, 2007 |
| EphA7 | ↑ | Poorer outcome | Correlation with microvascular density | Wang et al, 2008b |
| EphA8 | ↓ | Poorer outcome | Induces neuronal-like differentiation in rat glioma cells by inducing sustained MAPK activation | Gu et al, 2007 |
| Ephrin-A1 | ↓ | Poorer outcome | Negative regulator of EphA2/forced expression attenuates adhesion, migration and proliferation | Hatano et al, 2005; Liu et al, 2007 |
| Ephrin-A5 | ↓ | Tumour suppressive role via negative regulation of EGFR/ downregulated in glioma | Li et al, 2009 |

| EphB/ephrin-B | Expression | Clinical outcome | Mechanism | Reference |
|---------------|------------|-----------------|-----------|-----------|
| EphB2 | ↑ | Poorer outcome | Dichotomous role suggested where ↑ EphB2 increases migration and invasion while decreases proliferation/function mediated via R-Ras and FAK signalling/targeted by miR-204/↑ during GBM differentiation | Nakada et al, 2004, 2005; Wang et al, 2012; Ying et al, 2013 |
| EphB4 | ↑ | Poorer outcome | Promotes angiogenesis (venous) via the DLL-4-Notch signalling PW/co-expressed with ephrin-B2/ ↑ EphB4 correlates with increasing tumour grade/activates EGFR signalling to promote growth/mutated at low frequency/ ↑ on less-differentiated cells | Tu et al, 2012; Li et al, 2011; Li et al, 2012; Xiao et al, 2004; Chen et al, 2013; Erber et al, 2006 |
| EphB6v | ↑ | Poorer outcome | EphB6 variant lacking transmembrane and cytoplasmic domains/↑ in GBM cell lines/two unique antigenic peptide sequences of EphB6v were recognised by CTL in a HLA-A24 restricted manner/potential targets for immunotherapy | Jin et al, 2008 |
| Ephrin-B1 | ↑ | Poorer outcome | Expression promotes invasion in U87-MG cells | Nakada et al, 2010 |
| Ephrin-B2 | ↑ | Poorer outcome | ↑ ephrin-B2 correlates with increasing tumour grade/co-expressed with EphB4/regulated tumour angiogenesis (arterial) via VEGFR regulation/phosphorylation promotes cell migration and invasion/↑ on less-differentiated cells | Tu et al, 2012; Nakada et al, 2010; Sawamiphak et al, 2010; Li et al, 2011; Li et al, 2012; Xiao et al, 2004; Erber et al, 2006 |
| Ephrin-B3 | ↑ | Poorer outcome | Promotes cell invasion through activation of Rac1 | Nakada et al, 2006, 2010 |

Abbreviations: ↑ = upregulated; ↓ = downregulated; Akt = protein kinase B; CLAS = classical subtype; CTL = cytotoxic T-lymphocyte; DLL = delta-like ligand; EGFR = epidermal growth factor receptor; FAK = focal adhesion kinase; FGFR = fibroblast growth factor receptor; GSC = glioma stem cell; HLA = human leukocyte antigen; MAPK = mitogen-activated protein kinase; MES = mesenchymal subtype; miR = microRNA; Notch = neural-crest notch homologous protein; Phos = phosphorylation; PW = pathway; R-Ras = ras-related protein; Rac = ras-related C3 botulinum toxin substrate; VEGFR = vascular endothelial growth factor receptor.

proliferation and cell migration leading to decreased invasiveness of cancer cells. This idea appears to hold true in the case of GBM, recent findings suggest that EphA receptors are expressed in a gradient with high expression present on the more aggressive GSC mesenchymal phenotype. Conversely elevated ephrin-A expression correlates with a less-aggressive more-differentiated phenotype with a better patient prognosis (Figure 1). This has been well demonstrated for both EphA2 and EphA3 and their high affinity ligands ephrin-A1 and ephrin-A5, respectively (Wykosky et al, 2005; Liu et al, 2007; Wykosky et al, 2008b; Li et al, 2009, 2010; Binda et al, 2012; Day et al, 2013). The most widely studied EphA receptor to date has been EphA2 that has been shown to be highly expressed in GBM specimens but not in normal brain (Wykosky et al, 2005). EphA2 has also been found to be highly expressed on the tumour vasculature in GBM suggesting a role in neovascularisation (Wu et al, 2011). Moreover increased EphA2 expression has been correlated with pathological grade, proliferation and apoptosis in astrocytic brain tumours (Li et al, 2007). EphA2 overexpression has been shown to inversely correlate with GBM patient survival (Liu et al, 2006; Wang et al, 2008a). The high affinity EphA2 ligand, ephrin-A1, is lowly expressed in GBM especially in areas of EphA2 positivity (Hatano et al, 2005; Liu et al, 2007). Overexpression of ephrin-A1 was shown to downregulate both EphA2 and focal adhesion kinase (FAK).
leading to reduced migration, adhesion and proliferation of GBM cells (Liu et al., 2007). The preferred ligand for EphA3, ephrin-A5, has also been reported to be expressed at low levels in GBM and has been proposed as a tumour suppressor through its negative regulation of EGFR. Evidence shows a mutually exclusive expression pattern of ephrin-A5 and EGFR. It was shown that ephrin-A5 enhanced c-Cbl binding to EGFR, thus promoting ubiquitination and degradation of the receptor (Li et al., 2009).

