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Research Paper

VE-Cadherin Regulates EphA2 in Aggressive Melanoma Cells Through a Novel Signaling Pathway

Implications for Vasculogenic Mimicry

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KEY WORDS

vasculogenic mimicry, melanoma, VE-cadherin, EphA2

ABBREVIATIONS

VM	vasculogenic mimicry
FAK	focal adhesion kinase
PI3K	phosphoinositide 3-kinase
MT1-MMP	membrane type-1 matrix metallo proteinase
MMP-2	matrix metallorproteinase-2

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ABSTRACT

The formation of matrix-rich, vasculogenic-like networks, termed vasculogenic mimicry (VM), is a unique process characteristic of highly aggressive melanoma cells found to express genes previously thought to be exclusively associated with endothelial and epithelial cells. This study contributes new observations demonstrating that VE-cadherin can regulate the expression of EphA2 at the cell membrane by mediating its ability to become phosphorylated through interactions with its membrane bound ligand, ephrin-A1. VE-cadherin and EphA2 were also found to be colocalized in cell-cell adhesion junctions, both in vitro and in vivo. Immunoprecipitation studies revealed that EphA2 and VE-cadherin could interact, directly and/or indirectly, during VM. Furthermore, there was no change in the colocalization of EphA2 and VE-cadherin at cell-cell adhesion sites when EphA2 was phosphorylated on tyrosine residues. Although transient knockout of EphA2 expression did not alter VE-cadherin localization, transient knockout of VE-cadherin expression resulted in the reorganization of EphA2 on the cells' surface, an accumulation of EphA2 in the cytoplasm, and subsequent dephosphorylation of EphA2. Collectively, these results suggest that VE-cadherin and EphA2 act in a coordinated manner as a key regulatory element in the process of melanoma VM and illuminate a novel signaling pathway that could be potentially exploited for therapeutic intervention.

INTRODUCTION

In a process that resembles embryonic vasculogenesis, highly aggressive human melanoma cells, but not poorly aggressive melanoma cells, form patterned networks of matrix-rich tubular structures and express select endothelial-associated genes when cultured on three-dimensional matrices in vitro.[1,2] This unique characteristic of aggressive melanoma is termed vasculogenic mimicry (VM) and is clinically significant with a corresponding 50% increased risk of death from metastatic disease.[3] Furthermore, VM has been described in a variety of tumor types including breast, prostate, ovarian, and lung with similar clinical significance.[1,4-8] These observations suggest that, understanding the key molecular mechanisms that regulate VM would offer new avenues for therapeutic intervention for several different tumor types.

In an attempt to discern the molecular mechanisms that regulate melanoma VM, microarray analysis was performed to identify the differentially expressed genes between the highly aggressive compared to poorly aggressive melanoma cells. These data helped establish a molecular signature characteristic of the aggressive melanoma cells which revealed both endothelial and epithelial cell genotypes, including the expression of both VE-cadherin and EphA2, respectively.[1,9] VE-cadherin expression by endothelial cells plays an important role in regulating vascular morphology and stability.[10] In particular, the importance of VE-cadherin in maintaining vascular integrity was made evident from studies using a VE-cadherin knockout mouse which resulted in the death of embryos during midgestation due to large vascular malformations.[11] Additionally, EphA2—a receptor tyrosine kinase and a member of the Eph family of protein tyrosine kinases, has been found to play an important role in angiogenesis. Several different studies using both in vitro and in vivo angiogenesis models have demonstrated that EphA2, in conjunction with its membrane bound ligand, ephrin-A1, regulates key processes controlling tumor neovascularization.[12-15]

Previous work has also shown that both VE-cadherin and EphA2 play important roles in melanoma VM.[16,17] The present study extends those observations and identifies critical

events that link VE-cadherin and EphA2 in the regulation of this process. While immunocytochemistry of highly aggressive melanoma cells in vitro and in vivo show that both EphA2 and VE-cadherin are colocalized at cell-cell adhesion sites, likewise, coimmunoprecipitation experiments demonstrate that these two receptors interact during VM in vitro. Furthermore, transient knockout of VE-cadherin in the highly aggressive melanoma cells resulted in a reorganization of EphA2 on the cell surface and an accumulation of EphA2 in the cytoplasm, as well as its dephosphorylation on tyrosine residues. However, subsequent stimulation of EphA2 using a monoclonal antibody or transient knockout of EphA2 expression resulted in no change in the distribution or localization of VE-cadherin in these cells. Together, these results suggest that VE-cadherin regulates the function of EphA2 in highly aggressive human melanoma cells, and identifies a key regulatory mechanism for downstream signaling events underlying VM.

