SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

Deciphering the Signaling Events that Promote Melanoma Tumor Cell Vasculogenic Mimicry and Their Link to Embryonic Vasculogenesis: Role of the Eph Receptors

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During embryogenesis, the primordial microcirculation is formed through a process known as vasculogenesis. The term "vasculogenic mimicry" has been used to describe the manner in which highly aggressive, but not poorly aggressive melanoma tumor cells express endothelial and epithelial markers and form vasculogenic-like networks similar to embryonic vasculogenesis. Vasculogenic mimicry is one example of the remarkable plasticity demonstrated by aggressive melanoma cells and suggests that these cells have acquired an embryonic-like phenotype. Since the initial discovery of tumor cell vasculogenic mimicry by our laboratory, we have been focusing on understanding the molecular mechanisms that regulate this process. This review will highlight recent findings identifying key signal transduction events that regulate melanoma vasculogenic mimicry and their similarity to the signal transduction events responsible for promoting embryonic vasculogenesis and angiogenesis. Specifically, this review will focus on the role of the Eph receptors and ligands in embryonic vasculogenesis, angiogenesis, and vasculogenic mimicry. *Developmental Dynamics 236:3283–3296, 2007.* © 2007 Wiley-Liss, Inc.

Key words: EphA2; vasculogenic mimicry; melanoma; VE-cadherin; metastasis

Accepted 16 April 2007

INTRODUCTION

During embryonic development the primordial microcirculation is formed through a process known as vasculogenesis (reviewed in Conway et al., 2001). Embryonic vasculogenesis involves the differentiation of endothelial precursors, known as angioblasts, which then coalesce to form a primitive microcirculation, often resembling a "honeycomb-like network" of tubular structures that are homogenous in size and length. This primitive microcirculation is then remodeled through the process of angiogenesis, which involves the sprouting of new endothelial-lined vessels from pre-existing vessels. This remodeling process results in the formation of endothelial-lined vessels of varying sizes that eventually give rise to the adult vasculature. The factors that regulate the process of vasculogenesis and angiogenesis in the developing embryo are the result of coordinated signal transduction events in addition to physiological factors such as shear stress and hemodynamics occurring within the developing embryo (reviewed in Jones et al., 2006). Angiogenesis continues to occur throughout adulthood during periods of wound healing or tissue repair. Additionally, pathological forms of angiogenesis can occur such as that which takes place during tumor formation.

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Grant sponsor: Melanoma Research Foundation; Grant sponsor: US National Institutes of Health (NIH); Grant numbers: CA59702 and CA80318.
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DOI 10.1002/dvdy.21190
Published online 6 June 2007 in Wiley InterScience (www.interscience.wiley.com).

Several years ago, we described a unique characteristic of highly aggressive melanoma tumor cells and coined the term vasculogenic mimicry (VM) (reviewed in Hendrix et al., 2003; Zhang et al., 2007). VM describes the ability of highly aggressive melanoma tumor cells, but not poorly aggressive melanoma tumor cells, to express multiple cellular phenotypes, including endothelial and epithelial associated markers, and to form a vasculogenic-like network of matrix-rich patterns when cultured on a three-dimensional matrix in vitro in a manner similar to the process of embryonic vasculogenesis (Maniotis et al., 1999). Remarkably, the formation of these structures in vitro recapitulates matrix-rich patterned networks found in patients' melanoma tissues correlating with an increased risk of melanoma metastasis resulting in a poor clinical outcome (Folberg et al., 1993). Further studies revealed that the networks formed by aggressive melanoma tumor cells were capable of conducting fluorescent dyes in vitro (Maniotis et al., 1999). Additionally, it was established that these extravascular networks physiologically connected with the mouse vasculature in cutaneous melanoma xenografts (Ruf et al., 2003). Together, these data suggested that this primitive microcirculation may act both as a complimentary means of tumor perfusion as well as an additional conduit for metastasis. In recent years, VM has been reported in a number of different tumor types including breast, prostate, ovarian, Ewing sarcoma, and lung carcinoma (Kobayashi et al., 2002; Liu et al., 2002; Passalidou et al., 2002; Sharma et al., 2002; Shirakawa et al., 2002; Sood et al., 2002; van der Schaft et al., 2005). VM is one example of the plasticity of aggressive tumor cells and suggests a reversion to a more primitive embryonic phenotype.

To better understand the process of VM, our laboratory has been investigating the molecular mechanisms underlying this newly characterized form of tumor neovascularization as an attempt to identify new therapeutic targets, particularly for aggressive melanoma. We have focused our attention on the signal transduction mechanisms that promote VM and to date have identified various roles for EphA2, vascular endothelial cadherin (VE-cadherin), focal adhesion kinase (FAK), phosphoinositide 3-kinase (PI3K), and extracellular regulated kinase 1 and 2 (Erk1/2) in regulating the process of melanoma VM as well as melanoma aggressiveness in general (Hendrix et al., 2001; Hess et al., 2001, 2003, 2005, 2006). Interestingly, all of these signaling molecules play a role in embryonic vasculogenesis as well as adult angiogenesis, particularly with respect to the Eph receptors. Therefore, the goal of this review is to understand the link between the signal transduction mechanisms that regulate embryonic vasculogenesis and angiogenesis with that of melanoma vasculogenic mimicry, with a particular focus on the role of the Eph receptors and their downstream effectors.

EPH RECEPTOR SIGNALING DURING EMBRYONIC VASCULOGENESIS

The Eph family of receptor tyrosine kinases (RTKs) is the largest family of RTKs, consisting of 14 members. The receptors are divided into two classes, EphA and EphB, based on the homology of their extracellular domain (Eph Nomenclature Committee, 1997). The ligands for this family are also membrane bound and/or secreted (termed the ephrins) and are divided into two classes based on their membrane linkage: class A contains ligands that are bound by glycosylphosphatidylinositol (GPI) linkage, while the B class of ligands contains members that possess both transmembrane and cytoplasmic regions (Eph Nomenclature Committee, 1997). Receptor/ligand engagement results in phosphorylation of the receptor on tyrosine residues, thus resulting in an induction of the receptor's kinase activity. Additionally, it has been noted that several receptor/ligand pairs, particularly within the EphB/ephrin-B subclass, are capable of bi-directional signaling. The Eph family of receptors and ligands has been found to play key roles during development where they regulate various processes such as directing axonal (Henkemeyer et al., 1996; Orioli et al., 1996; Park et al., 1997; Wang and Anderson, 1997) and neural crest cell migrations (Krull et al., 1997; Smith et al., 1997; Wang and Anderson 1997), regulating axonal bundling (Orioli et al., 1996; Winslow et al., 1995), preventing mixing of different cell populations during embryogenesis (Mellitzer et al., 1999), and mediating angiogenesis (Adams et al., 1999; Brantley-Sieders and Chen, 2004; Brantley-Sieders et al., 2004, 2005; Chen et al., 2006; Cheng et al., 2002; Gale et al., 2001; Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998; Zhong et al., 2001).