Recent studies of EphA2 and EphA3 in GBM have shown that both receptors are expressed most highly on the cells responsible for tumour formation and were also found to be co-expressed with other reported GSC markers such as CD133 and integrin α6 (Binda et al., 2012; Day et al., 2013). In addition, these studies, using the TCGA database, showed differential expression of both EphA2 and EphA3 in molecular subtypes of GBM. Greatest expression was present in the most aggressive, de-differentiated, stem cell-like mesenchymal subtype, although elevated expression was also detected in the EGFR-driven classical subtype. These receptors, despite being relatively kinase-inactive in GBM, are not silent passengers, but through kinase-independent signalling, contribute to the malignant process. Functionally it appears that this is in part mediated through cross talk with growth factor receptors and adhesion molecules, such as integrins and cadherins, to promote angiogenesis, tumour cell proliferation and to support formation of the tumour microenvironment. Both EphA2 and EphA3 were shown to actively maintain GBM cells in a stem–like state by negatively regulating the MAPK pathway. Sustained ERK1/2 signalling was observed following knockdown of either EphA2 or EphA3, leading to increased GBM cell differentiation and reduced tumorigenic potential in GBM xenograft models (Binda et al., 2012; Day et al., 2013). These findings were at first counterintuitive given that the differentially EphA2/A3-low GBM cells proliferated significantly slower than in EphA2/A3-high GSCs, in which the MAPK pathway was only transiently activated. However, these findings fit with the observations of others showing that sustained MAPK activation mediated via the EphA8 receptor also led to neuronal differentiation (Gu et al., 2005). This idea, that transient versus sustained MAPK signalling can drive either proliferation or differentiation is not new (Marshall, 1995). It appears that the outcome of either proliferation or differentiation depends highly upon the duration of ERK activation and cell context. In the context of neuronal cells and GBM, receptor tyrosine kinases such as EphA2 and EphA3 appear to act as switches to regulate the duration of ERK activation and subsequent ability of these cells to undergo differentiation. Cues from the microenvironment and proximity to niche may also be critical in this process; EphA receptor expression diminishes as tumour cells expanded away from the vascular niche.

**Figure 1.** Model of EphA/ephrin-A expression in GBM. A model has emerged suggesting that EphA receptors and ephrin-A ligands may exist within an expression gradient in GBM. EphA receptor expressing cells are poorly activated due to low ligand expression and take on a more de-differentiated mesenchymal-like GSC phenotype, whereas elevated ephrin-A expression led to a less-aggressive more-differentiated epithelial phenotype. Functionally, EphA receptors appear to maintain this GSC phenotype by limiting the duration of MAPK signalling. Cues from the microenvironment and proximity to niche may also be critical in this process; EphA receptor expression diminishes as tumour cells expanded away from the vascular niche.

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EphA5 was also shown to be overexpressed in some astrocytomas and GBM, although functional activation of the receptor was not found to promote cell proliferation in the GBM cell line U118 (Bruce et al., 1999). Another study found loss of EphA5 in plasma correlated with increased tumour grade in glioma patients, and that this protein could be used as a novel biomarker in GBM (Almog et al., 2009). EphA7 has also been reported to be highly expressed in GBM and was a predictor of poor clinical outcome (Wang et al., 2008b). Interestingly in the absence of ligand activation, EphA8 induced neuronal differentiation in rat glioma cells by inducing sustained MAPK activation (Gu et al., 2005).