MATERIALS AND METHODS

Cell culture. The human metastatic cutaneous, C8161, and uveal, MUM-2B, melanoma cell lines used in this study have been previously characterized as highly aggressive and having an epitheliod phenotype.[18,19] These cell lines were maintained in RPMI 1640 (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA) and 0.1% gentamycin sulfate. Cell cultures were determined to be free of *Mycoplasma* contamination using the Mycoplasma PCR ELISA (Roche, Indianapolis, IN).

Immunoprecipitation, electrophoresis and immunoblotting. MUM-2B and C8161 cell lysates were collected from three-dimensional type 1 collagen matrices as previously described.17 A total of 500 mg of total protein was used for immunoprecipitation with an anti-EphA2 antibody (C-20; 5 mg per reaction; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-VE-cadherin antibody (160840; 5 mg per reaction; Cayman Chemical Co., Ann Arbor, MI). Immunoprecipitations were performed by continuous shaking at 4°C for 1 hour, followed by 1 hour incubation with protein-A sepharose beads (50 ml of a 50% slurry in PBS; Sigma Chemical Co. St. Louis, MO). Samples were washed three times with a modified 1x RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate) containing 25 mg/ml leupeptin (Sigma Chemical Co.), 10 mg/ml aprotinin (Sigma Chemical Co.), 1mM sodium ortho-vanadate, and 2mM EDTA, and the protein recovered by boiling for 4 minutes in 2x electrophoresis sample buffer.20 Samples were separated by 10% SDS-PAGE and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH). Western blots prepared from immunoprecipitation reactions or by using 25 mg whole cell lysates, were incubated in 5% nonfat dry milk in Tris buffered saline (TBS; 50 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 and 0.1% BSA (TBSTB) for one hour at room temperature. Blots were then incubated with anti-EphA2 antibody (1:500; clone D7; Upstate, Lake Placid, NY), anti-VE-cadherin (1:500; Cayman Chemical Co.), or anti-paxillin (1:1000; clone 349, BD Pharmingen, San Diego, CA) for 1 hour with continuous shaking, washed two times 10 minutes with TBS-TB, followed by incubation for one hour with a horseradish peroxidase-conjugated anti-mouse (anti-EphA2) or anti-rabbit (VE-cadherin) secondary antibody (both at 1:5000; Jackson Laboratory, Bar Harbor, ME). Blots were developed using enhanced chemiluminescence detection (ECL; Pierce Co, Rockford, IL).

For the antibody stimulation assays, the MUM-2B and C8161 cells were plated onto 60 mm dishes in serum-free media and incubated for 24 hours. The cells were washed once with PBS, then fresh serum-free media containing 3 μg/ml of EA2 antibody was added to the cells in a manner previously described.[21] Lysates were harvested at subsequent time points and immunoprecipitation performed using anti-EphA2 antibodies (5 μg per reaction; Santa Cruz Biotechnology). A western blot was prepared and probed with an anti-phosphotyrosine antibody (1:500, PY20, BD Pharmingen) as previously described,[17] stripped and subsequently reprobed with anti-EphA2 antibody (1:500; Upstate).

Transient transfections. The MUM-2B and C8161 cell lines were transiently transfected with either scrambled sense oligodeoxynucleotides (ODNs) (5'GACTGGAATGCAGATCGA3') or anti-sense ODNs (5'CATGAGCCTCTGCATCTT3') to knockout the expression of VE-cadherin, or inverted anti-sense (5'GCCGCGTCCCGTTCCTTCACCATGACGACC3') or anti-sense (5'CCAGCAGTACCACTTCCTTGCCCTGCGCCG3') to knockout the expression of EphA2, as previously described.[16,17] After initial transfection with ODNs, the cells were harvested and seeded onto 18 mm glass coverslips. Twenty-four hours after plating, the cells were fixed for 5 minutes in ice-cold methanol. Coverslips were incubated for 20 minutes in serum-free protein block (DAKO Cytomation, Carpinteria, CA). The coverslips were then incubated with an anti-EphA2 (1:100; Upstate) or anti-VE-cadherin antibody (1:100; Cayman Chemical Co.) for 1 hour, rinsed with PBS, incubated with an anti-rabbit Oregon Green 488 conjugate secondary antibody (for VE-cadherin) or an anti-mouse rhodamine conjugate secondary antibody (for EphA2;1:500 for each, Molecular Probes, Eugene, OR) for 1 hour at room temperature then rinsed with PBS. Pictures were taken using an Axioskop 2 microscope (Carl Zeiss, Inc., Thornwood, NY) using a Spot 2 camera (Diagnostic Instrument, Inc. Sterling Heights, MI) and Zeiss Axiovision 2.0.5 software (Carl Zeiss, Inc.).