Within the last decade, several reports of Eph RTKs and ephrins have indicated that these molecules play key roles during embryonic vasculogenesis, specifically, the EphB2, EphB3, and EphB4 receptors along with their respective ligands, ephrin-B1 and ephrin-B2. Studies have shown that ephrin-B2 marks endothelial cells destined to form the lining of arteries, whereas its principle receptor EphB4 marks endothelial cells destined to form the lining of veins (Adams et al., 1999; Gerety et al., 1999; Shin et al., 2001; Wang et al., 1998). The importance of these proteins in embryonic vasculogenesis is highlighted in homozygous knockout mice of either ephrin-B2 or EphB4, both of which result in embryonic lethality due to a failure in remodeling of the capillary plexus into functional veins and arteries (Gerety et al., 1999; Wang et al., 1998). The remarkable finding that homozygous knockouts of either ephrin-B2 or EphB4 resulted in essentially the same phenotypical outcome is indicative of bi-directional signaling between this ligand and receptor pair. Moreover, it has been demonstrated that the expression of both ephrin-B2 and EphB4 can persist throughout adulthood. Shin and colleagues utlized ephrin-B2$^{taulacZ/+}$ indicator mice to provide evidence suggesting that ephrin-B2 can mediate various processes of adult angiogenesis including tumor neovascularization (Shin et al., 2001).

In addition to the expression of EphB4 and ephrin-B2 during embryonic vasculogenesis and angiogenesis, it has been demonstrated that EphB2, EphB3, and ephrin-B1 are also expressed in the embryo and play a role during embryonic vasculogenesis and angiogenesis (Adams et al., 1999). In-

terestingly when the phenotype of double *ephB2/ephB3* knockout mice was compared with the phenotype of *ephrin-B2* knockout mice, striking similarities in vascular defects were found (Adams et al., 1999). Given that EphB2 was found to be expressed within the embryonic mesenchyme whereas EphB3 was found to be expressed in endothelial cells (Adams et al., 1999), it was suggested that the communication between the embryonic mesenchyme and endothelial cells is critical for embryonic vasculogenesis and angiogenesis and that this communication is in part mediated by the Eph receptors and ligands.

Although the role of EphB2, EphB3, EphB4, ephrin-B1, and ephrin-B2 in mediating embryonic vasculogenesis is clear, the role of EphA2 and its principle ligand ephrin-A1 remains to be determined. Early studies reported the presence of ephrin-A1 in areas of embryonic vasculogenesis and angiogenesis, suggesting a role for ephrin-A1 in this process; however, EphA2 has not been found to be expressed during embryonic vasculogenesis or angiogenesis, and in fact EphA2 knock-out mice are viable (Brantley-Sieders et al., 2004; Flenniken et al., 1996; McBride and Ruiz 1998). Despite a lack of evidence connecting EphA2 and ephrin-A1 with embryonic vasculogenesis, there is a significant amount of evidence for this Eph receptor/ligand pair in adult angiogenesis, which will be the focus of the next section.

THE ROLE OF EPHA2 AND EPHRIN-A1 IN ADULT ANGIOGENESIS

Angiogenesis, which involves the formation of a new endothelial-lined vasculature from a preexisting one, occurs throughout adulthood under both normal and pathological conditions. One of the first indications implementing EphA2 and ephrin-A1 as mediators of angiogenesis was reported by Pandey and colleagues who demonstrated that TNF-α could upregulate the expression of ephrin-A1 in human umbilical vein endothelial cells (HUVECs), resulting in EphA2 phosphorylation and stimulation of angiogenesis in vivo as well as endothelial cell chemotaxis in vitro (Pandey et al., 1995). Since those initial observations, there have been many published reports aiming to address the role of both EphA2 and ephrin-A1 in mediating angiogenesis.

Using various models for angiogenesis, it has been demonstrated that addition of soluble EphA2 can significantly inhibit ephrin-A1 and VEGF induced angiogensis in vivo (Chen et al., 2006; Cheng et al., 2002). Interestingly, addition of soluble EphA2 had no effect on bFGF-stimulated angiogenesis, suggesting that EphA2 signaling is specific to ephrin-A1- and VEGF-induced angiogenesis (Cheng et al., 2002). In vitro studies using isolated endothelial cells uncovered several potential mechanisms for how EphA2 promotes angiogenesis. Chen and colleagues demonstrated that soluble EphA2 inhibited both ephrin-A1 and VEGF induced endothelial migration and tube formation (Chen et al., 2006). Additionally, Cheng and colleagues demonstrated that soluble EphA2 abrogated the positive effects of VEGF on endothelial cell survival under serum-free conditions, suggesting that EphA2 serves as a survival factor for endothelial cells in conjunction with VEGF (Cheng et al., 2002). Interestingly, these investigators found that treatment of human microvascular endothelial cells (HMECs) and HUVECs with VEGF resulted in the upregulation of ephrin-A1 in these cells concomitant with EphA2 phosphorylation, similar to the observations reported by Pandey and colleagues with respect to TNF-α treatment (Cheng et al., 2002). Based on these data, it is apparent that signaling through the EphA2 receptor is important for mediating many of the properties characteristic of endothelial cells engaging in angiogenesis.

Several signal transduction pathways have been demonstrated downstream of EphA2/ephrin-A1 during angiogenesis. Recently, it was shown that ephrin-A1 stimulation of EphA2 phosphorylation on endothelial cells can induce PI3K activation concominant with an increase in Rac1 activity, thus promoting endothelial cell migration and vascular assembly (Brantley-Sieders et al., 2004). Moreover, there is evidence to suggest that PI3K-mediated activation of Rac1 GTPase may be partially regulated through Vav2 and Vav3 guanine nucleotide exchange factors (Hunter et al., 2006). Another study by Ojima and colleagues using bovine retinal endothelial cells (BRECs) demonstrated that VEGF-induced extracellular regulated kinase 1 and 2 (Erk1/2) and Akt phosphorylation was inhibited by pretreatment with ephrin-A1 (Ojima et al., 2006). Furthermore, this inhibition was found to reduce the effects of VEGF-stimulated endothelial cell migration, tube formation, and proliferation. Together, these data offer insight into the complexity of the signaling events that lie downstream of EphA2 and ephrin-A1, which act to promote angiogeneis.

THE ROLE OF EPHA2 AND EPHRIN-A1 IN TUMOR NEOVASCULARIZATION

As with any other tissue within the body, a growing tumor mass needs a constant supply of nutrients as well as an avenue for the removal of waste products; therefore, development of a functional vascular system is necessary to sustain the growth of a tumor. The notion that a tumor's blood supply can be acquired through a process of angiogenesis was first proposed over 30 years ago (Folkman, 1995). Since then, it is now recognized that tumors can acquire a blood supply through alternative means including vessel cooption, intussuseception, recruitment of endothelial progenitor cells, and vasculogenic mimicry (reviewed in Dome et al., 2007).

EphA2 is overexpressed in a large number of tumor types including melanoma, prostate, breast, ovarian, pancreatic, and lung and is often linked with a poor clinical outcome (Duxbury et al., 2004a; Kinch et al., 2003; Mudali et al., 2006; Thaker et al., 2004; Walker-Daniels et al., 1999; Zelinski et al., 2001; Zeng et al., 2003). Given that EphA2 is often overexpressed in various tumor types combined with increasing amounts of evidence supporting a role for EphA2 in promoting angiogenesis, it is natural to hypothesize that overexpression of EphA2 by tumor cells may promote tumor neovascularization. Recently, there have been a number of reports that support the hypothesis that upregulation of EphA2 promotes tumor neovascular-

ization. One of the earliest examples using immunohistochemistry (IHC) performed on xenografts of human MDA-MB-435 breast cancer cells or KS1767 Kaposi's sarcoma cells revealed that EphA2 and ephrin-A1 were expressed within the tumor vasculature as well as the tumor cells themselves (Ogawa et al., 2000). To confirm the clinical relevance of this expression pattern for EphA2 and ephrin-A1 in xenografted tumors, Ogawa and colleagues performed IHC on a number of different clinical specimens covering a wide variety of tumor types with similar results. These data set the stage for numerous reports supporting a role for EphA2 in mediating tumor neovascularization.

