

Characterization of A-Type Ephrin Signaling
by

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B.Sc, University of Victoria, 2004

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Abstract

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Membrane attachment of ephrin ligands plays an important role in Eph receptor activation. Membrane anchorage is thought to provide a clustering effect to ephrins that is necessary for stimulation of Eph receptor kinase activity. The presence of soluble A-type ephrin in conditioned media of numerous cultured cancer cell lines and normal endothelial cells prompted me to question the purpose of ephrin release. In this thesis I show that ephrin A1, a potent angiogenic factor, is released from several cancer cell lines and is a substrate for tissue transglutaminase, a multifunctional enzyme with the ability to form covalent crosslinks between substrate proteins. I show that tissue transglutaminase crosslinking primes soluble ephrin A1 to promote Eph A2 activity. These results suggest a role for soluble A-type ephrins in promoting Eph receptor activity at distant sites and also indicate that ephrin A1 may be acting as a soluble angiogenic factor during tumor neovascularization.

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Chapter 1 - Introduction

1.1 Overview and Hypotheses

In 1987, Hirai et al identified a novel putative tyrosine kinase receptor gene in an erythropoietin-producing human hepatocellular carcinoma cell line (ELT-1). They named the gene *eph*(erythropoietin-producing hepatocellular) and defined its protein product as the Eph receptor, a new class of receptor tyrosine kinase. Quickly, multiple family members of this receptor were identified, such that it now forms the largest class of mammalian receptor tyrosine kinases. There are 14 Eph receptors in mammals (Murai and Pasquale 2003). These receptors have been divided into two classes, A and B, based on sequence similarities and binding preferences for their membrane anchored ligands, the ephrins. There are 8 Eph A (Eph A1-8) and 6 Eph B receptors (Eph B1-6). A-type Eph receptors bind preferentially to A-type ephrins, whereas B-type Eph receptors bind to B-type ephrins, and binding within each class is promiscuous (Himanen et al 2004). There are exceptions to class specificity. For example Eph A4 has been shown to bind to ephrin B2, and Eph B2 has been shown to also bind to ephrin A5 (Himanen et al 2004) (Brors et al 2003). The ligands are distinguished from one another by their method of attachment to the cell membrane. A-type ephrins are attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor; B-type ephrins are single-span transmembrane proteins and contain a cytoplasmic C-terminal tail that is used for signaling. In mammals, there are five A-(ephrin A1 –A5) and three B-type (ephrin B1-B3) ephrins. Not surprisingly, given the size of the family and importance of receptor tyrosine kinases in

general, mammalian Eph receptors and ephrins are important for many developmental and adult processes.

The original function attributed to Eph receptors was to guide growing neural processes toward their appropriate targets through repulsive mechanisms (Flanagan et al 1998). In general, the Eph receptors and ephrins are thought to act at sites of cell-cell contact where they mediate signals between Eph expressing cells and ephrin expressing cells (Himanen and Nikolov 2003). Additional functions of Eph family of receptor tyrosine kinases have emerged, and these functions also require the Eph receptor-expressing cell to be in close contact with the ephrin-expressing cell. Eph receptor/ephrin expression is widespread, and they have been found to be involved in neural development, cell morphogenesis, tissue patterning, angiogenesis, neural plasticity, stem cell niche maintenance, brain size determination, and insulin secretion (Flanagan et al 1998) (Klein 2004) (Depaepe et al 2005) (Konstantinova et al 2007). The direct cellular consequences of Eph signaling generally include effects on the dynamics of cellular protrusions and affect cell migration by modifying the organization of the actin cytoskeleton and cell adhesion properties (Klein 2004). Also, some Eph receptors may influence cell proliferation and cell fate determination (Pasquale 2005). Interestingly, the Eph receptors and ephrins are overexpressed in several human carcinomas, which suggested that they are involved in neoplastic processes (Surawska et al 2004). This thesis is focused on the regulation of A-type ephrins, and their potential to be released from the cell surface and oligomerized by a covalent crosslinking enzyme, called tissue transglutaminase. Specifically I hypothesized the following:

- 1) A-type ephrins can be released from the cell surface and this soluble ligand is important for ephrin signaling.
- 2) Soluble A-type ephrins are substrates for tissue transglutaminase, a crosslinking enzyme, which forms covalent isopeptide bonds between glutamine and lysine side chains in its substrates.
- 3) Transglutaminase crosslinking of A-type soluble ephrin functions to “activate” A-type ephrins, effectively clustering ephrins such that they are able to bind to and stimulate Eph A receptor activity.

I have shown that ephrin A1 is released from the cell surface of cultured cells, and that ephrin A1 is a substrate for transglutaminase crosslinking. Furthermore, my data show that transglutaminase-oligomerized ephrin is able to promote the migration and invasion of Eph A2-expressing malignant carcinoma cells (HeLa). These results support my hypotheses. In broader terms, these results suggest that A-type ephrin and Eph receptor signaling may not be confined to sites of cell-cell contact and may also participate in paracrine signaling through the activity of transglutaminase. If this work can be extended *in vivo* to determine the extent and requirement for transglutaminase crosslinking of ephrin, this work will represent a significant paradigm shift in our view of how A-type Eph receptors and ephrins function. In this chapter, I review Eph receptor/ephrin signaling, specifically focusing on the molecules participating in Eph receptor/ephrin signaling. Chapter two is focused on my methodology while in chapter three I present my research results. In Chapter four, I discuss the implications of my results and in Chapter five I suggest future directions for this research.

1.2 Eph Receptor architecture

All Eph receptors have an extracellular region containing an N-terminal globular domain, which is necessary and sufficient for ligand binding, (Labrador et al 1997) and folds into a compact jellyroll β -sandwich (Himanen et al 1998) (Figure 1). The ligand-binding domain is followed by a cysteine-rich region and two fibronectin type three repeats. Although the cysteine-rich and fibronectin domains do not participate in ligand binding, they are thought to contribute to the stability of the Eph-ephrin complex by aiding in receptor dimerization, once the initial contact with ligand has been made (Lhotak and Pawson 1993) (Himanen et al 2001). Following a membrane-spanning region, the cytoplasmic part of the receptor contains a juxtamembrane segment containing key tyrosine residues that become phosphorylated upon receptor activation. The juxtamembrane sequences have two functions: the first is as a regulatory domain. The second is a substrate, which upon phosphorylation, acts to recruit downstream signaling molecules into a multi-protein complex. As a consequence of ephrin binding, Eph receptor kinase activity is stimulated. Three highly conserved autophosphorylation sites are particularly important for kinase activity (Figure 1). The first of these tyrosine residues is located in the kinase activation loop, while the remaining two are in the juxtamembrane region. Tyrosine phosphorylation within the activation loop of the kinase domain displaces the activation loop such that substrates are able to enter the catalytic cleft. However, phosphorylation of the juxtamembrane region also plays a role in regulation. Binns et al (2000) found that the phosphorylation of all three tyrosine residues is required for full kinase activity. Substitution of the juxtamembrane tyrosine residues of Eph A4 with phenylalanine creates a mutant receptor with a 10-fold decrease in its ability to phosphorylate substrates compared with wild-type Eph A4.

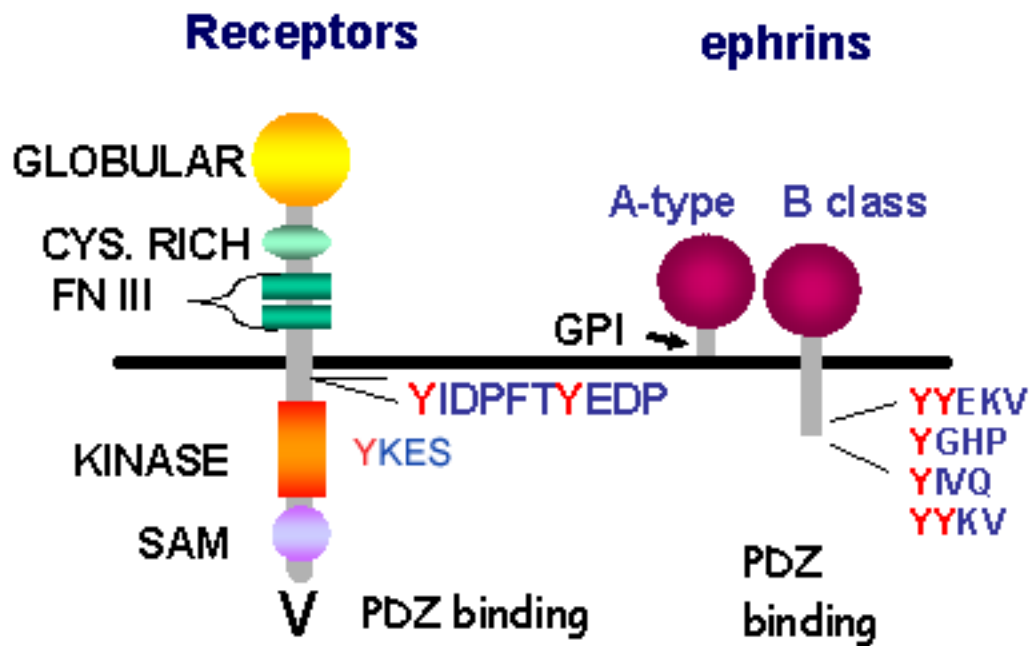


Figure 1. Conserved motifs in receptor and ligand.

The Eph receptors have a conserved domain structure. They are divided into A-type and B class based on sequence homology and binding preference for membrane bound ephrin ligands. A-type ephrins are bound to the membrane with a GPI anchor while B class ephrins exist as transmembrane proteins.

Wybenga-Groot et al (2001) provided the structural basis for this regulation. In the X-ray crystal structure of Eph B2, they found that in its unphosphorylated form, the juxtamembrane region of the receptor adopts a helical structure that distorts the small lobe of the kinase domain, which prevents the kinase domain from achieving an activated conformation. Phosphorylation of the juxtamembrane tyrosine residues relieves this catalytic inhibition, while at the same time providing docking sites for SH2 and PTB domains of signaling proteins.

The kinase domain is next to the juxtamembrane region, and has a typical bi-lobe kinase structure containing a small N-terminal domain and a larger C-terminal domain (Himanen et al 2001). The ATP binding and catalytic site are formed at the interface of the two subdomains. C-terminal to the kinase domain is a sterile alpha motif (SAM) domain and a postsynaptic density protein/disc large/zona occludens (PDZ) binding motif. The functional relevance of the latter two regions (SAM and PDZ) is unclear. Several protein partners have been identified for the SAM domain and PDZ motif, suggesting the two regions participate in downstream signaling. For example, Stein et al (1998) found that the SAM domain of Eph B1 is tyrosine phosphorylated and recruits low-molecular-weight phosphatase (LMW-PTP). The last 6 amino acids (PDZ motif) of several Eph receptors (Eph A7, B2, B3, B5, B6) have been shown to interact with the PDZ domain of the Ras-binding protein AF6 (Hock et al 1998). In addition the C-terminus of Eph B2 has been shown to bind to the PKC- α interacting protein, PICK1, and to GRIP, a protein that is a concatamer of 7 PDZ domains. The C-terminus of Eph A7 interacts with syntenin (Torres et al 1998). These PDZ-dependent interactions are thought to participate in receptor clustering.

In addition to a role in protein interactions, there is a potential role for the Eph SAM domain in receptor oligomerization. Stapleton et al (1999) examined the crystal structure of the Eph A4 receptor SAM domain and showed that the SAM domain formed a homodimer. They suggest that the SAM domain may contribute to the formation and stabilization of ligand-induced Eph oligomers through self-association. Similarly, Behlke et al (2001) found that at high concentrations the SAM domains from Eph B2 receptors were able to form higher order clusters. Taken together, these studies suggest a role for SAM domains in the activation of Eph receptor signaling by facilitating the formation of higher order clusters. Although there is a great deal of evidence to suggest the SAM domain and PDZ motif contribute to Eph receptor function, their role in Eph signal transduction is not essential. Using genetically engineered mice in which the SAM domain and PDZ motif are deleted, Park et al (2004) and Kullander et al (2001) have shown that loss of these regions has no significant impact on Eph function *in vivo*.

1.3 Ephrin architecture

The extracellular domains of A and B-type ephrins are structurally very similar to one another. They all comprise an 8-stranded β -barrel Eph binding domain, which is related to the multi-copper oxidase-like domain (Himanen et al 2001). The receptor-binding domain is held away from the membrane by 37-40 disordered amino acids that form a stalk, lifting the interaction interface away from the cell surface (Figure 1). In addition, within this stalk region, the ephrins contain an ADAM10 metalloprotease and disintegrin cleavage site. Protease cleavage of this site is dependent upon receptor binding. Hattori et al (2000) showed that the binding of clustered Eph A3 to ephrin A2 activates the cleavage of ephrin A2 from the cell surface by the metalloprotease

ADAM10/Kuzbanian. The release of ephrin A2 from the cell surface allows the disengagement of cells. Janes et al (2005) further investigated this process and found that the formation of functional Eph A3/ephrin A5 complex creates a new molecular recognition site for kuzbanian, which allows for effective ephrin cleavage. They further showed that kuzbanian is constitutively associated with the receptor on the receptor-expressing cell and that cleavage occurs in trans. This is the first demonstration of a trans cleavage mechanism for a metalloprotease.

A-type ephrin ligands are bound to the membrane through a GPI-anchor. A GPI anchor is a lipid anchor consisting of an inositol phospholipid, and an oligosaccharide with at least one phosphoethanolamine substitution. The anchor is synthesized within the endoplasmic reticulum (ER) prior to transfer, and is transferred in bulk to the C-terminus (ω site) of substrate proteins after cleavage of the C-terminal propeptide. Anchor attachment to the substrate occurs within the ER, and GPI anchored proteins are then sorted into vesicles destined for the plasma membrane. For example, in ephrin A1, the GPI anchor is attached to Serine 183. This modification results in deletion of 23 hydrophobic amino acids from the C-terminus of ephrin A1 (Figure 2).