Immunocytochemistry. The presence and location of EphA2 and VE-cadherin on the MUM-2B and C8161 cells was determined immunocytochemically. Cells were seeded onto 18 mm glass coverslips and allowed to reach confluence. The coverslips were fixed with ice-cold methanol for 5 minutes, rinsed with PBS and incubated for 20 minutes with serum-free protein block (DAKO Cytomation) followed by incubation with both anti-EphA2 (1:100; Upstate) and anti-VE-cadherin antibodies (1:100; Cayman Chemical Co.) for 1 hour and rinsed with PBS. The coverslips were then incubated with rhodamine conjugated anti-mouse (for EphA2) and an Oregon Green 488 conjugated anti-rabbit (for VE-cadherin) secondary antibodies (both at 1:500, Molecular Probes) for 1 hour followed by rinsing with PBS.

The presence of EphA2 and VE-cadherin in uveal melanoma tumor sections from patients with aggressive disease was performed as follows: slides were deparafinized and the tissue digested with Ficin (Digest-All 1, Zymed Laboratories, San Francisco, CA) for 20 minutes at 37°C. The samples were then blocked for 10 minutes with serum-free protein block (DAKO Cytomation) followed by rinse with TBST. After blocking, a primary antibody cocktail of mouse anti-human EphA2 antibody (1:50; clone B2D6, Oncogene Research Products, Boston MA) and rabbit anti-human VE-caderhin antibody (1:200; Cayman Chemical Co.) or a primary cocktail of mouse anti-human melanoma cocktail (1:40; contains antibodies to HMB-45 and MART-1; Biocare Medical, Walnut Creek, CA) and rabbit anti-human VE-cadherin antibodies (1:200) was added. As a negative control, an antibody cocktail prepared using Mouse IgG (1:50; DAKO) and Rabbit IgG (1:200; DAKO) was added and incubated for 1 hour. After a thorough rinse with TBST, a secondary antibody cocktail consisting of Alexa Fluor 568 goat anti-mouse (1:100, Molecular Probes) and Alexa Fluor 660 goat anti-rabbit (1:100, Molecular Probes) antibodies was applied to both sets of slides for 45 minutes, followed by a thorough wash in TBST. Images were captured using a BioRad 1024MRC confocal microscope (BioRad Laboratories, Hercules, CA).

For the EA2 antibody stimulation assay, MUM-2B and C8161 cells were seeded onto 18 mm coverslips in serum-free media and incubated for 24 hours. After 24 hours, cells were washed and fresh serum-free media containing 3 μg/ml EA2 antibody was added; at appropriate time points coverslips were fixed with ice-cold methanol for 5 minutes and then rinsed with PBS. Coverslips were incubated with both anti-EphA2 and anti-VE-cadherin antibodies for 1 hour, rinsed with PBS, and then incubated with rhodamine conjugated anti-mouse (anti-EphA2) and Oregon Green 488 conjugated anti-rabbit (anti-VE-cadherin) antibodies for 1 hour. Slides were mounted with permanent mounting media. Pictures were taken using an Axioskop 2 microscope as previously described.