Much of the work investigating a role for EphA2 in mediating tumor angiogenesis has been done using two different tumor models: RIP-Tag transgenic model of angiogenic dependent pancreatic islet cell carcinoma and the 4T1 model of metastatic mammary adenocarcinoma (Brantley-Sieders et al., 2005, 2006; Brantley et al., 2002; Cheng et al., 2003). Using these models, it has been shown that blocking EphA2 inhibits tumor angiogenesis manifested as a decrease in tumor volume and microvessel density. IHC analysis of tumors taken from these two models revealed that ephrin-A1 was predominantly expressed by the tumor cells whereas EphA2 was primarily expressed within the tumor vasculature, suggesting that ephrin-A1 expressed on the tumor cells was responsible for attracting EphA2 expressing endothelial cells. In support of this hypothesis, Brantley-Sieders and colleagues demonstrated that transplanting 4T1 mammary adenocarcinomas cells expressing ephrin-A1 into EphA2-deficient mice resulted in a significant decrease in tumor volume concomitant with a decrease in microvessel density (Brantley-Sieders et al., 2005). Additionally, EphA2-deficient endothelial cells displayed significantly reduced migratory capacity in response to 4T1 mammary adenocarcinoma cells in vitro (Brantley-Sieders et al., 2005). Moreover, downregulation of ephrin-A1 in metastatic mammary tumor cells using siRNA resulted in a reduction in tumor microvessel density in vivo as well as tumor cell induced endothelial migration in vitro (Brantley-Sieders et al., 2006). Lastly, as proof of principle, these authors overexpressed ephrin-A1 in non-metastatic mammary tumor cells, which resulted in an increase in microvessel density in vivo and tumor-cell-induced migration in vitro (Brantley-Sieders et al., 2006). Together, these results highlight an important role for both eprhin-A1 and EphA2 in promoting tumor neovascularization. However, the mechanism underlying this pathogenesis is just beginning to emerge.

To understand how EphA2 is mediating tumor neovascularization, Cheng and colleagues investigated the role of VEGF in promoting angiogenesis using the RIP-Tag transgenic model (Cheng et al., 2003). They found that conditioned media from a β islet carcinoma cell line induced endothelial cell migration, and that this response was inhibited by the addition of blocking antibodies to VEGF or addition of soluble EphA2 both in vitro and in vivo, suggesting that EphA2 can mediate VEGF induced angiogenesis in this model. Similar results have been reported using an ovarian cancer model where it was demonstrated that treatment with agonistic EphA2 antibodies, which act to decrease the levels of EphA2 expressed by tumor cells, resulted in a decrease in the levels of VEGF but not bFGF, and that the decrease in VEGF was concomitant with a decrease in Src phosphorylation as well as a decrease in microvessel density in vivo (Landen et al., 2006). Furthermore, it was recently reported that downregulation of ephrin-A1 in metastatic mammary cells resulted in a downregulation of VEGF expression in these cells; likewise, overexpression of ephrin-A1 in non-metastatic mammary cells resulted in a significant increase in VEGF production (Brantley-Sieders et al., 2006). These results suggested that ephrin-A1 can regulate angiogenesis through both the secretion of VEGF and by activation of EphA2 on the surface of tumor-associated endothelial cells.

THE ROLE OF EPHA2 IN MEDIATING MELANOMA TUMOR CELL VASCULOGENIC MIMICRY

As mentioned previously, a tumor's blood supply can be acquired through a number of different mechanisms. Vasculogenic mimicry describes the unique ability of highly aggressive melanoma tumor cells to express multiple cellular phenotypes, including endothelial and epithelial associated markers and matrix-rich vasculogenic-like networks when cultured on a three-dimensional matrix in vitro, mimicking embryonic vasculogenesis, and resembling the matrix-rich patterned networks found in patient tumors correlating with an increased risk of metastatic disease (Folberg et al., 1993; Maniotis et al., 1999). Studies using mouse xenograft models have suggested that VM can serve as a functional means for tumor perfusion that may complement classical angiogenesis (Ruf et al., 2003). Additionally, VM may serve as an additional route for tumor metastasis. Given that the most significant health threat to patients with melanoma is death due to metastatic disease (Balch et al., 2004), identifying the unique characteristics of aggressive melanoma cells that enable them to engage in VM may offer new therapeutic avenues in which to target malignant melanoma.

To compare the molecular signature of highly versus poorly aggressive melanoma tumor cells, microarray analysis was performed (Bittner et al., 2000; Seftor et al., 2002). These data revealed a dysregulated genotype whereby highly aggressive melanoma tumor cells expressed various endothelial and epithelial genes important for both embryonic vasculogenesis and angiogenesis, including many different Eph receptors and ligands (Table 1; Bittner et al., 2000; Seftor et al., 2002). Given the role of the Eph receptors and their ligands in mediating many aspects of embryonic development combined with reports identifying the expression of multiple Eph-family receptor tyrosine kinases in human and mouse embryonic stem cells (Baharvand et al., 2006; Lickliter et al., 1996) as well as in hematopoietic stem cells (Lazarova et al., 2006) suggested that the aggressive melanoma tumor cells had undergone transdifferentiation to acquire a more embryonic-like phenotype. These data, in combination with data supporting a role for Eph receptors in embryonic vasculogenesis as well as angiogenesis, suggest that expression of these Eph

TABLE 1. Expression of Eph Receptors and Ligands in Aggressive Versus Poorly Aggressive Human Melanoma Tumor Cells[a]

Gene	Unigene	Fold change
EphA2	Hs. 171596	↑ 14.8–58
EphA3	Hs. 123642	↓ 2.3–45
EphB1	Hs. 116092	↑ 4.3
EphB2	Hs. 523329	↑ 2.8–34.7
EphB4	Hs. 437008	↓ 1.6–2.3
Ephrin-A1	Hs. 516664	↓ 3.0–10.8
Ephrin-A4	Hs. 449913	↑ 1.3
Ephrin-B1	Hs. 144700	↑ 3.9
Ephrin-B2	Hs. 149239	↑ 3.7–7.5
Ephrin-B3	Hs. 26988	↓ 3.7–6.5

[a]Altered gene expression of Eph receptors and ligands was identified by cDNA and/or oligonucleotide microarray analysis. Data are reported as a fold change in gene expression between highly aggressive melanoma tumor cells versus poorly aggressive melanoma tumor cells.

receptors and ligands are playing a role in mediating melanoma VM.