B-type ephrin ligands are transmembrane proteins. Structurally they are related in their extracellular domains to the A-type ephrins, however there are important differences that account for the subtype specificity (Himanen et al 2001). Similar to the A-type ephrins, the β -barrel receptor-binding domain of B-type ephrins is held away from the cell surface by a stalk region. Following the stalk region, there is a single-span hydrophobic transmembrane domain. A short, highly conserved cytoplasmic tail extends into the cytoplasm and contains several conserved tyrosine residues that can serve as

phosphotyrosine docking sites for downstream signaling proteins (Figure 1) (discussed in reverse signaling section). The cytoplasmic tail also contains a C-terminal PDZ-binding motif, which has been shown to recruit various PDZ-domain proteins through a (YKV) target site (Torres et al 1998) (Lin et al 1999). For example, PDZ-containing proteins known to interact with B class ephrins include Syntenin, Pick1, Grip (Torres 1998), Fap-1, Phip (Lin et al 1999), PTP-BL (Palmer et al 2002), Grip2 (Bruckner et al 1999), Par-3 (Etemad-Moghadam et al 1995) and PDZ-RGS (Lu et al 2001). Many of these proteins also have roles in the control of the actin cytoskeleton and cellular guidance. For example, PDZ-RGS3 binds B-type ephrins through its PDZ domain and uses its regulator of heterotrimeric G protein signaling (RGS) domain to mediate signaling. This interaction of PDZ-RGS3 is proposed to inhibit SDF-1-stimulated chemoattraction of cerebellar granule cells (Lu et al 2001). The proposed mechanism is that PDZ-RGS3 recruitment to the cytoplasmic tail of B class ephrins inhibits heterotrimeric G protein signaling through the GAP activity of its RGS domain. Thus Eph/ephrin signaling can modulate signals from other receptors including G-protein coupled receptors.

1.4. Ephrin clustering through membrane effects and soluble ephrin.

Membrane attachment of both A and B-type ephrins plays an important role in Eph receptor activation. Membrane attachment is thought to provide a clustering effect to ephrins that is necessary for robust stimulation of Eph receptor kinase activity (Davis et al 1994). This limits the distance that this receptor-ligand pair can signal. In fact it is widely held that Eph-ephrins function strictly at points of close cell-cell contact. This is consistent with Eph receptor/ephrin expression patterns, which are frequently expressed in a complementary fashion between neighbouring cells (Gale et al 1996).

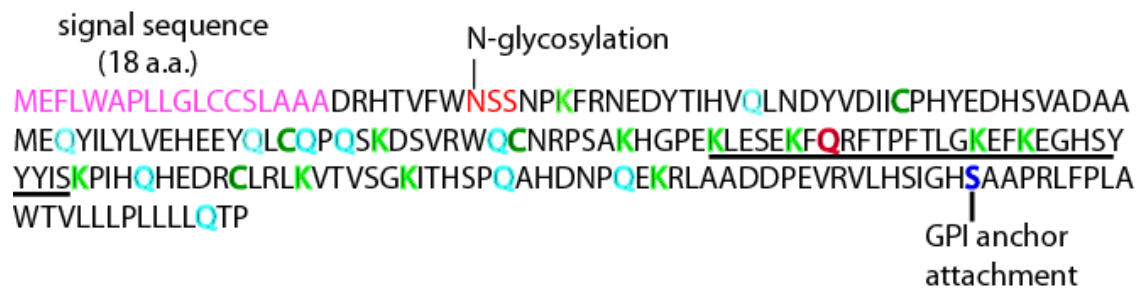


Figure 2. *Mus musculus* ephrin A1 protein sequence.

The ephrin protein is made as a pre-protein with an N-terminal signal sequence (pink) and a C-terminal GPI anchor attachment sequence. The signal and GPI anchor sequences are cleaved posttranslationally. Ephrins have a highly conserved N-glycosylation site, as well as 4 cysteine residues (dark green) that are present in all family members. Although ephrins are typically glycosylated, glycosylation is not necessary for receptor binding. Ephrin A1 is rich in lysine and glutamine residues. One glutamine residue (red) is present within a core-conserved region (underlined), which is present in all A-type ephrins. After removal of the signal sequence and hydrophobic C-terminus, ephrin A1 is a 165 amino acid protein with a predicted molecular weight of 18 kDa plus the mass of the GPI anchor and glycosylation.

Unlike other receptor tyrosine kinases that become active through dimerization, Eph receptors require higher order oligomerization. The membrane is thought to provide this oligomerization through microdomain organization and protein interactions between the Eph/ephrins and the cytoskeleton. A caveat to the requirement for ephrin membrane attachment is that A-type ephrins, in particular ephrin A1, ephrin A4, and ephrin A5, can be released from the cell surface through metalloproteases (Hattori et al 2000), phospholipases, or through alternative splicing (Aasheim et al 2000). The best studied of the soluble ephrins are ephrins A1 and A5. In fact, ephrin A1 was originally isolated as a secreted protein product of tumor necrosis factor alpha (TNF α)-stimulated endothelial cells, and was subsequently shown to be involved in TNF α induced angiogenesis (Dixit et al 1990) (Pandey et al 1995). This early work provided evidence for the existence of soluble ephrins (Bartley et al 1994). However, this work did not determine whether soluble or membrane attached ephrin A1 was actively involved in this angiogenic response, and subsequent studies showed that soluble monomeric ephrin was unable to activate Eph kinase activity (Davis et al 1994). Thus, based on this evidence, ephrin activity is proposed to be limited to points of cell-cell contact. In keeping with the inability of monomeric ephrin to stimulate Eph kinase activity, studies of soluble ephrin A5 showed that addition of soluble ephrin A5 to cortical neuron-astrocyte co-cultures inhibited the stimulation of axon bundling by clustered ephrin A5 (Winslow et al 1995). Thus, in certain instances soluble ephrins may antagonize Eph receptor signaling by binding to, but not activating, Eph receptor activity. These findings have led to the widely held view that soluble ephrins are a minor event, and either do not participate in Eph function or serve a minor regulatory role. An alternative, hypothesis is that soluble

ephrins are oligomerized by non-membrane-dependent mechanisms, which allows the soluble ephrins to actively engage Eph receptors at sites other than direct cell-cell contact.

1.5 Bidirectional signaling

The interaction of the Eph receptor with its cognate ephrin ligand brings about conformational change in the receptor, which results in activation of the kinase domain and autophosphorylation of the receptor. The signals that originate from the activated receptor are important for bringing about changes in the receptor-expressing cell, and frequently result in changes to the actin cytoskeleton. A unique feature of Eph/ephrin signaling is that, in addition to contact between Eph receptor and ephrin causing changes in the receptor-expressing cell, contact also causes signaling events in the ephrin-expressing cell. In essence, both the Eph receptor and ephrin act as receptors and ligands for one another. Engagement of the receptor-ligand pair generates a bi-directional response, eliciting a signal in both the ligand-expressing (reverse signaling) and the receptor-expressing (forward signaling) cells (Holland et al 1996) (Bruckner et al 1997).

1.5 a) Forward Signaling

The major consequence of Eph receptor activation is reorganization of the actin cytoskeleton. The effects of Eph receptors on the actin cytoskeleton are mediated by small GTPases of the Rho family, including RhoA/B, Rac1/2 and Cdc42. These GTPases control cell shape by promoting the formation of stress fibers (Rho), lamellipodia (Rac) and filopodia (Cdc42) (Ridley 2001). They alternate between an active GTP-bound state and an inactive GDP-bound state (Noren and Pasquale 2004). The GTPases are primarily regulated by the opposing effects of two classes of enzymes, the dbp family of guanine

nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Dbl GEFs activate GTPases by enhancing the exchange of GDP for GTP (Shamah et al 2001). In its GTP-bound state, GTPases are able to bind and regulate effector molecules. On the other hand, GAPs inactivate RhoGTPases by enhancing their intrinsic GTPase activity (Shamah et al 2001). Eph receptors and ephrins exert their influence on the cytoskeleton by recruiting and regulating the activity of GEFs and GAPs.

The principle target of Eph A receptors appears to be Rho. In culture experiments, inhibitors of RhoA and its downstream effector, serine/threonine kinase Rho kinase (ROCK), strongly reduced the rate of ephrin A5 induced growth cone collapse in retinal ganglion cells (Wahl et al 2000). One manner in which Eph A receptors regulate Rho GTPases is by recruiting and regulating the RhoGEF, ephexin. For example, expression of a dominant negative form of ephexin, interferes with ephrin A5 induced growth cone collapse (Shamah et al 2001). Ephexin has been shown to interact directly with Eph A4 in a manner independent of receptor kinase activity. The association with Eph A4 is dependent upon the catalytic Dbl homology-pleckstrin homology (DH-PH) domain of ephexin. In primary neurons, Eph A4 activation leads to an enhancement of ephexin activity towards RhoA, and an inhibition of ephexin activity towards rac1 and Cdc42 (Shamah et al 2001). These activities lead to changes in cell morphology. Specifically, the activation of RhoA and suppression of Rac1 and Cdc 42 activity by ephexin through Eph A4 activation leads to actin-myosin contractility, growth cone collapse, and axon retraction in neuronal cells (Noren and Pasquale 2004).

In vascular smooth muscle cells, the main RhoGEF responsible for RhoActivity is vascular smooth muscle-specific RhoGEF (VSM-RhoGEF) (Ogita et al 2003). This

RhoGEF is closely related to ephexin and has been shown to interact with Eph A4 in vascular smooth muscle cells. Similarly to ephexin, this interaction is mediated by VSM-RhoGEF's DH-PH domains and is independent of kinase activity. Ephrin A1 activation of Eph A4 in VSMCs increases the GEF activity of VSM-RhoGEF for RhoA, which increases RhoA activation and smooth muscle contractility in these cells (Ogita et al 2003). In human kidney epithelial cells, Eph A3 activates RhoA through a CrkII-dependent mechanism and causes cell rounding, blebbing and de-adhesion (Lawrenson et al 2002).

In addition to RhoA, Eph A receptors can also target Rac activity directly, in either a positive or negative manner. For example, Deroanne et al (2003) found a role for Rac and PAK in ephrin A1 induced inhibition of endothelial cell spreading. Inhibition of RhoA partially rescued the effect suggesting that RhoA is only a minor component. In contrast, blocking Rac1 signaling led to a significant amplification of the action of ephrin A1, implying ephrin A1 negatively regulates this GTPase. In contrast, in certain endothelial cell types, Eph A receptor activity towards Rac is PI3-kinase dependent and induces activation of Rac1. Expression of a dominant negative form of either PI3-kinase or Rac1 inhibits ephrin A1 induced endothelial cell migration (Brantly-Sieders et al 2004).

Eph B receptors exert their effects on the actin cytoskeleton primarily through regulating the activities of Rac1 and Cdc42. For example, several Eph B receptors have been shown to interact with the GEF, intersectin-1, and regulate Cdc42 activity. Similar to the interactions of ephexin and Vsm-RhoGEF with Eph A4, intersectin associates with the kinase domain of Eph B2 independently of receptor activation, through interactions

with the N-terminal region of intersectin-1 (Irie and Yamaguchi 2002). The interaction between Eph B receptors and intersectin is important for Cdc42 dependent reorganization of hippocampal dendritic spines, and suggests that Eph B receptors may participate in Long Term Potentiation (LTP), a model for learning and memory (Irie and Yamaguchi, 2002). Similarly, Kalarin, a Rac1 directed GEF, has also been shown to mediate Eph B signaling in neurons and thereby participate in dendritic spine morphogenesis (Penzes et al 2003).

Eph receptors/ephrins and Ras family proteins

The activity of most RTKs leads to the direct activation of the Ras/Mitogen activated protein kinase (MAPK) cascade. In contrast, Eph receptors typically do not activate Ras. In fact, in most instances, Eph receptors suppress Ras signaling. For example, Eph A2 activation by ephrin A1 has been shown to inhibit Ras/MAPK cascade in fibroblasts, prostatic epithelial cells, endothelial cells and tumor cells (Miao et al 2001). Similarly, Eph B2 suppresses Ras activity and induces neurite retraction in NG108 neuronal cells (Elowe et al 2001). The ability to suppress Ras activity can modulate the affects of other growth factors. For example, in human umbililcal vein endothelial cells (HUVECs) ephrin B2 stimulation of Eph B receptors can suppress Vascular Endothelial Growth Factor (VEGF) and Angiopoietin 1 (Ang-1) induced Ras/MAPK signaling (Kim et al 2002). The ability of Eph receptors to regulate Ras/MAPK signaling cascades appears to be mediated by the Ras GTPase activating protein, RasGAP (Kim et al 2002) (Elowe et al 2001) (Tong et al 2003). However, direct phosphorylation of R-ras by Eph B2 has also been shown inhibit Ras activity (Zou et al 1999).

For every rule in biology there are exceptions, and there are a few instances where Eph receptors appear to activate Ras/MAPK pathways rather than inhibit them. For example, Pratt et al (2002) found that active Eph A2 can interact with SH2 and PTB domains of SHC, which mediates an indirect interaction of Eph A2 with GRB2, a positive regulator of MAPK pathways. This Eph A2/SHC/GRB2 complex leads to an Eph A2 mediated activation of the ERK kinases in certain fibroblast cultures. It has also been shown that the overexpression of Eph B receptors in T cells leads to the activation of Erk1/Erk2 MAP kinases (Zisch et al 2000) (Vindis et al 2003) (Luo et al 2002). Thus spatial, temporal, and developmental context may influence the pathways activated downstream of Eph receptors.

Eph Receptors and interactions with other signaling molecules

In addition to members of the Rho and Ras family members, Eph receptors influence many other signaling molecules that regulate cell behavior. These include focal adhesion kinase (FAK) and Nck. Fak is constitutively associated with Eph A2 (Miao et al 2000) and Eph A2 phosphorylation of Fak can lead to its activation and stimulation of integrin inside-out signaling. For example, ephrinB1 stimulation of Eph B1 enhanced $\alpha_5\beta_1$ integrin-mediated cell adhesion in human embryonic kidney cells through a Fak-dependent mechanism (Cowan and Henkemeyer 2001). The recruitment of Nck to Eph B1 has similarly been shown to affect integrin signaling (Becker et al 2000) and is dependent upon Nck's ability to recruit Nck interacting kinase (Nik) (Becker et al 2000). In addition, Nck recruitment to Eph receptors can affect actin cytoskeleton remodeling by modulating Pak3 and WASP (Becker et al 2000) (Holland et al 1997). Finally, Eph

receptors have also been shown to recruit other protein tyrosine kinases such as Src family kinases and Abelson tyrosine kinase (Abl) (Wang and Kruh 1996).