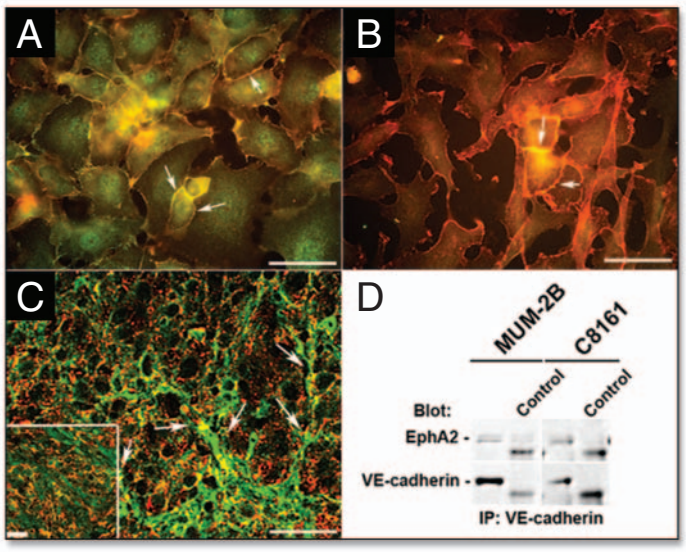

Figure 1. Immunolocalization of EphA2 and VE-cadherin in areas of cell-cell adhesion. Highly aggressive MUM-2B uveal (A) and C8161 cutaneous (B) melanoma cells stained with antibodies to both EphA2 (red) and VE-cadherin (green); arrows delineate immunostaining at cell-cell junctions. (C) Histological section of an aggressive uveal melanoma primary tumor incubated with both anti-EphA2 (red) and anti-VE-cadherin (green); arrows delineate networks present within tumor. Inset: Similar histological section incubated with both anti-melanoma cocktail (contains antibodies to HMB-45 and MART-1; red) and anti-VE-cadherin (green) antibodies. (D) MUM-2B and C8161 cells were cultured on a three-dimensional matrix for a period of 6 days; lysates were collected and immunoprecipitated with anti-VE-cadherin antibodies or rabbit IgG (control), run on a 10% polyacrylamide gel, and transferred to nitrocellulose membranes. The blots were probed with anti-EphA2 antibodies and reprobed with anti-VE-cadherin antibodies. (Bar = 50 μm).

RESULTS

EphA2 and VE-cadherin are co-localized at sites of cell-cell adhesion. Microarray analysis of the highly aggressive metastatic cutaneous melanoma (C8161) and uveal melanoma (MUM-2B) cell lines compared with their poorly aggressive counterparts revealed the overexpression of VE-cadherin, an endothelial-specific cell adhesion molecule, and EphA2, an epithelial cell receptor protein tyrosine kinase.[1,2,9,16,17] The present study provides direct immunocytochemisty evidence demonstrating that EphA2 and VE-cadherin are colocalized at areas of cell-cell contact in MUM-2B (Fig. 1A) and C8161 (Fig. 1B) cells. In addition, this staining pattern was confirmed in sections from patients' melanoma tumors characterized as aggressive, revealing that both EphA2 and VE-cadherin were colocalized at cell-cell contacts in situ, as shown in (Fig. 1C). To confirm that positive VE-cadherin-staining was localized in melanoma tumor cells, tissue sections were also costained with an anti-melanoma cocktail and VE-cadherin antibodies (Fig. 1C; inset). Furthermore, immunoprecipitation of VE-cadherin from MUM-2B and C8161 engaged in VM on three-dimensional matrices resulted in the co-precipitation of EphA2 (Fig. 1D).

EphA2 phosphorylation does not change the colocalization of EphA2 and VE-cadherin at sites of cell-cell adhesion. In order to determine whether the association of VE-cadherin with EphA2 involved the functional regulation of one by the other, MUM-2B and C8161 cells were stimulated with a monoclonal anti-EphA2 antibody EA2, which has been shown to induce EphA2 phosphorylation on tyrosine residues.[21] At subsequent time points, whole cell lysates were collected and analyzed for EphA2 phosphorylation on tyrosine residues. Additionally, cells were fixed in situ and stained with both anti-EphA2 and anti-VE-cadherin antibodies. Figure 2A demonstrates the effects of the EA2 antibody on EphA2 phosphorylation on tyrosine residues. Tyrosine phosphorylation of EphA2 peaks at 30 minutes and returns to baseline levels by 24 hours. Dual-labeling immunocytochemistry with both VE-cadherin and EphA2 antibodies revealed that after 30 minutes and 24 hours post-stimulation with EA2 antibody, the colocalization of VE-cadherin with EphA2 at cell-cell junctions remained unchanged in both the MUM-2B (Fig. 2B) and C8161 (data not shown) cells. These data suggest that colocalization of EphA2 and VE-cadherin in both the MUM-2B and C8161 cells does not change in response to EphA2 phosphorylation on tyrosine residues.

Knockdown of EphA2 expression does not affect the localization of VE-cadherin at sites of cell-cell adhesion. Given that there was no change in the localization of VE-cadherin in response to EphA2 tyrosine phosphorylation, we sought to determine if loss of EphA2 protein expression would have an effect on the localization of VE-cadherin. To test this, transient knockout of EphA2 expression was performed by treating the C8161 and MUM-2B (data not shown) cells with inverted anti-sense (IAS; Fig. 3A) or anti-sense ODNs (AS; Fig. 3B) followed by immunocytochemistry to examine VE-cadherin distribution within the cells. Western blot confirmation of EphA2 knockout is depicted in Figure 3C, with paxillin serving as a loading control. These results suggest that VE-cadherin localization at cell-cell adhesion sites is not changed when EphA2 expression is lost. Together, these results indicate that neither EphA2 expression nor its phosphorylation regulates the expression or function of VE-cadherin in these melanoma cells.