Our studies have utilized an in vitro model system in which to study the process of melanoma VM, whereby aggressive melanoma tumor cells are cultured on a three-dimensional type 1 collagen matrix for a period of 6 days, thus allowing the investigation of the signaling mechanisms regulating this process over time (Fig. 1). Using this model, we demonstrated that during VM, there is an increase in tyrosine phosphorylation within the aggressive melanoma tumor cells as they form vasculogenic-like structures in vitro, as illustrated in Figure 2, suggesting that aggressive melanoma cells engaged in VM have an increase in signal transduction events mediated by tyrosine phosphorylation (Hess et al., 2001). To confirm these findings, we analyzed the difference in tyrosine phosphorylated proteins between aggressive melanoma tumor cells engaged in VM and poorly aggressive melanoma tumor cells unable to engage in VM. We reported that aggressive melanoma tumor cells had a different profile of tyrosine phosphorylated proteins compared to poorly aggressive melanoma tumor cells (Hess et al., 2001), and identified EphA2 as a predominant receptor tyrosine kinase found to be phosphorylated in aggressive melanoma cells. These results, in combination with those obtained from the microarray analysis identifying EphA2 as the most overexpressed of all Eph receptors in the aggressive melanoma tumors cells, suggested that increased EphA2 may potentiate downstream signaling events necessary for melanoma VM. As proof of principle, we utilized antisense oligonucleotide strategies to transiently downregulate the expression of EphA2 in aggressive melanoma tumor cells, which resulted in the inability of these tumor cells to engage in VM (Hess et al., 2001). These data identified EphA2 as an important mediator of melanoma VM and likewise began to establish a signal transduction–mediated mechanism for this phenomenon.

In an effort to better understand the role of EphA2 in mediating melanoma VM and metastasis as a whole, we have since developed aggressive melanoma tumor cells that have stably downregulated levels of EphA2 and have been characterizing them based on VM potential, invasion, proliferation, and tumor formation potential in vivo. As our previous report suggested (Hess et al., 2001), downregulation of EphA2 inhibits the ability of aggressive melanoma cells to engage in VM in vitro (Fig. 3). Furthermore, downregulation of EphA2 resulted in a decrease in the invasive capacity of aggressive melanoma tumor cells in vitro, as demonstrated in Figure 4. To further understand the role of EphA2 in promoting melanoma aggressiveness, we utilized an orthotopic mouse model for cutaneous melanoma and challenged the aggressive melanoma cells having stably downregulated EphA2 to form tumors in vivo. The data presented in Table 2 demonstrate a significant inability of these aggressive tumor cells lacking sufficient amounts of EphA2 to form tumors in vivo, thus underscoring the importance of EphA2 in mediating melanoma tumor formation. The question remains as to the underlying mechanism resulting in tumor inhibition. There are at least two possibilities: (1) a decrease in microvessel density within the tumor, as a result of either inhibiting tumor angiogenesis and/or vasculogenic mimicry; and/or (2) tumor growth inhibited due to an inability of the tumor cells to proliferate within a mouse microenvironment.

Recently, there have been reports attempting to investigate a correlation between EphA2 and microvessel density in both colorectal and ovarian carcinomas (Kataoka et al., 2004; Lin et al., 2007). In both cases, there was a significant association between increased EphA2 expression in the tumor cells with increased microvessel density. Furthermore, Lin and colleagues reported that increases in EphA2 expression and microvessel density corresponded to a decrease in patient survival (Lin et al., 2007). These data suggest that the inability of aggressive melanoma cells lacking sufficient EphA2 to form tumors in mice may be due to impaired tumor neovascularization, especially given previous reports that firmly establish a role for EphA2 in mediating angiogenesis in conjunction with the inability of these cells to undergo VM in vitro.

Early reports first identifying the overexpression of EphA2 in melanoma suggested that eprhin-A1 could act as a growth factor for melanoma cells (Easty et al., 1995), likewise it has been reported that EphA2 may act as a survival factor for endothelial cells (Cheng et al., 2003). Additionally, Straume and Akslen (2002) found that expression of EphA2 in patient samples of melanoma was associated with increased tumor cell proliferation as measured by Ki-67 positively. To assess the role of EphA2 in mediating melanoma tumor cell proliferation, we examined the proliferation capacity of the aggressive melanoma tumor cells

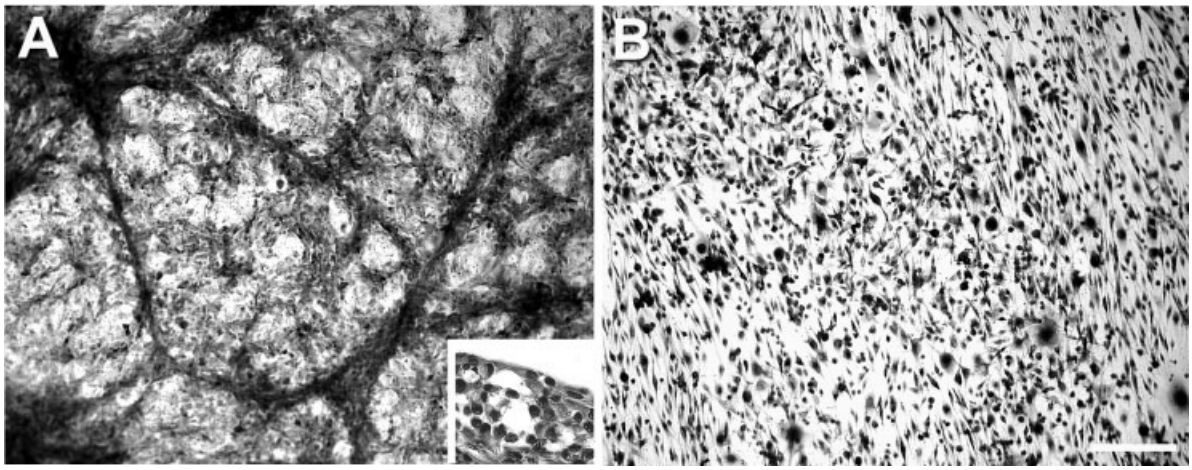

Fig. 1. Bright-field microscopy of aggressive human melanoma tumor cells (**A**) and poorly aggressive melanoma tumor cells (**B**) cultured on three-dimensional type 1 collagen matrix for 6 days and stained with Periodic-Acid Schiff reagent (PAS) without hematoxylin counterstain. The inset in A shows a cross-section of the extracellular matrix-rich networks that contain lumen-like structures. Scale bar = 100 μm (A,B). Inset: Image was viewed using a 63× oil immersion lens.

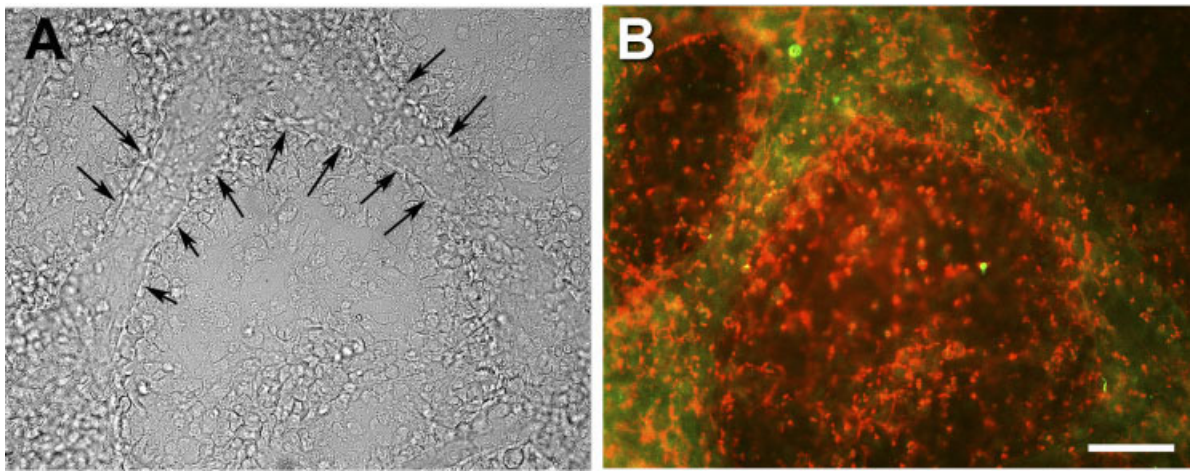

Fig. 2. Bright-field and immunofluorescence microscopy showing co-localization of phosphotyrosine and F-actin proteins within areas of VM in aggressive melanoma tumor cells cultured on three-dimensional type 1 collagen matrix for 4 days. **A,B:** Three-dimensional cultures demonstrating bright-field image (A) with corresponding fluorescent image (B), dual-labeled with phosphotyrosine proteins (PY-20; FITC) and F-actin (phalloiden; Texas Red). Arrows indicate matrix-rich VM networks. Scale bar = 100 μm for A and B.

with stably downregulated EphA2 and found a significant decrease in the ability of these cells to proliferate in vitro, as demonstrated in Figure 5. These data suggest that EphA2 may mediate melanoma tumor formation in vivo due to its direct effects on the proliferation capacity of these cells.

