Characterizing the interaction between VE-PTP, Tie2 and VE-Cadherin

Sharif Ossai Muhammad
Virginia Commonwealth University

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Characterizing the interaction between VE-PTP, Tie2 and VE-Cadherin

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Ossai Muhammad Sharif
Virginia Commonwealth University

Director: Dr William A Barton
Associate Professor, Department of Biochemistry and Molecular Biology

Virginia Commonwealth University
Richmond, Virginia
July 2012
Acknowledgements

I would like to thank my advisor and the biochemistry department.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Ang</td>
<td>angiopoietin</td>
</tr>
<tr>
<td>CFP</td>
<td>cyan fluorescent protein</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>IP</td>
<td>immuno-precipitation</td>
</tr>
<tr>
<td>mTQ</td>
<td>mTurquoise</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SYFP</td>
<td>super yellow fluorescent protein</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of measurement</td>
</tr>
<tr>
<td>Tie</td>
<td>tyrosine kinase with immunoglobulin-like and EGF-like domains</td>
</tr>
<tr>
<td>TIME</td>
<td>telomerase-immortalized human microvascular endothelial</td>
</tr>
<tr>
<td>U2OS</td>
<td>human osteosarcoma cell line</td>
</tr>
<tr>
<td>VE-PTP</td>
<td>vascular endothelial protein tyrosine phosphatase</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelia growth factor receptor</td>
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ABSTRACT

Many signaling pathways have been shown to be involved in the formation of the vascular system. Among them are the endothelial specific receptor families such as VEGF, Ang/Tie, as well as other signaling pathways such as semaphorins, which are also involved, in axonal guidance. It is known that the interaction between receptor tyrosine kinase, Tie2, VE-Cadherin, and VE-PTP mediate endothelial cell quiescence and adhesion. However, the structural basis of these interactions is not well understood. The aim of our study is to characterize the binding interactions between these players. Another important part of our study is describing the cross-talk between vasculature and nervous system by characterizing the Neuropilin/Plexin/Semaphorin system. VE-Cadherin along with neuropilins plays an essential role by directing VEGF signals to the appropriate location and coordinating the activation of downstream molecules.

We characterize the interaction between Tie2, VE-PTP and VE-Cadherin by (FRET)-based proximity assay, fluorescence lifetime imaging, and co-immunoprecipitation assays. Our data showed a consistent localization of the protein and FRET signal for Tie2 and VE-PTP prior to ligand recognition. We showed the association between Tie2 and VE-Cadherin complex by co-immunoprecipitation. However, our FRET data was not consistent. The examination of VE-PTP and VE-Cadherin for association and localization of the protein showed a very unique, mutually exclusive localization of the protein. Our study of Neuropilin/Plexin/Semaphorin system showed changes in the protein localization, FRET signal and morphology upon stimulation of HEK293 cells expressing Nrp/plexin with
Sema3D. In this system VE-Cadherin along with neuropilins plays an essential role by directing VEGF signals to the appropriate location and coordinating the activation of downstream molecules. The characterization of extracellular binding between Tie2, VE-PTP, and VE-Cadherin, will help to better understand the molecular mechanisms of normal and tumor angiogenesis to develop new anti-angiogenic therapies.
Chapter 1

Introduction

1.1 Blood Vessel Development

The formation of the vascular system is one of the earliest and most important events that occur in the developing embryo providing the growing organism with oxygen and nutrients as well as removing the waste products. Vasculogenesis and angiogenesis are two distinct processes that are required for the development of the vascular system. Vasculogenesis is the process of the de novo formation of blood vessels that consists of two distinct events: one is production of new endothelial cells in a developing embryo and second is the formation of blood vessels in adult avascular tissue area from precursor cells (hemangioblasts) which eventually differentiate into vascular precursors (angioblasts). These angioblasts migrate, coalesce into cords and assemble into a primary capillary plexus which expresses endothelial specific markers such as vascular endothelial protein tyrosine phosphatase (VE-PTP), vascular endothelia growth factor receptor 2 (VEGFR2), Tie1, Tie2, and vascular endothelium (VE)-cadherin (Nawroth, 2002; Moser et al., 2012). Angiogenesis is the process of formation of new blood vessels from already existing vessels by three mechanisms—non-sprouting (intussusceptive), sprouting, and stretching of existing vessels through mechanical forces (looping). The difference between the non-sprouting and sprouting mechanisms is that non-sprouting angiogenesis does not rely primarily on proliferation of endothelial cells during embryonic development. In contrast, sprouting angiogenesis depends on cell proliferation and occurs relatively slower (Makanya et al., 2008).
The formation of new blood vessels occurs through sprouting and looping. The understanding of looping mechanisms is very limited since it was only identified recently (Kilarski et al., 2009).

In this dynamic network of blood vessels, endothelial cells that line the vascular wall control the permeability of these blood vessel walls. The normal vascular permeability contributes to normal angiogenesis, blood pressure control, as well as immune responses. Abnormal increase in vascular permeability is often observed in many pathological conditions and human diseases, such as brain stroke, allergy, inflammation, and tumor-induced angiogenesis (Prager et al., 2012).

1.2 Tumor Angiogenesis

It is easy to forget that cancer is not a single disease, but instead hundreds of diseases that involve variety of molecules and signaling pathways. It starts when a cell from any part of our body starts multiplying out of control for no good purpose and, if there is no restraint, sooner or later it will create a mass of tissue called a tumor.