Knockdown of VE-cadherin expression results in a redistribution of EphA2 on the cell-membrane. To investigate whether VE-cadherin regulates the expression of EphA2 in the highly aggressive melanoma cells, a transient knockout of VE-cadherin expression was performed in the C8161 and MUM-2B cells and the effects observed by immunocytochemistry using an anti-EphA2 antibody. Untransfected C8161 and MUM-2B cells (data not shown) or cells transfected with scrambled-sense VE-cadherin (Scr; Fig. 4A) or anti-sense VE-cadherin (AS; Fig. 4B) ODNs were treated with an anti-EphA2 antibody and fluorescent secondary antibody. Western blot analysis (Fig. 4C) confirmed that the expression of VE-cadherin decreased in anti-sense treated cells relative to scrambled sense treated cells. Scrambled-sense transfected cells revealed EphA2 at cell-cell adhesion complexes (arrows; Fig. 4A), while cells transfected with anti-sense VE-cadherin showed a reorganization of EphA2 that appears to be a random scattering of the EphA2 on the cell surface, accompanied by a predominant loss of EphA2 from the cell-cell adhesion complexes, and the appearance of EphA2 in the cytoplasm of the transfected cells (Fig. 4B). In addition, loss of VE-cadherin expression by treatment with anti-sense ODNs resulted in the apparent decrease of phosphorylated EphA2 in these cells (Fig. 4D).

DISCUSSION

Interestingly, the molecular signature of aggressive melanoma cells is indicative of a dysregulated genotype aberrantly expressing genes normally associated with endothelial and epithelial cells, in addition to the other cellular phenotypes.[1,2,9,16,17] In particular, VE-cadherin and EphA2 are expressed by aggressive cutaneous and uveal melanoma cells and have been shown by transient knockout experiments to be essential for VM to occur in vitro.[16,17] In the present study, VE-cadherin and EphA2 are shown to be linked in melanoma cells expressing vasculogenic molecules and forming vasculogenic-like networks, thereby implicating their involvement in the initial signaling and regulation of this unique process.

The data presented in this study demonstrate that both EphA2 and VE-cadherin are colocalized at areas of cell-cell contact in aggressive uveal and cutaneous melanoma cells in vitro and in vivo. Furthermore, coimmunoprecipitation analysis indicates that EphA2 and VE-cadherin are associated with each other during the process of VM. These data correlate with other studies that report similar interactions between EphA2 and E-cadherin in various breast cancer cell lines and embryonic stem cells.[22,23] These studies found that E-cadherin homotypic adhesion is necessary for the phosphorylation

and localization of EphA2 to cell-cell adhesion sites which enables EphA2 to contact its ligand, ephrin-A1.[22,23] However, while these studies show that E-cadherin is required for the localization of EphA2 to areas of cell-cell contact, they were unable to show a direct interaction between EphA2 and E-cadherin.[22,23] It is important to note, however, that the interaction between EphA2 and E-cadherin described in this previous work may be indirect and involve additional signaling molecule(s) which link(s) these proteins together when EphA2 is phosphorylated. An excellent candidate for such a bridging protein is phosphoinositide 3-kinase (PI3K). This kinase binds phosphorylated EphA2 and is also thought to interact with VE-cadherin through β-catenin and VEGFR2.[11,24] However, it is unclear whether VE-cadherin or EphA2 serves as the receptor that recruits PI3K to the cell membrane.

Experiments were designed to address the question of whether EphA2 modulates the expression and function of VE-cadherin, or whether VE-cadherin modulates the expression and function of EphA2. The data indicate that there is no change in the distribution of VE-cadherin on the cells' surface when EphA2 is phosphorylated on tyrosines or after knocking out EphA2 expression, suggesting that EphA2 does not regulate the distribution of VE-cadherin in the cells. However, transient knockout of VE-cadherin expression in these cells resulted in the redistribution of EphA2 from the cell-cell adhesion complexes into a more random distribution over the cell surface. Furthermore disruption of VE-cadherin expression resulted in a dephosphorylation of EphA2, suggesting that VE-cadherin facilitates relocalization of EphA2 to cell-cell adhesion complexes and may potentiate its interaction with ephrin-A1 (EphA2's membrane-bound ligand), and its subsequent phosphorylation.