1.5 b) Reverse Signaling

One of the most unique and interesting features of Eph/ephrin signaling is bi-directional signaling. The Eph and ephrins are the only class of RTK that function in this manner. The concept of bi-directional signaling for Eph/ephrin molecules was first shown by Holland et al (1997) and Bruckner et al (1997). These two papers demonstrated that B-type ephrins become tyrosine phosphorylated upon stimulation with Eph-Fc ectodomains and elicit a response in the ephrin B2-expressing cells. The importance of reverse signaling *in vivo* was demonstrated by Henkemeyer et al (1996). Using a knockout and knockin approach, this group demonstrated that genetic disruption of Eph B2 in mice leads to an axon guidance defect in the commissural tract of the anterior commissure. Instead of crossing the midline and tracking laterally to innervate regions within the cortex, these axons mis-migrate and track ventrally towards the hypothalamus. By replacing the Eph B2 gene with an Eph B2- β -Gal fusion, in which the entire cytoplasmic region of the Eph B2 receptor was replaced with β -Gal, Henkemeyer et al (1996) were able to rescue the axonal migration defects of an Eph B2 knockout. They further showed that the misguided axons in the Eph B2 knockout expressed ephrin B1 ligand, and not the mutant receptor. Thus, in a non-autonomous manner, restoring the reverse signal with the Eph B2- β gal fusion was sufficient to rescue the migration defects. This was the first demonstration that reverse signaling through ephrins is functionally important *in vivo*.

In B-type ephrins a consequence of ephrin binding to an Eph receptor is the phosphorylation of key tyrosine residues on the cytoplasmic tail of the ephrin by cytoplasmic tyrosine kinases. Signal transduction pathways initiated from the B class ephrins involve interactions of SH2 domain containing adaptor proteins with these phosphotyrosine residues or PDZ domain containing proteins with the C terminal PDZ binding domain. Palmer et al (2002) found Src family kinases to be positive regulators of ephrin B phosphorylation in primary endothelial cells and cortical neurons. They also found that with delayed kinetics, B-type ephrins recruit a PDZ-containing protein tyrosine phosphatase PTP-BL to their PDZ binding domain and become dephosphorylated. This suggests a possible mechanism for the activation and deactivation of reverse signaling or a mechanism which allows a switch from phosphorylation-dependent signaling to PDZ-dependent signaling. Cowan and Henkemeyer (2001) showed that that Grb4/Nck2 is recruited to activated ephrin clusters and interacts with phosphorylated ephrinB1 tails through its SH2 domain. Consequences of the interaction between ephrinB1 and Grb4 include an increase in FAK catalytic activity, the loss of adhesive foci, and the disassembly of actin stress fibers. There are a multitude of signaling proteins that bind to the SH3 domains of Grb4 and could mediate these and other cellular responses. Most of these proteins have roles in regulating cellular adhesion and actin dynamics and include Abl (Coutinho et al 2000), CAP (Cowan and Henkemeyer 2001), p21 activated kinase (Pak) (Manser et al 1994) and a multidomain signaling protein DOCK 180 (Tu et al 2001).

Reverse Signaling and A-type Ephrins

As discussed, A-type ephrins are connected to the cell surface by a GPI anchor and therefore do not directly contact the cytoplasm. Therefore, it was unexpected to find that reverse signaling is also important with this class of ephrin. The first evidence that reverse signaling through A-type ephrins may be important came through cell culture studies of ephrin A5 expression in fibroblasts, which showed that receptor stimulation of ephrin A5 promoted cell adhesion. This was shown to be dependent upon the activation of Src family kinases, in particular, Fyn, and resulted in activation of integrins through an inside-out mechanism (Davy et al 1999). In follow up studies, Davy and Robbins (2000) and Huai and Drescher (2001) demonstrated that changes in cell adhesion were dependent upon the prolonged activation of Erk1/2 by ephrin A5. Intriguingly, Huai and Drescher (2001) isolated lipid rafts and showed that a 120-kDa protein becomes tyrosine phosphorylated after A-type ephrin activation. The identity of this molecule and the putative receptor remains unknown. Genetic evidence of reverse signaling through A-type ephrins was provided by studies of *Caenorhabditis elegans*. In *C. elegans*, there is a single Eph receptor, Vab-1, and 4 A-type ephrins. Loss of Vab-1 causes embryonic lethality due to a ventral closure defect. In a pair of papers, Chin-Sang et al (2002) and Wang et al (1999) showed that kinase activity of Vab-1 was not required, which suggested reverse signaling through the GPI ephrins may be important. How A-type ephrins communicate with Src family kinases is a mystery. One possibility is that A-type ephrins may interact with an unidentified transmembrane receptor. This would be similar to reverse signaling through contactin, a GPI-linked protein, which is mediated by Caspr, a transmembrane protein (Peles et al 1997).

1.6 Functions of ephrin/Eph family signaling

As discussed, the principle target of Eph/ephrin signaling is the actin cytoskeleton. However, cellular response of Eph/ephrin signaling can be difficult to predict and is complicated by the fact that engagement of an Eph/ephrin pair on adjacent cell surfaces can elicit either cell-cell adhesion or repulsion. Very little is known about the signaling events that trigger a switch from adhesion to repulsion. The distinction between repulsion and adhesion does not appear to be cell type-dependent but rather may be dependent on cellular context and degree of ephrin/Eph clustering. For example, Cooke et al (2005) found that both repulsive and adhesive cues mediated by Eph A4 are required for hindbrain segmentation in zebrafish. Cellular responses may be controlled by the degree of ephrin oligomerization, which may, in turn, influence the recruitment of different signaling complexes to the Eph/ephrin pair (Stein et al 1998). For example, Huynh-Do et al (1999) found that Eph B1 discriminates ephrinB1 surface density to direct integrin-mediated cell attachment. Also, Hansen et al (2004) showed that retinal axon response to ephrin A2 shifted from attraction to repulsion as the concentration of ephrin A2 increased on the surface of target cells. These two studies lend support to the idea that the degree of ligand clustering may play a role in the transition from adhesion to repulsion. Another study done by Dravis et al (2004) revealed that the transduction of both forward and reverse signals in the same cell is required for a pro-adhesion response. Furthermore, Yin et al (2004) propose that the adhesion to repulsion switch is made when both ligand and receptor are expressed on the same cell surface. They found that when an Eph A receptor and its ephrin A ligand are expressed on the same cell surface, it inhibits that receptor from interacting with its ligand on an adjacent cell. They suggest that this may mediate cell repulsion.

Early studies examining the expression patterns of Eph receptors and their ligands during embryogenesis revealed important roles for these molecules in forming and stabilizing the spatial organization of tissues. These studies also showed that in early development, the embryo is divided into broad domains defined by the complementary and mutually exclusive expression of Eph receptors and their corresponding ligands. Additionally, each ligand may have stage and tissue specific interactions with one or multiple receptors. For example, Gale et al (1996) stained whole mouse embryos with either soluble receptor bodies to show embryonic distribution of their ligands or soluble ligand bodies to define the embryonic distribution of their corresponding receptor. They found that the distribution of a given receptor subclass was complementary to the distribution of its corresponding ligand subclass. Also, the embryonic distribution of Eph receptors and ligands appeared to be dynamic, with dramatic changes occurring as development proceeded. The most striking compartmentalization seen by Gale et al (1996) was in the developing brain. For example, they found ventral expression of Eph B receptors in the developing forebrain, midbrain, and hindbrain with dorsal expression of ephrin B ligands in these areas. Compartmentalization of the midbrain along the anteroposterior axis by Eph A receptors and ligands was also seen.

1.6 a) Axon Guidance

One of the roles of Eph receptors and ephrins in the brain is in axon guidance. Both forward and reverse signaling are important for this process. Yokoyama et al (2001) found that ephrin B3 acts as a midline repellent through its ability to activate forward signaling through the Eph receptors in corticospinal axons. Conversely, Mendes et al

(2006) found that bidirectional signaling by B type Eph receptors and ephrins is important for axon guidance during the development of the corpus callosum.

The retinotectal system has been widely used to demonstrate how Eph receptors and ephrins regulate the formation of topographic maps through complementary expression gradients. In the mammalian retinotectal system, retinal axons project to the superior colliculus in the midbrain. Retinal ganglion cells in the nasal retina project their axons to the posterior part of the superior colliculus, while retinal ganglion cells in the temporal retina project to the anterior colliculus. Also, ventral axons project to the medial or dorsal colliculus. According to Sperry's chemoaffinity hypothesis, a graded distribution of molecules in the tectum and retina give positional information that guide retinal axons to the appropriate targets (Sperry 1963). Due to the graded expression of ephrins and Eph receptors in this area and the repulsive cues they provide, they are prime candidates for a role in retinotectal mapping. Indeed, Cheng et al (1995) found that each retinal ganglion cell expresses a different level of A-type Eph receptors along the nasotemporal axis, while two closely related ephrins, A2 and A5, are expressed in overlapping gradients in both the superior colliculus and lateral geniculate nucleus. Axons with high expression level of receptor terminate in areas with low ligand expression whereas axons with low levels of receptor expression terminate in areas with high ligand expression. Consistent with a requirement for ephrin/Eph gradients for this process, Frisén et al (1998) showed ephrin A5 knockout mice have mapping defects. Similarly, Nakamoto et al (1996) showed that overexpression of ephrin A2 in the chick tectum caused retinal axons to form abnormal retinotectal maps.

1.6 b) Segmentation and Cell migration

A second developmental process in which Eph receptors and ephrins have been shown to play an important role is in the segmentation of the developing embryo. Two major segmentation events that occur during vertebrate development are the segmentation of the paraxial mesoderm and the segmentation of the hindbrain. Both of these processes require Eph/ephrin mediated cell repulsion, which allows for the formation of sharp boundaries between cell populations.

The segmentation of the paraxial mesoderm leads to the formation of somites during vertebrate development. Somites later give rise to migration paths for neural crest cells and spinal nerve axons and eventually produce vertebrae, ribs, dermis of the dorsal skin, skeletal muscles of the back and skeletal muscles of the body wall and limbs (Palmer and Klein 2003). Several Eph receptors and ephrins are expressed in somites, and involved in somitogenesis. These include ephrin B2 (Bergemann et al 1995), ephrin A1, ephrin A5, ephrin A4, ephrin A2 (Gale et al 1996). The formation of somites requires changes in cell morphology and adhesive properties that are aided by Eph/ephrin mediated cytoskeletal rearrangements. For example, Durbin et al (1998) found that the disruption of Eph signaling in zebrafish results in the abnormal formation of somite boundaries. Schmidt et al (2001) found that the loss of Eph A4 signaling results in cells of irregular morphology and that somites fail to form.

Ephrins also play an important role in hindbrain segmentation during vertebrate development. During hindbrain development there are eight rhombomeres that form. Odd numbered rhombomeres have distinct cellular properties from even numbered rhombomeres, and cell movements between even and odd rhombomeres is restricted (Guthrie and Lumsden 1991). Repulsive interactions between Eph receptors and ephrins

are important in the formation and maintenance of rhombomeres. Mellitzer et al (1999) found that interactions between Eph receptors and ephrins regulate both the restriction of cell intermingling and communication between cells in different rhombomeres. Eph receptors and ephrins are expressed in complementary rhombomeres; ephrins in even numbered rhombomeres (Flenniken et al 1996) and Eph receptors in odd numbered rhombomeres (Becker et al 1994). Cooke et al (2001) found that in zebrafish repulsive interactions between Eph B4 and ephrin B2 are important for cell sorting and boundary formation in the caudal hindbrain. Xu et al (1994) used a dominant negative approach in *Xenopus* and zebrafish embryos to show that Eph A4 is necessary for rhombomere boundary formation.

1.6 c) Eph/ephrin signaling and stem cells

One of the most interesting recent developments in this field is the demonstration of the importance of Eph receptors and ephrins signaling to stem cells (Suenobu et al 2002) (Tumbar et al 2004) (Aoki et al 2004) (Lickliter et al 1996). Batlle et al (2002) found that in the small intestine Eph B/ephrinB signaling controls the positioning of stem cells along the crypt-villus axis, which in turn determines the exposure of these cells to proliferative factors which emanate from the bottom of the crypts. Furthermore, in a recent study by Holmberg et al (2006), Eph B receptors within the intestine were shown to control the proliferation of the progenitor cells within the crypts. In the hematopoietic system, Wang et al (2002) showed that Eph B4 signaling accelerates the transition of primitive cells from a stem cell to a lineage-restricted progenitor phenotype. Also, Eph B4 affects the rate differentiation in hematopoietic cells, endothelial cells and cardiomyoblasts (Wang et al 2004). Eph B4-deficient embryonic stem cells produced

significantly fewer of all of these cell types. It has also been revealed that certain hematopoietic progenitor cells express Eph B4 and when those cells are co-cultured with stromal cells expressing ephrin B2 these cells differentiate into mature erythroid cells (Suenobu et al 2002). In the brain, Holmberg et al (2005) found that interactions between ephrin A2 and Eph A7 negatively regulate progenitor cell proliferation. This response was mediated mainly by ephrin A2 reverse signaling. By negatively regulating progenitor cell proliferation, ephrins may provide a feedback between cells of different maturation states in the stem cell niche and modulate the number of cells generated in the brain. These studies give a just a few examples of the emerging roles of Eph receptors and ephrins in the regulation of the balance of stem cell renewal versus differentiation and fate determination.

1.6 d) Angiogenesis

Angiogenesis is the development of new blood vessels from existing vasculature and occurs during development and in adult tissues. In adult tissues angiogenesis normally only occurs during the female menstrual cycle and during wound healing yet it does also occur in many pathologies, such as diabetic retinopathy, arthritis, psoriasis and cancer.

During blood and lymphatic vessel formation, endothelial cells undergo shape changes including elongation, flattening and assembly into tubular structures. Eph receptors and ephrins play an important role in the control of actin cytoskeleton dynamics and contribute to angiogenesis in part by contributing to changes in endothelial cell shape. Wang et al (1998) also found that during development, ephrin B2, which is expressed in arterial endothelial cells, and Eph B4, which is expressed in venous

endothelial cells, control segregation and positioning of arterial and venous endothelial cells. Adams et al (1999) went further to show that Eph/ephrin signaling between endothelial cells and surrounding mesenchymal cells is also important in vasculogenesis, and that these signals involve Eph B2/B3 and ephrin B2 and Eph B4. Ephrin A1 has also been found to be expressed at sites of vascular development in the mouse (McBride and Ruiz 1998) and is present on adult endothelial cells (Pandey et al 1995). Ephrin A1 and its receptor Eph A2 have been shown to be potent angiogenic factors both *in vitro* and *in vivo*. Ephrin A1 was originally identified by Holzman et al (1990) as a soluble factor released from human umbilical vein endothelial cells (HUVECs) after TNF α stimulation. This led to the idea that these proteins participate in mediating the response of vascular endothelium to proinflammatory cytokines. Since then several studies have looked at the role of this ligand receptor pair in angiogenesis. Ephrin A1 has been shown to be upregulated by VEGF and that soluble Eph A2-fc inhibits VEGF induced endothelial cell migration, sprouting and survival *in vitro* and angiogenesis *in vivo* (Cheng et al 2002). Ephrin A1 is known to have a strong effect on endothelial cell behavior. For example, Pandey et al (1995) found that ephrin A1 induces endothelial cell migration and corneal neovascularization in rats. Cheng et al (2002) found similar results when they implanted hydon pellets impregnated with ephrin A1 into the corneas of mice. Daniel et al (1996) discovered that endothelial cells assembled capillary like structures in response to ephrin A1. It has also been shown Eph A2 is a major regulator of the angiogenic process in adult endothelial cells and that it is required for angiogenic remodeling in mature tissues in response to ephrin A1 (Brantley-Sieders et al 2004).