Angiogenesis and tumor progression are closely linked to each other. Just as embryo during development, tumor cells require a sufficient amount of blood vessels that supply nutrients and oxygen for their growth and expansion (Hanahan et al., 2006). In the
absence of angiogenesis, tumor is an avascular mass, and can remain in the state of dormancy for years. The tumor is referred to as the “angiogenic switch” (Bergers et al., 2003) when it becomes angiogenic. This switch is initiated when the balance between pro-angiogenic and anti-angiogenic factors is in favor of the pro-angiogenic molecules such as VEGF and Ang-2. This unbalance is triggered by genetic mutations, metabolic or environmental factors such as hypoxia (Carmeliet et al., 2000). In addition to genetic and epigenetic changes, the “angiogenic switch” is a process that allows rapid tumor progression. This increase in tumor vasculature can occur at any stage of tumor development depending on the tumor type and the environment. The temporal and spatial expansion of molecules that are involved in angiogenesis is not well regulated, leading to an unorganized and functionally abnormal vasculature (Figure 1). The understanding of how normal and tumor angiogenesis is regulated by these angiogenic factors is important to development of anti-cancer drugs. For example anti-cancer drug, Bevacizumab (trade name Avastin) is a humanized monoclonal antibody that inhibits the major pro-angiogenic factor, vascular endothelial growth factor A (VEGF-A) (Heinke et al., 2012).
Figure 1: Tumor Angiogenesis. Following somatic mutation (1) a small collection of tumor cells form that are avascular and remain dormant (2). The unbalance between activators and inhibitors of angiogenesis results in “angiogenic switch” and release of angiogenic factors (3) that stimulate rapid tumor growth via forming a mass of new blood vessels (4).
Tumor is dormant

1. Somatic Mutation

2. Small avascular tumor

Angiogenic switch

3. Tumor secretion of pro-angiogenic factors stimulates angiogenesis

4. Rapid tumor growth
1.3 Endothelial Cell quiescence and Adhesion

Pathological conditions such as inflammation, hypoxia and thrombosis are associated with increased vascular permeability. Endothelial cells possess several molecular mechanisms by which this permeability can be regulated. In mature vessels, the endothelium is quiescent and cells adhere tightly to each other resulting in inhibition of proliferation and a leak resistant vessel (Hordijk et al. 1999). Under these conditions cells are less sensitive to growth factors such as VEGF, angiopoietin-1 (Ang-1) and fibroblast growth factors (FGFs) and are protected from apoptosis. This condition is called endothelial cell quiescence and maintained at site of cell-cell junctions. The ability to adhere is one of the most important characteristics of endothelial cells and regulated by the proteins in adherens junctions (AJs) and tight junctions (TJs) (Taddei et al., 2008). Tight junctions seal adjacent epithelial cells in a narrow band just beneath their apical surface whereas adherens junctions provide strong mechanical attachments between adjacent cells and responsible for contact inhibition. AJs are primarily composed of VE-cadherin, which is capable of clustering and plays a vital role in endothelium integrity and regulates vascular permeability (Montera-Balaguer et al., 2009). Structurally, VE-cadherin consists of 5 extracellular domains, a transmembrane domain, and a cytoplasmic tail. The short
cytoplasmic tail acts as a docking site for signal-transduction molecules and the external domain regulates hemophilic interactions. VEGF is a fundamental regulator of vascular permeability through downstream signaling mechanisms. It has been shown that the binding of VEGF to its receptor VEGFR-2 activates the downstream phosphorylation of VE-cadherin and causes it to translocate from of cell-cell junctions thereby decreasing adhesive properties and enhancing permeability (Gavard et al., 2006). The primitive vascular plexus is formed in the absence of VE-cadherin, however mice that are null for VE-cadherin gene have major defects in vascular development, which results in embryo lethality around 9.5-10.5 E (Montera-Balaguer et al., 2009). In addition to being linked to VEGF receptors, VE-cadherin interacts with the endothelial receptor-type protein tyrosine phosphatase VE-PTP through their extracellular domains and enhances its adhesive function (Broermann et al., 2011). In the absence of VE-PTP, cell layer permeability enhances and VE-cadherin adhesive function decreases (Nottebaum et al., 2008). The fact that in VEGF stimulated cells VE-PTP dissociates from VE-cadherin, suggests that a precise balance is needed between the VEGF and VE-PTP to control the endothelial barrier integrity of VE-cadherin (Figure 2). These findings suggest that both VEGF and VE-PTP are attractive targets in treatment of pathological vascular permeability.
VE-PTP is found to associate with another cell surface membrane protein, Tie-2 that binds to the cytoplasmic phosphatase domain of VE-PTP and it is shown to dephosphorylate VE-PTP (Figure 2) (Baumer et al., 2006). Like VEGFRs, Tie-2 is a receptor tyrosine kinase (RTK), which is vital to the formation of blood vessels during the development and tumor angiogenesis. Structurally, RTKs consist of an extracellular ligand binding domain, a single transmembrane helix, a juxtamembrane domain, a cytoplasmic kinase domain and a C-terminal tail composed of multiple phosphorylatable tyrosines (Hubbard et al., 2007). RTK’s have evolved many signaling mechanisms that consist of cell surface protein receptors which serve to transduce signals from extracellular part of the cell to the inside of the cell. They initiate intacellular signals via their ability to phosphorylate themselves and other cytoplasmic proteins on tyrosine residues. Thus, understanding of how VE-PTP negatively regulates RTK’s has important therapeutic implications.
Figure 2: Schematic Representation outlining the molecular mechanism regulating the integrity of endothelial cell-cell contacts. Ang1 translocates Tie2 to cell-cell contacts, which in turn, promotes VE-PTP translocation to cell junctions. Here at cell-cell contacts, VE-PTP dephosphorylates Tie2 and VEGFR-2, which leads to inhibition of VEGFR-2 phosphorylation of VE-Cadherin resulting in increased stability of endothelial cell junctions and reduced permeability.
1.4 The VEGF/VEGFR/Neuropilin/Plexin/Semaphorin system