**EphB/ephrin-B family members.** EphB/ephrin-B signalling appears to differ from that of EphA/ephrin-A proteins. Indeed the function of these receptors is less well characterised in GBM, although it is clear that they do have functional roles in GBM cell migration, invasion and tumour angiogenesis. High levels of EphB2 have been observed in human glioma cells and strong kinase activation of EphB2 has been reported, specifically in migrating U87 GBM cells. Consistent with a role in glioma invasiveness, high levels of EphB2 reduced adhesion and increased GBM cell migration and invasion in vitro and using an ex vivo rat brain slice invasion assay (Nakada et al., 2004). Furthermore this effect was shown to be regulated by R-Ras, a small GTPase implicated as a signal mediator of Eph receptors. EphB2 decreased extracellular matrix adhesion through activation of R-Ras signalling (Nakada et al., 2005). A recent study has shown that miR-204 suppressed both glioma cell migration and reduced stem cell self-renewal and stem cell-like features, interestingly miR-204 was shown to target the stemness-inducing transcription factor SOX4 and EphB2 (Ying et al., 2013). These invasive effects are not only EphB-mediated, but also appear to be induced by ephrin ligands. Similarly, the EphB ligand, ephrin-B3 is also overexpressed in GBM cell lines and promotes cell migration and invasion via a Rac1-mediated effect (Nakada et al., 2006). Rac1, a small Rho GTPase, has roles in cytoskeletal organisation and plasticity in glioma migration and invasion (Chuang et al., 2004). Furthermore Nakada et al (2010) found that forced expression of ephrin-B2 enhanced migration and invasion and that high ephrin-B2 expression was a strong predictor of shorter survival. A recent finding highlighted an interesting dichotomous role of EphB2 in GBM (Wang et al., 2012). This evidence suggests that although elevated EphB2 increased migration and invasion, it also decreased proliferation in GBM stem-like neurospheres. This was further confirmed by EphB2 silencing, which conversely increased proliferation and decreased migration. The pro-migratory and anti-proliferative effects of EphB2 in GBM are intriguing: this behaviour was documented in GBM cells almost two decades ago showing that proliferation and migration are temporarily mutually exclusive behaviours (Giese et al., 1996). FAK appeared to be critical in this process and was heavily localised in the membrane of non-migrating cells and absent in migrating cells. FAK acts downstream of EphB2 and is a pivotal mediator of EphB2 induced cell migration. FAK and EphB2 have been shown to be co-localised in GBM and FAK pathway inhibitors prevented migration of EphB2 overexpressing cells. Although EphB2 has emerged from...
these studies as a candidate for therapeutic strategies to prevent GBM tumour invasion, one caveat would be that inhibiting EphB2 signalling may also increase GBM cell proliferation.

EphB4 has been shown to be expressed in endothelial cells of embryonic veins while ephrin-B2 is expressed in endothelial cells of arteries, in this instance acting in the guidance and formation of these tissues (Wang et al, 1998). It is not surprising then that EphB4/ephrin-B2 expression has been detected in GBM and linked to tumour angiogenesis (Xiao et al, 2004; Erber et al, 2006; Chen et al, 2013). EphB4 has been shown to promote venous angiogenesis via the DLL-4-Notch signalling pathway (Li et al, 2011, 2012), whereas ephrin-B2 promotes arterial angiogenesis through regulation of VEGFR2 function (Sawamiphak et al, 2010). Expression of these proteins has also been positively correlated with GBM progression and poor prognosis (Tu et al, 2012).

**Eph receptors as therapeutic targets in GBM**

**Potential Eph receptor targeting strategies.** Several potential therapeutic strategies have emerged to target Eph receptors in cancer (Figure 2). Some approaches aim to activate Eph kinase function through ligand stimulation, activating antibodies or ligand peptide mimetics strategies to deliver toxic payloads following receptor activation and internalisation include coupling of cytotoxic agents or radionuclides to Eph monoclonal antibodies.