1.6 e) Eph receptors/ephrins and Cancer

Many Eph receptors and ephrins are upregulated and/or overexpressed in cancer. One of the best characterized examples of this is Eph A2. Eph A2 had been found to be upregulated in several cancers, such as cancer of the breast, liver, ovary, and prostate, glioblastoma, melanoma and esophageal squamous cell carcinoma (Vogt et al 1998) (Fox and Kandpal 2004) (Walker-Daniels et al 1999) (Zelinski et al 2001) (Kinch et al 1998). Zelinski et al (2001) showed Eph A2 to be a powerful oncoprotein, which conferred malignant transformation and tumorigenic potential when overexpressed on non-transformed mammary epithelial cells. Rather than increasing cellular growth rates, Eph A2 overexpression increased the invasive and metastatic potential of these cells as illustrated by their behavior in semi solid agar and matrigel. Interactions of this Eph receptor with members of the Rac/Rho family of small GTPases, which alter the organization of the actin cytoskeleton may contribute to these effects. Also, due to the role of tissue disorganization and aberrant cellular adhesion in more advanced stages of cancer, Eph expression may increase a tumor's invasive and metastatic potential through interactions with cell adhesion molecules such as E-cadherin. Zantek et al (1999) found Eph A2 to be tyrosine phosphorylated and localized to sites of cell-cell contact in nontransformed epithelial cells with normal E-cadherin expression. However, in breast cancer cells, which lack E-cadherin, Eph A2 was redistributed to membrane ruffles and had a lower level of phosphorylation. When Eph A2 was activated by E-cadherin expression or pre-clustered ligand there was a decrease in cell-extracellular matrix adhesion and cell growth.

Another way in which Eph receptors and ephrins are involved in cancer is during the process of tumor neovascularization. As discussed previously, Eph A2 and its ligand

ephrin A1 are potent angiogenic factors. Ogawa et al (2000) found ephrin A1 and Eph A2 to be present throughout tumor vasculature of both xenograft tumors and surgically removed human tumors. They also found Eph A2 to be activated in xenograft tumors, which suggests an interaction with ephrin A1. A study by Brantley et al (2002) found complementary expression of ephrin A1 in tumor cells and Eph A2 in tumor associated blood vessel endothelium. They also showed that soluble Eph A2 receptors could inhibit tumor angiogenesis and progression in an *in vivo* model. Brantley-Sieders et al (2006) found that when ephrin A1 was knocked down in metastatic mammary tumor cells there was a reduction in tumor-induced endothelial cell migration *in vitro* and microvascular density *in vivo*. They also show that the overexpression of ephrin A1 elevated wild-type, but not Eph A2-deficient, endothelial cell migration toward tumor cells which indicates that the activation of Eph A2 on endothelial cells by ephrin A1 is important for ephrin A1 mediated angiogenesis. These are just a few studies, which highlight an important role for Eph A2 and its ligand ephrin A1 in tumor angiogenesis.

The presence of soluble A-type ephrins in conditioned media of numerous cultured cancer cell lines and normal endothelial cells prompted us to question the purpose of ephrin release, and to examine mouse tissues and embryos to determine the extent of soluble ephrin expression *in vivo* (Bartley et al 1994) (Dixit et al 1990) (Pandey et al 1995). Our findings led us to investigate whether soluble A-type ephrins are substrates for tissue transglutaminase, a multifunctional enzyme with the ability to form covalent crosslinks between amino acid side chains in its substrates. Transglutaminases are a family of enzymes that catalyze the formation of isopeptide bonds between the gamma-carboxamide groups of glutamine residues and the epsilon amino groups of

lysine residues in their target substrates (Mangala et al 2005). Expressed both intracellularly and extracellularly, tissue transglutaminase is unique from other family members in that it functions as a transglutaminase, a GTPase (Iismaa et al 1997), and a Serine/Threonine kinase (Mishra et al 2004). GTPase and kinase activities are independent of crosslinking activity and are regulated by calcium. Calcium binding promotes crosslinking activity and suppresses GTPase and kinase activities (Iismaa et al 1997) (Iismaa et al 2000) (Liu et al 2002). Additionally, extracellular tissue transglutaminase is a coreceptor for fibronectin binding to $\alpha_5\beta_1$ integrin (Akimov et al 2000) (Akimov et al 2001) and may be a ligand for GPR56, an orphan G protein-coupled receptor (Xu et al 2006). Tissue transglutaminase has a wide range of intercellular crosslinking substrates including latent transforming growth factor binding protein (LTBP) (Munger et al 1997), fibronectin, and insulin growth factor binding protein 1 (IGFBP1) (Sakai et al 2001). Originally it was thought that transglutaminase activity was only active during apoptosis (Piacentini et al 1991), however, it is now known that transglutaminase plays a role in several cellular processes, including adhesion, migration, and extracellular matrix remodeling (Mangala et al 2005).

My thesis work shows that A-type ephrins are substrates for tissue transglutaminase crosslinking activity and provide evidence that the soluble A-type ephrins can be oligomerized *in vivo*. I have shown that tissue transglutaminase oligomerizes soluble A-type ephrins to promote Eph receptor activity. Transglutaminase crosslinking of ephrin increases the migration of HeLa cells endogenously expressing Eph A2 kinase receptor. These results suggest a role for soluble A-type ephrins in

promoting Eph receptor activity at distant sites in addition to sites of direct cell-cell contact.

Chapter 2 - Methods

2.1 Membrane and tissue preparation

For western blot analysis, embryos and tissues from ICR mice were harvested and snap frozen in liquid nitrogen. Powdered extract was prepared by crushing the frozen samples using a chilled mortar and pestle. The extract was then lysed in PLC lysis buffer (50 mM Hepes pH 7.5, 150mM NaCl, 10% glycerol, 1% Triton X-100, 1.5mM MgCl₂, 1mM EGTA, 10mM sodium pyrophosphate, 100mM sodium fluoride, 10µg/ml aprotinin, 10µg/ml leupeptin, 1mM sodium vanadate, and 1mM PMSF) and analyzed by SDS-PAGE followed by western blotting.

For crude membrane fractionation, tissue was homogenized in buffer containing 50 mM Tris-HCl pH 8.0, 0.1mM EDTA, 150mM sodium chloride, 0.1mM sodium vanadate, 0.2mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, and 1mM dithiothreitol (DTT). The homogenate was centrifuged at 100,000 x g for 2.5 hours at 4°C. The supernatant containing the soluble fraction was brought to 0.5% NP-40 and 5% glycerol and set aside. The pellet containing crude membranes was dissolved in 50mM Tris-HCl (pH 8.0), 0.5 % NP-40, 10% glycerol, 0.1mM EDTA, 150mM sodium chloride, 0.1mM sodium vanadate, 0.2 mM PMSF, 10µg/ml aprotinin, 10µg/ml leupeptin, and 1mM DTT. This suspension was rotated on a nutator at 4°C overnight. The insoluble material was removed by centrifugation at 25 000 x g.

For GPI/lipid raft fractionation, crushed tissue was homogenized in 50mM Hepes (pH 8.0), 150mM sodium chloride, 1mM magnesium chloride, 1mM EGTA, 1% triton X-100, 100mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium vanadate, 10 µg/ml aprotinin, 10µg/ml leupeptin, and 1mM PMSF. The homogenate was centrifuged

at 1,300 x g at 4 °C for 20 minutes to remove cell debris. To the cleared supernatant, an equal volume of 85% sucrose (w/v) in 50mM Hepes (pH 8.0), 150mM sodium chloride, and 1mM magnesium chloride was added. A step gradient of 5%/30%/42.5% (with sample) was created and centrifuged at 200,000 x g in a SW 41 Beckman Rotor for 24 hours at 4°C. Fractions were collected from the bottom of the tube and examined by SDS-PAGE followed by western blot analysis.

2.2 Antibodies/Western blotting

A rabbit polyclonal antibody was raised against a peptide (FDVNKVENSLPAC) within the C-terminus of mouse ephrin A5. The antibody for ephrin A1 was purchased from Sigma (Oakville, On). This antibody was developed in goat using purified recombinant mouse ephrin-A1 extracellular domain, expressed in mouse NSO cells, as an immunogen. For western blot analysis, 50µg of sample was added to 2x sample buffer and separated by SDS-PAGE (7.5%, 10%, or 12% polyacrylamide). Proteins were then electroblotted onto PVDF or nitrocellulose, blocked with 5% milk in Tris-buffered saline, 0.1% Tween 20 (TBST), probed with the appropriate primary antibodies, and detected using horseradish-peroxidase (HRP)-conjugated secondary antibodies and Enhanced Chemiluminescence (Amersham, RPN2132).

2.3 Pull down and Transglutaminase Assays

The ligand-binding domain (LBD) of Eph A4 (residues 22-209) was cloned in-frame and C-terminal to GST using the pGEX 4T1 expression vector. Fusion proteins were expressed using a BL21 *E. coli* expression system and purified over glutathione sEph Arose. Ephrin A1-Fc (1 µg) or control Fc was crosslinked using 0.01 units of tissue

transglutaminase (Sigma) in 10mM Tris-HCl pH 7.4, 10mM CaCl₂, and 3mM DTT at 25°C for four hours. Reaction products were centrifuged at 20,800 x g for ten minutes to pellet any insoluble aggregates. Control reactions were also performed in the absence of transglutaminase or in the presence of 5mM EGTA to chelate Ca²⁺. For some pull down experiments, transglutaminase reactions were carried out in the presence of 14μM biotinylated-monocadaverine (Sigma-Aldrich). Monocadaverine, which is frequently used as an amine donor in transglutaminase reactions, did not block ephrin crosslinking, and incorporation of biotinylated-monocadaverine into the reaction mixture allowed for easy detection using streptavidin-HRP (Amersham). The interaction of crosslinked ephrin with GST-Eph A4 LBD was independent of monocadaverine addition, which was confirmed in pull down assays in which monocadaverine was omitted from the transglutaminase reaction. The supernatants of this reaction were incubated with 1.5μg GST-Eph A4 LBD or GST alone for five hours at 4°C with gentle shaking. Glutathione sepharose beads (pre-incubated with human Fc) were added to each reaction mixture and allowed to incubate overnight at 4°C. Glutathione beads were pelleted, washed three times with PBS, and the bound fraction was eluted with SDS-PAGE sample buffer. Eluates were resolved by SDS-PAGE and probed with streptavidin-HRP.

2.4 Ephrin A5 crosslinking

An adenovirus expressing human ephrin A5 was used to infect HeLa cells (MOI 100:1) and incubated at 37°C, 5% CO₂ for 24 hours. The next day the cells were washed twice with serum free media (SFM) and the incubated overnight with PI-PLCγ 10 units/ml (Boehringer Mannheim) in SFM containing 2.5 mM CaCl₂ and MgCl₂ each. The next day, the media was collected and concentrated 20 fold using a Centricon 10

ultrafiltration device. The concentrated filtrate was dialyzed against three changes of buffer (TBS and 5mM CaCl₂). A 25µl sample of this concentrated conditioned media was combined with 0.02 units of transglutaminase and the volume was brought to 50µl with dH₂O. The reaction was incubated overnight at 25°C.

2.5 Eph A2 Immunoprecipitation

NIH 3T3 cells were serum starved for one hour and then stimulated for 20 minutes with either Ephrin A1-Fc (300 ng/ml), transglutaminase treated Ephrin A1-Fc (300 ng/ml), or with ten fold dilutions (30ng/ml, 3ng/ml) thereof. The media was aspirated, and the cells were lysed in PLC lysis buffer. After removal of the insoluble material by centrifugation, the lysate was precleared with protein A-sepharose beads and incubated with anti-Eph A2, specific for an EphA2 extracellular domain, at 1µg/ml (R&D Systems) and 10µl of a 10% slurry of protein A-sepharose. The isolated beads were washed three times with PLC buffer and proteins were eluted by boiling in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and analyzed by western blot. Western blots were subject to densitometric analysis (NIH Image J). The fold increase in phosphorylation represents the average ratio of phosphorylated Eph A2 to Eph A2 in transglutaminase-crosslinked ephrin-Fc stimulated cells relative to cells stimulated with Ephrin A1-Fc alone. The data represents the results from three independent experiments (+/- standard deviation).

2.6 Invasion Assay

HeLa cells were serum starved for one hour and harvested. The cells were plated on a matrigel coated membrane insert (8 µM pore size) at a density of 5×10^4 cells/insert. In the lower chamber, SFM containing Ephrin A1-Fc (2µg/ml), Fc (2µg/ml),

transglutaminase alone (.02 units), transglutaminase crosslinked Ephrin-Fc, antibody clustered Ephrin A1-Fc, or 10 % serum, was added. The cells were incubated at 37 °C for 12 hours and the inserts were transferred to wells containing 4µg/ml Calcein AM in 1x Hank's balanced salt solution and incubated for 1.5 hours at 37°C, 5% CO₂. Inserts were washed twice with 1x Hank's balanced salt solution. Samples were imaged on a Leica DMIREZ inverted fluorescent microscope using a 5x objective (NA 0.12) equipped with Openlab 5.1 software and a Retiga 2000R fast cooled mono 12 bit digital camera. The images were converted to grayscale (8 bits/channel), saved as Tiff files and imported into NIH Image J. Images were binarized and the average % area occupied by cells was calculated. Statistical significance between Ephrin A1-Fc and Ephrin A1-Fc plus transglutaminase-treated cells was determined using a two-tailed student T-test.