The cardiovasculature and nervous system form a highly branched network extending to every part of the body. VEGF signaling has crucial effects on cross-talk between the two systems, but these signaling mechanisms must be regulated on many levels to ensure the correct biological outcome (Heinke et al., 2012). Co-receptors such as neuropilins (Nrp) and (VE)-cadherin direct VEGF signals to the appropriate location and coordinates the activation of downstream molecules (18). Nrps (Nrp-1 and Nrp-2) are single-pass transmembrane proteins with a large extracellular domain and a short cytoplasmic domain that presents a PDZ binding site. Having a very short cytoplasmic domain, Nrps are not able to transduce cell signals on their own and therefore, act as co-receptors and require plexins for signaling. They were initially characterized as non-tyrosine kinase receptors that mediate axon guidance and neuronal development through class-3 semaphorins. Now it is clear that Nrps are expressed by variety of cells including EC and associate with normal and disease-associated blood vessel formation (Prahst et al., 2008). The ability of Nrps to bind to two structurally and functionally unrelated ligands, VEGF and semaphorins, suggests a dynamic interaction between angiogenesis and nervous system. Semaphorins belong to a large family of cell-associated and secreted proteins.
characterized by an extracellular N-terminal sema domain and a cysteine rich PSI (plexin, semaphoring, and integrin) domain. They mainly function as chemorepellents that direct axons away from the tissues marked by their expression though in some cases they act as chemoattractants. Plexins are a large family for cell surface receptors for semaphorins. Similarly, they contain a sema domain in the extracellular portion as well as (in general) three copies of PSI domains and three IPT. Like Nrps, plexins are also widely expressed in vasculature and nervous system.
Chapter 2

Methods

2.1 Cell Manipulations and Transfections

Human Embryonic Kidney 293 (HEK293) and human osteosarcoma cell line (U2OS) cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Serum Source International) and 5% Pen/Strep (Invitrogen). Transfections were performed in complete medium lacking the antibiotics. Cells were consistently transfected at 70-90% using Lipofectamine 2000 (HEK293) or FuGENE HD (U2OS) and Opti-MEM (Invitrogen) according to the protocol provided by Lipofectamine 2000 kit. In all co-expression experiments the ratio of each construct was 1:1.

2.2 Cellular Imaging

For the analysis of interactions, in which two separate molecules are labeled with an appropriate pair of fluorophores, an interaction can be shown by observing FRET (the two molecules must be in close proximity (~10nm), the emission spectrum of the donor must overlap with the excitation spectrum of the acceptor, and the emission dipole moment
must not be orthogonal to the acceptor absorption dipole moment). By using fluorescence imaging microscopy, one can visualize the location of green fluorescent proteins within a living cell and follow the time course of the changes in FRET corresponding to cellular events at a millisecond time resolution. The observation of such dynamic molecular events in vivo provides vital insight into the action of biological molecules (Periasamy et al., 2005).

Cell imaging was performed 24-48 hours post-transfection using Zeiss Observer Z1 wide-field microscope equipped with violet HeNe (405 nm), Argon (458, 488, 514 nm), green laser (561nm), orange (594 nm), red HeNe (633 nm). The system includes mercury arc lamp excitation, a Zeiss AxioCamMRm CCD camera with 12-bit dynamic range, fully automated xyz stage, a glycerin-immersion objective lens, 63x. In all experiments, the conditions for live cells were 37°C and 5% CO2 humid atmosphere. Fluorophore-protein fusions were imaged using excitation and emission wavelengths of 433 nm and 527 nm for mTQ and sYFP2 respectively. The AOTF’s for 433 nm and 527 nm were set to 100% and 25% respectively to eliminate cross talk between the two channels. The FRET efficiency is calculated using precision FRET (pFRET) algorithm which performs pixel-by-pixel signal bleed-through (SBT) correction resulting in removal of donor and acceptor SBT on the basis of matched fluorescence levels between double and single labeled specimens (21).
The failure to detect FRET from a pair of labeled proteins may not always mean the absence of interaction between them. Because this detection signal relies on the efficiency of FRET and choosing a donor fluorophore that has a significant overlap with the acceptor absorption is important. When measuring living cells, one of the challenges is the concentration dependence of the measured proteins, determination of which is practically impossible in living cells. Another limitation of FRET is the intensity, which is more prone to artifacts at weaker energy transfer signals. Lifetime imaging of the donor and acceptor fluorophores offer significant improvement in sensitivity for determining the physical interaction between the proteins. Fluorescence lifetime imaging monitors lifetime independent of local fluorophore concentrations and intensity but sensitive to environmental conditions such pH and ion concentration (Gardella et al., 1993). One of the limitations is the existence of different lifetimes which makes FRET determination using FLIM very difficult in living cells.

For fluorescence lifetime imaging we used Zeiss LSM 510 META NLO multi-photon laser scanning microscope (fixed stage upright) with internal (descanned) detectors (including the META detector), two direct (non-descanned) detectors, a transmitted light detector and a Becker & Hickl Fluorescence Lifetime Imaging system with 2 hybrid GaAsP detectors (for FRET-FLIM). The system has four lasers: a Spectra-Physics Mia-Tai
broadband tunable Ti:sapphire laser (710-990 nm) for multi-photon imaging, as well as Argon (458, 476, 488, 514 nm), 561 diode (561 nm), and red HeNe (633 nm). We analyzed the lifetime data measured by Fluorescence Lifetime Imaging (FLIM) using SPCImage 3.2 (Becker & Hickl). This software analyzes the raw data using an exponential model function. During the fitting process, the chi-square value between the data and the model function is minimized.