VE-CADHERIN MEDIATES EPHA2 EXPRESSION AND PHOSPHORYLATION DURING MELANOMA VM

In addition to the upregulation of EphA2 in aggressive melanoma cells, microarray analysis also revealed the upregulation of VE-cadherin. VE-cadherin is an endothelial-specific cadherin that plays an important role in regulating vascular morphology and stability (Dejana et al., 1999). VE-cadherin is also critically important for embryonic vasculogenesis as VE-cadherin knock-out mice die midgestation due to large vascular malformations (Carmeliet et al., 1999). It is interesting to note that a link between EphA2 and VE-cadherin has never been established in endothelial cells, even though they both are expressed in endothelial cells and both play a role in tumor neovascularization; however, a link between EphA2 and E-cadherin has been established in breast cancer cells as well as in embryonic stem cells (Orsulic and Kemler 2000; Zantek et al., 1999). Studies have shown that E-cadherin is necessary for the stabilization of EphA2 on the surface of the membrane at cell–cell adhesions allowing for proper interaction between EphA2 and ephrin-A1. Restoring appropriate interactions between EphA2 and ephrin-A1 can downregulate many of the aggressive properties of breast cancer cells overexpressing EphA2. Based on these observations and the fact that both EphA2 and VE-cadherin are necessary for melanoma VM (Hendrix et al., 2001; Hess et al.,

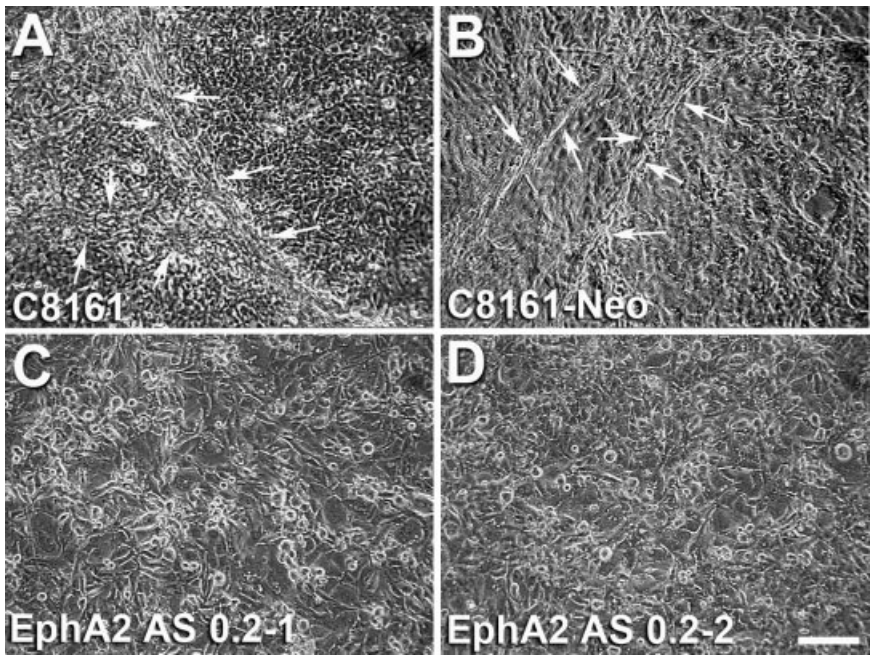

Fig. 3. Bright-field microscopy of untransfected human cutaneous C8161 metastatic melanoma cells (**A**; C8161), neo-transfected (**B**; C8161-Neo), or C8161 EphA2 knock-downs (**C**; EphA2 AS [AS; antisense] 0.2-1 and EphA2 AS 0.2-2) seeded on three-dimensional type 1 collagen matrix for 4 days. Arrows indicate VM networks. Scale bar = 100 μm (A–D).

2001), we sought to understand the relationship between EphA2 and VE-cadherin in aggressive melanoma cells. Using dual-labeled immunofluorescence, we observed that both VE-cadherin and EphA2 localized to areas of cell–cell adhesion, both in vitro and in patients' tumors classified as having a high metastatic potential (Hess et al., 2006). We validated these observations using co-immunoprecipitation experiments and found that EphA2 and VE-cadherin formed an association. Furthermore, increased phosphorylation of EphA2 or knock-down of EphA2 using anti-sense oligonucleotides resulted in no change in the localization of VE-cadherin on the surface of aggressive melanoma cells. However, knock-down of VE-cadherin resulted in a dramatic redistribution of EphA2 on the cell surface as well as a downregulation of EphA2 phosphorylation. These results suggested that VE-cadherin may act to stabilize EphA2 on the surface of the aggressive melanoma cells, thus potentiating signals important for melanoma VM. These results are contrary to those found in breast cancer cells, and suggest that tumors derived from non-epithelial cells use EphA2 signal transduction–mediated mechanisms to promote tumor cell aggressiveness, rather then suppress it. These data also suggest that perhaps EphA2 expression in aggressive melanoma signals in a manner similar to EphA2 expressed on the surface of endothelial cells undergoing angiogenesis, thereby promoting the formation of vasculogenic-like networks. The signal transduction pathways that lie downstream of these two important cell surface–associated proteins are currently under investigation. There are many possibilities including signaling through phosphoinositide 3-kinase and focal adhesion kinase, both of which have been found to play roles in tumor neovascularization and signal downstream of EphA2 and VE-cadherin (Carmeliet et al., 1999; Carter et al., 2002; Duxbury et al., 2004b; Miao et al., 2000; Qi and Claesson-Welsh, 2001).

PHOSPHOINOSITIDE 3-KINASE SIGNALING AS A MEDIATOR OF MELANOMA VM

Phosphoinositide 3-kinase (PI3K) is a cytoplasmic lipid kinase consisting of a regulatory subunit, p85, and a catalytic subunit, p110. Once activated, PI3K phosphorylates phosphoinositide-4,5-bisphosphate (PIP2) to form phosphoinositide-3,4,5-trisphosphate (PIP3), which regulates a variety of cellular functions (reviewed in Vanhaesebroeck and Waterfield 1999). PI3K activity has been implicated in the recruitment of a variety of cell-signaling components to the plasma membrane where they can induce cell motility and survival. The role of PI3K in promoting tumor progression through increased invasion, migration, survival, and induction of tumor angiogenesis has been reported in many different tumor types (reviewed in Brader and Eccles, 2004). PI3K and the signal transduction pathways that it activates have become important therapeutic targets for a number of different tumor types.