2.7 Kinase Assay

10⁵ NIH 3T3 cells were plated onto 10 cm tissue culture dishes and grown overnight in 10% FBS DMEM high glucose media 37°C, 5% CO₂. The next morning, cells were washed with SFM and serum starved for 1 hour. Serum starved cells were stimulated for 15 minutes with 2µg of either Fc, ephrin A1-Fc alone, ephrin A1-Fc pre-clustered with anti-human Fc, or ephrin A1-Fc clustered with transglutaminase in SFM. The media was aspirated and the cells were lysed in PLC lysis buffer. The insoluble material was pelleted by centrifugation, and Eph A2 was immunoprecipitated with 1µg/ml anti-Eph A2 (R&D Systems). The beads containing bound Eph A2 were washed 3 times in Enzyme dilution buffer (50 mM HEPES pH 7.2, 0.1% BSA, 0.01% Brij-35, 0.1mM EDTA, 1mM DTT) and resuspended in 25µl of Enzyme dilution buffer. 5µl of resuspended beads were added to a fresh tube and substrate was added to a final

concentration of 1mM. The reactions were started by the addition of 10 μ l of ATP (250 μ M). The reaction was incubated for 30 minutes at room temperature. The reactions were stopped with the addition of TK Stop mix, the beads were pelleted by centrifugation, and the supernatant was transferred to a 384 well plate. Eph A2 kinase activity was assayed according to the manufacturer's instructions (Upstate Eph A2 KinEASE). Each condition was performed in triplicate and the experiment repeated at least 4 times. Shown are the average data from one triplicate experiment (+/- standard deviation).

2.8 Immunofluorescence

NIH 3T3 cells were seeded onto glass coverslips in 6 well dishes and grown overnight in 10% FBS DMEM high glucose media 37C, 5%CO₂. The next morning, cells were washed with SFM and serum starved for 1 hour. Serum starved cells were stimulated for 15 minutes with either Fc, ephrin A1-Fc alone, ephrin A1-Fc pre-clustered with anti-human fc, or ephrin A1-Fc pre-clustered with transglutaminase. The cells were then washed with PBS and fixed with 4% paraformaldehyde/4% sucrose in PBS for 20 minutes at 4°C. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at 4°C. They were then washed with PBS and blocked overnight in 2% BSA in PBS. The next morning the cells were incubated with anti-Eph A2 in 2%BSA/PBS for 2 hours. Unbound antibody was washed away with PBS and the cells were incubated with FITC-conjugated anti-human Fc and with rhodamine-conjugated anti-goat for 2 hours. After washing 3 times with PBS 1 μ M Hoescht in d₂H₂O was added for 5 minutes at room temperature. The coverslips were then mounted onto glass slides with mounting medium. Localization was determined using a 100x oil objective with an automated Leica

DMIREZ inverted fluorescent microscope, equipped with Openlab 5.1 software and a Retiga 2000R fast cooled mono 12 bit digital camera.

Chapter 3 – Results

This work was the basis for the manuscript entitled “Tissue Transglutaminase Clusters Soluble A-type Ephrins into Functionally Active High Molecular Weight Oligomers ” which has been accepted for publication in the *Journal of Experimental Cell Research*. This was a collaborative project, with contributions from Spencer Alford, Perry Howard, Sabine Elowe, Heather Lorimer and myself, who are acknowledged throughout this chapter.

Ephrin A1 was originally identified as a soluble factor released from the surface of HUVEC cells in response to TNF- α . Independently, using a Biacore assay Bartley et al (1994) showed that conditioned media from numerous cancer cell lines contained Eph receptor binding activity. Our lab is interested in the role of soluble ephrin in cancer cells. It is surprising that cancer cells release ephrins when membrane-bound A-type ephrins can evoke angiogenesis. One might expect cancer cells to block ephrin release and thereby gain angiogenic ability. One possibility is that the release of A-type ephrins occurs only in cultured cells. To examine the extent of soluble A-type ephrins, we first sought to identify A-type ephrin from conditioned media of cancer cells lines. This was important as the original paper by Bartley simply showed that there was Eph binding activity in the media from cancer cell lines but did not show the cause of this activity. Conditioned media from breast cancer cell line SKBR3 was collected, concentrated 20x and examined for ephrin A1 expression. Ephrin A1 was chosen since it was ephrin A1 that was postulated to be present in conditioned media of cancer cell lines by Bartley et al (1994) and the existence of soluble ephrin A1 has been supported in the literature. A strong signal consistent with ephrin A1 was detected in the conditioned media of SKBR3 cells that was not present in media that had not been exposed to cells (Figure 3a). We expanded our study to include the ovarian cell line, SKOV3, an ErbB2-negative breast cancer cell line, MDA-MB231, and cervical cancer cell line HeLa. Prominent expression was seen in conditioned media from SKOV3 and HeLa cells but not MDA-MB231 cells (Figure 3b). To confirm that the band detected in the conditioned media was ephrin A1, an ephrin A1 knockdown experiment was performed using RNAi. HeLa cells were transfected with RNAi against ephrin A1, serum starved overnight, and the conditioned

media, and lysates from the cells were examined for ephrin A1 expression. RNAi to ephrin A1 reduced the expression of the 25 kDa band in lysates and conditioned media, confirming that this protein is indeed ephrin A1. This expression data confirms the results of Bartley et al (1994) and indicates that soluble ephrin A1 is present in conditioned media of several cancer cell lines.

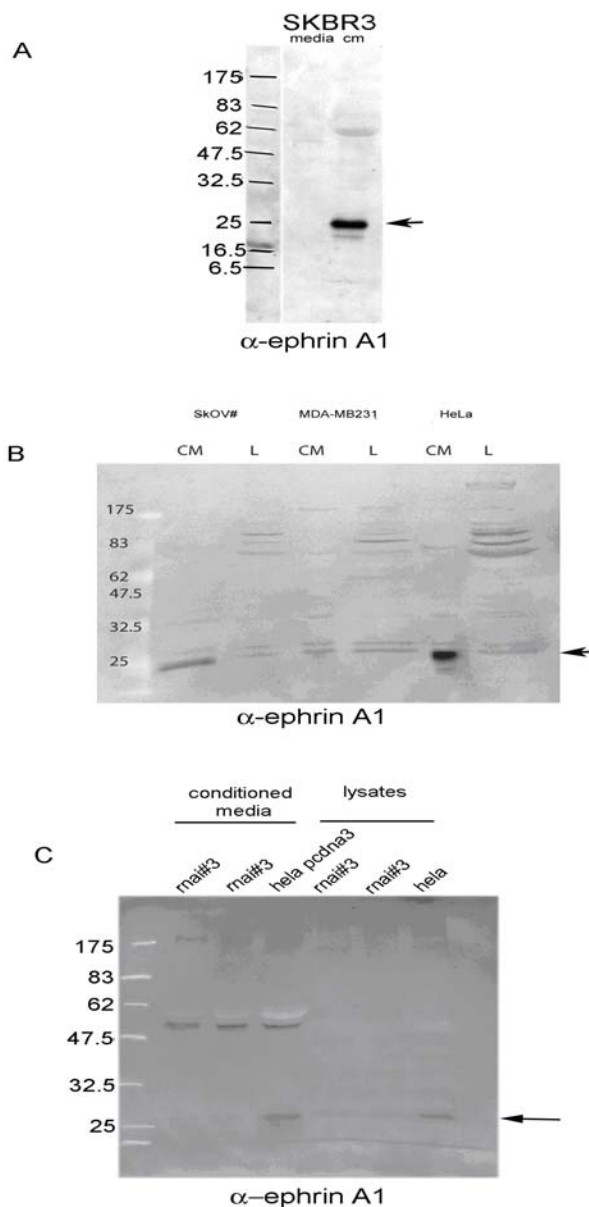


Figure 3. Ephrin is released from the cell surface of cancer cells in culture

a) Conditioned media (CM) from the breast cancer cell line SKBR3 was collected and concentrated 20x. Western blot analysis revealed a 25 KDa protein consistent with ephrin A1 (as indicated by arrow). This band is present in the conditioned media but not serum free media alone. b) Western blot analysis of conditioned media and cell lysates from three additional cancer cell lines; SKOV3, an ovarian cancer cell line, MDA-MB 231, a breast cancer cell line and HeLa, a cervical cancer cell line. A strong signal consistent with ephrin A1 is present in the conditioned media of SKOV3 and HeLa cells but not MDA-MB231 cells. c) Western blot analysis of conditioned media and lysates from HeLa cells after transfection with RNAi against ephrin A1. The 25 KDa band corresponding to ephrin A1 is reduced in both conditioned media and lysates of cells transfected with RNAi. This figure was contributed by Jessa Bazowski. Chris Zroback, a former summer student, assisted with the RNAi experiment.

Membrane bound ephrin A1 is a potent angiogenic factor, so it is surprising that cancer cells would secrete ephrin A1 when soluble ephrin is proposed to block Eph receptor signaling. To determine the extent of soluble ephrin *in vivo* we characterized the expression of A-type ephrins during development and in adult mouse tissues. Initially, we focused on ephrin A5 protein expression during development, since this is the only isoform for which there is functional data supporting a regulatory role for soluble ephrin. Mouse embryos were collected, and lysates were examined by western blotting. A monomeric ephrin A5 band was detected as early as embryonic day 6.5 and continued through all stages tested (Figure 4a). To confirm that our antibody was specific for ephrin A5, we infected 293 cells with an ephrin A5 adenovirus. 293 cells do not endogenously express ephrin A5 and therefore non-infected cells serve as a negative control. Western blotting of 293 cells infected with an ephrin A5 adenovirus, identified a single protein at the expected size of ephrin A5 and confirmed that our antibody recognizes ephrin A5 protein (Figure 4b). To determine whether ephrin A5 in mouse embryos was membrane attached, we utilized sucrose density gradients to isolate the low buoyant density fraction, which contains the lipid raft and GPI linked membrane proteins. As shown in figure 4c, in comparison to Fyn, which is enriched in the low buoyant density fraction, ephrin A5 was split between the low buoyant density fraction and the soluble fraction, which suggests that a portion of ephrin A5 is not membrane associated during mouse development. This result extends previous results reporting soluble ephrin in media from cultured cells, and suggests a portion of ephrin A5 *in vivo* may be released from the cell surface (Figure 4c).

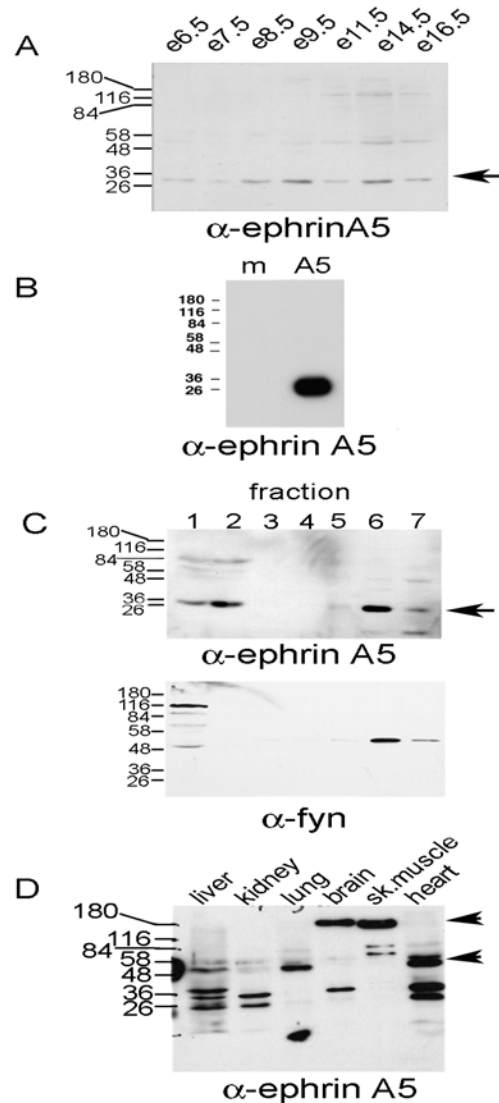


Figure 4. Western of embryo and tissue lysates with anti-ephrin A5

a) Western blot showing ephrin A5 is expressed from embryonic day (e) 6.5–16.5. Mouse embryo lysates from embryonic day 6.5–16.5 were western blotted with anti-ephrin A5, which is directed against a unique peptide within the C-terminus of ephrin A5. Arrow identifies ephrin A5. b) Specificity of anti-ephrin A5 towards ephrin A5. Anti-ephrin A5 detects a single band at 25 kDa in infected 293 cells but not in mock infected (m) cells with adenovirus expressing GFP. c) Ephrin A5 fractions with the lipid raft. (Top) anti-ephrin A5 western blot of fractions from a sucrose density gradient on embryo lysates. (Bottom) anti-fyn western control showing location of lipid raft fraction. The % low buoyant density was calculated as amount of ephrin in the low buoyant density fraction relative to the total amount of ephrin in the sample using NIH Image J software. d) Anti-ephrin A5 western of mouse liver, kidney, lung, brain, skeletal (sk) muscle, and heart lysates. In addition to bands consistent with monomeric ephrin A5, high molecular weight bands at approximately 50 and 175 kDa were detected in some tissues. Arrows indicate bands corresponding to ephrin A5. Numbers indicate molecular weight markers. This figure was contributed by Perry Howard.

We next examined ephrin A5 expression in adult mouse tissues. Western blotting of adult tissue revealed a unique expression pattern. Whereas proteins consistent with ephrin A5 were detected in liver, kidney, brain, and heart tissues, additional proteins at approximately 50 or 200 kDa were detected in heart, brain, liver, lung and skeletal muscle tissues (Figure 4d). The abundance and molecular weight of the cross-reacting proteins was tissue dependent, with brain and skeletal muscle expressing particularly high levels of the 200 kDa protein. Several explanations are possible for the presence of the high molecular weight proteins. Non-specific cross-reactivity is an obvious possibility. However, the fact that the cross-reacting bands were tissue specific, developmentally regulated, and detected with different antibodies (data not shown), suggested that the bands may not represent non-specific cross-reactivity and warranted further investigation. We next asked whether the high molecular weight proteins were specific to our ephrin A5 antibody, or whether they could also be detected with other ephrin antibodies. We reasoned that if ephrin A5 were being modified to generate high molecular weight forms, the other A-type ephrins might also show similar modifications. Western analysis with commercially available antibodies to ephrin A1- A4, also identified higher molecular weight proteins that were consistent with dimerization, trimerization, and high order oligomerization of ephrins (Figure 5 a,b). The presence of the high molecular weight proteins was dependent upon the developmental stage, and the tissue being examined, with high molecular weight proteins predominating during development for ephrins A1- A4 and in adults for ephrin A5. The finding that five different ephrin antibodies all detected high molecular weight proteins coupled with developmental and tissue specific

regulation of these proteins suggested that at least some of the cross-reacting bands may represent alternative forms of A-type ephrins rather than non-specific cross-reactivity.