![Figure 2. Potential therapeutic strategies to target Eph receptors in GBM. Several approaches exist to target Eph receptors. Kinase inactivating strategies include kinase inhibitors or blocking peptides or antibodies. Kinase-activating strategies include ligand stimulation, activating antibodies or ligand peptide mimetics strategies to deliver toxic payloads following receptor activation and internalisation include coupling of cytotoxic agents or radionuclides to Eph monoclonal antibodies.](image-url)
ligand peptide mimetics, these strategies aim to take advantage of Eph forward signalling to induce tumour suppressive functions. In situations where Eph activation leads to tumour promotion, strategies that block active kinase signalling are sought. These typically include kinase inhibitors or the use of ephrin-blocking peptides or antibodies. It is now well established that Eph receptor activation typically leads to rapid internalisation and degradation of receptor complexes. For clinical application protein targets with rapid turnover can be exploited to deliver cytotoxic payloads using antibody drug conjugates (ADCs). The most commonly used ADCs include Maytansine (USAN) and monomethyl auristatin E (MMAE) also known as Vedotin, these are potent antimitotic agents, which inhibit cell division by binding to tubulin and blocking microtubule assembly. Another effective approach is to couple monoclonal antibodies (mAbs) to a radionuclide, which delivers a lethal dose of radiation. Radio-immunotherapies are attractive for a number of reasons: they induce potent killing in aggressive cancers such as GBM and, depending upon the half-life and radiation spectra of the radionuclide chosen, can induce significant ‘bystander’ effects killing adjacent tumour cells and tumour stromal cells (for a detailed review of Eph receptors as therapeutic targets see (Boyd et al. 2013)).

Proven pre-clinical successes. EphA2 has been effectively targeted in GBM animal models using strategies which rely on receptor activation. Studies using treatment with the preferred EphA2 ligand, ephrin-A1 showed that both unlabelled ephrin-A1-Fc (Binda et al., 2012), and ephrin-A1 coupled to the pseudomonas aeruginosa exotoxin (Wykosky et al., 2007, 2008a) were effective. Our recent studies have also shown that targeting the EphA3 receptor using an EphA3 mAb with activating properties coupled to the beta-emitting radionuclide lutetium (\(^{177}\)Lu) was an effective strategy to target both subcutaneous and orthotopic GBM tumour-bearing animals with minimal toxicity (Day et al., 2013). Importantly, this antibody binds with equal affinity to both mouse and human EphA3 and no toxicity in normal tissues was observed.

EphA2 has also been investigated as a possible target for novel immunotherapeutic vaccines. A vaccine comprising autologous dendritic cells loaded with a synthetic EphA2 peptide was shown to induce an interferon response and specific cytotoxic T-lymphocyte (CTL) responses in glioma cell lines, highlighting the potential of molecularly targeted glioma vaccines (Hatano et al., 2005). Studies in melanoma patients have shown that EphA3 may be a source of tumour-specific antigens recognised on tumour cells that express HLA class II molecules (Chiari et al., 2000). A variant of EphB6 (EphB6v) was shown to be overexpressed in a panel of brain tumour cell lines (Jin et al., 2008). Interestingly EphB6v has a unique C-terminal 54 amino-acid sequence. Jin et al. (2008) generated two antigenic peptides to this region for the purpose of developing an immunotherapeutic agent. The two EphB6v-derived peptides bound HLA-A0201 molecules and were shown to induce CTLs in vitro in the peripheral blood mononuclear cells of HLA-A2+ glioma patients.

Directions for the future. Other EphA receptors such as EphA7, which has been shown to be a predictor of poor clinical outcome in GBM, may also hold significant potential. EphA7 has been shown to be ubiquitously expressed on quiescent ependymal cells and was co-expressed with nestin, a marker associated with neural stem cells (Holmberg et al., 2005). Holmberg et al. (2005) describe both ephrin-A2 and EphA7 as negative regulators of neural progenitor cell differentiation. These findings suggest a potential role of EphA7 in promoting GSCs. The discrete expression of EphA7 on ependymal cells is also of interest and may be significant in the maintenance of ependymomas. Eph receptors may also have a functional role in other brain cancers such as medulloblastoma. Only a handful of studies to date have reported Eph receptor expression in these predominantly paediatric tumours, such as a recent meta-analysis which identified EphA3 as a potential therapeutic target (Haeberle et al., 2012).

Eph receptors are often expressed on migrating tumour cells especially at the leading edge where GBM cells are actively invading into the brain parenchyma. This observation has led to the investigation of Eph mAbs as potential imaging agents, which might accurately delineate tumour borders and better define areas of active invasion allowing more complete resection and better patient outcomes (Cai et al., 2007). mAbs against EphB2 are an obvious candidate given the strong link between this receptor and actively migrating GBM cells. Enhanced imaging agents are at present an unmet clinical need in neuro-oncology, this area of Eph related research is still in its infancy and may hold significant potential for the future.

In summary, recent advances in our understanding of Eph receptor biology in GBM has unleashed Eph receptors as genuine therapeutic targets in this disease. This has now been validated
in pre-clinical studies and early phase clinical testing appears imminent.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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