Taken together, these observations suggest that VE-cadherin and EphA2 play important roles in melanoma vasculogenesis and possibly in embryonic vascular networks formation as well.[10,11,14-17] The work presented here demonstrates that knocking out VE-cadherin or EphA2 expression affects EphA2 phosphorylation and the signal transduction pathways downstream, and results in an inability of the cells to form vasculogenic structures. It has been shown in epithelial cells that elevated PI3K activity can mediate the process of tubulogenesis by activating unknown downstream effectors, and EphA2 has been shown to interact with PI3K using a yeast two-hybrid study.[24,25] In this latter study, induced phosphorylation of EphA2 resulted in its binding to PI3K and its subsequent activation of PI3K.[24] Additionally, our laboratory has recently shown an important role for PI3K activity in promoting melanoma VM by regulating the expression and function of MT1-MMP and MMP-2, and consequently the cleavage of laminin 5γ2 chain.[26,27] Furthermore, VE-cadherin has been shown to interact with PI3K through β-catenin and VEGFR2 in endothelial cells, thus contributing to their survival.[11] Moreover, endothelial cell migration is dependent on the activation of PI3K which occurs downstream of focal adhesion kinase (FAK) activation in VEGF-A stimulated cells expressing VEGFR-2.[28] Other reports have suggested an interaction between FAK and EphA2 in PC-3 prostate cancer cells.[29] We have found that EphA2 can interact with phosphorylated FAK in aggressive melanoma cells (unpublished observations) and that FAK is phosphorylated exclusively in these cells undergoing VM.[30] It is tempting to speculate that in highly aggressive melanoma cells, VE-cadherin promotes the interaction between EphA2 and FAK by regulating the ability of EphA2 to translocate to the membrane. This would facilitate EphA2 interaction

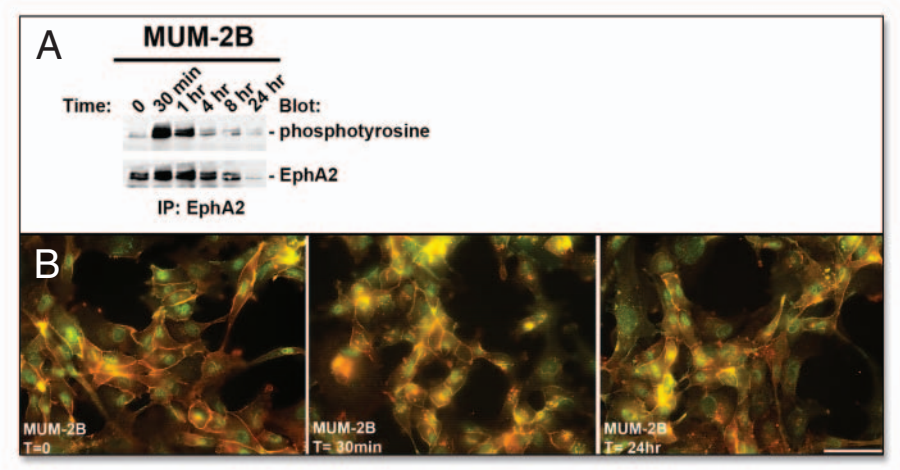

Figure 2. Western blot confirmation of the level of phosphorylated EphA2 following stimulation with EA2 antibody; which recognizes the extracellular domain of EphA2. (A) MUM-2B cells were seeded onto 60 mm dishes and allowed to adhere, treated with 3 μg/ml EA2 antibody, and cell lysates collected at the appropriate time points. Lysates were immunoprecipitated with anti-EphA2 antibodies, run on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The blots were probed with anti-phosphotyrosine antibodies and with anti-EphA2 antibodies. Immunofluorescence microscopy shows that stimulation of EphA2 with the monoclonal antibody EA2 does not change the distribution of VE-cadherin in the aggressive MUM-2B cells (B). MUM-2B cells were seeded onto glass coverslips and allowed to adhere, followed by addition of EA2 antibody in serum-free media. After various time points the cells were fixed and stained with both EphA2 (red) and VE-cadherin (green) antibodies. (Bar = 50 μm).