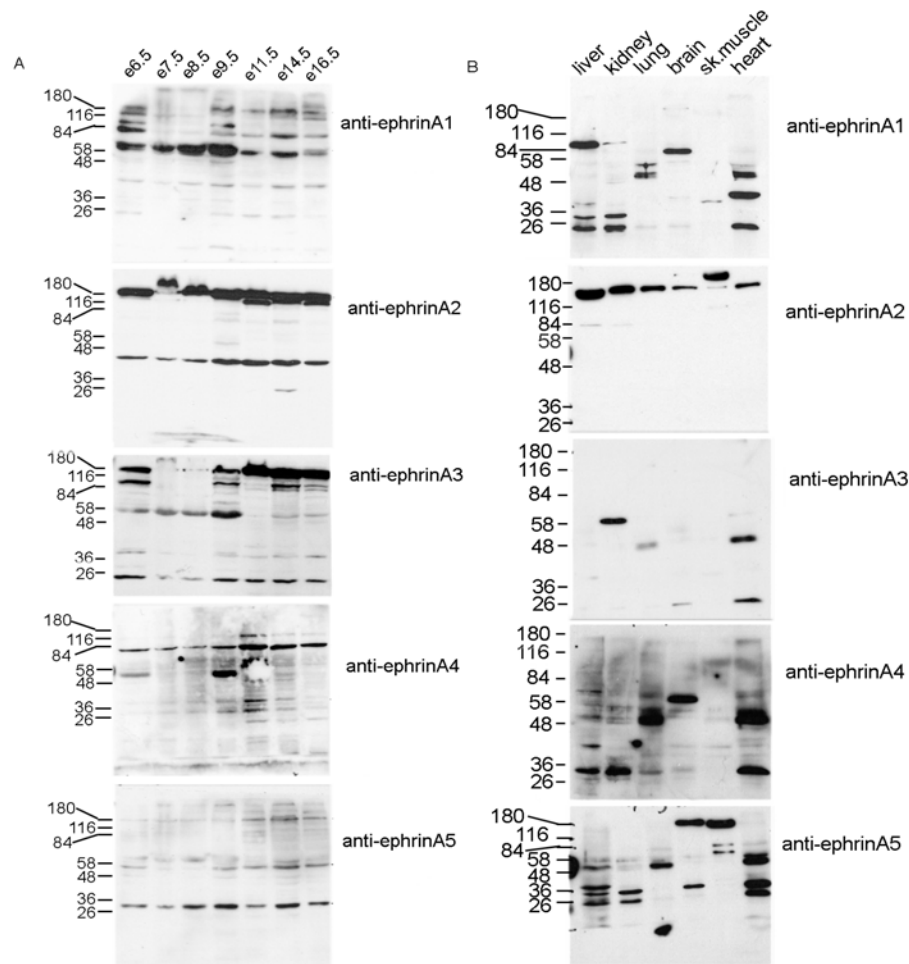


Figure 5. Western blot of embryo and tissue lysates using a panel of ephrin antibodies.

a) Mouse embryo lysates from day 6.5–16.5 were separated by SDS-PAGE and individually blotted with antibodies to ephrin A1, A2, A3, A4, and A5. b) As above, except mouse liver, kidney, lung, brain, skeletal muscle, and heart lysates were used instead of embryo lysates. High molecular weight bands appear to be tissue dependent and developmentally regulated. This figure was contributed by Perry Howard

To determine whether these high molecular weight proteins represented ephrin molecules, we performed a pull-down assay with Eph A5-Fc (Figure 6). We reasoned that this would distinguish between bonafide A-type ephrin, which should bind to the extracellular domain of an Eph A5, and non-specific cross-reacting proteins. Eph A5-fc fusion protein precipitated high molecular weight proteins that were detected with antibodies to ephrin A4 and ephrin A5. Bands at similar sizes could be detected on western blots of these tissues with anti-ephrin A4 and A5 antibodies. These bands were not present in negative controls, including immunoprecipitates using Fc alone, or ProteinA sepharosebeads alone. These observations argue that at least some of the high molecular weight proteins represent alternative forms of ephrin molecules and suggest that A-type ephrins may be subject to oligomerization *in vivo*, in a manner that is resistant to SDS-PAGE denaturation.

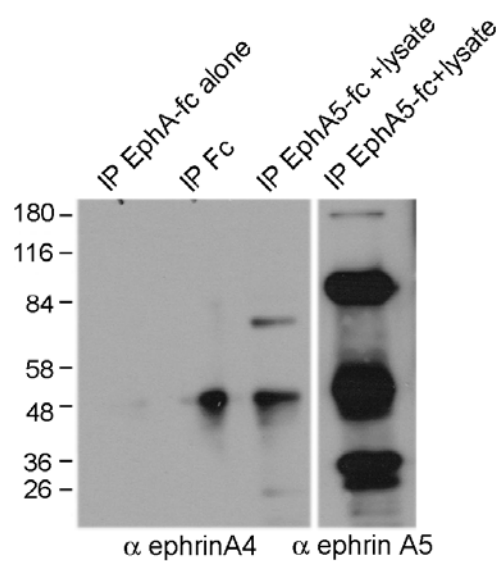


Figure 6. Immunoprecipitation of high molecular weight ephrin A4 or A5 with Eph A5-Fc. Lysates were incubated with Eph A5-Fc and precipitated with ProteinA-sepharose. Negative control lysates were incubated with Fc alone. Precipitated material was separated by SDS-PAGE and examined by western blotting with anti-ephrins A4 or A5 antibodies. Samples were run on the same gel, transferred to nitrocellulose and stained with Ponceau S. The blot was cut in two for incubation with the different primary antibodies. This figure was contributed by Perry Howard

The finding of high molecular weight cross-reacting proteins that can bind to an Eph receptor, prompted us to ask whether the high molecular weight ephrins were membrane attached or soluble. To answer this question, we examined whether the oligomerized ephrin fractionated with the membrane or soluble fraction, in membrane preparations from homogenates of mouse embryos, brain, or new born pup lysates. The oligomerized ephrins A1-A5 are enriched in the soluble fraction (Figure 7). As a control for our fractionation we included ephrin B2, which contains a transmembrane domain and is therefore expected to fractionate with the membrane fraction. As expected, ephrin B2 is highly enriched in the membrane fraction. This suggests that the soluble pool of ephrin, in particular, may be subject to covalent oligomerization.

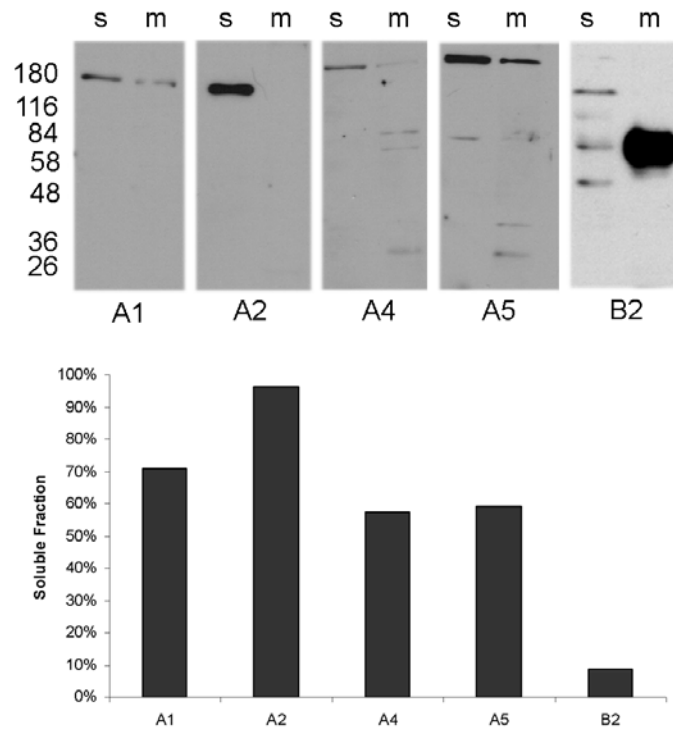


Figure 7. High molecular weight ephrins are enriched in the soluble fraction.

Mouse brain lysates were separated by centrifugation into membrane (m) and soluble (s) fractions and examined by western blotting using anti-ephrins A1-A5 or anti-ephrin B2 (anti-ephrin A3 not shown). The percent soluble was calculated by analyzing densitometry data using NIH Image J software and represents the soluble ephrin relative to the total amount of ephrin in the sample. This Figure was contributed by Perry Howard

Based on our analysis of A-type ephrin expression, we hypothesized that A-type ephrins may be oligomerized *in vivo* by covalent crosslinking. Since covalent oligomerization of many proteins is dependent upon transglutaminases, we examined whether ephrins are substrates for tissue transglutaminase. To this end, recombinant ephrin A1-Fc was incubated with purified transglutaminase and then examined by silver staining and western blotting. As shown in Figure 8a, treatment of ephrin A1-Fc fusion protein with transglutaminase resulted in high molecular weight covalent multimers of ephrin A1 that were stable when electrophorised by reducing SDS-PAGE. Oligomers consistent with trimers and greater were readily detected. Oligomerization was not detected when Fc alone was treated with transglutaminase, indicating that transglutaminase activity was specific for the ephrin A1 substrate and not the Fc portion of the fusion protein. Similarly, treatment in the presence of EGTA also prevented ephrin oligomerization. Western blotting with anti-ephrin A1 antibody confirmed that the high molecular weight bands are multimers of ephrin A1-Fc (Figure 8b). To show that ephrin can be oligomerized by transglutaminase *in vivo*, we infected cells with an adenovirus expressing ephrin A5. Addition of transglutaminase to conditioned media from ephrin A5 expressing cells treated with PLC- γ resulted in cross-linking of ephrin A5 as detected by western blotting (Figure 8c). This result confirmed that cross-linking was not caused by the fusion of ephrin to Fc, and further showed that ephrin A5 is also a substrate for tissue transglutaminase.

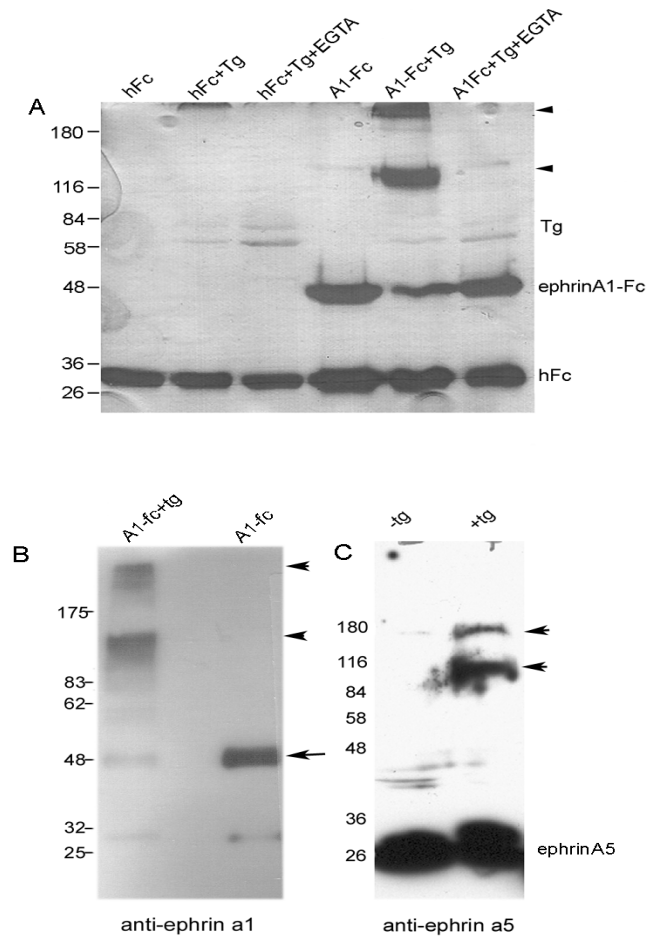


Figure 8. Transglutaminase crosslinking of ephrin A1-Fc.

a) ephrin A1-Fc (A1-Fc) or control human Fc (hFc) were either treated with transglutaminase (5 mM CaCl₂) or transglutaminase (10mM EGTA). Samples were separated by SDS-PAGE and silver stained. Transglutaminase specifically oligomerizes ephrin A1-Fc in the presence of calcium. Oligomerization of control hFc or ephrin A1-Fc in the presence of EGTA was not detected. b) Anti-ephrin A1 western blot of either transglutaminase treated ephrin-Fc, or untreated ephrin-Fc. c) Ephrin A5 is a substrate for transglutaminase. Anti-ephrin A5 western blot of HeLa cells infected with adeno-ephrin A5. Cells were incubated overnight. Arrows indicate bands corresponding to ephrin A1. This figure was contributed by Jessa Bazowski (a,b) and Sabine Elowe (c).

As an additional support to our argument that the high molecular weight bands detected by western blotting represent covalently oligomerized forms of ephrin, Spencer Alford in our lab has shown that ephrin A5 is oligomerized into a >200 kDa form during the differentiation of C2C12 cultured myoblasts. Using RNAi, he has further shown that the oligomerization of ephrin A5 is dependent upon the expression of tissue transglutaminase (Alford et al 2007). Taken together with the data presented in this thesis, these results strongly support hypotheses 1 and 2. We next examined the purpose of ephrin oligomerization. There are two possible explanations for transglutaminase crosslinking of soluble A-type ephrins. The first is that crosslinking may be a method of inactivating soluble ephrins so that they do not interfere with membrane bound ephrin signaling through Eph receptors. The second is that crosslinking may be a mechanism to oligomerize and thereby activate soluble ephrins such that crosslinked ephrins can stimulate Eph kinase activity.

Based on the requirement for ephrin clustering, we hypothesized that oligomerization of ephrin by transglutaminases may function to cluster ephrins to make them competent to stimulate Eph A receptor activity. To characterize the role of transglutaminase oligomerization of soluble ephrin, we first asked whether transglutaminase crosslinked ephrin could bind to an Eph receptor, to rule out the possibility that transglutaminase crosslinking of ephrin is a mechanism of ephrin inactivation. As shown in Figure 9a, Gst-Eph A4 ectodomain interacts with oligomerized ephrin A1-Fc. This indicates that transglutaminase crosslinking of ephrin does not block the ability of ephrin to bind to the Eph receptor.

We next examined the localization of Eph A2 after stimulation with either transglutaminase-treated or untreated ephrin to determine the effect of transglutaminase crosslinking of ephrin on Eph receptor localization. Anti-human-Fc was used as a positive control for oligomerizing ephrin A1-Fc. Regardless of whether ephrin was presented as a Fc dimer or an oligomer, ephrin treatment resulted in a redistribution of Eph A2 receptors from points of cell-cell contact, to discrete clusters located more centrally in the cell (Figure 9b). However, there was a noticeable increase in the size and intensity of the Eph receptor clusters when ephrin was pretreated either with anti-human Fc or with transglutaminase. Since the degree of ephrin clustering has been shown to alter ephrin signal transduction, this result suggests transglutaminase crosslinking of ephrin may lead to Eph receptor activation.