2.3 Immunoprecipitation studies

HEK293 were seeded in 10 cm dishes and allowed to grow to 80-90% confluence. Prior to transfection the media was changed to DMEM medium without penicillin. Cells were transfected with Lipofectamine and Opti-MEM (Invitrogen) according to the protocol provided by Lipofectamine 2000 kit with the appropriate vector DNA. After 48 hours the transfected cells were washed briefly and gently with PBS. Whole cell lysates were harvested by adding lysis buffer (20 mM Hepes, 150 mM NaCl, 1 mM EDTA, 1% NP-40 detergent, 1 mM Sodium Orthovanadate, complete protease inhibitor (Roche), and 1.2 mg/mL Aprotinin (Sigma-Life Science). To get rid of cellular debris the cell lysate was centrifuged for 10 minutes at 15,000 rpm and the supernatant was collected for
immunoprecipitation. Lysates were precipitated with Protein A agarose beads and incubated with Tie2, VE-Cadherin or myc antibodies (depending on the experiment performed) overnight at 4C. After washing with HBST and HBS, bounded protein was eluted from the beads by boiling in 2x Sample Buffer (10 mM Tris pH8.0, BM-t, 1% bromophenol). Cell lysates and elutes from Protein A beads were separated by 10% SDS-PAGE gel electrophoresis and electrotransferred to a nitrocellulose membrane (VWR). The membrane was blocked with milk for 1 hour and then probed with primary monoclonal antibody in TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween20) overnight. After a 15 minutes wash with TBST, the blot was probed with secondary antibody coupled to HRP for 1 hour. The blot was developed with the chemiluminescent substrate (Pierce).
Results

Chapter 3

VE-PTP and Tie2 complex formation

To observe the localization and extracellular interaction between VE-PTP and Tie2, we transiently expressed full length VE-PTP and VE-PTP lacking the phosphatase domain with Tie2 lacking the tyrosine kinase domain in transfected human embryonic kidney cell line (HEK293). The proteins were tagged with fluorophores and the localization was monitored using a Zeiss Observer Z.1 wide-field microscope coupled with fluorescence resonance energy transfer (FRET)-based proximity assay. The Tie2 construct was fused with a cyan fluorescent protein donor (mTurquoise-mTQ) to the carboxy termini in place of the catalytic tyrosine kinase domain and a yellow fluorescent protein acceptor (sYFP2) was linked to the carboxyl terminus of a full-length VE-PTP ectodomain construct (phosphatase domain present). Figure 3.1 depicts that energy transfer between the donor (mTQ) and acceptor (sYFP2) only occurs if the receptors are in close proximity (<10nm) which results in emission of sYFP2 following excitation of mTQ. The FRET efficiency is calculated using the precision FRET (pFRET) algorithm (developed by Ammasi Periasamy) which performs pixel-by-pixel signal bleed-through (SBT) correction resulting in removal of donor and acceptor SBT on the basis of matched fluorescence levels between double and single
labeled specimens (Elangoan et al., 2003). Figure 3.2 A illustrates a representative image of fluorescence intensity and membrane localization of VE-PTP-full length (FL)-sYFP2 and Tie2-mTQ. Based on our FRET-based proximity we are able to observe VE-PTP-FL and Tie2 of an average FRET efficiency value of 15.2 ± 0.89 SEM. This FRET signal suggests that the ectodomain of VE-PTP and Tie2 interact. We observed this association in the absence of angiopoietin ligand demonstrating that VE-PTP-FL and Tie2 form complex prior to ligand recognition. In addition to examining VE-PTP-FL, we also looked at the VE-PTP-FRET (Figure 3.2 B) construct, which lacks the phosphatase domain. Similarly, the protein was co-localized uniformly but did not interact based on the FRET efficiencies were significantly lower. To make sure that the strong FRET signal between VE-PTP-FL and Tie2 was not due to over-expression, we co-expressed Tie2 receptor with the functionally unrelated receptor Plexin-YFP shown in Figure 3.3 B and observed a 5.3% FRET efficiency (negative control was generated by another member of the lab, Annamarie Dalton). These findings suggest that the association between VE-PTP and Tie2 is specific and that FRET efficiency observed is not due to overexpression and nonspecific interaction. For the positive control Tie2-CFP construct was transiently transfected into HEK293 with Tie1-YFP. This known association between Tie2 and Tie1 gave a very high FRET efficiency of 66.4±2.8 SEM (Figure 3.3 B).
To confirm the association between Tie2 and VE-PTP with another technique we performed Fluorescence Lifetime Imaging (FLIM) using SPCImage 3.2 software (Becker & Hickl). When measuring living cells, one of the challenges is the concentration dependence and different intensities of the fluorophores. However, fluorescence lifetime imaging monitors lifetime independent of local fluorophore concentrations and intensity but sensitive to environmental conditions such as pH and ion concentration (Gardella et al., 1993). The viability of live-cells is critical when using high intensity light sources such as lasers. Although, high speed time-gated FLIM reduces light that gets to cells, measurements at very low light affect quantitative FLIM results. In two component FRET system, non-radiative energy transfer occurs from donor to acceptor if they are in close proximity and the fluorescence lifetime of the donor decreases. Figure 3.4 illustrates the lifetime data for Tie2-mTQ alone (A) and Tie2-mTQ co-expressed with VE-PTP (B). In average the lifetime of the double labeled cells does not decrease in our experiments (2.9 ns for both donor alone and donor in the presence of acceptor). Since numerically our lifetime data did not confirm the interaction between Tie2 and VE-PTP, we ran both the donor alone and the double labeled cells as monoexponential decay to show that the lifetime distribution histogram clearly indicates that the donor in the presence of the acceptor is not monoexponential decay. This “shoulder like” peak observed in the
histogram suggests the quenching of the donor and the possible occurrence of FRET between VE-PTP and Tie2. In addition, we have made several attempts to show the interaction between Tie2 and VE-PTP by co-immunoprecipitation assays. HEK293 cells, which lack endogenous Tie2 and VE-PTP receptors, were transiently transfected with Tie2-myc alone, VE-PTP-myc alone or a combination of two. As seen in Figure 3.5A, western blotting of whole cell lysates with anti-myc antibodies demonstrate expression of Tie2, VE-PTP and both in combination. The co-immunoprecipitation of Tie2, VE-PTP and both Tie2 and VE-PTP are shown in 3.5 B (total protein loaded in each lane was 1 mg). All three were IP’ed with Tie2 antibody and probed with anti-myc and therefore we expected to observe Tie2 expression (lane 1) and no VE-PTP expression (middle lane). When both Tie2 and VE-PTP were co-expressed (lane 3), we expected to see two bands (one for Tie2 and one for VE-PTP) since both constructs were fused to myc, IP’ed with Tie2 antibody and probed with myc. The relative ratio was determined and the calculated value was arbitrarily set equal to 1.0 for VE-PTP alone. Values are graphically displayed below each respective lane.
Figure 3.1: Schematic Representation of FRET-based proximity assay of interaction between VE-PTP and Tie2. The extracellular domains are fused to GFP variants, the VE-PTP construct tagged with sYFP2 and Tie2 tagged with mTQ. Due to the proximity of the receptors, upon excitation of mTQ at 433nm, non-radiative energy transfer occurs to sYFP2, resulting in sYFP2 emission.
Figure 3.2: In vivo FRET images of VE-PTP-Tie2 interaction. Tie2-mTQ construct was transiently transfected into HEK293 with VE-PTP-FL-sYFP2 (A) or VE-PTP-FRET-sYFP2 (B). FRET efficiency values were determined within the regions of interest (ROI) and averaged with ±SEM.
**A**