PI3K has been shown by two independent laboratories to interact with EphA2, using a yeast two-hybrid screen, and has been reported to play a role in mediating angiogenesis (Brantley-Sieders et al., 2004; Pandey et al., 1994). Based on these data we began investigating the role of PI3K in mediating melanoma VM. We found that addition of LY294002, a specific inhibitor of PI3K, inhibited the ability of aggressive melanoma tumor cells to engage in VM (Hess et al., 2003). In order to elucidate a mechanism for this apparent role of PI3K in mediating melanoma VM, we investigated the ability of PI3K to mediate the activities of membrane type 1 matrix metalloproteinase (MT1-MMP) and matrix metalloproteinase-2 (MMP-2), and the cleavage of the laminin 5γ2 chain into promigratory fragments, events previously shown to play a role in promoting melanoma VM (Hess et al., 2003; Seftor et al., 2001). We found that treatment with LY294002 reversibly blocked the activity of MMP-2 as well as the expression and activity of MT1-MMP, and the cleavage of the laminin 5γ2 chain. These results suggested that signaling through PI3K could promote melanoma VM by regulating the activities of MT1-MMP and MMP-2 and, ultimately, the cleavage of the laminin 5γ2 chain into promigratory fragments.

FOCAL ADHESION KINASE SIGNALING AS A MEDIATOR OF MELANOMA VM

Focal adhesion kinase (FAK) is a cytoplasmic kinase largely responsible for signaling events downstream of integrins (extracellular matrix receptors). Integrin clustering as triggered by binding to extracellular matrix components results in FAK phosphorylation and subsequent activation of this kinase domain. Activation of FAK in turn activates a plethora of signaling events that act to promote many different cellular processes, including cell survival, migration, and invasion (reviewed in Cox et al., 2006; Schlaepfer et al., 1999). Unregulated increases in cell survival mechanisms, migration, and invasion can contribute to a tumor's ability to grow and metastasize to distant sites within the body. Therefore, FAK is often found to be overexpressed and/or constitutively active in numerous tumor types including melanoma, prostate, thyroid, colorectal, ovarian, and oral tumors (Han et al., 1997; Maung et al., 1999; Owens et al., 1996; Schneider et al., 2002; Sood et al., 2004; Tremblay et al., 1996). Additionally FAK mediates many aspects of angiogenesis by regulating endothelial cell proliferation, survival, and migration. The importance of FAK signaling in mediating these events is highlighted in a recent report by Shen and colleagues who demonstrated that endothelial cell specific knockout of FAK resulted in defective angiogenesis during late embryogenesis resulting in embryonic lethality (Shen et al., 2005).

Several reports have linked EphA2 and ephrin-A1 to FAK signaling. Miao and colleagues first reported that in PC-3 prostate cancer cells, stimulation of EphA2 with ephrin-A1 resulted in a decrease in FAK phosphorylation concomitant with a decrease in integrin-mediated cell adhesion, spreading, and migration, suggesting that signaling through EphA2 acts to negatively regulate FAK function (Miao et al., 2000). In contrast to these observations, Carter and colleagues demonstrated that ephrin-A1 induced spreading in EphA2 expressing NIH3T3 cells or mouse embryonic fibroblasts (MEF) derived from $FAK^{+/+}$ or $p130^{Cas+/+}$ mice, but not in NIH3T3 cells or mouse embryonic fibroblasts derived from $FAK^{-/-}$ or $p130^{cas-/-}$ mice (Carter et al., 2002). Moreover, expression of constitutively active EphA2 in NIH3T3 cells allowed them to spread when plated on poly-L-lysine in a manner similar to that observed when plated on ephrin-A1 suggesting that eprhin-A1 and EphA2 signaling through FAK can promote cell adhesion and spreading (Carter et al., 2002). In support of these observations, Duxbury and colleagues demonstrated that in pancreatic adenocarcinoma cells, overexpression of EphA2 could induce a FAK-dependent increase in MMP-2 expression relative to an increase in invasive potential (Duxbury et al., 2004b). Furthermore, treatment of these cells with ephrin-A1 resulted in the downregulation of EphA2 and subsequent dephosphorylation of FAK concomitant with a decrease in MMP-2. These results demonstrated that EphA2 signaling through FAK can promote cellular adhesion and invasion. Similar results were recently reported by Liu and colleagues, who observed that forced expression of ephrin-A1 in glioma cell lines resulted in the degradation of EphA2 concomitant with a downregulation of FAK resulting in an inhibition of cellular migration, proliferation, and anchorage independent growth (Liu et al., 2007). Collectively, these studies suggest that signaling through EphA2 and ephrin-A1 can result in either promotion or inhibition of cellular adhesion, migration, and invasion depending on the cell type.

Given that FAK plays a role in mediating angiogenesis and that signaling between EphA2 and FAK can promote tumor cell aggressiveness, we investigated a role for FAK in mediating many of the characteristics of an aggressive melanoma tumor cell including VM (Hess et al., 2005; Hess and Hendrix, 2006). We showed that

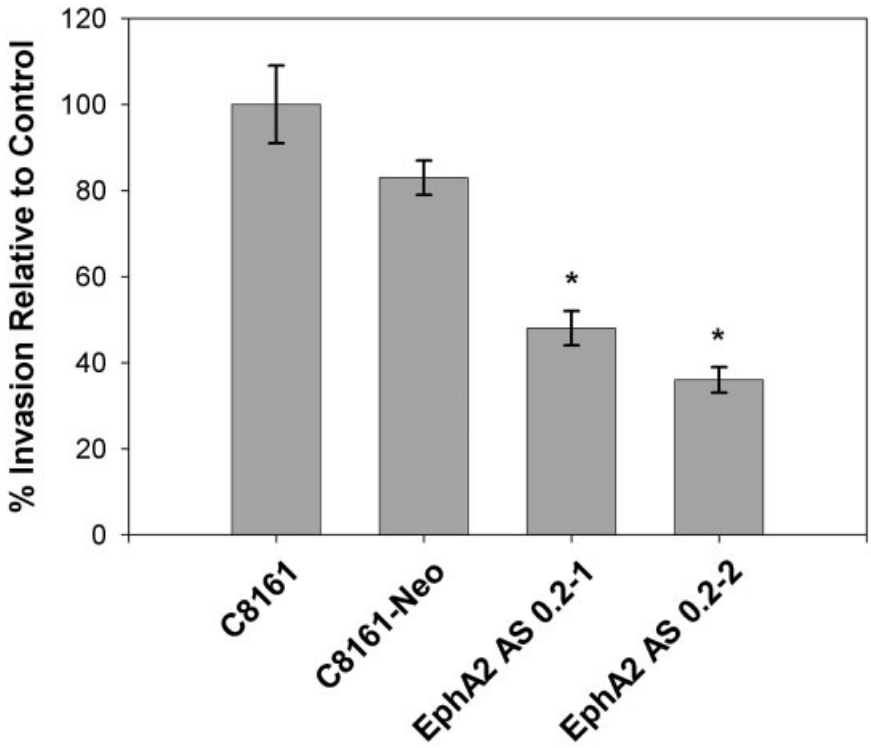

Fig. 4. Percent invasion for untransfected C8161 cells (C8161), neo-transfected C8161 cells (C8161-Neo), or C8161 EphA2 knock-down cells (EphA2 AS 0.2-1 and EphA2 AS 0.2-2) was calculated as a percentage of cells able to invade through a matrix (collagen IV, laminin, and gelatin)-coated polycarbonate membrane within a 24-hr period using an in vitro invasion chamber compared with the total number of cells seeded and normalized to control. The 50–60% decrease in invasion in both C8161 EphA2 AS clones compared to untransfected C8161 cells was found to be statistically significant using a student's *t*-test (*$P < 0.001$).