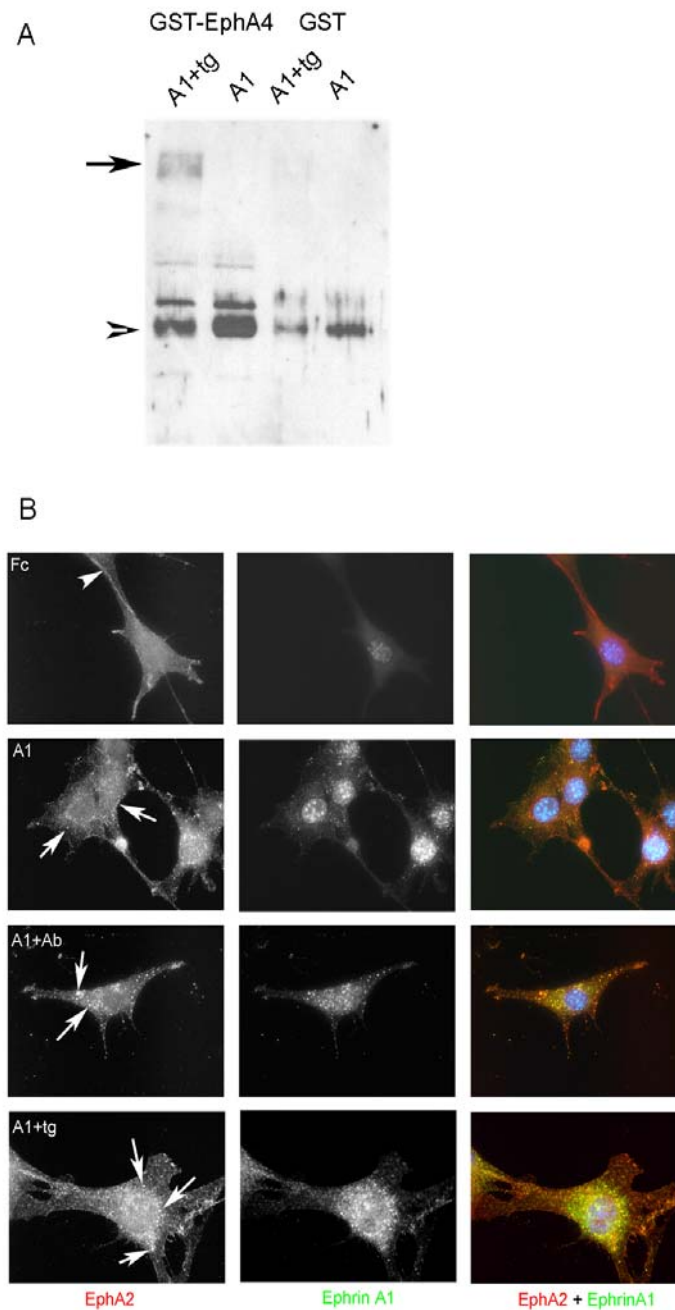


Figure 9. Oligomerized soluble ephrin A1 interacts with Eph receptors and promotes receptor clustering.

a) Eph A4-GST interacts with oligomerized ephrin A1. Glutathione sEph Arose pulldown of either Eph A4-GST, or GST alone, incubated with transglutaminase or untreated ephrin A1-Fc. B) Oligomerization of ephrin A1 promotes Eph A2 clustering. NIH 3T3 cells were serum starved and stimulated with either Fc, ephrin A1-Fc, ephrin A1-Fc clustered with anti-human Fc, or ephrin A1-Fc clustered with transglutaminase. Arrows indicate localization of Eph A2. This figure was contributed by Spencer Alford (a) and Jessa Bazowski (b)

As a measure of the effect of transglutaminase crosslinking of ephrin on Eph signal transduction, we sought to determine the activation of endogenous Eph A2 in NIH 3T3 cells following stimulation by ephrin A1-Fc, transglutaminase crosslinked ephrin A1, or antibody clustered ephrin. Immunoprecipitation of Eph A2 was performed and Eph A2 kinase activity was determined using a fluorescence polarization based assay, which measures the phosphorylation of a peptide substrate. In this assay a decrease in fluorescence polarization correlates to an increase in Eph A2 kinase activity. This assay was developed commercially to measure kinase activity of purified Eph A2, and its use in measuring Eph A2 activity from immunoprecipitates has not been reported. The principle advantage of this procedure is that it is not based on radiolabeling and secondly the kinase activity can be measured directly in a 384 well format. This means that there are fewer steps, less material is required, and the procedure is amenable to high throughput analysis. To develop this assay, I first determined the dose-response of Eph A2 activity to antibody-clustered ephrin A1. As shown, Eph A2 activity corresponded closely with ephrin concentration (Figure 10A). Although there was a high degree of variability between samples, and the dynamic range of detection was low, we decided to further develop the test. Based on the dose response, we chose to stimulate with 2 $\mu\text{g/ml}$ of ephrin for subsequent experiments. We next determined the optimum incubation time for the kinase assay, and tested between ten and fifty minutes. Based on this analysis, we chose 30 minutes as the appropriate incubation time for the assay, which would allow for maximum phosphorylation of substrate, but still remain sensitive to kinase activity (Figure 10b). As shown in Figure 10c, transglutaminase crosslinking of ephrin

stimulated Eph A2 kinase activity. The level of activity obtained was comparable to the activity obtained by antibody pre-clustering but was not statistically significant.

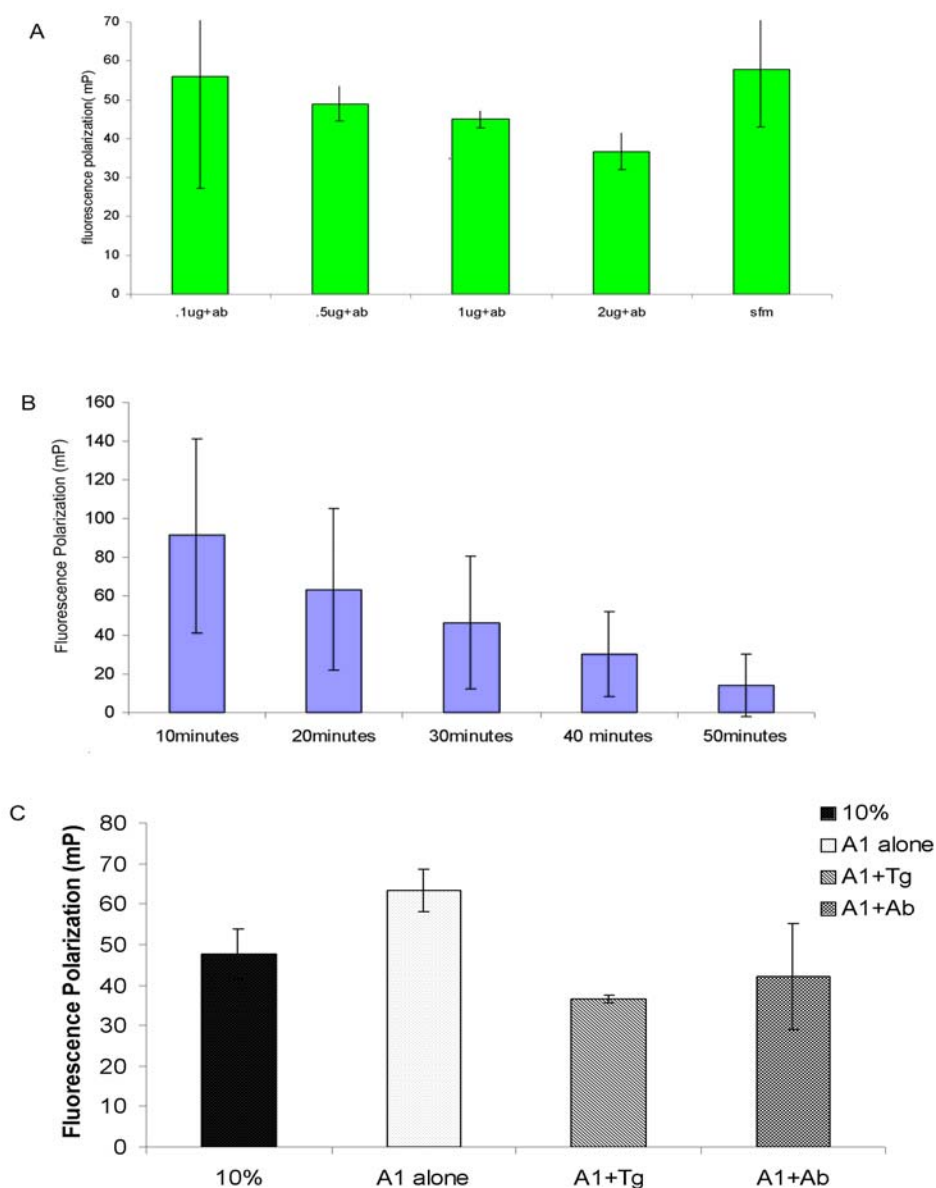


Figure 10. Oligomerized soluble ephrin A1 activates the kinase activity of Eph A2.

A fluorescence polarization based kinase assay was used to detect Eph A2 activity from immunoprecipitates a) Dose-response of Eph A2 activity to antibody clustered ephrin A1. Eph A2 activity, as measured by a decrease in fluorescence polarization, corresponded closely with ephrin A1 concentration. The greatest amount of activity occurred when Eph A2 was stimulated with ephrin A1 at 2 ug/ml. b) Determination of optimal incubation time for the kinase assay. 30 minutes allowed for maximum phosphorylation of substrate while preserving sensitivity to kinase activity. c) Transglutaminase crosslinked ephrin A1 stimulates the kinase activity of Eph A2. This figure was contributed by Jessa Bazowski.

As a consequence of Eph A2 kinase activation, tyrosine residues within the juxtamembrane region of the receptor become auto-phosphorylated. These phosphorylated tyrosine motifs serve as docking sites for downstream SH2 and PTB containing proteins, which propagate ephrin/Eph signaling. We next examined Eph A2 phosphorylation status after stimulation with transglutaminase treated ephrin A1. Although phosphorylation is typically used as an indirect measure of kinase activity, Eph A2 phosphorylation has not correlated well with kinase activity (Zantek et al 1999). However, as shown in Figure 11b, transglutaminase-oligomerized ephrin A1 enhanced the tyrosine phosphorylation of Eph A2 when compared to non-crosslinked ephrin A1, suggesting that ephrin cross-linking promotes downstream signaling through Eph receptors (Figure 11b).

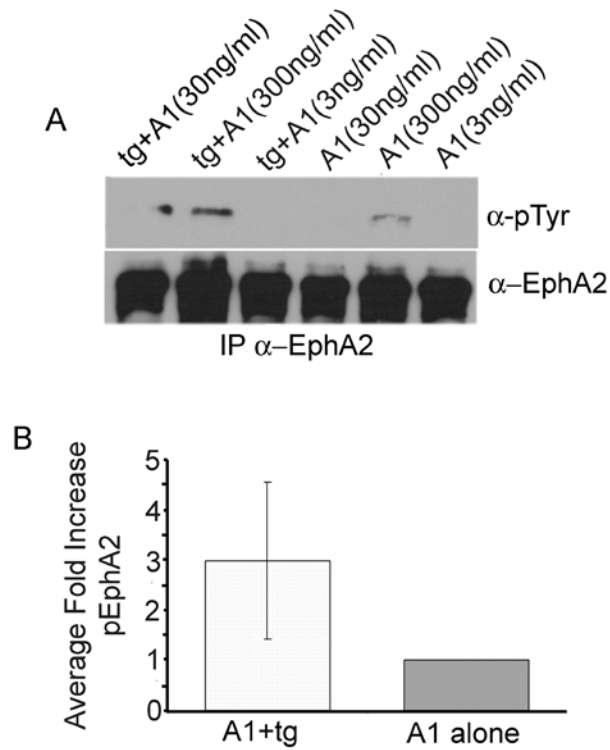


Figure 11. Transglutaminase crosslinked ephrin A1 enhances receptor phosphorylation.

a) Western blot of Eph A2 immunoprecipitation after stimulation with transglutaminase crosslinked or monomeric ephrin at 300 ng/ml, 30 ng/ml or 3 ng/ml. b) Average fold increase of Eph A2 phosphorylation showing that transglutaminase crosslinked ephrin A1 enhances tyrosine phosphorylation of Eph A2. This figure was contributed by Jessa Bazowski.

The degree of clustering of ephrin can have important consequences to cell behaviour. For example, in PC 19 cells expressing Eph B2, adhesion is promoted by tetrameric but not dimeric or higher order oligomerized ephrin B2 (Stein et al 1998). To test whether transglutaminase crosslinked ephrin is interpreted in a different manner than non- oligomerized ephrin, we performed an invasion assay with migration competent HeLa cells, which endogenously express Eph A2. The cells were serum starved and plated onto matrigel matrix in the upper chamber. In the lower chamber, either ephrin A1-Fc or ephrin A1-Fc that had been treated with transglutaminase was added. For controls, the lower chamber was filled with 10% serum, SFM, Fc alone, or transglutaminase alone. As shown in Figure 12, stimulation of HeLa cells with transglutaminase clustered ephrin but not ephrin A1-Fc alone resulted in the invasion and migration of HeLa cells into the lower chamber. These results show that transglutaminase oligomerization of ephrin changes the way cells respond to ephrin, and that crosslinked ephrin promotes the invasiveness and migration of Eph A2 expressing cells.