![Image](image1)

Tie2-mTQ  
VE-PTP-FL-sYFP2  
Merged

FRET efficiency  
15.2 ± .89% SEM

**B**

![Image](image2)

Tie2-mTQ  
VE-FRET-sYFP2  
Merged

FRET efficiency  
2.9 ± .92% SEM
Figure 3.3: In vivo FRET images positive and negative controls. A) Tie2-CFP construct was transiently transfected into HEK293 with Tie1-YFP as a positive control. B) Tie2-mTQ and Plexin-YFP were transiently transfected into HEK293 as a negative control. FRET efficiency values were determined within the regions of interest (ROI) and averaged with ±SEM.
A

Tie2-CFP

Tie1-YFP

Merged

FRET efficiency
66.4±2.8 SEM

B

Tie2-mTQ

Plexin-YFP

FRET efficiency
5.3%
Figure 3.4: The lifetime data of Tie2-mTQ and VE-PTP. Tie2-mTQ alone (A) and Tie2-mTQ co-expressed with VE-PTP (B) measured by Fluorescence Lifetime Imaging (FLIM) using SPCImage 3.2 software (Becker & Hickl).
A

Tie2-mTQ

B

Tie2-mTQ VE-FL-PTP-sYFP
Figure 3.5: Cell Lysate and Immunoprecipitation of Tie2 and VE-PTP. (A) Cell Lysate of Tie2 alone, VE-PTP alone and both combined. (B) HEK293 cells were transfected with Tie2-myc alone, VE-PTP-myc alone or both proteins combined, harvested and lysates probed with anti-myc. The relative ratio was determined and the calculated value was arbitrarily set equal to 1.0 for VE-PTP alone. Values are graphically displayed below each respective lane.
A

<table>
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<tr>
<th>Cell Lysate</th>
<th>Tie2</th>
<th>VE-PTP</th>
<th>Tie2-VE-PTP</th>
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B

<table>
<thead>
<tr>
<th>Tie2-myc</th>
<th>+</th>
<th>-</th>
<th>+</th>
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<tbody>
<tr>
<td>VE-PTP-myc</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Blot: α-myc | IP: Tie2