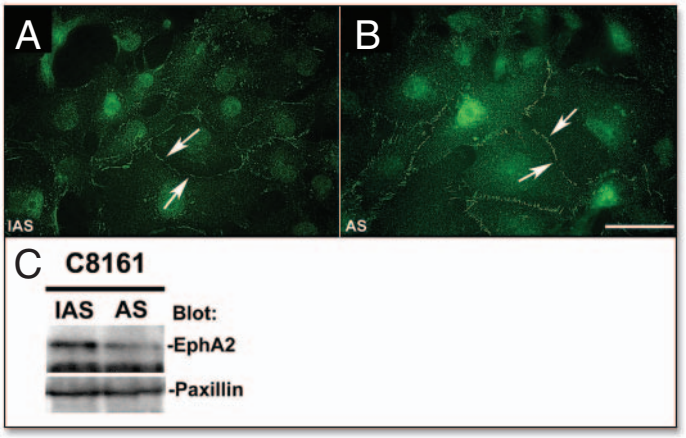

Figure 3. Immunofluorescence microscopy demonstrates that transient knockout of EphA2 resulted in no change in the distribution or localization of VE-cadherin to areas of cell-cell adhesion in highly aggressive melanoma cells. The C8161 melanoma cells were seeded onto 18 mm coverslips, transfected with (A) inverted anti-sense (IAS) EphA2 ODNs or (B) transfected with anti-sense (AS) EphA2 ODNs (arrows delineate immunostaining at cell-cell junctions). Twenty-four hours after transfection, the cells were fixed and stained with anti-VE-cadherin antibodies followed by incubation with Oregon Green 488 conjugated secondary antibody. (C) Western blot analysis of cell lysates showing the level of EphA2 in cells treated with inverted anti-sense (IAS) or anti-sense (AS) ODNs. Paxillin serves as a loading control. (Bar = 50 μm).

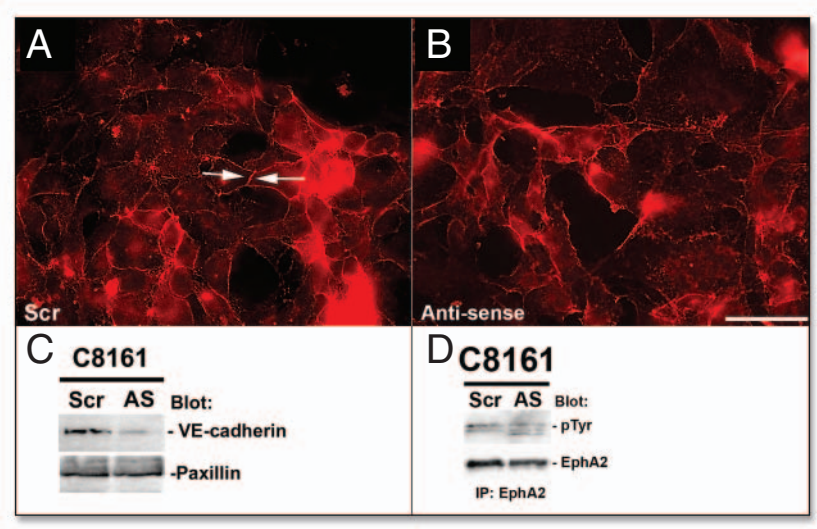

Figure 4. Immunofluorescence microscopy demonstrates that transient knockout of VE-cadherin resulted in the reorganization of EphA2 in highly aggressive melanoma cells. The C8161 melanoma cells were seeded onto 18 mm glass coverslips, transfected with scrambled-sense (Scr), VE-cadherin ODNs (A) or anti-sense (AS) VE-cadherin ODNs (B). 24 hours after transfection, cells were fixed and stained with anti-EphA2 antibodies followed by incubation with rhodamine conjugated secondary antibody; (A) Arrows delineate immunostaining at cell-cell junctions. (C) Western blot analysis of cell lysates showing the level of VE-cadherin in cells treated with scrambled-sense (Scr) or anti-sense (AS) ODNs. Paxillin serves as a loading control. (D) Western blot detecting the level of phosphorylated EphA2 in cells treated with scrambled-sense (Scr) or anti-sense (AS) ODNs. Lysates were immunoprecipitated with anti-EphA2 antibodies, run on a 10% polyacrylamide gel, and transferred to nitrocellulose membranes. The blots were probed with anti-phosphotyrosine antibodies, followed by probing with anti-EphA2 antibodies. (Bar = 50 μm).

with its membrane bound ligand resulting in its phosphorylation, and potentially forming an interaction with FAK leading to its phosphorylation and activation. The signal transduction pathways activated by both EphA2 and VE-cadherin could converge and result in the activation of PI3K, and lead to the promotion of melanoma VM through activation of MMP-2 resulting in the cleavage of the laminin 5γ2 chain. A hypothetical model describing the critical signaling cascade mediating melanoma VM is depicted in Figure 5.