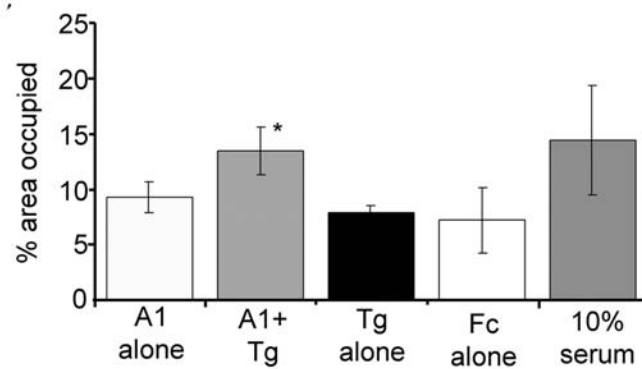
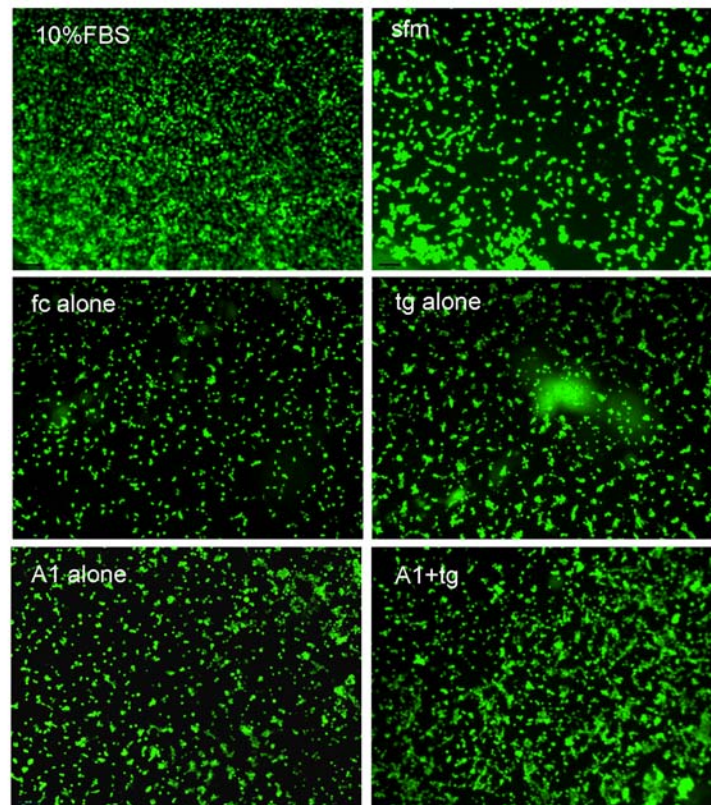


Figure 12. Transglutaminase crosslinked ephrin A1 stimulates invasion and migration of HeLa cells.

Equal numbers of serum starved HeLa cells were plated in the upper compartment of a modified boyden chamber. The bottom chamber contained either 10% serum, sfm, hFc, transglutaminase, ephrin A1-Fc, or transglutaminase crosslinked ephrin A1. (* p-value = 0.0001)

Chapter 4 – Discussion

Typically, Eph receptors and ephrins signal bidirectionally at sites of cell-cell contact, and the degree of ephrin clustering on the membrane influences cellular responses (Vearing et al 2005). Membrane attachment may facilitate Eph /ephrin clustering and association with downstream targets through recruitment to lipid rafts and possibly promote the association of PDZ containing proteins to the cytoplasmic tails of Eph receptors and B-type ephrins, which may also participate in clustering (Bruckner et al 1999) (Gauthier et al 2003). Clustering has been shown to alter the outcome of ephrin/Eph engagement by changing the signaling complexes that are formed. For example, the degree of ephrin B2 oligomerization has been shown to alter capillary-like assembly in human renal microvascular endothelial cells, and cell attachment of P19 cells through differential recruitment of low molecular weight phosphatase (LMW-PTP) (Stein et al 1998). The basis for oligomerization has been elucidated by co-crystal structure of ephrin B2 and Eph B2, which showed the pair form a tetrameric ring complex with each ligand binding to 2 receptors and each receptor interacting with 2 ligands (Himanen et al 2001). This is thought to be the minimal complex necessary for bi-directional signaling. However, in many cancer and endothelial cells (Bartley et al 1994), A-type ephrins are shed, which creates a potential pool of monomeric ephrin of unknown function. Indeed we have shown that the amount of soluble monomeric ephrin A5 during mouse development is equal to the amount associated with the GPI lipid raft fraction, which indicates shedding of A-type ephrins is not restricted to cells in culture. This raises an important question as to whether these shed ephrins are functional.

We have found that soluble A-type ephrins can be covalently cross-linked, forming high molecular weight oligomers that can be detected on denaturing and reducing SDS-PAGE. The presence of the high molecular weight ephrins is developmentally regulated and tissue dependent. Several lines of evidence indicate that the high molecular weight bands represent a posttranslational modification of A-type ephrins and not non-specific cross-reactivity. First, multiple antibodies to different A-type ephrins each detected bands consistent with ephrin multimers. Second, the high molecular weight bands are developmentally regulated and tissue dependent, with each ephrin antibody detecting a different pattern and extent of oligomerization. This suggests that the bands are not simply a common cross-reacting protein shared between the different antisera. Thirdly, we have shown by RNAi that C2C12 cells express oligomerized ephrin A5 during differentiation in a manner that is dependent upon tissue transglutaminase expression. Finally, we were able to show that Eph A5 interacts with high molecular weight proteins that are detected by anti-ephrin A4 and anti-ephrin A5 antibodies. Therefore, not only do the high molecular weight proteins cross-react with ephrin antibodies, they also bind to an Eph receptor. Formal proof of this will require biochemical purification and sequencing. Although we cannot conclude that all of the bands present are oligomerized forms of ephrin, our results strongly suggest that A-type ephrins are subject to oligomerization. Characterization of ephrin-deficient mice as they become available will assist characterizing the degree and extent of ephrin oligomerization *in vivo*.

Our results indicate that the mechanism of ephrin oligomerization is through the activity of transglutaminases. Both ephrin A1 and A5 were substrates for tissue

transglutaminase *in vitro*, and the ephrin oligomerization detected in mouse tissue is consistent with transglutaminase crosslinking A-type ephrins to form covalent multimers, ranging in size from dimers, to high order structures of seven or eight ephrin molecules. Tissue transglutaminase is one of eight family members and is the most widely expressed isoform. Until recently, tissue transglutaminase has been primarily associated with widespread ultrastructural changes during apoptosis, which is thought to stabilize apoptotic cells prior to phagocytic clearance (Mehta et al 2006). However, it is increasingly clear that tissue transglutaminase is multifunctional and has a variety of cellular roles, including apoptosis, cell adhesion, matrix remodeling and regulation of cell signaling (Mehta et al 2006) (Chen et al 1999) (Mangala et al 2005). Ephrins are the first example of a tyrosine kinase ligand serving as a direct substrate of tissue transglutaminase. Our data reveals an unappreciated role for this enzyme in signaling from a receptor tyrosine kinase.

Based on our results and the results of others, it is clear that there is an emerging role for tissue transglutaminase in growth factor regulation. Tissue transglutaminase has also been shown to regulate both TGF β and insulin-like growth factor (Munger et al 1997) (Nunes et al 1997) (Akimov et al 2001) (Sakai et al 2001). TGF β is secreted in a latent form, sequestered in an inactive complex by latent -TGF β - binding protein (LTBP) and TGF β propeptide (LAP) (Munger et al 1997). Tissue transglutaminase activates TGF β by crosslinking LTBP to the extracellular matrix. This is thought to release TGF β from its association with LTBP and LAP, freeing the ligand to bind to its receptor. Similarly, transglutaminase regulates the activity of insulin-like growth factor by modifying a key regulator, IGF binding protein 1 (IGFBP1) (Sakai et al 2001).

Oligomerization of IGFBP1 prevents its binding to IGF, thereby releasing the ligand from negative regulation by IGFBP1 (Sakai et al 2001). In contrast, the crosslinking of ephrin is distinct since the ephrin itself is being oligomerized, rather than a regulatory protein, and ephrin oligomerization promotes ephrin activity rather than inhibiting it. Given the wide tissue distribution of oligomerized ephrin that we detected and the importance of ephrin/Eph signaling to many developmental and tissue processes, our results suggest that tissue transglutaminase may participate in ephrin regulated processes such as angiogenesis, axon guidance, growth, and differentiation.

How does oligomerization of ephrins by transglutaminase contribute to ephrin-Eph signaling? Although it is possible that oligomerized ephrin is involved in sequestering ephrin in an inactive form, we favour a model in which oligomerization of soluble ephrin by transglutaminase clusters the ligand for recognition and stimulation of Eph receptors. This is supported by the ability of crosslinked ephrin to stimulate Eph receptor tyrosine kinase activity, the ability of Eph A5-Fc to interact with oligomerized ephrin A4 and A5 from brain tissue, and by the ability of crosslinked ephrin to stimulate the invasiveness and migration of Eph A2 expressing HeLa cells. These findings suggest that transglutaminases may regulate the ability of soluble ephrin to promote cellular responses in a paracrine fashion, a feature that hereto has not been reported. Given the amount of oligomerized ephrin detected in some tissues, there is a potentially large pool of ephrin regulated in this manner.

In summary, we have identified a novel mechanism of activating soluble A-type ephrins through oligomerization by transglutaminases. Our results suggest that transglutaminase may promote the oligomerization of soluble ephrins *in vivo* in a manner

that promotes Eph receptor activity. Based on our findings, it appears that ephrins may function at more than just sites of cell-cell contact.

Chapter 5 - Future Directions

Although my research has produced several interesting findings with regards to the signaling capabilities of soluble ephrin A1 and its relationship with tissue transglutaminase, several questions still remain. One of the next steps will be to investigate ephrin expression and release from tumor cells *in vivo*. To do this one could obtain tumor tissue from different cancers and use a combination of northern blotting or RT-PCR to look for ephrin A1 expression. Tissue fractionation and western blotting could be used to look at soluble versus membrane bound forms of ephrin A1. In addition, it will be important to look at tissue transglutaminase expression and how it correlates with ephrin expression and release in these tumor tissues. Loose correlations between ephrin A1 upregulation and transglutaminase upregulation have been reported. For example, Fok et al (2006) found that tissue transglutaminase is selectively upregulated during advanced stages of malignant melanoma, while Easty et al (1999) also identified an upregulation of ephrin A1 in malignant melanomas. Furthermore, Easty et al (1995) found that ephrin A1 was secreted by all melanoma cell lines that they tested. Subsequent studies which look quantitatively at tissue transglutaminase and ephrin A1 levels in the same tumor tissue will be important to properly examine a correlation such as this. It will also be important to determine the localization of ephrin A1 and transglutaminase in these samples. One could examine tissue, using immunogold labeling and electron microscopy to determine if transglutaminase and ephrin are found together in the extracellular matrix, and whether ephrin is soluble or membrane bound.

Another avenue to explore is the method by which ephrin is released from the cell surface. Ephrin is attached to the cell surface with a lipid anchor therefore it would be

reasonable to expect that a lipase may be responsible for this. However, it has also been shown that A -type ephrins have an association with Kuzbanian, a metalloprotease, and this could also be responsible for releasing A-type ephrins from their membrane anchor. To test for this one could use inhibitors or metal chelators to inhibit these enzymes and then look at the level of ephrin released from the cell surface. Also, creating a lipid free form of A-type ephrin which would be a transmembrane protein would tell us if ephrins GPI anchor is necessary for cleavage. Alternatively one could examine the amino acid sequence of the ephrin released from the cell surface through purification and mass spectrometry and draw a conclusion as to the method of release from this. During the course of my research I have tried to purify the ephrin present in the conditioned media of cells in culture using a Rotofor which separates proteins using isoelectric focusing. While I was able to separate small quantities of ephrin from conditioned media, I was unable to get enough to conduct any further analysis.

Although several proteins have been shown to be substrates for transglutaminase, there is no known consensus sequence for this enzyme. What is known is that transglutaminase forms covalent crosslinks between glutamine and lysine residues of substrate proteins. When looking comparatively at A-type ephrin sequences, only one site which contains both amino acids and is conserved among all ephrins within this class can be found.(See chapter 1, figure 2). This site is LSEKFQ which is located within the ligand binding domain. How crosslinking affects the ability of these molecules to bind their receptor is an interesting question. To determine whether this is the crosslinking site, it will be important to generate mutants that lack the glutamine residue and determine whether crosslinking still occurs. If this is the site, examining the structural

interactions of transglutaminase crosslinked ephrin and its receptor is another area in which further investigation is required.

It is important that investigations are conducted to further characterize the signaling properties of transglutaminase crosslinked ephrin. These studies would include comparisons between Eph A2 phosphorylation and kinase activity after interacting with oligomerized soluble ephrin A1. I found evidence that the phosphorylation level of the receptor did not correlate with kinase activity. Similar results were found by Zantek et al (1999) when they looked at Eph A2 activity in several breast cancer cell lines. They found that while receptor phosphorylation was decreased in some cell lines, kinase activity in those same cell lines remained high. These findings tell us that receptor phosphorylation level alone cannot be used as a measure of receptor activity.

Stein et al (1998) found that different ephrin multimers were capable of recruiting different proteins to active receptors, thus it would be necessary to test oligomers of different sizes in order to identify all pathways activated by transglutaminase crosslinked ephrin. Stein et al (1998) used exclusion chromatography to separate these multimers based on their mass. It would be interesting to examine the various multimers obtained by transglutaminase crosslinking to determine whether these exhibit differences in cellular response. I have shown that transglutaminase treatment of ephrin A1 promotes migration of HeLa when compared to ephrin A1-Fc alone. Investigation of the degree of crosslinking and migration response will be important. This will require purifying the oligomeric forms of ephrin.

Finally, *in vivo* assays will help to further assess the contributions of soluble crosslinked ephrin to tumor angiogenesis. Xenograft transplants combined with RNAi

knockdown of transglutaminase, could be used to determine whether transglutaminase crosslinking of ephrin is important for tumor angiogenesis.

Although several steps still need to be taken to further characterize the properties and contributions of transglutaminase crosslinked ephrin, my research has provided the framework necessary to initiate these further studies.

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Appendix A – Abbreviations

Abl	Abelson tyrosine kinase
ADAM	A disintegrin and metalloprotease
Ang-1	Angiopoietin 1
CAP	c-Cbl-associating protein
DH-PH	Dbl homology-pleckstrin homology
Eph	Erythropoietin-producing hepatocellular
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAP	Fas-associated phosphatase
FAK	Focal adhesion kinase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factors
GRB2	Growth factor receptor-bound protein 2
GRIP	Glucocorticoid receptor interacting protein
GPI	Glycosylphosphatidylinositol
GST	Glutathione-S-transferase
HRP	Horseradish-peroxidase
HUVEC	Human umbilical vascular (or vein) endothelial cells
IGFBP1	Insulin growth factor binding protein 1
LTBP	Latent transforming growth factor binding protein
LMW-PTP	Low molecular weight protein tyrosine phosphatase
MAPK	Mitogen-activated protein kinase
NIK	Nck interacting kinase
PAR-3	Protease-activated receptor 3
PAK	p21-activated kinase
PDZ	Postsynaptic density protein/disc large/zona occludens
PKC	Protein kinase C
PTB	Polypyrimidine tract-binding protein
PTP-BL	Protein tyrosine phosphatase
RGS	Regulator of heterotrimeric guanine nucleotide-binding protein (G protein) signaling
RNAi	RNA interference
SAM	Sterile alpha motif
SDF-1	Stromal cell-derived factor 1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src-homology 2 domain
TNF- α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
WASP	Wiskott-Aldrich syndrome protein