[Graph showing data points]
Chapter 4

VE-Cadherin and Tie2 complex formation

To observe the localization and extracellular interaction between VE-Cadherin and Tie2 in vivo similar FRET assay as described above for VE-PTP and Tie2 was used. Both proteins were linked to GFP variant fluorophores to visualize the localization. The Tie2 construct was fused with a cyan fluorescent protein donor (mTurquoise-mTQ) and a yellow fluorescent protein acceptor (Venus) was linked to the VE-Cadherin construct. Both proteins were expressed in transiently transfected HEK293 and imaged to look for the occurrence of FRET. The FRET efficiency is calculated similarly using precision FRET (pFRET) algorithm. Couple different scenarios were observed with this complex. We saw a co-localization with FRET efficiency of 14.9±1.3 SEM which suggests a possible association between of VE-Cadherin and Tie2. However, we also observed the protein not co-localizing in cells co-expressed with these two constructs. Figure 4.1 illustrates the protein co-localizing with FRET signal of 14.9±1.3 SEM in not a confluent dish (A) or at cell-cell junctions (B). We also saw the protein not co-localizing at either confluent (data not shown) or cell-cell junction (C). Our studies in U2OS suggested no interaction between VE-Cadherin and Tie2 since the protein did not co-localize and therefore there was no FRET signal (4.1D). Figure 4.2 illustrates the lifetime data for Tie2-mTQ alone (A) and Tie2-mTQ
co-expressed with VE-Cadherin-Venus (B). In average the lifetime does not decrease in our experiments (2.9 ns for both donor alone and donor in the presence of the acceptor). However, although we ran both the donor alone and the double labeled cells as monoexponential decay, the lifetime distribution histogram clearly shows that the donor in the presence of the acceptor is not monoexponential decay. This "shoulder like" peak observed in the histogram suggests the quenching of the donor and the possible occurrence of FRET between VE-Cadherin and Tie2. We were able to show the interaction between VE-Cadherin and Tie2 by co-immunoprecipitation assays shown in Figure 4.3. Telomerase-immortalized human microvascular endothelial (TIME) cells which has endogenous VE-Cadherin and Tie2 were harvested, lysed, and probed with anti-Tie2 (4.2A) As expected, we were able to observe Tie2 expression in whole cell lysate (lane 3) and no expression in the negative control (middle lane). Lane 1 was IP'ed with VE-Cadherin antibody, probed with Tie2. The reciprocal was performed with anti-VE-Cadherin antibody. Similarly, we were able to express VE-Cadherin (lane 1) and observed no expression in the negative control (middle lane). The relative ratio was determined and the calculated value was arbitrarily set equal to 1.0 for the negative control. Values are graphically displayed below each respective lane (total protein loaded for IP was 1mg).
Figure 4.1: In vivo FRET images of VE-Cadherin-Venus with Tie2 interaction. Tie2-mTQ construct was transiently transfected into HEK293 with VE-Cadherin-Venus showing co-localization of the protein on the cell membrane (A) or at cell-cell junction (B) or no co-localization (C). Tie2-mTQ construct was transiently transfected into U2OS with VE-Cadherin-Venus showing no co-localization (D). FRET efficiency values were determined within the regions of interest (ROI) and averaged with ±SEM.
A

Tie2-mTQ  VE-Cadherin-Venus  Merged

FRET efficiency
14.6 ± 1.3% SEM

B

Tie2-mTQ  VE-Cadherin-Venus  Merged

FRET efficiency
14.6±1.3%SEM
Figure 4.2: Lifetime data of Tie2 and VE-Cadherin. Tie2-mTQ alone (A) and Tie2-mTQ co-expressed with VE-Cadherin-Venus (B) measured by Fluorescence Lifetime Imaging (FLIM) using SPCImage 3.2 software (Becker & Hickl).
A

Tie2-mTQ

B

Tie2-mTQ-VE-Cadherin-Venus
Figure 4.3: Immunoprecipitation of VE-Cadherin and Tie2. TIME cells were harvested, lysed, and probed with anti-Tie2 (A) and the reciprocal was performed with anti-VE-Cadherin (B). The relative ratio was determined and the calculated value was arbitrarily set equal to 1.0 for the negative control. Values are graphically displayed below each respective lane.
A

IP: α-VE-Cadherin  IP (-)  Cell Lysate

Blot:Tie2

B

IP: α-Tie2  IP (-)  Cell Lysate

Blot: VE-Cadherin
Chapter 5

VE-Cadherin and VE-PTP complex formation

We performed similar assay to examine the localization and extracellular interaction between VE-Cadherin and VE-PTP in vivo. We examined few differently tagged constructs of VE-PTP and VE-Cadherin and all showed a very unique mutually exclusive localization of the protein. VE-PTP-FL-mTQ was co-expressed with VE-Cadherin-Venus showing mutually exclusive localization of the protein when the cells are confluent (5.1 A) and mutually exclusive behavior in isolated cells (5.1 B). This unusual mutually exclusive localization of the protein was also observed with VE-Cadherin-mTQ co-expressed with VE-PTP-FL-sYFP2 and VE-FRET-sYFP2 (data not shown). Figure 5.2 illustrates the lifetime data for VE-PTP-FL-mTQ alone (A) and VE-PTP-FL-mTQ co-expressed with VE-Cadherin-Venus (B). In average the lifetime does not decrease in our experiments (2.8 ns for both donor alone and donor in the presence of the acceptor). However, although we ran both the donor-acceptor and the donor alone as monoexponential decays, the lifetime distribution histogram clearly shows that the double labeled specimen is not monoexponential decay.

In addition, we made several attempts to co-express all three, VE-PTP, VE-Cadherin, and Tie2 in HEK293. VE-PTP-FL-sYFP2 and VE-Cadherin-mTQ were co-expressed with Tie2 fused to myc. We observed mutual exclusive localization of the protein as shown in figure 5.3 but unfortunately Tie2-myc appeared to be toxic to cells and we were unable to obtain statistically enough data to fully characterize the behavior of the protein.
Figure 5.1: In vivo FRET images of VE-Cadherin-Venus and VE-PTP interaction. VE-Cadherin-Venus construct was transiently transfected into HEK293 with VE-PTP-FL-mTQ showing mutually exclusive localization of the protein in a confluent dish (A) or mutually exclusive behavior in isolated cells (B) FRET efficiency values were determined within the regions of interest (ROI) and averaged with +/- SEM.
A

VE-PTP-FL-mTQ  VE-Cadherin-Venus  Merged

B

VE-PTP-FL-mTQ  VE-Cadherin-Venus  Merged
Figure 5.2: The lifetime data of VE-PTP and VE-Cadherin. VE-PTP-mTQ alone (A) and VE-PTP-mTQ co-expressed with VE-Cadherin-Venus (B) measured by Fluorescence Lifetime Imaging (FLIM) using SPCImage 3.2 software (Becker & Hickl).
A