TABLE 2. Ability of EphA2 Knock-Downs to Form Tumors In Vivo[a]

Cell line	Number of animals	Tumor volume (mm^3)[b]	P*
C8161	5	3,034 ± 385	1.0
C8161-Neo	5	3,727 ± 673	0.8
EphA2 AS 0.2–1	5	114 ± 34	≥0.001
EphA2 AS 0.2–2	5	190 ± 75	≥0.001

[a]2.5×10^5 untransfected human cutaneous metastatic melanoma cells (C8161), neo-transfected (C8161-Neo), or C8161 EphA2 knock-downs (EphA2 AS [AS, antisense) 0.2–1 and EphA2 AS 0.2–2), were injected into the subscapular region of nu/nu female mice.

[b]After 5 weeks, mice were sacrificed and tumor volume was calculated by multiplying the height, length, and width of each tumor, as measured using a microcaliper, and volume is presented as mm^3

*Statistical significance was determined using a Student's t-test analysis.

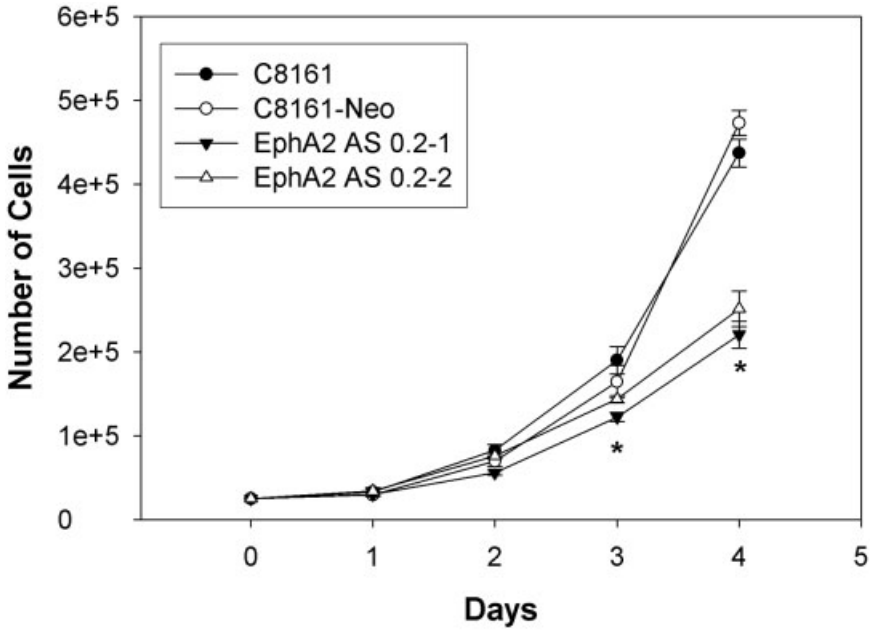

Fig. 5. To assess proliferation, 2.5×10^4 untransfected C8161 cells (C8161), neo-transfected C8161 cells (C8161-Neo), or C8161 EphA2 knock-down cells (EphA2 AS 0.2-1 and EphA2 AS 0.2-2) were plated on 24-well tissue culture plates in RPMI containing 5% serum. Cells were harvested with trypsin/EDTA and counted every 24 hr for a period of 4 days. Statistical significance was determined using the student's t-test. *$P < 0.01$; for each time point n = 12.

by expressing FAK-related non-kinase (FRNK), which acts as a dominant negative FAK protein, in aggressive melanoma cells we could inhibit melanoma VM, and significantly reduce tumor cell invasion, migration, proliferation, and clonogenicity (Hess et al., 2005; Hess and Hendrix, 2006).

As an attempt to understand a mechanism for how FAK signaling may be regulating melanoma tumor cell invasion, migration, and VM, we first examined the proteolytic enzymes known to be important for these biological functions in several tumor cell types including melanoma. In particular, there have been reports linking both the urokinase-type plasminogen activator receptor (uPAR)/urokinase system and matrix metalloproteinases (MMPs) with FAK signaling (guirre Ghiso, 2002; Hauck et al., 2001, 2002; Nguyen et al., 2000). We found urokinase activity to be greatly reduced by FRNK expression in aggressive melanoma cells; however, we did not see an effect on MMP-2 or MT1-MMP (Hess et al., 2005). These data are in contrast to other reports linking FAK signaling with the regulation of MMPs. However, this discrepancy could be due to the specificity of FAK signaling in different cell types (epithelial vs. mesenchymal) or in response to stimulation from different extracellular matrix components (i.e., fibronectin vs. collagen). Together these results support previous observations linking EphA2 and FAK signaling in tumor cell aggressiveness; however, the signal transduction events that lie downstream of EphA2 and FAK remain to be identified.

MITOGEN ACTIVATED KINASE SIGNALING AND MELANOMA VM

Extracellular regulated kinase 1 and 2 (Erk1/2) is one of the mitogen-activated protein kinase (MAPK) family members. It is a component of the Ras-Raf-Mek1/2-Erk1/2 signal transduction pathway and is activated in response to growth factors, cytokines, and hormones. Activation of this pathway results in numerous cellular responses including, survival, proliferation, adhesion, invasion, and migration (reviewed in Gray-Schopfer et al., 2005). Mutations of Ras and/or Raf resulting in constitutive activation of Erk1/2 have the capacity to transform mammalian cells in vitro (Cowley et al., 1994; Mansour et al., 1994). Furthermore, an association has been reported between activating mutations of both Ras and Raf and tumorigenesis in numerous tumor types including melanoma (Davies et al., 2002; Giehl, 2005).

Recently, there have been several reports linking EphA2 signal transduction events with Erk1/2 phosphorylation. Studies by Pratt and colleagues in addition to those initiated by Macrae and colleagues utilizing various breast cancer cell lines indicate that ligand (ephrin-A1) binding of EphA2 results in an increase in Erk1/2 phosphorylation, and subsequent upregulation of EphA2 mRNA, suggesting an autocrine feedback loop between these pathways (Giehl, 2005;