Highly aggressive uveal and cutaneous melanoma cells express genes which have previously been thought to be specific to endothelial and epithelial cells. The work presented here demonstrates that these endothelial and epithelial-specific genes contribute to the formation of vasculogenic-like structures similar to the process of embryonic vasculogenesis. While VE-cadherin and EphA2 have been previously shown to be important in the process of VM,[16,17] this work extends these observations and provides a novel finding that VE-cadherin regulates the distribution and function of EphA2 in highly aggressive human cutaneous and uveal melanoma cells. In summary, the identification that EphA2 function can be regulated by VE-cadherin, and that this may serve as a critical mediator of VM, provides new insights for the therapeutic intervention of tumor associated vasculature.

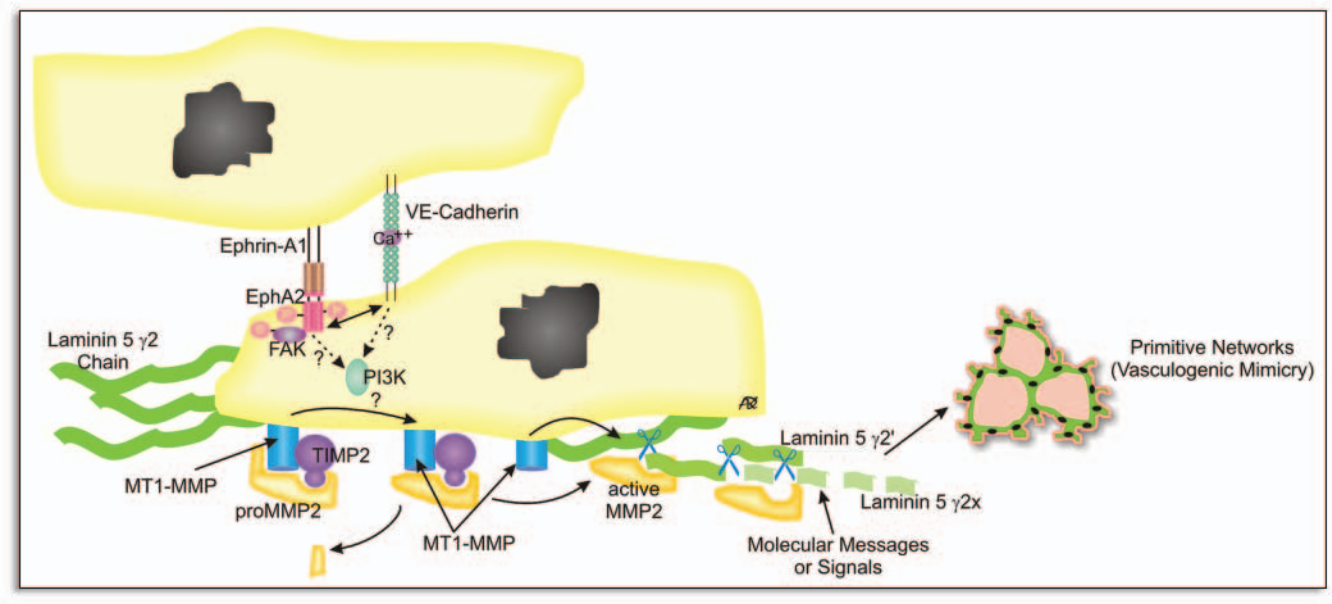

Figure 5. Hypothetical model for the regulation of EphA2 by VE-cadherin in highly aggressive melanoma cells. In this model, VE-cadherin association with another VE-cadherin molecule on adjacent cells facilitates the organization of EphA2, either by interacting directly or indirectly with EphA2 on the cell membrane. Once organized on the cell membrane, EphA2 is able to bind to its ligand, ephrin-A1, resulting in the phosphorylation of the receptor. Phosphorylated EphA2 can then bind to phosphorylated FAK. Additionally, VE-cadherin and EphA2 may converge to activate the PI3K pathway leading to the activation MMP-2 and the consequent cleavage of the laminin 5γ2 chain.[26] The ability of EphA2 to signal thorough FAK and PI3K may play an important role in triggering the signaling pathways underlying melanoma cell vasculogenic mimicry.

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