VE-PTP-FL-mTQ

B

VE-PTP-FL-mTQ-VE-Cadherin-Venus
Figure 5.3: In vivo FRET images of VE-Cadherin, VE-PTP and Tie2 interaction. VE-PTP-FL-sYFP2, VE-Cadherin-mTQ and Tie2-myc constructs were transiently transfected into HEK293 showing mutually exclusive localization of the protein.
Merged (VE-Cadherin-mTQ+
VE-PTP-FL-sYFP2+Tie2-myc)

VE- Cadherin-mTQ
VE-PTP-FL-sYFP2
Chapter 6

Neuropilin/Plexin/Semaphorin system

We tested several constructs of Nrps and Plexins with various linker lengths to obtain the best FRET efficiency signal in HEK293. In addition to difference in linker size, we tested different members of PlexinA family. Neuropilin-1 with relatively small linker size of five residues and Plexin-A1 with nine amino acid residues gave the most reliable results. Nrp-1 tagged with mYFP was co-expressed with PlexinA1 tagged with mCFP in HEK293. We observed a co-localization of the protein with FRET efficiency 13.8±5.3SEM HEK293 cells expressing Nrp-1 and PlexinA1 were induced with Semaphorin3D (Sema3D) and the images were taking every five minutes for one hour. The role of Sema3D on this Nrp/Plexin complex is not well understood. Based on evidence provided by studies on the nature of Nrp-1/Plexin-A1 complex formation, we hypothesized that Sema3D has the ability to change the morphology of the cells. Class 3 semaphorins are known to have a high-potency growth cone-collapsing activity in neuronal cells and morphologic changes in nonneuronal cells (Luo et al., 1993; Tamagnone et al., 1999). Based on our observations, Sema3D caused expansion in size and rounding up of Nrp-1/PlexinA1 expressing cells. In addition, we observed the protein dissociation and clustering in some parts of the cells as shown in figure 6.1. This morphological change was observed over the first 5-10 minutes after the ligand addition. The addition of Semaphorin3D also induced FRET signal in the two experiments we performed. However, we did not average this increase since in one experiment the FRET signal increased by two fold and in the other one by six fold. The FRET signal before adding the ligand was significantly different for both experiments as
well. From our experiments it is clear that addition of Sema3D to Nrp-1/Plexin complex is able to mediate the morphologic changes in nonneuronal HEK293 cells.
Figure 6: In vivo FRET imaging of Nrp-1 and PlexinA1 complex induced with Semaphorin 3D in HEK293. Nrp-1-mYFP construct was transiently transfected into HEK293 with PlexinA1-mCFP. Semaphorin 3D was added and images were taken every five minutes for 60 minutes (the images of every 20 minutes are shown).
Sema3D (500ng/mL)

mPlxnA1
mCFP

mNrp-1
mYFP

Merged

0min  20min  40min  60min
Chapter 7

Conclusions

Fluids, cells, nutrients and oxygen are normally exchanged between the blood compartments and the surrounding tissues. In this dynamic network, endothelial cells that line the vascular wall control the permeability of these blood vessel walls. The normal vascular permeability contributes to normal angiogenesis, blood pressure control, as well as immune responses. Abnormal increase in vascular permeability is often observed in pathological conditions and human diseases, such as tumor-induced angiogenesis, inflammation, allergic reaction, and brain stroke. This vascular permeability is regulated by adhesive molecules and receptors that co-localize at cell-cell contacts (Kilarski at el., 2009).

What happens when the adhesion molecules meet the receptors? Receptors such as RTK’s are located on the cell surface helping cells to communicate with their environment and carry signals to the intracellular machinery. It used to be known that the binding of ligands triggers the activation of the receptors, now it is clear that cell adhesion molecules significantly contribute to this activation process (Hanahan at el., 1996). The understanding of this tight collaboration between cell adhesion molecules and RTK’s has important therapeutic implications.

It has been shown that receptors such as VE-PTP and Tie2 regulate permeability through its interaction with an adhesive molecule VE-Cadherin. Ang-1 is an important player in this scenario by translocating Tie2 to cell-cell contacts, which, in turn, promotes VE-PTP translocation from vesicular compartments in the cell, to the cell-cell contacts. Here
at cell-cell contacts, VE-PTP dephosphorylates Tie2 and VEGFR-2 which leads to inhibition of VEGFR-2 phosphorylation of VE-Cadherin and inhibition of EC proliferation. The clear picture of the role of these players in normal angiogenesis and tumor angiogenesis is still unclear. Further investigation of VE-PTP, VE-Cadherin and Tie2 will reveal possible applications in treatments of angiogenic diseases (Winderlich et al., 2009).

It is known that VE-PTP interacts with Tie2 primarily in contacting cells decreasing permeability. For ease of transfection and cellular imaging, we have done most of our experiments in HEK293 cells based upon their lack of endogenous Tie2 and VE-PTP (Vestweber et al., 2008). Our data shows the association between VE-PTP and Tie2 in HEK293 with FRET efficiency of 15.2 ± 0.89 SEM, compared to the negative control, which gave a result of 5.3% FRET efficiency. This FRET signal leads us to believe that the ectodomain of VE-PTP and Tie2 interact. As discussed above Ang1 translocates Tie2 to cell-cell contacts and therefore Ang1-activated Tie2 decrease permeability by introducing more VE-PTP to the intercellular junctions. We observed VE-PTP-Tie2 association in the absence of Ang1 demonstrating that VE-PTP-FL and Tie2 form a complex prior to ligand recognition. Our positive control (co-expressed Tie1 and Tie2) gives a very high FRET efficiency of 66.4 ± 2.8 SEM (Figure 3.3B) indicating that further biochemical and biophysical studies have to be done to characterize the interaction between the Tie2 and VE-PTP ectodomains. One of the essential future directions is to show the formation of VE-PTP-Tie2 complex in endothelial cells, which express the proteins endogenously.