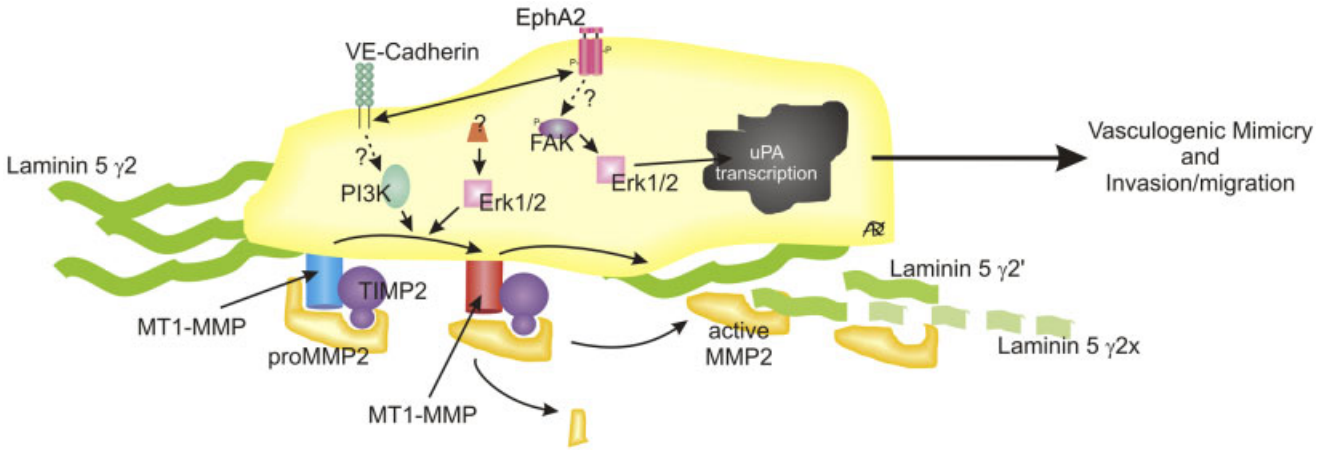

Fig. 6. Hypothetical model illustrating signal transduction pathways that promote melanoma VM. In this model, EphA2 and VE-cadherin co-localize on the surface of aggressive melanoma cells at sites of cell-cell adhesion. This association may allow for signal transduction events to be mediated through either PI3K and/or FAK. Increased FAK phosphorylation results in an increase in Erk1/2 phosphorylation thus promoting an increase in urokinase production. Additionally, increased Erk1/2 phosphorylation can increase the activities of MT1-MMP and MMP-2 through, as yet unidentified, upstream effectors. Lastly, activation of PI3K can regulate the expression and activity of MT1-MMP, which in turn promotes the conversion of MMP-2 into its active confirmation through an interaction with TIMP-2. Both enzymatically active MT1-MMP and MMP-2 may then promote the cleavage of the laminin 5γ2 chain into pro-migratory fragments. The combination of these signaling events work in concert to promote properties of aggressive melanoma tumor cells, including invasion, migration, proliferation, and VM, which are associated with an increased risk for metastasis.

Macrae et al., 2005; Pratt and Kinch, 2002, 2003). On the contrary, Miao and colleagues have reported that stimulation of EphA2 with its ligand ephrin-A1 results in a decrease in Erk1/2 phosphorylation in prostate cancer and endothelial cells (Miao et al., 2001). Additionally, Maio and colleagues reported that the stimulation of the MAPK pathway using growth factors such as placental derived growth factor (PDGF) and epidermal growth factor (EGF) can be attenuated when simultaneously treated with ephrin-A1 (Miao et al., 2001). These latter results are in agreement with the study initiated by Macrae and colleagues, who reported similar results using EGF to stimulate Erk1/2 phosphorylation in a panel of breast cancer cells, and found those effects to be attenuated by ephrin-A1 (Macrae et al., 2005). Furthermore, they found that cells containing mutations in Ras resulted in Erk1/2 phosphorylation that was independent of EGF stimulation and likewise unresponsive to the attenuation effects of ephrin-A1 stimulation (Macrae et al., 2005). Coincidently these same cells expressed high levels of EphA2 expression. Together these results suggest that constitutively active Erk1/2, possibility due to mutations in Ras and/or Raf, are responsible for the high levels of EphA2 expression found in certain tumor types.

Although we have yet to establish a link between EphA2 and Erk1/2 phosphorylation in our aggressive melanoma cells, we have established a role for Erk1/2 in promoting an aggressive melanoma phenotype with respect to FAK signaling. We found that expressing FRNK, a dominant negative antagonist of FAK signaling, in aggressive melanoma cells reduced Erk1/2 phosphorylation (Hess et al., 2005). Furthermore, we found that treatment of the aggressive cutaneous melanoma cells with specific inhibitors to Mek1/2 (PD98059 and U0126), thus decreasing the levels of Erk1/2 phosphorylation, resulted in a decrease in the invasive capacity and VM potential of these cells. Interestingly, we failed to see a decrease in the migratory behavior of these cells, suggesting that Erk1/2 is necessary for some but not all aspects of melanoma aggressiveness. Subsequently, we explored specific proteolytic enzymes that are important for invasion and VM. Using the MEK1/2 inhibitor, U0126, to down-regulate Erk1/2 phosphorylation, we found decreases in secreted urokinase, MMP-2 and MT1-MMP activity, which could result in the decreases observed in invasion and VM. We hypothesize that increased levels of secreted urokinase, MMP-2, and MT1-MMP, mediated through Erk1/2 signaling, allow aggressive melanoma cells to remodel, degrade, and invade the extracellular matrix enabling VM and metastasis.

Based on our investigations into the molecular mechanisms that mediate melanoma VM, we have developed a hypothetical model that illustrates our current understanding of the signaling events involved in this process, as shown in Figure 6. Although we have made considerable progress in deciphering the molecular mechanisms underling various characteristics of aggressive melanoma cells with a particular focus on melanoma VM, there remain many unanswered questions. Current studies are aimed at linking the signaling events mediated by PI3K, FAK, and Erk1/2 with that of EphA2. Additionally, we are interested in understanding the molecular mechanisms involved in promoting the deregulated genotype characteristic of aggressive melanoma cells, specifically the aberrantly regulated signaling events that are responsible for the increased expression of EphA2. It is interesting to note that many of the different tumor types that have been found to engage in VM, also have increased EphA2, suggesting that signaling events regulated by EphA2 may mediate VM in several tumor types.

FUTURE DIRECTIONS

EphA2 has been implicated in mediating two different avenues of tumor neovascularization: angiogenesis and vasculogenic mimicry. The overexpression of EphA2 in several different tumor types and its correlation with poor clinical outcome makes it a prime target for the development of therapeutic intervention strategies. Recently, reports have investigated the feasibility of downregulating EphA2 in breast, ovarian, and pancreatic tumors using agonistic antibodies and siRNA technologies (Carles-Kinch et al., 2002; Duxbury et al., 2004a; Landen Jr. et al., 2005, 2006). Using orthotopic mouse models and systemic delivery of either agonistic antibodies or EphA2 targeted siRNA, there has been success in reducing tumor burden and increasing survival with no toxic side effects. Our current understanding of the consequences of EphA2 over-expression in melanoma clearly demonstrated that EphA2 is a potential therapeutic target for metastatic melanoma as well. Thus, we are currently investigating the feasibility of targeting melanoma tumors using similar siRNA strategies.

Understanding the ability of aggressive melanoma cells to acquire a dysregulated genotype and engage in VM is just one example of the plasticity potential of aggressive tumor cells, and suggests a reversion to a more embryonic phenotype. As additional reports emerge linking overexpression of EphA2 or other Eph receptors with tumor aggressiveness, new information regarding the convergence of embryonic and tumorigenic signaling pathways may provide new insights into the regulation of the plastic tumor cell phenotype. Deciphering the molecular underpinnings promoting tumor cell plasticity will offer promise for the development of new therapeutic intervention strategies to target a wide variety of tumor types.

ACKNOWLEDGMENTS

We thank Dr. Kevin Knudston at the University of Iowa DNA sequencing facility in Iowa City, IA, for performing the microarray analysis and Dr. Dawn A. Kirschmann for her artistic contribution of the hypothetical signaling model. This work was supported in part by the Melanoma Research Foundation (to A.R.H) and the US National Institutes of Health (NIH) grants CA59702 and CA80318 (to M.J.C.H)

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