VE-PTP-Tie2 complex formation was also shown using Fluorescence Lifetime Imaging Microscopy (FLIM) which is a powerful tool to map protein-protein interactions in living specimens. As discussed in chapter1 fluorescence lifetime is unaffected by the
change in probe concentration or excitation intensity. However, FLIM is a technically challenging technique because it is influenced by any small changes in the cellular environment and the existence of different lifetimes makes FRET determination using FLIM very difficult in living cells. Although, it is expected to observe the decrease of lifetime in the presence of the acceptor, we were unable to show that numerically. However, the lifetime distribution histogram clearly showed the quenching of the donor in the presence of the acceptor, which indicates the possible FRET occurrence between VE-PTP and Tie2.

Several attempts were made to detect Tie2-VE-Cadherin binding by transiently expressing them in HEK293 and performing co-immunoprecipitation assays. Unfortunately, despite repeated attempts in changing the concentrations, using different constructs and different antibodies, we were unable to show a clear indication of binding of these two receptors in transient system. One of the most promising data was obtained when both Tie2 and VE-PTP were fused to myc and we were able to see two bands (one for Tie2 and one for VE-PTP) when both were transiently overexpressed in HEK293 and co-immunoprecipitated (Figure 3.5).

Among all the mechanisms that regulate vascular permeability of endothelial cells, tyrosine phosphorylation plays an essential role. It is generally accepted that this tyrosine phophorylation of VE-Cadherin is observed in weak junctions, which usually leads to pathological conditions. The extent of this phosphorylation is declined when cells reach confluency (Lambugnani et al., 1997). However, the exact mechanism of VE-Cadherin phosphorylation has not been fully clarified. The extracellular interaction between VE-PTP and VE-Cadherin has been shown (Nawroth et al., 2002). Surprisingly, our data showed a very unique mutually exclusive interaction (Figure 5.1A and B) in both confluently
isolated cells. We believe that this relationship between tyrosine phosphorylation and AJ opening had an effect on our data because we observed localization and no-localization in both confluent and not very confluent cells when with VE-Cadherin and Tie2 complex. Our FRET data of this complex showed a co-localization with FRET efficiency of 14.9±1.3 SEM and a significant part of our data showed no co-localization of the protein in both HEK293 and U2OS cell lines. Similarly, the numeric part of the lifetime data was not promising but the lifetime distribution histogram clearly showed the quenching of the donor in the presence of the acceptor and possible occurrence of FRET between VE-Cadherin and Tie2. For immunoprecipitation studies, crude protein lysates were prepared from TIME cells expressing endogenous Tie2 and VE-Cadherin. As shown in Figure 4.3 when used for immunoprecipitation, we were able to precipitate endogenous Tie2 and VE-Cadherin.

In addition, we made several attempts to co-express all three, VE-PTP, VE-Cadherin, and Tie2 in HEK293. VE-PTP-FL-sYFP2 and VE-Cadherin-mTQ was co-expressed with Tie2 fused to myc. We observed mutual exclusive localization of the protein (Figure 5.3) but unfortunately Tie2-myc appeared to be toxic to cells and we were unable to obtain statistically enough data to fully characterize the protein behavior. The failure to detect FRET from a pair of labeled proteins may not always mean the absence of interaction between them. Mainly because we are working with overexpressed system and there are many factors that have effects on our data such as the transfection efficiency, fluorophore concentrations, intensity, environment and etc.

Another focus of our studies was to better understand the Neuropilin/Plexin/Semaphorin system. This signaling system is involved in the regulation of various developmental events including axonal extension, cell migration in the nervous system,
growth of endothelial cells, and cardiac development. Neuropilins are known for their ability to bind to class 3 semaphorins using plexin as a co-receptor to activate downstream signal transduction cascades (Capparuccia at el., 2009). Not much is known about the effect of Sema3D on Nrp/plexin complex. The aim of our study is to characterize the protein localization, morphological changes and FRET signal upon of this complex upon stimulation with Sema3D. We first examined several constructs of Nrp and plexin with various linker lengths to determine the optimal pair that gave the best FRET efficiency signal (13.8%±5.3 SEM, this is a negative control with no Sema added). HEK293 cells expressing Nrp-1 and PlexinA1 showed a morphology change after addition of Semaphorin 3D. Based on our observations, Sema3D caused expansion in size and rounding up of Nrp-1/PlexinA1 expressing cells. In addition, we observed that the protein dissociated and clustered in some parts of the cells and the FRET signal in the two experiments we performed was 22.5%±4.5 SEM upon stimulation with Sema3D. (Figure 6). Based on our observations Sema3D has effects on the association of Nrp and plexin. As we discussed in the introduction Nrps have the ability to bind to both semaphorins and VEGF, which are two structurally and functionally, unrelated ligands suggesting a tight collaboration between angiogenesis and the nervous system. In this process VE-Cadherin along with neuropilins plays an essential role by directing VEGF signals to the appropriate location and coordinating the activation of downstream molecules. Further studies are needed to investigate the Nrps/Plexin complex behavior upon addition of semaphorins and VEGF.
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Vita

In 2010 graduated with Bachelor of Science in Chemistry and enrolled in a graduate program at Virginia Commonwealth University, in the department of Biochemistry and Molecular Biology.